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LIVING WITH GLOBAL CHANGES: PHYSIOLOGICAL AND MOLECULAR MECHANISMS AS THE BASIS FOR SEAGRASSES RESILIENCE IN A CHANGING WORLD

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DOTTORANDA
JESSICA PAZZAGLIA

COORDINATORE
PROF. GIORGIO ALBERTI

SUPERVISORE DI TESI
PROF. ANTONIO TERLIZZI

CO-SUPERVISORI DI TESI
DOTT. GABRIELE PROCACCINI

DOTT. LÁZARO MARÍN-GUIRAO

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Living with global changes: physiological and molecular mechanisms as the basis for seagrasses resilience in a changing world

Jessica Pazzaglia

University of Trieste – Stazione Zoologica Anton Dohrn

Supervisored by:

Professor **Antonio Terlizzi**

Dipartimento di Ecologia Marina Integrata, Stazione Zoologica Anton Dohrn (SZN), Italy;
Dipartimento di Scienze della Vita, Università degli studi di Trieste (UNITS), Italy

Co-Supervised by:

Dr. **Gabriele Procaccini**

Dipartimento di Ecologia Marina Integrata, Stazione Zoologica Anton Dohrn (SZN), Italy

Dr. **Lázaro Marín-Guirao**

Oceanographic Center of Murcia, Spanish Institute of Oceanography, Spain
Dipartimento di Ecologia Marina Integrata, Stazione Zoologica Anton Dohrn (SZN), Italy;

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ABSTRACT

The intensification of seawater warming and the co-occurrence of different anthropogenic stressors are threatening coastal marine habitats, including seagrasses which form a unique group of marine plants supporting diverse and productive ecosystems. However, seagrasses are declining globally and are one of the most threatened ecosystems on earth. The simultaneous presence of sea warming with local pressures can result in antagonistic, additive, or synergistic effects depending on their interactions. One of the main concerns of rapid environmental shifts is that these changes do not allow species to react swiftly enough in order to cope with and survive in the new more stressful environment. Thus, the analysis of the degree of phenotypic plasticity could reveal important insights into seagrasses' persistence. The main aim of this doctoral research was to investigate the resilience capacity of *Posidonia oceanica*, endemic of the Mediterranean Sea, to environmental changes. Plants' performances were analyzed exploring the effect of local environmental conditions in driving different plants' responses to single and multiple stressors. To this end, I previously reviewed the concept of phenotypic plasticity suggesting mesocosm experiments and reciprocal transplants as useful approaches to assess the phenotypic plasticity that allows discriminating the effect of local adaptation and acclimation in plants' responses to common stress conditions. Starting from these considerations, I performed a mesocosm experiment where plants growing in oligotrophic (Ol plants) and eutrophic (Eu plants) environments were exposed to single (nutrients and temperature increases) and multiple stressors (nutrients combined with temperature increases). Plants' performance was assessed applying an 'omic approach', exploring physiological and transcriptional responses with the focus on the dynamics of DNA methylation during the exposure to stress conditions. Physiological analysis revealed that the exposure to nutrients induced the worst effect in the leaf in both Ol and Eu plants while antagonistic effects with temperature were found in Eu plants for some parameters. Accordingly, the analysis of the whole battery of transcribed genes revealed an organ-specific response depending on the plants' origin and stress exposure. I also aimed to investigate the dynamics of DNA methylation selecting key genes and analyzing the global DNA methylation levels during the exposure to stresses in both Ol and Eu plants. DNA methylation levels changes according to the plants' origin and environmental stresses, demonstrating that DNA methylation changes dynamically with the surrounding environmental conditions contributing to the regulation of stress responses in *P. oceanica* plants. In the framework of designing appropriate restoration strategies, approaches to assisted evolution can be implemented. In

this thesis, I applied the thermo-priming treatment to *P. oceanica* seedlings through exposure to a simulated warming event. This priming process modifies the phenotypic state of an organism favouring phenotypic-plastic adjustments to future environmental stress conditions. Primed seedlings performed better during the re-occurring stress event than un-primed ones. This possibility provides important implications for restoration and conservation management. During the Ph.D. thesis, I also authored a review paper, highlighting the importance of the genetic component in seagrass restoration, where the hypotheses and the knowledge acquired during the study, were integrated for providing a conceptual framework to serve future restoration plans. The integration of studies related to local adaptation and acclimation, local environmental disturbances with the analysis of the genetic and epigenetic component, should always be considered to select the most appropriate donor site to restore degraded habitats, guaranteeing the success of the restoration plan.

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INTRODUCTION

Global and local threats to coastal ecosystems and their interactions

Coastal marine ecosystems, such as seagrass meadows, mangroves, salt marshes, estuaries, coral reefs, and continental shelves, are among the most important of the world for providing services and benefits to humans (Fisher et al., 2015). Being in regions where sea, atmosphere, and land processes interact, these coastal ecosystems are characterized by high complexity and dynamism that render them also among the most threatened habitats, particularly vulnerable to global and local pressures (Zhang et al., 2004). In the era of the Anthropocene, climate change (e.g. warming) is provoking profound and irreversible changes on coastal marine environments at all levels of biological organization (He and Silliman, 2019). Globally, the estimates of the increase of mean coastal Sea Surface Temperature (SST) is 0.17± 0.11 °C/decade with larger increases and more heterogeneous trends than those predicted for open oceans (Liao et al., 2015). Human-induced stressors and their consequences, including the increase of greenhouse gas concentrations, are worsening the impacts of climate changes enhancing atmosphere warming, and the occurrence of extreme weather and climatic events such as marine heatwaves (MHWs) (IPCC, 2012). The frequency of prolonged periods of anomalously warm seawater (i.e. MHWs) has increased since the early twentieth century (Oliver et al., 2018), and their impacts on marine ecosystems are driving biodiversity loss especially among habitat-forming species (e.g. seagrasses, Thomson et al. 2015; coral reefs, Hughes et al. 2019; kelp forests, Thomsen et al. 2019). Although climate change is considered the major threat to marine ecosystems, its effect on marine biota can change according to ongoing local anthropogenic pressures. Globally, most of the metropolises with higher population density are located along coastlines, with over 1.2 billion people living within 100 km of the coast (Marone et al., 2017). Being so populated, coastal habitats are threatened by different kinds of stressors that can interact with each other either exuberating or buffering the negative impacts of climate changes. As a result, nearly 97.7% of the entire oceans are affected by multiple stressors (Halpern et al., 2015). The intensity of these effects varies according to the scale of the stress occurrence (Fig. 1), where densely populated areas tend to be affected by more intense stresses, especially for industry-derived pollution (e. g. toxic contaminants) and nutrient inputs (e. g. eutrophication) (Breitburg et al., 2019). In other specific cases, such as after extreme weather events, large rivers can discharge enormous amounts of sediments and nutrients to the coastal ocean impacting wide areas, and thus the possibility of an interaction between eutrophication and other local factors is more

likely to occur (He and Silliman, 2019). Synergistic effects arise when the intensity of the impacts of co-occurring stressors is greater than the sum of each single stressor (fig. 1). Conversely, when the interaction is antagonistic the cumulative impacts of stressors is reduced (Folt et al., 1999). Synergistic interaction between climate change and local human pressures is usually linked to the greatest impact on biodiversity and ecosystems, whereas antagonistic effects are predicted to enhance resilience capacity to warming reducing the possibility to exceed species tolerance ranges (He and Silliman, 2019). However, the interpretation of the interactions observed between multiple stressors is even more complicated considering the dominant effect that one stressor may have on another which could lead to better explain the contribution of single stressors on the observed combined response (Brown et al., 2014; Coté et al., 2016). Hence, predicting the effect of multiple stressors on marine environments is a complex task that depends on local environmental conditions and pressures. Priorities must be given to cumulative impact assessment studies to identify the typology of the stress and eventual hotspot areas to warming, which is necessary to predict environmental shifts and their consequences in a more realistic future scenario of environmental changes.

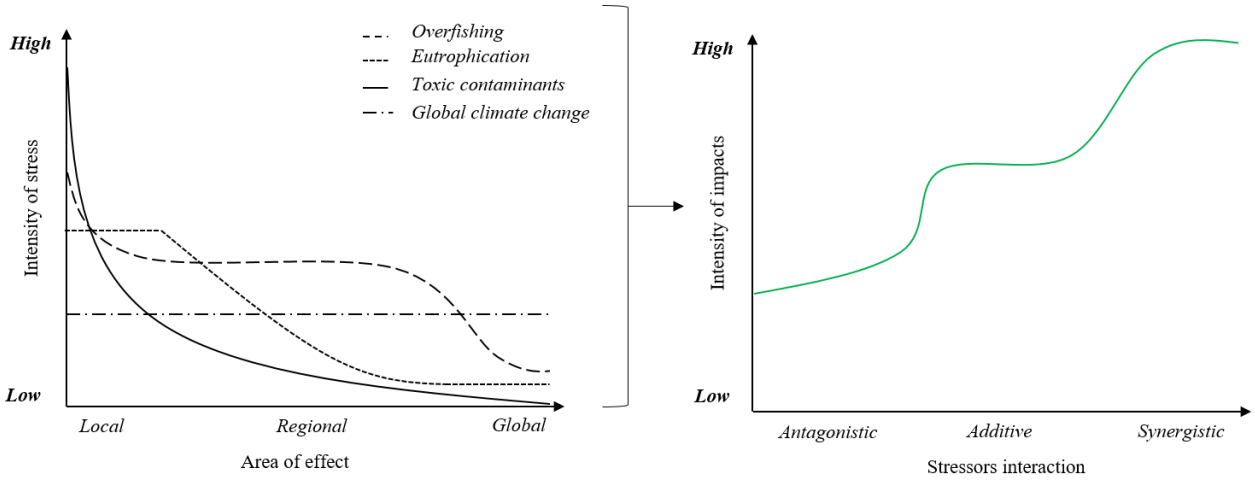

Figure 1. Hypothetical scale of different stressors co-occurring at local, regional and global areas (left), Modified from Breitburg et al. (2019), and variation in antagonistic, synergistic and additive interactions between climate change and local human impacts (right).

Biology and ecology of marine plants

Seagrasses are marine flowering plants belonging to the polyphyletic group of monocotyledonous angiosperms of the order Alismatales which includes 11 families of aquatic– freshwater species and four families that are fully marine (Posidoniaceae, Zosteraceae, Hydrocharitaceae, and Cymodoceaceae; Les et al. 1997). These marine plants

have evolved peculiar adaptation capacities to return to a completely submerged lifestyle in marine waters (Den Hartog, 1970). Phylogenetic analysis revealed that seagrasses returned into the sea at least three times through their evolution from a common aquatic-freshwater ancestor of terrestrial origin (Les et al. 1997). Living in seawater requires peculiar morphological adjustments such as a thin cuticle layer in place of stomata, which allows direct gasses and nutrients diffusion, and a buried rhizome that anchors the plants to the sediment. The rhizomes of one of the largest species (i.e. *Posidonia oceanica*), together with roots and leaf sheath remains form dense mats (Kuo and Den Hartog 2007). Contrary to terrestrial plants, in seagrasses epidermis of the leaf blade is the major site for photosynthesis, containing high concentrations of chloroplasts, mitochondrial, lipid droplets, dictyosomes, endoplasmic reticulum, and microbodies (Kuo and Den Hartog 2007). However, a huge phenotypic diversity exists among seagrass species, which is the result of the acclimation to different environmental conditions.

Seagrasses fulfill a series of different ecosystem services, which are fundamental for the maintenance of benthic ecosystem functions, and whose benefit extend across the terrestrial the marine systems. Seagrass ecosystems are important primary producers and despite they occupy only 0.1% of the ocean surface, they have been estimated to store 27–44 Tg organic carbon (Corg) year–1 globally, which correspond to 10–18% of the total carbon burial in the oceans (Duarte et al., 2005; Fourqurean et al., 2012). Additionally, seagrass beds influence numerous biogeochemical processes trapping suspended particles from the water column through the leaf canopy. Thus, enriched seagrass sediments with particulate organic matter (POM) enhances sediment microbial activity, favouring associated fauna (i.e. macro suspension feeders or epibionts) and sustaining marine food webs (Marbà et al. 2007). They provide nursery habitats for fish and a diversity of marine organisms (e.g. invertebrates) representing one of the most valuable ecosystems on Earth with an estimated value of $1.9 trillion per annum (Costanza et al., 2014; Waycott et al., 2009). The functional role of seagrasses render them as a potential solution to mitigate climate change (Gattuso et al., 2018).

Besides the species diversity of seagrasses is low (<60 species), they are distributed worldwide occupying thousands of kilometers of coastline. Seagrasses are spread across different parts of the globe, colonizing tropical and temperate regions, shallow and deep waters (Short et al., 2007). Most seagrass species exhibit a mixture of reproduction, shifting from clonal growth (i.e. vegetative propagation) to sexual reproduction according to different environmental conditions. Clonal growth results in a hierarchy of different organizational levels (i.e. ramets) that through the vegetative fragmentation allows plants to extend spatially at the expense of

genetic diversity, contrary to sexual reproduction that favours the maintenance of high genetic diversity through seeds production. The ramet, which represents the most elementary level of organization, typically consists in a leaf bundle, a piece of rhizome, and a root bundle (Waycott et al. 2006). Several ramets can form physiologically integrated clusters (the second organization level), that may comprise up to several hundreds of individuals in some genus (e.g. Posidonia). Thus, the sexual individual (i.e. genet) comprises all ramets originated from the same zygote (Waycott et al. 2006). In seagrasses, the sexual reproductive system has evolved unique structural adaptations for marine submerged pollination and seeds development. Once seeds are released, they can float or remain buried into the sediment, and generally, their dispersion occurs through winds and marine currents that transport floating reproductive fragments or fruits for long distances (Orth et al., 2007). Seedling's establishment is one of the most vulnerable life stage of seagrasses that can be affected by different biotic and abiotic agents preventing the germination or altering the suitable condition for growing (McMahon et al., 2014; Pereda-Briones et al., 2020). Since clonal growth is widely diffused among long-lived species (e.g. *Posidonia oceanica*), it is considered a winner strategy allowing species to survive under unfavourable conditions (Honnay and Bossuyt, 2005). The success plants clonality is related to unique ecological advantages including resource and risk and economies of scale among ramets (Ruocco et al., 2021), all issues that may favour their tolerance to environmental changes (Dodd and Douhovnikoff, 2016). However, the rapid occurrence of environmental changes is forcing seagrasses to exceed their tolerance ranges resulting in a worldwide decline of seagrass meadows (Waycott et al., 2009).

Posidonia oceanica

Posidonia oceanica (L.) Delile (fig. 2) is endemic of the Mediterranean Sea, where it forms wide-spread underwater meadows (Telesca et al., 2015). This species is characterized by long persistence genotypes, slow rhizome elongation rates (1–10 cm per year), low genetic diversity, and a mixed reproductive strategy (Arnaud-Haond et al., 2012; Procaccini et al., 2002). Along the Mediterranean coast, *P. oceanica* colonizes rocky and sandy bottoms where it forms extensive monospecific meadows (Procaccini et al. 2002). It ranks amongst the slowest-growing and longest-lived plants, with single genotypes that can persist for millennia through asexual (clonal) reproduction (Arnaud-Haond et al. 2012). Populations of *P. oceanica* can display high phenotypic plasticity as the result of local adaptation. A clear genetic structure was observed among populations distributed along bathymetrical and latitudinal gradients where peculiar environmental conditions occur (e.g. light availability) (Dattolo et

al., 2017; Jahnke et al., 2015). Despite this intrinsic property has favoured the spread of this iconic species across different Mediterranean areas, estimates of *P. oceanica* extent indicate that up to 50% might have been lost in the last 50 years due to rapid environmental changes that forced fragmentation of the remaining meadows increasing their vulnerability to further stress exposure (Marbà et al., 2014).

Figure 2. *P. oceanica* adult plants with associated biodiversity. Photo credit: J. Pazzaglia

Phenotypic plasticity in a changing environment

Populations of marine clonal plants show high genetic and genotypic variability depending on the interplay between clonal and sexual reproduction in addition to latitudinal gradients and geographical regions (Bricker et al., 2011; Jahnke et al., 2016). In the era of environmental changes, understanding how genotypes interact with the surrounding environment is crucial for assessing the degree of phenotypic plasticity. Phenotypic plasticity represents the main response to environmental changes, being a property of organisms to produce different phenotypes (Kelly et al., 2012). Besides controversies arise in defining phenotypic plasticity concept and all related terms, phenotypic variation rising from the interaction of a genotype with environmental variations can be defined by its "phenotypic curve" or "reaction norm", a basic and highly useful concept to understand the interrelations among phenotype, genome, and environment (Woltereck and WOLTERECK, 1909). The slope of this curve describes the degree of plasticity and thus explains how that genotype can be more or less plastic under stressful conditions (Schlichting and Pigliucci, 1998). Since global environmental changes are occurring too fast, likely preventing appropriate plant responses, displaying plastic proprieties

may facilitate seagrass persistence under environmental shifts through their alignment with the new conditions (i.e. acclimation) or by increasing their dispersal abilities (i.e. migration). Seagrasses display a different degree of phenotypic plasticity. Although phenotypic differences are genetically induced, recent evidence suggests that part of these responses is also dependent on epigenetic variations, which include all DNA and chromatin modifications environmentally induced that do not involve changes in the DNA structure (Bossdorf et al., 2008; Douhovnikoff and Dodd, 2015). Therefore, the epigenetic modifications resulting from environmental cues are not isolated events but are closely-linked processes that affect chromatin structure, and hence, DNA organization into the nuclei, regulating and modifying gene expression (Holliday, 2006; Kouzarides, 2007). Epigenetic marks can also be inherited across generations representing a reflection of lifetime stressor exposures of species (i.e. epigenetic memory, Mirbahai and Chipman 2014). In this sense, these mechanisms act as a regulatory machine enhancing stress acclimation responses that can be subsequently selected and fixed for a rapid adaptation (Douhovnikoff and Dodd, 2015; Richards et al., 2017). Investigating how the phenotypic plasticity of seagrasses will affect their responses to different stressors is crucial not only to better understand intrinsic mechanisms that may have favoured their survival in the past but also to apply appropriate conservation managements to ensure their persistence under the ongoing environmental changes. However, it is not even clear how to manage these complex tasks, especially in marine clonal plants, giving rise to the following question: **what are the best methods to approach the study of phenotypic plasticity in seagrasses?**

Seagrasses experiencing single and multiple stresses

Climate change and local pressures associated with human activities are threatening seagrass meadows that are declining worldwide (Waycott et al., 2009). The environmental conditions (i.e. temperature and CO2 concentrations) that seagrasses are currently experiencing are less severe than the conditions experienced by their ancestors (Beer and Koch, 1996). However, seawater warming and other anthropogenic-induced environmental changes are (co-)occurring faster than previously, during the evolutionary history of the lineage, and are accelerating the rates of regression of seagrass populations. Warming represents one of the greatest challenges for the future persistence of seagrasses, as it negatively affects the morphology, physiology, and gene expression (Nguyen et al., 2021). Different studies have been performed analyzing seagrass responses to a single stress factor, mostly to temperature increase (Nguyen et al., 2021). Exposure to high-temperature conditions alters the

performance of the photosynthetic apparatus and accelerates plant respiration, resulting in plant carbon imbalances (Collier and Waycott, 2014; Marín-Guirao et al., 2016). High temperatures also enhance the production of reactive oxygen species (ROS) that tend to accumulate, leading to membrane, proteins, and DNA damages. Since ROS are normally produced by plants as a product of the aerobic metabolism and are generated in several cellular compartments, mainly in chloroplasts and mitochondria, plants are equipped with antioxidant defence systems that involved the expression of specific genes like superoxide dismutase (SOD) and ascorbate peroxidase (APX) acting as ROS scavengers (Reusch et al., 2008; Winters et al., 2011). Temperature increase also promotes flowering in *P. oceanica* adult plants, which can be interpreted as an escape strategy adopted by plants to track for more suitable environmental conditions (Ruiz et al., 2018; Marín-Guirao et al. 2019). While the analysis of seagrass's responses to single stresses is useful to describe the main impacts at different organization levels, information on the potential interaction among different stressors and the effects on marine plants is needed for describing a more realistic scenario. In this context, few studies revealed synergistic interactions when plants are exposed to a combination of stressors (Collier et al., 2011; Ontoria et al., 2019; Touchette and Burkholder, 2000; Villazán et al., 2015). For instance, seagrasses from temperate regions exposed to the co-occurrence of nutrients enrichment (i.e. eutrophication) and warming showed higher deleterious effects on plant functions (photosynthesis, growth, demographic balance) than those caused by the exposure to single stresses, suggesting that the combined effects of warming and eutrophication may further impact plant survival (e.g. *Cymodocea nodosa*, Ontoria et al. 2019). When warming and eutrophication are combined with the increased level of acidification, the overall plant's response is different, improving nutrients assimilation and thus the production of carbon reserves, and buffering the enhanced respiration promoted by temperature (Egea et al., 2018; Nguyen et al., 2021). On the contrary, the early life-stage of tropical species (e.g. *Enhalus acoroides* seedlings) showed weak response to the interactions among warming and eutrophication, suggesting that the responses to multiple stressors are highly species-specific and vary according to the life-stage of the plant. In this context, it is worth underlining that different populations of the same species are locally adapted to environmental conditions, and respond differently when they are exposed to common stressful conditions (Dattolo et al., 2017; Hämmerli and Reusch, 2002; Marín-Guirao et al., 2016). Comparative analysis of different seagrass populations revealed a huge variability of morphological and physiological responses to stressors (carbon balance, carbohydrates content, growth, and mortality), that could be at the basis of a geographical heterogeneity of

the potential negative impacts on the functions and services offered by seagrass meadows (Marín-Guirao et al., 2018). All these early evidences open the following question: are the **local pressures and environmental conditions experienced by seagrasses in their home environment influencing their response to single and multiple stresses, modifying thus, their resilience capacity to global changes?**

Gene expression approaches for studying seagrass stress responses

The assessment of gene expression represents a fundamental tool to describe morphological and physiological responses to environmental changes in non-model species. Different approaches include the selection of target genes (e.g. RT-qPCR) and transcriptomic analysis which can be carried out using different techniques among which the most dominant is RNAseq, an high-throughput sequencing technology (Lowe et al., 2017). In seagrasses, these approaches were applied mainly to investigate gene expression responses of locally-adapted plants exposed to single stresses (Bergmann et al., 2010; Dattolo et al., 2017; Franssen et al., 2011, 2014; Gu et al., 2012; Marín-Guirao et al., 2016, 2017; Reusch et al., 2008; Winters et al., 2011). The transcriptomic response to multiple stressors remains unexplored, and only few studies integrate gene expression analysis with morphological and physiological measurements (Ceccherelli et al., 2018; Ravaglioli et al., 2017; Ruocco et al., 2019). Ravaglioli and colleagues (2017) observed that *P. oceanica* plants growing close to CO_2 vents showed different responses when exposed to nutrient loadings. In fact, nutrients additions at low pH levels induced the overexpression of nitrate transporters while reduced the expression of antioxidant genes. However, since the exposure to both acidification and nutrients additions did not exuberate the impacts of plants performances, this interaction was not defined as synergistic. By contrast, Ceccherelli et al. (2018) observed synergistic effects of nutrients additions and burial treatment on *P. oceanica* adult plants. Under such conditions, shoot loss increased about 60% and different regulation of stress-related genes was suggested to function as anticipatory of the immediate seagrass collapse. The absence of interaction among nutrients enrichment and herbivory was observed by Ruocco et al. (2018) in a field experiment, where the only presence of high herbivore pressure induced the greatest molecular response. Additionally, differential regulation of genes involved in carbon fixation and N assimilation was also observed in response to pulse nutrient loadings and chronic exposure. These studies provided new insights into molecular signatures that regulate physiological and morphological responses of seagrasses to multiple stresses. However, most of them investigated only selected genes and did not consider the role that local acclimation may play in differentiating the whole

transcriptomic response to multiple stresses, giving rise to the following question: **how seagrasses respond at transcriptomic level when exposed to multiple stresses?**

The evolution of epigenetic regulation under environmental stressors

The term epigenetics was first coined in the context of developmental biology playing a crucial role in the differentiation and maintenance of specialized cells (Waddington, 1940). The analysis of epigenetics is currently incorporated in ecological studies to investigate phenotypic plasticity changes in response to environmental disturbances (Bossdorf et al., 2008). Part of the organism's epigenetic landscape is genetically determined and depends on the activity of enzymes (e.g. MET1, dnmt1 and acetyltransferases) and proteins (e.g. SET-domains and Trithorax groups) encoded by specific genes that catalyzed epigenetic modifications (Suzuki et al., 2017). Together with chromatin conformational modification, DNA methylation is one of the most documented epigenetic marks in both plants and animals and consists in the addition of a methyl group to the C-5 position of the cytosine (Li et al., 2018). Particularly, DNA methylation can also be modulated by the surrounding environment due to the influence of biotic (e.g pathogens and microbial communities) and abiotic factors (e.g temperature, drought, and chemicals; Bossdorf et al. 2008; Feil and Fraga 2012). Variation in DNA methylation plays a fundamental role in many biological processes, regulating the gene expression, growth, development, and protecting from environmental stresses (Kumar, 2017; Zilberman et al., 2006). Stress-induced DNA methylation can be reversible and DNA returns to the basal levels once the stress is relieved, which can be considered as the first response of an organism under stress promoting short-term acclimation. When DNA methylation occurs in specific regions, it can be fixed and inherited across generations to function as a "stress memory" (Chinnusamy and Zhu, 2009). Different components are involved in the *de novo* DNA methylation, maintenance, and demethylation (i.e. writers, readers and erasers) and a "cross-talk" between DNA and histone marks also exists underling the complexity of epigenetic regulation (Holliday, 2006; Kouzarides, 2007; Nicholson et al., 2015). Recent findings from terrestrial studies, revealed that DNA methylation levels change in response to stressful conditions and higher levels of methylation were related to heat-sensitive genotypes compared with the more tolerant ones (Gao et al., 2014). By contrast, other evidence showed more DNA demethylation events in the heat-tolerant genotype concerning the sensitive genotype (Akhter et al., 2021). In seagrasses, epigenetic mechanisms remain unexplored, and only recently new approaches have been performed for a restricted number of species (*Z. marina*, Jueterbock et al. 2019; *P. oceanica*,

Ruocco et al. 2021; *P. oceanica* and *C. nodosa*, Entrambasaguas et al. 2021). What emerge from these recently analysis studies is that several epigenetic mechanisms can modulate gene expression patterns in seagrasses depending on plant's origins, and that DNA methylation regulates the cellular status of plants and the possibility to memorize stress events with important implications for regulating seagrass plasticity under changing conditions. All these findings suggest that understanding the evolution of DNA methylation in plants under stress could be critical for better exploring not only the potential role of epigenetics in driving gene expression regulation but also to investigate the dynamic of epigenetic marks behind the appearance of different phenotypic responses. Thus, the further question to be addressed is the following: **Is DNA-methylation a dynamic process in marine plants influenced by the plant's origin and the timing of the stress occurrence?**

Thermal priming to assist marine plants in a changing environment

Under the ongoing environmental changes, the existing conservation tools such as reducing exploitation and restoring degraded habitats, are effective only to some degree as they can be compromised by the rapidly changing ocean conditions (Hobbs, 2013; Seastedt et al., 2008). Novel applications, like *assisted evolution* approaches, are now available to implement the possibility to survive environmental stresses. In fact, this term refers to all active human interventions on organisms that accelerate the rate of natural evolutionary processes conferring higher resilience to environmental changes (Jones and Monaco, 2009). Building more resistant genotypes can be performed using genome editing manipulations (i.e. CRISPR) or through less invasive techniques such as the selection of more tolerant genotypes or *priming* approaches (Jisha et al., 2013). These lasts are value-added techniques widely applied among land plants species of commercial interests (Wang et al., 2017). In plant stress biology, priming or hardening strategies can be applied to individuals through their exposure to a first (mild-)stress event, which confers a *priming status*, improving their performance under subsequent (future) stress conditions (*triggering stimulus*, Fig. 3, Hilker et al. 2016). These processes are based on the capability of plants to store information of the past stress event, which is known as stress memory. Recently, new findings revealed that stress memory is closely associated with epigenetic regulation (Friedrich et al., 2019; Lämke and Bäurle, 2017). Stress-induced epigenetic modifications during the priming stimulus can modify the expression of stress-responsive genes that can be maintained mitigating the negative impacts of the re-occurring stress event (Chang et al., 2020). This kind of approach is only recently applied in seagrasses in one study performed on adult plants of tropical species (*Posidonia*

australis and *Zostera muelleri*, Nguyen et al. 2020). Since this study provided new insights on the possibility to confer stress memory in seagrasses, applying this approach to early–life stages of marine plants could be fundamental for enhancing resilience capacity in younger seedlings. Furthermore, this approach could be essential for the possibility of inducing a long-last stress memory during the adult stage, enhancing the survival chance of marine plants in a changing environment.

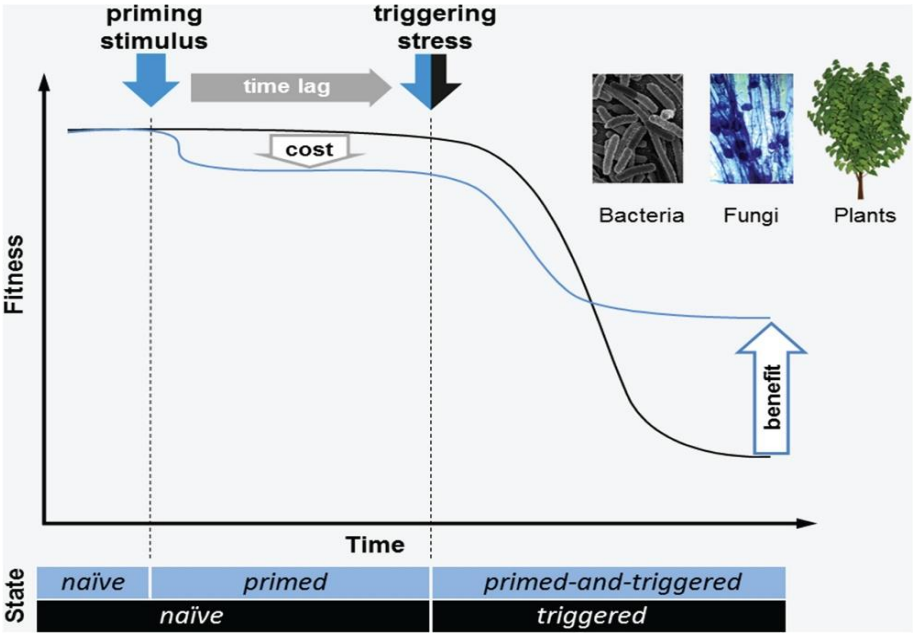

Figure 3. Schematic representation of the relationship between fitness and priming of a stress response by bacteria, fungi or plants over time. An organism which have experienced a prming stimulus shows less severe response to the triggering stimulus in respect to non-primed organism (from Hilker et al. 2016).

Implications on seagrass restoration strategies

The analysis of seagrass responses to single and multiple stresses, the role that local acclimation/adaptation may have on seagrass responses to stress, and the possibility to induce a priming status during early-life stages in seagrasses are all crucial aspects to take into consideration for developing improved and successful restoration plans. Restoring degraded seagrass meadows required a clear and delineated plan, which starts from the analysis of the genetic structure of natural populations in order to select the appropriate donor site. Since marine plants reproduce mostly via clonal propagation, an initial analysis of genetic and genotypic diversity ensures the selection of more diverse populations. This is fundamental as

seagrass studies had revealed that high genetic diversity enhances stress tolerance, and populations showing high genotypic diversity are more likely to include resistant genotypes with wider tolerance ranges (Ehlers et al., 2008; Hughes and Stachowicz, 2004; Jahnke et al., 2015). However, these preliminary considerations and all consequences that can derive from the application of inappropriate strategies (e.g genetic pollution) are not always considered in seagrass restoration. Since restoration and conservation programs are regulated by national laws and international conventions, a central role for the elaboration of a well-organized plan requires a constant dialogue among scientists, stakeholders, and policymakers in order to identify new opportunities limiting potential risks for the environment (Farber et al., 2006). Thus, a comprehensive view of the considerations necessary to perform seagrass restoration plans is determinant to merge all scientific, ethical, and legal knowledge for delineating clear restoration actions.

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AIMS OF THE THESIS

My doctoral thesis is a collection of scientific papers that analyzed the responses of different life stages of the iconic seagrass species of the Mediterranean Sea, *Posidonia oceanica*, (i.e. adult and seedlings) to stresses. The general objective of the thesis was to explore the resilience capacity of *P. oceanica* to single and multiple stresses. In detail, the effect of different abiotic stressors and their combination (temperature, nutrients and temperature + nutrients) was assessed in adult plants of *P. oceanica* species, while the effect of the priming treatment induced by only temperature (thermo-priming) was assessed in *P. oceanica* seedlings. Two main experiments have been performed using the indoor mesocosm system offered by Stazione Zoologica Anton Dohrn in Naples and the indoor mesocosms facility of the Oceanographic Center of Murcia (Spain). Plant responses were assessed at different levels of organization, considering morphological, physiological and transcriptomic responses focusing also on epigenetic modifications that can be linked to the phenotypic responses observed. The thesis is structured in six chapters each of them includes original papers with specific aims.

In **Chapter I**, I reviewed the concept of phenotypic plasticity and all related terms (acclimation and adaptation) to better clarify their meaning and role in the face of environmental changes. This allowed me to explore the most recent literature on seagrass responses to environmental stressors to analyse and describe pros and cons of different techniques and approaches utilized. Thus, the most suitable approaches to explore the role of plastic responses in seagrasses under global climate changes and local environmental stressors have been suggest.

In **Chapter II,** I evaluated if *P. oceanica* plants undergoing chronic cultural eutrophication (Eu, eutrophic site) and plants growing in relatively pristine waters (Ol, oligotrophic site) were more (or less) sensitive to heat stress, nutrient load and the combination of both stressors. Plants performance were assessed analysing morphological and physiological traits.

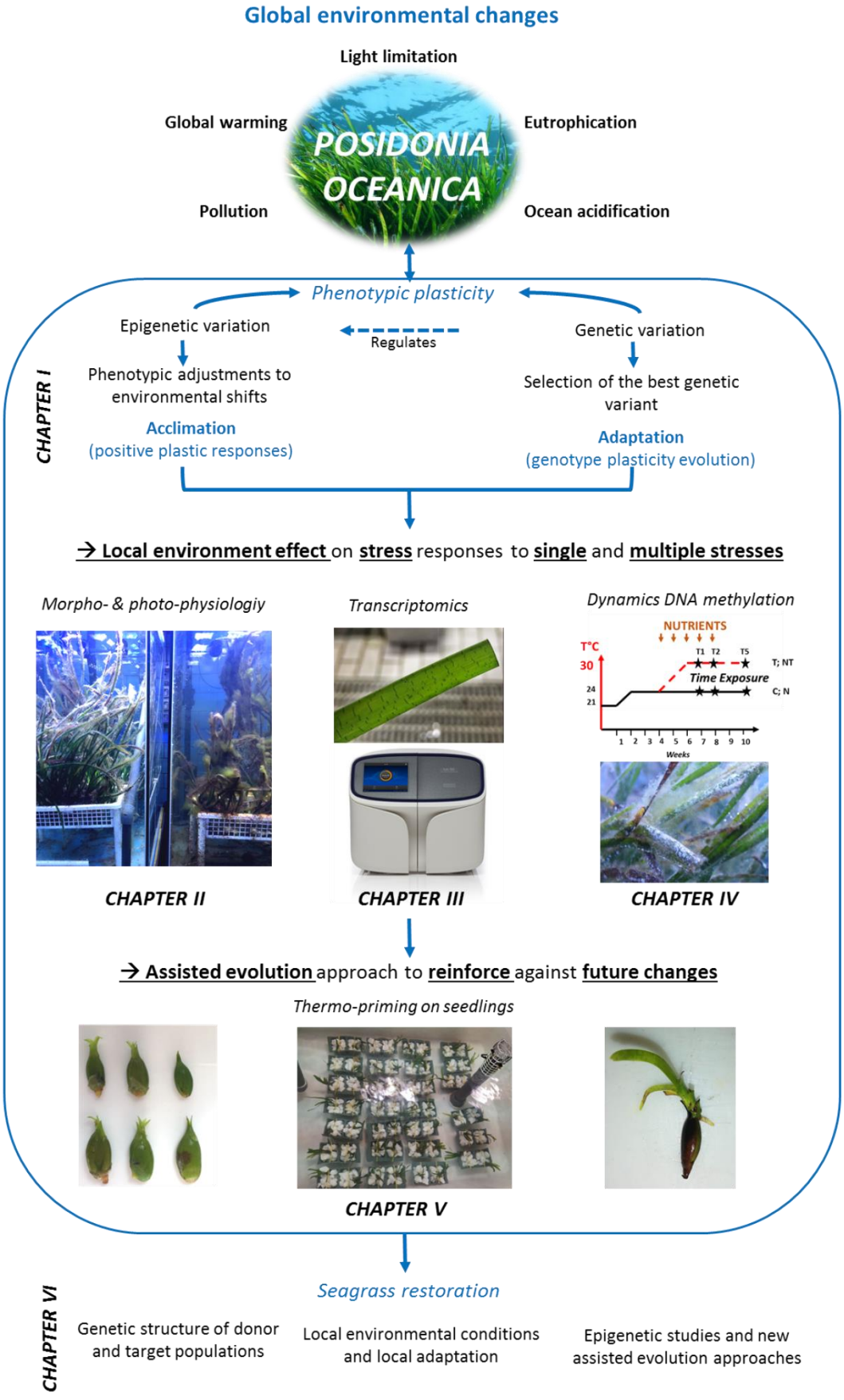
In **Chapter III,** starting from previous physiological assessments, I aimed to investigate on transcriptome rearrangements occurring in different organs (leaf and shoot-apical meristem) of *P. oceanica* plants with a different history of nutrient loads and exposed to single and multiple stressors. I first screened transcriptomic profiles of both plants and organs to explore differential genes regulation according to plant's origin and the exposure to single and multiple stresses. Then, I focused on biological processes activated under different experimental conditions exploring the potential of epigenetics in regulating stress responses

to stresses. I assessed the dynamics DNA methylation of selected genes in **Chapter IV**, where key genes involved in the DNA methylation in plants were investigated during the exposure to stressful conditions in both Ol and Eu plants, from the initial exposure to single and multiple stressors to the end of the experiment.

In **Chapter V**, I aimed to explore if primed seedlings of *P. oceanica* were more tolerant under the short-term re-occurrence of a thermal-stress event. To achieve that, the thermo-priming stimulus was induced on *P. oceanica* seedlings, and the induction of the priming status was assessed by analyzing the photo-physiological and growth performance of primed and non-primed seedlings, as well as their gene expression responses of a selected set of genes (i.e. stress-, photosynthesis- and epigenetics-related genes) during their exposure to extreme high temperature.

In **Chapter VI**, I explained the relevance of seagrass studies related to phenotypic plasticity, local adaptation, exposure to multiple stressors and the application of novel techniques knowns as *assisted evolution* approaches that on the success of seagrass restoration managements.

GRAPHICAL THESIS OVERVIEW



ORIGINAL PUBBLICATIONS

CHAPTER I

(Paper I)

Jessica Pazzaglia, Thorsten B. H. Reusch, Antonio Terlizzi, Lázaro Marín- Guirao and Gabriele Procaccini. Phenotypic plasticity under rapid global changes: the intrinsic force for future seagrasses survival.

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REVIEWS AND SYNTHESES

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Phenotypic plasticity under rapid global changes: The intrinsic force for future seagrasses survival

Jessica Pazzaglia[1,2] | Thorsten B. H. Reusch[3] | Antonio Terlizzi[2,4] | Lázaro Marín-Guirao[1,5] | Gabriele Procaccini[1]

[1]Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy

[2]Department of Life Sciences, University of Trieste, Trieste, Italy

[3]Marine Evolutionary Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

[4]Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples, Italy

[5]Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, Murcia, Spain

Correspondence
Gabriele Procaccini and Lázaro Marín-Guirao, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy.
Emails: gpro@szn.it; maringuirao@gmail.com

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Abstract

Coastal oceans are particularly affected by rapid and extreme environmental changes with dramatic consequences for the entire ecosystem. Seagrasses are key ecosystem engineering or foundation species supporting diverse and productive ecosystems along the coastline that are particularly susceptible to fast environmental changes. In this context, the analysis of phenotypic plasticity could reveal important insights into seagrasses persistence, as it represents an individual property that allows species' phenotypes to accommodate and react to fast environmental changes and stress. Many studies have provided different definitions of plasticity and related processes (acclimation and adaptation) resulting in a variety of associated terminology. Here, we review different ways to define phenotypic plasticity with particular reference to seagrass responses to single and multiple stressors. We relate plasticity to the shape of reaction norms, resulting from genotype by environment interactions, and examine its role in the presence of environmental shifts. The potential role of genetic and epigenetic changes in underlying seagrasses plasticity in face of environmental changes is also discussed. Different approaches aimed to assess local acclimation and adaptation in seagrasses are explored, explaining strengths and weaknesses based on the main results obtained from the most recent literature. We conclude that the implemented experimental approaches, whether performed with controlled or field experiments, provide new insights to explore the basis of plasticity in seagrasses. However, an improvement of molecular analysis and the application of multi-factorial experiments are required to better explore genetic and epigenetic adjustments to rapid environmental shifts. These considerations revealed the potential for selecting the best phenotypes to promote assisted evolution with fundamental implications on restoration and preservation efforts.

KEYWORDS

acclimation, adaptation, genetic diversity, global changes, phenotypic plasticity, reaction norm, seagrasses

1 | INTRODUCTION

In the context of global environmental changes, studying the ability of species to cope with environmental shifts is fundamental for predicting their fate. The possibility to rapidly respond to environmental changes is exacerbated by the occurrences of different human pressures, which can have critical effects at the ecosystem level, forcing ecological systems into an alternative stable state (Beisner et al., 2003; Harley et al., 2006). The marine coastline is particularly vulnerable to environmental disturbances, such as sea level rise, acidification, increase of temperature, and intensity of heat waves events and storms (IPCC, 2019). Additionally, climate-derived environmental changes and their consequences on habitats can potentially be intensified by regional anthropogenic pressures, including overfishing and nutrient pollution among others (Chaturvedi et al., 2017; Zaneveld et al., 2016). The resulted exposure to multiple stressors forces coastal marine environments to drastic changes as a consequence of the alteration of species biodiversity, distribution, and ecosystem functioning (Gunderson et al., 2016). Importantly, rapid and extreme environmental changes strongly affect the performance of foundation species (i.e., species with a structural role within an ecosystem) altering the resilience capacity (i.e., the ability to recover and continue functioning after a disturbance) of the entire ecosystem (Thrush et al., 2009). The degree of the ecosystem transition into different states strongly depends upon the foundation species' tolerance and resistance to environmental variability or disturbances (Scheffer & Carpenter, 2003). These abilities, in turn, rely on different physiological and molecular mechanisms that drive individual or population responses in the presence of relatively rapid environmental changes (Sih et al., 2011; Summers et al., 2012; York et al., 2013). One of the main concerns of rapid shifts is that these changes do not allow species to react swiftly enough in order to cope with and survive in the new more stressful environment. Analyzing how species traits change with the environment becomes thus of crucial importance.

Among higher plants, seagrasses are the only group that has returned to a completely submerged marine life (Shepherd et al., 1989). Although fossil evidence for marine plants is limited, some records indicate that seagrass' ancestors likely evolved more than 100 Ma ago in the Cretaceous Period, whereas modern seagrass families beginning to diverge more than 70 Ma ago (Hedges & Kumar, 2009). Seagrasses are a polyphyletic group of monocotyledons, belonging to the order of Alismatales which includes 11 families of aquatic–freshwater species and four families that are fully marine (Posidoniaceae, Zosteraceae, Hydrocharitaceae, and Cymodoceaceae; Les et al., 1997). Among the hundreds of thousand species of angiosperms today, there are currently only 12 genera and ca. 65 species of seagrasses (Chase et al., 2016). Seagrasses are widely recognized as key ecosystem engineering or foundation species, supporting diverse and productive ecosystems in the photic zone of the marine coastline around all the continents except Antarctica (Bos et al., 2007). These marine plants fulfill a series of important ecosystem services worldwide, including oxygen production and CO_2 sequestration (Champenois & Borges, 2019; Duarte & Krause-Jensen, 2017). Although they occupy only 0.1% of the ocean surface, it is estimated that seagrasses can store 27–44 Tg organic carbon (C_{org}) year^{-1} globally, corresponding to the 10–18% of the total carbon stock in the oceans (Fourqurean et al., 2012). As in terrestrial plants, where clonal species are the most abundant members among perennial grasslands (Klimeš et al., 1997), seagrasses are also mostly herbaceous even if stiff and hard stems and rhizomes occur in some families (e.g., Posidoniaceae). A huge variability exists among seagrasses, ranging from species characterized by short-lived shoots, with a quicker cycle of growth and death of shoots (i.e., Cymodoceaceae), to slow-growing and long-lived plants (i.e., Posidoniaceae) (Larkum et al., 2006). Seagrasses often exhibit a mix of sexual and clonal reproduction that has been a crucial aspect of their evolutionary history. Seagrass meadows show high-genetic variability depending on the interplay between sexual reproduction and clonal growth and by latitudinal and geographical regions (Bricker et al., 2011; Jahnke et al., 2016). As for terrestrial plants, seed dispersal is critical for population distribution, contributing to the maintenance of genetic diversity and the shaping of spatial genetic structure (Kendrick et al., 2012). Theory predicts that the lack of genetic variation leads to the accumulation of deleterious mutations that negatively affect plants' persistence under environmental shifts (Silvertown, 2008). However, sexual reproduction has a drawback, since it is a costly energetic process that requires resource allocation and depends on surrounding conditions (Diaz-Almela et al., 2006).

In seagrasses, vegetative (= clonal) reproduction occurs through rhizome extension and branching in space, leading to the formation of extensive underwater meadows (Larkum et al., 2006). The success of clonal propagation is related to different and unique ecological advantages, such as resource and risk sharing, and economies of scale among ramets within a genotype (Dodd & Douhovnikoff, 2016; Ruocco et al., 2020). Thus, clonal plants appear to be more resistant than plants lacking clonal reproduction and are likely more buffered against habitat deterioration (Pennings & Callaway, 2000). This capacity has allowed clonal plants to colonize diverse terrestrial and marine ecosystems, and include many of the most important crops and invasive plants, and some of the earth's largest and oldest plant species (Honnay & Bossuyt, 2005; Pan & Price, 2002).

Seagrass meadows are particularly susceptible to environmental changes. They are exposed to the effects of single and multiple stressors, due to local and global threats, including changes of environmental parameters (i.e., light and salinity levels) and nutrient condition of the water column (Moreno-Marín et al., 2018; Pereda-Briones et al., 2019; Salo & Pedersen, 2014). The intensifying destruction of the marine environment is promoting a huge decline of seagrass meadows with knock-on effects for the entire coastal benthic ecosystem (Boudouresque et al., 2009; Gacia et al., 1999). A complete analysis performed by Waycott et al. (2009) revealed that seagrass loss rates have increased to 7% year^{-1} since 1990, placing seagrasses among the most threatened ecosystems on earth.

How marine clonal plants with low-genetic recombination have been able to survive to past environmental changes and which are the main implications for current and future environmental shifts are still to be clarified and becomes mandatory for assessing their fate and adopting proactive management actions. An aspect that has to be considered is the longevity that characterizes genets in some species (Arnaud-Haond et al., 2012; Ruggiero et al., 2002). This points to an intrinsic ability of single genotypes, including mostly clonal populations, to survive and persist across environmental changes (Arnaud-Haond et al., 2012; Ruggiero et al., 2002). Plastic responses represent an individual property that allow genotypes to accommodate and react to fast environmental changes (Donelson et al., 2019). According to the climate variability hypothesis, seagrass populations living in more dynamic environments and/or at their tolerance limits (e.g., lagoons characterized by unstable salinity or temperature conditions) may better perform in face of environmental changes (Ashander et al., 2016; Botero et al., 2015; Chevin & Hoffmann, 2017; Tomasello et al., 2009). Thus, organisms growing in highly variable environments are more plastic (tolerant) than organisms from more stable environments (Tuya et al., 2019).

Although environmental cues trigger phenotypic differences, the ability to respond is genetically based. Recent evidence suggests that part of this capacity is also due to epigenetic variations (Douhovnikoff & Dodd, 2015) or to somatic mutations that have been shown to segregate among ramets (Yu et al., 2020; see Box 1; Figure 1). If the latter is true, plasticity may interact with "hard-wired" genetic changes that thus far have been neglected.

Here, we focus on marine angiosperms (aka seagrasses) and describe the concept of phenotypic plasticity and its role in the face of rapid environmental changes as a potential way to overcome future environmental shifts. To do that, we focussed in particular on the most recent literature on seagrass responses to environmental stressors that has been critically analyzed also underlining the pros and cons of the technical approaches utilized. As an outlook, we suggest the most suitable approaches to analyze the role of plastic responses in seagrasses under global climate changes and local environmental stressors. A glossary of more specific terms utilized in this review is given in Table S1.

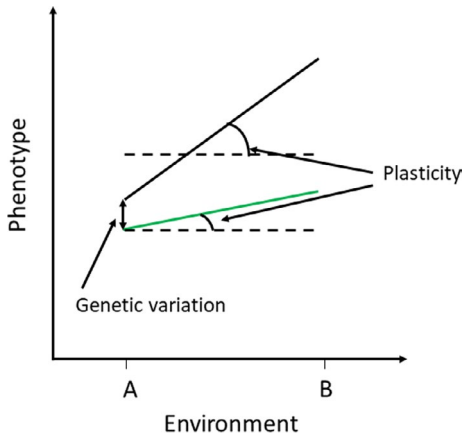

FIGURE 1 Schematic representation of plasticity resulting from the interaction of linear reaction norms and environments (line slopes). Black and green solid lines refer to different genotypes characterized by genetic variation (line heights); dashed lines refer to the mean phenotypic value across environments (A and B) (from Schlichting & Pigliucci, 1998, modified)

2 | THE CONCEPT OF *PHENOTYPIC PLASTICITY*

The concept of "phenotypic plasticity" was applied for the first time by Nilsson-Ehle (1914) in a case study of plant' phenotypic changes resulting from different environments. Since then, the term itself evolved according to the development of new studies that document changes in environmental conditions, moving the scientific interest on organismal responses to environmental shifts. Not surprisingly, a broad literature defined concepts related to plasticity in plants during recent years. Different ways to define plasticity have been utilized, along with a variety of associated terms and more specific terminology (Kelly et al., 2012). Plasticity was also described with philosophical significance, as the 'plastic nature' of organisms or 'plastic properties' inherent to life. According to West-Eberhard (1989), phenotypic plasticity can be defined as the ability of an organism to produce different phenotypes when it is exposed to different biotic and abiotic environmental conditions. In other words, a single genotype has the possibility to adjust its response to environmental changes, modifying its phenotypic state in terms of chemistry, physiology, morphology, and gene expression. The exposure to environmental variations enhances the development of different phenotypes (i.e. phenotypic plasticity) within and among individuals of the same population. The window of phenotype changes of a genotype along environmental variations defines its "phenotypic curve" or "reaction norm", a basic and highly useful concept to understand the interrelations among phenotype, genome, and environment (Woltereck & Woltereck, 1909). Importantly, the reaction norm itself is under genetic control (Schlichting & Pigliucci, 1998) and can be defined as a function that relates the environment with the phenotype resulting from a particular genotype across an environmental gradient. This function can take any shape, and for continuously distributed traits, such as many physiological, morphological, and life-history traits, it is typically visualized as a line or curve on a plot of the environment *vs* the phenotype (Gabriel & Lynch, 1992; Schlichting & Pigliucci, 1998). Evidently, deciphering more complex threshold/saturation type responses requires more than two measurements of the environment. Being a property of the reaction norm of single genotypes, plasticity is described by comparing the slope of the phenotype curve with the mean phenotypic value resulting from the external conditions. Consequently, the greater the slope of the curve, the more it deviates from the mean phenotypic value and the more the phenotype is plastic (Figure 1). Assessing the genetic basis of the reaction norm slope (i.e., phenotypic plasticity) is fundamental to explore the genotype–environment relation. In this regard, the genetic variation of a genotype is displayed by the "height" of the reaction norm plot. Thus, genotypes differing in terms of heights

BOX 1 Linking genetics to epigenetics

The term *epigenetics* refers to all DNA and chromatin changes that can be inherited by the next generations and that do not involve changes in the DNA sequence (Bossdorf et al., 2008). These intrinsic mechanisms include methylation of cytosine residues, chromatin structure changes through chemical modifications of histone proteins, and a possible "crosstalk" between modifications at different levels (Holliday, 2006; Kouzarides, 2007). Overall, these modifications can be environmentally induced, promoting phenotypic plasticity through gene regulation and its heritability (*epigenetic plasticity*) (Feil & Fraga, 2012; Verhoeven et al., 2016). Since epigenetic marks can promote down- and up-regulation of genes that can also be inherited to next generations, epigenetic alterations could be referred to as a regulatory machine, firstly for the acclimation response, and then through the fixation of that "epigenetic acclimation", for a rapid adaptation (Dodd & Douhovnikoff, 2016; Richards et al., 2017). In this context, epigenetics could be the link between genetic diversity, phenotypic plasticity and the environment (Zhang et al., 2013). Another relevant issue in epigenetic mechanisms is related to the inheritance of histone modifications, as the possibility in plants to "remember" past stress events. This is already demonstrated for terrestrial clonal plants (Latzel et al., 2016; Verhoeven et al., 2016). Therefore, one of the most important epigenetic contributions in individual plasticity is the possibility to pass specific environmental information to the next generations and to regulate fast responses to ongoing environmental perturbations. This "learning process" could contribute to the accumulation of memory mechanisms, altering plant–environmental interactions in future generations. The epigenetic memory as plastic behavior seems to be partially responsible also for rapid phenotypic adjustments following fast environmental changes (Dodd & Douhovnikoff, 2016). For instance, *Arabidopsis thaliana* showed that a single genotype can display different epigenetic states, meaning that probably a widely epigenetic variation takes place between different ramets as a result not only of environmental conditions but also of the connection that exists among phenotypes, environment, and progenitors (Johannes et al., 2009). Recently, evidence about epigenetic mosaics within a genotype has been also shown in marine clonal plants, where epigenetics has been suggested as a key molecular mechanism enhancing phenotypic plasticity conferring thermal tolerance and the evolution of (pre-) adaptive strategies (Marín-Guirao et al., 2017, 2019). In particular, a case study performed on a clonal meadow of *Zostera marina* described epigenetics as the potential advantage to enhance beneficial phenotypic variations under environmental stressors without costs of clonal reproduction (Jueterbock et al., 2020). In seagrasses, new evidence pointed out the appearance of more tolerant phenotypes to contrast fast environmental shifts as a mechanism regulated by epigenetic rearrangement that occurs through genetic regulation (Jueterbock et al., 2020). Thus, the activation/inactivation of this regulatory machinery is strongly dependent on the environment triggering the existence of a stress memory with important implications for seagrasses exposed to future factors of stress (Nguyen et al., 2020). In *Posidonia oceanica*, differences in global DNA methylation has also been found among leaf tissue of different age in the same shoot, highlighting its role in the response to changes in environmental conditions (i.e., light availability and water temperature; Ruocco, De Luca et al., 2019; Ruocco, Marín-Guirao et al., 2019). Additionally, an in silico gene–body–methylation approach showed that house-keeping genes are hyper-methylated, while genes with more inducible expression are widely hypo-methylated (Entrambasaguas et al., *under review*).

(genetic variation) and slopes (degree of plasticity) of their reaction norms are more likely to evolve (see next paragraph, Pigliucci, 2001; Schlichting & Pigliucci, 1998). Another important issue is that the shape of the reaction norm can be the result of a different organismal response along the biological hierarchy. Responses at the gene expression level, for example, may be plastic, that is, exhibit a strong slope when stress genes (HSPs) are activated (e.g., in seagrasses: Bergmann et al., 2010; Traboni et al., 2018). At the higher organizational level, however, this results in the maintenance or resilience of organismal function, for example photosynthesis, so essentially in a flat reaction norm with increasing stress (as in a generalist response) (Reusch, 2014).

In general, the analysis of processes involved in phenotypic plasticity and the possibility that such plastic responses might or might not be adaptive is complex. Currently, phenotypic plasticity is not unequivocally defined in seagrasses, and the approaches to assess the adaptive potential of phenotypic plasticity have not been standardized. Long-life cycles and slow growth, which characterize most of the seagrass species, impede manipulative experiments and trans-generation assessments. Additionally, advanced genetic tools, such as recombinant technologies (e.g., CRISP), are currently unavailable for all seagrass species and a complete sequenced genome is only available for two species, that is, *Zostera marina* (Olsen et al., 2016) and *Z. muelleri* (Lee et al., 2016).

3 | THE GENETIC COMPONENT OF PHENOTYPIC PLASTICITY

Seagrass populations can be more resilient or resistant to environmental changes as the expression of individual or population plasticity. In general, the process can be addressed at two different but interconnected levels: genetic diversity displayed among genets, and number and distribution of genets at a particular location that can

be summarized as genotypic diversity. Genetic diversity depends on the allelic variation and heterozygosity resulting from the sexual reproduction and the immigration of new genetic variants from other populations, whereas the genotypic diversity depends on the size structure and persistence of clones (or genets, consisting of many ramets) at a location, through vegetative propagation (i.e., clonal diversity) (Procaccini et al., 2007). Experimental studies have demonstrated that genetic and genotypic diversity of populations are a good proxy of population resilience and plasticity to changes (Ehlers et al., 2008; Hughes et al., 2008; Jahnke et al., 2015) since population reaction norm results in a broad sense from the amplitude of the reaction norms of single genotypes.

Since seagrass genotypes can persist for a long time (>>100 years) as in long-living species such as *P. oceanica* (Arnaud-Haond et al., 2012) and *Z. marina* (Olsen et al., 2016), the genetic diversity among the genet level is maintained by the interplay between sexual reproduction and clonal growth (Arnaud-Haond et al., 2020; Kendrick et al., 2012). This results in the formation of submerged meadows ranging from almost monoclonal to highly genetic diverse (e.g., *P. oceanica*: Arnaud-Haond et al., 2012; *Z. marina*; Ferber et al., 2008; Figure 2).

Despite the importance of population size, low-genetic variation has been found as a winner strategy in different plant species (e.g., clonal invasive species; Lambertini et al., 2010; Li et al., 2006), particularly in long-lived ones (e.g., *P. oceanica*; Arnaud-Haond et al., 2012; Ruggiero et al., 2002; *Z. marina*: Reusch et al., 1999). Population size (the level of genetic variation within populations) is considered a major constrain for the adaptation of natural populations to environmental changes, as the higher is the number of genotypes, the higher is the possibility that some of them can be positively selected (Bell & Gonzalez, 2009; Matesanz & Valladares, 2014). Effective population size can be decreased by the selection of plastic genotypes (adaptive phenotypes), in presence of rapid environmental changes, through changes of allele frequencies of specific loci and globally on the genome (Grenier et al., 2016).

Any adaptation to a new environment at population level is a process resulting from the natural selection of better-suited genotypes across generations, changing the genetic composition of populations.

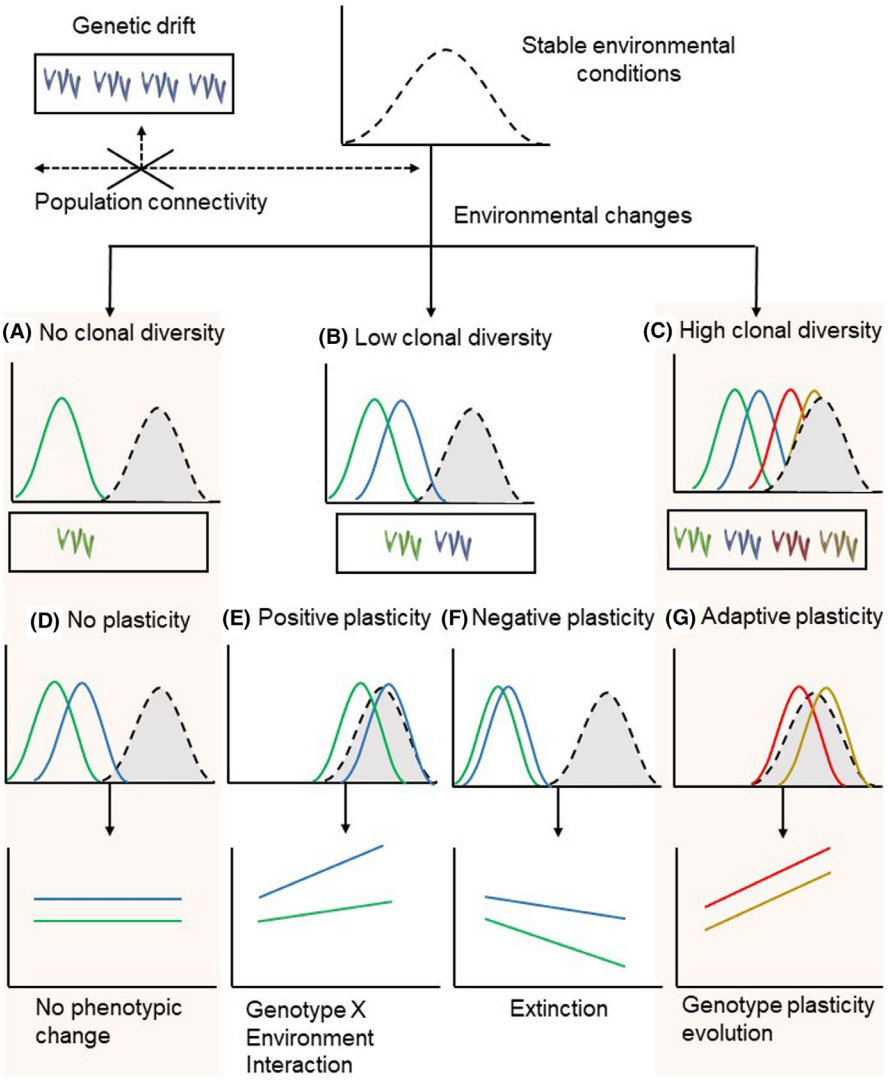

FIGURE 2 The role of genetic diversity and its effect on phenotypic plasticity in face of prompt environmental changes (see the text for more details)

This process can be slow, inducing an initial decline in population fitness and size and experiencing a subsequent increase once adaptive genotypes exhibit appropriate phenotypes (Hamilton & Miller, 2016; Valladares et al., 2014). The selection of these genotypes leads to changes in the frequency of alleles that confer greater fitness under the new altered conditions, promoting adaptive evolution (Grether, 2005). For this reason, the slope or shape of reaction norms is continuously evolving in most cases, rendering the mutually exclusive distinction of plasticity *vs*. adaptation meaningless (Schlichting & Pigliucci, 1998). The occurrence of somatic DNA mutations in single individuals can provide a readily available extra source of variation that was previously not considered and that can be maintained via clonal growth in long-living genotypes (Whitham & Slobodchikoff, 1981). The role of somatic mutations in seagrasses was initially assessed by Reusch and Boström (2011) (Reusch & Boström, 2011). Recently, high somatic genetic variation was detected among ramets of a single genet of *Z. marina* plants (Yu et al., 2020).

Similar to genotypes within a population, populations from contrasting environmental conditions also showed different plasticity which is indicative of local adaptation (Sánchez-Gómez et al., 2011). Thus, the existence of populations locally adapted to natural environments showing more adaptive genotype curves (i.e., reaction norms) results in population divergence in plasticity patterns representing an evolutionary potential for the species. In this sense, plasticity has the potential to drive population divergence as the environment changes (Pfennig et al., 2010). The capability of an individual to adapt and the timing of evolutionary adaptation is intrinsically related to its plasticity.

Even in species with high clonal persistence, such as *P. oceanica*, stochastic events of sexual reproduction and migration of genetic variants through populations via sexual propagules seem to suffice to promote genetic rearrangements and enhance selectively advantageous genetic variations (Arnaud-Haond et al., 2014; Jahnke et al., 2015; Kendrick et al., 2012; Procaccini et al., 2007). The connectivity among populations depends on the existence of geographic or oceanographic barriers and the different features of dispersal vectors, that is, sexual or clonal propagules (e.g., Jahnke et al., 2016; McMahon et al., 2014; Serra et al., 2010). High-resolution genetic data for the seagrass *Thalassia testudinum* along the western tropical Atlantic coasts revealed high-genetic diversity as the result of high connectivity between subpopulations (i.e., gene flow) which in turn favored the appearance of different phenotypes (Bricker et al., 2011). Isolated meadows, instead, can progress toward genetic drift lowering allelic diversity and making populations even more fragile against changes in environmental conditions (Figure 2a). When environmental conditions change only more diverse populations could harbor genotypes able to face the new extreme conditions, while monoclonal or less diverse populations could disappear (Figure 2b–d). This is the reason for the higher sensitivity to environmental changes of marginal populations concerning central populations of the species distribution (e.g., Billingham et al., 2003). The alternative would be to move toward conditions that are more favorable or to adapt, requiring times that are not achieved against fast environmental changes as we are facing nowadays.

3.1 | Genotype by environment interactions

Being a characteristic of individual genotypes, the amount of phenotypic variation across the environment describes the degree of genotype plasticity (genotypes by environment interactions – GxE; Li et al., 2018). Different reaction norms arise according to the degree of the interaction between individual genotypes and the environment (which is represented by the slope of each reaction norms in Figure 2). When environmental conditions change, populations with low genotypic diversity (Figure 2a,b) can react in different ways: (i) genotypes are stable and show no plastic behaviors (the reaction norms are parallel with the same shape, Figure 2d). Phenotypic changes do not occur, meaning that the mean of the phenotypic value of genotypes is enough to support environmental changes; (ii) genotypes re-shape their phenotypes to the new environmental condition exhibiting positive phenotypic plasticity (Figure 2e). This results in different positive plastic responses depending on the individual genotype interaction with the new environmental factor; (iii) genotypes interact with the new environment showing phenotypic changes that are maladaptive or not able to accommodate new conditions (Figure 2f). Negative phenotypic plasticity could result in population extinction. Contrary, more diverse populations have the potential to exhibit more plasticity if most plastic genotypes bring phenotypes closest to the new optimum conditions (Figure 2e). Then, if the plastic response is positively correlated with plant fitness, phenotypic plasticity can evolve by natural selection (Valladares et al., 2014) leading to genotype plasticity evolution (Figure 2g).

4 | PLASTIC RESPONSES TO RAPID ENVIRONMENTAL CHANGES: ACCLIMATION AND MIGRATION

Currently, global environmental changes may be too fast to allow for selection and evolutionary changes to occur in long living species, resulting in mean decline in population fitness. Thus, the persistence of species in the age of global climate changes will mainly depend on their intrinsic abilities that facilitate their persistence under environmental shifts adjusting to new conditions (i.e., *acclimation capacity*) or increase their dispersal capacity to find a more suitable environment to which they are adapted (i.e., *movement capacity*, Figure 3).

Here, we refer to *acclimation capacity* as the most relevant short-term response derived from pre-existing phenotypic plasticity, which allows organisms to adjust to rapidly changing environments extending their tolerance ranges (De Los Santos et al., 2009; Sharon et al., 2009). Phenotypic responses to environmental changes occur at different organizational levels that may include highly specific developmental, morphological, and physiological adjustments enhancing survival and persistence in the novel environment (Bercovich et al.,

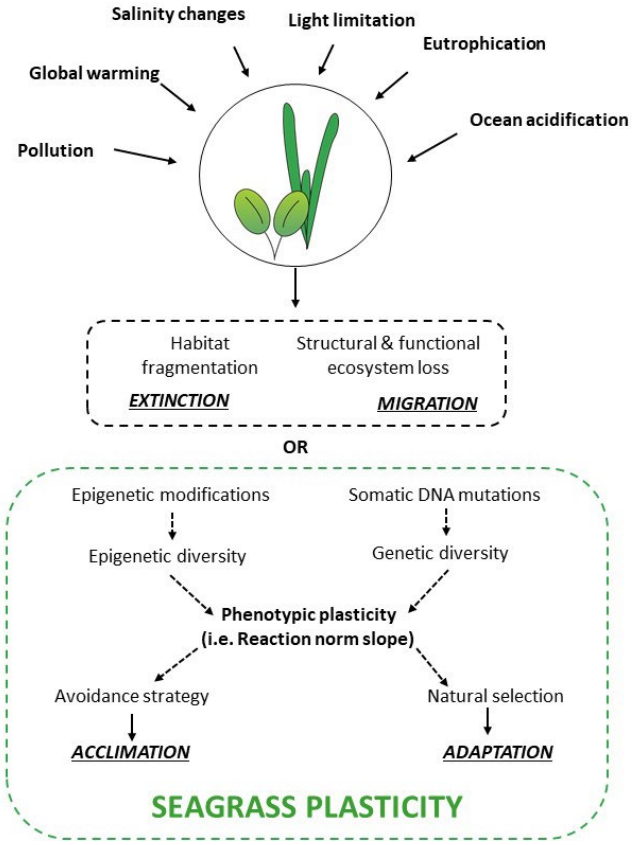

FIGURE 3 Representation of seagrass reactions to environmental changes. In the presence of environmental perturbations as global changes, seagrass survival is compromised, through habitat fragmentation and structural and functional ecosystem loss with consequent species extinction. Alternatively, intrinsic forces can increase their dispersal capacity to find more suitable environments (i.e., migration) or facilitate their persistence in the new environment through phenotypic plasticity. This adjustment to external conditions can be enhanced by epigenetic modifications or somatic DNA mutations, which increase epigenetic and genetic diversity, respectively. The resulting phenotype will favor the acclimation to the new environment and can be naturally selected. Thus, acclimation and adaptation are interrelated strategies of the seagrass plasticity representing intrinsic forces for their survival to future environmental changes

2019; Zhang et al., 2014). The degree of plasticity, as stated above, is related to the slopes of reaction norms that are variable among individuals, populations, and species. Thus, the steeper is the slope of the reaction norm, the more an organism is able to acclimate to different environmental conditions, and the more it is plastic. The process of acclimation resulting from phenotypic adjustments to environmental cues can occur during the early development of an organism that persists also on the adult stage, or as reversibly acclimation occurring during the lifetime (Beaman et al., 2016). The continuous alignment of phenotypes to the environment involves associated costs to perform strategies for sensing and responding without affecting individual performances (Vialet-Chabrand et al., 2017; Zimmerman, 2017). In fact, phenotypic plasticity is constrained by the energetic costs required for the sensory and regulatory mechanisms that ensure the processing of information and the development of the best phenotype–environment match (Gibbin et al., 2017). This is especially true for fast environmental changes and highly variable environments that force continuous prompt adjustment. The cost of switching the phenotype related to individuals and populations could be determinant for the ability of organisms to withstand environmental changes and to sustain the provision of ecological functions (Auld et al., 2010; Forsman, 2015; Murren et al., 2015). The high energetic costs involved in producing the optimum phenotype and the presence of a trade-off diverting energetic costs to support other traits and/or functions (DeWitt et al., 1998) can also result in the appearance of phenotypes that are less fit to the new environment (non-adaptive or maladaptive phenotypes) and hence not positively selected by evolutionary processes (see Figure 2 and previous paragraph; Palacio-López et al., 2015). Non-adaptive phenotypes would induce the decline of many species, including seagrasses, as fast global changes are currently increasing environmental stochasticity. However, costs related to phenotypic plasticity are currently under-studied in seagrasses.

Short-term responses occur through acclimatory mechanisms. In seagrasses, it has been described that these involve the modulation of gene expression profiles, which in turn depend on stress intensity, time of exposure to stressful conditions (Pernice et al., 2016; Ruocco et al., 2018), and morphometric plasticity in relation to geographical distributions and nutrient *status* (De los Santos et al., 2016; Soissons et al., 2018). At the individual level, plasticity can buffer environmental changes throughout the plant's lifetime, further increasing its tolerance to stress (e.g., short-term acclimation to light conditions; Olesen et al., 2002). It has been shown that plants of the Mediterranean species *Posidonia oceanica* have plastic responses to different light conditions as a consequence of regulatory mechanisms that allow them to acclimate to low-light environments (Dattolo et al., 2014; Mazzuca et al., 2009; Procaccini et al., 2017).

Plasticity at lower levels of the biological organization needs to be integrated at the level of individual and/or population fitness to evaluate how this influences the fitness and therefore alters the structure and the functioning of seagrass ecosystems. As an example, the stenohaline *P. oceanica* is able to thrive in environments with highly fluctuant salinity regimes thanks to high plasticity at the physiological (e.g., photosynthesis, carbohydrates metabolism) and morphological (e.g., plant size) levels (Marín-Guirao et al., 2017). These adjustments permit the species to keep unaltered plant density and population growth rates, as a plastic response to maintain population fitness. Nevertheless, the reduced size of plants weakens the physical structure of the leaf canopy and thus, its functionality, affecting the provision of ecological services.

At the population—and ultimately species—level, plasticity can allow colonization and establishment in diverse habitats and therefore influences the species' ecological breadth (Gimeno et al., 2009; Pigliucci, 2001; Sultan, 2003). In the presence of environmental stressors, plasticity could increase the dispersal capacity favoring the *migration* to more comfortable conditions or increase the reaction norm slope of a particular trait to cope with new environmental

conditions. In this sense, organisms could adopt an escape mechanism to avoid unfavorable conditions due to environmental changes. Thus, the migration capacity can be described as an alternative strategy to local acclimation, which allows organisms to track more favorable conditions (Bulleri et al., 2018). It is important to emphasize that the movement capacity can be a consequence of trait plasticity when its reaction norm is defined by the interaction with the environment.

The migration capacity of seagrasses is related to clonal growth, sexual reproduction and dispersal of sexual propagules, and vegetative fragments (McMahon et al., 2014). This means that the motion capacity is very different among species and even within the same seagrass species given that the frequency of sexual reproduction, the dispersion of seeds (floating vs buried seeds), the rates of clonal elongation, and the persistence of plant fragments greatly vary among populations, let alone species (Orth et al., 2007). For instance, settling velocities of fragments are important for successful seagrass movements, which allow plants to disperse spatially. Thus, rapid settling capacities can be the result of an adaptive process that reduces the risks for plants of being away from theirs optimal habitats (Weatherall et al., 2016).

Overall, large and long-lived species mainly rely on slow vegetative growth and have infrequent sexual reproduction events, which may potentially result in a reduced migratory success since they spread extremely slowly over large distances and seldom produce sexual propagules (McMahon et al., 2014). However, some species have shown high plasticity in reproductive phenology in response to environmental changes that increase their movement capacity. For instance, in terrestrial plants, it has been observed that different natural populations grown in common environments showed different flowering time in response to wet and dry conditions (e.g., *Brassica rapa*; Franks, 2011), a way to produce dispersal vectors (i.e., sexual propagules) and escape from the existing environment. *B. rapa* genotypes growing from seeds that experienced drought anticipate flowering in further dry conditions, in respect to seeds collected before the stressing event (Franks et al., 2007). This evidence suggests that the escape strategy adopted by these plants could be an indication of a rapid evolutionary shift to early flowering rather than the modification of the phenotypic state through trait adjustments (i.e., phenotypic plasticity). Thus, the potential to adopt plastic strategies is mostly the result of a trade-off between avoidance (through phenotypic plasticity) and escape (through early flowering). Similar evidence was recently described for seagrasses, where flowering phenotypes resulted in response to warming (i.e., *Z. marina*, Blok et al., 2018; *P. oceanica*, Ruiz et al., 2018). Collecting and storing seeds from seagrass populations growing in different conditions would allow testing evolutionary processes in face of future environmental scenarios.

Small and more ruderal species, such as *Halophila stipulacea*, are able to migrate fast into new environments adjusting their dispersal ability through phenotypic plasticity. This species, a native from the Red Sea, rapidly spread and colonized new environments, as the Mediterranean and Caribbean seas, locally adapting through phenotypic changes, such as changing sex ratio (Nguyen et al., 2018; Winters et al., 2020). The rapid establishment and spread of this species in cooler regions are mediated by its great plasticity for shifting the thermal tolerance during the Mediterranean invasion (Georgiou et al., 2016; Nguyen et al., 2020; Wesselmann et al., 2020). Despite the migration being a valuable strategy to avoid species extinction, losers can be the native species that are potentially outcompeted by colonizing species (e.g., *H. stipulacea*; Winters et al., 2020).

It is noteworthy to mention that plasticity is an underlying attribute to these processes, which in turn are not mutually exclusive since acclimation, adaptation, and distributional changes are interrelated to some extend (Donelson et al., 2019; Kelly, 2019).

5 | ASSESSING PHENOTYPIC PLASTICITY IN SEAGRASSES

The analysis of plasticity and the discrimination between adaptive or acclimation processes in plants has been mostly approached in model species, where the appropriate molecular and manipulative tools have been developed (Bossdorf et al., 2010; Matesanz et al., 2020). Seagrasses are a polyphyletic and unique group of plants, with convergent morphology due to constraining imposed by the adaptation to a fully submerged life in the marine environment (Les et al., 1997; Olsen et al., 2016). Sexual reproduction is adapted to the marine environment and its experimental manipulation has not been developed for most of the species. Hence, the dissection of the different drivers of plasticity can mostly be assessed based on indirect evidence. The complex and multidisciplinary information needed for disentangling plasticity components can be obtained through field observations, experimental manipulations, and laboratory approaches that are described in detail in the following paragraphs. The most recent seagrass literature has been reviewed to present the strength and weaknesses of each approach (Table 1; Table S2).

5.1 | Field observations

Phenotypic plasticity, and in particular whether it is adaptive and which are the energetic costs involved, can be first approached by comparing performances between populations subjected to different environmental conditions (Forsman, 2015). Variation in single or multivariate trait plasticity along environmental gradients can inform about factors and conditions potentially promoting the evolution of phenotypic variation and give insights into how plasticity can contribute to evolutionary differentiation within species (Donelson et al., 2019).

Analysis of functional traits selected for plants combined with genetic data is a helpful approach to investigate genotype–environment interactions (Haseneyer et al., 2009). For instance, Maxwell et al. (2014) observed that physiological and morphological characteristics of *Zostera muelleri* varied along a gradient of water quality according to well-known light acclimation responses. They

TABLE 1 Summary of pros and cons of approaches used to assess phenotypic plasticity in seagrasses (see the main text for more detail)

Approaches	Pros	Cons
Field observations	Inform about factors that potentially promote the evolution of phenotypic variation and how plasticity can contribute to evolutionary differentiation within species	Limited to observations
Field experiments	Quantify the degree of plastic responses, analyzing phenotypic changes in relation to the environment	Natural environmental variation leads to misleading interpretations
Mesocosm experiments	Simulate the effect of the stress factor of interest for analyzing intraspecific and interspecific responses and the genetic basis of phenotypic plasticity	Require sophisticated systems. Results cannot be automatically transferred to natural conditions
Reciprocal transplant experiments	Identify the genetic component of plastic responses	Sensitive to environmental forces and regional stressors
Common garden experiments	Allow discriminating the contribution of genetic and plastic effects comparing genetically distinct families or populations	Require long acclimation phases and an accurate experimental design

also observed a consistent response in all meadows to a severe flooding event increasing freshwater run-off along the gradient. Plants maintained population productivity unaltered (i.e., biomass, shoot, or leaf density alterations) through physiological adjustments, suggesting high phenotypic plasticity and a reaction norm with a large positive slope. In another example, the congeneric seagrass species *Z. noltii* showed the capacity to acclimate to local environmental conditions exhibiting different phenotypes in terms of mechanical and morphological traits during one growing season and across the latitudinal range of the species. The presence of stronger and stiffer leaves under oligotrophic as compared to more eutrophic conditions suggested that the species suffers in nutrient-enriched environments without evolving a potentially adaptive phenotype (Soissons et al., 2017).

Phylogeny-based comparative analyses can be used to infer the role of plasticity for evolutionary diversification among species and for speciation (Coyer et al., 2013; Olsen et al., 2004). Candidate genes that are indirectly related to environmental gradients, providing evidence of local adaptation, can be identified through genome-wide transcriptomic analysis performed on wild populations (Jahnke et al., 2019), though the identification of real causation among genes and the environment is not trivial. This could be approached by combining genome-wide analysis with manipulative stress experiments (e.g., Anderson et al., 2014).

The analysis of spatial variation across environments by comparing ecosystems and populations along gradients is a useful approach to extrapolate temporal dynamics and to infer about future ecosystem responses (i.e., space for time substitution; Fukami & Wardle, 2005). This is a valid approach, which states that environmental factors vary over time in the same way as they vary in space providing new opportunities to explore the potential success of plastic species (Buyantuyev et al., 2012). The analysis of samples along a wide spatial range allows to assess relationships between phenotypic variations and the environmental gradient without the constraints of time (Banet & Trexler, 2013). As showed by Bricker et al. (2011), *T. testudinum* individuals from different populations across north–south physiochemical environmental gradients in the Florida Bay was an effective method to discriminate plasticity as the main driver for phenotypic variations across sites. The space-for-time substitution approach is helpful not only to analyze populations' plasticity through natural gradients and thus to assess long-term consequences of human impacts, but also to infer temporal dynamics by comparing multiple sites with different disturbance gradients (Fukami & Wardle, 2005). For instance, Yang et al. (2018) showed, under different stress regimes, different degree of plasticity for physiological and morphological traits in *Z. marina* plants collected across regions that displayed diverse eutrophic gradients. New potential bio-monitoring metrics, which may help the management of seagrass meadows in monitoring and predicting phenotypic variations, can derive from this kind of study.

5.2 | Experimental manipulation of selected parameters

Observational studies can offer important insights in order to generate further hypotheses and testable predictions. However, demonstrating causal relationships and mechanisms, linking either variation in the capacity for plasticity itself or plasticity induced phenotypic variation to aspects of the individual or population fitness, is complex, as it requires experimental manipulation, replication, and controlled comparisons (Forsman, 2015).

The experimental manipulation of one or more environmental factors can be performed directly in the field or in the laboratory under controlled conditions. The last option requires a deep analysis of the relevant environmental factor to establish the correct experimental design, which in turn reflects the environmental variation that occurs under natural conditions. This is not an easy task, since many environmental factors act and interact with each other in natural conditions.

5.2.1 | Field experiments

Field experiments allow quantifying the degree of plastic responses, analyzing phenotypic changes in relation to the environment (Merilä & Hendry, 2014), and predicting shifts in species compositions under environmental changes (La Nafie et al., 2013). This can be realized through the artificial modulation of one or more factors, to compare control and treatment under natural environmental conditions, in order to investigate the potential drivers for the observed phenotypic changes. One of the major strengths of this approach is the inclusion of natural variability and processes that are difficult to reproduce under controlled conditions. In this respect, individual responses measured in situ provide more reliable results than those performed in the laboratory. Different studies have been carried out in the field, exploring phenotypic responses of seagrass species to single (e.g., Bité et al., 2007; Collier et al., 2012; Cox et al., 2015; Darnell & Dunton, 2017; Silva et al., 2013; Table S2) or to multiple environmental factors (e.g., Ceccherelli et al., 2018; La Nafie et al., 2013; Ravaglioli et al., 2017). For instance, Ruocco et al. (2018) showed that in *P. oceanica* plants, herbivory increases under nutrients addition, with a clear effect on seagrass productivity. In such environmental conditions, the species can enhance growth to compensate for the increase of herbivory, or can increase the accumulation of deterrent substances and the translocation of nutrients to underground tissues to protect them against external pressures (Alcoverro & Mariani, 2005; Ruocco et al., 2018; Sánchez-Sánchez & Morquecho-Contreras, 2017).

Tuya et al. (2019) assessed the tolerance of *C. nodosa* to low-light levels across different populations located in the Canary Islands and the Mediterranean Sea by manipulating the light intensity directly in the field. Results demonstrated biogeographical variability among populations in the degree of shading tolerance, with Canary Island populations being less tolerant in respect to the others. As suggested by authors, the lower plasticity of Canary Island populations can be related to the lower genetic diversity of these populations, living at the range edges of species' distribution. Salo et al. (2015) also found different gene expression and physiological performance of *Z. marina* genotypes to light reduction. The experimental manipulation in the field offers also the opportunity to study plastic responses of plants locally adapted to particular environmental conditions. In order to model the response to eutrophication in a future ocean acidification scenario, Ravaglioli et al. (2017) evaluated the performances of *P. oceanica* plants adapted to long-term acidification by exposing them to in situ nutrient enrichment. The field experiment revealed that the increased CO_2 benefits plants facilitating the absorption and assimilation of nutrients.

Although experimentation in the field is helpful for quantifying the plasticity in the response to environmental stressors, the natural environmental variability can lead to misleading interpretations of the specific drivers responsible for the resulted phenotypic changes. Additionally, these experiments provide results that are difficult to replicate and compare with similar studies because regional stressors and biotic interactions may modify the final outcome (e.g., Garrote-Moreno et al., 2016).

5.2.2 | Mesocosm experiments

One of the main advantages of performing experimental manipulations under controlled conditions is the possibility to simulate the effect of the stress factor of interest, isolating it from all the other variables that are naturally occurring, and to analyze intraspecific and interspecific responses. Additionally, controlled experiments offer the opportunity to evaluate the degree of phenotypic plasticity in the form of a genetically determined reaction norm. An example from terrestrial plants refers to the manipulation of temperature, utilized for assessing thermal tolerance variability across latitudes (Molina-Montenegro & Naya, 2012). In this case, the authors measured the phenotypic plasticity of an invasive species (*Taraxacum officinale*) to different environmental temperatures, confirming that higher thermal tolerance at higher latitudes is related to an improved phenotypic expression. Different studies performed under laboratory conditions assessed phenotypic plasticity of seagrass species, such as the mesocosm experiments performed on the most abundant Mediterranean species, *Posidonia oceanica* and *Cymodocea nodosa*. These studies have confirmed that these two species have different tolerance to hypersaline stress (i.e., *C. nodosa* > *P. oceanica*), consistent with their physiological and morphological plasticity (Piro et al., 2015; Sandoval-Gil et al., 2012, 2014). Furthermore, *C. nodosa* also showed higher tolerance and higher plasticity to warming, possibly related to the tropical affinity of the genus (Marín-Guirao et al., 2018; Olsen et al., 2012; Tutar et al., 2017). Controlled experiments also allow the manipulation of multiple stressors simulating realistic environmental changes affecting coastal marine habitats (Artika et al., 2020; Egea et al., 2018; Pazzaglia et al., 2020; Viana et al., 2020). Through the manipulation of temperature and nutrients concentration, Ontoria et al. (2019) investigated individual and population responses in *C. nodosa* plants. Different phenotypes arose depending on the interaction among temperature-ammonium and temperature-organic carbon suggesting that the exposure to multiple stressors triggers phenotypic responses in relation to stress-specific thresholds. The analysis of the recovery after stressing conditions, allowed to point out contrasting resilience abilities of seagrass populations living in different environments, as a result of their adaptation to local climatic conditions (e.g., Franssen et al., 2011; Winters et al., 2011; Table S2). This represents an important

advantage of experimental manipulations, as offers the possibility to understand if plants are able to turn back to their original natural state after extreme events providing new insights into the long-term survival of seagrasses to environmental changes.

5.3 | Transplantation experiments

Transplantation experiments fall into two distinct approaches. A reciprocal transplant experiment entails the movement of phenotypes between contrasting natural environments along with on-site transplantation controls. In common garden experiments, genotypes coming from different environments are planted into the common environmental conditions of a single site.

5.3.1 | Reciprocal transplant experiments

This experimental approach allows for a direct test of local adaptation by comparing two sites with each other (Kawecki & Ebert, 2004). Thus, provided proper acclimation and control for carry-over effects (see below), a potential genetic component of the plastic response (as reaction norm) can be quantified by comparing the phenotypic performances of transplants in native vs. foreign environments. Local adaptation and plastic abilities of different populations can be addressed using two different comparisons. First, local populations can be compared within habitats, that is, "local" vs "immigrant" design; second, plants can be compared across habitats, that is, "home" vs "away" design (Svensson et al., 2019). The final expectation of such experimental conditions is that plants perform better in their "home" environment in respect to the "away" ones, showing direct indications of a local adaptation. In this case, the degree of plasticity of genotypes locally adapted to their home site and transplanted to reciprocal environments within their environmental tolerance range can be assessed. A recent review summarizing 75 years of plant experiments on local adaptation revealed that indeed, local populations almost always showed higher performance than non-local ones, especially in traits related to reproductive output, suggesting a notably local adaptation in terrestrial plants (Baughman et al., 2019).

Factors other than local adaptation can affect transplants performance. Evans et al. (2018) designed a reciprocal transplantation experiment of two genetically and geographically distinct populations of *P. australis* in southeastern Australia. They assessed local adaptation by comparing plant productivity of low- and high-genetic diversity meadows using the "home" vs "away" approach. After 6 months, they found higher survival rates and productivity for high-genetic diversity plots, which outperformed less genetically diverse plants both at home and away sites. This means that more genetically diverse plots included also more plastic genotypes that performed better than less diverse plots, allowing them to survive after transplantation. This high genetic demonstrated that both high-genetic diversity and local adaptation play a crucial role in enhancing transplant success (Hämmerli & Reusch, 2002; Jahnke, Serra, et al., 2015; Procaccini & Piazzi, 2001; Reusch et al., 2005; Reynolds et al., 2012; Williams & Davis, 1996; Williams, 2001).

A reciprocal transplantation approach has also been used to evaluate seagrass short-term acclimation along environmental gradients. Sharon et al. (2009) transplanted shoots of *H. stipulacea* between the depth extremes of its distribution, to evaluate the plastic response to different irradiance regimes (Table S2). After 2 weeks of exposure to reciprocal environments, they found fast changes in photosynthetic performance supporting the high plasticity of the species.

The long-term maintenance of field experiments can be jeopardized by environmental forces (storms, salinity, and temperature fluctuations), regional stressors (anchoring boats and anthropic inputs), or other technical problems (van Katwijk et al., 2009). As an alternative, reciprocal transplant experiments in controlled conditions are a valid tool to overcome logistical issues with transplantation in the field that also includes the risk of introducing as yet unknown pathogens over longer distances. In *P. oceanica*, plants from shallow and deep environments were transplanted in individual pots and exposed to their reciprocal light regimes in a controlled mesocosm approach (Dattolo et al., 2017). *P. oceanica* genotypes showed some degree of photo-physiological and morphological plasticity. Nevertheless, after several weeks under reciprocal light environments, genotypes showed performances that were similar to those shown by plants from their original depth, suggesting local adaptation to their home environment.

5.3.2 | Common garden experiments

Common garden experiments are particularly relevant to investigate the nature of plastic responses and to discriminate the contribution of genetic and plastic effects on phenotypic variation. In fact, these experiments allow comparing distinct genotypes or populations from different environments by growing them under identical environmental conditions (De Villemereuil et al., 2016; Merilä & Hendry, 2014). This approach is commonly used to test for local adaptation, as it enables to unravel the genetic basis of phenotypes from different populations excluding the effects of the corresponding environments (Cruz et al., 2019; De Villemereuil et al., 2016; Lepais & Bacles, 2014; Vermaat et al., 2000).

In seagrasses, Franssen et al. (2011) performed a common garden stress experiment to assess transcriptomic profiles of *Z. marina* populations from two contrasting thermal environments (Venice Bay, Italy, vs. Limfjord, Denmark) to a simulated heat wave. They found a strong divergence in terms of gene expression profiles between populations only in the recovery phase, while the immediate stress response was similar and showed the typical heat shock protein-encoding genes with overexpression. This was consistent with local adaptation to the local natural thermal environment. One caveat of such studies is that even under a long acclimation phase of about 1 month, this may not be sufficient to overcome long-term acclimatization to the home environment. We can thus not fully conclude that observed stress responses resulted from a genetically based

adaptation (as stated by Bergmann et al., 2010; Winters et al., 2011; Table S2). One way forward to overcome such limitations is raising the experimental plants from seeds. This was done in seagrasses, for the first time to our knowledge, in eight populations across the distribution range of the seagrass *C. nodosa*. Seeds were germinated and subsequently grown for sixteen months in a common garden before being exposed to two marine heat waves of different intensity (Pereda-Briones et al., *under review*). The positive relationship observed between the resilience and local thermal regimes of the studied populations strongly evidenced local adaptation of the populations to their thermal regime. Such studies provide strong evidence for the existence of underlying genetic variation resulting from divergent selection, representing the evolutionary potential of the species within the frame of global warming, although the attainable rates of change remain obscure (Reusch & Wood, 2007). This "adaptive transgenerational plasticity" is not only the result of the development of specific traits in response to environmental stresses passed from parental individuals to the progeny, but also the inheritance of regulatory epigenetic machinery enhancing offspring to activate regulatory mechanisms under the same stresses (King et al., 2018). Despite the relative long acclimation imposed on plants under common conditions, it remains still difficult to conclude on the genetic and/or epigenetic basis of the observed plasticity. Long acclimation phases and phenotypic responses of individuals under common conditions over one or more generations are necessary to test for adaptive traits in order to reset plants' experiences of their place of origin (as in terrestrial model species; Raabová et al., 2007; Watson-Lazowski et al., 2016). However, as stated above, the reproduction of seagrasses under controlled conditions is challenging, and life cycles are often too long to allow experimentation over multiple generations.

Common garden experiments can also be designed based on a space-for-time substitution approach. A case in point is the study by Winters et al. (2011) that compared plant responses to a heat wave originating from three populations of *Z. marina* across a latitudinal thermal gradient. The differential thermal response in terms of growth and photo-physiology was consistent with local adaptation and could be integrated into seagrass models to predict the future persistence of this species in different regions affected by climate changes.

Some common garden experimental designs are a merger of all approaches described above (Jueterbock et al., 2016; Marín-Guirao et al., 2018). Jueterbock and colleagues tested temperature adaptation of *Z. marina* populations collected from contrasting and phylogenetically independent thermal clines (North vs South in Mediterranean and Atlantic areas), using a common garden experiment combined with a space-for-time substitution design in anticipation of rapid ocean warming predicted for the next decades. Upon exposure of plants to a marine heat wave, full transcriptome profiles were obtained and mapped onto the genome. Results revealed a stronger adaptive transcriptomic differentiation between the Mediterranean and the Atlantic samples that is likely due to the reduced gene flows that characterized the smaller and isolated Mediterranean populations, favoring adaptive differentiation (Olsen et al., 2004; Procaccini et al., 2007).

6 | FUTURE PERSPECTIVES: ENHANCING PLASTICITY FOR BOOSTING SEAGRASS ADAPTATION

Ascertained the importance of phenotypic plasticity and its role in driving short-term responses and evolution, it is now necessary to explain how all this information can be integrated into seagrasses research. In the framework of conservation and restoration management, understanding the phenotypic plasticity of selected meadows to restore a disrupted habitat strongly boosts the success of restoration plans (Falk et al., 2001; Paulo et al., 2019). In fact, the selection of highly plastic and tolerant/resilient genotypes of foundation species could be a valid approach to restore marine ecosystems (Abelson et al., 2020; Coleman et al., 2020; Kettenring et al., 2014; van Katwijk et al., 2009). Selected genotypes should present a set of positive traits in order to increase their plasticity for successfully facing coming fast environmental changes.

In order to support the restoration, better performing genotypes can not only be identified and selected but can also be experimentally manipulated. A possible way is to use gene-editing approaches, though their ethical implications are currently under debate (Rodriguez, 2016). After the identification of genes that directly affect seagrass ability to thrive in a changing climate, genetic engineering techniques, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), can be used to produce genotypes with higher plasticity and the ability to acclimate and adapt to strong and stochastic environmental changes (Scheben et al., 2016).

Another way, which does not involve genetic manipulation, is the experimental hardening. The terms "hardening" or "priming" define phenomena that induce temporally limited environmental stimulus in order to prepare and modify the response to future stress (Hilker et al., 2016). This is a well-known concept among botanists, which used to harden plants taking advantage of their ability to "remember" their ancestral environments via phenotypic plasticity, revealing a mechanism by which past experience affects future evolution (Gibbin et al., 2017; Ho et al., 2020). The capability of genotypes to save memory of past stress events and to better perform when the stress re-occurs has recently been observed in seagrasses (i.e., *P. australis* and *Z. muelleri*, Nguyen et al., 2020). This process is one element of "assisted evolution" strategies to promote individual and population resilience against environmental changes without genetic manipulation constraints (Filbee-Dexter & Smajdor, 2019). Genotypes with improved resistance (i.e., hardening response, Bruce et al., 2007) can represent preferential material for the restoration of endangered or disturbed populations. In terrestrial studies, the ability to "remember" past stressful events is currently investigated for model crop species, especially through the assessment of epigenetic modifications induced by the exposure to stress (Liu et al., 2015). Although the field of *ecological epigenetics* is gaining momentum, due

to the application of increasingly specific and sophisticated molecular techniques (Ay et al., 2014; Bossdorf et al., 2010; Popova et al., 2013; Rendina Gonzàlez et al., 2018; Richards et al., 2017), the study of the epigenetic "stress memory" is still at the beginning, for both marine and terrestrial plants.

7 | CONCLUSIONS

Our main goal was to present an overview on the importance of plasticity in the face of rapid environmental changes for a group of marine plants with long generation times owing to clonality that lives in an environment with very steep environmental gradients, subject to alarming rates of global change. The rapid occurrence of global changes forces marine plants to react in order to prevent population declines. Species react acclimating to new conditions, through phenotypic plasticity, evolutionary adaptation, or migration (Bulleri et al., 2018). The acclimation abilities as one major form of phenotypic plasticity are widely explored in seagrasses' studies (Bité et al., 2007; Dattolo et al., 2017; Duarte et al., 2018; Maxwell et al., 2014). This acclimation process can be based on genetic and epigenetic processes, the last fostering rapid adaptive evolution (Douhovnikoff & Dodd, 2015), but it is so far unstudied in seagrasses. Equally, unstudied is the adaptive significance of a large degree of standing somatic genetic variation detected in seagrass clones that could be the basis for adaptation within a genet or clone (Yu et al., 2020). The adaptation occurs through natural selection and requires too long times to react in the face of rapid changes. Nevertheless, the selection of more plastic genotypes could prevent population declines, as they are more likely to contrast dynamic changes (Bricker et al., 2011; Table S2).

We explored several main approaches that allow us to infer the nature of plastic responses to global changes and discussed pros and cons. The experimental approaches implemented in seagrass studies, whether performed with controlled or field experiments and space for time designs, were instrumental for exploring the basis of plasticity. One future avenue is clearly more multi-factorial experiments that would be required to understand seagrass responses under more realistic present and future scenarios. Another important way forward is the integration of different phenotypic and genomic approaches to study the interaction among the genetic and plastic components of phenotypic variation, including the study of epigenetic mechanisms. Considering the importance that plasticity may have in response to rapid environmental changes, future promising research in seagrasses should involve the analysis of relationships between gene expression profiles resulting from environmental stresses and epigenetic regulatory machinery. The majority of seagrass studies employing molecular approaches involve gene expression and transcriptomic analysis, while being limited to few species and mostly related to thermal and light responses (Davey et al., 2016; Gu et al., 2012; Marín-Guirao et al., 2017; Procaccini et al., 2017; Tutar et al., 2017). We also observed that many recent transcriptomic studies in response to environmental stressors lack consideration of molecular elements that may have strongly regulatory roles in stress responses, such as transposable elements and micro-RNA (miRNAs) (e.g., Barghini et al., 2015). The improvement of molecular approaches in seagrasses could play a crucial role not only in studying their plasticity but also in digging on the basis of stress memory and on its potential evolutionary role under global climate changes (Chinnusamy & Zhu, 2009; Lämke & Bäurle, 2017).

In conclusion, we strongly suggest that the evaluation of plastic adaptive responses should be moved from a local to a global scale. The future implementation and evolution of seagrass observatories will foster this process. Next-generation marine observatories should make it possible to collect multivariate time series synchronously in different sites or regions and to exploit the information by integrating data through multivariate statistics and/or machine-learning algorithms (Crise et al., 2018; Danovaro et al., 2016). Real-time multivariate monitoring in seagrass observatories will enable assessing environmental and seagrass trait changes and inferring adaptive potential of the observed processes in seagrass populations.

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CONFLICTS OF INTEREST

There is no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

ORCID

Jessica Pazzaglia https://orcid.org/0000-0002-8677-7712
Thorsten B. H. Reusch https://orcid.org/0000-0002-8961-4337
Antonio Terlizzi https://orcid.org/0000-0001-5968-4548
Lázaro Marín-Guirao https://orcid.org/0000-0001-6240-8018
Gabriele Procaccini https://orcid.org/0000-0002-6179-468X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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CHAPTER II

(Paper II)

Jessica Pazzaglia, Alex Santillán-Sarmiento, Stephanie B. Helber, Miriam Ruocco, Antonio Terlizzi, Lázaro Marín-Guirao and Gabriele Procaccini. Does Warming Enhance the Effects of Eutrophication in the Seagrass *Posidonia oceanica*?

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Does Warming Enhance the Effects of Eutrophication in the Seagrass *Posidonia oceanica*?

Jessica Pazzaglia[1,2†], Alex Santillán-Sarmiento[2,3†], Stephanie B. Helber[4], Miriam Ruocco[2], Antonio Terlizzi[1,5], Lázaro Marín-Guirao[2,6‡] and Gabriele Procaccini[2‡]*

[1] Department of Life Sciences, University of Trieste, Trieste, Italy, [2] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy, [3] Faculty of Engineering, National University of Chimborazo, Riobamba, Ecuador, [4] Department of Ecology, Leibniz Centre for Tropical Marine Research, Bremen, Germany, [5] Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples, Italy, [6] Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, Murcia, Spain

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Nuria Marba,
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Carmen B. De Los Santos,
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Fernando Tuya,
University of Las Palmas de Gran Canaria, Spain

***Correspondence:**
Gabriele Procaccini
gpro@szn.it

[†]These authors share first authorship

[‡]These authors have contributed equally to this work

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Seagrass meadows are disappearing at rates comparable to those reported for mangroves, coral reefs, and tropical rainforests. One of the main causes of their decline is the so-called cultural eutrophication, i.e., the input of abnormal amounts of nutrients derived from human activities. Besides the impact of eutrophication at a local scale, the occurrence of additional stress factors such as global sea warming may create synergisms in detriment of seagrass meadows' health. In the present study, we aimed to evaluate if plants undergoing chronic cultural eutrophication and plants growing in relatively pristine waters are more (or less) sensitive to heat stress, nutrient load and the combination of both stressors. To address this question, a mesocosm experiment was conducted using *Posidonia oceanica* collected from two environments with different nutrients load history. Plants were exposed in controlled conditions to high nutrient concentrations, increased temperature and their combination for 5 weeks, to assess the effect of the single stressors and their interaction. Our results revealed that plants experiencing chronic cultural eutrophication (EU) are more sensitive to further exposure to multiple stressors than plants growing in oligotrophic habitats (OL). OL and EU plants showed different morphological traits and physiological performances, which corroborates the role of local pressures in activating different strategies in response to global environmental changes. EU-plants appeared to be weaker during the treatments, showing the greatest percentage of mortality, particularly under increased temperature. Temperature and nutrient treatments showed opposite effects when tested individually and an offset response when combined. The activation of physiological strategies with high energetic expenses to cope with excess of nutrients and other stressors, could affect plants present and future persistence, particularly under eutrophic conditions. Our results represent a step forward in understanding the complex interactions that occur in natural environments. Moreover, unraveling intraspecific strategies and the role of local acclimation/adaptation in response to multiple stressors could be crucial for seagrass conservation strategies under a climate change scenario.

Keywords: seagrasses, multiple stressors, *Posidonia oceanica*, global warming, eutrophication, plant physiology

INTRODUCTION

Seagrasses are important aquatic angiosperms that form meadows of great ecological and economic value for marine and global ecosystems. The productivity of seagrass meadows and their ability to capture carbon are comparable to those of terrestrial forests (Fourqurean et al., 2012). These macrophytes are considered ecosystem engineers due to their habitat-forming capacity, providing food and shelter for a range of organisms such as finfish, shellfish, waterfowl, and herbivorous mammals (Boudouresque et al., 2009). There is strong evidence that seagrasses are disappearing at rates comparable to those reported for mangroves, coral reefs, and tropical rainforests (Waycott et al., 2009). Such decline has been reported worldwide especially in populations occurring in sheltered embayments and lagoons, where water recirculation is low and prone to nutrient loading from neighboring human population, the so-called "cultural eutrophication" (Touchette and Burkholder, 2000). Besides the impact of local anthropic pressures such as cultural eutrophication, additional global stressors (e.g., sea warming) may generate synergisms, thus increasing loss rate of these precious ecosystems (Lloret et al., 2008). However, little is known about the potential interplay between multiple sources of stress and the response that meadows growing in different environmental conditions may have.

Eutrophication or over-enrichment of nitrogen and phosphorus in the water column has several indirect and direct effects on the physiology of seagrasses (Unsworth et al., 2015; Ceccherelli et al., 2018). Excessive inorganic nitrogen (N_i, as NO_3^- and NH_4^+) concentrations can indirectly inhibit seagrass growth and survival by reducing light availability due to stimulation of phytoplankton, macroalgae and epiphytic algae overgrowth (Touchette and Burkholder, 2000). Additionally, N_i enrichment can directly affect growth in several seagrass species by altering their cellular function and generating a negative physiological response (Burkholder et al., 2007). Some studies suggest that feedback inhibitory mechanisms for N_i uptake are missing in seagrasses as a result of an evolutionary adaptation to oligotrophic habitats (Burkholder et al., 1992; Touchette and Burkholder, 2000). Thus, as observed in the eelgrass *Zostera marina* (Burkholder et al., 1992), plants continuously uptake N_i even during dark periods, when surrounding water is rich in nitrate. This uncontrolled uptake of N_i generates metabolic imbalances because N assimilation is a highly energy-demanding process (Touchette et al., 2003). The toxic effect of ammonium addition was observed to be stronger during winter in *Zostera noltii* (Brun et al., 2002), suggesting interactions between different environmental drivers. In the latter, the addition of NH_4^+ implied a mobilization of non-structural carbohydrates accompanied by a reduction in growth. Nitrate can be stored in vacuoles or reduced in the cytosol to nitrite and subsequently in the chloroplast to ammonium, which is then converted to glutamine (Touchette and Burkholder, 2000). The tissue content of carbon and nitrogen has provided good indication of the nutritional status of seagrasses, integrating the long-term nutrient exposure history of plants (Lee et al., 2004). Variations in N content can arise from specific environmental conditions. For instance, Mediterranean *Posidonia oceanica* growing in high CO_2 natural conditions showed a clear up-regulation of nitrate transporter genes, when fertilized with nutrients, suggesting a plastic response of plants for balancing C:N ratio (Ravaglioli et al., 2017). Although the C content shows low variability across species (Duarte, 1990), the rate of change in C:N ratio should shift from high to small as nutrient supply meets the plants' demands. However, inferences on a particular species should be taken carefully as substantial inter- and intra-specific variation is common (Touchette and Burkholder, 2000).

In addition to eutrophication, global increase of sea temperature also threatens seagrasses. Rapid sea warming is occurring at unprecedented alarming rates due to anthropogenic activities altering climatic conditions worldwide (Francour et al., 1994; Bianchi, 2007; Vargas-Yáñez et al., 2008; Coma et al., 2009; Lejeusne et al., 2010; Marbà et al., 2015). The Intergovernmental Panel on Climate Change (IPCC) predicts a global increase of 2.58°C in mean sea surface temperatures (SST) by the end of the century (IPCC, 2019). In the short term, SST anomalies in the form of heat waves have also been observed, e.g., in 2010/11 in the southeast Indian Ocean, where a heatwave hit the Western Australian coast, experiencing record water temperatures of 2–4°C above long-term averages for about 10 weeks during late summer (Pearce and Feng, 2013; Kendrick et al., 2019). In the Mediterranean Sea, warming due to heatwaves, particularly from late spring/early summer to early autumn, are expected to be stronger, lasting up to a whole month (Diaz-Almela et al., 2007; Hobday et al., 2016; Oliver et al., 2018). For instance, in 2003 the average temperature reached at 1 m depth was 25.6 ± 1.5°C, with a maximum of 28.6°C for the Gulf of Naples (Garrabou et al., 2009). The increasing frequency of those events raised the alarm on their impacts to marine biota, including seagrass meadows. For instance, mortality of *P. oceanica* has been observed in the Balearic Islands in 2003 and 2006 after summer heatwaves (Díaz-Almela et al., 2009; Marbà and Duarte, 2010). Moderate temperature increase can stimulate biochemical reactions such as photosynthesis and respiration (Olsen et al., 2012; Marín-Guirao et al., 2018), or even promote flowering (Ruiz et al., 2018; Marín-Guirao et al., 2019). However, beyond certain thresholds, thermal stress affects the stability of photosystem II (PSII) reaction centers in the chloroplasts and reduces electron transport rates (York et al., 2013; Marín-Guirao et al., 2016; Repolho et al., 2017; Ruocco et al., 2019a). The acceleration of respiration over photosynthetic rates can also lead to carbon imbalances under heat stress (Collier and Waycott, 2014; Marín-Guirao et al., 2018). In any case, the effect of temperature is highly variable and the specificity of a particular species or population responses will be highly dependent on the natural regimes where plants grow (Collier et al., 2011; Winters et al., 2011; Marín-Guirao et al., 2018).

Interactions between multifactorial environmental conditions are indeed influencing the physiology of marine organisms. However, experiments assessing the interactions between temperature and nutrients are rare due to experimental and budgetary constraints. Some studies on seagrasses demonstrated that synergistic interactions occur when plants are exposed to a combination of stressors, such as osmotic, light, temperature

and eutrophication (Touchette and Burkholder, 2000; Collier et al., 2011; Villazán et al., 2013, 2015; Ontoria et al., 2019b). However, the responses were highly specific for the species assessed and varied according to the life stage of the plant. For instance, in *Enhalus acoroides* seedlings, the effect of interaction between temperature and nutrient enrichment was weak (Artika et al., 2020). On the other hand, an antagonistic interaction was observed in *Cymodocea nodosa* adult plants, where acidification improved ammonium assimilation and buffered the enhanced respiration promoted by temperature (Egea et al., 2018).

The endemic seagrass *P. oceanica* forms extensive monospecific meadows on rocky and sandy bottoms, representing one of the most significant benthic species in the Mediterranean Sea (Serra and Mazzuca, 2011). Declines in *P. oceanica* populations over the last decades have been attributed, among many other causes, to both sea warming and eutrophication (Pergent et al., 1999; Seddon et al., 2000; Ruiz et al., 2001; Pergent-Martini et al., 2006; Díaz-Almela et al., 2009; Marbà and Duarte, 2010; Marbà et al., 2014; Moore et al., 2014; de los Santos et al., 2019). However, the consequences of the combined effect of both stressors on the plant physiology are virtually unknown. A recent microcosm study on *P. oceanica* examined the interactions between ammonium addition and high temperature (Ontoria et al., 2019a). In this case, synergism between factors caused a decrease of about 70% in photosynthetic performance indicating the harming potential of eventual heat waves in areas exposed to high anthropic pressures. Hence, the presence of meadows in highly impacted and nutrient enriched areas (e.g., the Gulf of Naples, Italy; Cianelli et al., 2012) is cause of concern and raises questions about the physiological strategies eventually adopted to respond to additional stress. For instance, *Halophila ovalis* plants growing in turbid waters were more sensitive to further shading in respect plants growing in clear waters that were able to decrease shoot density and increase photochemical efficiency (Fv/Fm; Yaakub et al., 2013).

In this study, we aimed to evaluate whether plants undergoing chronic cultural eutrophication and plants from relatively pristine sites are more (or less) sensitive to heat stress, nutrient load and the combination of both stressors. To address this question, a mesocosm experiment was conducted using *P. oceanica* plants from two environments with different nutrients load history. Plants were exposed in controlled conditions to high nutrient concentrations, increased temperature and their combination for 5 weeks, to assess the effect of single stressors and their interaction. At the end of the exposure period, we analyzed morphological, photo-physiological and biochemical endpoints including the content of C and N to corroborate nutrient uptake.

MATERIALS AND METHODS

Sampling Sites

Fragments of *P. oceanica* consisting of a horizontal rhizome bearing 10–20 vertical shoots, were collected by SCUBA diving on May 15 – 16th 2019, from shallow-water meadows (7–9 m depth) from two locations with different history of nutrient loads, located at 8 nautical miles apart (**Figure 1A**). Plants were collected from a meadow in the vicinity of Spiaggia del Poggio (Bacoli) in the Gulf of Pozzuoli (Italy, 40°47.930′ N; 14°05.141′ E) and from a meadow in the surrounding area of Castello Aragonese in the Island of Ischia (Italy, 40°44.114′ N; 13°57.866′ E). Because of their proximity, both locations experienced similar Sea Surface Temperature (SST) regimes. The island of Ischia is a marine protected area since 2007, whereas the Gulf of Pozzuoli is heavily impacted by human activities (Tornero and Ribera d'Alcalà, 2014; Chiarore et al., 2019). The Bacoli sampling site was close to the city of Bacoli and to the one of Baia, an important commercial harbor since Roman age. Sediments in the area of the Bacoli sampling site are classified as sandy with mud being an abundant component (Celia Magno et al., 2012), whereas *P. oceanica* meadows around Ischia are settled on a "matte," a dense mixture of rhizomes, roots and accumulated sediment (personal observation). The marine area around Baia/Bacoli is particularly impacted through dense urban settlements, intense maritime traffic and mussel aquaculture (Appolloni et al., 2018). Additionally, along the region of the Gulf of Pozzuoli, untreated or not appropriately depurated sewage discharges are still present (Margiotta et al., 2020). The different exposure to anthropogenic pressures determines the current conditions of *P. oceanica* meadows at both locations. According to the percentage of N leaf content, a value that integrates the eutrophic status of coastal areas over time (Yang et al., 2018), the site where the Bacoli plants grow appears more eutrophic. The N leaf content value, in fact, was almost twice in Bacoli (%N leaves = 1.89 % ± 0.2; C/N ratio = 16.7 ± 0.9) than in Ischia (%N leaves = 0.97% ± 0.2; C/N ratio = 33.2 ± 2.4) (**Supplementary Table 1**). The same applies to the sediment pore water that also integrates nutrients value over time. The Bacoli site shows values almost double than the Ischia site (DIN [μM] = 47.9 ± 4.4 in Bacoli, and 26.7 ± 8.9 in Ischia site; PO_4^- [μM] = 4.3 ± 1.0 in Bacoli, and 2.1 ± 0.4 in Ischia site; Helber et al., unpublished data). Meadows demography also confirms their different ecological status (Buia et al., 2004). Meadow percentage cover is almost 30% higher in Ischia than in Bacoli. Additionally, in the Bacoli site the shoot density (122.6 ± 70.3 number of shoots per m^{-2}) is half than the minimum value defining a "highly disturbed meadow" (see **Supplementary Table 1**). Rhizomes of Ischia plants were strong and thick, while rhizomes from Bacoli plants were brittle and broke off easily (personal observation). Epiphyte cover on leaves was on average almost 4 times higher in Bacoli compared to Ischia plants (Helber et al., unpublished data). Based on all the above observation, we consider plants collected in Bacoli as more exposed to eutrophic conditions, compared to plants collected in Ischia. Hereafter, we refer to plants collected in Bacoli as relatively eutrophic (EU plants), and plants collected in Ischia as relatively oligotrophic (OL plants). From a genetic point of view, comparative analysis of *P. oceanica* populations at the two sites using 9 microsatellite markers (SSRs) (Jahnke et al., 2015), revealed they are not fully distinct, sharing most of the alleles (F_{ST}: 0.092) and possess a very similar genotypic richness (Ischia, $R = 0.30$; Bacoli, $R = 0.37$) (data not shown).

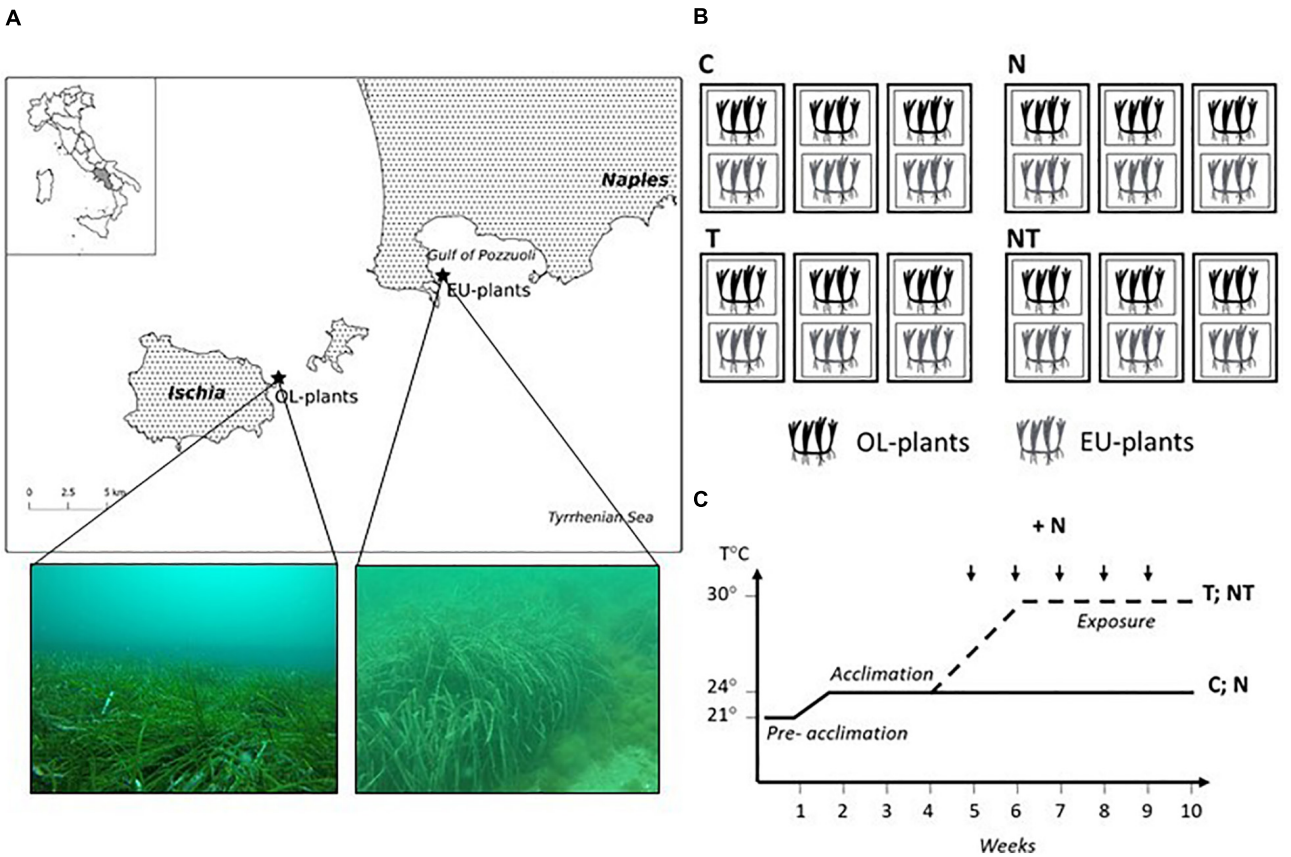

FIGURE 1 | (A) Sampling area for OL (oligotrophic) and EU (eutrophic) plants in the Tyrrhenian Sea. **(B)** Experimental layout of the SZN mesocosms system included three tanks per treatment (C = Control, T = Temperature, N = Nutrients, NT = Nutrients plus Temperature). In each tank, one basket for each EU and OL plants was placed, each basket containing two plant fragments. **(C)** Experimental timeline showing main events: exposure to high temperature (T; NT) and nutrient additions (N; NT) (see text for more details). Arrows indicate periodical nutrient additions to keep nutrients levels within the selected experimental concentrations.

Experimental Design

After collection in the field, plant material was kept in darkened cooler containers filled with ambient seawater and rapidly transported to the indoor mesocosm facility at Stazione Zoologica Anton Dohrn (SZN, Naples, Italy). A detailed description of the experimental mesocosm system can be found in Ruocco et al. (2019b). Two plant fragments consisting of 15–20 connected shoots for each EU- and OL-plants were individually attached to the bottom of one plastic net basket (base 34 × 24 cm, height 10 cm) filled with coarse sediment. Twelve baskets for each site were arranged randomly in 12 glass aquaria (500 L) filled with natural seawater from a nearby-unpolluted area and following an orthogonal experimental design (**Figure 1B**). In this way, plants from both locations were placed in the same tank and in triplicates per experimental treatment/condition. The four treatments were: Control (C), Temperature (T), Nutrients (N) and Nutrients plus Temperature (NT). Stressful environmental factors for our experiment were set according to previous mesocosm experiments and environmental features of sampling sites. Experimental temperature level was selected from the SST data recordings obtained from a MEDA (Monitoring and Environmental Data Unit) type buoy placed in Bagnoli, Gulf of Naples (IRM, RIMAR department, SZN) about three miles far from the sampling site, within the Gulf of Pozzuoli. In years 2017 and 2018, the maximum temperatures reached in August were 30 and 28°C, respectively, whereas the average temperatures measured for the same month were 24.6°C in 2017 and 25.3°C in 2018 (**Supplementary Figure 1**). Thus, it was decided to expose seagrasses to a temperature of 30°C, which is 4–5 degrees above the seasonal average, a sub-lethal temperature level for the species in the mid-term (Traboni et al., 2018). There was an initial pre-acclimation (1 week) into the mesocosms under the environmental conditions found at the time of plant collection (21°C). Then temperature was gradually increased (0.5°C day^{-1}) in all tanks to reach the experimental control temperature of 24°C, at warming rates naturally occurring in late spring/early summer according to MEDA recordings. Plants were allowed to acclimate to the new temperature for an additional week. Then, in T treatment tanks, temperature was gradually increased (0.5°C day^{-1}) until reaching 30°C in a 2-week period (**Figure 1C**). Light was provided by two LED lamps (max. noon irradiance = ca. 400 μmol photons m^{-2} s^{-1} above the canopy; 14:10 h light:dark photoperiod) in accordance to the natural levels measured in the field during

plant sampling; for a detailed description of the LED system and settings see Ruocco et al. (2019b). Seawater was circulated using a 10,000 l/h self-priming pump, allowing continuous seawater circulation and filtration as well as a continuous replacement of water in the system. Additionally, within each aquarium, incoming seawater was spread through a diffuser in order to favor water mixing and to create a homogenous movement of water. Temperature in aquaria was controlled and maintained by cooler/heaters (Teko TK 2000). Salinity was measured daily in each aquarium using a WTW Cond 3310 portable conductivity meter and kept constant (within the range 37.3–37.7) by adding freshwater to compensate for evaporation. Aquaria were cleaned every week, and a 50% (filtered 45 μm and UV irradiated) seawater was also renewed to maintain seawater quality in the system.

According to the literature, NO_3^-, PO_4^{-3} and NH_4^+ levels follow different trends among seagrass meadows with low concentrations at canopy level (0.1 – 4 μM) and higher concentration into sediments, where NH_4^+ can also reach 180 μM (Touchette and Burkholder, 2000, 2002). Dynamics of nutrient concentration in our mesocosm system and frequency of nutrient addition was previously determined in a trial, where nutrient concentration was observed to decrease starting 3 days from the initial spike (data not shown). Hence, we decided to add nutrients weekly, to maintain the value almost constant during the experiment (**Figure 1**). To avoid a sudden increase, the stock solution (170 mM total nitrogen) was added during two consecutive days at the beginning of each week, for a 5-week experimental period (**Figure 1**). We aimed to reach a nominal nutrient concentration of 100 μM (total nitrogen) in the nutrient treatment tanks. Since nutrient concentration was assessed in the tanks at the end of each week, measured values reflected the weekly nutrient consumption in the experimental system. The stock nutrient solution was prepared using Osmocote® Pro (6 months release: 19% N – 3.9% P – 8.3% K, ICL Specialty Fertilizers) fertilizer pellets (Ravaglioli et al., 2017, 2018). Water analysis of dissolved organic nitrogen (DIN) confirmed nutrient enrichment in N and NT treatments (26.8 ± 4.0 μM) and normal levels in C ant T (1.7 ± 1.1 μM). According to the European Environmental Agency for the Mediterranean Sea, total nitrogen levels lower than 2.28 μM are considered "low" (class boundaries concentration is determined by the 80/20 percentiles of the DIN dataset for the years 2007 to 2012), while values higher than 26.0 μM are considered "high" and are indicative of eutrophic waters[1].

Plant Traits

Plant samples for biochemical analysis were collected at the end of 5 weeks and photo-physiology measurements were performed every week to assess the status of plants. In order to reduce within-shoot and within-leaf variability of *P. oceanica* responses to warming (Ruocco et al., 2019a), parameters were measured in the middle portion of young fully developed leaves (second- and third-rank leaves). The aquarium was our true experimental unit, hence measurements performed on shoots of the same aquarium (i.e., 'pseudoreplicates') were averaged to obtain an independent replicated value. Therefore, the number of replicates used in all statistical tests was $n = 3$.

Carbon and Nitrogen Content

The leaf total carbon and nitrogen content was measured on a 6-cm segment collected starting 14 cm above the ligule of the third-rank leaf of four randomly sampled shoots per treatment. The analysis was carried out on epiphyte-free dried and homogenized tissue using an automatic elemental analyser FlashEA 1112 (Thermo Fisher Scientific, Waltham, MA, United States). Acetanilide was used as standard. Carbon and nitrogen content were expressed as a percentage of dry weight and the values were used to calculate the C:N ratio.

Photochemical Efficiency and Chlorophyll Content

Photochemical efficiency was estimated on the basal healthy and epiphytes-free part of leaves (10–15 cm from ligule) of the same shoots selected for morphological and physiological analysis. Chlorophyll *a* fluorescence measurements were conducted with a diving-PAM portable fluorometer (Walz, Germany). Rapid light curves (RLC) were performed after 4 h of illumination to calculate maximum electron transport rates (ETR_m) as an estimate of photosynthetic efficiency. During RLC, a saturation pulse was applied after 20 s incubation at increasing irradiance levels to determine the basal (F) and maximal light-adapted fluorescence (F_m'). These parameters allowed for the calculation of the effective quantum yield (Y). Relative electron transport rate (rETR) at each irradiance level of RLC was calculated according to the formula: $rETR = Y \times E_i \times 0.84 \times 0.5$ (Beer et al., 2014), where Y is the effective quantum yield in the light-adapted state, E_i is the incident irradiance (μmol photons m^{-2} s^{-1}), 0.84 is the default average light absorptance of leaves (Ralph et al., 1998) and 0.5 is the fraction of photons absorbed by PSII in plants (Beer et al., 2014). rETR data (μmol electrons m^{-2} s^{-1}) were plotted against incident irradiance and the resulting curve was fitted to the model described by Eilers and Peeters (1988) to obtain ETR_m values.

Following light-adapted chlorophyll *a* fluorescence measurements, selected leaves were immediately frozen at −80°C for subsequent pigment analysis. Leaf segments (1 cm^2) were homogenized using 80% acetone solution buffered with $MgCO_3$ to prevent acidification of the extract. Then, extracts were stored in the dark at 4°C for 24 h and finally centrifuged (1000 *g* for 10 min at 4°C). The absorbance of extracted pigments was read spectrophotometrically at 470 nm, 646 nm, 663 nm and 725 nm, using 3 mL quartz cuvettes. Total chlorophyll content (chlorophyll *a* and *b* concentrations) was calculated using the equations described by Lichtenthaler and Wellburn (1983) and expressed as μg cm^{-2}.

Leaf Morphology and Relative Growth Rate

Morphometric measurements (leaf length and width) were performed on the same shoots used for photo-physiological analyses and carbon/nitrogen content, using a ruler. The necrotic surface area of leaves was measured considering the portion

[1]https://www.eea.europa.eu/data-and-maps/figures/winter-dissolved-inorganic-nitrogen-ammonium

of leaves with necrotic marks for each shoot and reported as percentage. The remaining surface of healthy tissue was measured and estimated as total photosynthetic surface (TPS, cm^2 $shoot^{-1}$). Two weeks before the end of the experiment, five randomly selected shoots from both EU and OL were marked using the method described by Zieman (1974), i.e., piercing the boundary limit between the leaf and the ligule with a fine needle. Shoots were collected at the end of the experiment to calculate the leaf area of newly growth tissue. Relative growth rate (RGR) was estimated by the ratio between the newly produced leaf area (width × length) and the initial area measured before marking (cm^2 cm^{-2} day^{-1}).

Carbohydrate Content

The content of carbohydrates was analyzed in leaf and rhizome tissues following the method described by Dubois et al. (1951). A portion of 6 cm from the third leaf (epiphytes removed) and a 3 cm of clean rhizome apex were collected from all treatments and plants locations (EU and OL). Leaf and rhizome samples were dried at 50°C for 24 h and the extraction of soluble sugars and starch was performed using a phenol-sulphuric acid reaction. Soluble sugars (sucrose, fructose and glucose) were suspended from 50 mg of the ground tissue using subsequently heated 80% ethanol reactions (80°C for 15 min). Samples were centrifuged (3000 rpm for 10 min) and then soluble sugars were extracted from the supernatant using 3% phenol and sulfuric acid. The absorbance of extract was read spectrophotometrically at 750 nm and 490 nm using a 1 mL quartz-glass cuvette. The analysis of starch content was performed adding 3 mL of NaOH to the remaining solid pellet after the extraction of soluble sugars and stored at 4°C for 24 h. Total non-structural carbohydrates (TNC) were calculated according to Sørensen et al. (2018) and expressed on a dry weight basis (mg g^{-1} DW).

Plant mortality

Plant mortality was assessed as change in shoot number between the beginning (T1) and the end (T4) of the experiment. All shoots were counted and the net change in shoot number was calculated for OL and EU. For graphical representation, average shoot number of the three replicates/treatment was considered and expressed as percentage.

Statistical Analysis

Morphological data (TPS, necrotic area), physiological data (carbon and nitrogen content, photochemical efficiency and chlorophyll content, carbohydrate content), relative growth rate and the shoot mortality were analysed with 3-way analysis of variance (ANOVA) to detect significant differences between the responses of EU and OL plants to experimental treatments (see **Supplementary Table 2**). The model included plants (P, with two levels: EU and OL), temperature (T, with two levels: control and high) and nutrients (N, with two levels: control and high) as fixed factors. When significant differences were found for the factor plants, EU and OL plants were analyzed individually with 2-way ANOVA. In this case, the model was similar to the previous one, including temperature and nutrient as fixed factors (**Table 1**).

Before carrying out ANOVA analyses, normality and homoscedasticity were checked using the Shapiro–Wilk and Levene's tests and data subsequently transformed where necessary. Student-Newman-Keuls (SNK) *post hoc* test was used whenever significant differences ($P < 0.05$) among treatments were detected using the statistical package STATISTICA (StatSoft, Inc., v. 10). All variables measured for EU and OL were plotted in a Principal Component Analysis (PCA) to analyze differences in similarity patterns among treatments and plants with the software PAST v.3.03 (Hammer et al., 2001). A correlation matrix was performed to standardize measurement scales of different variables.

RESULTS

Carbon and Nitrogen Content and Ratio

Leaf nitrogen content was significantly higher in treatments with addition of nutrients (N and the combined NT) compared to control (C) and high temperature (T) treatment in both EU and OL ($P < 0.01$) (**Figure 2A** and **Table 1**). In contrast, carbon content was similar among treatments. *Post hoc* comparisons indicated significant differences in leaf nitrogen content among treatments, where NT (2.84%) and N (2.37%) were significantly greater than C in EU (NT vs. N, $P < 0.05$), but not in T treatment, which was similar to C conditions in both plant groups (T vs. C, $P > 0.05$). This variability in terms of nitrogen content was reflected in the Carbon:Nitrogen (C:N) ratio (**Figure 2B**). C:N ratio decreased with the increase of nitrogen in treatments enriched with nutrients (−70% in N and −45% in NT). This pattern was similar for EU and OL, although EU showed a lower C:N ratio under NT treatment (12%) compared to OL (15%; $P < 0.01$). In the latter, C:N ratio in the NT treatment was similar to the N treatment.

Photochemical Efficiency and Chlorophyll Content

The maximum electron transport rate (ETR_m) differed between OL and EU plants (3-way ANOVA; **Supplementary Table 2**). ETR_m was higher in EU than in OL plants (35.3 ± 2.2 vs. 24 ± 1.9 μmol electrons m^{-2} s^{-1}; $P < 0.01$) (**Figure 3A**). In both OL and EU plants, photochemical efficiency decreased in N and NT treatments compared to controls (N vs. C −23%, NT vs. C −14% in EU; N vs. C −48%, NT vs. C −24% in OL), whereas the presence of high temperature alone did not influence photosynthetic performances. Total chlorophyll (Chl *a* + Chl *b*) content was significantly higher in EU than OL (+12%; $P < 0.05$). In contrast to OL plants, which maintained constant chlorophyll levels in all treatments, EU plants increased their content under nutrient addition (N, +30%) and temperature increases (T, +9%), whereas NT treatments showed similar levels to controls (**Figure 3A**).

Morphology and Relative Growth Rate

The highest percentages of necrotic area was recorded in treatments enriched with nutrients (i.e., N and NT) in both

TABLE 1 | Results of two-way ANOVA analysis in OL and EU for factors "Nutrients" (N) and "Temperature" (T) for nitrogen content (%N), carbon and nitrogen ratio (C:N Ratio), Total chlorophyll, maximum electron transport rate (ETR_m), total non-structural carbohydrates in leaf (TNC leaf) and rhizome (TNC rhizome), % necrotic surface, total photosynthetic surface, relative growth rate and shoot mortality.

ANOVA 2-WAY			**OI-Plants**			**Eu-Plants**		
Variables	**Factors**	**df**	**MS**	***F***	***P***	**MS**	***F***	***P***
%N	**Nutrients (N)**	1	7.26	83.87	**0.00**	6.43	37.93	**0.00**
	Temperature (T)	1	0.42	4.86	**0.04**	1.48	8.73	**0.01**
	N X T	1	0.00	0.00	0.97	0.01	0.07	0.80
	Error	28	0.09			0.17		
C:N Ratio	**Nutrients (N)**	1	1.48	1.20	0.28	404.80	35.94	**0.00**
	Temperature (T)	1	0.75	0.61	0.44	151.48	13.45	**0.00**
	N X T	1	2.09	1.70	0.20	4.22	0.37	0.55
	Error	28	1.23			11.26		
Total chlorophyll content	Nutrients (N)	1	24.68	0.60	0.45	40.95	1.22	0.29
	Temperature (T)	1	25.61	0.62	0.45	76.97	2.29	0.16
	N X T	1	1.37	0.03	0.86	453.43	13.50	**0.00**
	Error	12	41.34			33.60		
ETR_m	**Nutrients (N)**	1	268.02	7.91	**0.02**	190.26	5.54	**0.05**
	Temperature (T)	1	16.98	0.50	0.50	20.45	0.60	0.46
	N X T	1	71.15	2.10	0.19	3.39	0.10	0.76
	Error	8	33.90			34.31		
TNC leaf	**Nutrients (N)**	1	1246.91	5.37	**0.04**	1893.25	11.94	**0.00**
	Temperature (T)	1	63.24	0.27	0.61	15.67	0.10	0.76
	N X T	1	0.91	0.00	0.95	2162.36	13.63	**0.00**
	Error	12	232.41			158.615		
TNC rhizome	Nutrients (N)	1	56.69	0.09	0.77	163.953	0.22	0.65
	Temperature (T)	1	0.35	0.00	0.98	382.626	0.51	0.49
	N X T	1	17.61	0.03	0.87	2897.03	3.84	0.07
	Error	12	612.42			754.437		
% Necrotic surface	**Nutrients (N)**	1	1289.71	12.48	**0.00**	0.83804	14.87	**0.00**
	Temperature (T)	1	509.32	4.93	**0.04**	0.19036	3.38	0.08
	N X T	1	69.10	0.67	0.43	0.13628	2.42	0.14
	Error	16	103.32			0.05634		
Total photosynthetic surface	Nutrients (N)	1	36128.30	23.70	**0.00**	2245.29	0.84	0.37
	Temperature (T)	1	4294.80	2.82	0.11	4148.06	1.55	0.23
	N X T	1	1462.67	0.96	0.34	6748.04	2.53	0.13
	Error	15	1524.65			2668.99		
Relative growth rate	Nutrients (N)	1	0.00	0.01	0.90	2.6E-07	0.29	0.60
	Temperature (T)	1	0.00	1.09	0.31	1.2E-08	0.01	0.91
	N X T	1	0.00	5.05	**0.04**	1.6E-06	1.76	0.20
	Error	16	0.00			8.9E-07		
Shoot mortality	Nutrients (N)	1	11.74	0.09	0.77	87.1574	1.68	0.23
	Temperature (T)	1	151.06	1.20	0.31	527.964	10.15	**0.01**
	N X T	1	9.74	0.08	0.79	208.391	4.01	0.08
	Error	8	125.81			52.03		

Values in bold indicate significant differences ($P < 0.05$).

OL (33 and 27%, respectively) and EU plants (28 and 32%, respectively; $P < 0.01$) (**Figure 3B**). In contrast, the percentage of necrosis was lower in OL plants under T treatment, in respect to the control (−14%; $P < 0.01$). Total photosynthetic surface (TPS) measured in OL displayed an inverted pattern in respect to necrotic area, whereas EU followed a different trend especially for T and NT treatments, where total photosynthetic surface remained similar to control conditions even if necrosis increased (+23 and +13%, respectively; **Figure 3B**). The relative growth rate (RGR) showed a decrease under N (−58%) and T (−30%) in OL plants, although not statistically significant. Growth was affected by the combined treatment (NT) only in EU plants (**Table 1**), even if the *post hoc* comparison was not significant. Statistical analysis revealed no significant differences between plants and treatments (**Figure 3D** and **Supplementary Table 2**).

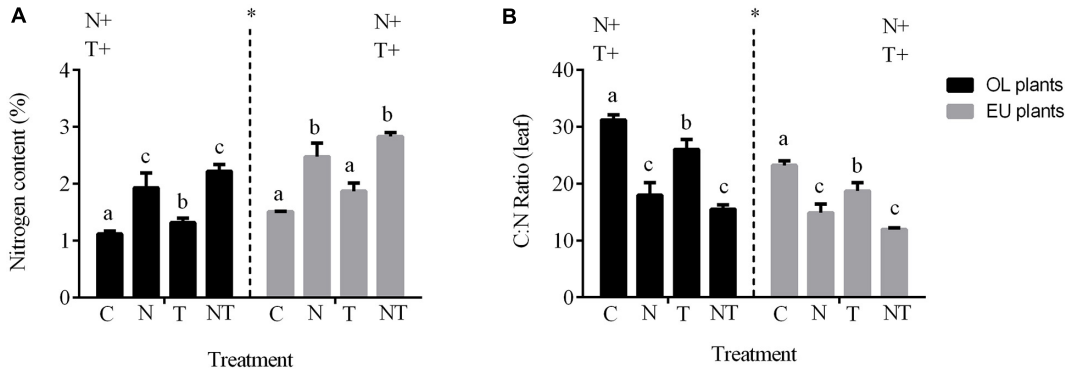

FIGURE 2 | (A) Nitrogen content measured in OL (oligotrophic) and EU (eutrophic) plants and **(B)** C:N ratio after 5 weeks of nutrient (N), temperature (T) and their combination (NT) exposure. Asterisks indicate differences between OL and EU plants (ANOVA 3-way); (+) indicates experimental factors significantly affecting the response variable (2-way ANOVA). Different lowercase letters indicate the groups of homogeneous means obtained in the *post hoc* ($P < 0.05$).

Carbohydrate Content

Total non-structural carbohydrates (TNC) in leaves showed a different pattern compared to rhizomes (**Figure 3C**). In leaves, TNC measured from OL plants decreased in the N (−32%) as well as in the NT treatment (−24%) compared to control ($P < 0.01$). In contrast, TNC decreased in EU plants for all treatments especially under N treatment (−70 %). At rhizome level, EU plants showed the greatest reduction in N treatment (−38%) followed by NT (−10%) (**Table 1**). This is in contrast with OL plants, where no clear trend was observed. TNC were higher in rhizomes compared to leaves in both OL and EU plants (+29 and +45%, respectively).

Shoot Mortality

The net change in shoots measured in EU and OL plants significantly changed during the experiment ($P < 0.01$, 3-way ANOVA; **Figure 4** and **Table 1**). Temperature was the main factor that affected shoot mortality along the experiment ($P < 0.05$). The highest mortality (−41.6%) was observed in EU plants under high temperature conditions (T treatment), followed by NT (−27.9%) and N (−23%). OL plants showed an overall lower mortality. As for EU plants, T induced the highest mortality (−15.7%), followed by NT (−15.6%) and N (−7%). Along the experiment, the highest mortality was observed between T2 and T4 (**Supplementary Figure 2**).

Principal Component Analysis

Considering EU plants, the first PCA axis (PC1), which explains 43.5% of the total variance, clearly placed treatments where nutrients were involved (N and NT) on the negative side of the axis, away from control (C) and temperature (T) treatments that are on the positive side (**Figure 5A**). The second axis (PC2; 25.6% of total variance) segregated nutrient treatment from the combined stress treatment, being positively related with TNC measured in the rhizome and negatively related with the total chlorophylls. The PCA performed on OL plants (**Figure 5B**) showed a different distribution pattern compared to EU plants. The first axis (PC1; 40.8% of total variance) clearly separates temperature from control and the combined treatment. This axis was negatively related with N content (%N) and positively related with TNC measured in the leaf. The second axis (PC2; 20.1% of total variance) segregated nutrient treatment from the combined one even if in this case treatments appeared more widely distributed, with a positive relation with ETR_m and a negative relation with TNC measured in the rhizome.

DISCUSSION

Being sessile organisms, seagrasses must cope with possible changes of local environmental conditions, modifying their morphological and mechanical traits, diverting energy for the maintenance of photosynthesis and changing their physiological traits (La Nafie et al., 2012, 2013; de los Santos et al., 2013; Marín-Guirao et al., 2016; Roca et al., 2016). Our results revealed that plants undergoing chronic cultural eutrophication (EU) are more sensitive to a further increase of nutrients, particularly when in presence of a temperature increase, than plants growing in oligotrophic water conditions (OL). OL and EU plants showed different morphological and physiological performances, which corroborates the role of local conditions in activating different strategies in response to environmental changes.

We also found that temperature and nutrient treatments showed opposite effects when tested individually and an offset response when combined (**Figure 6**). We found antagonistic interactions resulting from the combination of chronic nutrient increase and temperature rise for chlorophyll content and total non-structural carbohydrates in rhizomes, only in plants growing in eutrophic conditions. Intraspecific variations in terms of resistance and performance are widely documented in seagrasses (e.g., Dattolo et al., 2014; Sandoval-Gil et al., 2014; Zhang et al., 2014; Marín-Guirao et al., 2016; Procaccini et al., 2017; Beca-Carretero et al., 2019; Tuya et al., 2019) The differential responses showed in our study further confirm that *P. oceanica* can have different levels of plasticity in response to changes of environmental conditions. According to our results, plants from nutrient enriched conditions, which are very fragile and

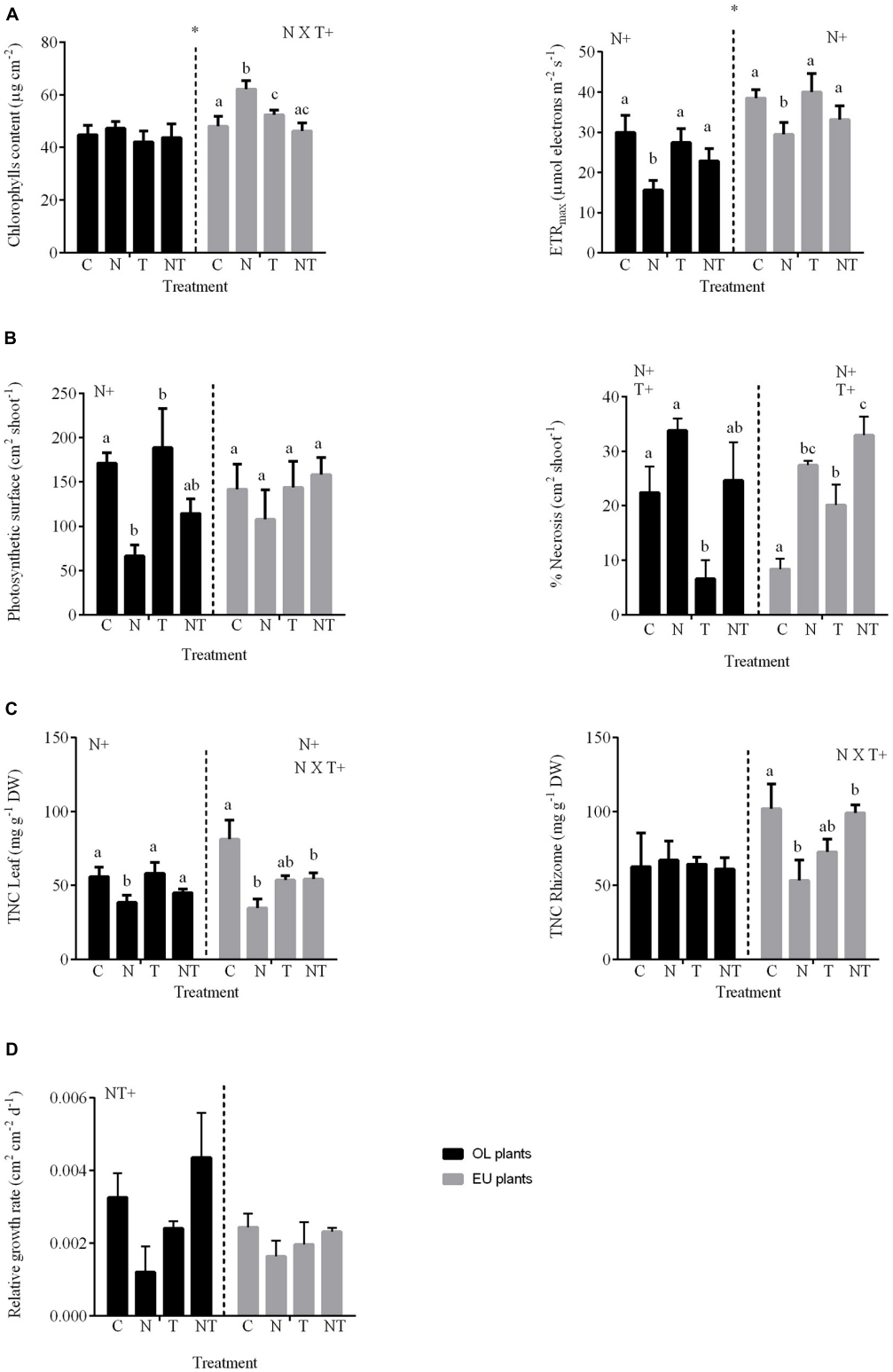

FIGURE 3 | Endpoints measured in OL (oligotrophic) and EU (eutrophic) plants after 5 weeks of the exposure to high temperature and nutrient enrichment. **(A)** Chlorophyll content and maximum electron transport rate (ETR_m); **(B)** Necrotic area and photosynthetic surface; **(C)** Total non-structural carbohydrates (TNC) measured in leaves and rhizomes; **(D)** Relative growth rate. Asterisks indicate differences between OL and EU plants (ANOVA 3-way); (+) indicates factors where significant differences were found between levels of factor after ANOVA 2-way and *post hoc* test. Different lowercase letters indicate the groups of homogeneous means obtained in the *post hoc* ($P < 0.05$).

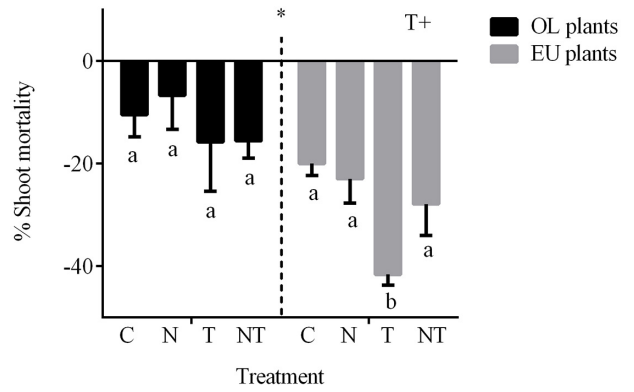

FIGURE 4 | Shoot mortality measured as percentage comparing the mortality between the beginning (T1) and the end of the experiment (T4) in OL and EU plants exposed to nutrient (N), temperature (T) and their combination (NT). Asterisks indicate differences between OL and EU plants (ANOVA 3-way); (+) indicates factors where significant differences were found between levels of factor after ANOVA 2-way and *post hoc* test. Different lowercase letters indicate the groups of homogeneous means obtained in the *post hoc* ($P < 0.05$).

break off easily, may be at higher risk, compared to plants from more oligotrophic conditions. It has also been observed by studies on *Z. noltei* that nutrient enrichment is able to reduce the strength of leaves (La Nafie et al., 2012). Although plants undergoing a particular stress factor could acclimate to the environment or even improve their resistance to a subsequent unfavorable pressure (Alexieva et al., 2003), individuals growing under cultural eutrophication conditions could already be at the limit of their biological capacity. Thus, the high energetic costs for nutrient excess acclimation might have compromised their tolerance to a further nutrient enrichment and to thermal stress. OL plants showed higher C:N values than EU plants, suggesting the presence of specific nutrient-balancing strategies, that may be related to the different nutrient levels chronically experienced by two populations in natural conditions. The activation of contrasting acclimation mechanisms to face high nutrient loads has been already demonstrated in *P. oceanica* exposed to chronic or pulse enrichments in a long-term field experiment (Ruocco et al., 2018). In our experiment, plants living in eutrophic conditions (EU plants) treated with high nutrient concentrations, regardless of temperature, increased the percentage of nitrogen content followed by a decrease of TNC reserves in leaves and rhizomes. A similar response was observed in OL plants, but only for leaves. The reduction of carbohydrate reserves is thought to be related to the assimilation of NH_4^+, which needs C skeletons to create new amino acids, an energetically costly process (Alcoverro et al., 2000). This strategy is more evident in EU plants, where TNC in rhizomes were higher than in leaves, suggesting translocation processes as a response to nutrient enrichment. A possible explanation for the observed TNC reduction only in leaves of OL plants, could be the lack of an active mechanism to cope with nutrient excess due to their low natural nutrient exposure. From a different perspective, since carbohydrate reserves allow *P. oceanica* to maintain leaf growth and to assimilate C and N from the environment (Invers et al., 2004), the absence of carbohydrate reallocation could also be considered as an alternative strategy: as OL plants appeared healthier than the EU ones and their rhizome stronger, probably OL plants reduced carbon reserves in the leaves to cope with nutrient excess, maintaining constant reserves at rhizome level. In contrast, EU rhizomes were weaker due to chronic cultural eutrophication at their natural environment and used more carbohydrates to deal with nutrient assimilation.

Carbohydrate reserves in temperate species are fundamental for survival under low-light conditions, especially during winter (Alcoverro et al., 2001; Soissons et al., 2018). A proportion of the carbohydrates used to produce amino acids during nutrient assimilation will certainly go to the production of other compounds such as chlorophylls. In EU plants under only nutrient treatment, total chlorophyll content increased significantly compared to control and the combined treatment, suggesting that the accumulated amino acids, such as glutamate, were used toward the production of chlorophyll molecules. Similar effects have been reported for seagrasses under low pH conditions and nutrient additions, where the presence of nutrients implied higher concentrations of chlorophylls compared to control (Roca et al., 2016; Ravaglioli et al., 2017). The change in chlorophyll concentrations was not observed in OL plants, indicating once again the lack of response compared to EU plants, which may produce more chlorophylls as a strategy to capture more light in eutrophic (reduced) light environments. Despite a greater concentration of chlorophylls due to the increased nutrient treatment, the photochemical performance (ETR_m) decreased in both EU and OL plants. However, as suggested above, the reduction in light availability may be responsible for this effect, as PSII modulates quickly according to light changes (Beer et al., 2014) and this may not be reflected in biochemical variables. It is important to highlight that ETR_m values in EU plants were overall higher than OL plants.

There is evidence that *P. oceanica* responses to warming vary among plants experiencing different thermal conditions in their natural range and some are able to recover after short-term warming events (Marín-Guirao et al., 2016, 2018). The high temperature treatment in the present study could have been below the thermal tolerance threshold of the sampled individuals, as not many physiological parameters responded to this factor. Optimum growth temperature for this temperate species range from 11.5 to 26°C (Olsen et al., 2012). However, this optimal range probably depends on other co-occurring environmental factors, such as nutrient availability, leaf senescence and nutrient partitioning within plant tissues (Ontoria et al., 2019a). Indeed, photochemical efficiency and carbohydrate reserves of OL plants were not affected by warming in this study. This is in contrast with EU plants, where high temperature led to the reduction of TNC, which reflects the metabolic adjustment of plants accelerating leaf senescence. Different to other parameters measured after 5-week exposure, the shoot mortality under high temperature in EU plants was higher (−41.6%) than OL plants (−15.7%). A possible explanation is that plants exposed to high temperature tried to acclimate to stressful conditions diverting

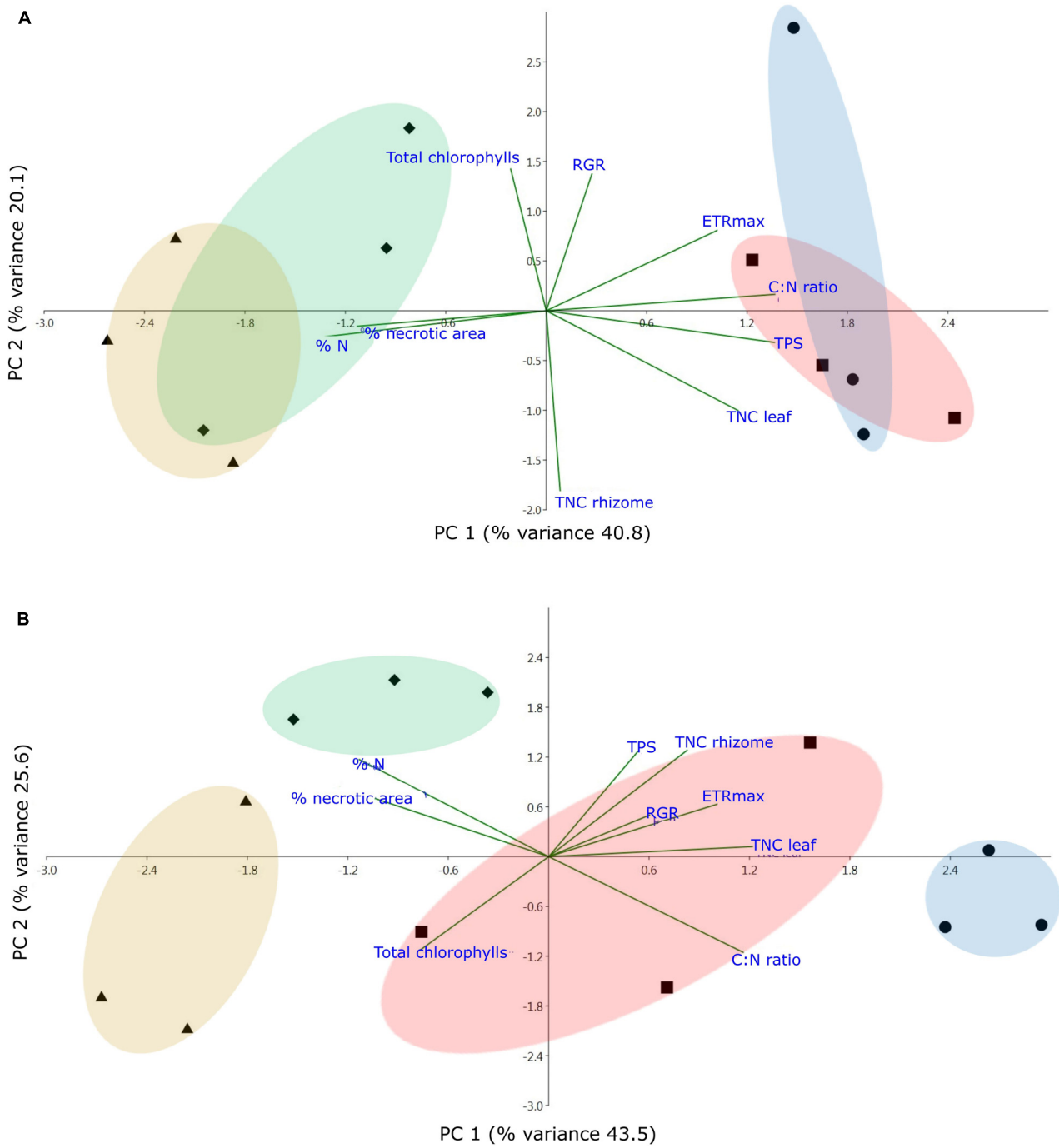

FIGURE 5 | Principal Component Analysis (PCA) performed on **(A)** EU (eutrophic) and **(B)** OL (oligotrophic) plants at the end of the experiment considering all variables: nitrogen content (%N), carbon and nitrogen ratio (C:N), total chlorophyll, maximum electron transport rate (ETR_m), total non-structural carbohydrates in leaf (TNC leaf) and rhizome (TNC rhizome), % necrotic area, total photosynthetic surface (TPS) and relative growth rate (RGR). Different colored circles and symbols refer to different treatments (● = Control; ▲ = Nutrients; ■ = Temperature; ♦ = Nutrients + Temperature).

energy for the maintenance of high metabolic rates (Olsen et al., 2012). Thus, plant growth decreased due to high-energy costs (Marín-Guirao et al., 2018). Accordingly, we found lower relative growth rates (RGR) in EU plants and reductions of RGR in nutrient and temperature treatments for OL plants. This is in agreement with previous studies performed on *P. oceanica*, where shoot survival decreased after exposure to high temperature (Marín-Guirao et al., 2018) and high nutrient (Ceccherelli et al., 2018) concentrations, respectively. While the physiological changes may be subject to a rapid regulation, the observed

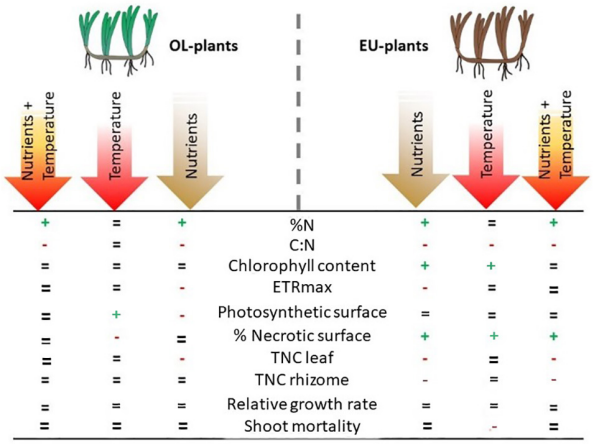

FIGURE 6 | Summary of the overall physiological responses of *P. oceanica* from oligotrophic (OL) and eutrophic (EU) environments to nutrient excess, temperature increase and their combination. Signs (+, −,=) reflect statistical significance and direction of the difference in respect to the control.

mortality may indicate that temperature could be a determinant factor in the long-term survival of the whole ramet.

Few multi-factorial experiments have been performed on *P. oceanica*, including some assessing nutrient (ammonium) input and temperature (Gera et al., 2013; Ontoria et al., 2019a). In these studies, different levels of synergism have been found between stress factors, influencing negatively the survival, photosynthesis and plant growth. In contrast, we found antagonistic interactions resulting from the combination of chronic nutrient increase and temperature rise for pigment content and TNC in EU and for growth in OL. Indeed, both EU and OL plants exposed to the combined stressors showed similar leaf nitrogen contents as plants that were only exposed to increased nutrients, with no change in TNC at rhizome level. This suggests that despite nutrient excess negatively affected plant performance, the simultaneous exposure to increased temperature accelerated metabolic rates to cope with the impact induced by nitrogen assimilation. These results are in agreement with those obtained by Egea et al. (2018) on *Cymodocea nodosa* plants, where the simultaneous exposure to NH_4^+ enrichment and the high temperature had an antagonistic response. In contrast, Guerrero-Meseguer et al. (2020), found a synergistic effect of temperature increase in *P. oceanica* seedlings development when occurring concomitantly with other stressors, such as seed burial and grazing. In our experiment, plants were adversely affected by the combined treatment although the overall evidence showed a less negative effect in comparison to single nutrient and temperature treatments for the physiological parameters. Similarly, in *Zostera capensis* it was shown that nutrient enrichment and warming had a limited interaction, and that eutrophication was a stronger stressor (Mvungi and Pillay, 2019). It would be interesting to assess if *P. oceanica* seedlings would show similar response since Artika et al. (2020) found that in *E. acoroides* seedlings rely more on seed nutrient resources and are less affected by experimental nutrient addition.

Overall, our results suggest that ongoing eutrophic conditions in the Gulf of Pozzuoli are weakening the local *P. oceanica* meadow, since these plants showed the greatest percentage of mortality, particularly under increased temperature. Our findings do not seem to be a general rule for seagrasses, since Connolly et al. (2018) found the opposite trend in *Z. muelleri*. In that study, plants growing in more disturbed sites were more resilient to further disturbance, although with a lower genotypic diversity, suggesting a genotypic selection. In our analysis, the two studied meadows are only slightly distinct and did not show differences in genetic or genotypic variability, indicating that factors other than genotypic selection can explain our results. The data we presented indicate that eutrophication is likely inducing severe effects on the local seagrass meadows, which have activated physiological strategies to cope with excess of nutrients. However, the lack of replication of relative EU and OL populations of *P. oceanica* in our experiment precludes the extrapolation of our findings to other than the studied populations. The strategy adopted by these plants probably implied large energetic costs affecting their present and future persistence. Considering the high level of cultural eutrophication of a large part of the coastlines, and taking into account the predicted increase of SST and heat waves frequency, our results highlight the intraspecific vulnerability to environmental changes of seagrass meadows and the importance of performing studies at a local scale. Unraveling intraspecific strategies and the role of local acclimation/adaptation in the response to multiple stressors could be crucial for seagrass conservation strategies under a climate change scenario (Duarte et al., 2018). It becomes fundamental to perform multi-factorial experiments on seagrass populations from different environments, in order to understand their resilience to future local and global environmental changes and to support management strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JP, AS-S, LM-G, and GP conceived and designed the experiments. SH performed initial field assessment. MR contributed to the analysis of carbohydrates and assessment of plant morphology. JP and AS-S performed the experiment, all the other analyses and drafted the manuscript, and all authors reviewed it critically.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.564805/full#supplementary-material

Supplementary Figure 1 | Sea surface temperature (SST) data recordings for years 2017 and 2018 obtained from a MEDA (Monitoring and Environmental Data Unit) type buoy placed in Bagnoli, Gulf of Pozzuoli (IRM, RIMAR department, SZN).

Supplementary Figure 2 | Net shoot change measured as percentage of T0 (beginning of the experiment) in OL and EU plants after one (T1), two (T2), three (T3) and five (T4) weeks exposure to nutrient (N), temperature (T) and their combination (NT).

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CHAPTER III

(Paper III)

Jessica Pazzaglia, Alex Santillán-Sarmiento, Miriam Ruocco, Luca Ambrosino, Emanuela Dattolo, Lazaro Marín-Guirao, Gabriele Procaccini. Local environment modulates the whole-transcriptome expression in the seagrass *Posidonia oceanica* under warming and nutrients excess.

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Local environment modulates the whole-transcriptome expression in the seagrass *Posidonia oceanica* under warming and nutrients excess

Jessica Pazzaglia[1,2], Alex Santillán-Sarmiento[1,3], Miriam Ruocco[1], Luca Ambrosino[4], Emanuela Dattolo[1], Lazaro Marín-Guirao[1,5*], Gabriele Procaccini[1*#]

[1] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy

[2] Department of Life Sciences, University of Trieste, 34127, Trieste, Italy

[3] Faculty of Engineering, National University of Chimborazo, Riobamba, Ecuador

[4] Department of Research Infrastructure for Marine Biological Resources, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy

[5] Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, Murcia, Spain

*these authors contributed equally to the paper

#corresponding author: gpro@szn.it

Abstract

The intensification of anomalous events of seawater warming and the co-occurrence of local anthropogenic stressors are threatening coastal marine habitats, including seagrasses, which form extensive underwater meadows. Among the others, eutrophication highly affects coastal environments, potentially summing up to the widespread effects of global climate changes. In the present study, we investigated for the first time in seagrasses, transcriptional responses of different plant organs (i.e., leaf and shoot apical meristem, SAM) of the Mediterranean seagrass *Posidonia oceanica* growing in environments with a different history of nutrients enrichment. To this end, a mesocosm experiment exposing plants to single (nutrient enrichment or temperature increase) and multiple stresses (nutrient enrichment plus temperature increase), was performed. Results revealed a differential transcriptome regulation of plants under single and multiple stressors, showing an organ-specific sensitivity depending on plants' origin. While leaf tissues were more responsive to nutrients stress, SAM revealed a higher sensitivity to temperature treatments, especially in plants already impacted in their native environment. The exposure to stress conditions induced the modulation of different biological processes. Plants living in an oligotrophic environment were more responsive to nutrients compared to plants from an eutrophic environment. Evidences that epigenetic mechanisms were involved in the regulation of transcriptional reprogramming were also observed in both plant' organs. These results represent a further step in the comprehension of seagrass responses to abiotic stresses pointing out the importance of local pressures in a global warming scenario.

Keywords: Seagrasses, multiple stressors, global warming, eutrophication, gene expression, epigenetics

Introduction

Coastal marine environments are among the most threatened marine habitats (Worm et al., 2006). The continuous increase of human urbanization along the coastline, with the extensive use of marine resources and services, has amplified the number and diversity of anthropogenic stressors. Among different local pressures, eutrophication due to nutrient inputs from land-based pollution sources (e.g. agriculture, urban/industrial development and aquaculture) is one of the greatest concern for coastal habitats, especially for environments characterized by dense urbanization such as most of the Mediterranean basin (Liquete et al., 2016). The dominant components of nutrients inputs are nitrates and phosphorus that are considered the main nutrients sources intensifying water hypoxia and acidification, as a consequence of phytoplankton and microbial proliferation (Gobler and Baumann, 2016). Additionally, different indirect effects are linked to nutrients increase such as the reduction of light penetration along the water column, which compromises biological performances of photosynthetic organisms and in general the benthic production (Touchette and Burkholder, 2000). In an Era of global warming, the effects induced by these local disturbances can be much more complex depending on their interaction with ongoing climate changes, which are globally threatening marine ecosystems (He and Silliman, 2019; Nguyen et al., 2021). The intensification of anomalous events of seawater warming and the increase of sea surface temperature at unprecedented rates can induce synergic or antagonistic effects when more eutrophic conditions occur (Ceccherelli et al., 2018; Paerl and Scott, 2010). Thus, local pressures may have the potential to exacerbate or buffer the effects of climate change on marine habitats (Bowler et al., 2020). Understanding how marine organisms can overcome the potential cumulative impacts by multiple stresses is becoming of fundamental importance especially for sessile organisms such as marine plants (Micheli et al., 2013).

Seagrasses are marine angiosperms belonging to the order of *Alismatales*, representing a unique group of higher plants that re-colonized marine environments, forming extensive underwater meadows (Les et al. 1997). These habitat forming-species provide important services and benefits to ecosystems and human livelihoods (Nordlund et al., 2018). Similar to their terrestrial counterpart, seagrasses have a high carbon storage capacity, which underlines their potential contribution to climate change mitigation (Duarte et al. 2013; Gattuso et al. 2018). Despite their importance, seagrasses are declining globally at alarming rates (Waycott et al., 2009). New projections estimate a huge reduction of marine habitat-forming species as a consequence of global warming by the end of 2050, stressing that environmental changes are occurring too fast, preventing their capacity to react properly (Trisos et al. 2020).

The evolutionary success of marine plants derive from their extraordinary adaptation capacity, which allowed them to colonize heterogeneous environments including temperate and tropical regions with different environmental conditions (Short et al., 2007). Single species display peculiar strategies from physiological to gene expression rearrangements for adapting along wide bathymetric and latitudinal gradients (Dattolo et al. 2017; Jahnke et al. 2019). These emerging plastic properties that characterize some seagrass species are at the basis of the appearance of different phenotypes according to local environmental settings (Bergmann et al., 2010; Franssen et al., 2011; Pazzaglia et al., 2020; Soissons et al., 2017). Among seagrasses, *Posidonia oceanica* (L.) Delile is an iconic species widely distributed in the Mediterranean basin forming large meadows across the photic zone (Telesca et al., 2015). Being one of the oldest-living organisms on our planet and due to the prominent clonal propagation, *P. oceanica* is an ideal target species for studying plastic responses to environmental changes (Arnaud-Haond et al., 2012).

Molecular signatures at the basis of phenotypic responses to single stressors have been explored in seagrasses, especially in relation to different light and thermal regimes (Dattolo et al. 2017; Marín-Guirao et al. 2016; Massa et al. 2011; Ruocco et al. 2021). In general, large-scale gene expression studies in response to abiotic stresses have revealed the regulation of specific stress genes that modulate different phases of the cellular stress response, as protein folding and degradation (Franssen et al. 2011; Reusch et al. 2008; Traboni et al. 2018). Warming, in particular, can also induce oxidative stress enhancing the accumulation of reactive oxygen species (ROS) causing membrane, protein and DNA damages leading to homeostatic imbalance. Under such conditions, seagrasses activate their antioxidant system, which includes key ROS-scavenging enzymes (Franssen et al. 2014; Purnama et al. 2019; Traboni et al. 2018; Tutar et al. 2017; Winters et al. 2011). Additionally, photosynthesis is one of the most heat-sensitive processes and the modulation of genes encoding for crucial enzymes of the photosynthetic apparatus is part of the machinery that regulates primary metabolism under heat stress (Marín-Guirao et al. 2017; Ruocco et al. 2019a; Wang et al. 2018). In seagrasses, the analysis of transcriptional profiles in populations experiencing diverse thermal regimes in their home environments has revealed differential responses, reflecting the contribution of local adaptation to gene expression divergence (e.g. Franssen et al. 2011). Thus, plants living in more dynamic and variable environments (e.g. southern regions and shallow waters) showed higher thermal tolerance and can be more resilient to environmental changes than plants living in more stable environments (Ashander et al., 2016; Botero et al., 2015; Chevin and Hoffmann, 2017; Pazzaglia et al., 2021; Tomasello et al., 2009).

While modulation of gene expression in seagrasses under thermal stress has been extensively investigated (Nguyen et al., 2021), much less emphasis has been given to gene-expression changes in response to high nutrients conditions. Most of the literature is focused on nutrient assimilation and physiology, pointing out the importance of leaf tissues in nutrient uptake (Touchette and Burkholder, 2000). Direct effects induced by the excess of nutrients on growth and survival have been showed in seagrasses (Burkholder et al. 2007), while mechanisms behind nutrient toxicity and gene expression regulations are still unclear.

NH_4^+ is the primary form of nitrogen that can be assimilated by seagrasses, through high- or low-affinity transporters, depending on external nutrients concentrations. Since the assimilation of nutrients differs among above- and below-ground tissues, this is also reflected in the regulation of specific responsive genes that tend to be activated earlier in the leaf in respect to below-ground tissues (Pernice et al., 2016). In *P. oceanica*, the regulation of genes playing a key role in nutrient assimilation is influenced by the co-occurrence of other kind of stressors, such as herbivory (Ruocco et al., 2018) and acidification (Ravaglioli et al., 2017). All this highlights that interactions among different stresses and local disturbances need to be considered for a complete understanding of the effects of global changes on seagrasses. However, only few studies have investigated the effects of nutrients in a global warming scenario, focusing mainly at plant physiological responses (Artika et al., 2020; Campbell and Fourqurean, 2013; Mvungi, 2011; Pazzaglia et al., 2020).

Epigenetic mechanisms, such as chromatin modifications have recently been recognized to play a crucial role in gene regulation in response to abiotic stressors (Bhadouriya et al., 2021; Lindermayr et al., 2020). Chromatin accessibility can be regulated by the exclusion or inclusion of different histone variants and various histone modifications (e.g., acetylation/deacetylation, methylation/demethylation) can be influenced by environmental variations. In plants, chromatin modifications induced by specific environmental stress can regulate the transcriptional machinery at somatic level (within the same generation), and have the potential to be stored or memorized for future reoccurring events (Bäurle and Trindade 2020; Dai et al. 2017; Kumar et al. 2017; Tasset et al.

2018). While epigenetic changes have been extensively investigated in terrestrial plants, they remain mostly unexplored in seagrasses. Indeed, only few studies have recently analysed epigenetic responses to abiotic stressors, especially DNA methylation marks (*P. oceanica*, Greco et al 2012; Greco et al. 2013; Ruocco et al., 2019b; Entrambasaguas et al., 2021; *Zostera marina*, Jueterbock et al. 2019; *Posidonia australis* and *Zostera muelleri*, Nguyen et al. 2020).

The present study aims to investigate the transcriptome rearrangements occurring in *P. oceanica* plants with a different history of nutrient loads and exposed to single and multiple stressors. Starting from previous physiological assessments (Pazzaglia et al., 2020), here we proceeded with a further step, exploring the whole transcriptome profile of leaf and shoot-apical meristem (SAM) in plants with a different origin, and provided a functional characterization of biological processes activated in response to temperature increase, nutrients addition, and their combination. In general, the SAM is considered the most sensitive plant organ with a lowest tolerance threshold, playing a crucial role in the maintenance of growth and survival under abiotic and biotic stresses (Fulcher and Sablowski, 2009). Recently, a gene expression study performed on SAM revealed the activation of an early molecular response in respect to the leaf, besides a much more complex and specific response (Ruocco et al., 2021). We hypothesize that leaves and SAMs of plants growing in environments with a different history of nutrient loads would show a divergent gene expression signature and the activation of specific biological processes in response to the same stress conditions. We also expected different effects induced by nutrients and thermal stresses, which should modulate the transcriptional profile of *P. oceanica* plants. Furthermore, since epigenetic mechanisms are involved in gene regulation, we also expected different activation of related processes. Overall, we aim to assess plant response in a future scenario of local human driven pollution and global increase of seawater temperature.

2. Methods

2.1 Plant collection and experimental design

The sampling sites and the experimental design for this study are the same of Pazzaglia et al. (2020). Briefly, large fragments of *P. oceanica* bearing 10-20 vertical shoots were collected by SCUBA diving on May 15 – 16th 2019 from shallow-water meadows growing in two locations with different history of nutrient loads: Spiaggia del Poggio (Bacoli) in the Gulf of Pozzuoli (Italy, 40 47.9300 N; 14 05.1410 E), and Castello Aragonese in the Island of Ischia (Italy, 4044.1140N; 1357.8660 E). The former (Bacoli) is considered an impacted site as it is close to a highly urbanized area with more eutrophic conditions in respect to the later site (Ischia), which is in a marine protected area (for a comprehensive description of sampling sites see Pazzaglia et al., 2020). Because plants growing in the two sites were exposed to different anthropogenic pressures, here we refer to plants collected in Bacoli as relatively eutrophic (Eu plants), and plants collected in Ischia as relatively oligotrophic (Ol plants). After sampling, plants were exposed to multiple stressors in an indoor mesocosm facility at Stazione Zoologica Anton Dohrn (SZN, Naples, Italy) (Ruocco et al. 2019a) following a multi-factorial design, including four treatments: Control (C), Nutrients (N), Temperature (T) and Nutrients + Temperature (NT). The experimental set-up consisted of 12 glass aquaria (500 L) filled with natural seawater. Two plant fragments for each Eu- and Ol- plants were allocated in the same tank using a basket filled with coarse sediment. Stress levels were set according to a previous mesocosm experiment and different environmental observations at the sampling sites (Pazzaglia et al. 2020). The temperature treatments (T and NT) consisted in the gradual increase (0.5 C day^{-1}) of temperature from control conditions (measured during the sampling, 24°C) to 30°C, which is 4–5 degrees above

the summer average. The nutrient treatments (N and NT) consisted in the increase of nutrient concentrations adding a stock solution (170 mM total nitrogen) that was prepared using Osmocote Pro fertilizer pellets (6 months release: 19% N – 3.9% P – 8.3% K, ICL Specialty Fertilizers). The solution was added every week in order to maintain a nutrient enrichment condition in N and NT treatments (DIN = 26.8 ± 4.0 mM).

2.2 RNA extraction and 3'Tag sequencing

After two weeks from the initial exposure to stress conditions (T2), three samples per treatment of *P. oceanica* leaf and shoot-apical meristem (SAM) were collected (n = 3). A portion of 6 cm of the second leaf was cleaned from epiphytes and immediately submerged in RNA later© tissue collection solution (Ambion, life technologies). Leaf samples were kept at 4 °C overnight to let the solution penetrate into the tissue, and finally stored at - 20 °C. The first most apical 0.5 cm of the rhizome tip, containing the SAM, were also collected from the same shoots and preserved in liquid N_2, since previous trials demonstrated that RNA later solution does not permeate appropriately in the meristem tissue. Total RNA was extracted with the Aurum™ Total RNA Mini Kit (BIO- RAD). RNA purity and concentration was assessed by using NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies) and 1% agarose gel electrophoresis, while RNA integrity was assessed by means of 2100 BioAnalyzer (Agilent). Twenty-four libraries (3 replicates × 4 treatments × 2 different plant conditions) were constructed for each tissue (24 leaf and 24 SAM) with the QuantSeq 3' mRNA-Seq Library Prep Kits (Lexogen) and sequenced using Ion Torrent technology (Ion Torren GeneStudio). The QuantSeq protocol produces only one fragment per transcript, generating reads towards the poly (A) tail. In contrast to the traditional RNA-Seq, TagSeq approach directly reverse transcribed cDNAs from the 3′ end of the mRNAs, without a fragmentation step. It represents a cost-effective approach applicable to model species and has also been successfully applied to non-model for which reference transcriptomes are available (Marx et al., 2020; Moll et al., 2014). Hereinafter, we refer to leaf and SAM of Ol plants as 'Ol leaf' and 'Ol SAM', respectively, and to leaf and SAM of Eu plants as 'Eu leaf' and 'Eu SAM', respectively.

2.3 Data filtering and functional annotation

Raw reads were quality checked using FASTQC (Andrews, 2010) and then subjected to a cleaning procedure using Trimmomatic (Bolger et al. 2014), setting the minimum quality per base at 15 phread score and minimum length of the read after cleaning at 50bp. All cleaned reads were then mapped, independently, on the reference transcriptome of *P. oceanica* (Ruocco et al., 2021) using the Bowtie2 aligner (default settings, Langmead and Salzberg, 2012). Reads count and FPKM (fragments per kilobase of exon model per million reads mapped) calculation per transcript for each replicate were performed using the eXpress software (Roberts et al., 2011). Functional annotation of the reference transcriptome was carried out through sequence similarity search against the Swiss-Prot database using the BLASTx software (Camacho et al., 2009), setting as minimum *E*-value threshold $1e^{-3}$ and getting only the best hit detected.

2.4 Differentially Expressed Genes (DEGs) and Gene Ontology (GO) enrichment analysis

DEGs analysis was performed using two tools implementing two different statistical approaches: DESeq2 (Love et al. 2014) and edgeR (Robinson et al. 2010). For each transcript, the mean of the $\log_2$ fold change values (Log_2FC) obtained with the two tools was calculated. The thresholds for the DEGs calling were FDR $\leq$0.05 or *P*-adjusted $\leq$0.05, and Log_2 fold change $\leq|1.5|$. Differential gene expression profiles resulted from the comparison between all treatments (N, T and NT) *vs* control in

both organs and plant conditions. A graphical representation of shared and unique DEGs across samples was obtained using DiVenn 2.0 interactive tool (Sun et al., 2019). DEGs-related GO-terms were retrieved by using InterProScan (version 5.33, Jones et al., 2014) and G.O. enrichment analysis was performed using the Ontologizer software (Bauer et al., 2008). The threshold used to identify significantly enriched functional terms was $P \leq 0.05$. DEGs and GO enrichment results are discussed separately for leaf and SAM organs, comparing Ol and Eu plants. GO enriched terms for both Ol and Eu plants are reported in the supplementary table S3 and S4. Additionally, GOs enriched terms related to epigenetic mechanisms (epi-GOs) were screened for leaf and SAM organs independently from the treatments, and unique/shared biological processes and molecular functions for Ol and Eu plants are explained separately.

3. Results

3.1 General overview of transcriptomic responses

Different transcriptomes obtained for both tissues of plants collected in different environmental conditions (Ol leaf, Ol SAM, Eu leaf and Eu SAM) showed a comparable number of transcripts and significantly matched to Swiss-Prot database (**Table 1**). DEGs results are included in the supplementary **Table S1**, whereas GO terms associated with biological processes, cellular components and molecular functions obtained for all treatments are reported in the **Table S2**.

Table 1. *Summary description of the number of transcripts for each dataset (N = Nutrients, T = Temperature, NT = Nutrients + Temperature). The % of annotated transcripts for each dataset is also showed (BLASTx).*

Unique datasets	**N. of transcripts**				**% of annotated transcripts**
	N	**T**	**NT**	**Tot.**	
Ol leaf	10,016	10,024	10,014	30,054	66.4
Ol SAM	10,037	10,012	10,014	30,063	67.2
Eu leaf	10,020	10,013	10,029	30,062	65.7
Eu SAM	10,015	10,177	10,041	30,233	66.6

3. 2 Leaf-specific transcriptomic responses

3.2.1 Differentially expressed genes (DEGs) and GO enrichment analysis

Leaf showed the largest transcriptomic response in treatments with nutrients addition (N and NT), whereas a less severe effect was observed under the increase of only temperature (T), which is similar between Ol and Eu plants (**Fig. 1**). However, while Ol leaf showed the highest percentage of DEGs in N treatment, Eu leaf appeared more responsive to NT (**Fig.1**). The comparison of up and down-regulated DEGs among treatments, highlighted a larger and unique transcriptome rearrangement occurring in the leaf under nutrients addition, in particular in Ol plants exposed to N (Fig. 2 and Fig. 3), where most of the unique DEGs were up-regulated (**Fig. 2a; Table S1**). Contrarily, T treatment induced only a limited and less specific response (**Fig 2a**). Eu leaf displayed a distribution pattern of DEGs similar to Ol leaf, with larger counts of unique DEGs under N and NT (higher in NT), in comparison to T treatment (**Fig. 2b, Table S1**).

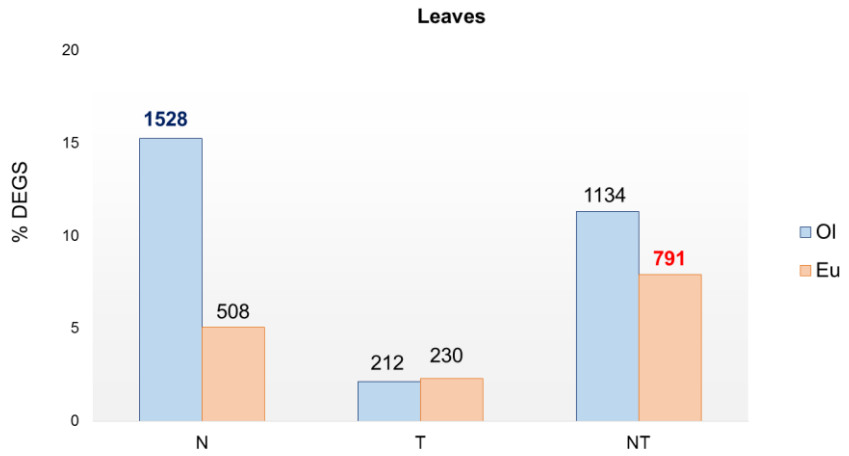

Figure 1. *Percentages of DEGs (down and up-regulated) over the total number of transcripts counted for each unique dataset (Ol leaf and Eu leaf). The total n° of DEGs is shown on the top of each histogram. The greatest n° of DEGs are underlined in bold with different colours for Ol (blue) and Eu plants (red).*

The GO enrichment analysis of the leaf revealed similar patterns in both Ol and Eu plants, activating more processes under nutrient additions (N and NT, **Fig. 3**; **Table S2**). However, unique GO enriched terms found in Ol leaf under N conditions were twice of those counted for Eu leaf for the same treatment (**Fig. 3a**, **supplementary Table S3**). In Ol leaf, different transcripts belonging to the transport category like *Nuclear transport factor 2B (NTF2)* and *Zinc transporter 4 (ZIP4)* were overexpressed in presence of nutrients (N and NT) (**Table S1**). One of the most significant GO enriched term in the N treatment was related to "protein kinase activity" including enzymes involved in protein degradation such as *Putative U-box domain- containing protein 50 (PUB50)* and the *RING-H2 finger protein (ATL13)* that were up- and downregulated respectively. Ol leaf activated also defence processes regulating e.g., *Leucine-rich repeat-like serine/threonine/tyrosine protein kinase (SOBIR1)* and the *Stromal cell-derived factor 2-like protein (SDF2).* In addition, DEGs of NT and N treatments shared different GO terms including "photosynthesis", pointing out the downregulation of genes that play a crucial role in photosystem assembly and functions (*HCA6-Chlorophyll a-b binding protein CP26, PSBS-Photosystem II 22 kDa protein 1*). The presence of nutrients activated also processes related to metabolism like "nitrogen cycle metabolic process" and "reactive nitrogen species metabolic processes", where key genes of nitrate assimilation were downregulated (*NR2-Nitrate reductase [NADH] 2* and *NRT2.5-High affinity nitrate transporter 2.5*). Several transcripts within this category were also upregulated in NT, including key enzymes involved in the lipid biosynthesis pathway like the *Allene oxide synthase 1 (AOS), Delta(8)-fatty-acid desaturase 2 (SLD2)* and S*NF1-related protein kinase regulatory subunit beta-1 (AKIN subunit beta-1)* (**Table S1**). In this treatment (NT), Ol leaf activated also processes related to flavonoid synthesis (i.e. *Chalcone and Squalene synthase*). The exposure exclusively to temperature (T) induced the lowest activation of specific biological processes (**Fig. 3a; Table S2**). In this case, Ol leaf regulated processes related to defence mechanisms and Ubiquitin-conjunctions ("regulation of biological quality, chaperone binding") that include transcripts encoding for positive regulators of basal defence such as *Protein SGT1 homolog A and B* that were downregulated. In general, few processes were shared among all treatments, mostly including categories related to metabolisms ("oxidoreductase activity", "small molecule metabolic process") and flavonoids ("flavonoid biosynthetic process" and "flavonoid metabolic process").

Similarly, Eu plants showed the highest counts of GOs uniquely enriched in treatments with nutrients addition, especially in the combined treatment (NT, **Fig. 3b**; **Table S3**). In this case, "structural constituent of chromatin", "oxidoreductase activity" and "generation of precursor metabolites and energy" were the most significant categories (**Table S3**). Genes belonging to these terms are involved in the modulation of chromatin structure (*HMGBs, high mobility group proteins*), mitochondrial electron transport chain (*Cytochrome c oxidase subunit 1, COX1* and *Ubiquinol oxidase 1b, AOX1B*), and starch synthesis (*Glucose-1-phosphate adenylyltransferase small subunit 1, AGPC*), and were highly downregulated. In contrast to Ol plants, in Eu leaf different processes related to transcriptional regulation were also activated in the presence of only nutrients (N, *regulation of nucleobase-containing compound metabolic process* and *transcription).* Different Transcription factors (TFs) belonging to these categories were differentially regulated, including transcriptional activators such as *WRKY22-transcription factor 22* and *MED16- Mediator of RNA polymerase II transcription subunit 16* that were downregulated, and the *SARD1- Protein SAR DEFICIENT 1* which was upregulated. The exposure to T treatment induced a less pronounced response activating processes involved in stress responses and photosynthesis ("photosystem", "phosphoprotein binding" and "carbohydrate derivative binding"). Associated genes encode for chaperone proteins (*HSP70-1- Heat shock 70 kDa protein 1*) and photosystem proteins (*PSBS1-Photosystem II 22 kDa protein 1*). Overall, treatments shared common processes related to transport and defence activities ("nitrate transport", "small molecule metabolic process", "reactive nitrogen species metabolic process") downregulating genes involved in the response to nitrate (*Protein NRT1/ PTR FAMILY 6.4, NIA2- Nitrate reductase [NADH] 2*) and oxidation (*DOX1- Alpha-dioxygenase 1*).

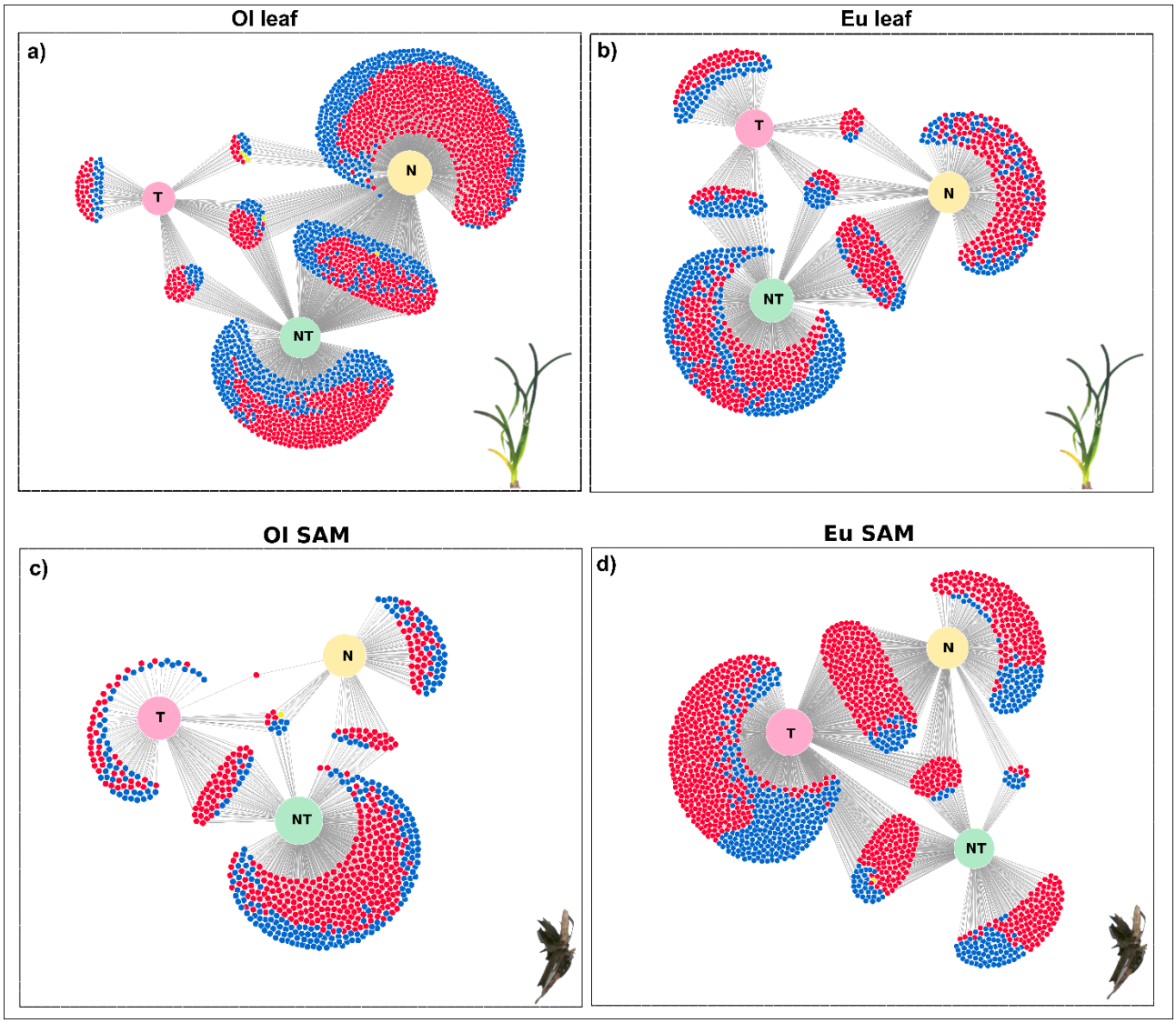

Figure 2. *DiVenn diagrams showing unique and shared differentially expressed genes (DEGs) among treatments (N = Nutrients, T = Temperature and NT = Nutrients + Temperature) in Ol leaf (a), Eu leaf (b), Ol SAM (c) and Eu SAM (d). Red and blue nodes refer to up- and down-regulated DEGs respectively, whereas yellow nodes refer to shared DEGs among treatments that were up-regulated in one sample but down-regulated in another one.*

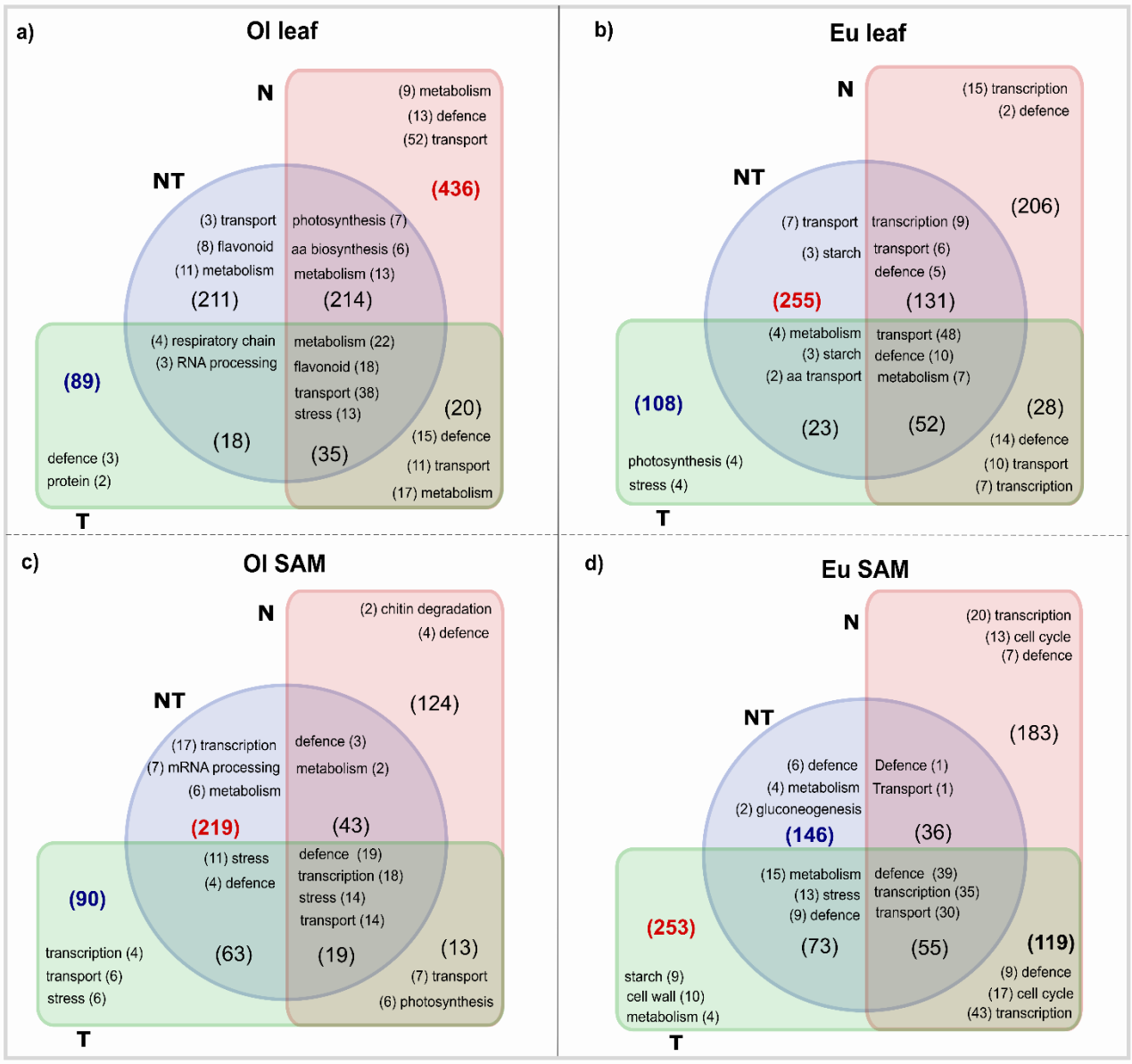

Figure 3. *Venn diagrams showing unique and shared GO enriched terms in Ol leaf (a), Eu leaf (b), Ol SAM (c) and Eu SAM (d). The number of unique and shared GOs are shown in brackets. Red and blue numbers identified the largest and lowest counts, respectively. The number of DEGs associated to the most significant GOs were also reported in brackets with the associated category which corresponds to keywords derived by the Retrieve/ID mapping tool of UNIPROT database.*

3.3 SAM-specific transcriptomic responses

3.3.1 Differentially expressed genes (DEGs) and GO enrichment analysis

Contrary to leaf, SAM showed a greater response to temperature treatments (T and NT) with clear differences between Ol and Eu plants (**Fig. 4**). While Ol plants showed the higher counts of DEGs under the combined treatment (NT), Eu plants revealed a huge gene activation under the exposure to only temperature (T), followed by N and NT treatments (**Table S1)**. Differences in terms of DEG

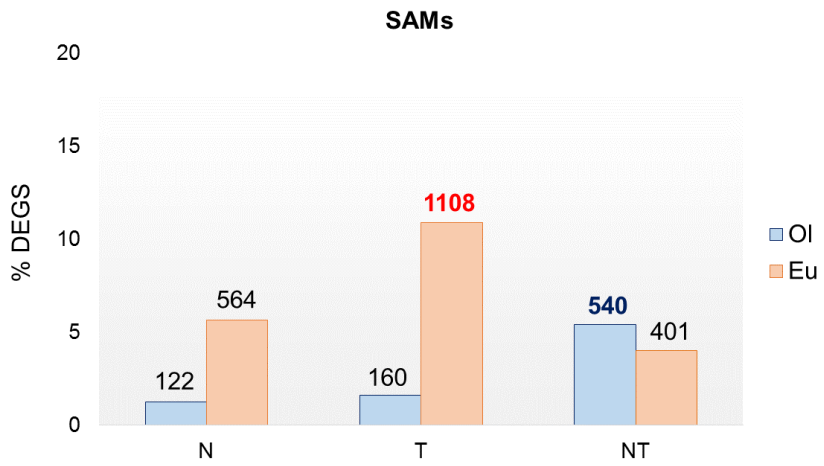

Figure 4*. Percentages of DEGs (down and upregulated) normalized by the total number of transcripts counted for unique datasets (Ol SAM and Eu SAM). The total n. of DEGs is shown on the top of each histograms. The greatest counts of DEGs are underlined in bold with different colors for Ol (blue) and Eu plants (red).*

distributions among treatments in Ol and Eu plants were more evident for SAM organs (**Fig. 2**). Ol SAM showed a higher number of DEGs under NT treatment that were mostly up-regulated (**Fig. 2c**; **Table S2**). On the other hand, T treatment induced the highest transcriptomic response in Eu SAM, sharing most of DEGs with N treatment (**Fig. 2d; Table S2**). Eu plants expressed a lower number of DEGs in the combined treatment (NT), that were mostly shared with T treatment.

Surprisingly, SAM response to treatments was less pronounced with respect to the leaf, with a general lower number of distinct enriched GOs terms (**Table S2**). However, GO terms and related processes in the SAM were significantly different between Ol and Eu plants (**Fig. 3**; **Table S2**). In detail, Ol SAM responses were more pronounced in treatments with nutrients (N and NT), highlighting the downregulation of different transcripts mostly related to defense mechanisms, like *Alpha-dioxygenase (DOX1)* and *Nodulin-related protein 1 (NRP1)* (**Table S1**). In Ol SAM, "aminoglycan metabolic process", "cell wall macromolecule metabolic process" and "chitinase activity" were the most significantly enriched terms in N treatment, where other similar processes related to nutrients-induced stress ("cellular response to nitric oxide") were shared with NT treatment (**Fig. 3c; Table S3**). Notably, distinct processes related to transcription were activated in NT ("gene expression") modulating TFs involved in gene expression regulation like *Transcription factor MYB7*, which was up regulated, and *Protein LNK1* and *SWI/SNF complex component SNF12* that were repressed. Different processes related to stress response were also shared among NT and T treatments ("unfolded protein binding" and "heat shock protein binding") with the expression of key genes encoding for chaperone proteins (*HSP83, HSP90-5* and *Chaperonin CPN60-1*). T treatment induced a less pronounced response, which is in contrast to Eu SAM where the presence of temperature alone showed the largest number of unique GOs enriched terms (**Table S3**). At these conditions, Eu SAM activated processes mainly related to starch synthesis ("glucose-1-phosphate adenylyltransferase activity" and "starch biosynthetic process") and cell wall biogenesis ("cellular carbohydrate metabolic process"). DEGs related to these categories, all overexpressed, are key genes involved in starch synthesis (*AGPP-Glucose-1-phosphate adenylyltransferase small subunit 2, WAXY - Granule-bound starch synthase 1* and *ISA3-Isoamylase 3*) and cell wall construction (*XTH28-Probable xyloglucan endotransglucosylase* and *CSLD5- Cellulose synthase-like protein D5*) (**Table S1**). Contrarily to Ol SAM, Eu SAM shared most of the GO enriched terms with N treatment where the most representative categories were related to transcription ("protein-DNA complex", "DNA

binding" and "chromatin"). Here, associated DEGs include different histone variants (*H2B, H3.2, H3.3*) and several TFs belongs to different families with transcription regulatory activities (*MYBS2, BHLH35, NFYB5, HHO5*) (**Table S1**).

3.4 Insights into epigenetic regulation

Different unique epigenetics-related GOs (epi-GOs) were activated in treatments with nutrients in both Ol and Eu leaves (**Table 2**). In Ol plants, leaf and SAM activated unique epigenetic-related functions (**Fig S1a** and **b**). In detail, Ol leaf regulated processes related to "RNA methylation activity" and "methylated histone binding" that included the largest count of associated transcripts (**Table 2**). Here, important chromatin remodelers and RNA methyltransferases were overexpressed especially under nutrients stress conditions (*Chromatin remodeling protein, Putative tRNA (cytidine(32)/guanosine(34)-2'-O)-methyltransferase*). In Ol SAM, different unique epi-GOs related to terms such as "chromatin organization" and "histone modification" were the most representative biological processes including the largest counts of transcripts (**Table 2**). Associated DEGs included DNA methyltransferase (*DNA (cytosine-5)-methyltransferase DRM1*) and chromatin remodelers (*CH5-Protein CHROMATIN REMODELING 5*), which were upregulated under T treatment.

Contrarily to Ol plants, Eu leaf and Eu SAM shared several processes related to DNA binding functions. Regulated genes in Eu leaf belong to the category of "sequence-specific DNA binding" which showed the largest counts of transcripts (**Table 2**). In such a case, different DEGs involved in transcription regulation were regulated in treatments with nutrients like *WRKY transcription factor 22* and *SARD1-Protein SAR DEFICIENT 1* that were highly overexpressed, and *ALKBH10B-RNA demethylase* which was repressed in the treatment with only nutrients (N, **Table S1**). In Eu SAM, "chromatin binding" was the most representative molecular function considering the number of associated transcripts (**Table 2**). Here, genes involved in transcription regulation were differentially expressed such as *AHL16-AT-hook motif nuclear-localized protein 16* which was overexpressed under single treatments (N and T), and DNA methylation including *MET1-DNA (cytosine-5)-methyltransferase*) that was upregulated in N and NT (**Table S1**).

Table 2. *Unique and shared GOs enriched terms related to epigenetic mechanisms in Ol plants (leaf – SAM) and Eu plants (leaf – SAM). The GO identification (GO ID), category (GO cat.), description, P value and the number of associated transcripts are reported.*

Ol leaf					Eu leaf				
GO ID	**GO cat.**	**GO description**	**P value**	**N. Transcripts**	**GO ID**	**GO cat.**	**GO description**	**P value**	**N. Transcripts**
GO:0102741	MF	paraxanthine:S-adenosyl-L-methionine 3-N-methyltransferase	4.10E-08	6	GO:0031062	BP	positive regulation of histone methylation	2.42E-02	137
GO:0004161	MF	dimethylallyltranstransferase activity	9.65E-03	20	GO:0070989	BP	oxidative demethylation	9.51E-03	34
GO:0002128	BP	tRNA nucleoside ribose methylation	9.81E-03	37	GO:0070734	BP	histone H3-K27 methylation	3.03E-02	126
GO:1990258	BP	histone glutamine methylation	1.09E-02	9	GO:0061087	BP	positive regulation of H3-K27 methylation	4.42E-02	46
GO:0035064	MF	methylated histone binding	2.29E-02	192	GO:0031058	BP	positive regulation of histone modification	2.60E-02	203
GO:1990259	MF	histone-glutamine methyltransferase	2.39E-02	9	GO:0035513	BP	oxidative RNA demethylation	1.38E-04	28
GO:0008898	MF	S-adenosylmethionine-homocysteine S-methyltransferase	2.42E-02	43	GO:0043982	BP	histone H4-K8 acetylation	3.29E-02	22
GO:0008173	MF	RNA methyltransferase	3.96E-02	618	GO:0043565	MF	sequence-specific DNA binding	2.34E-04	4743
-	-	-	-	-	GO:0035515	MF	oxidative RNA demethylase activity	4.66E-04	28
-	-	-	-	-	GO:0043984	BP	histone H4-K16 acetylation	1.30E-02	14
-	-	-	-	-	GO:0080182	BP	histone H3-K4 trimethylation	4.02E-02	68
Ol SAM					**Eu SAM**				
GO ID	**GO cat.**	**GO description**	**P value**	**N. Transcripts**	**GO ID**	**GO cat.**	**GO description**	**P value**	**N. Transcripts**
GO:0016576	BP	histone dephosphorylation	1.58E-04	13	GO:0035404	BP	histone-serine phosphorylation	2.64E-02	16
GO:0006325	BP	chromatin organization	3.63E-03	2963	GO:0009008	MF	DNA-methyltransferase activity	2.65E-02	71
GO:0031498	BP	chromatin disassembly	4.52E-03	6	GO:0003682	MF	chromatin binding	9.49E-03	946
GO:0032986	BP	protein-DNA complex disassembly	5.04E-03	7	GO:0006342	BP	chromatin silencing	5.39E-04	273
GO:0140658	MF	ATP-dependent chromatin remodeler activity	5.49E-03	361	GO:0000819	BP	sister chromatid segregation	3.22E-02	515

GO:0009008	MF	DNA-methyltransferase activity	1.33E-02	71
GO:0051052	BP	regulation of DNA metabolic process	1.56E-02	645
GO:0000018	BP	regulation of DNA recombination	2.17E-02	204
GO:0006304	BP	DNA modification	2.71E-02	663
GO:0008172	MF	S-methyltransferase activity	2.95E-02	67
GO:0016570	BP	histone modification	2.98E-02	1628
GO:0016569	BP	covalent chromatin modification	3.48E-02	1649
GO:0003886	MF	DNA (cytosine-5-)-methyltransferase activity	3.50E-02	47
GO:0000792	CC	heterochromatin	3.14E-02	114
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-	-	-	-	-
-	-	-	-	-
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-	-	-	-	-
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GO:0061712	MF	tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase	9.00E-05	15
GO:0006346	BP	DNA methylation-dependent heterochromatin assembly	4.86E-02	51
GO:0071824	BP	protein-DNA complex subunit organization	1.57E-03	776
GO:0035600	BP	tRNA methylthiolation	2.72E-04	18
GO:0035174	MF	histone serine kinase activity	3.99E-02	14
GO:0071204	CC	histone pre-mRNA 3'end processing complex	4.23E-02	16
GO:0065004	BP	protein-DNA complex assembly	1.34E-04	617
GO:0070828	BP	heterochromatin organization	1.98E-02	204
GO:0034401	BP	chromatin organization involved in regulation of transcription	1.51E-02	441
GO:0000785	CC	chromatin	8.92E-03	1910
GO:0006306	BP	DNA methylation	4.39E-02	509
GO:0031938	BP	regulation of chromatin silencing at telomere	9.09E-03	1
GO:0003886	MF	DNA (cytosine-5-)-methyltransferase activity	3.50E-02	47

Eu Leaf – Eu SAM

Go ID	GO cat.	GO description	P value	N. Transcripts
GO:1903231	MF	mRNA binding - posttranscriptional gene silencing	1.92E-02	5
GO:0044815	CC	DNA packaging complex	7.35E-04	239
GO:0032993	CC	protein-DNA complex	1.32E-02	471
GO:0150100	MF	RNA binding - posttranscriptional gene silencing	1.23E-02	5
GO:0003677	MF	DNA binding	3.92E-02	11285
GO:0006333	BP	chromatin assembly or disassembly	1.07E-02	431
GO:0030527	MF	structural constituent of chromatin	2.58E-07	16

4. Discussion

Here we describe, for the first time in seagrasses, the whole-transcriptome response of different organs (leaf and shoot apical meristem) of plants (i.e. *P. oceanica*) living in two contrasting environments with a different history of nutrient loads and exposed to single and multiple stressors. Our comparative transcriptomic analysis provides clear evidences for an effect of the local (native) environment in determining/influencing the ability of the species to cope with global stress factors, in agreement with previous physiological and morphological evidences (Pazzaglia et al. 2020). The exposure to single and multiple stresses differentially affected plants' transcriptomic response and highlighted an organ-specific vulnerability of plants depending on their origin. Leaf appeared to be more responsive in presence of nutrients whereas SAM organs showed more vulnerability to temperature treatments. Below, principal outcomes from leaf and SAM analyses are discussed separately, considering the effects of treatments and plant origin.

4.1 The effects of local environments in driving different response to stress

4.1.1 Leaf vulnerability to stress conditions

A large transcriptomic reprogramming was activated in leaves of plants coming from both oligotrophic (Ol) and eutrophic (Eu) environments, when exposed to high nutrient loads alone or in combination with warming (Fig. 5). The exposure to only warming induced instead a less pronounced response, which is in line with physiological responses reported in Pazzaglia et al. (2020), where the presence of nutrients induced the greatest effects on both Ol and Eu *P. oceanica* plants. This is probably due to the high nutrient affinity of leaves, which bear the primary responsibility for the assimilation of dissolved inorganic nitrogen (e.g., NH4+ and NO3-) in the species (Lepoint et al., 2002; Romero et al., 2006). Contrary to terrestrial plants, seagrasses live in more oligotrophic environments and the maintenance of high productivity through high nutrient incorporation is operated by Na+-dependent nitrate, phosphate and amino-acids transport systems that favour nutrient assimilation from the surrounding environments, regulating plants' nutrient budget (Alcoverro et al. 2000; Rubio et al. 2018). In our study, transcriptomic responses to nutrient enrichment also differed in plants according to their origin. Thus, leaves of plants from oligotrophic conditions (Ol) showed a more complex transcriptome reprogramming under nutrient enrichment than leaves from eutrophic conditions (Eu). The number of DEGs was indeed more than four times higher in Ol leaves than in Eu leaves.

Ol plants required a considerably higher level of gene expression regulation in treatments with nutrients, activating processes related to transport activities to cope with the new stress condition. These plants downregulated high affinity nitrate transporters (NRTs and NIAs), which can be interpreted as a need to prevent the excess of nutrient assimilation. Similar strategies have been already observed in terrestrial plants, where the excess of nutrients modulated the assimilation of nitrate through an inhibitory mechanism that temporally blocks its activity favouring the subsequent adaptation to stress conditions (Reyes et al., 2018; Stitt et al., 2002). Moreover, different modulation of NRTs has already been observed in *P. oceanica* plants exposed to different temporal regimes of nutrient loading (Ravaglioli et al. 2017; Ruocco et al. 2018). Ruocco et al. (2018) showed that the leaves of plants under discrete/pulse nutrient addition enhanced the activity of genes involved in nitrate uptake and reduction (NRT2 and NR); while the leaves of plants chronically exposed to nutrient additions repressed the expression of these genes. This regulatory mechanism allowed plants to take advantage of pulse nutrients events, while their down-regulation was considered as a strategy adopted by plants to avoid excessive nitrogen uptake and assimilation. Other low affinity nitrate transporters were overexpressed in both Ol and Eu leaves, which could explain the higher nitrogen

content previously measured at the end of the experiment (Pazzaglia et al 2020). The excessive assimilation of nitrates by Ol leaf induced the modulation of processes related to reactive nitrogen species activating defence mechanisms that are typically involved in plants responses to abiotic stresses. Genes functioning as E3 ubiquitin ligase like PUB50 and ATL13 were up- and down-regulated respectively under high nutrient conditions. These genes are reported to participate in many cellular functions, playing a role in the regulation of abiotic and biotic stresses and in the modulation of hormone signalling (Seo et al., 2012; Sharma and Taganna, 2020; Yee and Goring, 2009). In addition, Ol leaf specifically regulated processes related to flavonoid synthesis that are representative of stress-induced conditions in *P. oceanica* plants (Migliore et al., 2007). In this experiment, leaves exposed to the combination of nutrients addition and temperature increase showed an up regulation of Squalene and Chalcone (CHL) synthases, which could reveal a different degree of sensitivity by leaves in comparison with the exposure to only nutrients. Chalcones are key enzymes of the flavonoid biosynthesis pathway in angiosperms (Heglmeier and Zidorn, 2010; Hu et al., 2019; Mannino and Micheli, 2020). They play important roles in plant defence against biotic and abiotic stress factors (e.g., UV light and pathogens, (Dao et al., 2011). The induction of CHLs expression depends on environmental stimuli resulting in the accumulation of secondary metabolites (Besseau et al., 2007). The overexpression of these genes suggest the presence of an altered natural metabolism in Ol plants that could be the result of the accumulation of reactive oxygen species (ROS) (Fini et al., 2011). In line with this evidence, high nutrient levels impaired the photosynthetic performance of Ol plants, down-regulating components of light harvesting complexes (e.g., LHCA6) and subunits of the photosystem II (e.g., PSBS). For these genes, a differential regulation was already observed in *P. oceanica* plants from meadows with different light regimes and exposed to reciprocal light conditions (Dattolo et al. 2017). In that case, the variation in light availability induced plants to adopt contrasting photo-acclimatory strategies to improve the utilization of the available light, maintaining a high photosynthetic efficiency (Dattolo et al. 2014, 2017). Ultimately, Ol plants experiencing for the first time acute eutrophic conditions, suffered more than Eu plants that have faced direct and indirect effects of eutrophic waters during their life history (Pazzaglia et al., 2020).

By contrast, leaves of Eu plants were less responsive to the presence of only nutrients, while a largest transcriptomic modulation was observed in the combined treatment. Since these plants already experienced nutrients stress conditions in their local environments, they appeared more vulnerable when nutrients were combined with temperature increases, and thus in the presence of new stress typology that required a large transcriptomic response. However, the variation in nutrients availability induced substantial transcriptomic reprogramming of different transcription factors, as already reported in model plant species (Brumbarova and Ivanov, 2019). On the other hand, in the combined treatment, Eu leaf regulated processes related to the generation of precursor metabolites and energy, where a key gene involved in starch synthesis (AGPC) was down regulated. This gene synthetizes ADP-glucose from glucose 1-phosphate and ATP which is required as a glucose donor for starch synthesis in the plastid (Patron et al., 2004). Starch synthesis plays an important role in plant metabolism supporting growth and productivity under abiotic stresses (Thalmann and Santelia, 2017). The regulation of starch biosynthesis observed in Eu leaf suggests that these plants instead of activating large metabolic processes to counteract stress from nutrient excess modulated their energetic reserves to provide more energy for sustaining growth (Marín-Guirao et al. 2018; Krasensky and Jonak, 2012). Eu leaf also regulated genes with oxidoreductase activity (COX1 and AOX1) under the combined treatment. In *P. oceanica* plants, heat stress modulated the expression of alternative oxidase 1a (AOX1) which plays a key role in the maintenance of the redox homeostasis in the mitochondrial respiratory chain (Marín-Guirao et al. 2017; Ruocco et al. 2019a; Tutar et al. 2017). Furthermore, other transcripts involved in the regulation of the Salicylic acid (SARD1), which is a

defence hormone for local and systemic acquired resistance in plants (Zhang et al., 2010), was up regulated in the presence of nutrients. All these evidence support the existence of a regulatory defence machinery in plants that had already experienced stress conditions in their local environments, giving prominence to different strategies adopted by plants to counteract stress conditions previously observed in Pazzaglia et al. (2020).

4.1.2 SAM response to single and multiple stresses depends on plants' origin

The transcriptomic response of shoot apical meristems (SAMs) was less pronounced and differed substantially from the response of leaves in the experimental treatments, which contrasts with the pattern observed for the same species under severe light limitation (Ruocco et al., 2021). In addition, while the leaf transcriptomic response was mostly triggered by nutrients, the SAM mainly responded to warming with differences between Ol and Eu plants (Fig. 5). Eu SAM was more responsive to temperature alone, while in Ol SAM the strongest transcriptomic response was observed in the combined treatment (NT). Transcriptional profiles followed opposite patterns in Ol SAM and Eu SAM, especially in terms of activated processes. While Ol SAM was more responsive to NT, showing a lower vulnerability to T, Eu SAM showed a huge activation of specific processes in T, whereas NT induced the lowest response.

Stress categories related to chaperon activities (*unfolded protein binding* and *heat shock protein binding*) were among the most representative ones in Ol plants under temperature treatment, and in Eu plants under both T and NT treatments, where also metabolic processes were highly differentially regulated. In Ol SAM, temperature induced the over-expression of Heat shock proteins (HSPs) that are a group of highly conserved proteins involved in the protection of cells against harmful consequences of a diverse array of stresses (Beere, 2004). This evidence is in line with previous studies performed on *P. oceanica*, where HSPs were upregulated in response to heat stress (Marín-Guirao et al. 2016; Ruocco et al. 2021; Ruocco et al. 2019b; Traboni et al. 2018). Different HSPs were also regulated in Eu SAM as a stress response shared between N and T treatments. Particularly in this case, more transcripts encoding for HSPs were highly regulated, confirming the higher vulnerability to temperature increase of Eu plants. Although heat stress signals are particularly evident in Eu plants, important processes related to cell wall construction and starch metabolism appeared to be modulated under warming conditions. In Eu SAM, different enzymes involved in starch metabolism were overexpressed (e.g., AGPC, ISA3 and WAXY). Their regulation in Eu plants suggests that these plants were energetically active to contrast thermal stress and therefore they modulated carbohydrate metabolism to provide more energy. This evidence could also explain carbohydrate modulation previously observed at the rhizome level only for Eu plants (Pazzaglia et al. 2020).

In agreement with this evidence, Eu SAM also overexpressed key genes involved in cell wall biogenesis and organization, including cellulose synthase (CSLD5) and xyloglucan endotransglucosylase/hydrolase (XTH28). In terrestrial plants, these genes have a fundamental role in load-bearing cell wall framework, showing also different regulation to environmental stimuli (Sasidharan et al., 2014; Xu and Huang, 2000; Yan et al., 2019). In fact, the integrity of cell wall provides important mechanical strengths to counteract abiotic stresses (Kesten et al. 2017). These findings support the fact that Eu plants were metabolically active especially in the presence of a new stress factor. However, this strategy probably implied large energetic costs, especially under chronic exposure to stress conditions that could explain the huge increase of shoot mortality observed in the T treatment several weeks later, at the end of the experiment (-40%, Pazzaglia et al. 2020). Stress responses observed in SAMs also confirmed the high sensitivity of the shoot apical meristem to acute

stresses already detected in *P. oceanica* under different experimental conditions (Ruocco et al., 2020). Furthermore, the transcriptomic profiles of the SAMs observed in the present study revealed different levels of response, which depends on the stress typology. The molecular pattern observed after two weeks of the initial exposure to stresses may also be considered as an anticipatory signal of physiological and morphological response observed at the end of the experiment. Similarly, the altered expression of stress-related genes anticipated morphological changes and population collapse in *P. oceanica* under eutrophication and burial stress (Ceccherelli et al., 2018).

4.2 Evidence of gene expression regulation due to epigenetic mechanisms

In seagrasses, little is known about the role that epigenetic mechanisms have in driving gene expression responses to environmental stimuli. Only few studies have suggested that epigenetic mechanisms are involved in the regulation of stress responses in marine plants, pointing out their potential role in the regulation of phenotypic plasticity to environmental changes (Entrambasaguas et al., 2021; Jueterbock et al., 2019; Marín-Guirao et al., 2017, 2019; Nguyen et al., 2020; Pazzaglia et al., 2021; Ruocco et al., 2019b). Additionally, epigenetic marks could also be linked to the ability for creating a stress-memory in plants pre-exposed to stress (Nguyen et al., 2020), and different epigenetic states exists among different plant tissues as well as among portions of different age of the same tissue (Ruocco et al. 2019b). Here, Ol and Eu plants showed a substantial regulation of processes related to chromatin modifications in both leaf and SAM organs. In particular, epigenetics mechanisms were mostly activated in organs where Ol and Eu plants showed the largest transcriptomic modulation, suggesting a potential epigenetic regulation in gene expression responses to stresses.

Ol leaf mainly regulated genes involved in the modification of the chromatin structure. Chromatin remodelling complexes are conserved proteins that harbour ATPase/helicase of the SWITCHING DEFECTIVE2/SUCROSE NON-FERMENTING2 (SWI2/SNF2) to control DNA accessibility regulating gene expression (Clapier and Cairns, 2009). Recently, these complexes were also found to regulate nitrate responsive genes in maize (Meng et al., 2020). In that case, the core subunit of the SWI/SNF-type ATP-dependent chromatin remodelling complex interacted with high affinity nitrate transporters repressing their expression in the presence of nitrate supply. Similarly, Ol leaf increased the expression of transcripts encoding for chromatin remodelling proteins under high nutrient conditions. As mentioned above, nutrients induced the greatest transcriptomic response in Ol leaf and most of the genes involved in epigenetic modifications were differentially expressed under such conditions. Although it is hard to find a functional relation between gene expression changes and epigenetic variations, this study provides new insights into the potential key role played by chromatin modifications in the regulation of target genes under environmental disturbances. Likewise, different GO enriched terms related to chromatin remodelling and modifications were also observed in Eu plants. These plants showed a great transcription regulation under stress conditions, especially in the SAM where different transcription factors were shared between N and T treatments. Notably, processes related to protein-DNA binding and chromatin modifications were modulated in response to single stresses. In this case, gene encoding for AT-hook motif nuclear localization (AHL) proteins, which belongs to a family of transcription factors, was overexpressed in N and T. The AT-hook motif is a small DNA-binding motif, which recognizes specific DNA structures activating or inhibiting the expression of different genes (Nagano et al., 2001). In plants, it is overexpressed under various abiotic stresses, including drought, salinity and temperature (Zhou et al., 2016). Furthermore, in Eu SAM, different histone variants were mostly regulated under single stressors (H2B, H3.2, H3.3), where the larger number of DEGs was observed. In *A. thaliana*, histone proteins, especially H3.3 was found to be preferentially enriched in the 3’ end of the transcribed regions, which was also related to gene

body methylation (Wollmann et al., 2017). Further observations revealed that the recruitment of these complexes induced transcriptional reprogramming during the differentiation of plant cells in response to biotic and abiotic stresses (Tripathi et al., 2015). In this study, eutrophic (Eu) plants activated transcriptional reprogramming to contrast nutrient stress for counteracting also the negative effect induced by the exposure to a new stress factor, which was temperature. Similar regulation involving physiological, genetic and epigenetic responses was previously observed in *P. oceanica* plants during warming (Marín-Guirao et al., 2019). In that case, plants showed altered expression levels of genes involved in epigenetic modifications that are at the intersection between stress tolerance and flowering processes. As stated by the authors, this regulation could be related to different response mechanisms adopted by plants to survive to warming conditions. Moreover, it is worth to underline that stable epigenetic states regulating phenotypic variations can be inherited across generations favouring stress memorization (Bruce et al., 2007). Since plants previously exposed to stress stimuli can store stress information to be primed and more active to cope the reoccurrence of stress events (Bäurle and Trindade 2020; Friedrich et al., 2019), this study provide epigenetic signatures that could suggest the existence of a transcriptional memory in plants that had already experienced stressful conditions due to local pressures.

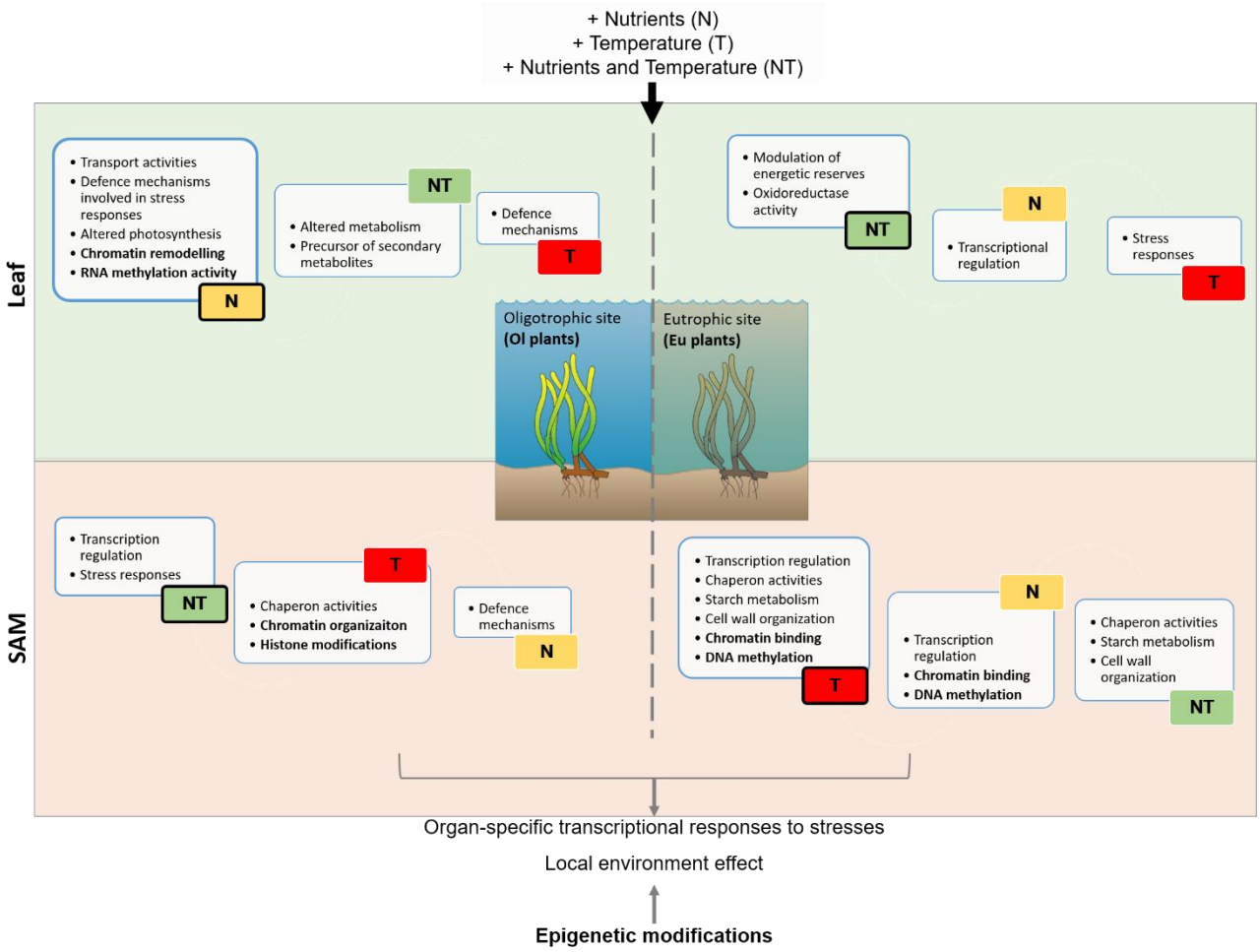

Figure 5. *Summary description of main results for leaf and SAM in Ol and Eu plants exposed to single (nutrients addition and temperature increases) and multiple stresses (nutrients addition plus temperature increases). In the leaf of Ol plants, N induced the greatest transcriptomic reprogramming followed by NT and T, contrary to the SAM where NT induced the larger transcriptomic regulation. In Eu plants, leaf showed a greatest reprogramming under NT followed by N and T, while the SAM showed a larger transcriptomic regulation in T. Transcriptomic data revealed an organ-specific vulnerability to stresses, which depends on*

the local environmental conditions with the potential role of epigenetic regulation (see the main text for more detail).

5. Conclusions and perspectives

In conclusion, the present work represents a further step in the comprehension of *P. oceanica* responses to single and multiple stresses. The transcriptomic profiles of plants under single and multiple stress conditions provide a valuable playground for further studies and future insights on the response of marine plants to realistic and complex scenarios as those already occurring under the framework of climate change. Local pressures experienced by plants in their home environment have a marked influence on plants' transcriptional responses under unprecedented stress conditions, influencing their ability to withstand current and future challenges. This study also highlighted an organ-specific vulnerability to stress, with a higher sensitivity of the leaf to high nutrients addition, in contrast to SAM that was more responsive to temperature increases. This contrasting sensitivity/responsiveness opens the possibility to improve our ability to manage and protect the valuable seagrass meadows by selecting appropriate plants' organs with specific responsiveness to particular stressful conditions in monitoring plants responsiveness to occurring threats. Plants that experienced for the first time eutrophic waters needed to be more active to cope with the new stress conditions expressing different genes related to metabolic, detoxification and photosynthesis processes, contrary to plants pre-exposed to eutrophic waters that only required the activation of basic processes to withstand high nutrient levels. In the latter case, the activation of specific processes related to starch synthesis and its degradation and cell wall organization suggests that eutrophic plants invested energy to counteract the exposure to a new stress condition (i.e. increased temperatures) increasing shoot mortality in the case of chronic stress exposure. The "pre-adaptation" to local environmental conditions influences the degree of transcriptomic responses of the SAM to single and multiple stresses. In this case, plants already experiencing local pressures at their home site resulted more vulnerable to temperature increases. In a global warming scenario, these results suggest that meadows that are already impacted by local pressures (e.g. eutrophic conditions) will be compromised by future temperature increases.

Chromatin remodelling seems to be involved in plant responses to different stresses, since a different regulation of epigenetic-related genes was observed among plants and treatments. However, more studies on chromatin modifications are required to better understand the function of epigenetic changes in driving stress responses in seagrasses and to identify specific "actors" involved in the process. This could also provide new insights into the mechanisms that regulate the transcriptional memory of the SAM, which is fundamental for understanding seagrass survival to future environmental changes. Moreover, the molecular pattern observed in the SAM differed according to stress typology and plants' origin, and anticipated the high shoot mortality observed several weeks later after chronic exposure to warming, suggesting its strong potential as a sentinel-organ to monitor seagrass meadows under direct and indirect human pressures. Since *P. oceanica* is widely distributed along the Mediterranean coasts, from pristine to highly disturbed sites, it is important to bear in mind that local conditions can play an important role on their ability to withstand regional and global climate change-related stresses. In the framework of the UN decade of ecosystem restoration, similar studies are necessary to improve conservation and restoration managements of seagrasses.

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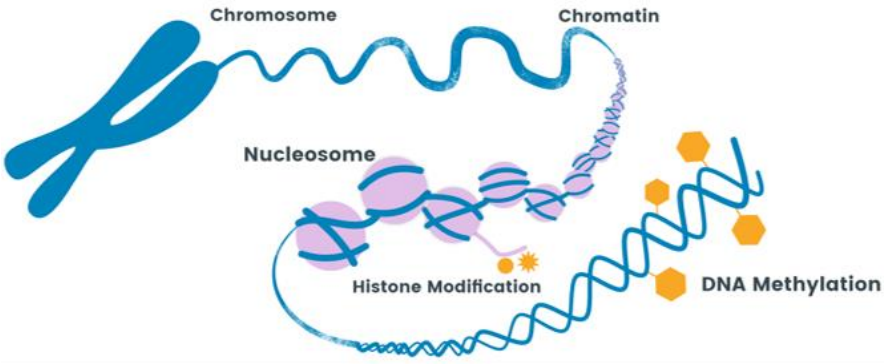
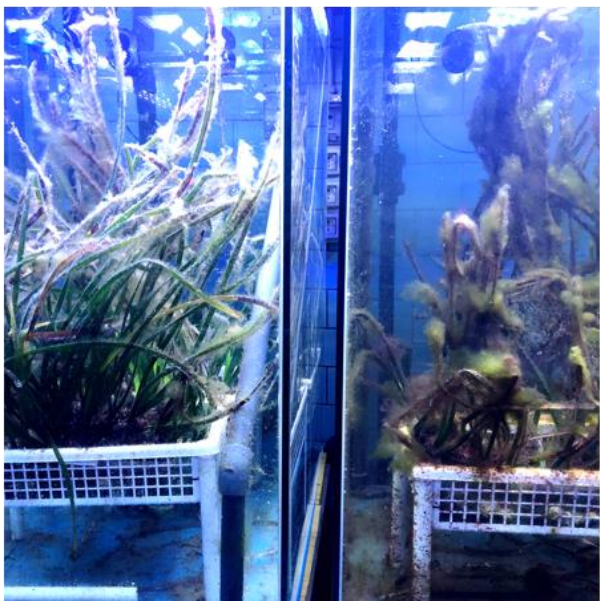
CHAPTER IV

(Paper IV)





Jessica Pazzaglia, Emanuela Dattolo, Miriam Ruocco, Alex Santillán-Sarmiento, Lazaro Marin-Guirao, Gabriele Procaccini. Plants' origin influences the dynamics of DNA methylation in Posidonia oceanica exposed to altered nutrient and temperature levels.

Plants' origin influences the dynamics of DNA methylation in the seagrass *Posidonia oceanica* exposed to altered nutrient and temperature levels

Jessica Pazzaglia[1,2], Emanuela Dattolo[1], Miriam Ruocco[1], Alex Santillán-Sarmiento[1,3], Lazaro Marin-Guirao[1,4]*, Gabriele Procaccini[1]*

[1] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy

[2] Department of Life Sciences, University of Trieste, 34127, Trieste, Italy

[3] Faculty of Engineering, National University of Chimborazo, Riobamba, Ecuador

[4] Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, Murcia, Spain

*These authors have contributed equally to this work

Abstract

DNA methylation is an epigenetic modification that can be influenced by different organisms' development stages and environmental changes. Epigenetic variations modulate phenotypic responses through gene expression regulation, allowing organisms to adjust to new environmental conditions. Although DNA methylation has been intensively studied in terrestrial plants, the dynamics of *de novo* methylation, its maintenance, removal, and the cross-talk with histone methylation remained unexplored in marine plants. Seagrasses form a unique group of angiosperms that have colonized coastal marine environments forming extensive underwater meadows. Among seagrasses, the Mediterranean endemic *Posidonia oceanica* ranks amongst the slowest-growing and longest-living plants on earth. However, sea warming and the co-occurrence of different local anthropic pressures are threatening these highly valuable ecosystems compromising their future survival in the frame of global changes. In the present study, we aimed to analyze the dynamics of DNA methylation in plants growing in contrasting environments (oligotrophic, Ol; eutrophic, Eu) and exposed to stress conditions. To this end, plants were exposed to single (nutrients addition and temperature increase) and multiple stressors (nutrients and temperature combination,) and the global DNA methylation levels together with the level of expression of key genes involved in DNA methylation were assessed after one, two and five weeks of exposure to stresses. Results revealed a clear differentiation between Ol and Eu plants depending on environmental stimuli, plants' origin, and time of exposure, with the temperature being the main driver for the observed differences. Total DNA methylation levels were higher at the initial exposure to stresses, especially in Ol plants that overexpressed almost all genes involved in de novo DNA methylation and its maintenance. Contrarily, Eu plants showed lower gene expression levels in respect to Ol plants that tend to increase with chronic exposure to stresses, particularly in temperature treatments. These findings showed, for the first time in seagrasses, the dynamics of DNA methylation during stress exposure underling its potential role in the regulation of phenotypic responses to environmental changes.

Keywords

Seagrasses, epigenetics, dynamics DNA methylation, gene expression, stress responses

Introduction

Epigenetic mechanisms such as DNA methylation, histone modifications and regulation by non-coding RNA (ncRNA) are important processes influencing chromatin structure and accessibility to genetic information and thus regulating gene expression (Gibney and Nolan 2010). Epigenetic variations may occur during the development of the organisms, be related to surrounding environmental conditions or arise stochastically (Feinberg and Irizarry 2010; Mirouze and Paszkowski 2011; Pikaard and Scheid 2014). Yet, they can be flexible, inducing short-term regulations in response to environmental stimuli, or stable during the lifetime of an organism, being eventually heritable through multiple generations (Verhoeven et al. 2010). DNA methylation is a conserved mechanism, which occurs in both plants and animals (Kumar and Mohapatra 2021). In animals, this process is characterized by the addition of a methyl or hydroxymethyl group to the C5 position of cytosine to form 5-methylcytosine (5-mC), which mainly occurs in the context of CpG (or GC) dinucleotides, while in plants it is also found in CHH and CHG contexts (where H = A, C or T) (Gruenbaum et al. 1981). This reaction is mediated by methyltransferases using S- adenosyl-l-methionine as donors of a methyl group, and the dynamic of its establishment, maintenance and removal is highly regulated through different pathways involving various enzymes (Bossdorf et al. 2008; Li et al. 2018; Kumar et al. 2018). According to the sequence context, methylation can either activate or repress gene expression (Bossdorf et al. 2008; Niederhuth and Schmitz 2017).

In plants, *de novo* DNA methylation is mediated by the RNA- directed DNA methylation (RdDM) pathway, which is based on small-interfering RNAs (siRNAs), scaffold RNAs, and many accessory proteins (Greenberg et al. 2011; Kumar and Mohapatra 2021). Once DNA methylation is established, its maintenance is regulated by different methyltransferases, depending on the sequence context. For instance, the DNA METHYLTRANSFERASE 1 (MET1) regulates CG cytosine methylation during the replication adding a methyl (CH_3) group at the fifth carbon of cytosine in the daughter strand, while methylated CHG is maintained mainly by CHROMOMETHYLASE 3 (CMT3) and CHROMOMETHYLASE (CMT2). The CHG methylation attracts H3K9-specific methyltransferases (i.e., SUVH4, SUVH5 and SUVH6) that favor the CMT3–H3K9me2 interaction (Du et al. 2015). Their recruitment induces di-methylation of H3K9 (H3K9me2) and facilitates CMT3 and CMT2 functions in a cross-talk mechanism between CHG methylation and H3K9 methylation (Du et al. 2012, 2014). In model plant species, this interaction is known to be crucial for maintaining methylation, as mutations in SUVH4 unable CMT3–H3K9me2 interactions, prevent H3K9me2, and reduce CMTs activities, decreasing CHG methylation (e.g., in *Arabidopsis thaliana*, Jackson et al. 2002; and maize, Du et al. 2012). The removal of 5-mC can be a passive process occurring by lower expression of MET1 (Kawashima 2014) or it can be regulated by the activity of bifunctional 5-mC DNA glycosylases including REPRESSOR OF SILENCING 1 (ROS1), TRANSCRIPTIONAL ACTIVATOR DEMETER (DME), DEMETER-LIKE PROTEIN 2 (DML2) and DML3 (Gong et al. 2002; Gehring et al. 2006). Importantly, DNA methylation and histone modification can store information of environmental cues comprising the establishment of stress memory in many plants species (Jiang et al. 2014; Lu et al. 2016).

Since the epigenetic landscape influences gene regulation and thus phenotype, the interplay between genome-wide DNA methylation and gene expression levels is at the basis of phenotypic adjustment to environmental conditions, possibly resulting in local adaptation (Kawakatsu et al. 2016). Analyzing DNA methylation variation and its implications in short-term stress responses and stress memory can be fundamental, especially for foundation species, such as marine plants that are declining worldwide due to climate changes and local pressures (Waycott et al. 2009). The rapid occurrence of environmental changes and the potential interaction with global warming are forcing seagrasses to

exceed their tolerance and resilience capacity. Seagrasses form a unique group of angiosperms that diverged from terrestrial plants more than 70 Ma ago (Hedges and Kumar 2009). Being the only group that returned to a completely submerged lifestyle in marine waters, they have evolved peculiar molecular, physiological and morphological adjustments that have favored the colonization of different habitats along the marine coastlines (Den Hartog 1970; Olsen et al. 2016). The degree of phenotypic plasticity observed among seagrass species reflects the interaction between genotypes and the surrounding environments, which is considered a crucial property for their survival to environmental shifts (Pazzaglia et al. 2021). While genetic diversity have the potential to increase resilience capacity of seagrass meadows under long-term environmental changes (Jahnke et al. 2015), epigenetic modifications such as DNA methylation may contribute to the regulation of phenotypic plasticity favouring short-term responses (i.e., acclimation) to rapid environmental changes (Bossdorf et al. 2008; Jueterbock et al. 2019; Pazzaglia et al. 2021). However, the physiological and morphological response to single or multiple stresses can also depend on the environmental conditions locally experienced by plants in their native habitats (Pazzaglia et al. 2020). Recently, Entrambasaguas and colleagues (2021) explored gene body DNA methylation (gbM) patterns in different *P. oceanica* ecotypes, revealing the existence of a relationship between gbM and gene expression flexibility depending on the origin of plants. Similarly to terrestrial plants, genes with low levels of methylation showed inducible expression in relation to environmental conditions. Hence, the genetic–epigenetic control already described in terrestrial plants, could also regulate the interaction of seagrass genotypes with the surrounding environments allowing their survival to environmental changes.

In plants, the dynamics of DNA methylation is strongly affected by environmental stimuli and is associated to the inheritance of chromatin modifications. As already observed in terrestrial plants, the non-stressed progeny can inherit the DNA methylation landscape from parental plants exposed to stress with the potential to improve their stress tolerance (Boyko et al. 2010). Most of the epigenetic research in plants exposed to abiotic stresses such as drought, cold or heat stress and salinity, have provided important evidence of stress-induced DNA methylation and demethylation both at the genome and specific loci levels (Li et al. 2014; Zhang et al. 2018). For instance, in cotton plants heat stress triggers DNA demethylation especially in the CHH context of heat-sensitive line, whereas higher DNA methylation levels were observed in heat-tolerant line (Ma et al. 2018).

It is evident that the dynamic regulation of DNA methylation in response to stress conditions is a key process to be addressed in the era of global environmental changes, especially in marine clonal plants that are particularly vulnerable to environmental shifts. Although great progress in the understanding of epigenetic processes in plants has been achieved by using well established terrestrial model species, little is known about the dynamics of epigenetics mechanisms in aquatic or marine non-model plants such as seagrasses.

Being sessile organisms, plants are frequently exposed to chronic or recurring disturbances in natural environments. The storage of past stress events can occur through the regulation of a specific set of genes known as stress memory genes (Bäurle and Trindade 2020). Plants exposed to thermal stress showed the activation of specific heat shock factors (i.e., heat-stress memory genes) involved in transcriptional memory as they resulted re-activated during the recurring stress (Lämke et al. 2016). Their induction is maintained by epigenetic mechanisms (i.e., histone methylation) and by the interaction of specific genes with the chromatin structure, like FORGETTER1 in *A. thaliana* (Brzezinka et al. 2016). The ability to acquire a stress memory for enhancing resilience against further stress has also been shown in seagrasses (Nguyen et al. 2020).

In this study, we aimed to investigate the dynamics of DNA methylation analysing the expression profiles of key genes involved in *de novo* and maintenance DNA methylation and demethylation, as well as histone methylation in adult plants of *P. oceanica* with a different history of nutrient loads. This analysis was compared with the analysis of global DNA methylation level (% 5-mC) of plants at different time points of the exposure to nutrients addition, temperature increase and their combination. We also aimed to investigate on pre-acquired memory by analysing a specific gene involved in heat-stress memory. According to previous observations in terrestrial plants and in seagrass studies, our initial hypothesis is that DNA methylation can be modulated according to specific environmental stresses and local pressures at their site of origin.

2. Methods

2.1 Experimental design and plant collection

Leaf material of *P. oceanica* used for this study was collected during the experiment performed by Pazzaglia et al. (2020). Briefly, large plant fragments bearing 10-20 vertical shoots were collected by SCUBA diving on May 15 – 16th 2019 from shallow-water meadows growing in two locations with different history of nutrient loads: Spiaggia del Poggio (Bacoli) in the Gulf of Pozzuoli (Italy, 40 47.9300 N; 14 05.1410 E), and Castello Aragonese in the Island of Ischia (Italy, 4044.1140N; 1357.8660 E). The former is considered an impacted site with eutrophic conditions due to local pressures, contrary to Ischia site, which is in a marine protected area (for a detailed description of sampling sites see Pazzaglia et al. 2020). Thus, two plant fragments (a rhizome portion bearing a minimum of eight vertical shoots) for each eutrophic (Eu) and oligotrophic sites (Ol) were allocated in each tank of an indoor mesocosm system at Stazione Zoologica Anton Dohrn (SZN, Naples, Italy) (Ruocco et al. 2019), and exposed to single and multiple stresses. The experiment was designed including four treatments as follows: Control (C), Nutrients (N), Temperature (T) and Nutrients + Temperature (NT). Temperature and nutrient treatments were set according to a previous mesocosm experiment and environmental data taken at the sampling sites (see Pazzaglia et al. 2020). After a first acclimation phase, temperature was gradually increased (0.5 C day^{-1}) in the T and NT treatments to 30°C, whereas temperature in the C conditions was maintained at 24°C. In N and NT treatments a stock solution (170 mM total nitrogen) was added weekly to maintain a nutrient enrichment (DIN = 26.8 ± 4.0 mM). The solution was prepared using Osmocote Pro® fertilizer pellets (6 months release: 19% N – 3.9% P – 8.3% K, ICL Specialty Fertilizers). Leaf material was obtained from the middle section of second-rank leaves collecting a portion of 6 cm above and below the established height (20 cm) for RNA and DNA extractions, respectively. A total of 72 leaf samples (n = 3 biological replicates for each condition) were collected for gene expression and DNA methylation analysis from both Eu and Ol plants after one week (T1), two weeks (T2) and five weeks (T5) from the initial exposure to stresses (N, T and NT). Leaf tissue for gene expression analysis was completely cleaned from epiphytes and submerged in RNA later© (Ambion, life technologies) collection solution, then samples were kept at 4 °C overnight to let the solution penetrate into the tissue, and finally stored at -20 °C. Leaf samples for DNA extractions were accurately cleaned from epiphytes and stored in silica gel.

2.2 Reverse Transcription-quantitative Polymerase Chain Reaction

RT-qPCR analysis was used to explore differences in expression levels of target genes in control vs. treatments (N, T and NT) in both Eu and Ol plants during the course of the experiment (T1, T2 and T4). Total RNA was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD) following manufacturer's instructions. RNA purity and concentration were checked using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was assessed through 1.0%

(w/v) agarose gel electrophoresis. Then, total RNA (500 ng) from each sample was retro-transcribed into cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to manufacturer's protocol. Five genes of interests (GOIs) were selected according to their roles in DNA methylation processes from previous studies performed on terrestrial plants (Kumar and Mohapatra 2021): *de novo* DNA methylation (DRM and AGO), DNA methylation maintenance (MET1 and CMT2), DNA demethylation (ROS1), Histone methylation (SUVH4) and stress memory (FGT1) (**Table 1**). GOIs were specifically designed based on a *P. oceanica* transcriptome (Ruocco et al., 2021) with the primer analysis software Primer3 v. 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) setting primer length to 18-20 bp, product size to 100-250 bp and Tm = 59-61◦C. Two reference genes (elf4A and GADPH) were selected and used for target gene-expression normalization considering their stability in previous works with the same species under abiotic stresses (Serra et al. 2012; Lauritano et al. 2015). Primer's sequences, efficiencies (*E*) and regression coefficients (R^2) of GOIs are showed in **Table 1**. Primers with efficiencies (*E*) within the range 90-110% and correlation coefficient >0.95 were used in the study (**Table 1**). RTqPCR efficiencies for all primer pairs were calculated from the slopes of standard curves of the threshold cycle (CT) vs. cDNA concentration, with the equation $E = 10^{-1}$/slope. RT-qPCR reactions were performed in triplicates in a Viia7 Real Time PCR System (Applied Biosystems) using Fast SYBR® Green MasterMix (Applied Biosystems) as fluorescent detection chemistry and MicroAmp Optical 384-well reaction plates (Applied Biosystems). Reactions were carried out in a 10µl final volume with 5µl MM SYBR® Green, 2µl of 1.4 pmol μl^{-1}primers and 1µl of 1:5 cDNA: nuclease-free water dilution as template. The thermal profile of the reactions was as follows: 95°C for 20 s, 40 times 95°C for 1 s and 60°C for 20 s. Relative quantification of gene expression was obtained following Marín-Guirao et al. (2016). The amplification data were analysed using the ViiA7™ Software v.1.0 (Applied Biosystems) and the differential expression parameters were manually calculated as follows: the cycle threshold (C_T), the negative difference in cycles between the reference genes (RGs) and the respective GOI ($-\Delta C_T = C_T$ RGs - C_T GOI), the fold expression change = ±2 ($|(-\Delta C_T$ treatment)-$(-\Delta C_T$ control)|).

2.3 Global DNA methylation assessment

Genomic DNA was isolated using the NucleoSpin® Plant II kit (Macherey–Nagel). DNA quality was checked through 1.0% agarose gel electrophoresis and the concentration was accurately determined by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Global DNA methylation was assessed colorimetrically in duplicate by an ELISA-like reaction with the 5-mC DNA ELISA Kit (Zymo Research) starting from 50 ng DNA per sample and reported as % of methylated CpG (% 5-mC) relative to the standard input of DNA quantity. Absorbance at 450 nm was read using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

Table 1. List of housekeeping genes and genes of interest (GOIs) analyzed in this study by RT-qPCR. For each GOI, gene category, gene acronym and protein name, primer sequences, amplicon size (S), percent efficiency (E), regression coefficients (R^2) and reference, is reported.

Gene category	Gene	Protein	Forward sequence (5->3)	Reverse sequence (3->5)	S	*E* (%)	R^2
Houskeeping	[a]GADPH	Glyceraldehyde-3-phosphate dehydrogenase	AGGTTCTTCCTGCTTTGAATG	CTTCCTTGATTGCTGCCTTG	138	93	0.99
	[b]elF4A	Eukaryotic initiation factor 4°	TTCTGCAAGGGTCTTGACGT	TCACACCCAAGTAGTCACCAAG	192	85	0.99
De novo DNA methylation	[c]AGO	Argonaute	GCCTCCTCCTGTGATACCTC	AGTAGCCATCCACATTGCCT	179	99	0.99
	[c]DRM	DNA (cytosine-5)-methyltransferase DRM	CCCTTTGGAACCTGATGAGAT	AAGGGCCATTTCAGCTCCA	216	100	0.99
DNA methylation mantainance	[c]MET1	DNA methyltransferase 1	ACTGTTCGTGAGTGTGCAAG	AGGAGTTTTGCCGCTTTCTG	166	100	0.99
	[c]CMT2	Chromomethylase 3	CGTAAAGGGTGTTGAAGGACA	CAGCCCTGAAGAACCATTGA	107	100	0.99
DNA demethylation	[c]ROS1	Repressor of silencing 1	GCACTGTTTCTGGAAAGGCT	CCTTGCTTGCTGGGAAATGT	102	99	0.99
Histone methylation	[c]SUVH4	Histone-lysine N-methyltransferase	TGCTGCCAACAAGAACAACA	ACGGTGCCAGCATCTATACA	162	98	0.99
Stress memory	[c]FGT1	Forgetter 1	TACCGCCACCTTCAACAGAT	ACGCTCTTTTGCTGCTTCAA	137	96	0.98

[a] Serra et al. (2012); [b] Lauritano et al. (2015); [c] Primers designed in this study

2.4 Data analysis

Multivariate statistics was used within both plant groups (Eu and Ol) and for each time point (T1, T2 and T5) individually, to explore significant differences among treatments (N, T and NT) affecting DNA methylation (gene expression and % 5-mC). A repeated measures ANOVA (3-way RM-GLM) was conducted to investigate the effect of single stress factors (N and T) and their interaction on gene expression and % 5-mC data in both Ol and Eu plants. The model included 'time' as a within-subject factor, 'plants' with two levels (Eu and Ol), 'N' and 'T' both with two levels (control and high). To assess the dynamics of gene expression and % 5-mC for each group of plants (Eu and Ol), a second repeated measures ANOVA (2-way RM-GLM) excluding 'plants' as factor was performed using only treatments (N and T) as fixed factors with two levels (control and high) and time as within-subject factor. Data were checked for the assumptions of normality and homoscedasticity and transformed when necessary. In the case of RM-GLMs, the assumption of sphericity was assessed using Mauchly's sphericity test. A *post-hoc* mean comparison test (Student-Newman-Keuls, SNK) was performed when significant differences were found ($p < 0.05$). All ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10).

3. Results

Dynamics of global DNA methylation

Overall, mean DNA methylation levels (% 5-mC) of Ol plants were higher than those observed for Eu plants during the whole experiment ($P < 0.05$, **Table 2, Figure 1**). In both plant typology, values varied along the experiment ($P < 0.01$; Supplementary Table S1). Nutrients were the main driver of % 5-mC differences over time only in Eu plants (Time×N, $P < 0.05$; **Table 2**), where the % 5-mC measured in treatments with high nutrients decreased from T1 (3.79%) to T2 (2.41%) and then increased again at the end of the experiment (2.94%, **Table 2, Figure 1**).

Table 2. Results of the two-way RM-GLM performed on -ΔCT values of GOIs and % of methylated cytosine (% 5-mC) measured in Ol and Eu plants for factors "N" (Nutrients) and "T" (Temperature) with "Time" as within-subject factor. Significant factors and values are in bold and italics.

Two-way RM-GLM

			OL plants				EU plants			
Variable	**Factor**	**df**	**MS**	**F**	**P**	**Factor**	**df**	**MS**	**F**	**P**
% 5-mC	N	1	0.074	0.068	0.800	N	1	0.391	7.900	0.023
	T	1	0.931	0.860	0.381	***T***	1	2.031	41.007	***0.000***
	N×T	1	2.443	2.255	0.172	N×T	1	0.173	3.501	0.098
	Error	8	1.083			Error	8	0.050		
	Time	2	4.367	5.997	***0.011***	***Time***	2	3.452	10.289	***0.001***
	Time×N	2	1.166	1.601	0.232	***Time×N***	2	1.423	4.242	***0.033***
	Time×T	2	0.768	1.055	0.371	Time×T	2	0.258	0.770	0.480
	Time×N×T	2	1.057	1.452	0.263	Time×N×T	2	0.139	0.414	0.668
	Error	16	0.728			Error	16	0.335		
AGO	N	1	0.873	2.053	0.195	N	1	0.869	1.903	0.205
	T	1	4.342	10.210	***0.015***	***T***	1	6.068	13.287	***0.007***
	N×T	1	0.001	0.002	0.966	N×T	1	0.000	0.000	1.000
	Error	8	0.425			Error	8	0.457		
			171.5	188.41						
	Time	2	29	0	***0.000***	***Time***	2	2.924	3.661	***0.049***
	Time×N	2	0.209	0.230	0.798	Time×N	2	0.349	0.437	0.654
	Time×T	2	1.348	1.481	0.261	Time×T	2	1.037	1.299	0.300
	Time×N×T	2	0.351	0.385	0.687	Time×N×T	2	0.311	0.390	0.684
	Error	16	0.910			Error	16	0.799		
DRM	N	1	0.126	1.312	0.304	N	1	0.268	0.295	0.602
	T	1	1.275	13.307	***0.015***	T	1	1.546	1.700	0.229
	N×T	1	0.014	0.145	0.719	N×T	1	0.372	0.409	0.540
	Error	8	0.096			Error	8	0.909		
	Time	2	0.706	6.362	***0.017***	Time	2	1.606	1.614	0.230
	Time×N	2	0.088	0.791	0.480	Time×N	2	1.796	1.806	0.196
	Time×T	2	0.183	1.649	0.240	Time×T	2	1.017	1.023	0.382
	Time×N×T	2	0.065	0.584	0.576	Time×N×T	2	1.558	1.566	0.239
	Error	16	0.111			Error	16	0.995		
MET1	N	1	4.250	2.504	0.152	N	1	1.935	1.499	0.256
			40.25							
	T	1	9	23.719	***0.001***	T	1	3.128	2.423	0.158
	N×T	1	4.209	2.480	0.154	N×T	1	0.020	0.016	0.903
	Error	8	1.697			Error	8	1.291		
									354.01	
	Time	2	9.460	2.518	0.112	***Time***	2	416.847	3	***0.000***
	Time×N	2	1.536	0.409	0.671	Time×N	2	0.095	0.080	0.923
	Time×T	2	0.608	0.162	0.852	Time×T	2	1.024	0.870	0.438
	Time×N×T	2	0.749	0.199	0.821	Time×N×T	2	0.244	0.208	0.815
	Error	16	3.757			Error	16	1.177		
CMT2	N	1	0.583	0.742	0.414	N	1	0.000	0.000	0.998

			15.340							
	T	1		19.530	***0.002***	T	1	1.420	3.623	0.093
	N×T	1	2.054	2.616	0.144	N×T	1	0.052	0.133	0.725
	Error	8	0.785			Error	8	0.392		
	Time	2	0.514	0.459	0.640	Time	2	0.425	0.779	0.476
	Time×N	2	0.065	0.058	0.944	Time×N	2	0.346	0.635	0.543
	Time×T	2	0.308	0.275	0.763	Time×T	2	0.349	0.641	0.540
	Time×N×T	2	0.963	0.861	0.441	Time×N×T	2	0.107	0.196	0.824
	Error	16	1.119			Error	16	0.545		
ROS1	N	1	0.063	0.083	0.781	N	1	0.039	0.084	0.779
			10.912							
	T	1		14.416	***0.005***	***T***	1	2.830	6.035	***0.040***
	N×T	1	0.734	0.970	0.354	N×T	1	0.139	0.297	0.601
	Error	8	0.757			Error	8	0.469		
									597.032	
	Time	2	2.442	7.217	***0.006***	***Time***	2	201.417		***0.000***
	Time×N	2	1.172	3.463	0.056	Time×N	2	0.671	1.988	0.169
	Time×T	2	0.161	0.476	0.630	Time×T	2	0.658	1.951	0.175
	Time×N×T	2	0.099	0.294	0.749	Time×N×T	2	0.472	1.399	0.276
	Error	16	0.338			Error	16	0.337		
SUVH4	N	1	0.040	0.034	0.858	N	1	0.017	0.061	0.811
	T	1	6.708	5.727	***0.044***	***T***	1	2.723	9.523	***0.015***
	N×T	1	0.002	0.001	0.971	N×T	1	0.886	3.101	0.116
	Error	8	1.171			Error	8	0.286		
			15.055						807.204	
	Time	2		16.129	***0.000***	***Time***	2	219.272		***0.000***
	Time×N	2	0.398	0.426	0.660	Time×N	2	0.446	1.643	0.224
	Time×T	2	0.252	0.270	0.767	Time×T	2	0.379	1.394	0.277
	Time×N×T	2	0.443	0.475	0.630	Time×N×T	2	0.387	1.425	0.269
	Error	16	0.933			Error	16	0.272		
FGT1	N	1	0.004	0.009	0.929	N	1	25.378	0.393	0.548
	T	1	7.417	17.561	***0.003***	T	1	340.355	5.269	0.051
	N×T	1	2.913	6.897	***0.030***	N×T	1	311.501	4.822	0.059
	Error	8	0.422			Error	8	64.595		
								6473.313	102.321	
	Time	2	0.437	0.539	0.594	***Time***	2			***0.000***
	Time×N	2	0.111	0.136	0.874	Time×N	2	25.083	0.396	0.679
	Time×T	2	0.363	0.447	0.647	***Time×T***	2	315.683	4.990	***0.021***
	Time×N×T	2	0.594	0.732	0.496	***Time×N×T***	2	298.435	4.717	***0.025***
	Error	16	0.812			Error	16	63.265		

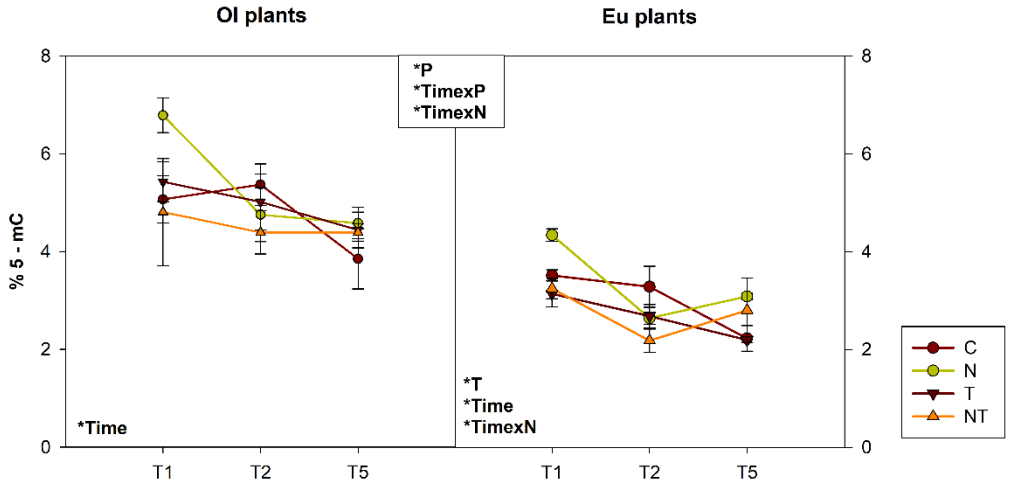


Figure 1. Global % of methylated cytosine (% 5-mC) measured in Ol and Eu plants during the experiment (T1 = one week of the exposure; T2 = two weeks of the exposure; T5 = five weeks of the exposure) in the different treatments (C = control; N = nutrients; T = temperature; NT = nutrients + temperature). Significant differences resulting from 3-way RM-GLM are reported in the central square while results of 2-way RM-GLM performed individually for Ol and Eu plants, are showed in the bottom left corner of each graph. Data are mean ± SE (n = 3).

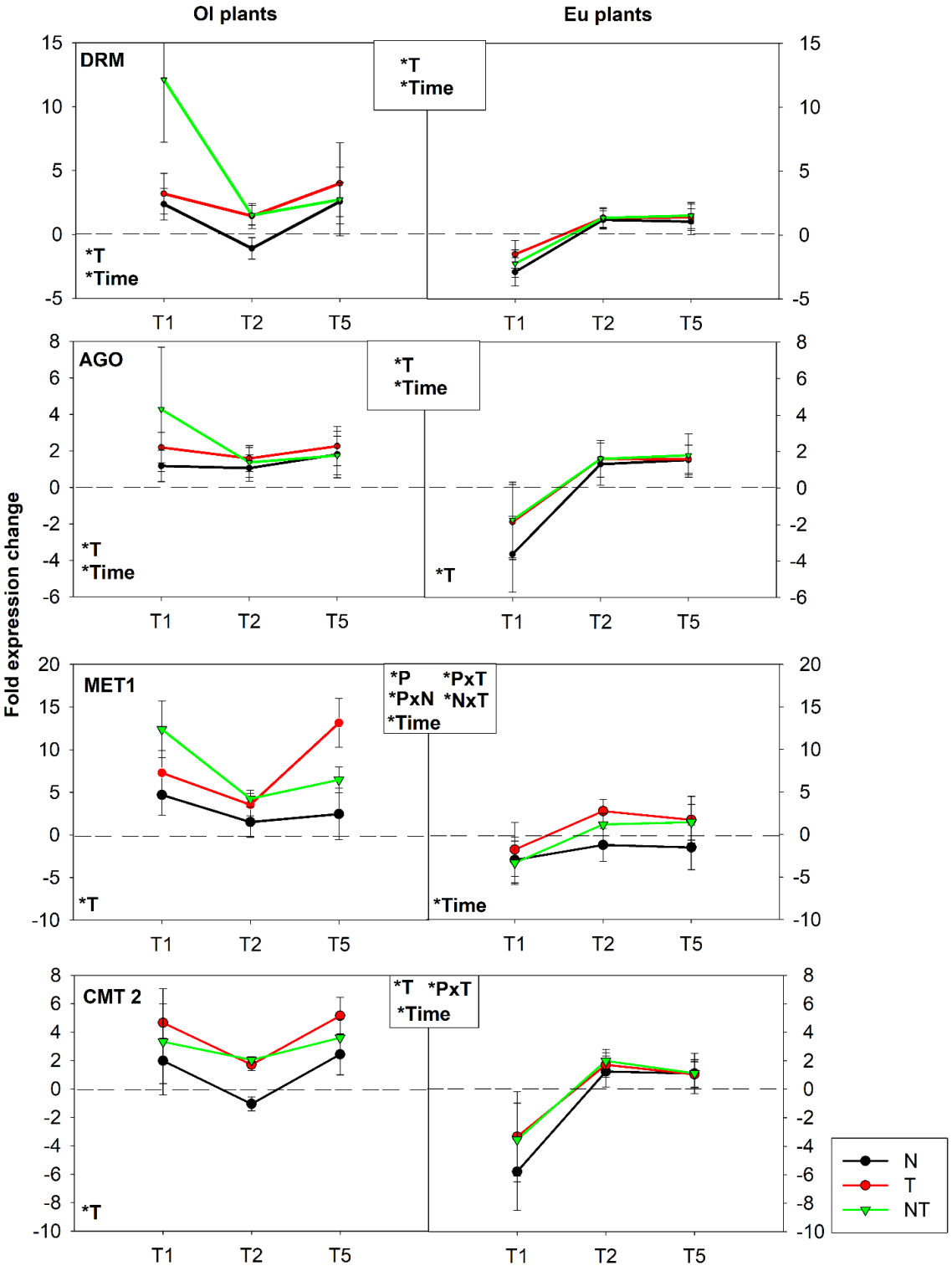


Figure 2. Expression dynamics of GOIs involved in *de-novo* DNA methylation (DRM and AGO) and its maintenance (MET1 and CMT2) measured in both Ol and Eu plants under different stress conditions (N = nutrients; T = temperature, NT = nutrients + temperature) compared to Control (dashed line). Significant differences resulting from 3-way RM-GLM are reported in the central

square, while outputs of 2-way RM-GLM performed individually for Ol and Eu plants are showed in the bottom left corner of each graph. Data are mean ± SE (n = 3).

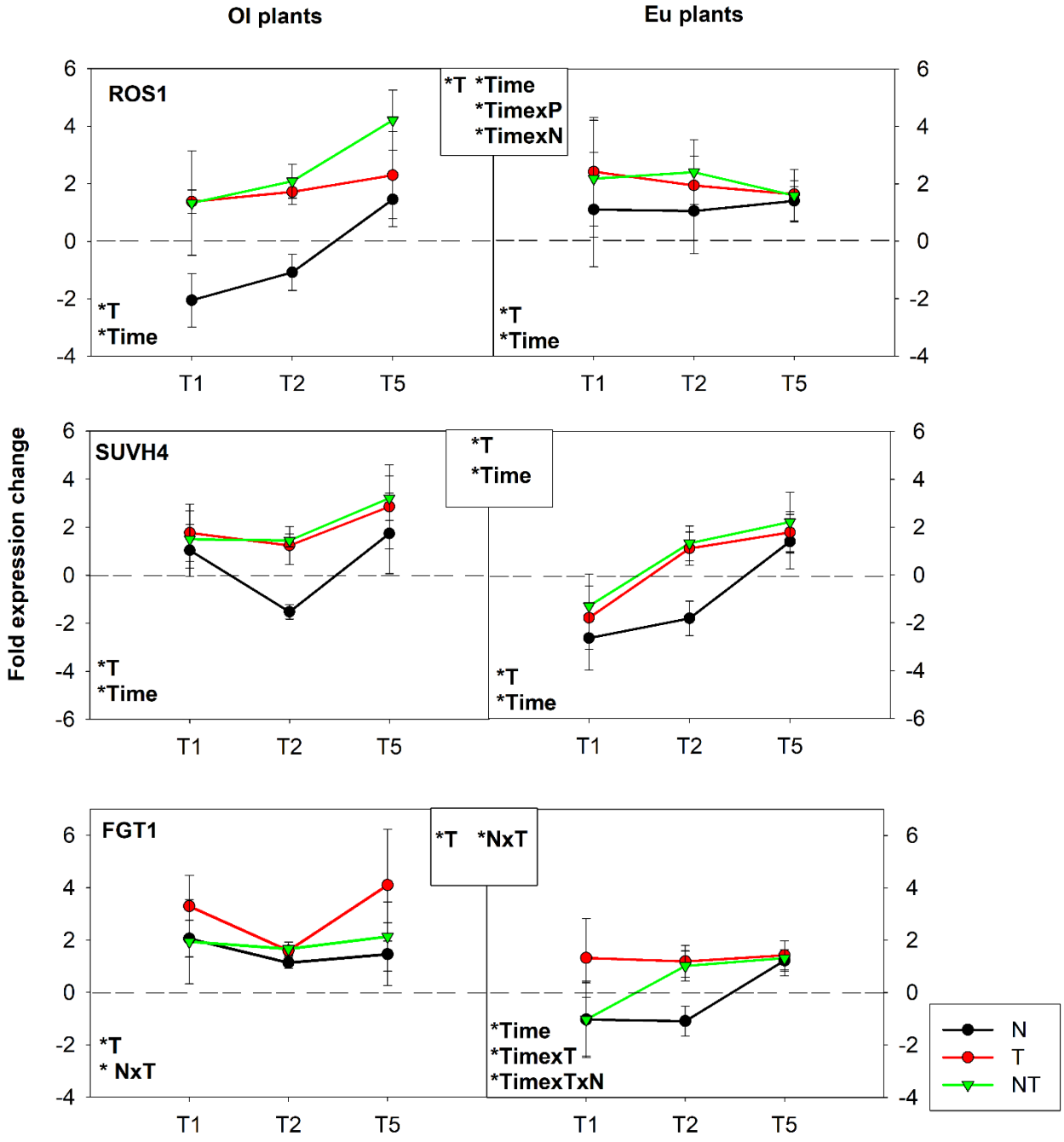


Figure 3. Expression dynamics of GOIs involved in DNA demethylation (ROS1), histone methylation (SUVH4) and heat stress memory (FGT1) measured in both Ol and Eu plants under different stress conditions (N = nutrients; T = temperature, NT = nutrients + temperature) compared to Control (dashed line). Significant differences resulting from 3-way RM-GLM are reported in the central square, while results of 2-way RM-GLM performed individually for Ol and Eu plants are showed in the bottom left corner of each graph. Data are mean ± SE (n = 3).

Expression dynamics of DNA methylation and thermal memory-related genes under stresses

Gene expression results revealed that time and temperature were the main factors in driving significant differences in both Ol and Eu plants (**Table 2**). However, temporal overall patterns of gene expression differ between Ol and Eu plants (**Figures 2** and **3**). In Ol plants, most of the genes analysed showed higher expression levels at T1 that tend to decrease over time, contrary to Eu plants that displayed an opposite overall pattern, increasing or maintaining high fold expression values during the exposure to stress conditions in six of the seven genes analysed (DRM, AGO, MET1, CMT2, SUVH4 and FGT1, **Table 2, Figure 2 and 3**). In detail, Ol plants overexpressed genes involved in *de novo* DNA methylation (DRM and AGO) in T1, while Eu plants repressed their expression showing a different pattern compared to Ol plants although not significant (**Figure 2**, 3-way RM-GLM **Table S1**). Interestingly, DRM and AGO expression changes were much higher significant in Ol plants in respect to Eu ones. Similar patterns were also observed for genes involved in DNA methylation maintenance (MET1 and CMT2) that were significantly different between Ol and Eu plants depending on temperature (**Figure 2**; $P<0.05$, RM-GLM **Table S1)**. Although MET1 and CMT2 did not show a significant expression modulation over time in Ol plants (**Table 2, Figure 2)**, they were highly expressed in T1 especially in treatments with temperature (T and NT), following an opposite pattern in respect to Eu plants that downregulated these genes in all treatments. Gene involved in histone methylation (SUVH4) was also overexpressed in Ol plants especially in temperature treatments (T and NT), contrary to Eu plants where its expression increased over time (**Figure 3**; **Table 2**). The temporal pattern observed for ROS1 significantly differed between Ol and Eu plants ($P<0.05$; **Table S1**). Ol plants increased ROS1 expression over time in all treatments especially in response to nutrients treatments (N and NT). Instead, in Eu plants ROS1 was overexpressed in all treatments (**Figure 3**). The expression patterns observed for the gene involved in thermal stress memory (FGT1) were influenced by temperature exposure and its interaction with nutrients in Ol plants with no significant changes over time although the general observed patter was similar to all the other genes ($P<0.05$; **Table 2**). By contrast, in Eu plants the expression of FGT1 changed significantly over time depending on temperature and its interaction with nutrients ($P<0.01$; **Table 2**).

Discussion

The DNA methylation state of a particular genome reflects the dynamics of its establishment and maintenance. In plants, this process is highly coordinated by different enzymes that actively methylate the DNA increasing the whole methylation level or passively remove methylated cytosine by decreasing their activity. The establishment of DNA methylation (DNAm) and its removal depends on environmental stimuli that have the potential to mediate plant response through gene regulation (Thiebaut et al. 2019). Here we observed, for the first time in seagrasses, the dynamics of DNAm in *P. oceanica* plants collected from environments with a different history of nutrient loads. Our results revealed that DNA methylation was influenced by the time of exposure to stress conditions and by plants' origin, with temperature (T) being the main driver for the observed differences. Thus, the present study underlined that DNA methylation and its dynamics could play a fundamental role in regulating physiological responses to single and multiple stresses according to environmental conditions experienced by plants in their home environment (Pazzaglia et al. 2020).

DNA methylation levels change dynamically over time

The total DNA methylation levels measured after one week from the initial exposure to stress were higher than at the end of the experiment. The stepper decrease was from one week to two week of stress exposure. Moreover, plants collected from environments with more oligotrophic conditions (Ol

plants), showed higher total DNA methylation levels (% 5-mC) in comparison with plants that have already experienced stress conditions in their home environments (Eu plants). This is in line with previous studies performed on terrestrial plants, where the exposure to abiotic stresses induced a reduction of DNAm levels over time (Peng and Zhang 2009). A study performed on *Brassica napus,* revealed a different degree of DNAm levels between heat-tolerant and heat-sensitive genotypes. In that case, tolerant genotypes showed lower levels of DNAm contrary to sensitive ones that displayed a general increase in methylation levels (Gao et al. 2014). Changes in DNAm levels in response to temperature increases were also observed for different plant model species, where DNAm rises as a stress response mechanism regulated by the expression of specific genes (i.e. *Arabidopsis thaliana*, Naydenov et al. 2015; *Gossypium hirsutum*, Ma et al. 2018). Importantly, epigenetic variations were also found to be correlated with local environmental conditions in different plants revealing the contribution of epigenetics to phenotypic plasticity, in the absence of genetic diversity (Vanden Broeck et al. 2018; Medrano et al. 2020). In our study, the higher % 5-mC value measured in T1 suggests that DNA methylation was implicated in the initial response to stress conditions. Moreover, since Ol plants were supposed to experience the combined effect of Nutrients and Heat stress for the first time, they showed a more pronounced activation in respect to Eu plants that were collected in a site with higher nutrients load. DNA methylation levels vary widely among angiosperms (Niederhuth et al. 2016), and intra- and inter-specific variability was also showed in seagrasses, according to species and populations life history (Entrambasaguas et al. 2021). Methylome variation was also recently observed among ramets of the same genet in the seagrass *Zostera marina* under heat stress that appeared to be linked with photosynthetic performance and thus phenotypic plasticity (Jueterbock et al. 2020). In *P. oceanica* plants, global DNA methylation changes were also observed in relation to different leaf developmental stages and temperature increase (Ruocco et al. 2019b, a), and light conditions (Greco et al. 2013; Ruocco et al. 2021).

In this study, the stress-induced dynamic of DNA methylation in both Ol and Eu plants changed over time showing a strong reduction after two weeks from the initial exposure to stress conditions and tends to increase again or remained constant after five weeks of exposure. This suggests that besides the involvement of DNAm at the initial phase of the stress exposure, it could be dynamically involved in the regulation of stress responses to prolonged exposure. Moreover, since both plants showed similar patterns of DNAm changes, this underlined the existence of a common mechanism of epigenetic regulation in *P. oceanica* plants against chronic stress exposure. It is interesting to note that the dynamics of DNAm was different in control plants, stressing the relation between DNAm and response to stress. Although our results revealed that DNA methylation is a dynamic process influenced by the time of exposure to abiotic stresses, it remains difficult to correlate these results with plants' performance previously observed at the end of the experiment (Pazzaglia et al., 2020). However, since Ol and Eu plants did not show differences in genetic or genotypic variability (Pazzaglia et al., 2020), it is reasonable to suppose that the strong differentiation in the levels of DNA methylation observed in this study could contribute to the regulatory machinery driving different phenotypic responses in these plants.

The expression of DNA methylation, demethylation and maintenance related-genes depends on time exposure to stresses and plants' origin

In the present study, temperature was the main factor influencing DNAm changes and the expression of related genes. The expression levels of genes involved in *de novo* DNA methylation and its maintenance were in line with previous considerations reported above for % 5-mC. Here, we analysed the expression of DOMAINS REARRANGED METHYLASE (DRM) and ARGONAUTE (AGO) genes whose interaction catalyse *de novo* DNAm in plants thought the RdDM pathway (Cao and

Jacobsen 2002; Zhong et al. 2014). In *A. thaliana*, the RdDM pathway is involved in the production of small-interfering RNAs (siRNAs) produced by DNA polymerases that are subsequently loaded onto Argonaute proteins (AGO) mediating the recruitment of DRM for methylation of the target locus (He et al. 2009; Zhong et al. 2014). In Ol plants, both genes were overexpressed in T1, especially in treatments with temperature increases (Fig. 4a). Similarly, ROS1, which catalysed the removal of DNAm, was also overexpressed in T1 under the same treatments (Fig. 4c). These findings are in line with previous observations obtained for terrestrial plants exposed to temperature increase (Naydenov et al. 2015). In that case, the authors reported simultaneous increases of ROS1 and DRM2 genes that could result in a target-specific deposition and removal of DNA methylation. In plants, the ROS1 gene promoter includes a sequence termed DNA methylation monitoring sequence (MEMS), which allows the coordination between DNAm and active demethylation through the transcription regulation of ROS functioning as a "methylstat" (Lei et al. 2015). The increase of DNA methylation at the MEMS sequence favours the increases of ROS1 expression. Since the DNAm at the MEMS is regulated by RdDM, ROS1 itself, and also METHYLTRANSFERASE 1 (MET1) activities, the ROS1-dependent DNA methylation under environmental stresses could monitor DNA methylation state regulating and maintaining the dynamics of DNAm and demethylation balanced (Zhang et al. 2018). Contrary to Ol plants, Eu plants showed an opposite regulation pattern, downregulating genes involved in DNAm and its maintenance in T1, whereas ROS1 was overexpressed. This evidence underlined different DNAm regulation depending on local environmental conditions. Plants that were already impacted by local disturbances had probably already activated these genes prior to the exposure to experimental conditions. In fact, the gene expression pattern observed in T1 was similar to that found in Ol plants after five weeks of the exposure to stress conditions. However, since DNA methylation analyses were not performed before the exposure phase this evidence cannot fully supported by data.

In line with the expression patterns observed in Ol plants for genes involved in *de novo* DNA methylation, MET1 was highly overexpressed in treatments with high temperatures (NT and T; Fig. 4b). A similar regulation was already demonstrated in terrestrial plants exposed to thermal stress, where the overexpression of MET1 controls the maintenance of cytosine methylation at symmetrical CG positions, while loss of MET1 induced a strong reduction of cytosine methylation marks (Brocklehurst et al. 2018). By contrast, MET1 followed an opposite behaviour in Eu plants, which was similar to that observed for the other genes. This suggests that the local concentration of this MET1 transcript is necessary for maintaining high DNA methylation levels and that probably it functions in coordination with other proteins forming dense methylation marks. Evidence of different responses to stresses based on local acclimation/adaptation to different environments were already described for different seagrass species (Franssen et al. 2011; Marín-Guirao et al. 2016; Dattolo et al. 2017). In particular, different epigenetic-related genes were already found to be differentially regulated in *P. oceanica* plants under thermal stress, revealing higher vulnerability to temperature in more sensitive plants (i.e. cold-adapted, Marín-Guirao et al. 2019).

As already reported above, since heat-tolerant genotypes in plants tend to show lower DNA methylation, the lower expression values of genes involved in DNA methylation and its maintenance reported for Eu plants could be related to the presence of local disturbances that already induced epigenetic regulation in these plants. In these plants, only the chronic exposure to further stresses increased the modulation of DNAm-related genes, contrary to Ol plants that promptly activated these genes after one week of exposure. This new finding can have important implications for understanding the degree of stress perception in seagrasses, and since their regulation was strongly

dependent on local environmental conditions, these genes could be suggested as molecular epigenetic markers of stress response in *P. oceanica* plants.

Here, we also investigated a specific plant DNA methyltransferases (CMT2) involved in both maintenance and *de novo* methylation (Kenchanmane Raju et al. 2019). Chromomethylase can also be targeted by H3K9me2 due to the dual recognition mechanism mediated by its BAH and chromo domains (Du et al. 2012). High expression levels of chromomethylases were already observed in *P. oceanica* plants exposed to cadmium toxicity (Greco et al. 2012). In that case, DNA hypermethylation was associated with chromatin condensation increasing the heterochromatic nuclear fraction. In Ol plants, CMT2 and SUVH4 followed the same expression patterns, showing higher expression values in T1 and T5, contrary to Eu plants that repressed these genes in T1. This evidence suggests that DNA methylation and histone modifications are cooperating to regulate stress responses acting especially at the initial exposure to stresses.

Plants responses depend on stress-memory genes?

In addition to genes involved in DNA modifications, we also analysed the expression levels of FORGETTER1 (FGT1) which was identified as a relevant gene for heat stress memory in *A. thaliana* (Brzezinka et al. 2016). FGT1 is required for heat stress memory thought the interaction of FGT1 with chromatin remodelers that regulate the DNA accessibility (Brzezinka et al. 2016; Friedrich et al. 2019). In this study, the expression of FGT1 changed over time and was particularly susceptible in presence of heat stress in both Ol and Eu plants. However, in Eu plants, FGT1 was repressed in treatments with high nutrients additions and overexpressed in T only after one week of the initial exposure to stressful conditions. Thus, its regulation appeared to be significantly influenced by temperature and nutrients interactions at the early phase of the exposure to stressors. Generally, genes involved in the memorization of past stress events (i.e memory genes, Liu et al. 2015), show lower activation before the occurrence of a stress. During the exposure to stress conditions, they become active regulating the transcription of target genes involved in the stress response. Thus, in the presence of another stress event they can be more quickly re-induced (Oberkofler et al. 2021). It is worth to underline that a similar regulation seems to be activated in Eu plants, where the presence of temperature induced high expression levels that remained constant over time, whereas the exposure to nutrients activated FGT1 later, as the exposure time to stress conditions increases. This new finding revealed the potential role of FGT1 in regulating nutrients-memory responses in plants that already experienced high nutrient conditions in their home environments. Although this observation cannot be fully supported by these results as it requires the analysis of other genes involved in stress-memory responses (i.e heat-shock proteins, Liu et al. 2015), they represent a first evidence of a transcriptional memory in *P. oceanica* plants that needs to be further investigated.

Conclusions

In this study, the dynamics of DNA methylation of plants living in different environments and exposed to nutrient and temperature stresses was assessed through the analysis of total DNA methylation levels and the expression level of selected epigenetic-related genes during the experiment. We demonstrated that DNA methylation is a dynamic process influenced by environmental stresses and plants' origin, which may have important implications in regulating stress responses. The in-deep characterization of epigenetic mechanisms (e.g. context-specific DNA methylation changes) integrated with studies of plants from contrasting environments is necessary to better explore seagrass vulnerability to future environmental changes. Further investigations are also required to investigate on the potential link between epigenetic regulation and gene expression, which could provide new findings on potential markers for addressing seagrass vulnerability to stress

conditions. These findings represent a significant step forward in the study of epigenetic regulation in seagrass biology.

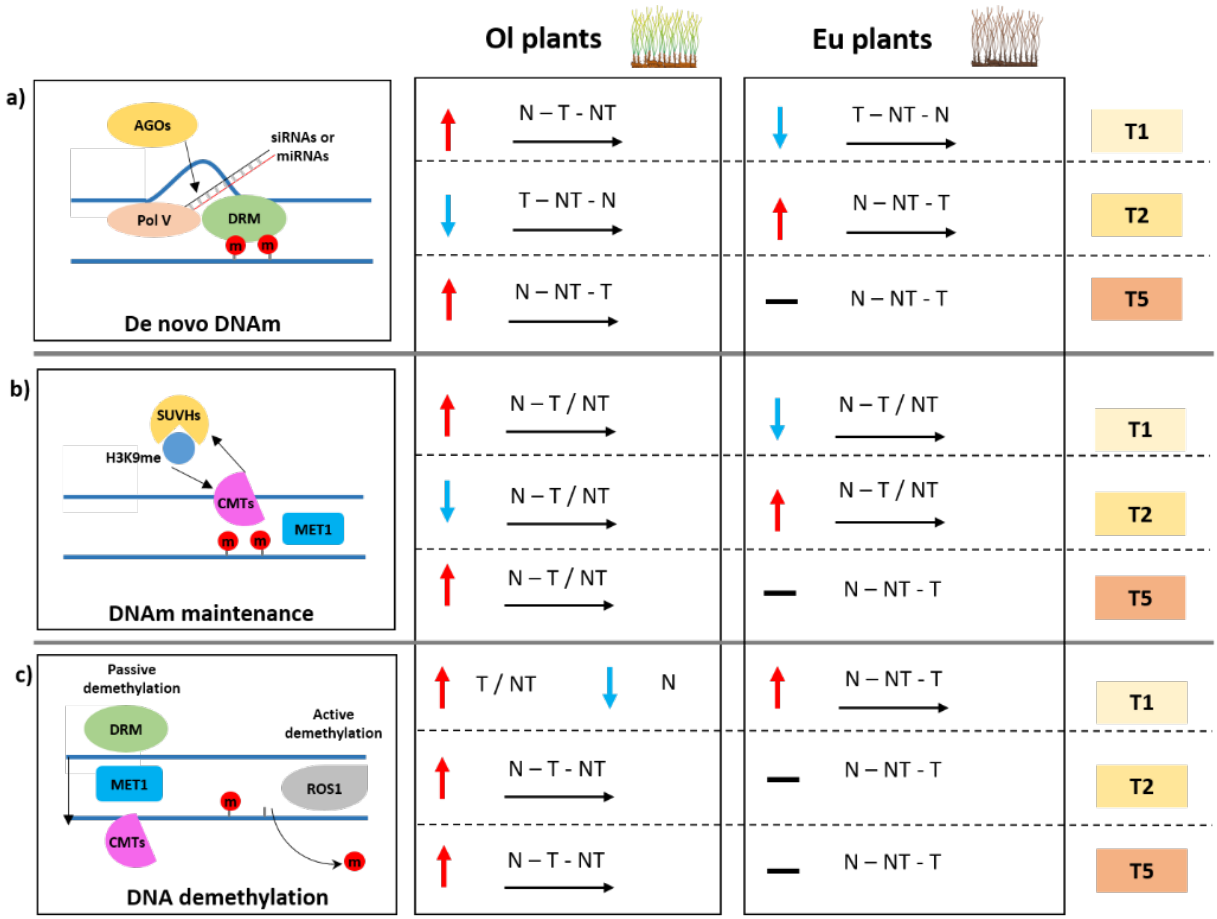


Figure 4. Summary graph showing expression patterns observed in both Ol and Eu plants during the exposure phases (T1 = one week; T2 = two weeks; T5 = five weeks). **a)** *De novo* DNA methylation catalyzed by DRM-AGO; **b)** DNA methylation maintenance catalyzed by MET1/CMTs and the cross-talk with histone methylation operated by SUVHs; **c)** DNA demethylation. **Red arrows** refer to gene expression increases; **blue arrows** refer to gene expression decreases; **black lines** refer to constant gene expression levels; **black arrows** and their direction indicate the trend from the highest to the lowest expression levels measured among treatments (N = nutrients; T = temperature; NT = nutrients + temperature).

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CHAPTER V

(Paper V)

Jessica Pazzaglia, Alex Santillán-Sarmiento, Miriam Ruocco, Luca Ambrosino, Emanuela Dattolo, Lazaro Marín-Guirao, Gabriele Procaccini. Thermal priming increases heat-stress tolerance in *P. oceanica* seedlings.

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Thermal priming increases heat-stress tolerance in *P. oceanica* seedlings

Jessica Pazzaglia[1,2], Fabio Badalamenti[1,3], Jaime Bernardeau-Esteller[4], Juan M. Ruiz[4], Vincenzo Maximiliano Giacalone [5], Gabriele Procaccini[1*], Lazaro Marín-Guirao[1,4*]

[1] Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy

[2] Department of Life Sciences, University of Trieste, 34127, Trieste, Italy

[3] CNR-IAS, Lungomare Cristoforo Colombo 4521, 90149 Palermo, Italy

[4] Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, Murcia, Spain

[5]CNR-IAS, Via del Mare 3, 91021 Torretta Granitola, Italy

*These authors have contributed equally to this work

Keywords: ***priming, seagrass seedlings, respiration, photosynthesis, gene expression, epigenetics***

Abstract

Seawater warming and the increased incidence of marine heatwaves (MHW) are threatening the integrity of coastal marine habitats including seagrass meadows, which are particularly vulnerable to climate changes. Novel stress tolerance-enhancing strategies, including thermal priming, have been extensively applied in terrestrial plants for enhancing resilience capacity under the re-occurrence of a stress event. We applied, for the first time in seagrasses, a thermo-priming treatment to *P. oceanica* seedlings. We analyzed the photo-physiological and growth performance of primed and non-primed seedlings, and the gene expression responses of selected genes (i.e. stress-, photosynthesis- and epigenetic-related genes). Results revealed that during the re-occurring stress event, primed seedlings performed better than unprimed ones, showing unaltered photo-physiology supported by high expression levels of genes related to stress response, photosynthesis, and epigenetic modifications. These findings offer new opportunities to improve conservation and restoration efforts in a future scenario of environmental changes.

1. Introduction

In recent decades, the rates of changes including human pressures and climate change have been rapidly forcing organisms to exceed their resilient capacity and thus the potential to quickly respond and adapt to environmental changes (Doney et al., 2012). In the marine realm, sea warming is increasing at alarming rates inducing severe and significant consequences on ocean physical features as documented in the most recent IPCC assessment (2019). Sea-surface temperature changes include prolonged anomalous high temperature events that last for five or more days known as marine heatwaves (MHWs; Hobday et al., 2016). The occurrence of these events varies globally and regionally, with high intensity in the western part of the globe (+2-5°C), followed by the central and eastern equatorial Pacific Ocean (+1–4 °C) and eastern regions (+1–3 °C) considering boundaries of Northern Hemisphere oceans (Oliver et al., 2018). In the Mediterranean Sea, which is considered a hotspot for environmental changes, climatic events represent the main drivers that caused the largest impacts, especially on coastal areas and coastal ecosystems (Micheli et al., 2013). In this framework, it is fundamental to improve new strategies that allow to better estimate and mitigate future impacts on coastal marine environments.

To date, different approaches are being developed to improve conservation and restoration strategies of natural resources by human interventions, generally known as assisted evolution approaches (Filbee-Dexter and Smajdor, 2019). These interventions vary according to the level of organism manipulation, ranging from active-genome editing (e.g. CRISPR, Hsu et al., 2014) to less manipulative methods such as priming treatments (Jisha et al., 2013). In plant stress biology, the term "priming" refers to a stimulus, which prepares an organism for upcoming environmental challenges by improving its response capacity (Conrath et al., 2015). Hence, this

priming process modifies the phenotypic state of an organism (i.e. priming stimulus), favoring phenotypic-plastic adjustments to future environmental stress conditions (i.e triggering stimulus) (Hilker et al., 2016). The maintenance of these phenotypic responses constitute the "memory" of the past stress event that may be temporary or persist for several months, depending on the typology of the priming stimulus and on its duration (Pastor et al., 2013; Walter et al., 2013). Thus, the memorization of the past stress event consists in the recognition of the reoccurring event as a stress in order to activate the appropriate response (Friedrich et al., 2019). Terrestrial plants can be primed during young life stages (i.e. seeds and seedlings), improving seed germination, seedling establishment and growth (i.e. *Solanumly copersicum* seeds, González-Grande et al., 2020; *Arabidopsis thaliana* seedlings, Leuendorf et al., 2020). Numerous priming techniques have been applied (chemical, thermal or biotic, Rakshit and Singh, 2018), and all contribute to allow plants to better respond to re-occurring stress, minimizing the investments of resources. Primed plants show faster and stronger activation of defense mechanisms typically involved in stress responses, including expression of key responsive genes, epigenetic mechanisms and signaling pathways such as those involving hormones such as jasmonic acid, salicylic acid and ethylene (Bruce et al., 2007; Kreps et al., 2002).

Molecular mechanisms that regulate the priming process modulate genes transcription during the priming stimulus, producing much higher levels of transcripts during the subsequent stress (triggering stimulus), and resulting in the potential induction for a long-term "transcriptional memory" (e.g *A. thaliana*, Kotak et al. 2007; Liu et al. 2014). This defense response, which is enhanced by stress-memory, is arbitrated by epigenetic changes and the accumulation of signaling proteins with inactive configuration (Bruce et al., 2007). Epigenetic marks include DNA modifications operated by cytosine methyl-transferases that leave the DNA sequence unchanged, and acetylation, methylation, phosphorylation and ubiquitinylation of the nucleosome core histones (H2A, H2B, H3, H4) (Duncan et al. 2014). These last modifications induce changes in the chromatin structure, regulating the activation or repression of gene expression (Reyes et al. 2002). Thus, the priming of genes may be achieved through chromatin modifications that promote long lasting regulation favoring epigenetic memory (Borg et al., 2020). For instance, vernalization is an epigenetic-regulated process that involves the repression of the gene FLOWERING LOCUS C (FLC) maintained by histone H3 lysine 27 trimethylation (H3K27me3) (Hepworth and Dean, 2015). In plants, histone modifications occur in the presence of different abiotic stress, including heat-stress, which modify the fluidity of the membrane, the interaction of DNA with the nucleosome as well as the folding of chromatin proteins allowing the regulation of stress-responsive genes (Bäurle and Trindade 2020; Chen et al. 2011; Chinnusamy and Zhu 2009; Kumar et al. 2020).

Seagrasses are marine flowering plants that form extensive underwater meadows in most coastal areas, representing one of the most valuable ecosystems on earth (Costanza et al., 2014). Despite clonal growth is the most diffuse propagation typology among seagrass species, sexual reproduction through seed fertilization and seedling establishment is crucial for maintaining high genetic diversity, which enhances population resilience to environmental changes (Jahnke et al., 2015; McMahon et al., 2014). Similar to terrestrial forests, seagrasses represent a highly productive system supporting different ecosystem services such as O2 production and CO2 sequestration (Champenois and Borges, 2019; Fourqurean et al., 2012). The high degree of phenotypic plasticity that characterizes seagrass species favored their extensive distribution allowing adaptation to different marine environments (Pazzaglia et al., 2021b). However, rapid environmental changes can exceed their tolerance capacity preventing appropriate responses. Seagrasses are declining globally and estimates indicate an increased meadows loss rate of 7% year−1 since 1990 (Waycott et al., 2009). Projections estimate the functional extinction of some seagrass species in the next decades, including *Posidonia oceanica* (L.) Delile (Chefaoui et al., 2018), which is endemic to the Mediterranean Sea and one of the largest and long-lived plant species in the world (Arnaud-Haond et al., 2012). Increased temperature trends and MHWs negatively affect seagrass performances, accelerating respiration rates in a higher proportion than photosynthetic rates, eventually resulting in plant carbon imbalances (Collier and Waycott, 2014; Marín-Guirao et al., 2016; Nguyen et al., 2021). Species responses to heat stress are variable depending on local environmental conditions where plants grow and thus on local (pre-) adaptation (Marín-Guirao et al., 2017; Pazzaglia et al., 2020). MHWs have also the potential to affect flowering, seeds germination, seedlings development and survival thereby compromising the future of natural populations (Ruiz et al., 2018; Salo and Pedersen, 2014; Xu et al., 2016). Seedlings represent one of the most vulnerable life stages of seagrasses

(Balestri et al., 2009) and are particularly sensitive to MHWs. The experimental exposure to simulated heat waves induced negative effects on growth and seed germination, increasing mortality and the occurrence of indirect effects such as herbivory (Guerrero-Meseguer et al., 2017; Hernán et al., 2017; Pereda-Briones et al., 2019). Despite these early evidences, there is a lack of research conducted on seedlings and seeds with the aim to explore the effects of warming on these early life stages and more studies are required especially for improving seagrass conservation and management practices. This is particularly relevant in the frame of restoration and reinforcement of natural populations. Using seeds or seedlings as transplant material guarantees high genetic diversity levels, and novel approaches boosting resilience to environmental changes have been proposed in seagrasses (Pazzaglia et al., 2021a). In the present era of environmental changes, seagrass restoration has the potential to slow-down habitat degradation and fragmentation mitigating the negative impacts of the ongoing climate change (Duarte et al., 2020).

In seagrasses, different studies investigated the degree of phenotypic plasticity under different abiotic stressors, including thermal stress (Nguyen et al., 2021). Besides the expression of key stress-related genes under thermal stress conditions (e.g. HSPs), seagrass's responses include also the activation of epigenetics-related genes (i.e DNA and histone methylation, Marín-Guirao et al., 2017; Marín-Guirao et al., 2019). The methylome assessment of adult *Zostera marina* genets have underlined the existing relation of DNA methylation changes with phenotypic variation of fitness-related traits and heat stress resilience (Jueterbock et al., 2020). Moreover, a recent study has pointed to a relationship between gene body DNA methylation and the transcriptomic responsiveness of Mediterranean seagrasses to warming conditions, together with warming-induced changes in the level of global DNA methylation (Entrambasaguas et al., 2021). These evidences revealed the flexibility of the methylome in response to heat stress and the possibility of marine plants for memorizing heat responses. Only very recently in seagrasses, thermal priming has been successfully tested in adult plants of two species (*P. australis* and *Z. muelleri*) and the activation of key epigenetic-related genes seems to be involved in the process (Nguyen et al. 2020). Nevertheless, more studies are necessary to assess the potential role of epigenetic mechanisms in seagrass responses and stress-memory.

Here we applied, for the first time in seagrass seedlings, a thermo-priming stimulus to *P. oceanica* through the exposure of seedlings to an anomalous warming event (priming treatment: 30.5ºC). The induction of the priming status was subsequently assessed after two weeks by analyzing the photo-physiological and growth performance of primed and non-primed seedlings, as well as their gene expression responses of a selected set of genes (i.e. stress-, photosynthesis- and epigenetics-related genes) during their exposure to extreme high temperature (triggering treatment: 32ºC). The hypothesis is that young *P. oceanica* seedlings experiencing a seawater warming event during their first summer (thermo-primed seedlings) are better equipped to respond and resist to a subsequent more intense and longer-lasting warming event than seedlings grown under normal/average summer temperatures (non-primed seedlings).

2. Methods

Seedlings collection and experimental design

Beach-casted *P. oceanica* seeds were collected in June 2019 along the coasts of Marsala (West Sicily), where one of the largest *P. oceanica* meadow of the western Mediterranean Sea is located. Seeds were germinated and grown at Torretta Granitola/C.N.R. laboratory (N/W Sicily), during early- and mid-summer, in two circular outdoor tanks (2.5 m diameter; 4000 l) with flow-through natural seawater (ca. 22l min $^{-1}$) drawn from a well. During this period, seedlings were exposed to irradiance levels ranging from 50 to 80 (μmoles m^{-2} s^{-1}) by shading tanks with neutral screens to mimic the irradiance levels existing inside natural *P. oceanica* meadows in the region at 8-10 m depth.

In late summer (mid-September), seedlings were shipped by plane in thermos flasks with clean moist paper to the Oceanographic Center of Murcia (Spain) within about 12 hours' time. Upon arrival, seedlings were immediately transplanted in individual small seed pots (5x5x6 cm) filled with coarse gravel (2.5 cm) (Fig. 1). Subsequently, they were allocated randomly into nine tanks of an indoor mesocosms facility, where temperature was adjusted to 24.5ºC according to the natural summer temperature present in the sampling region

(SST was on average 24ºC and 26 °C in July and August, respectively). Tanks were filled with natural seawater from an oligotrophic, unpolluted area. Each tank was equipped with its own circuit of seawater, temperature and irradiance (see Marín-Guirao et al. 2018; Lázaro Marín-Guirao et al. 2013 for a complete description of the system). The system allows for an accurate control of the water temperature in tanks (± 0.2 °C) which was checked daily during the experiment by using a handheld mercury thermometer. Salinity was also checked daily and maintained constant at 37.5 (± 0.2) by adding purified fresh water to compensate for evaporation. Seawater quality was maintained throughout the experiment by continuous physical and chemical filtration and weekly partial (30-40%) water renewal. Irradiance in tanks was adjusted to 70 µmol quanta m^{-2} s^{-1} with a 12h:12h light:dark photoperiod according to the daily photosynthetic photon flux density measured within natural *P. oceanica* meadows (Marín-Guirao et al. 2015). Temperature in all tanks was progressively increased (0.3º C/day) from 24.5º C to 26º C, and seedlings were allowed to acclimate for ten days. After the acclimation period, temperature in three tanks (n=3) was progressively increased (0.5º C/day) up to 30.5º C to induce a priming stimulus, while the remaining of tanks were kept under control temperature. Previous studies have shown that this temperature level caused heat stress to 3-5 months old *P. oceanica* seedlings from different locations of the western Mediterranean Sea (Guerrero-Meseguer et al., 2017; Hernán et al., 2017; Pereda-Briones et al., 2019). This priming treatment lasted a total of 11 days, after which the temperature was progressively lowered to the control level of 26º C (1º C/day). Seedlings were kept at control temperature (26º C) for two weeks. After this period, seedlings from the three priming tanks and from three non-priming tanks were exposed to triggering treatment (i.e. extreme warming event), while the other three non-primed tanks continued growing under control temperature. The triggering treatment was applied by increasing temperature up to 32ºC (0.5º C/day) and lasted a total of 2 weeks since the beginning of temperature ramping. The response of primed (P), non-primed (NP) and control (C) seedlings was studied at the end of the simulated MHW (figure 1). Measurements performed on seedlings from the same tank (i.e. 'pseudo replicates') were averaged to obtain an independent replicated value since the experimental tank is the true experimental unit in our experiment. Therefore, the number of replicates used in statistical tests was n=3.

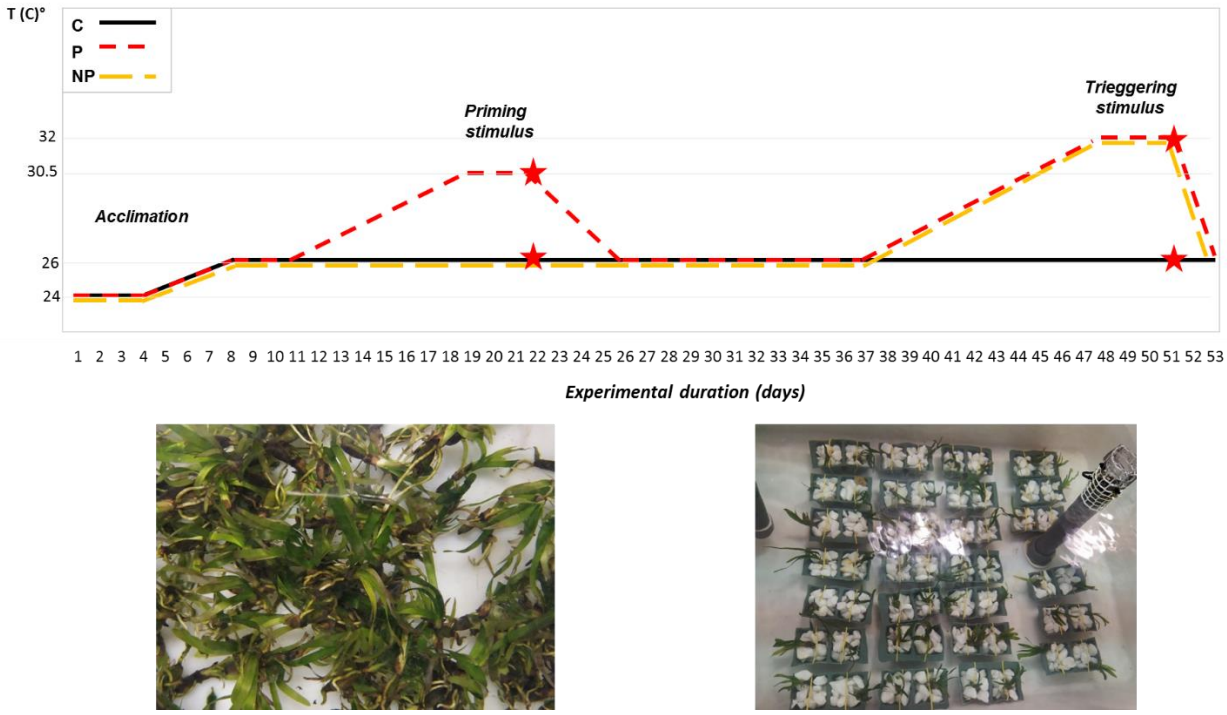

Figure 1. Experimental design. Seawater temperature during the course of the experiment (above panel). Black line refers to control (C), dashed red line refers to primed seedlings (P), and dashed yellow line refers to non-primed seedlings (NP), while red stars refer to sampling points. Pictures of 4-month old *P. oceanica*

seedlings upon arriving to the IEO mesocoms facility (lower-left panel) and after their transplantation and allocation in an experimental tank (lower-right panel).

Chlorophyll a fluorescence

Measurements of chlorophyll-fluorescence emissions were performed on four seedlings per tank using a pulse amplitude modulation portable fluorometer (diving-PAM; Walz, Germany). The light saturation pulse method was used to characterize the performance of the photosynthetic apparatus at the level of photosystem II (PSII). Measurements of basal (F_0) and maximum (F_m) fluorescence were conducted on whole-night dark-adapted seedlings to calculate the maximum photochemical efficiency of PSII ($F_v/F_m = F_m-F_0/F_m$). The method was applied again in the same seedlings after 5 hours of illumination in aquaria to determine the basal (F) and maximum (F_m') fluorescence of light-adapted leaves in order to calculate the effective photochemical efficiency of PSII ($\Delta F/F_m' = F_m' - F/F_m'$).

Photosynthetic and respiratory rates

Determination of the maximum photosynthetic and respiratory rates was carried out on two seedlings from each tank using an incubation chamber with a Clark-type O_2 electrode (Hansatech, UK) connected to a controlled temperature-circulating bath. From each seedling, a 2cm long leaf segment, taken from the middle part of the first mature leaf, was used in incubations. Leaf segments were first incubated in darkness for 10 min to determine dark respiration rates (R_d) and then exposed to six increasing irradiances (from 10 to 500 µmol quanta m^{-2} s^{-1}) to determine maximum photosynthetic rates (net-P_{max}). Gross photosynthesis (gross-P_{max}) was then calculated as the sum of net-P_{max} and R_d, and the ratio of gross-P_{max}:R_d was used as a proxy of the leaf metabolic carbon balance.

Leaf pigments content

Leaf pigment content (chlorophyll *a*, chlorophyll *b* and total carotenoids) was analyzed in the same leaf segments used in photosynthetic and respiratory measurements. Leaf pigments were extracted from each leaf segment in 10 ml of 80% acetone and examined spectrophotometrically at 470, 646, and 663 nm. The concentration of chlorophyll *a* and *b* along with total carotenoids was calculated from the readings using the equations of Lichtenthaler and Wellburn (1983).

Seedlings growth and morphology

Seedlings growth was determined by using the punch-hole technique (Zieman, 1974). The leaves of three seedlings from each tank were marked with a needle at the height of the ligule before the beginning of the warming exposure. Marked seedlings were collected two weeks later at the end of the warming period (triggering treatment) and the surface area of newly formed leaf tissues (i.e. those below the mark) was measured to estimate leaf growth (cm^2 d^{-1}). Seedlings size (i.e. leaf surface area; cm^2) was also characterized by measuring the length and width of all leaves form each marked seedling.

Gene expression analysis

Total leaf tissue of seedlings was collected per each condition in triplicates to perform gene expression analysis. Samples were cleaned from epiphytes and entirely submerged in RNAlater© tissue collection (Ambion, life technologies), stored overnight at 4 °C to let the solution penetrate into the tissue and finally stowed at − 20 °C until RNA extraction. Total RNA was extracted with Aurum™ Total RNA Mini Kit (BIO-RAD) following manufacturer's protocol. Purity and concentration of RNA were checked using the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific) and 1.5% agarose gel electrophoresis. Subsequently, 500ng of RNA from each sample and condition was retro-transcribed into cDNA with the iScript™ cDNA synthesis kit (BIO-RAD), according to manufacturer's instructions.

Quantitative Real-Time PCR (RT-qPCR) on genes of interest (GOIs)

Primers for 15 genes of interest (GOIs) were selected from previous studies or designed based on *P. oceanica* transcriptomes (D'Esposito et al., 2017; Marín-Guirao et al., 2017) with the primer analysis software Primer3

v. 0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012). In detail, 10 GOIs were selected from previous studies according to specific functional categories including stress-related and photosynthesis-related genes. General-stress response was assessed targeting heat shock genes (HSP90 and SHSP), key gene involved in mitochondrial energy dissipation mechanisms (AOX), antioxidant response (MSD) and DNA repair response (DDB) (Table 1). A number of genes involved in light reaction functions of photosynthesis (psbA, PSBS, FD), chlorophyll *a-b* binding proteins (CAB-151), and a key enzyme involved in the chlorophyll biosynthetic pathways (POR) were also targeted. Epigenetics-related genes with specific methylation activities (ATX2, ATRX7, ASHL2, SETD3) and the Transcriptional activator-DEMETER, which catalyzes the release of 5-methylcytosine (5-meC) from DNA, were designed setting the primer length to 18-20bp, product size to 100–200 bp and Tm = 59–61◦C. Three reference genes (18S, elf4A and GADPH) were selected and used to normalize gene expression of target genes according to previous related works based on plant response to thermal stress conditions or abiotic stresses (Dattolo et al., 2014; Lauritano et al., 2015; Serra et al., 2012). The best reference genes for our experimental conditions were identify by using the web-based tool RefFinder (Xie et al., 2012). Primers efficiency was assessed with different cDNA dilutions and using a linear regression model to calculate the percentage of efficiency as follows: $E\ (\%) = (10^{-1}/\text{slope}^{-1}) \times 100$ (Radonić et al. 2004; Table 1). RT-qPCR reactions were performed in triplicates in a Viia7 Real Time PCR System (Applied Biosystems) using Fast SYBR®Green MasterMix (Applied Biosystems) as fluorescent detection chemistry and MicroAmp Optical 384-well reaction plates (Applied Biosystems). Reactions were carried out in a 10µl final volume with 5µl MM SYBR ® Green, 2µl of 1.4 pmol µl-1primers and 1µl of 1:50 cDNA template. The thermal profile of the reactions was as follows: 95°C for 20 s, 40 times 95°C for 1 s and 60°C for 20 s. Relative quantification of gene expression was obtained using the following equations

$$\Delta CT = CT\text{reference gene} - CT\text{GOIs}$$

to evaluate the negative differences in cycles to cross the threshold value between the reference and the target GOI (-ΔCT). Subsequently, ΔΔCT were calculated on ΔCT means for each GOI by comparing ΔCT of treatments (2HW and 1HW) with the control (C). The Fold change expression was assessed according the following equation:

$$\text{Fold expression change} = +2(|(\Delta\Delta CT\text{treatment}) - (\Delta\Delta CT\text{control})|)$$

Statistical analysis

One-way analysis of variance (ANOVA) was conducted to detect significant differences in the response to the simulated MHW among experimental *P. oceanica* seedlings (i.e. primed, non-primed and control seedlings). Before carrying out ANOVA analyses, Shapiro–Wilk and Levene's tests were applied to assess the normality and homoscedasticity of the data and transformed where necessary. Subsequently, Student-Newman-Keuls (SNK) *post hoc* test was used whenever significant differences ($P < 0.05$) among treatments were detected using the statistical package STATISTICA (StatSoft, Inc., v. 10). Photo-physiological and gene expression results of GOIs were also analyzed using Permutational Multivariate Analyses of Variance (PERMANOVA) that were carried out on Euclidean distances of data, using 9999 permutations of the residuals under a reduced model. Significant differences were investigated using a posteriori pair-wise test. P values in the PERMANOVA and pairwise tests were obtained from Monte Carlo asymptotic distributions, because of the restricted number of unique permutations. The analysis was performed using Primer 6 v.6.1.16 and PERMANOVA + v.1.0.6 software package (PRIMER-E Ltd) (Anderson et al., 2008). Data is presented as average values ± standard error (n=3).

Table 1. List of housekeeping genes and genes of interest analyzed in this study.

Gene category	Gene	Protein	Forward sequence (5->3)	Reverse sequence (3-->5)	S	E (%)	R2	Reference
Huskeeping genes	GADPH	Glyceraldehyde-3-phosphate dehydrogenase	AGGTTCTTCCTGCTTTGAATG	CTTCCTTGATTGCTGCCTTG	138	93	0.99	Serra et al., 2012
	18S	Ribosomal RNA 18S	AACGAGACCTCAGCCTGCTA	AAGATTACCCAAGCCTGTCG	200	100	0.99	Serra et al., 2012
	elF4A	Eukaryotic initiation factor 4A	TTCTGCAAGGGTCTTGACGT	TCACACCCAAGTAGTCACCAAG	192	85	0.99	Lauritano et al., 2015
Stress-related genes	HSP90	Heat shock protein 90	CTCCATCTTGCTTCCCTCAG	TCAGTTTGGAGGAACCGAAC	146	100	0.99	Lauritano et al., 2015
	SHSP	Small heat shock protein	ACCGGAGGATGTGAAGATTG	AGCTTGCTGGACAAGGTGAT	125	99	0.99	Lauritano et al., 2015
	AOX	Alternative oxidase 1a	TGCTGCATTGCAAGTCTCTAC	GTTGTGACACCTCCATGAAGGTC	116	100	0.99	Procaccini et., 2017
	MSD	Manganese superoxide dismutase	GGCGGAGGTCATATAAACCA	ATAAGCAAGCCACACCCATC	192	0.93	0.99	Lauritano et al., 2015
	DDB	Damaged DNA binding protein	TCTCAGGTCCGGCACTAATC	GAAAGGCTTGCTCGTATTGC	224	100	0.99	Lauritano et al., 2015
Photosynthesys-related genes	CAB-151	Chlorophyll a-b binding protein 151, chloroplastic	AAGCCCATTAGCACAACCTG	GGGCAATGCTTGGTACTCTC	199	93	0.99	Dattolo et al., 2014
	POR	Protochlorophyllide reductase	AGTTCCACAGACGGTTCCAC	AATCACCACCTGAGCGAGTC	194	98	0.99	Ruocco et al., 2018
	FD	Ferredoxin-1, chloroplastic	TCAGACTGGGGGTAAGCAAC	TCTACATCCTCGACCACTGC	187	100	0.98	Dattolo et al., 2014
	psbA	Photosystem II protein D1	GACTGCAATTTTAGAGAGACGC	CAGAAGTTGCAGTCAATAAGGTAG	137	92	0.99	Dattolo et al., 2014
	PSBS	Photosystem II 22 kDa protein, chloroplastic	CCGCTCCTGTTGTTCTTCAT	GGACCTCCTTCCTTGAGACC	158	100	0.99	Dattolo et al., 2014
Epigenetic-related genes	ATX2	Histone-lysine N-methyltransferase ATX2	CCAGATACAAAGCTGCACCA	GCATTGTCATCCCCTTGAGT	170	94	0.99	This study
	ATRX7	Histone-lysine N-methyltransferase ATXR7 isoform X1	CGAGTAGGGTCGAATGTGGT	ATCCATCCAGTCACACACGA	149	95	0.98	This study

ASH2L	Ash2 histone methyl transferase complex subunit ash-2	CTATCCTGCTGCCTCCATGT	TCAACTGCACCTTCAACTCG	170	94	0.99	This study
SETD3	Histone-lysine N-methyltransferase setd3	TGGGCTTGRGAACTGTGGTA	CGAATGATTGAGTCGTCCAG	200	99	0.99	This study
DME	Transcriptional activator DEMETER	CAACTGTTCCCCTCACTGGT	CCACAGGTTCAGGTTCTGGT	162	94	0.99	This study

3. Results

The multivariate analysis (PERMANOVA) showed that the overall photo-physiological and gene expression responses of non-primed seedlings (NP) significantly differed from the response of control (P (MC) = 0.008) and thermo-primed seedlings (P (MC) = 0.040), Table 2). The overall response of the latter was also significantly different from C (P (MC) = 0.048), though with the weakest significance value.

Table 2. Output of the PERMANOVA analysis carried out on photo-physiological and gene expression data obtained from different treatments (C = Control, P = Primed, NP = Non-Primed). Df = degrees of freedom; MS=mean square; Pseudo-F=F statistic; P (MC) = probability levels obtained from Monte Carlo asymptotic distributions.

PERMANOVA

Suorce of variation	**Df**	**MS**	**Pseudo-*F***	**P (MC)**
Treatment	2	202550	13.351	**0.005**
Residulas	6	15171		
Total	8			
Pairwise test	***T***	**P (MC)**		
C, P	2.6887	**0.048**		
C, NP	4.7513	**0.008**		
P, NP	2.9555	**0.040**		

Values in bold indicate significant differences (P (MC) < 0.05).

Effects of heat priming on leaf pigment responses and photochemical responses to warming

The triggering treatment significantly affected chlorophylls (Chl *a* and Chl *b*) and total carotenoids content of *P. oceanica* seedlings (Table 2). All analyzed pigments showed a generalized reduction after the warming exposure, being stronger and more significant in non-primed seedlings (NP) with respect to primed (P) seedlings (Fig. 2; Table 2). In fact, the percentage of reduction of Chl *a*, Chl *b* and total carotenoids against controls was respectively of 16.6, 18.6 and 15.5% in P seedlings, while the corresponding values for NP seedlings were of 26.7, 28.9 and 25.1% , respectively.

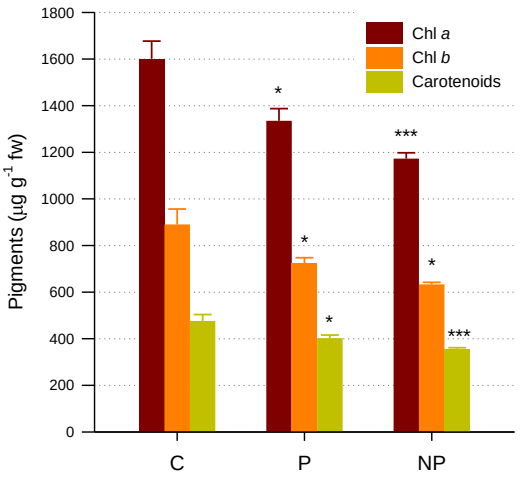

Figure 2. Pigment responses. Pigments content in leaves of control (C), primed (P) and non-primed (NP) *P. oceanica* seedlings at the end of the simulated MHW. Asterisks denote significant differences with respect to controls as derived from the Newman-Keuls post-hoc test. * $p<0.05$; *** $p<0.001$.

At the end of the extreme warming event, the maximum photochemical efficiency of PSII (Fv/Fm) of P and NP seedlings was significantly lower with respect to controls (Fig. 3a; Table 2). However, primed seedlings showed 14% and 20% greater effective photochemical efficiency (light-adapted $\Delta F/F_m'$) than controls ($p = 0.019$) and non-primed seedlings ($p = 0.011$), respectively (Fig. 3b).

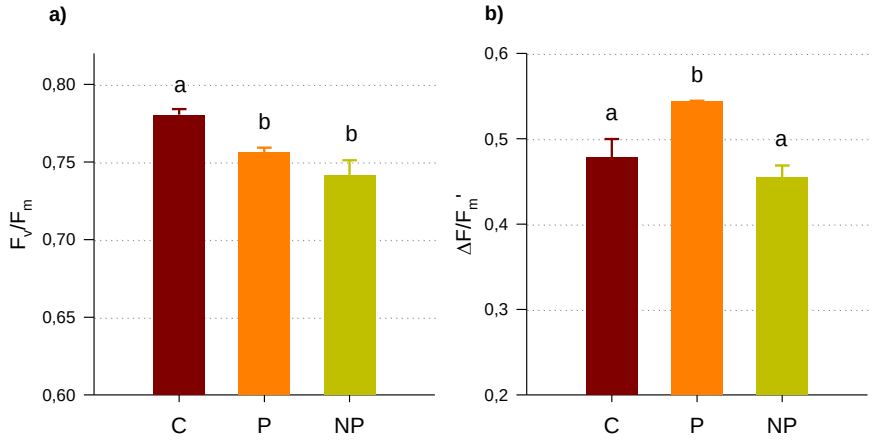

Figure 3. Photochemical responses. Maximum (F_v/F_m; a) and effective quantum yield ($\Delta F/F_m'$; b) of control (C) primed (P) and non-primed (NP) *P. oceanica* seedlings at the end of the triggering treatment. Different letters indicate significant differences obtained in the post hoc Newman-Keuls test ($p < 0.05$).

Effect of heat priming on photosynthetic and respiratory responses to warming

The exposure to an extreme warming event did not significantly affect the maximum gross-photosynthetic rates of *P. oceanica* seedlings**,** although the average rates of primed (P) and non-primed (NP) seedlings were respectively 27% and 15% lower than controls (gross-P_{max}; Fig. 4a; Table 3). This triggering treatment significantly increased the respiratory rates of NP seedlings by 48% ($p<0.001$) while, on the contrary, the respiration of P seedlings was reduced by 13%, although not significantly different from control (Rd; Fig. 4b; Table 3). As a consequence of the above responses, C and P seedlings showed a similar leaf carbon balance, whereas the carbon balance of NP seedlings was 43% and 35% lower than C and P seedlings, respectively (P:R ratio; Fig. 4c; Table 3).

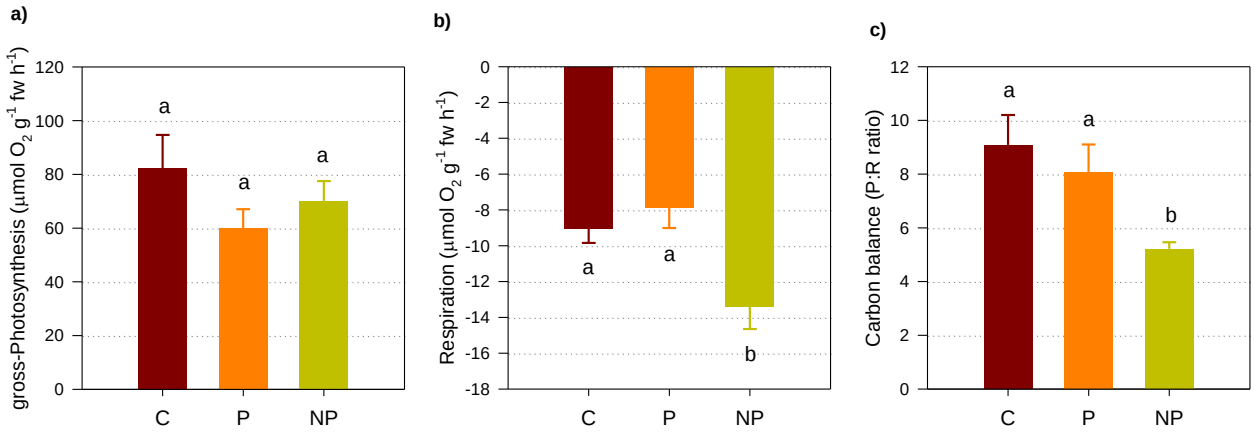

Figure 4. Photosynthetic and respiratory responses. Gross-Photosynthesis (a), respiratoration (b) and carbon balance (c) of control (C), primed (P) and non-primed (NP) *P. oceanica* seedlings at the end of the triggering treatment. Different letters indicate significant differences obtained in the post hoc analysis once significant effects were detected in the ANOVA analysis.

Effect of heat priming on seedlings growth and seedlings size

The warming exposure significantly affected leaf growth rates of *P. oceanica* seedlings (Fig. 5a; Table 3). Primed seedlings (P) showed 27% higher growth rates than control seedlings ($p = 0.034$), whereas the rates of non-primed seedlings (NP) were similar to controls ($p = 0.545$)**.** At the end of the simulated MHW the leaf surface area of P seedlings was 21% and 24% higher than C and NP, although the differences were not statistically significant (Fig. 5b; Table 3).

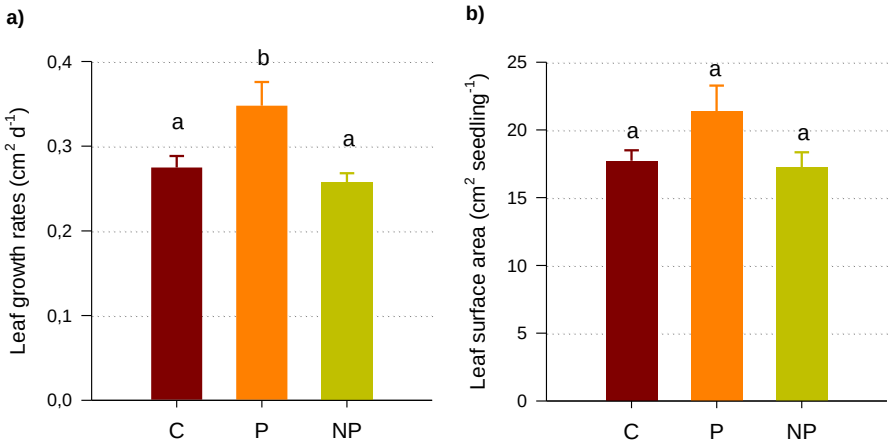

Figure 5. Morphological responses. Leaf growth rates **(a)** and leaf surface area **(b)** of control (C), primed (P) and non-primed (NP) *P. oceanica* seedlings at the end of the triggering treatment. Different letters indicate significant differences ($p < 0.05$) from Newman-Keuls post hoc test once significant effects were detected in the ANOVA analysis.

Table 3. Results of one-way ANOVA analysis for factor "Tratment" (T) for leaf growth rate, leaf surface area, maximum quantum yield (Fv/Fm), effective quantum yield ($\Delta F/F_m$), chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids, dark respiration rate (Rd), gross maximal photosynthesis (Gross-Pmax) and carbon balance (P:R).

One-Way ANOVA

Variable	Factor	df	MS	F	P	SNK pairwise tests
Relative growth (biomass)	Treatment (T)	2	0.00	3.87	0.083	
	Error	6	0.00			

Leaf surface area	Treatment (T)	2	15.33	2.83	0.137	
	Error	6	5.42			
F0	**Treatment (T)**	2	2211.47	9.96	**0.012**	**P, NP ≠ C (P < 0.05)**
	Error	6	222.06			P = NP (P > 0.05)
Fm	Treatment (T)	2	4400.06	3.81	0.086	
	Error	6	1155.00			
Fv/Fm	**Treatment (T)**	2	0.00	10.65	**0.011**	**P, NP ≠ C (P < 0.05)**
	Error	6	0.00			P = NP (P > 0.05)
DF/Fm'	**Treatment (T)**	2	0.01	9800.00	**0.013**	**P ≠ C, NP (P < 0.05)**
	Error	6	0.00			
ETRmax	Treatment (T)	2	4.45	3.65	0.092	
	Error	6	1.22			
NPQ	Treatment (T)	2	0.21	4.47	0.065	
	Error	6	0.05			
Chl a	**Treatment (T)**	2	140159.62	15.41	**0.004**	**P, NP ≠ C (P < 0.05)**
	Error	6	9094.68			P = NP (P > 0.05)
Chl b	**Treatment (T)**	2	50991.87	10.27	**0.012**	**P, NP ≠ C (P < 0.05)**
	Error	6	4963.11			P = NP (P > 0.05)
Carotenoids	**Treatment (T)**	2	10915.02	11.43	**0.009**	**P, NP ≠ C (P < 0.05)**
	Error	6	954.93			P = NP (P > 0.05)
Rd	**Treatment (T)**	2	25.48	15.62	**0.004**	**NP ≠ C, P (P < 0.01)**
	Error	6	1.63			
Gross-Pmax	Treatment (T)	2	374.42	2.64	0.151	
	Error	6	141.93			
P:R	**Treatment (T)**	2	12.05	11.25	**0.009**	**P, NP ≠ C (P < 0.05)**
	Error	6	1.07			P = NP (P > 0.05)

Values in bold indicate significant differences (P < 0.05).

Gene expression responses

Among 15 selected GOIs, nine showed significant fold expression changes, especially those included in the stress-related and epigenetics categories. Expression level of stress-related genes was significantly affected by the triggering treatment. Specifically, heat shock proteins (HSP90 and SHSP) were significantly over-expressed in both P and NP (Table 3; Fig. 6) in respect to control conditions. Despite no statistical differences were observed between P and NP, the former showed twice the expression level of the latter. The Alternative oxidase 1a (AOX) increased its level of expression up to 10-fold in both P and NP, with particular relevance in NP. Photosynthesis-related genes were over-expressed in P and NP, but significant differences were observed only for Ferredoxin-1 (FD) in P. Interestingly, photosynthetic pigment-related genes (CAB-151 and POR) followed an opposite regulation in P respect to NP, even if this was not supported by a statistical significance. Epigenetics-related genes were all up-regulated in both P and NP, where ASHL2 and SETD3 genes were commonly over expressed among treatments, contrary to ATRX7 and DME genes that showed significantly expression levels only for P seedlings. Overall, epigenetics-related genes showed a higher activation in P seedlings without significant differences among treatments.

Table 4. Results of one-way ANOVA analysis conducted on −ΔCT values for primed (P) and not-primed (NP) seedlings.

One-Way ANOVA							
Gene category	**Variable**	**Factor**	**df**	**MS**	**F**	**P**	**SNK pairwise tests**

Stress-related genes	*HSP90*	**T**	2	4.74	37.77	**0.00**	**NP ≠ C (P < 0.01)** **P ≠ C (P < 0.01)**
		Error	6	0.13			P = NP (P > 0.05)
	SHSP	**T**	2	10.23	6.98	**0.03**	**NP ≠ C (P < 0.05)** **P ≠ C (P < 0.05)**
		Error	6	1.47			P = NP (P > 0.05)
	AOX	**T**	2	14.32	12.89	**0.01**	**NP ≠ C (P < 0.01)** **P ≠ C (P < 0.01)**
		Error	6	1.11			P = NP (P > 0.05)
	MSD	T	2	2.15	4.83	0.06	
		Error	6	0.44			
	DDB	**T**	2	2.83	14.06	**0.01**	**NP ≠ C (P < 0.05)** **P ≠ C (P < 0.01)**
		Error	6	0.20			P = NP (P > 0.05)
	CAB-151	T	2	1.49	3.68	0.09	
		Error	6	0.40			
	POR	T	2	0.89	1.44	0.31	
		Error	6	0.62			
Photosynthesis-related genes	*FD*	**T**	2	3.47	5.80	**0.04**	NP = C (P > 0.05) **P ≠ C (P< 0.05)**
		Error	6	0.60			P = NP (P > 0.05)
	psbA	T	2	2.30	3.27	0.11	
		Error	6	0.70			
	PSBS	T	2	1.29	3.15	0.12	
		Error	6	0.41			
	ATX2	T	2	2.02	1.65	0.27	
		Error	6	1.22			
	ATRX7	**T**	2	2.23	6.46	**0.03**	**P ≠ C (P< 0.05)** NP = C (P > 0.05) P = NP (P > 0.05)
		Error	6	0.35			
Epigenetics-related genes	*ASHL2*	**T**	2	3.32	14.23	**0.01**	**NP ≠ C (P< 0.05)** **P ≠ C (P< 0.01)**
		Error	6	0.23			P = NP (P > 0.05)
	SETD3	**T**	2	3.16	6.69	**0.03**	**NP ≠ C (P< 0.05)** **P ≠ C (P< 0.05)**
		Error	6	0.47			P = NP (P > 0.05)
	DME	**T**	2	1.56	5.07	**0.05**	**P ≠ C (P< 0.05)**
		Error	6	0.31			P = NP (P > 0.05)

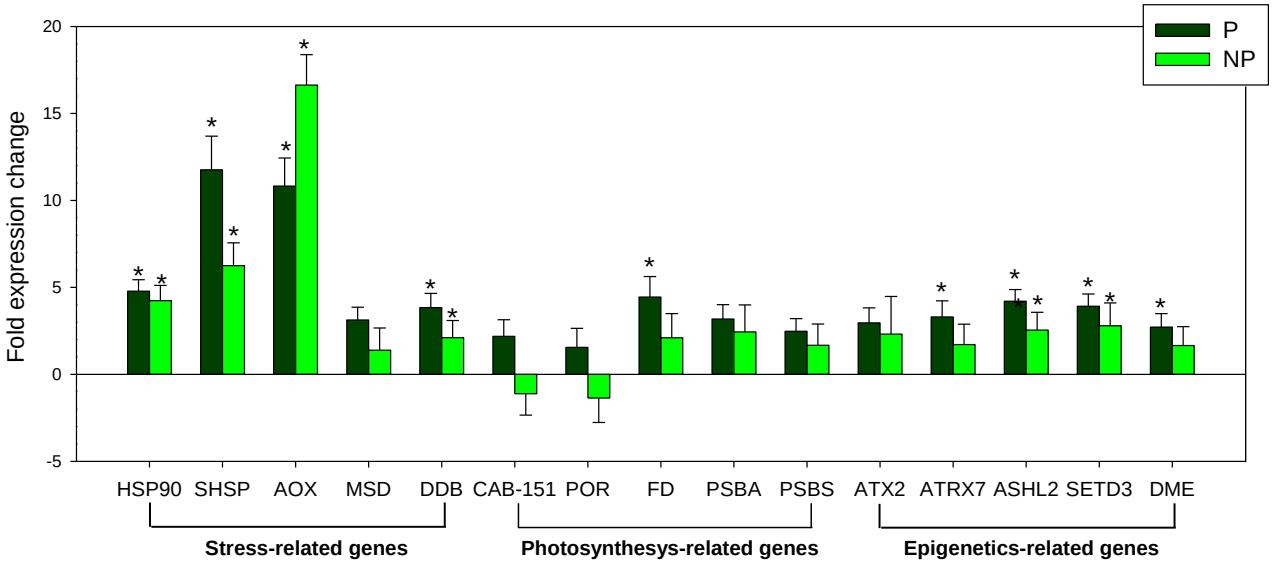

Figure 6. Gene expression. Relative expression of GOIs selected for stress-related category (HSP90, SHSP, AOX, MSD, DDB), photosynthesis category (CAB-151, POR, FD, psbA, PSBS) and epigenetic category (ATX2, ATRX7, ASHL2, SETD3, DME) in primed (P) and not-primed (NP) seedlings vs. control conditions (x-axis). Asterisks indicate post hoc significant differences of the treatment respect to the control.

Discussion

Phenotypic plasticity is the main factor behind the capability of organisms to cope and survive with changes in their niches, being crucial for the species to withstand and survive the ongoing climate change (Merilä and Hendry, 2014). This property is particularly relevant in long-lived organisms, such as several seagrass species (i.e. *P. oceanica*), as it modifies individuals' phenotype, through physiological and molecular changes, in order to adjust their performance under changing environmental conditions (Pazzaglia et al. 2021b). Findings from this study provide experimental evidences about the potential of *P. oceanica* seedlings to acquire a thermo-primed status that eventually confers an enhanced tolerance and resistance to an extreme warming event. Thermo-primed seedlings performed better during the re-occurring heat stress event than non-primed seedlings and offered some insights into the molecular basis of thermal priming in seagrass seedlings. During the triggering stimulus (i.e. the second exposure to high temperatures), these seedlings experienced lower thermal pigment degradation than non-primed seedlings, kept their carbon balance unaltered through a complete respiratory homeostasis and increased their growth rates leading to larger seedlings. In addition, the altered expression levels of epigenetic-related genes pointed to the potential involvement of chromatin remodeling processes as the basis of the acquired primed status revealing that early life stages of seagrasses may have the potential for long-term storage of stress responses.

In this study, the maximum quantum yield (Fv/Fm) of both P and NP experienced a significant reduction during the trigging stimulus confirming that 32 ºC is a stressful temperature for *P. oceanica* seedlings, likely close to the lethal temperature limit of the species (Guerrero-Meseguer et al., 2020; Hernán et al. 2017; Olsen et al., 2012; Pereda-Briones et al., 2019). Interestingly, the effective photochemical capacity (ΔF/Fm') of thermo-primed seedlings (P) was higher than controls and NP seedlings indicating an improved capacity to move electrons along the photosynthetic electron transport chain (ETC). These seedlings, contrarily to NP, increased the expression level of Ferredoxin (FD) which is a key gene of the chloroplast electron transport chain encoding for the photosynthetic electron carrier, and thus, likely responsible of the observed photochemical enhancement. FD plays an important role in the final step of the linear electron flow, thanks to its ability to divert electrons to cyclic or alternative electron flow pathways, sustaining photosynthesis and minimizing damaging ROS production (Munekage et al., 2004). In *P. oceanica* adult plants, thermal stress affects the regulation of ROS production and their removal altering defense mechanisms and plant

performances (Mittler et al., 2004). Here, despite the enhanced photochemistry, P seedlings showed lower photosynthetic capacity (O_2 production), although not significantly, suggesting the lack of acclimation in photosynthetic carbon fixation to imposed warming conditions. The inconsistency between the two photosynthetic parameters (i.e. photochemical efficiency and photosynthetic capacity) can be due to the potential activation/deactivation of alternative electron transport pathways (e.g., photorespiration, water–water cycle, cyclic electron transport; Niyogi, 2000) under stressful conditions, as already described in seagrasses under different abiotic stress conditions (e.g. Dattolo et al. 2017; Marín-Guirao, Sandoval-Gil et al., 2013; Silva et al. 2013). Alternatively, it might be related to changes in leaf absorbance, which can be promoted by changes in leaf pigment content and leaf morphology (Enríquez, 2005). During the exposure to anomalous high temperatures, thermo-primed seedlings experienced a lower generalized pigment degradation than non-primed seedlings, evidencing an improved tolerance to warming. We cannot determine whether this response is or not the result of *de novo* synthesis of thermally stable isoforms of proteins during the warming exposure (Somero, 1995). In fact, only primed seedlings activated genes (although not significantly) for the synthesis of photosynthetic pigments. Although the expression of pigment-related genes was not statistically significant, it could be reflecting a stronger activation during the early responses to warming. Significant changes in protein abundance through gene expression requires time, as the increase in protein abundance of the thermally stable isoform can take several days since the strong activation of the related gene (Degen et al., 2021).

The evidence of the increased heat tolerance in thermo-primed seedlings was also supported by the complete respiratory homeostasis achieved by these seedlings under warmed waters. Respiratory homeostasis is a functional trait associated with the tolerance to heat in seagrasses, as previously shown for *P. oceanica* and other seagrass species (e.g. Collier et al., 2011; Marín-Guirao et al., 2018, 2016). Through this metabolic acclimative response P seedlings were able to maintain the carbon balance unaltered (i.e. photosynthetic to respiratory ratio) under warming conditions, allowing the availability of fixed carbon for primary plant processes, such as growth and carbon storage. Carbon storage is a key process for the species survival since the ability of plants for overwintering tightly depends on the energy reserves stored during the summer growing season (Alcoverro et al., 2001). The evidence that P seedlings performed better during the triggering stimulus than NP seedlings was clearly reflected by their higher growth rates that ultimately led to larger seedlings. As demonstrated for terrestrial plants, one of the main advantages for inducing a priming status is the activation of thermo-tolerance mechanisms allowing the generation of more productive and larger individuals able to better cope with stressful conditions (*Triticum aestivum*, Wang et al. 2014). In contrast, non-primed seedlings experienced a dramatic carbon balance reduction driven mainly by an increased respiratory activity. However, they grew similarly to controls and attained also a similar size. This result suggests that seedling growth was sustained by the mobilization of carbohydrate reserves stored on seeds, which constitute a functional part of seedlings for several months, making young seedlings relatively independent from external conditions (Celdrán and Marín, 2013). Moreover, since the photosynthetic activity of seeds also contribute to seedlings growth and their metabolic (respiratory) activity varies with environmental changes (e.g. Temperature; Celdrán & Marín, 2011), it would be interesting to also study seed responses when exploring thermal priming strategies in seagrass seedlings. As commented above, the contrasting ability to regulate respiration under increased temperatures between thermo-primed and unprimed seedlings pointed to a lower heat-sensitivity in the former (Marín-Guirao et al. 2016, 2018). An accelerated respiratory metabolism leads to excessive production of ROS causing progressive oxidative damage and ultimately cell death (Mittler et al., 2004). The strong activation in heated seedlings of the alternative oxidase (AOX) pathway of the mitochondrial ETC can be interpreted as a metabolic response for alleviating ROS production. AOX activity appears to increase under stressful conditions that cause oxidative stress, including heat stress (Del-Saz et al., 2018; Saha et al., 2016), and helps to dissipate excessive reducing equivalents and limit respiratory ROS production (Scafaro et al., 2021). The induction of AOX under heat stress supports their pivotal role in mediating seagrass stress acclimation as previously suggested in *P. oceanica* adult plants (Marín-Guirao et al. 2017; Ruocco et al. 2019a; Tutar et al. 2017). Moreover, since the induction of AOX is dependent on ROS accumulation, the much higher AOX induction in non-primed with respect to thermo-primed seedlings, could be revealing that the former were suffering a greater heat-stress level and thus, greater heat-induced ROS production. Primed seedlings, indeed, activated a stronger antioxidant defense when subjected to increased temperatures and induced a stronger

expression of small heat shock proteins (SHSP), doubling the expression level of nonprime seedlings. SHSPs, as ubiquitous molecular chaperones, are involved in the heat stress response of plants and provide an effective and low-cost thermo-protection, responsible for downstream plant thermo-tolerance (Sun et al., 2002; Wang et al., 2004). In *P. oceanica*, the SHSP seems to be the HSP with higher responsiveness under heat stress (Tutar et al 2017; Traboni et al 2018, Marín-Guirao et al 2016). Additionally, the higher constitutive expression level of SHSP observed in shallow thermal-tolerant genotypes in comparison to deep-sensitive genotypes suggest its role in a pre-adaptive defense strategy of the species against heat stress (Marín-Guirao et al 2017, Tutar et al 2017).

While heat stress-inducible genes are essential to investigate the acquired priming status (He and Li, 2018; Lin et al., 2014), exploring epigenetic regulation under priming treatment is essential for the analysis of the subsequent storage of the information of priming cues which is known as stress-memory (Lämke and Bäurle, 2017; Liu et al., 2015). In this regard, methylation/demethylation of the histone H3 is known to be linked to gene regulation and memory of stress responses in primed plants (Ding et al., 2012), conferring to modifications of the chromatin structure a crucial role in driving the memorization of past stress events (Bäurle and Trindade, 2020). Here, we tested genes involved in chromatin modifications (ATX2, ATRX7, ASH2l, and SETD3) and a regulator of the active DNA methylation (DME) (Li et al. 2018; Pontvianne et al. 2010). In general, P seedlings showed slightly higher gene expression levels of all epigenetics-related genes in respect to NP seedlings. This is in contrast with what emerged from a recent study performed on *Posidonia australis* and *Zostera muelleri* adult plants (Nguyen et al. 2020), where methylation-related genes showed an opposite pattern and only ATX2 significantly differed among treatments in both species. It is worth underlining that in plants, trimethylation of lysine 4 of histone 3 (H3K4me3) is an important epigenetic mark that is associated to active chromatin states (Zhang et al. 2009) and transcriptional memory (Bhadouriya et al., 2021). In this study, the higher overexpression of genes related to H3K4 marks measured for P seedlings (ATRX7, ASH2l, and SETD3) gives first insights on: i) epigenetic changes induced by environmental stimuli in *P. oceanica* seedlings, ii) different epigenetic regulation under priming treatment of adult plants and seedlings, iii) the potential for juvenile stages of *P. oceanica* plants to memorize stress information through chromatin remodeling, overexpressing key genes in histone methylation and DNA demethylation. Regarding the first observation, in terrestrial plants dynamic epigenetic changes occur during embryo/seed development, germination, and early seedling development (Bouyer et al., 2017; Hannoufa et al., 2018; Kawakatsu et al., 2017). Thus, our results suggest that seedlings are more responsive to priming treatments in respect to adult plants. Moreover, since H3K4 marks tend to be accumulated after the exposure to heat stress in primed plants (*A. thaliana*, Lämke et al. 2016) and different regulation of key histone-modification related genes was already observed in *P. oceanica* during a heat-stress induced flowering event (Marín- Guirao et al., 2019), it appears that these genes are directly involved in driving epigenetic responses under stressful conditions, with the potential for storage of stress information.

In conclusion, this study revealed, for the first time in seagrass seedlings, that thermal priming conferred higher tolerance to the occurrence of an extreme seawater-warming event. All the responses measured in our experiment at the physiological, metabolic and molecular levels, pointed to the acquisition of a priming status in *P. oceanica* seedlings by a previous exposure to increased temperatures. In fact, during the triggering stimulus, primed seedlings performed better than unprimed ones. They were able to enhance the photochemical efficiency, to attain respiratory homeostasis, to keep their carbon balance unaltered and to grow faster reaching larger sizes compared to non-primed seedlings. Since the induction of the thermo-priming status depend on the level of heat-stress experienced by plants during the priming stimulus, exploring the influence of different temperature levels and the duration of the exposure to these conditions could be a critical point and a further step for understanding the acquisition of a thermo-primed status in *P. oceanica* seedlings. Our findings about the acquisition of a thermo-priming status in *P. oceanica* seedlings were supported by the expression levels of key genes related to stress response, photosynthesis, and epigenetic modifications. The overexpression of key genes in histone modifications suggests that primed seedlings have the potential to store priming stress information for long-lasting memorization of the past stress event. However, more studies are required to investigate specific stress-memory genes including epigenetic regulators to describe molecular mechanisms behind the acquisition of the priming status and to better describe the extent of the memorization of the past

stress event. Since priming approach have been utilized in terrestrial systems to reinforce plants against different kind of abiotic stresses (Kerchev et al., 2020), further studies are necessary to better explore tolerance-enhancing strategies in seagrasses, with important implications for improving conservation and restoration management of this highly valuable marine ecosystems.

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JP, FB, LMG and GP conceived and designed the experiment. FB and VG collected and maintained seedlings. LMG, JBE and JMR performed the experiment and analysed photo-physiological variables. JP performed all the molecular analyses. JP and LMG analysed the data. JP wrote the first draft and all other co-authors contributed to review the manuscript.

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CHAPTER VI

(Paper VI)

Jessica Pazzaglia, Hung Manh Nguyen, Alex Santillán-Sarmiento, Miriam Ruocco, Emanuela Dattolo, Lázaro Marín-Guirao and Gabriele Procaccini. The genetic component of seagrass restoration: what we know and the way forwards.

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Review

The Genetic Component of Seagrass Restoration: What We Know and the Way Forwards

Jessica Pazzaglia [1,2,†], Hung Manh Nguyen [1,†], Alex Santillán-Sarmiento [1,4], Miriam Ruocco [1], Emanuela Dattolo [1], Lázaro Marín-Guirao [1,3,‡] and Gabriele Procaccini [1,*,‡]

1 Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, 80121 Napoli, Italy; jessica.pazzaglia@szn.it (J.P.); hung.nguyen@szn.it (H.M.N.); alex.santillan@szn.it (A.S.-S.); miriam.ruocco@szn.it (M.R.); dattolo@szn.it (E.D.); lazaro.marin@ieo.es (L.M.-G.)
2 Department of Life Sciences, University of Trieste, 34127 Trieste, Italy
3 Seagrass Ecology Group, Oceanographic Centre of Murcia, Spanish Institute of Oceanography, C/Varadero, 30740 San Pedro del Pinatar, Spain
4 Faculty of Engineering, National University of Chimborazo, Riobamba 1407, Ecuador
* Correspondence: gpro@szn.it; Tel.: +39-081-5833363
† These authors have contributed equally to the work.
‡ These authors have contributed equally to the work.

Abstract: Seagrasses are marine flowering plants providing key ecological services and functions in coasts and estuaries across the globe. Increased environmental changes fueled by human activities are affecting their existence, compromising natural habitats and ecosystems' biodiversity and functioning. In this context, restoration of disturbed seagrass environments has become a worldwide priority to reverse ecosystem degradation and to recover ecosystem functionality and associated services. Despite the proven importance of genetic research to perform successful restoration projects, this aspect has often been overlooked in seagrass restoration. Here, we aimed to provide a comprehensive perspective of genetic aspects related to seagrass restoration. To this end, we first reviewed the importance of studying the genetic diversity and population structure of target seagrass populations; then, we discussed the pros and cons of different approaches used to restore and/or reinforce degraded populations. In general, the collection of genetic information and the development of connectivity maps are critical steps for any seagrass restoration activity. Traditionally, the selection of donor population preferred the use of local gene pools, thought to be the best adapted to current conditions. However, in the face of rapid ocean changes, alternative approaches such as the use of climate-adjusted or admixture genotypes might provide more sustainable options to secure the survival of restored meadows. Also, we discussed different transplantation strategies applied in seagrasses and emphasized the importance of long-term seagrass monitoring in restoration. The newly developed information on epigenetics as well as the application of assisted evolution strategies were also explored. Finally, a view of legal and ethical issues related to national and international restoration management is included, highlighting improvements and potential new directions to integrate with the genetic assessment. We concluded that a good restoration effort should incorporate: (1) a good understanding of the genetic structure of both donors and populations being restored; (2) the analysis of local environmental conditions and disturbances that affect the site to be restored; (3) the analysis of local adaptation constraints influencing the performances of donor populations and native plants; (4) the integration of distribution/connectivity maps with genetic information and environmental factors relative to the target seagrass populations; (5) the planning of long-term monitoring programs to assess the performance of the restored populations. The inclusion of epigenetic knowledge and the development of assisted evolution programs are strongly hoped for the future.

Keywords: seagrasses; restoration; genetic diversity; donor sites; transplantation; provenance; monitoring

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1. Introduction

Global climate change, along with local disturbances, are enhancing habitat degradation and biodiversity loss at an alarming rate and extension that is comparable only with past mass-extinction events [1]. Historically, restoration science has played a crucial role in the recovery of ecosystem properties and functions. However, with the current acceleration of environmental degradation, traditional restoration practices may no longer be sufficient [2,3]. Ecological restoration has become a major focus of conservation and natural resource management, as well as a strategy that can potentially provide realistic, context-specific pathways to a sustainable future. A meta-analysis estimated that global restoration practices had increased the provision of biodiversity and ecosystem services by an average of 25%–44% of what had been degraded [4], and some ecosystem services did recover with the success of restoration activities [5]. However, the restoration of marine ecological systems (including seagrasses) is still underdeveloped compared to terrestrial environments [6]. Although progress in restoration has been achieved for important marine ecosystems such as coral reefs, kelp forests, and seagrasses [7–10], the genetic research required for a proper restoration plan is not always applied, remaining more as a theoretical assumption rather than a practical action. In addition, legal issues on how to manage the genetic component of restoration are unclear [3,11].

Seagrasses are marine flowering plants that form extensive meadows in temperate and tropical waters of all continents except for Antarctica [12]. These meadows provide key ecological functions and ecosystem services to coastal areas and human livelihoods [13,14], ranking among the most valuable ecosystems on Earth along with coral reefs and tropical rainforests [15]. Seagrasses reproduce both clonally and sexually, these two strategies being dependent on external environmental conditions and internal cues [16–19]. Sexual reproduction ensures the rise of new genetic variants and boosts the plastic response of genotypes and populations to environmental changes [20]. Nevertheless, clonal (vegetative) propagation also plays a crucial role in the existence of seagrass species, contributing to important advantages, such as the colonization of vast areas and resource/risk sharing under unfavorable conditions [21–24]. In some species, sexual reproduction infrequently occurs, thus negatively affecting genetic diversity distribution within and among populations [25–27].

The decline of seagrass meadows reported in several regions of the world following extreme climate events (e.g., marine heatwaves and/or storms) is expected to occur more frequently in the coming decades [28,29]. It has been estimated that at least 1.5% of seagrass meadows are lost every year, and nearly 29% of their areal extent has disappeared since 1879 [30]. On the IUCN's Red List (International Union for Conservation of Nature), 24% of seagrass species have been classified as either 'threatened' or 'near-threatened' [31]. The concurrent action of local and global stressors is impacting seagrass performances [32,33], consequently affecting associated organisms and communities [34] as well as goods and services provided by them [13]. In the light of accelerated decline, restoration has become a priority strategy to slow-down seagrass degradation and to repopulate degraded meadows, thus protecting and ultimately recovering their ecosystem functions and services [5,8]. The survival of restored populations will strongly depend on future climatic events, which could jeopardize the heavy investment in time and money associated with restoration programs. This situation is currently opening a debate of whether to restore coastal vegetation-based ecosystems to historical baselines or to use a restoration to facilitate adaptation to climatic scenarios expected in the future [35–37]. To increase their effectiveness, seagrass restoration efforts should improve predictive models combining environmental and genomic data (Figure 1) to have a reliable guideline for helping decision-making in the development of restoration plans [38].

As we are approaching a new decade of ecosystem restoration [39], the need to rebuild marine life for a sustainable future has become more urgent than ever before [8]. Here, we aimed to provide a comprehensive review about genetic issues to be considered to perform a successful re-establishment of populations and for recovering lost ecosystem functions. To this end, we first reviewed conceptual frameworks related to genetic components

in restoration, with a particular emphasis on seagrasses. We then discussed different genetic-related aspects to be considered for restoring degraded environments, including the choice of whether replicate or reinforce the extant genetic structure, the importance of having genetic diversity and connectivity maps, the selection of donor sites as well as the monitoring efforts after transplantation. We also investigated the actual situation of legal and ethical issues dealing with seagrass restoration at a regional, national and international scale. Finally, we discussed novel approaches and future directions for seagrasses genetic research that could improve the success of restoration activities.

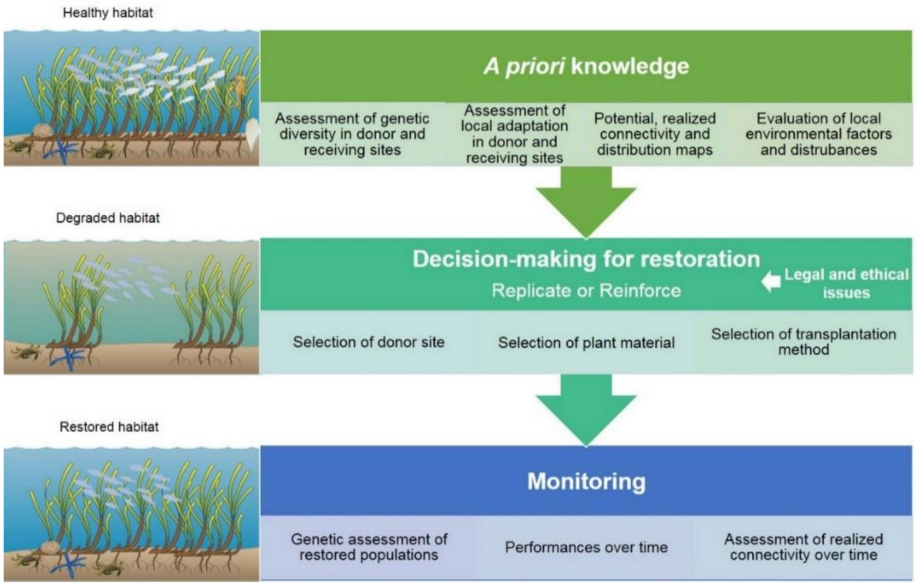

Figure 1. Diagram showing different aspects of seagrass restoration. The restoration plan should include different steps. The "a priori" knowledge includes the assessment of genetic diversity and local adaptation in donor and receiving sites. Moreover, maps of potential and realized connectivity and the evaluation of local environmental status over the whole distribution area of the species are necessary to have a comprehensive baseline to perform a successful restoration plan and to select suitable donor sites. The restoration itself can be aimed to either replicate or reinforce genotypes in target sites and can be performed with different plant material and thorough different restoration methods (always according to the evaluation of legal and ethical issues). In order to assess the restoration success, genetic traits (diversity and connectivity) and performances (physiological, demographic, and growth traits) of newly established meadows must be monitored over time. Symbols were taken from courtesy of the Integration and Application Network, university of Maryland center for environmental science (http://ian.umces.edu/symbols/ accessed on 10 March 2021).

2. A Brief Glance at Factors Shaping Genetic Diversity and Population Structure in Seagrasses

Genetic diversity is the basis for all biological diversity, which affects evolutionary and ecological processes at population, community, and ecosystem levels. It can be assessed in different ways and encompasses traits such as *allelic richness* (i.e., the average number of alleles per locus), *heterozygosity* (i.e., the average proportion of loci that carry two different alleles at a single locus within an individual), or *genotypic richness* (i.e., the number of genotypes within a population) [40]. Different methods used to quantify genetic diversity are explained in Box 1. Below, we briefly summarize the main factors shaping genetic variability and differentiation of seagrass populations, which should be taken into consideration for restoration purposes and should be a target for future research efforts.

2.1. Reproductive Strategies, Mutations

The level of genetic diversity in seagrass populations results from the balance between their sexual reproduction and clonal propagation, which in turn is related to different

factors, including environmental conditions, dispersal abilities, and population connectivity [17,41,42]. Most seagrasses are dioecious [43] and therefore are outcrossed, while other species, such as Posidoniaceae and several Zosteraceae, are monoecious [44,45] with highly variable outcrossing rates [46–49]. As a result, seagrass meadows can range from almost monoclonal, with very low genetic and genotypic diversity [21], to extremely diverse [41]. Clonal growth has been recognized as a winner strategy in seagrasses, avoiding the potential accumulation of deleterious mutations and maintaining the most suitable genotype over time [50]. An important source of genetic variation in marine clonal plants is represented by somatic DNA mutations resulting in genetic mosaicism [51]. In clonal plants, genetic mosaics can occur at different levels of the ramet (i.e., the morphological individual [52]) organization, including (1) within the same module; (2) within connected modules; (3) between different modules that belong to the same clone. Recently, it has been demonstrated that the mosaic genetic variation in a large seagrass clone of *Zostera marina* was greater within than among ramets, pointing out the importance that somatic mutations have in structuring genetically unique modules [53].

2.2. Level of Genetic Connectivity, Population Size, and Genetic Drift

For species with a wide distribution range, different factors can contribute to population isolation [54]. Moreover, despite the apparent spatial uniformity of the sea, marine habitats are characterized by clear discontinuities, and the presence of dispersal barriers may create a genetic breakdown in marine populations due to local selective pressures [55]. Nevertheless, dispersal vehicles such as buoyant fruits and vegetative propagules can travel long-distance transported by marine currents (potential connectivity), and new genotypes or allelic variants can establish in disjoint populations (realized connectivity [56–58]). This implies that even if sexual reproduction occurs at a low rate, passive transport of sexual propagules can play an important role in maintaining population connectivity and in the colonization of new habitats [59]. Isolated and small populations are more prone to undergo genetic drift and bottleneck events, increasing allele loss and the possibility of fixation for deleterious alleles compromising their persistence in the future [49,60]. This is even more relevant considering the fragmentation of populations resulting from the current destruction of natural habitats [61]. These processes may thus lead to genetic erosion, reducing the fitness of individuals and increasing the chance populations can disappear [62].

2.3. Phenotypic Plasticity and Local Adaptation

Different populations of the same species distributed over environmental and geographic gradients can be locally adapted, depending on selection and patterns of gene flow. Local adaptation occurs when individuals have higher average fitness in their local environment compared to individuals from elsewhere [63]. The measurement of adaptive genetic diversity is more difficult than neutral genetic diversity and requires an accurate analysis of genotype-by-environment interactions [20]. Disentangling plasticity from environmentally driven adaptation requires experimental approaches such as reciprocal transplants and common garden experiments [20,64–66] that have been performed in few seagrass species. Experiments carried out on *Z. marina* and *Posidonia oceanica* populations from divergent climatic regions highlighted a high divergence in their phenotypes in response to environmental stressors (e.g., heat stress [67–69]). Within populations, variations in acclimation to warming were observed among *P. oceanica* individuals collected along a depth cline [70], while a reciprocal transplant in a common garden [71] of plants coming from different depths (i.e., contrasting light-environment) showed clear indications of local adaptation. Thus, a deep knowledge of eco-physiology of plants at the donor and target sites is also required to perform restoration programs. Although genetic linkage mapping [72] is not applicable for most seagrass species, due to the scarcity of genomic resources, a genetic-environment association analysis, using a genome scan approach and a genome-wide transcriptome analysis, started to identify genetic loci and functions potentially associate with the selective environmental factors along either a latitudinal and

a bathymetric gradient [73]. Collectively, these studies suggest that local adaptation might play a role in shaping the divergence of seagrasses across environmental clines, even if it is not yet possible to assess how much of the observed phenotypic differences are heritable.

2.4. Disturbances

High genotypic diversity has been demonstrated to enhance the resistance and resilience of seagrasses to physical disturbances [17,74–76] or other stressful conditions such as heat stress or shading [76–78]. The level of genetic diversity of seagrass populations has also been shown to correlate with species richness and productivity [79,80] and ultimately with the associated community structure [79]. A high disturbance level can affect genetic diversity, leading to a decline in allelic or genotypic diversity or even to complete population extinction. Intermediate level of disturbance, instead, can boost sexual reproduction, increasing both allelic and genotypic diversity [17]. In general, the relationship between disturbance and genetic diversity is not simple, and the reciprocal causality of the two phenomena renders it difficult to assess the relative contribution of disturbance strength and frequency in relation to its effects on genetic components of diversity [17,40].

3. Integration of Genetic Research into Seagrass Restoration

How should a restored meadow be in order for it to successfully perform and persist? It should be genetically diverse and composed of genotypes locally adapted or able to adapt to the local environmental conditions. It should be connected, through a sufficient level of gene flow, with surrounding populations, in order to avoid negative effects of inbreeding depression, but it should not disrupt the local gene pool. It should be established to limit the damage to existing populations in providing source material and should comply with ethical and legal issues. Here we present and comment on key aspects to consider for a correct restoration plan.

3.1. Selection of Donor Sites

Genetic diversity is at the base of phenotypic diversity, which determines how restored populations will perform and respond to environmental stimuli at restored sites [74,75,81]. Prior to any restoration project, an accurate understanding of local environmental conditions and potential disturbances, the genetic makeup of populations nearby the transplantation site, and policies and legislation guidelines should be acquired in order to select proper donor sites. Many studies have investigated the relationship between genetic diversity (of both source and transplanted meadows) and the success of seagrass restoration plans (see Table 1). Those studies indicate that the selection of donor sites displaying a high level of genetic diversity as well as the choice of plant materials (e.g., adult plants, seeds, or seedlings) is crucial for maximizing restoration success.

Table 1. List of the most relevant studies investigating the effects of genetic diversity on seagrass restoration plans. Data were collected from Google Scholar using "seagrass restoration" plus "seagrass genetic" as keywords together with personal knowledge from the authors. Year: year when transplantation started; *: multiple restorations; related ref: see related reference for more details.

Species	Year	Donor Location	Restored Location	Plant Material	Area	Duration	Genetic Diversity Assessment	Ref.
Posidonia australis	2013	Jervis Bay (Australia) reciprocal transplant study	St. Georges Basin (Australia) reciprocal transplant study	Adult plants	na	6 months	Eight microsatellites	[11]
Zostera noltei	2009	Carteau in the Gulf of Fos (France)	Berre lagoon (France)	Adult plants	450 m^2	4 years	Nine microsatellites	[82]

Table 1. *Cont.*

Species	Year	Donor Location	Restored Location	Plant Material	Area	Duration	Genetic Diversity Assessment	Ref.
Zostera marina	2007	Mobjack Bay, Chesapeake Bay, South Bay, USA	Hog Island Bay, USA	Seeds	128 m^2	20 months	Eight microsatellites	[83]
Zostera marina	2006–2007 *	Chesapeake Bay (USA)	Virginia coastal bays (USA)	Seeds	na	2–3 years	Eight microsatellites	[84]
Posidonia australis	2004	Parmelia Bank, Cockburn Sound (Australia)	Southern Flats, Cockburn Sound (Australia)	Adult plants	3.2 ha	4 years	Seven microsatellites	[85]
Zostera marina	2001–2008 *	Related ref	related ref	Adult plants	related ref	10 years	Seven microsatellites	[86]
Zostera marina	2000	Two sites along the German Baltic Coast	Two sites along the German Baltic Coast	Adult plants	450 m^2	11 weeks	Four microsatellites	[87]
Zostera marina	Late 1990s	Chesapeake Bay	Twenty-three meadows along the eastern coast of North America	Seeds	1600 ha	15 years	Seven microsatellites	[88]
Posidonia oceanica	1994	Gorgona Island, Pantelleria Island (Italy)	Vada (Italy)	Adult plants	na	3 years	Six microsatellites	[89]
Halodule wrightii	1993–2000 *	Related ref	Related ref	Adult plants	na	2–7 years	98 AFLPs	[90]
Zostera marina	1993	South San Diego Bay (USA)	North San Diego Bay (USA)	Adult plants	na	2 years	Allozyme electrophoresis	[81]
Zostera marina	related ref *	Related ref	Related ref	Adult plants	Related ref	3–16 years	Allozyme electrophoresis	[91]

To date, the most widely applied approach of restoring a former local gene pool is by sourcing the plant material from nearby or well-connected donor sites, i.e., local provenance (Figure 2). The reason is that locally adapted plants are believed to fit the condition of the site being restored. However, trying to replicate what is already lost is inappropriate in highly degraded environments, and better environmental conditions should be achieved first.

Native genotypes that have already suffered past environmental disturbances could also be unable to overcome the recurrence of such perturbation or new stressful conditions in the future [36]. Sgrò et al. [92] identified critical problems of this "local is best" practice, including (1) the risk of establishing populations that do not exhibit sufficient genetic variation and evolutionary potential; (2) the possibility that particular environmental conditions driving local adaptation can change very quickly, hampering the advantage of using locally adapted genotypes. This is particularly important and can cause serious impacts on restoration outcomes, considering the speed at which environmental changes are occurring. On the other hand, the introduction of novel genotypes from distant sources (assisted gene flow) has the potential to restore levels of genetic diversity (genetic restoration), increasing the overall fitness of inbreeding-depressed populations (genetic rescue). Nevertheless, it may also result in deleterious effects as a consequence of outbreeding depression and maladaptation [93,94]. According to a modeling approach by Aitken and Whitlock [95],

the risks and consequences of outbreeding depression and contamination of the local gene pool are minor in respect to potential advantages.

Another important aspect to consider in the selection of donor sites is the taxonomic uncertainty, which characterizes some seagrass groups, such as, for example, the Halophila genus [96,97]. The high morphological plasticity of species and the presence of locally adapted morphotypes could lead to erroneous species identification. This could favor in turn, the hybridization with native species, the breakdown of locally adapted ecotypes, and the establishment of hybrids (i.e., genetic swamping), potentially compromising the entire ecosystem functioning [98]. In this case, species identification should also be performed at a genetic level to overcome taxonomic ambiguity.

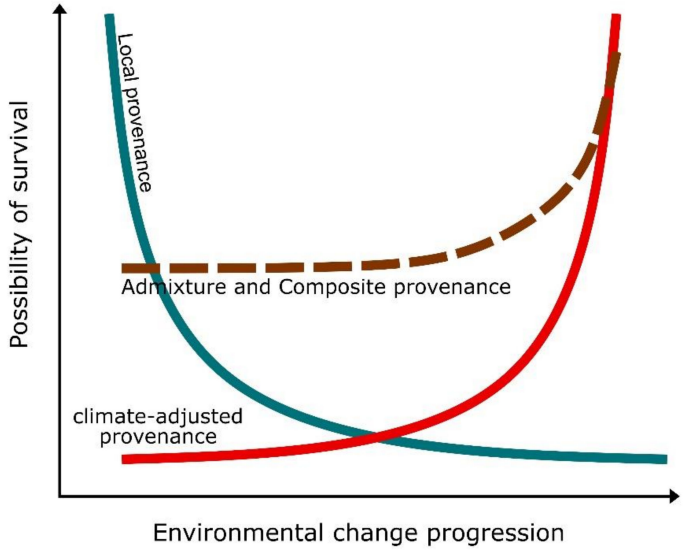

Figure 2. Graph showing the conceptual relationship between local (blue line), climate-adjusted (red line), admixture and composite (dashed line) provenance (*sensu* Prober et al. [99]) with the possibility of survival of the restored seagrasses under environmental change (e.g., ocean warming, eutrophication, etc.).

In order to utilize donor material potentially able to respond to projected climate changes, source populations can be selected within the distribution range of the species in areas experiencing environmental conditions as projected in the near future for the transplantation site, i.e., climate-adjusted provenance (Figure 2, red line) [88]. This source material could be utilized together with material coming from healthy local populations or from multiple sources across the species range, i.e., a composite and admixture provenance (Figure 2, dashed line) [99]. The latter is especially suitable for most seagrass species, where information about genotypic plasticity and potential response to changes is scarce. Furthermore, many seagrass species exhibit wide latitudinal ranges of distribution [100], making the selection of climate-adjusted or admixture provenance easier. These strategies may not result in a high survival rate of restored populations in the short-term as they can experience intraspecific hybridization with local and non-local genotypes (i.e., outbreeding depression) or maladaptation [93,94,101]. However, in the long-term, the introduction of "future climate-adapted" genotypes can enhance the survival and longevity of the restored meadows [6,10,99]. Even holding great potentials for seagrass restoration, there are still limitations in choosing non-local donor material approaches that require further investigation. For instance, to apply admixture provenance, it is important to establish the right proportion of local and climate-adjusted plant material and the number of donor populations to select. Sometimes, this is also highly dependent on the availability of material at both source and receiving sites (see Section 3.2).

3.2. Integration of Biogeographic and Genetic Data

Integrating genetic diversity information with biogeographical and oceanographic data into connectivity maps can be very helpful in the selection of donor sites and the monitoring of restoration efforts (Figure 3). This becomes particularly useful for species with the potential to disperse over long distances via ocean currents during various life stages [59,102] and for species with a highly variable level of meadow genetic diversity over their distribution range (e.g., *P. oceanica* [103,104]). These maps, together with habitat suitability and site selection models [105], are important to identify whether or not seagrass recovery can naturally occur or whether the targeted population would remain isolated after being restored (in this case, restoration is not advisable). In the first case, it is the result of a high level of connectivity and gene flow between degraded and neighboring sites, or in the second case, resulting from the absence of population connectivity. In the last case, the integration of genetic diversity, connectivity, and environmental data could reveal the reasons behind the isolation of the target area and the possible way of restoring dispersal and connectivity networks ([106]). Recently, Mari and colleagues [58] built maps of potential connectivity for *P. oceanica,* modeling the dispersal and potential exchange of propagules between sites evaluating environmental features. The resulting patterns could be integrated with genetic data of target populations useful for choosing potential donor populations. Survival data from seagrass restoration can also be used to investigate fundamental niches and model the persistence potential of restored seagrass meadows [107,108]. Recently, Oreska et al. [109] analyzed the presence and absence data of seedlings from restored plots in the Virginia Coast Reserve through Species Distribution Models (SDMs) to identify potential environmental factors that affect the survival rate of different sites. This offered the opportunity to compare the extent of the realized and fundamental niche of the restored and natural sites, improving management efforts to accelerate seagrass coverage and recovery.

The integration of information from genetic diversity into connectivity maps may also help to keep track of historical gene flow and local adaptation while at the same time, avoid the loss of genetic variation at the restored sites [110]. Seagrass genetic diversity tends to decrease in populations that locate at the range-edge of the species' distribution range [49,102]. This phenomenon has been suggested as the result of reduced seed production and pollen limitation [10,49] and limited connectivity of populations [111–113]. Range-edge populations often exhibit smaller effective population sizes, making them unsuited as donor sites [111,112,114]. Indeed, many studies have recommended that populations with large effective population sizes are the most appropriate donor sites [114]. These populations actually possess the genetic potential to better adapt to more extreme environmental conditions (e.g., marine heatwaves) and could be used as potential restoration materials for the future as ocean warming continues to rise [10,111]. However, these populations could also be at high risk of extinction if the speed of environmental change overrides their capacity to adapt [115].

Distribution and connectivity maps together with *a priori* knowledge of population structure should be integrated with the reproductive characteristics of related seagrass meadows [111,116]. For example, after studying reproductive and genetic profiles of *P. australis* meadows across Western Australia, Sinclair et al. [111] showed flower and fruit production variability between northern range-edge meadows and center range ones, with the first showing mixed mating system and lower sexual productivity. This evidence suggests that future restoration activities may benefit from sourcing plant material from multiple reproductive meadows. Future efforts on making complete maps (or georeferenced databases) as guidelines to restoration should also include information regarding intraspecific differences in genetic diversity, e.g., among different depths of the same population as seen in the case of the seagrass *Z. marina* [115] and *P. oceanica* [117,118], which can have potential implications in the collection of plant material.

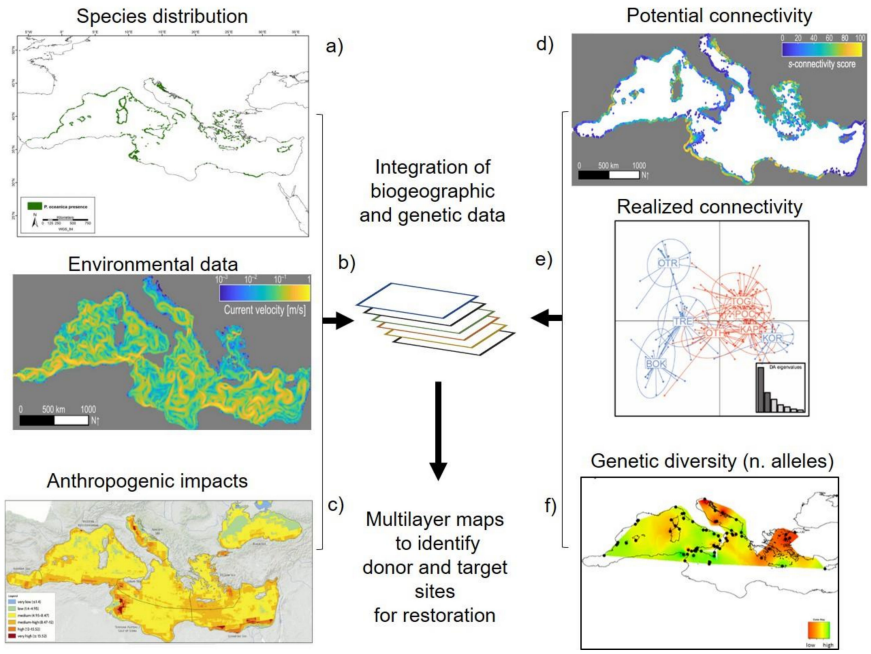

Figure 3. Examples of models and distributions maps and genetic data from seagrass' studies of the Mediterranean Sea. The integration of species distribution (**a** [119]), environmental data (**b** [58]), anthropogenic impacts (**c** [120]), with potential (**d** [58]) and realized connectivity maps (**e** [57]) and genetic diversity analysis (**f** [121]) could be combined to develop multilayers maps for the identifications of donor and target sites in seagrass restoration (see text for more detail). The figure was modified from the studies cited above.

3.3. Selection of the Plant Material

Different species of seagrasses have different morphological and reproductive traits, affecting in a different way restoration success. Moreover, restoration plans have mainly focused on species with higher ecosystem value (providing more valuable ecosystem services) and also forming monospecific meadows. Only one-third of the extant seagrass species have been utilized in restoration programs, with *Z. marina* present in more than 50% of the trials [122]. Other species highly utilized in restoration plans are the ones from the *Posidonia* genus in the Mediterranean and in Australia. Most of the restoration plans occur in temperate areas of the United States, Europe, Australia, and Eastern Asia [122].

Seagrass restoration can be performed by using different parts of the plant, such as rhizome fragments, seedlings, or seeds [122]. The most common approach implies the collection of adult plants with well-developed shoots and roots [85,122]. However, adult plant-based methods are often labor-intensive and costly, as the survival rate of transplanted shoots is strongly related to the amount of planted material used [10]. In contrast, the use of seed-based methods instead of adult shoots, particularly in large restoration plans, can result in a much lower impact on existing meadows (i.e., donor sites) [10]. Moreover, seed-based transplantation approaches are less expensive and more logistically feasible when restoring larger areas [88,123]. As reported by van Katwijk et al. [122], large-scale restoration trials (> 100,000 shoots/seeds planted) perform better than small trials, and part of these results depend on the initial sourcing material, which should have high genetic and genotypic diversity. One of the best examples of large-scale restoration in seagrasses was performed along the mid-western Atlantic coast, where over 70 million *Z. marina* seeds were planted from 1999 to 2010 [124]. In this case, the collection of a large number of seeds from multiple parents did offset potential genetic bottlenecks ensuring high genetic diversity of donor plants and thus of restored sites [84]. Orth et al. [125] also demonstrated that a large restoration plan not only restored local seagrass coverage but also improved water quality and ecosystem functioning, supporting other restoration programs (e.g., scallops). Seed-based methods can quickly facilitate the recovery of populations with

higher genetic diversity [83,90] and have the advantage of maintaining genetic variation mimicking natural ecological and evolutionary processes [92,123]. Thus, it is considered as a valid approach to restore and redefine populations that are more capable of persisting to changing environmental conditions. However, it is still unclear if and how massive seed collection can impact the survival and genetic composition of donor populations in the long-term. Although the acquisition and processing of large amounts of seeds is a limiting factor in most seagrass species, other species, such as *Z. marina*, produce large quantities of seeds that are released in a short time, allowing the implementation of different approaches to store and maintain collected seeds viable [126].

Nevertheless, seed-based methods still have limitations that deserve further efforts from the scientific community. For example, more information is needed about sexual reproduction and other biological characteristics of plants, such as flowering time, seed production strategies, dormancy, and germination condition. Furthermore, it has been found that in *P. australis* new seedlings have a low initial establishment rate, which depends on local environmental conditions [127], while in *Z. marina* in natural conditions, only around 5%–10% of seeds can survive and germinate [128].

3.4. Genetic Assessment of Transplantation Success

The success of seagrass restoration has historically been evaluated by demographic monitoring, which only informs about population processes such as recruitment, survival, and reproductive success, but that do not provide insights into the evolutionary resilience of restored populations or about the consequences of reproductive processes following restoration actions [129]. Genetic monitoring evaluates the success in restoring genetically viable populations and whether the positive effects of the restoration are maintained over time (i.e., across successive generations). Thus, well-designed monitoring programs are required, including also evaluation of changes in environmental conditions of the restored site and referring to comparable time frames for the same species [130]. Monitoring genetic changes in restored populations can be done retrospectively by using pre-disturbance genetic population datasets or for evaluating ongoing changes in their status and persistence (i.e., mid-and long-term restoration outcomes). Measuring changes in population allele frequencies or levels of linkage disequilibrium over time, using neutral markers, can provide information about absolute changes in the restored population (e.g., effective population size) and can be relevant for digging into the genetic processes driving these changes (e.g., selection, genetic recombination, mutation, genetic drift, mating system, and genetic linkage).

Genetic monitoring can also be useful to inform about the factors and processes underlying the success or failure of a restoration action, which could be critical to adjust management practices accordingly [131]. For instance, when mixed source populations are used in restoration, genetic monitoring has the potential to inform whether genotypes from different origins have been admixed or if the local genetic characteristics are maintained and not completely replaced by the newly introduced foreign genotypes. In the latter case, this would involve a reduction in the overall genetic diversity of the restored population, compromising its evolutionary potential in future environmental scenarios. In species with high clonal propagation, genetic monitoring could also inform whether the establishment of new recruits is the result of clonal spread or sexual reproduction, the latter being indicative of successful population rejuvenation [132].

Combining molecular markers with fitness-related phenotypic traits can provide a quantification of genetic variability and structure, as well as further valuable information about the progression of the restored population and the likely existence of constraints to recovery. For instance, genetic monitoring just several generations after the completion of a restoration action can reveal the existence of reduced fitness of inbred offspring (inbreeding depression; *Z. marina* [68]) or reduced fitness of progeny involving an admixture of different sources or of native and foreign genotypes (outbreeding depression; *Z. noltei* [133]). Additionally, monitoring the genetic structure of restored populations can identify the

re-establishment of a gene flow between the restored and closely populations (e.g., *Z. muelleri* [134]) as well as factors that have the potential to alter the future population genetic structure (e.g., *Z. marina* [135]). Whether selection pressures in the restored habitat with mixed source populations have the potential to result in population differentiation in the long term can also be inferred. Since fitness of transplants may depend both on the source origin and the particular environmental conditions of the restored habitat, the higher fitness in critical traits (e.g., sexual reproduction) of locally adapted genotypes might result in within-population differentiation. This can also result from heterosis, as heterozygous individuals are relatively fitter than homozygous individuals [136].

In addition, genetic monitoring could also shed light on the genetic basis influencing the provision of ecosystem services [133], which is a major outcome pursued in restoration programs. Reynolds et al. [84] found that a small increase in genetic diversity in transplant plots of the seagrass *Z. marina* improved restoration success, but also the provision of valuable ecosystem services (i.e., habitat provision, primary productivity, and nutrient cycling). The authors argued that the mechanism behind this ecosystem services enhancement was the increase in shoot density promoted by high genetic diversity in transplant plots.

For all the above, monitoring of transplants is essential to identify timely evidence-based information that can ultimately enhance the long-term success rates of transplantation efforts by establishing additional actions and modifications (see Figure 1). This information can also uncover mechanisms limiting transplantation success to inform future projects [124]. As the recovery of seagrass meadows can take from two to over 30 years to reach a fully functional state [6], and negative impacts of improper donor sites (e.g., in genetic aspects) can also take decadal times to be detectable [101,137]. All these make long-term seagrass monitoring essential. Unfortunately, most agencies typically fund restoration projects over a short period (e.g., in Australia from one to 10 years [6]) that is usually not enough for appropriate monitoring. Besides the devoted efforts of the scientific community, restoration programs require the involvement and commitment of all stakeholders in the industry, local communities, citizen-science projects, non-governmental organizations, states, and federal government agencies to establish multi-year to decadal funded restoration projects in order to progressively improve seagrass restoration outcomes and to complete the ambitious restoration goals set out for the present decade.

4. Future Directions in Seagrass Restoration

4.1. Improving Transplant Performances through Assisted Evolution

The ability for impacted or vulnerable seagrass populations to successfully adapt to environmental changes depends on their standing genetic variation and the pace at which genetic changes are incorporated [138]. However, in the context of accelerated climate change, the genetic adaptation of populations is considered slow compared to the celerity of climatic changes [139]. Different approaches with diverse levels of intervention intensity have been proposed within the concept of "assisted evolution" (or assisted adaptation) to accelerate the rate of naturally occurring evolutionary processes (e.g., corals [140], terrestrial plants [141]). Although such human interventions are under strong ethical debate (as discussed in Section 5), it is timely to start exploring and discussing the potential possibilities we have to secure a sustainable future for seagrasses.

The use of resistant genotypes in seagrass restoration is an approach with the potential for improving the extant genetic baselines of natural populations and for enhancing the resilience of the restored population to present and future stressors. Resistant genotypes can be identified through manipulative selection experiments and by identifying local adaptation (i.e., selection) in natural populations. Genotyping by sequencing of single nucleotide polymorphisms (SNPs) now enables to explore genome-environment interactions and to characterize both neutral and functional (adaptive) genetic diversity of organisms without a reference genome (see Box 1), which is the case of most seagrass species. The identification of putative heritable loci under selection for a given stressor (e.g., thermal stress) could then be combined with manipulative stress experiments to confirm candidate

gene function and to examine the resilience and the potential trade-off of genotypes possessing such loci [142]. This information could be crucial for improving seagrass restoration outcomes by facilitating an informed decision-making process about the provenance and genetic background of the transplant material. Furthermore, this can also be relevant in the future thanks to the recent development of novel technologies in genome editing, which are opening up new opportunities for molecular ecologists to achieve specific manipulation of genes of interest for improving restoration outcomes and for enhancing the overall resilience of restored populations [143,144]. However, these approaches require a high level of human intervention that are more socially and ethically controversial and still far from being applied in seagrasses, although they are common in terrestrial plants and animals and have been proposed in certain cases of coral reef restoration [35,140]. However, legal guidance on how to define organisms produced by exploring novel genome editing techniques and how to distinguish them from genetically modified organisms (GMOs) is still under construction [145].

The selection of more tolerant genotypes to improve restoration success can be performed by growing wild specimens under controlled conditions. These practices that include a culture phase are widely applied for coral restoration, where fragments or larvae are collected from the environment in order to prevent coral damage during their most vulnerable stages [7]. In seagrasses, the use of aquaculture systems to grow plants of *Z. marina* has been improved and representsd a way to obtain plant material alternative to harvesting plants from donor sites when vegetative shoots are required [146]. Additionally, growing plants under controlled conditions is useful to overcome acclimation to the home environment, avoiding problems related to the choice of a local or non-local site [20]. Although this approach can also be applied to other seagrass species, several constraints regarding reproductive cycles of the species, germination of seeds under control conditions, and slow growth rate limit its application.

Resistant genotypes can also be produced with a lower level of intervention through the use of priming/hardening methods [147]. Pre-exposing individuals to mild stress have the potential to induce stress memory, giving rise to genotypes with enhanced tolerance to subsequent stressful events. Whether stress memory is set by stress-induced epigenetic modifications (see Section 4.2), the acquired resistance can be passed to offspring leading to new generations with acquired resistance [148]. The first evidence of the existence of stress memory in seagrasses has very recently been published [149]. Adults of two seagrass species with contrasting biological attributes (pioneer vs. climax) have shown the capacity to acquire thermal-stress memory and to better resist and perform in a successive stressful thermal event. Primed plants also showed the activation of methylation-related genes suggesting the involvement of epigenetic modifications on stress memory in seagrasses, as also suggested in a recent paper on *Z. marina* [150].

4.2. Potential of Epigenetics in Seagrass Restoration

Plasticity provides a buffer against rapid climate changes and also assists the rapid adaptation of species and populations to the ongoing climatic change [20,139]. Among mechanisms promoting and regulating phenotypic plasticity, epigenetic modifications, which include potentially heritable changes of genomes that do not alter the DNA sequence itself, have been widely considered as key candidates [151,152]. Epigenetic variations can arise from genetic control, environmental induction, or spontaneous epimutations [151,153]. Epigenetically induced phenotypic variations could be transiently reversible or transgenerationally heritable within one or multiple generations through meiosis and/or mitosis [154]. Especially clonal plants such as seagrasses could benefit from epigenetic variations and their adaptive potential as an alternative to the slower mechanisms of adaptation through natural selection [155,156]. In addition, under clonal growth, epigenetic changes (e.g., DNA methylation patterns) are expected to be more stably inherited than under sexual reproduction [157].

In seagrasses, different studies pointed out the potential role of epigenetic mechanisms in regulating gene expression following stress events, thus promoting stress acclimation and increasing tolerance of individuals [19,24,70,158–160]. A very recent study in the seagrass *Z. marina* tested the hypothesis that clonal seagrass meadows could display epigenetic variation that compensates for low genetic variation [150]. Clonal shoots displayed DNA methylation variations independent from underlying genetic variations and associated with changes in plant performance under experimental conditions [150]. This demonstrates that epigenetic variation could play a similar role to genetic diversity in meadows dominated by a single or a few genotypes and that seagrass stress resilience could be much higher than expected considering only the genetic makeup of populations. Especially in long-living seagrass species (e.g., *P. oceanica* [21]), epigenetic responses can build through time, thus increasing the fitness of individuals over a number of ramet generations [155].

Consequently, when dealing with clonal plants, conservation and restoration management should consider 'epigenetic diversity' as an indicator of stability and functioning of the ecosystem equal to genetic diversity [161,162]. In a framework of restoration, the assessment of the epigenetic variation of populations could be potentially as informative as the assessment of their genetic status, thus being a reliable tool for the evaluation of suitable donor sites as well as for establishing the success of replanted shoots to overcome natural variability and stress events. As recently stated by Rey et al. [163], DNA-methylation, which is the most studied epigenetic modification, could contribute to improving ecological restoration, including the development of biomarkers, the study of wild populations' ecological structure, the improvement of translocation strategies, and the study of functional landscape connectivity. Introducing epigenetics into conservation and restoration practices, especially in seagrasses, would contribute to better understanding the plasticity of these unique plants and their adaptive potential in the face of environmental changes, thus improving conservation and restoration strategies [163].

5. Legal and Ethical Issues Related to Genetic Aspects of Seagrass Restoration

Conservation and restoration programs are generally regulated by national laws and international conventions with a central role of maintaining biological diversity [164,165]. Biodiversity conservation is regulated in the framework of the Convention on biological diversity (CBD) that was signed by 150 government leaders in Rio de Janeiro in 1992. The CBD aimed to stem the worldwide biodiversity loss, focusing on the conservation of biodiversity, sustainable use of the components of biological diversity, and sharing benefits derived from genetic resources. Importantly, an explicit goal of the CBD was the conservation of genetic diversity, as the persistence and evolutionary potential of species depend on it [166]. Other conventions with a role in seagrass conservation and management are the Berna Convention (1979), which deal with the conservation of wild species and European natural habitats, the Barcelona Convention (1995), which was recognized as the convention for the protection of marine habitats, and a series of other legislations related to fisheries and aquaculture [167]. In addition, the inclusion of seagrass ecosystems in national and international policies is a recommended action for the maintenance of marine ecosystems and biodiversity (UNEP, UN Environment Program 2020). The UN decade on ecosystem restoration [168] is an international call that aims to massively restore degraded ecosystems worldwide during the period 2021–2030, as well as to promote their resilience to climate and anthropogenic changes. The UNEP offers regional and international collaborations in broad thematic areas, including the protection and restoration of coastal 'blue carbon' ecosystems, like mangroves and seagrasses.

However, no specific regulations and practical implementations exist on the management of the genetic component in seagrass restoration practices. One exception is Article 15 of the Convention on biological diversity (CBD), where terms and conditions for access to genetic resources were recognized, such as the sovereignty of States over their natural environments (see Secretariat of the Convention on Biological Diversity 2002 [169]). The introduction of some countries' specific restrictions on access to genetic resources could

limit the possibility of choosing donor sites. One recent addition to the protocols of the CBD was the implementation of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization that was adopted on 29 October 2010 [170]. This new protocol introduced legal transparency for both providers and users of genetic resources by sharing benefits arising from the utilization of genetic resources contributing to the conservation and sustainable use of biodiversity as stated by the CBD.

Important management measures on genetic issues related to conservation have been applied for agrobiodiversity, especially for crop species of economic and commercial interest [171,172]. In this regard, different regulations and specific institutions already exist, which aim to conserve plants' genetic resources [173]. In 1971, Food and Agriculture Organization (FAO), with the World Bank and the UN Development Program, founded the Consultative Group on International Agricultural Research (CGIAR). Today, the CGIAR is primarily responsible for the international germplasm collections and includes governments, private foundations, and regional development banks (http://www.cgiar.org accessed on 10 October 2020). The conservation of plant genetic resources consists of the storage of crop genetic materials, usually as seeds or vegetative material. This approach, known as ex situ conservation and widely applied for terrestrial plants, consists of the collection of seeds and their storage for future use of plants [174]. The possibility to preserve seeds in seedbanks is strongly species-dependent and population-specific [175] and has not been considered to date for seagrasses. Different guidelines have been developed to improve translocation of plants (i.e., reintroduction) and breeding in restoration [176]. In the presence of highly degraded habitats, where the native species are almost disappearing, a strict sampling strategy must be followed considering the number of populations and individuals within populations to create a sufficient initial gene pool [177]. These guidelines can also be applied to seagrasses, even if more studies performing transplant experiments and addressing genetic diversity effects over the years are needed. Uncertainty related to the management of genetic issues in restoration is exacerbated by ethical questions that arise from novel approaches, such as assisted evolution and genome manipulation [36,178]. The artificial selection of more suitable genotypes or the release of genetically modified genotypes into wild populations opens a debate on the potential consequences that modified genomes can have on native populations. Furthermore, improving populations' performances to human-modified environmental conditions make it harder to define clear interventions' rules. In this context, the lack of long-term outcomes derived from these manipulations raises concerns about the appropriate use of assisted approaches. A constant dialogue among scientists, stakeholders, and policymakers is fundamental to identify opportunities from new technologies and potential risks for the environment.

6. Recommendations and Conclusions

In this review, we provide a comprehensive view of the importance of genetic knowledge to seagrass restoration. Whether a restoration program aims to replicate or to reinforce target populations, a proper restoration plan would require: (1) information about the genetic structure of both donor and restored meadows; (2) the analysis of local environmental conditions and disturbances that affect the site to be restored; (3) the analysis of local adaptation constraints influencing performances of donor and native plants; (4) the integration of distribution and connectivity maps with genetic information and environmental factors relative to the target seagrass populations; (5) long-term monitoring programs to assess the performance and the variability of the restored populations over time. In addition, we encourage the inclusion of an 'epigenetic conservation and restoration' perspective together with a genetic one. Further studies in the field of epigenetics in seagrasses are needed to broaden our knowledge on this emerging topic that can ultimately benefit future restoration and conservation activities. These kinds of studies are also crucial for the integration of assisted evolution strategies into seagrass restoration, which needs to be further reasoned and developed in a similar way to what has been done for other marine foundation species

such as corals [35,140] and kelps [36]. It is urgent and imperative to integrate and develop the concepts and methods of assisted evolution in seagrass restoration to reinforce seagrass ecosystems, avoiding rapid deterioration and promoting their adaptation to local and global pressures.

Bringing solid scientific knowledge from biologists to policymakers is essential to define clear restoration actions, delineating priority areas to restore, and making adequate funds available. Altogether, we expect to ensure a sustainable future for seagrasses and the multiple life forms they support worldwide to our future generations.

Box 1. Current molecular methods to assess seagrass genetic/epigenetic diversity, adaptation, and population structure.

Different molecular methods have been developed to quantify genetic diversity and structure within and among plant populations (see review by [179]). Early studies in seagrasses have largely relied on traditional molecular markers (e.g., Random Amplified Polymorphism DNA, Amplified Fragment Length Polymorphisms, and Restriction Fragment Length Polymorphisms). Some of these marker categories have limited power in assessing population genetics indices and in resolving the geographical differentiation of populations and a limited consistency among studies. Simple Sequence Repeats (or microsatellites) represented the reference markers in population genetics for many years and are still widely utilized, considering that whole-genome sequencing techniques are still too expensive, especially when dealing with tens or hundreds of samples and species with large-sized genomes.

The rapid progress in next-generation sequencing technologies (NGS) speeded up the development of various reduced-representation genome-sequencing (RRS) methods based on restriction enzyme digestion of genomic DNA (for a review see [180]), and genotyping-by-sequencing (GBS) is currently increasingly applied to ecological and evolutionary studies [181]. GBS methods can produce thousands to millions of single nucleotide polymorphisms (SNP_S), which allow resolving patterns of genetic diversity, genotyping, and spatial structure of populations at a very fine scale [180].

Restriction-site-associated DNA sequencing (RAD-Seq) is a family of techniques in which DNA is digested with restriction enzymes, and the resulting fragments are size-selected and sequenced via NGS. The resulting NGS reads are mined across individuals for SNPs that occur immediately adjacent to common restriction sites [182]. RAD-Seq provides high-resolution population genomic data for outliers scan, linkage mapping, and demographic analysis at relatively low cost and requires minimal starting material [183]. Diverse RAD-Seq techniques e.g., RAD based on fragments produced by type IIB restriction endonucleases (2b-RAD [184]), Isolength restriction site-associated DNA (isoRAD [185]), or Double digest RADseq (ddRAD [186]), have been developed in recent years (for a review see [187]). Importantly, they can be easily applied to non-model species without prior genomic knowledge [183], as most seagrass species (with the only exception of *Zostera marina* and *Z. mulleri*, whose genomes have been recently released [188,189]). A RAD-Seq approach has been recently applied in *Zostera capensis* to obtain SNPs data and examine the neutral genomic variation of populations [190,191].

NGS platforms can also be used to study genome-wide DNA methylation patterns across the genome to improve the assessment of epigenetic diversity in ecological settings and provide functional insights [192]. Bisulfite sequencing applied to whole genomes (WGBS) allows the evaluation of methylation status for every cytosine in a genome [193], and it represents the 'gold standard' of all available techniques, but it is only applicable to species with a high-quality reference genome, besides having prohibitive costs for large experimental designs. Several cost-effective reduced representation bisulfite-sequencing approaches (RRBS) have been recently developed, as for instance, Methylation-dependent restriction-site associated DNA sequencing (MethylRAD [194]), epi-genotyping by sequencing (EpiGBS [195]) or bisulfite-converted restriction site associated DNA sequencing (BsRADseq [196]) that can be applied to non-model organisms lacking a well-annotated reference genome. The Methyl-RAD technique has been recently applied to characterize the methylome of the seagrass *Z. marina* [150].

The integration of the various "omics" or "high-throughput" technologies now allows to achieve a comprehensive understanding of the link between genotype, phenotype, and the environment through the application of system biology approaches (for a complete review of new technologies in restoration and conservation, see reviews by [197] and [198]). Moreover, many commercial services are currently available to perform most of the 'genetic work', from library preparation to sequencing and bioinformatics analysis, allowing research in restoration and conservation genetics without access to a fully equipped molecular laboratory.

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Glossary

Admixture provenance: Plant materials for restoration collected as a mixture of Local provenance and Climate-adjusted provenance (i.e., from a variety of provenances from sources across a species range).

Assisted evolution: Conservation strategy adopted for vulnerable species and based on human intervention, which aims to accelerate the rate of natural evolutionary processes enhancing population resilience and the rapid adaptation to environmental changes.

Assisted gene flow: An active intervention which involves transplanting genotypes of a given species from distant sources to new locations within the species range with the potential to restore levels of genetic diversity.

Climate-adjusted provenance: Plant material for restoration collected along a climate gradient in line with climate change projections.

Composite provenance: Plant materials for restoration collected as a mixture from healthy local provenances together with smaller amounts of material from more distant sites.

Effective population size: A measure proposed by Montalvo et al. [199] to evaluate genetic diversity in populations by considering the percentage of reproductive individuals, sex ratio, and fluctuations in population density.

Genetic restoration: The process of assisting the recovery of an ecosystem that has been degraded by restoring or improving the genetic baselines of vulnerable populations.

Genetic rescue: Introduction of genetic materials from other populations to increase the fitness of small, isolated, and imperiled populations.

Heterosis: Increased fitness of hybrid product from two different genotypes in comparison with parental genotypes.

Inbreeding depression: Reduction in survival, fitness and reproduction of offspring of genetically related individuals.

Local provenance: Plant material for the restoration collected near to and in similar environmental conditions as the planting site, which gives new plants the best chance of survival.

Linkage disequilibrium: Non-random association of alleles at different loci. The frequency of association of different alleles is higher or lower than what would be expected if the loci were independent and randomly associated.

Outbreeding depression: Reduction in survival, fitness and reproduction of offspring of genetically distant individuals.

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CONCLUSIONS

The overall aim of the thesis was to evaluate the resilience capacity of the Mediterranean seagrass *Posidonia oceanica* to future environmental changes. The main findings indicate that local conditions in which plants grow affect the response capability to environmental stress. The eutrophic conditions existing in the Gulf of Pozzuoli (Naples, Italy), in fact, weaken local populations, thereby compromising their resilience and survival in the face of seawater warming that is ongoing in the context of global climate change. In fact, the physiological and transcriptomic characterization of *P. oceanica* adult plants exposed to simulated eutrophic conditions and sea warming (i.e. simulated marine heatwave) in a multi-factorial experiment revealed the higher vulnerability to marine heatwaves of plants growing under eutrophic conditions with regard to plants from pristine environments. Local environmental conditions also play a crucial role in the response specificity to single and/or multiple stressors in natural *P. oceanica* populations. The complex plants' responses to single and multiple stress also underlined that the occurrence of different local pressures has the potential to interact with the ongoing seawater warming, either exacerbating or buffering the impact on seagrass ecosystems. Dissimilar metabolic mechanisms are adopted, as highlighted by the activation of different genic pathways, to cope with stress factors in plants with a different history of exposure to stress. My results highlight the relevance of these type of experimental approaches (multifactorial designs in controlled mesocosm systems) and analytical techniques (transcriptomic and eco-physiology) for exploring the resilience of seagrasses populations in a realistic scenario of environmental changes and human pressures. Moreover, I also revealed a different organ-specific vulnerability in *P. oceanica* plants exposed to different stress factors. Leaves were more vulnerable to nutrients enrichment and modulated different nutrients-balancing strategies and transcriptional reprogramming depending on the nutrient exposure history of populations, while shoot-apical meristems (SAMs) were particularly affected by heat stress, whose intensity also depends on the plants' origin. Plants that have already experienced local pressures at their home site followed a greatest transcriptional reprogramming in the presence of new stress typology, which is temperature. Thus, SAMs seems to be a preferential tissue to analyse, if we aim in detecting early warning signals of heat stress in *P. oceanica* populations. This is of great importance for the possible implementation of monitoring plans of this vital resource for the dynamics and productivity of the Mediterranean coastline.

Plants exposed to single and multiple stressors also displayed a relevant modulation of epigenetic mechanisms, especially in organs where the largest transcriptomic regulation was observed. The dynamic regulation of epigenetic-related genes was remarkably different between plants depending on environmental conditions pre-experienced at their local sites and stress typology. Since epigenetic mechanisms regulated phenotypic variations that can also be inherited across generations favouring stress memorization, these results could provide the first epigenetic signatures of the existence of a transcriptional memory in *P. oceanica*. This is again variable, based on plants' origin.

These evidences were also supported by different dynamics of DNA methylation observed at the leaf level and pointed out the potential role of epigenetic modifications in regulating gene expression and the phenotypic plasticity of the species in presence of environmental changes. My results revealed that DNA methylation is a dynamic process in *P. oceanica* plants which is influenced by environmental stresses and plants' origin, with important implications in regulating stress responses.

My thesis also opened a new frontier in seagrass restoration and conservation, as it demonstrated the possibility to produce heat-primed *P. oceanica* plants during their early life stages (i.e. seedling stage) for increasing their heat tolerance to further stressful warming events. Furthermore, molecular analysis conducted on primed *vs* non-primed seedlings also suggested the potential of epigenetic modifications in stress-memory acquisition as well as for activating a suitable heat stress response. Reinforcing seagrasses by using non-invasive manipulative approaches could rewrite the fate of these important ecosystems improving restoration plans for marine plants. Moreover, the emerging role of epigenetic regulation of phenotypic responses to stressful conditions, underlined the importance to study epigenetic processes as active mechanisms that regulate the degree of phenotypic plasticity favouring the organisms adjustment to environmental changes.

To conclude, this thesis contribute to explain the impact that local disturbance, in particular eutrophication, has in a sea warming scenario, suggesting the interrelation existing among local environmental stress factors and global stress factors induced by Global Climate changes. Populations that are locally affected by environmental stresses will possibly suffer more for the effects of global changes. I also revealed the importance of transcriptional and epigenetic studies in describing molecular signals of stress perceived by plants. Overall, these findings underlined the importance to perform 'omic' approaches integrating epigenetic studies to describe seagrass responses to single and multiple stressors. The results of my thesis also offer new opportunities to explore resilience capacity of seagrasses to environmental

changes with important implications for future directions of restoration and conservation managements of these valuable ecosystems.