FACULTAD DE CIENCIAS DEL MAR

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Posidonia oceanica restoration: bibliographic review and data field analysis

Aleida Escandell Westcott
47260567Y

Prof. Dr. Rodrigo Riera Elena
Biodiversity and Conservation Group (BIOCON)
IU-ECOAQUA

Dra. Noelia Hernández Muñoz
(Vellmarí Association)

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ABSTRACT

Seagrasses like *Posidonia oceanica* provide a wide range of benefits for the ocean and for the planet. Efforts in conservation and restoration have increased in the last 20 years to preserve *P. oceanica* meadows; a plenty of works have been conducted to develop restoration and planting actions as a way to offset the regression of this seagrass in the Mediterranean Sea. We here compile the information on these studies to make a bibliographic review. Additionally, we collected seeds in Formentera (Balearic Islands, NW Mediterranean Sea) and cultured them for 1-2 months to measure them. Measuring these seedlings allowed us to compare annual data to prove seedlings annual size variability. As a way to calculate the growing rate in 1-year-old seedlings, we measured the leaves of seedlings planted last year. The review results showed that seedlings grow better on dead matte and rock covered with algae in calm waters with moderate depths. The PERMANOVA and Kruskal-Wallis tests showed significant differences in seedlings size between the two years of this study. All the means of the measurements were higher in 2021 than in 2022, and the n-MDS support this, showing significant differences. The growth rate showed a value of 9.8 cm year^{-1} in leaf length and 0.3 cm year^{-1} in leaf width in 1-year-old seedlings. More results and strong conclusions are necessary and will be obtained by extending the study for more years. Furthermore, with intensive and integrative work more gaps in knowledge could be filled in the upcoming years.

Keywords: *Posidonia oceanica,* seedlings, restoration, bibliographic review, Mediterranean Sea

RESUMEN

Las fanerógamas marinas como la *Posidonia oceanica* aportan una amplia gama de beneficios al océano y al planeta. Los esfuerzos por conservar y restaurar se han incrementado en los últimos 20 años para preservar las praderas de *P. oceanica*; se han realizado numerosos trabajos para desarrollar acciones de restauración y plantación como forma de compensar la regresión de esta pradera marina en el Mar Mediterráneo. Aquí recopilamos la información de estos estudios para hacer una revisión bibliográfica. Adicionalmente, recogimos semillas en Formentera (Islas Baleares, NW Mar Mediterráneo) y las cultivamos durante 1-2 meses para medirlas. Medir estas plántulas nos permitió comparar los datos anuales para comprobar la variabilidad anual del tamaño de las plántulas. Para calcular la tasa de crecimiento en plántulas de 1 año de vida, medimos las hojas de las plántulas plantadas el año pasado. Los resultados de la revisión mostraron que las plántulas crecen mejor en el sustrato mata (dead matte) y en las rocas cubiertas de algas en aguas tranquilas con profundidades moderadas. El PERMANOVA y el test de Kruskal-Wallis mostraron diferencias significativas en el tamaño de las plántulas entre los dos años de este estudio. Todas las medias de las mediciones fueron mayores en 2021 que en 2022, y el n-MDS lo corroboró mostrando diferencias significativas. La tasa de crecimiento mostró un valor de 9.8 cm año^{-1} en el largo de hoja y 0.3 cm año^{-1} en el ancho de hoja en las plántulas de 1 año. Se podrán obtener más resultados y conclusiones necesarios ampliando el estudio a más años. Además, con un trabajo intensivo e integrador se podrían cubrir más lagunas de conocimiento en los próximos años.

Palabras clave: *Posidonia oceanica,* plántulas, restauración, revisión bibliográfica, Mar Mediterráneo

1. INTRODUCTION

Seagrasses are plants that colonized marine ecosystems, about 100 million years ago, evolving from terrestrial monocotyledons (Zenone et al., 2020). They are one of the most productive and valuable ecosystems on Earth (Murphy et al., 2021; Valdez et al., 2020; van Katwijk et al., 2009). Numerous studies have recognized its ecological and economic value, which would disappear along with them, as they provide important ecosystem services such as: nutrient cycling, sequestration of carbon, a habitat for thousands of fish and invertebrate species and a source of food for many of them, among others (Bidak et al., 2021; Terrados et al., 2013). Seagrasses are globally threatened by direct (dredging, fishing or anchoring) and indirect human impacts, e.g. global change. (Bidak et al., 2021; Duarte, 2002; Waycott et al., 2009). A global estimation found that 29% of the known surface area has disappeared since 1879. This results in rates of decline accelerating from an average of 0.9% year^{-1} before 1940 to 7% year^{-1} by the end of the 20th century (Waycott et al., 2009). In Europe, seagrasses showed a slowdown in the declining rates due to an improvement on the coastal management to reduce nutrient supply from urban waters and agricultural pollution (Grizzetti et al., 2021). These enhancements led the decadal rate of area loss to a deceleration: in the 1980s (27% decade^{-1}), in the 1990s (16% decade^{-1}) and in the 2000s (8.3% decade^{-1}) (de los Santos et al., 2019). In the Balearic Islands, 70% of the seagrasses show stable or improving trends (Ruíz et al., 2015). This loss could be due to the fact that the sexual reproduction of phanerogams is very limited (Balestri & Cinelli, 2003) and, therefore, recovery is very slow.

Seagrasses can reproduce both sexually and asexually (clonation), but prefer the latter (Duarte, 2002; Terrados et al., 2013). Vegetative propagation, which does not involve critical phases like germination and establishment, offers ecological advantages because it allows the colonization through ramets, which have the same genetic information as the mother plant (Ferrer et al., 2011). This lack of recombination could explain their regression and diminish their efficiency in migrating and evolving (Dodd & Douhovnikoff, 2016; Vangelisti et al., 2020). Sexual reproduction is more difficult (Meinesz et al., 1993) and depends on many factors (flower and fruit production and frequency, current directions, fruit buoyancy or prosperous anchorage of the seedling) (Balestri & Cinelli, 2003; Vermaat, 2009). The dispersal from a distant source leads to an establishment of the propagule and a prosperous recolonization. This recolonization leans on current directions and on the floating of seeds and fruits, which normally is unlikely to occur. In addition, seagrass with annual shoots may change its extension and density every year because the seed production and the recruitment varies every year (Vermaat, 2009). Although seagrasses are widespread all over the world, the requirements of each species influence reproductive success. Seagrasses can be found on muddy, sandy or rocky sediments and in shallow or platform waters, forming huge and dense meadows from 0 to 35 m depth (Duarte, 2002). Water movement alters the seedling establishment because of the sediment movement; the roots fix them to the soil to counteract the pressure waves make. However, water movement often leads to wear, tear and loss of plant tissue, mainly leaves, because the drag forces augment with leaf area (Infantes et al., 2011). They need nutrients, carbon and light to survive, so it’s rare to see them in deep water (Vermaat, 2009).

Posidonia oceanica is an endemic seagrass in the Mediterranean Sea. Its meadows can be up to 100,000 years old, so it is considered a long-lived and slow-growing plant. It uses dispersal propagules for sexual reproduction by forming floating fruits with non-dormant seeds in its interior (Guerrero-Meseguer et al., 2018). Strategies based on seeds are an advantage for the donor because they depend on biological material, that in most cases don't survive, and provide genetic diversity for the new plant (Alagna et al., 2020). *Posidonia* seeds are moved by water currents after they leave the donor meadow, and they can be found as drift material on sandy or rocky beaches. The seed is embedded in a pulpous ovoid pericarp which will open and discharge the seed that will sink to the seabed (Terrados et al., 2013). The seeds have photosynthetic activity, thanks to chloroplasts found in the external layers of the fruit, to produce oxygen when the fruit is floating on the surface and will continue during seed germination (Guerrero-Meseguer et al., 2018). *Posidonia* seeds contain enough nutrients and carbon to survive from 6 to 8 months. (Terrados et al., 2013). When the seed reaches the seabed, it needs to establish, this phase between germination and establishment is the most difficult one (Pereda-Briones et al., 2020). In order to establish, seedlings must form a root system and enter deep into the substrate (Balestri et al., 2015). Adhesive root hairs must be in contact with the substrate to fix the seedling and grow, also increasing photosynthetic rates will help seedlings establish. Root hair morphology will rely on the seabed topography (Alagna et al., 2020; Balestri et al., 2015; Guerrero-Meseguer et al., 2018).

The present study was carried out on the island of Formentera (Balearic Islands, NW Mediterranean Sea), in Ses Salines Natural Park. Here *Posidonia* covers 70-80% of the seabed forming one of the largest and oldest meadows (Fig. 1). This location is selected because of the health status of this *Posidonia* meadow, manifested with annual flowering and presence of dense meadows (Ramón-Cardona et al., 2021). In the Balearic Islands, this seagrass has shown a rate of loss of 7.4 ± 1.8 % annual (Ruiz et al., 2015). Broadly speaking, the purpose of all this study is to improve the knowledge about *Posidonia oceanica* restoration methodology to enhance its protection and conservation. The first goal of this paper is to make a revision of papers about *Posidonia oceanica* culture to increase our understanding about it. In fact, this revision is done to find the best way to grow *Posidonia* seedlings that will help restoration projects. In order to study temporal variability of seedling size, we will use data analysis of the data collected in 2021 and 2022. So, the second aim is to study differences in the size of seedlings in 2021 and in 2022. The third objective is to evaluate the growth rate of 1-year-old seedlings restored in a pilot project in Formentera with "Asociación Vellmarí".

2. MATERIAL AND METHODS

The present study is based on a bibliographic research conducted between April and June 2022. The data was obtained from ResearchGate, Scopus and Google Scholar, a web search engine that works with citation indexing through papers, books and studies. The keywords applied to identify the most suitable works were: "*Posidonia oceanica*", "seedlings", "seed" and "restoration". All papers were included in this search, regardless of the date of publication. All the studies were analyzed, but only those focusing on restoration with seeds

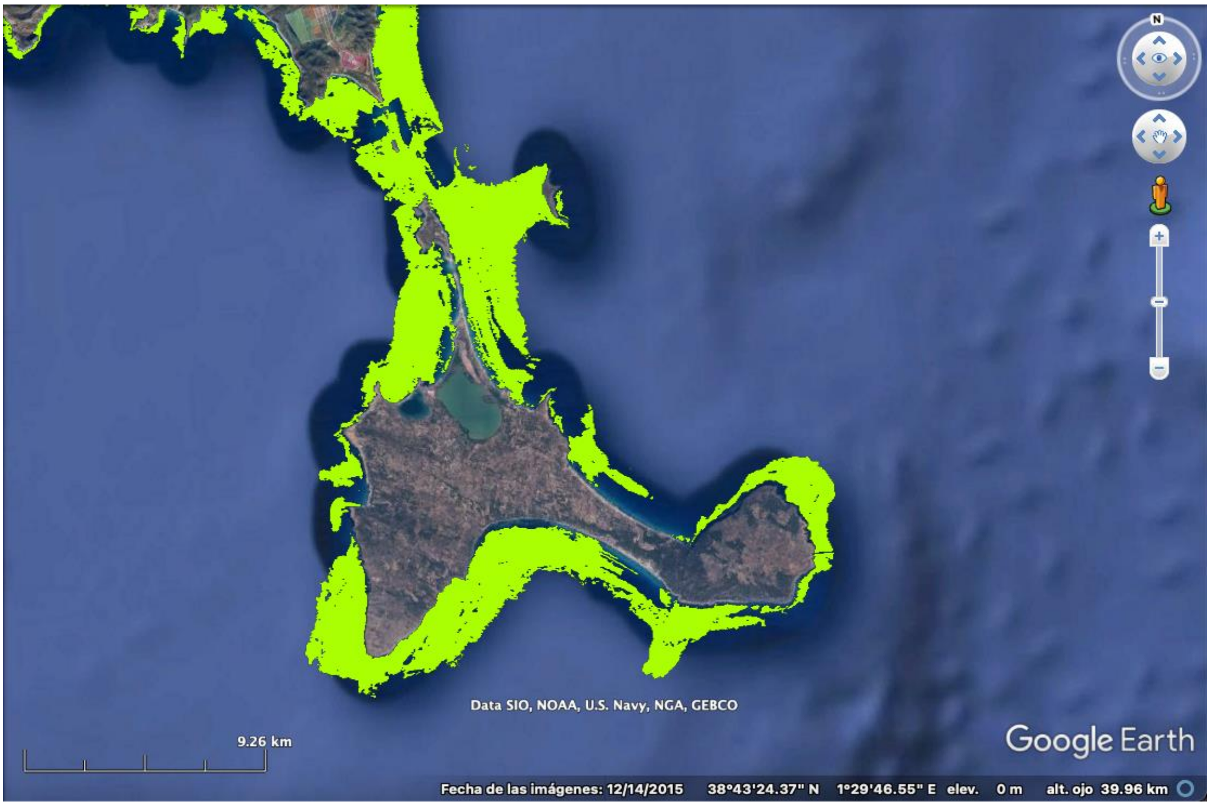


Figure 1. Posidonia oceanica distribution area in Formentera, Balearic Islands, Spain. Source: Cartografía del Atlas Posidonia de la Conselleria de Medi Ambient i Territori del Govern de les Illes Balears.

and not with *Posidonia* cuttings were retained. In particular, the greatest effort was focused on those that studied the factors involved in seedling culture. Later, parameters to study needed to be chosen. First, the author and year of publication were chosen to know better which authors are the most recurrent ones. Then, we start analyzing the experiment: factor/parameter, culture and experiment time, year of study, location, season, replicates and study area. The factor studied in the experiment was the most important issue, because it allows the reader to understand what and why it is done. The result could help others in new restoration projects. Culture time allows us to know the age of the seedlings at the time of the experiment. The experiment time is also important because a 1-year study is not the same as a 4-year study. A longer investigation makes it possible to verify that the seedlings have survived and grow after restoration, because survival and the factors that affect them can change over the years. The year of study indicates how much time has passed between the experiment and the publication of the article. The location gives an idea of the section of the Mediterranean that is being analyzed and, consequently, its contamination, exposure or regression of *Posidonia* in the area. The season will directly affect seawater temperature and mixture. Replicates and study areas help to perceive the reliability of the study by the number of times an experiment has been repeated.

Complementarily, a study regarding *Posidonia oceanica* restoration has been done since last year in Formentera by "Asociación Vellmarí". For the purpose of comparing the seedlings of this year with the ones collected and planted last year, fruits of *Posidonia* were

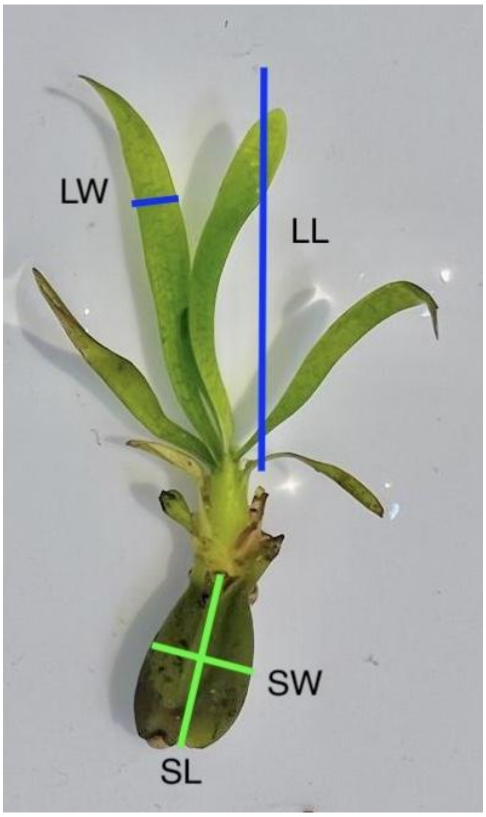


Figure 2. Seedling measurements. LW: Leaf width, LL: Leaf length, SW: Seed width, SL: Seed length, SRL: Secondary root length and RL: Root length.

collected from beaches of Formentera during May and June 2022. After this, fruits were left to germinate in fruit boxes with a net on top to avoid seed loss. As the seeds were so little, they were placed into a fruit net for growing during 2 months. With these two-month-old seedlings, all measures were taken: length and width of seed, length of primary and secondary roots and length and width of leaves (Fig. 2). One-year-old plants planted last year were monitored, measuring leaves length and width by scuba diving.

2.2. Data analysis

We execute an ANOVA (Analysis of variance) with the fixed factor "Year" (with two levels: 2021 and 2022) on seed length and width, leaves length and width and length of the primary root. Using Rstudio software, for each seedling, we average the leaves length and width. After the ANOVA, to evaluate normality and homoscedasticity, Shapiro-Wilk and Levene's test were performed. Residues of the test were not normal, so we decided to use Kruskal-Wallis' test to detect differences in each variable between years.

To test divergences on the seedlings in years, we used the PERMANOVA (Permutational multivariate analysis of variance). PERMANOVA analyzed differences in all variables (seed length and width, leaves length and width and length of the primary root) per year. It uses the adonis function in the *vegan* package from R software (Oksanen et al., 2019), with 999 permutation and the distance of Bray-Curtis. Permutation is used to find the dispersion between groups and the distance to calculate the dissimilarity matrix, measuring from 0 to 1 the difference. As a way to understand which parameter mainly contributed to the differences in years, a SIMPER analysis was used. To visualize graphically the variance of seedlings among the two years, a two-dimensional n-MDS (Non-metric multidimensional scaling) layout was used. Annual growth rate was calculated comparing leaf length and width from seedlings planted in 2021 and measured again in 2022.

3. RESULTS

3.1. Bibliographic review

The 27 studies included in the present study are shown in (Suppl. Mat.). All the studies have been done in countries around the Mediterranean Sea (i.e., Italy, France and Spain), as the *Posidonia oceanica* is an endemic species. After the analysis, new factors were revealed to be of utmost importance such as, variables regarding the substratum, i.e., complexity, roughness or slope.

A wide range of substrata were compared: dead matte, *Posidonia oceanica* meadow, rock, sand, gravel, pebble, fiberglass or tiles with different complexity or shape. The most suitable substratum type is dead matte or consolidated ones like rocks. Dead matte complexity allows the seedling to anchor in all dead and uprooted roots. The maximum survival rate was 92% on rock covered with algae and 75% on dead matte. The minimum survival was recorded on sand, where seedlings find much more difficult to establish. Substratum complexity and roughness favors seedlings anchorage, like the dead matte, where roots can penetrate in the interstitial space of the substratum. High complexity substrata had a survival rate as high as 55%. Another study evaluated the influence of pre-experimental substratum comparing between hard glass and sand but found out that it does not affect seedlings survival, just seedlings growing (4 times higher growth rate on sand).

Exposing seedlings to 25 (control), 27 and 29ºC revealed that at the control the survival rate was 94-100%. Another study proved the effects of thermal-printing in seedlings. Primed seedlings were exposed to 30.5ºC for 11 days and then exposed to 32ºC for 2 weeks. Non-primed seedlings were just exposed to 32ºC. “Priming” made the seedlings ready to withstand the extreme temperatures and, therefore, primed seedlings had more growth rates and pigment content. As the depth increases, *Posidonia oceanica* is becoming less abundant, so depth is an important factor for seedling culture. When cultured at 2 and 10 m depth, seedlings at 2 m died; only the ones cultured in matte at 10 m survived. The higher survival rate was on dead matte at 10 m (69%), this indicates that *Posidonia oceanica* needs to grow on moderate depths.

Studying the planting level (above or below ground) demonstrated that it does not influence seed and leaves growing. Neither does natural anchoring nor with “mesh pots" on survival. Seawater hydrodynamics hinders establishment; how much the seedling is affected by the drag force depends on the size of the leaves and the strength of the roots. This is explained in a paper where seedlings with less exposure had a survival rate of about 80%. Association with macroalgae like *Caulerpa cylindracea* or with seagrass *Cymodocea nodosa* enhances seedling anchorage by augmenting the substratum roughness and complexity.

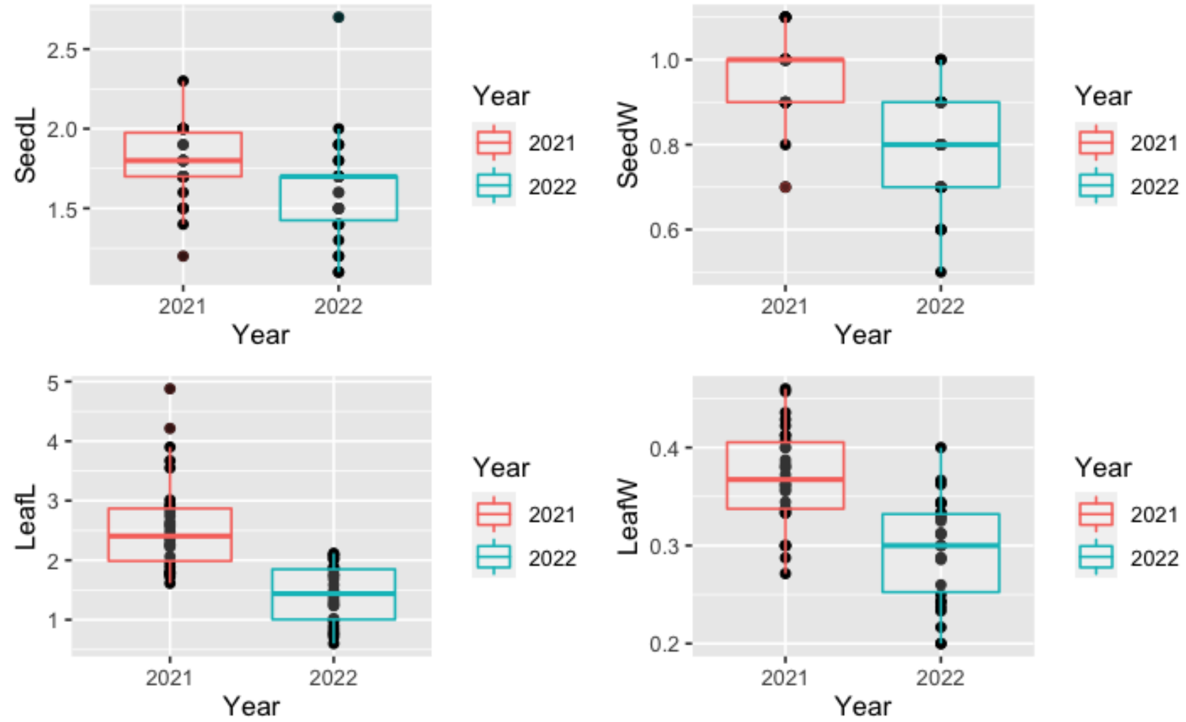
Table 1. SIMPER analysis results showing contribution of each parameter to differences in years.

Variable	Mean 2021	Mean 2022	Cumulative contribution
Primary root length	1.62	0.38	0.415
Leaf length	2.59	1.43	0.801
Seed length	1.78	1.61	0.906
Seed width	0.96	0.78	0.972
Leaf width	0.36	0.3	1.000

Rocks covered by crustose algae or brown seaweed, such as *Cystoseira spp.*, reached a survival rate of 96 and 81%, respectively.

Experiments in dark and light conditions were performed in order to understand how much photosynthesis affects the seedlings' growth. In illuminated conditions, leaves, roots and seeds grown more and better, with more nutrients mobilized. However, a study stated that seedlings that were exposed to direct sunlight more than 1 day showed irreparable morphological and physiological damage. Herbivorism (i.e., fish *Salpa salpa* or sea urchin, *Paracentrotus lividus*) is a problem that may be important, but a study found out that protecting the seeds with cages or nets (67-85% and 70-90% survival) or leaving them unprotected (60-70% survival) do not influence seedling survival and development. Interestingly, when seedlings were in high pCO_2 conditions, more herbivore feeding by sea urchins was detected. In addition, these extreme conditions did not affect seedlings survival, but it produced larger seedlings with more photosynthetic activity. For the purpose of knowing if nutrients are important, a study fertilized the sediments where seedlings were growing. Seedlings in fertilized sediments presented more leaf development, but it did not change survival, that range between 55-65%.

Salinity is also a factor of utmost importance in *Posidonia oceanica* culture. An experiment proved the effects of salinity in seeds germination and growth. In the germination period, salinity did not significantly affect survival (90% in the control salinity (37 PSU) and 63-80% in greater salinities (40-49 PSU)). Yet, in 50 days of growth, leaves in the control situation showed significantly more leaves and leaf and root proliferation. (Suppl. Mat.) integrates the above-mentioned information on each of the analyzed papers.



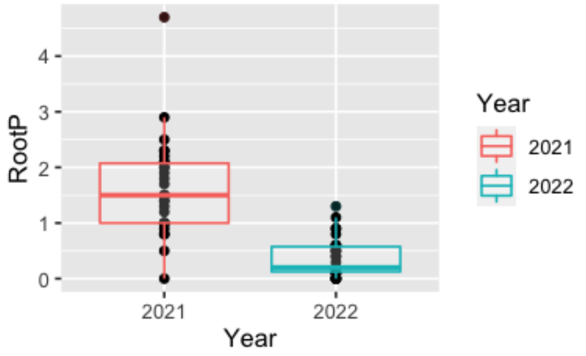


Figure 3. Differences in seedlings size per year. Differences in (A) seed length, (B) seed width, (C) leaf length, (D) leaf width and (E) primary root length.

3.2. Comparative data (2021-2022)

The second aim of this study was to analyze the differences in seedling size between 2021 and 2022. During the seeds collection and culture, differences in size compared to the ones cultured last year were observed. When collecting fruits in 2022, fruits and seeds inside them were smaller and the pericarp was already open in many of them. After culture time, seedlings showed shorter and narrower leaves. Also, the primary and secondary roots were shorter and, in some cases, there was no primary root. All morpho-anatomical variables (seed length and width, leaf length and width and primary root length) showed higher values in 2021 than in 2022 (Figure 3). Seed length: 1.78 ± 0.04 cm in 2021 and 1.61 ± 0.06 cm in 2022; seed width: 0.96 ± 0.02 cm in 2021 and 0.78 ± 0.02 cm in 2022; leaf length: 2.59 ± 0.14 cm in 2021 and 1.43 ± 0.09 cm in 2022; leaf width: 0.37 ± 0.01 cm in 2021 and 0.3 ±0.01 cm in 2022 and primary root length: 1.63 ± 0.16 cm in 2021 and 0.38 ± 0.06 in 2022 (Table 1). Standard error was very low in all calculated means, this indicated that the data was very homogenous in each year. The Kruskal-Wallis test indicated significant differences between years in all studied parameters. The results for the Kruskal-Wallis test were: seed length ($H = 8.25$, $p = 0.004$), seed width (H = 24.29, p = 0.00000083), leaf length (H = 31.23, p = 0.000000023), leaf width (H = 21.74, p = 0.0000031) and primary root length (H = 33.83, p = 0.000000006).

PERMANOVA showed that integrating all variables, significant differences were found in seedlings of the two studied years (2021 *vs.* 2022) (F = 49.6, p = 0.0001). SIMPER analysis demonstrated that primary root length contributed the most (41.5%) to differences in seedlings size between years. Leaf length also contributed significantly (38.6%) to the differences (Table 1). The n-MDS clearly showed a separation between the seedlings of the two years (Figure 4). Seedlings of 2021 are on top, whilst seedlings of 2022 are in the half down of the ordination. They formed two separate groups with almost no overlapping between 2021 and 2022. The mean growth rate per year calculated in seedlings was 9.8 cm year^{-1} in leaf length and 0.3 cm year^{-1} in leaf width. This rate is very high because there is a big difference in leaf length and width between the two years. This value can be explained by the fact that the plant is in its first year of life, where growth is normally faster.

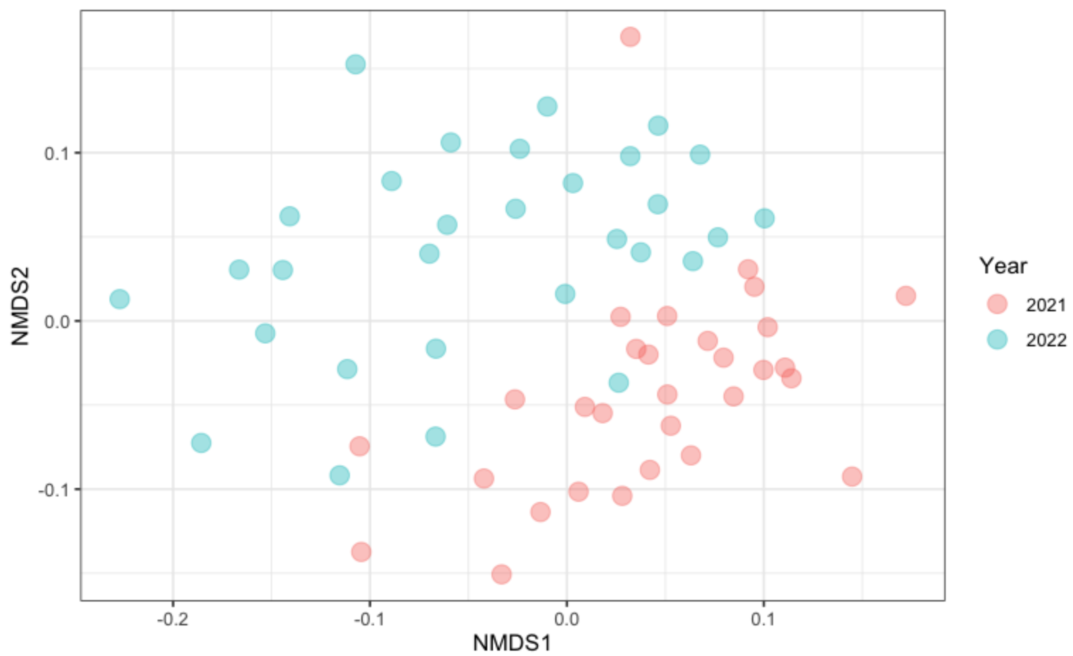


Figure 4. nMDS showing differences in seedlings size between 2021 and 2022

4. DISCUSSION

This study comprised a bibliographic review of the papers concerning *Posidonia oceanica* restoration by seedlings. Sexual reproduction is difficult, and it is not likely to succeed (Meinesz et al., 1993), because it has a lot of critical phases (i.e., germination and establishment) so, the factor affecting seedlings in these phases need to be studied (Alagna et al., 2020; Ferrer et al., 2011). The most suitable substratum are consolidated substrata (dead matte or rocks), where survival is higher than 75%. Association with algae or seagrasses increases substratum roughness and complexity and, therefore, helps establishment. Moderate depths (10 m) proved to be the most appropriate for seedling planting. Temperatures over 25ºC showed an increase of seedling mortality and herbivorism whilst at 25ºC survival rates were at least 95%. Hydrodynamics also affects seedling establishment and survival, low exposed areas had an 80% of survival rate. Higher salinities (40-49 PSU) decreased leaves and roots proliferation. Our analysis gave an overall view to help others understand all the parameters that could affect seedlings in any way. After the statistical analysis, significant differences were found in seedlings size between years, in 2021 seeds were larger with longer and wider leaves and longer roots. Length of the primary roots turned out to be the parameter that most contributed to this difference, followed by leaf length. The growth rate obtained in this analysis was 9.8 cm $year^{-1}$ in leaf length and 0.3 cm $year^{-1}$ in leaf width.

Improving coastal ecosystems protection and preservation can palliate the losses on a small scale, but *Posidonia* is disappearing faster than it is growing (van Katwijk et al., 2016). Therefore, *Posidonia oceanica* restoration is an important instrument to balance losses and recovery rates. Our review could help understand how to manage this restoration by seedlings on a larger scale, understanding what can affect their growing and survival. The most studied parameter in the analyzed papers was the substratum, which is one of the most important parameters during seedling anchorage; 15 of the 27 studies (55.6%) analyzed proved the effects of the substratum type. Pereda Briones et al. (2020) compared seedlings growth in consolidated substratum (rock and dead matte) and in unconsolidated ones (sand and gravel) and found that consolidated ones are the most suitable. They also found that macroalgae cover (crustose algae) improves the conditions for seedling success, because with its complexity and roughness facilitates seedling settlement. Alagna et al. (2013) also observed this trend, where higher seedling survival was observed in rocks covered with *Cystoseira* spp. Other studies compared rock with sand or gravel, sand with gravel, fiberglass with sand or pebble or matte with vegetated pebbles and reinforce the idea that more consolidated substrata help seedlings anchorage and establishment (Balestri et al., 1998, 2015; Domínguez et al., 2012; Guerrero-Meseguer et al., 2018; Piazzi et al., 1999). Balestri & Lardicci (2008) do not approve this idea and establish that seedlings can grow in consolidated and unconsolidated substratum, because they found that there were no significant differences in seedlings establishment and abundance between substrata (rock *vs* sand). Donor populations play an important role in restoration, healthy meadows are needed for survival of seedlings and shoots, for this reason protection of *Posidonia* is also necessary. Donor meadows can improve seedlings survival if they accomplish some criteria. The transplant site must be located close to the donor plant, since its presence indicates that it is a suitable site for its development. In addition, transplanted plants must have proper gene attributes to survive on a long-term scale that can be obtained by gene flow from nearby plants (van Katwijk et al., 2016). The seedling size analysis revealed significant differences that could be explained because in February and March in Formentera there were a lot of storms that could have caused the seedlings to detach sooner from the donor plant, and so the seedlings were smaller. However, the growth rate could not be compared with any papers, because it is a rate of the seedlings' growth in its first year of life and, to our knowledge, no papers regarding this topic were found.

Marine restoration is a new challenge for scientists, but it has proved its potential to restore destroyed ecosystems (Duarte et al., 2020, McAfee et al., 2021). The small-scale success in restoration project is what contributes to building a solid foundation of models, political disposition and agreement to advance to a bigger scale, like global scale (McAfee et al., 2021). Ecological restoration as a way for conservation is on the rise, so biotic and abiotic factors and culture methods need to be studied and understood to fill the gaps in restoration knowledge (Valdez et al., 2020). Our review found that, even if *Posidonia oceanica* restoration by seedlings started about 20 years ago, there are gaps in the literature that need to be reinforced since there are parameters that have been overlooked or poorly studied by former researchers. As mentioned above, the most studied parameter was the substratum, followed by seawater temperature, but the remaining parameters were poorly studied. Two studies compared seedlings growing at three different temperatures (25, 27 and 29ºC) and found out that seedlings showed the highest growth at 25ºC. Higher temperatures decreased success in seedlings establishment and their carbohydrate stock. In warmer conditions, more herbivorism was seen, grazers selected seedlings with lower content of fiber in its leaves (Hernán et al., 2017; Pereda-Briones et al., 2019). Another study proved that thermo-printing helps seedlings to be ready to extreme conditions, i.e., if the seedlings had an adaptation period with progressive temperature increases, they would be able to adapt to high temperatures (Pazzaglia et al., 2022). Increased concern about global warming may explain why it is the second most studied parameter, but the results are not encouraging. Association with macroalgae and seagrasses was also evaluated in four papers, and was proven to increase the probability of survival. Algae coverage (crustose algae, *Cystoseira* spp. and *C. cylindracea*) increased rate of survival (Alagna et al., 2013; Pereda-Briones et al., 2020; Pereda-Briones et al., 2018), as did the presence of seagrasses, like *C. nodosa* (Balestri et al., 2021). Planting level and seed burial, exposure to seawater hydrodynamics and effects of nutrients and hormones are studied in three papers each. Planting level and seed burial were not significant in seedlings cultures (Domínguez et al., 2012; Guerrero-Meseguer et al., 2017; Terrados et al., 2013). The three studies evaluating hydrodynamics showed that low exposure areas are appropriate for seedling culture, because drag forces could uproot them (Infantes et al., 2011; Pereda-Briones et al., 2020; Zenone et al., 2022). Studying the effects of nutrients in the sediments revealed that they improve leaf development (Pereda-Briones et al., 2018). Another study proved that seed germination stimulant (Sprintene) does not make the seed germinate earlier, however hormone presence caused the roots to emerge earlier and to grow longer (Balestri & Bertini, 2003). The other parameters taken into account in this review (effects of invasive algae presence, photosynthesis, light, herbivorism, high pCO2, epiphyte cover, salinity, depth and seedling density) have been scarcely studied to date.

Our work allowed us to sum up the information about *P. oceanica* seedlings culture, with many studies carried out in the Mediterranean Sea. However, there are still too many things to study and understand about this seagrass. Restoration projects with seedlings are being carried out in order to fill these gaps of knowledge and improve restoration success. The study conducted in this paper is part of a pilot project for *Posidonia* restoration by seedlings in the Mediterranean Sea carried out by "Asociación Vellmarí". At the same time, researchers from the University of Western Australia started a two-years pilot project to restore *Posidonia australis* with seedlings, a subject on which there are still no published results (Tan et al., 2020). These studies and others that are being conducted around the world will provide solid information to reach strong conclusions and remove the lack of knowledge. This analysis is only the beginning of a project that could help us learn some important parameters about *Posidonia* sexual reproduction. To fill the gaps in *Posidonia* restoration knowledge, more years and efforts are necessary. We need to explore thoroughly about *Posidonia* flowering and its variability, to understand how it works and why seedlings showed interannual variability.

Parameters affecting seedling culture, establishment and its further development should be more studied with field and laboratory experiments to fill the gaps or to reinforce the existing information. In addition, the growth rate should be given more consideration because it is a reflection of the seedling's health and growth. This study is a first step that could be strengthened with integrative work in the next years.

5. CONCLUSIONS

- Our analysis stated that for the best seedling growth, they need to establish in consolidated substratum (dead matte or rock).
- Algae or seagrass coverage enhances seedlings establishment by increasing substratum roughness and complexity.
- Low exposed areas with moderate depths are more suitable for seedlings to grow.
- High temperatures (>25ºC) and salinities (>39 PSU) decrease seedlings' success.
- In 2021 the flowering produced more and bigger seeds that produced bigger seedlings. All the parameters studied in seedlings were higher in 2021 compared with the ones collected in 2022.
- The large differences between years could be explained because of the storms that occurred in February in Formentera, but the limited data we have does not allow us to infer more conclusions.
- Growth rate obtained in 1-year-old seedlings planted in 2021 is 9.8 cm $year^{-1}$ in leaf length and 0.3 cm $year^{-1}$ in leaf width, but the lack of knowledge in this subject does not allow us to compare it.
- Future studies will have to increase the number of years of study to have a clear representation of what is happening.
- There are a lot of gaps to fill in this study area, *Posidonia oceanica* seedlings culture must be thoroughly studied in order to fill these gaps.

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SUPPLEMENTARY MATERIAL

ID	Title	Year of publication	Authors	Factor	Type	Experiment time	Culture time	Year of study	Location	Season	Results	% success	Study area	Replicate
1	Use of Posidonia oceanica seedlings from beach-cast fruits for seagrass planting	2013	Terrados	substratum type	dead matte vs meadow	3 years	2-3 months	2008	Hornillo Bay, Águilas, Spain	Summer	dead matte	44%	12 areas of 60m2=720 m2	3 areas meadow 3 areas dead matte; each area 24 seed (12 above/12 below)
				planting level	above vs below substratum surface	3 years		2008			no afecta	no significant differences		
				anchoring	“mesh-pot” vs. “natural” anchoring	2 years		2009			no afecta	no significant differences		6 areas dead matte; each area 24 seed (12 natural/12 mesh pot)
2	Influence of biotic and abiotic factors of seagrass Posidonia oceanica recruitment: Identifying suitable microsites	2020	Pereda Briones	Hydrodynamics	low exposure and high exposure	2 years	No culture time, seedlings grown on the field (9-	2015	Palma de Mallorca, Spain	All year	low exposure	70-85%	3 sites along Palma Bay	168 seedlings
				substratum type	consolidated (rock and matte) vs unconsolidated (sand and gravel)						Consolidated (rock and matte)	92% rock and 67% matte		
				association with macroalgae	corticated foliose, corticated, articulated calcareous, crustose						crustose	96%		
3	Influence of microhabitat on seedling survival and growth of the mediterranean seagrass posidonia oceanica (L.) Delile	2013	Alagna	substratum type	rock vs sand or gravel	2 years	No culture time, seedlings grown on	2004	Sicily, Italy	Summer	rock	81%	1m2	8 replicates for each combination of factors
				association with macroalgae	sand/gravel without algae vs rock with algae						rock with algae (Cystoseira)			
4	Understanding the sexual recruitment of one of the oldest and largest organisms on Earth, the seagrass Posidonia oceanica	2018	Guerrero-Meseguer	light direction influence	positive phototropism towards light?	2 months	1 week	2016	Múrcia, Spain	Summer	seeds turned to light	100%	Laboratory	8 seeds x treatment = 24 seeds
				photosynthesis activity	dark and light incubations in fruits, in seeds extracted from the fruits and in 1-week-old seeds	1 minute					1-week-old seeds	more photosynthetic efficiency		5 seeds x treatment = 25 seeds
				substratum type	sand, pebble, s+p, fibreglass	2 months					fibreglass, pebble and s+p	100%, 95% and 90%		8 seeds x treatment = 24 seeds
5	First evidence of root morphological and architectural variations in young Posidonia oceanica plants colonizing different substrate typologies	2015	Balestri	root morphology in substratum type	sand vs rock with algae	5 years	1 month	2004	Livorno, Italy	Summer	substratum affect root distribution and depth, but do not affect root biomass and length complex	No differences in survival rates, just in the morphology	150 m2	100 seedlings x substratum = 200 seedlings
6	Assessing Tolerance to the Hydrodynamic Exposure of Posidonia oceanica Seedlings Anchored to Rocky Substrates	2022	Zenone	Hydrodynamics/current efect on rocky substratum	exposure to a wave and current flume	4 months	1 month	2016	Sicily, Italy	Summer	No seeds were damaged or detached, but no seedling anchored on LC tiles	100%	240 seedlings in 100cm2 tiles = 2,4 m2	240 seedlings
				substratum complexity	(LC), medium (MC) and high (HC) complexity t						HC	55% anchored		
7	Biological adhesion in seagrasses: The role of substrate roughness in Posidonia oceanica (L.) Delile seedling anchorage via adhesive root hairs	2020	Zenone	substratum roughness	0, 0.3, 3, 12, 18, 26, 52, 162 µm of roughness	6 months	15 days	2018	Sicily, Italy	Spring/Summer	anchorage aumemts with roughness, , seedlings anchored in 7 of 8 substratum types	87,50%	2 x 40L aquaria	72 (9 each roughness)
				anchorage strength							max force in 12 µm			
				root morphology in substratum type							more branched when in contact with substratum			
8	Posidonia oceanica and Cymodocea nodosa seedling tolerance to wave exposure	2011	Infantes	Hydrodynamics/current effect in P. oceanica and C. nodosa	species subjected to waves and currents in the flume and in the field (meadow and sandy bottom)	6 months	1-2 weeks	2009	Mallorca and Ibiza, Spain	Spring/Summer/winter	Anchorage depends on the roots force and and the forces to which the leaves are exposed to	Drag forces are higher in P. oceanica	the flume and the field (six plots, 3 for each sp)	27 (flume); 144 (field)
9	The perfect microsite: How to maximize Posidonia oceanica seedling settlement success for restoration purposes using ecological knowledge	2020	Alagna	substratum complexity	Holder type: cube, crevice, star,tile	6 months	1 month	2018	Sicily and Naples, Italy	Spring/Summer	high complexity helps seedlings establish, no anchorage occured in tile (the lesss complex holder)	100%	2 tanks 1m3	25 seedlings x treatment = 100 seedlings
				substratum slope	2 holders with 90º and 2 holder with 135-225º						do not affect anchorage sucess			
10	Survival and growth of transplanted and natural seedlings of Posidonia oceanica (L.) Delile in a damaged coastal area	1998	Balestri	substratum type	"matte" vs pebbles without vegetation	3 years	Seedlings found on the field were 1 month old	1994	Livorno, Italy	All year	matte	70%	12 units (5x5 cm) = 300 cm2	5 seedling x unit = 60 seedlings
				herbivore exclosure	caging vs. fencing vs. unmanipulated control						do not affect survivorship	60-70%	18 units (5x5) = 450 cm2, 6 cages	5 seedling x unit = 90 seedlings

11	Substrate Type Influences the Structure of Epiphyte Communities and the Growth of Posidonia oceanica Seedlings	2021	De Battisti	epiphyte cover on leaves	internal vs external sides of the leaf	5 years	1 month	2004	Livorno, Italy	Summer	do not affect, both leaf sides are equally covered	100%	Laboratory	
				epiphyte cover on leaves between substratum							rock, encrusting algae	57%		5 seedling x treatment = 10 seedlings
				epiphyte cover on rhizomes between substratum							do not affect	34-38%		
				epiphyte cover on seedling growth							do not affect	there was no correlation		
12	Field transplantation of seagrass (Posidonia oceanica) seedlings: Effects of invasive algae and nutrients	2018	Pereda-Briones	sediment nutrients	control vs fertilized	6 months	11 months	2014	Palma de Mallorca, Spain	Spring	fertilized, nutrients enhance leaf development	do not affect seedlings mortality	12 x 9m2 plots = 108m2	9 seedling x plot = 108 seedlings
				Caulerpa cylindracea presence	present vs removed						present			
13	Growth and development of Posidonia oceanica seedlings treated with plant growth regulators: possible implications for meadow restoration	2003	Balestri & Bertini	seed germination stimulant (Sprintene)	control vs Sprintene	24 h	No culture time	2000	Livorno, Italy	Spring/Summer	do not affect	57-42%	Laboratory	40 seedlings x treatment = 80 seedlings
				hormone presence in root growth	control, Naftal 5ml/l, Naftal 10 ml/l, IBA 5mg/l, IBA 10 mg/l y 66 F 0.2 ml/l	1 h exposure, 10 months observation					Naftal 5 ml/l and IBA 10 mg/l	90-100% produced roots		10 seedlings x experiment = 60 seedlings
14	In situ survival and development of Posidonia oceanica (L.) Delile seedlings	1999	Piazzi	substratum type	dead matte, rock, gravel	2 years	No culture time, study carried out	1994	Livorno, Italy	All year	dead matte	69% on dead matte at 10 m	4 stations x substratum (2 each depth) = 12	25 seedlings x station = 300 seedlings
				depth	2m vs 10m						10m			
15	Assessing Posidonia oceanica Seedling Substrate Preference: An Experimental Determination of Seedling Anchorage Success in Rocky vs. Sandy Substrates	2015	Alagna	substratum firmness	sand vs rock	5 months		2010	Sicily, Italy	Summer/Autumn	rock	89%	24 boxes of 50x30x27 cm	6 boxes x level = 360 seeds
				substratum complexity	sand, rock low, rock medium, rock high						do not afffect anchorage, but seedling retention rate increased with complexity	100%		
16	Seagrass (Posidonia oceanica) seedlings in a high-CO2 world: from physiology to herbivory	2016	Hernán	effects of CO2	control vs high CO2 exposure	3 months	1 month	2013	Palma de Mallorca, Spain	Summer	do not affect seedlings mortality, but seedlings in high CO2 exposure have more size, seed biomass, photosynthetic activity, CO2 content and herbivore feeding (sea urchins)	7.6-8.4%	7 x 9L aquarium	17 seeds x aquarium=119 seeds
17	Effects of salinity on seed germination and early seedling growth of the Mediterranean seagrass Posidonia oceanica (L.) Delile	2013	Fernández-Torquemada	effect of salinity in seed germination	control (37), 40, 43, 36 and 49	2 weeks		2004	Alicante, Spain	Summer	control (37), but not significant	90% vs 63-80%	15 x 12 cm glass Petri	10 seeds x Petri glass = 150 seeds
				effect of salinity in seedlings growth	control (37), 25-51 separated by 2 units	50 days	2 weeks				control (37), more leaves, leaf length and rooth growth	90%	filter paper in Petri glass	10 seadlings x level = 140 seedlings
18	First evidence of a massive recruitment event in Posidonia oceanica: Spatial variation in first-year seedling abundance on a heterogeneous substrate	2008	Balestri & Lardicci	substratum type	sand vs rock	3 months	No culture time, seedlings grown on the field (1-year-old)	2003	Corsica, France	Summer	sand, but no significant	75-60%	80 quadrats (25x25 cm) per site (A and B)	site A: 35 sand, 45 rock; site B: 20 sand, 60 rock
				abundance per substratum type	sand vs rock						do not affect		15 quadrats (25x2	15 quadrats per substratum
				mortality and patch density	comparing sand patches						no significant diferences	30-70%	16 patches with 25x25 quadrats	16 patches with 25x25 quadrats
19	Seed photosynthesis enhances Posidonia oceanica seedling growth	2013	Celdrán & Marín	effect of photosynthesis on leaves and root growth	Plastic control (PC), full light illumination (FL), partial light illumination (PL) and full dark (FD)	3 months		2012	Múrcia, Spain	Spring/Summer	PC and FL, photosynthesis enhances leaves and growth		5 x 60 L aquarium	5 seedlings x treatment = 20 seedlings
				mobilization of nutrients and reserves							in FD more starch so there is less nutrients mobilization			

				effect of photosynthesis in seedling biomass							PC and FL, photosynthesis enhances seedling biomass			
20	Experimental Evaluation of the Restoration Capacity of a Fish-Farm Impacted Area with Posidonia oceanica (L.) Delile	2012	Domínguez	substratum type	dead matte vs meadow	1 year	2-3 months	2008	Hornillo Bay, Águilas, Spain	All year	dead matte	75%	3 areas of 140m2=420 m2	48 seedlings x area (24 dead matte/24 meadow) (24 above/24 below) interleaved = 144
				planting level	above vs below surface						do not affect	30-50% vs 25-30%		
21	Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae	2019	Pereda-Briones	effect of seawater warming	25ºC (control), 27ºC and 29ºC	3 month	4 month	2015	Palma de Mallorca, Spain	Summer/Autumn	25º (control), temperature decreases roots length and number of leaves	100%	40 x 20 L aquarium	4 replicates (80 seedlings) x treatment = 800 seedlings
				effect of presence of invasive algae	absence, C. cylindracea and L. lallemandii						do not affect, but they can palliate the negative effect of the temperature. In presence of L. lallemandii at 29º number of leaves show a stabilization			
22	Future warmer seas: increased stress and susceptibility to grazing in seedlings of a marine habitat-forming species	2017	Hernán	effect of seawater warming	25ºC (control), 27ºC and 29ºC	3 month	3 month	2013	Palma de Mallorca, Spain	Spring/Summer/Autumn	25º (control), temperature increases seedling mortality due to stress, decreases anchoring success and carbohydrate storage. In addition, higher palatability is seen, grazers favour seedlings with low content of fiber in its leaves.	94%	30 x 25 L aquarium	10 replicates (30 seedlings) x treatment = 900 seedlings
23	Influence of substrate and burial on the development of Posidonia oceanica: implications for restoration	2017	Guerrero-Meseguer	effect of culture (pre-experimental) substratum type	hard (glass slide) vs soft (sand)	4 months		2013	Ibiza, Spain	Spring/Summer	Sand, more root system development but a slower leaf development	70%	2 x 10L aquarium with plastic pots 9x9x3 cm3	2 aquarium with 30 seed each = 60 seeds
				effect of seed burial	nonburied, half-buried and full-buried	4 months	1 month				do not affect	100%	6 x 10L aquaria	6 aquaria with 10 seedling each = 60 seedlings
				legacy of culture substratum type	hard (glass slide) vs soft (sand)						sand, more leaves growth but the same roots growth	4 times greater growth rate		
24	Esperimenti di coltura de semi di Posidonia oceanica in acquario	1997	Bedini	effect of direct sunlight	exposure to direct sunlight during 1 day vs 3 days	1/3 days		1994	Livorno, Italy	Spring/Summer	1 day, more exposure causes irreparable damage	89%		34 seedlings
				effect of temperature	4 ºC	15 days					all seedlings died after 15 days	0%	laboratory refrigerator	10 seedlings
				effect of light	80% natural light vs 10% natural light	1 year					80% natural light, after 3 months all seedlings in 10% natural light died	70%	2 x 60L aquaria	160 seedlings
25	Poor success of seagrass Posidonia oceanica transplanting in a meadow disturbed by power line burial	2021	Castejón-Silvo y Terrados	substratum type after a power line burial	sand vs burlap bags with coarse gravel	4 years	2 months	2015	Palma de Mallorca, Spain	All year	gravel, seedling survivorship after 1 month is very bad. After 6 months there is no survivorship in sand and 15% in gravel	<15%	18 plot 625 cm2	3 plot with 25 seedlings at 3 depth (15, 20 and 25 m) = 450 seedlings

26	Thermo-priming increases heat-stress tolerance in seedlings of the Mediterranean seagrass P. oceanica	2022	Pazzaglia	effects of thermo-printing treatment	control (C), primed (P) and non-primed (NP). P seedlings are exposed to 11 days at 30.5ºC and an extreme temperature of 32ºC for 2 weeks, and NP just to the extreme conditions	53 days	2 months	2019	Sicily, Italy/Múrcia, Spain	Summer	P showed bigger growth rates an more stock of pigments	27% higher growth rates in P and only 16% pigment reduction	pots of 5x5x6 cm	3 tanks x treatment = 9 tanks
27	Managing biotic interactions during early seagrass life stages to improve seed-based restoration	2021	Balestri	effects of seed density in culture	low (1 seed/mesocosm), medium (2 seeds/mesocosm) and high (4 seeds/mesocosm)	2 years	15 days	2018	Livorno, Italy	All year	do not affect, but medium and high density with C. nodosa grow better	91-97%	circular pots 45 cm diameter and 5 cm deep	12 replicates x level
				effects of Cymodocea nodosa	presence vs absence							63-92%		