Dannie O’Brien

Improving Seagrass Production for Transplants

Micropropagation, adventitious root development, and artificial substrates

Master in Marine and Coastal Sciences
Work performed under the supervision of:
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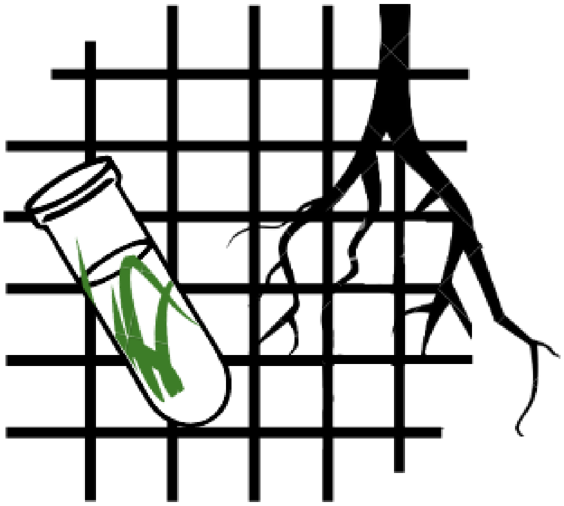

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Declaração de Autoria de Trabalho

Declaration of authorship of work

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

Dannie O'Brien

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Abstract

Degradation of seagrass meadows is countered by restoration to secure the important ecosystem services they provide. Restoration outcomes are highly variable, and there are many failures. Restoration methods frequently require harvesting from remnant meadows, with associated ecological risks. In the temperate North Atlantic, *Zostera marina*, *Cymodocea nodosa*, and *Zostera noltii* are the most important seagrass species, and targets for restoration. I tested different transplant approaches to understand and improve seagrass restoration initiatives for these species.

Zostera shoots were treated with 0.5 % NaOCl and successfully initiated into the *in vitro* growth environment for micropropagation. *Z. noltii* was maintained on enriched seawater media for up to 60 days, but *Z. marina* survived only 22 days. Mortality was likely related to yeast-like contamination. Cytokinin 6-benzylaminopurine (BAP) in five concentrations between 0.001 mg/L and 3 mg/L did not enhance shoot development in either species, but did maintain health.

To assess the impact of transplantation on root development, health, and survival, seagrass shoots were transplanted with roots either removed or intact. This did not affect survival or health. Both *Zostera* species regrew adventitious root length and mass in only three weeks, but regrowth was almost completely absent in *C. nodosa*. Auxin Indole-3-butyric (IBA) did not enhance adventitious root development, but rather inhibited root development above 5 mg/L.

Two tested seagrass transplant methods, sod and textile substrate, were equally successful and shoots demonstrated similar survival, height, leaf turnover and relative photosynthetic capacity.

Sod methods, while popular and successful, are not possible without large donor populations, and their harvest threatens remnant meadows. The successful initiation of *Zostera* spp. provides high potential to micropropagate shoots for future restoration. Non-rooted shoots can survive transplantation and rapidly establish roots, and these can be integrated with textile substrates to create artificial planting units with similar restoration outcomes to sods.

Keywords*:* Marine coastal systems, *Zostera marina, Zostera noltii*, *Cymodocea nodosa*, restoration, transplant, micropropagation, *in vitro* propagation, root development, artificial substrate.

Resumo

A degradação das pradarias de ervas marinhas é contrariada pela restauração de ecossistemas para garantir os importantes serviços ecossistémicos que elas fornecem. Os resultados da restauração são altamente variáveis e existem diversas falhas associadas. Os métodos de restauração requerem frequentemente a colheita de pradarias remanescentes com os derivados riscos ecológicos. No Atlântico Norte temperado, as espécies mais importantes e alvo de restauração são a *Zostera marina*, a *Cymodocea nodosa*, e a *Zostera noltii*. Nesta tese, foram testadas diferentes abordagens de transplantação para entender e melhorar iniciativas de restauração de ervas marinhas.

Rebentos de Zostera spp. foram tratados com 0.5% NaOCI e iniciados com sucesso no ambiente de tratamento in vitro para micropropagação. *Z. noltii* pôde ser mantido em meios de água salgada enriquecida até 60 dias, enquanto que a *Z. marina* sobreviveu apenas 22 dias. Está mortalidade poderá estar relacionada com a contaminação por parte de organismos não-alvo de “tipo fermento”. Tentativas para estimular o crescimento de plantas através do teste de 6-benzylaminopurine (BAP) em cinco concentrações entre 0.001 mg/L e 3 mg/L não estimularam o desenvolvimento de rebentos em nenhuma das espécies. Porém, a sua saúde foi mantida.

Para avaliar o impacto da transplantação no desenvolvimento das raízes, saúde da planta e sobrevivência, rebentos das três espécies foram transplantados com raízes removidas ou intactas, o que não afectou a sua sobrevivência ou saúde. Onde espécies de Zostera regeneraram comprimento e massa de raízes adventícias, a regeneração foi completamente ausente em C. nodosa. O factor de crescimento Indole-3-butyric (IBA) não aumentou o desenvolvimento de raízes adventícias, mas antes inibiu o desenvolvimento de raízes acima de 5 mg/L.

Dois métodos de transplante, unidades de tipo “sod” e unidades de substrato têxtil, demonstraram resultados semelhantes de sobrevivência, peso, substituição de folhas e capacidade fotossintética relativa para as espécies testadas. O sucesso de futuros projetos de restauração é determinado, tanto pelo desempenho aquando de trabalho de campo, como também pelo seu custo e mão de obra associada.

A bem-sucedida iniciação de Zostera spp. fornece alto potencial para micro-propagar rebentos para futura restauração. Rebentos sem raízes podem sobreviver à transplantação e rapidamente estabelecer raízes, e estes podem ser integrados com substratos têxteis para criar unidades de plantação artificiais com resultados de restauração para sods similares.

Palavras-chave: Sistemas Marinhos e Costeiros, *Zostera marina, Zostera noltii*, *Cymodocea nodosa,* trabalho de restauração, transplante, micropropagação, *in vitro* propagação, desenvolvimento de raízes, substratos artificiais.

Sumário

Melhorar a produção de ervas marinhas para transplantes:

Micropropagação, desenvolvimento de raízes adventícias e substratos artificiais.

Introdução

Uma vez danificadas, pradarias de ervas marinhas são lentas a recuperar passivamente (Park et al., 2009). Uma restauração activa pretende substituir valores de ecossistema perdidos (Bayraktarov et al., 2015), aumentar a biodiversidade (Lefcheck et al., 2017), providenciar habitat para espécies ameaçadas (van Katwijk, et al., 2016) e aumentar os stocks comerciais de peixe (Blandon & zu Ermgassen, 2014). Adicionalmente, há um crescente interesse na criação de novas pradarias de ervas marinhas tanto para compensação de habitat (Ganassin & Gibbs, 2008) como para alternativa a soluções tradicionais de engenharia costeira para estabilização de zonas costeiras limítrofes (James et al., 2019), uma vez que as pradarias de ervas marinhas atenuam a altura das ondas, reduzem a velocidade de correntes e aumentam a sedimentação (Koch, et al., 2006; Christianen et al., 2013; Reidenbach & Thomas, 2018). Pradarias de ervas marinhas são cada vez mais vistas como uma valiosa ferramenta para mitigar a mudança climática devido ao seu alto potencial de sequestro de CO_2 (Duarte et al., 2013), actuando como áreas de sequestros de carbono altamente eficazes, tanto dentro da pradaria como nos sedimentos adjacentes (Duarte & Krause-Jensen, 2017).

Tem havido uma grande variabilidade nas taxas de sucesso de restauração de pradarias marinhas e projectos de criação (Cunha, et al., 2012; van Katwijk et al., 2016), e o sucesso a largo prazo pode ser difícil de medir devido à limitação dos programas de monotorização após a conclusão dos projectos.

As técnicas de restauração são variadas, mas normalmente dependem na transplantação de material doador de pradarias existentes. Uma variedade de métodos de transplante de material doador pode ser utilizado. Métodos de género “*plug*” ou “*sod*” têm frequentemente atingido melhores resultados a longo prazo do que outros métodos (Paling, et al., 2001; Christensen et al., 2004; Fishman, et al., 2004; Suykerbuyk et al., 2016) mas dependem de camadas de ervas marinhas naturais abundantes e adequadas para providencial material doador para transplante.

Reduzir a dependência de pradarias naturais como doadoras deveria ser uma alta prioridade, já que a colheita pode expor o local doador a perturbação e degradação de valores existentes (Harris, 2012; Statton et al., 2015). A urgência para restaurar áreas suporta uma abordagem heurística em muitos casos, mas também é importante entender porque há um crescente sucesso com estes métodos e não com outros, pois limitações de tempo e orçamentos (Bayraktarov et al., 2015) e crescente reconhecimento dos valores de pradarias de ervas marinhas (Nordlund et al., 2016) exigem o melhor resultado possível para qualquer projecto. Este projecto tem como objectivo experimentar uma variedade de métodos de propagação de erva marinha, tanto in vitro com em ambientes protegidos mesocosm de forma a identificar alguns dos factores que podem ser realçadores ou limitativos para a sobrevivência de rebentos de ervas marinhas transplantados durante actividades de restauração.

Objectivos do projecto

Esta tese pretendeu testar uma nova abordagem concebida para aumentar o sucesso de transplantes de ervas marinhas. Os objectivos específicos foram utilizar a espécies modelo *Z. noltii, Z. marina* e *C. nodosa* em três investigações alvo:

1. Investigar a adequação de técnicas de micropropagação in vitro simples, rápidas e acessíveis para propagar material de transplante de *Zostera marina* e *Zostera noltii*.
2. Avaliar o grau de crescimento de novas raízes adventícias em rebentos de *Zostera marina, Zostera noltii* e *Cymodocca nodosa* depois da remoção de raízes e aferir o efeito da hormona de crescimento IBA nos parâmetros de crescimento.

3. Avaliar a sobrevivência e crescimento de *Zostera marina, Zostera noltii*, e *Cymodocca nodosa* transplantada em dois substratos têxteis (malha grossa e malha fina) comparada com rebentos um unidades de transplante *"sod"*.

Métodos

Foram recolhidos rebentos de erva marinha utilizando mergulho de garrafa de uma pradaria doadora numa lagoa costeira da Ria Formosa perto da ilha da Culatra, de onde material doador para transplante foi previamente recolhido para actividades de restauração (Paulo et al., 2019). O material for recolhido como "*sods*" (20 cm x 20 cm x 5-8 cm depth) no seu sedimento natural utilizando tubos plásticos e uma pá. Estes foram transferidos por barco em tubos com água para o centro de pesquisa do Ramalhete (demorando cerca de 30 minutos) e imediatamente colocados em grandes mesocosms exteriores onde foram fornecidos com água marinha corrente filtrada à temperatura ambiente e salinidade.

Rebentos saudáveis de *Z. marina* e *Z. nolti* foram seleccionados e foram iniciados no ambiente *in vitro* após esterilização com NaCIO para avaliar a sua resposta à fase-1 do protocolo de micropropagação standar. Foram feitos meios de crescimento utilizando água marinha e Murashige & Skoog Basal Salt Mixture (MS) (1962; Sigma- Aldrich; Appendix 2), açucar e factor de crescimento 6-benzylaminopurine (BAP) em concentrações de 0.001, 0.01, 0.1 e 1 mg/L. Os "iniciados" foram então mantidos numa camara de crescimento de plantas climatizada a 18°C sob luz branca num ciclo luz:escuridão 12:12. O objectivo foi de alcançar culturas axênicas de *Z. marina* e *Z. noltii*, estabelecer estas em *in vitro* por um período de tempo (vários meses) e começar a desenvolver um protocolo para multiplicação de folhas, caule órgãos rizomas por acrescento de BAP ao meio de crescimento.

Para aferir o impacto do dano de raízes e regeneração de raízes adventícias, foram seleccionados rebentos de material recolhido e replantados em mesocosms experimentais. O seu crescimento foi monitorizado para aferir os valores de crescimento sobre e abaixo do solo. Os rebentos de erva marinha tiveram todas as suas raízes removidas com um bisturi o mais próximo do rizoma quanto possível e foram tratados com 0, 3, 5, ou 10 mg/L de Indole-3-butyric acid (IBA). Após 14-60 dias os valores de crescimento dos diferentes tratamentos foram comparados entre eles e com plantas que não tiveram as suas raízes removidas. Foram comparadas taxas de mortalidade utilizando Pearson's Chi-squared tests e as outras medições utilizando PERMANOVA em Primer+ programas

Rebentos de erva marinha foram inseridos numa malha fina de 15 cm x 15 cm e numa malha grossa têxtil tipo serapilheira para criar unidades de plantação para serem comparadas com "*sod*s" de 15 cm x 15 cm. Estas foram crescidas durante 5 - 8 semanas em mesocosms. Medições de crescimento e fotossíntese (Pulse Amplitude Modulated (PAM) fluorometry) foram tiradas a cada 1 semana (*Z. noltii*) ou 2 semanas (*Z. marina e C. nodosa*) e comparadas usando PERMANOVA.

Resultados

Z. noltii foi iniciada com sucesso no ambiente de micropropagação *in vitro* após esterilização com 0.5 % NaClO por 1 minuto. Pode ser mantida em meios de água marinha enriquecida por um máximo de 60 dias, enquanto que Z. marina sobreviveu apenas 22 dias. Mortes foram provavelmente relacionadas com contaminação por organismos não alvo de tipo fermento. Tentativas de estimular o crescimento de plantas através do teste de 6-benzylaminopurine (BAP) em 5 concentrações entre 0.001 mg/L e 3 mg/L não aumentaram o desenvolvimento de rebentos em nenhuma das espécies mas mantiveram saúde.

Para as experiências de crescimento de raízes adventícias, as espécies *Zostera* regeneraram comprimento de raízes e massa em apenas 3 semanas, mas a regeneração de raízes foi quase totalmente ausente em *C. nodosa*. Factor de crescimento Indole-3-butyric (IBA) não aumentou o desenvolvimento de raízes adventícias em nenhuma das espécies, mas antes inibiu o crescimento de raízes acima de 5 mg/L e aparentemente inibiu o crescimento de folhas acima de 10 mg/L.

Para a experiência de transplante de substrato artificial, a sobrevivência de rebentos não foi uniforme ente os tratamentos (χ2 $P < 0.03$) para todas as espécies. Para *Z. marina* a taxa total de mortalidade foi de 28% para todos os tratamentos e foi maior do que esperado no substrato fino artificial e mais baixa do que esperado no tratamento de transplante "*sod*".

Para *C. nodosa* a taxa total de mortalidade foi de 7% consistindo na morte de 8 de 11 rebentos num único mesocosm de tratamento "*sod*". Para *Z. noltii*, a taxa de mortalidade entre todos os tratamentos foi de 23% e todos em mesocosms impactados por herbivoria. Uma herbivoria não identificada removeu todas as folhas dos rebentos 4-5cm acima da superfície de sedimento. Observou-se que estes rebentos estavam danificados e observou-se que muitos mais tarde morreram durante o período experimental. Outros parâmetros de crescimento e saúde variaram sobretudo temporalmente e houve pouca diferença entre tratamento de substratos (ver números 3.3.i, ii e iii).

Discussão

Era espectável que todas as espécies respondessem de forma diferente à transplantação uma vez que têm diferentes morfologias, história de vida, estratégia principal reprodutiva, faixa biológica e requisitos de habitat (Borum & Greve, 2004; Ondiviela et al., 2014). Comparando sods a substratos artificiais nas tentativas de transplantação, as taxas de mortalidade de rebentos eram difíceis de relacionar com o tratamento como outros factores como um evento herbívoro para *Z. noltii*. Diferenças de espécies foram encontradas; *Z. noltii* era apenas adequada em têxtil fino e transplantes tipo sod porque cai fora da malha grossa e rebentos de *Z. marina* danificam-se quando inseridos na malha grande, mas o uso de substratos artificiais, em si mesmo, não deu pior saúde ou resultados de mortalidade do que os sods.

Uma vez que foi agora demonstrado que a danificação de raízes e stress de transplante dos diferentes métodos não cria variações entre estes eles, outras características de técnicas de sod – tamanho do grão de sedimento, nutriente e microbioma associado com estes rebentos de sedimentos com eles transferido - requer investigação adicional. Outros estudos pretenderam testar a integridade abaixo do solo como um factor plantando raízes simples vs. transplantes de sod no campo (Suykerbuyk et al., 2016), mas ao não considerar que sítios alvo de transplantes têm frequentemente características de sedimento muito diferentes do sítio doador, e como tal dos sods, eles conseguiram apenas demonstrar que ancoragem dos rebentos é fundamental. Agora, a capacidade destas espécies de sobreviver e recuperar de danificação de raízes até completa remoção está confirmada. Este factor pode também ser eliminado como uma possível vantagem de métodos sod. Adicionalmente, demonstrei que o stress de inserir rebentos em têxteis, por sim mesmo, não conduz a taxas de sobrevivência ou de saúde mais baixas do que utilizando métodos sod, desde que o tamanho correcto da malha para a morfologia de espécie tenha sido seleccionado.

Enquanto que o desempenho possa variar em ambiente aberto, esta experiência demonstrou que onde os factores tipo de sedimento, componentes biológicos e orgânicos associados, exposição e população doadora forem controlados, rebentos transplantados dão-se bem e recuperam bem do stress de transplante em unidades de substrato artificial e em unidades sod. O próximo passo para criar unidades artificiais de plantas bem-sucedidas é compreender a contribuição destes factores para a sobrevivência do transplante. Isto ajudará a estabelecer se unidades de plantas artificiais podem vir a ser uma alternativa adequada a transplantes de sod onde métodos de plantação sod foram bem-sucedidos. Investigação adicional é necessária para aferir a viabilidade de usar unidades de plantas artificiais em vez de métodos sod, que podem não ser alcançáveis onde camas doadoras desadequadas estão disponíveis ou podem ser ameaçadas por colheita.

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1- Introduction

Once damaged or destroyed, seagrass meadows are slow to recover passively (Park *et al.*, 2009). Active restoration intends to replace lost ecosystem service values (Bayraktarov *et al.*, 2015), increase biodiversity (Lefcheck *et al.*, 2017), provide habitat for threatened species (van Katwijk *et al.*, 2016), and support commercial fish stocks (Blandon & zu Ermgassen, 2014). Additionally, there is an increasing interest in the creation of new seagrass meadows both for habitat compensation (Ganassin & Gibbs, 2008), or as an alternative to traditional coastal engineering solutions for foreshore stabilisation (James *et al.*, 2019), as seagrass meadows attenuate wave height, reduce current velocity, and increase sedimentation (Koch *et al.*, 2006; Christianen *et al.*, 2013; Reidenbach & Thomas, 2018). Seagrass meadows are increasingly valued as a tool to mitigate climate change due to their highly productive CO_2 sequestration potential (Duarte *et al.*, 2013), acting as highly effective carbon sinks both within meadow and in adjacent sediments (Duarte & Krause-Jensen, 2017).

There has been large variability in the success outcomes of seagrass meadow restoration and creation projects (Cunha *et al.*, 2012; van Katwijk *et al.*, 2016), and success can be difficult to measure in the long term due to limited monitoring programs after projects are completed. Restoration techniques are varied, but usually rely on transplanting donor material from existent meadows. A variety of methods of transplanting donor material can be used. Plug or sod type methods have often achieved better medium to long-term results than other methods (Paling *et al.*, 2001; Christensen *et al.*, 2004; Fishman *et al.*, 2004; Suykerbuyk *et al.*, 2016) but rely on plentiful and suitable natural seagrass beds to provide donor material for transplanting. Reducing reliance on natural meadows as donors should be a high priority, as harvesting can expose the donor site to disturbance and to degradation of existing values (Harris, 2012; Statton *et al.*, 2015). The urgency to restore areas supports a heuristic approach in many cases, but it is also important to understand why there is increased success with these methods than with others, as time and budget limitations (Bayraktarov *et al.*, 2015) and increasing recognition of seagrass meadow values (Nordlund *et al.*, 2016) demand the best possible outcomes for any project. This project aimed to trial a variety of seagrass propagation methods both *in vitro* and in protected mesocosm environments to identify some of the factors that may be enhancing or limiting the survival of translocated seagrass shoots during restoration activities.

1.1- Seagrasses

Seagrasses are an ecological grouping of 6 families of approximately 72 species of flowering plants (Short *et al.*, 2011) which complete their entire lifecycle in shallow marine environments (Kuo & den Hartog, 2001) to a depth where 10 % of surface light still reaches the bottom. They are found worldwide in coastal areas except for Antarctica, and the greatest diversity of species is generally associated with tropical regions (Short *et al.*, 2011). They form complex and highly productive meadows and interact with and support a broad variety of other organisms, forming the primary carbon source in many food webs, and acting as benthos or protection for a variety of other organisms (Orth *et al.*, 2006). Though they inhabit only 0.1 % to 0.2 % of the global ocean (Duarte, 2002), seagrass meadows are ecologically, economically, and socially important (Cullen-Unsworth *et al.*, 2014). They supply valuable ecosystem services (Nordlund, *et al.*, 2016; 2018), but are still an overlooked and under-researched habitat in many regions (van Katwijk *et al.*, 2016). Provisioning, regulating, and supporting services are both diverse and numerous (Nordlund *et al.*, 2016), and include coastal protection (James *et al.*, 2019), habitat for commercial species (Tuya, Haroun & Espino*,* 2014), food and habitat for threatened species (Hughes *et al.*, 2009), carbon storage (Duarte *et al.*, 2013), reduction of water pathogen load (Lamb *et al.*, 2017), local buffering of pH (Hendriks *et al.*, 2014), and protecting other important coastal and marine habitat; such as coral reefs through water filtration services, and mangroves through wave attenuation (Reidenbach & Thomas, 2018).

Seagrass have specific depth, light, nutrient, sediment, and water velocity requirements (Greve & Binzer, 2004) that limit their growth to shallow coastal regions and coastal estuaries (Ralph *et al.*, 2006). This habitat requirement also corresponds closely with the aggregation of human activities. Human inputs have increased turbidity; and changed hydrological regimes and this has put increasing pressure on seagrasses, which has been linked with historic and potential future losses (Orth *et al.*, 2006; Short & Wyllie-Echeverria, 1996). Land reclamation or use of coastal areas for aquaculture is a major threat to seagrass habitat, and eutrophication has been linked to significant and large scale losses (Walker & McComb, 1992).

Natural losses of seagrass meadows have been attributed to storms, flooding, and hurricane events (Preen *et al.*, 1995); to plant diseases and pathogens (Ralph & Short, 2002); and to overgrazing by fish, turtles or marine mammals (Rose *et al.*, 1999; Fourqurean *et al.*, 2010). Due to their growth patterns and environmental stressors, decline and recovery of area and density are likely a natural dynamic of seagrass meadows over the scale of years to decades (Hemminga, 1998), but anthropic disturbance increases seagrass meadow vulnerability and has been increasingly linked to long term seagrass meadow losses (Hemminga, 1998). It has been suggested seagrasses should be considered “coastal canaries” or “biological sentinels” due to their sensitivity to anthropic pressures (Orth *et al.*, 2006; Short *et al.*, 2011) in coastal and marine environments. Societies have a significant stake in conserving and protecting seagrass meadows for environmental, ecological, and cultural values; and this is supported by increasing regional and local regulation on water quality and associated ecological values including seagrass protection, such as the Water Framework Directive in Europe (Borja, 2005).

1.2- Study target species

There are four species of seagrass found in Europe, and several species of halotolerant aquatic plants (Borum & Greve, 2004). Three of these species, those found on the south coast of Portugal, will be included in this study. Target species for this study will be collected from existent populations in the Ria Formosa, a shallow mesotidal lagoon on the south coast of Portugal, and from connected artificial ponds, usually remnants from past salt pan or fish farm production. The Ria Formosa is home to three of the four main European seagrass species and an active research community which has studied their distribution, requirements, productivity, and potential threats.

Zostera marina is located subtidally to approximately 15 meters depth in cool to temperate waters globally. In Europe, *Z. marina* is distributed from Icelandic waters to southern Portugal, with only very sparse distribution in the Mediterranean (Borum & Greve, 2004). *Z.*

marina populations severely declined due to eelgrass wasting disease in the 1930s, with losses of up to 90 % across the Atlantic (Ralph & Short, 2002). The disease was discovered to be caused by a slime mould, *Labyrinthula zosterae* only in the late 1980s (Muehlstein, Porter & Short, 1991). There are still known occurrences in the present day, but not at the scale of loss as the events of the early decades of the 1900s. Many locations have not had any recordable natural recovery of lost meadow area (e.g. Godet *et al.*, 2008), due to low reproduction, slow growth rates, and increasing anthropic pressures such as reduced water quality. Additionally, meadow populations may have crossed a minimum threshold below which their ability to alter the biological and physical environment to their advantage is compromised (Suykerbuyk *et al.*, 2016), preventing their re-establishment in areas they previously inhabited (Moksnes *et al.*, 2018).

The population of *Z. marina* in the Ria Formosa is seen as a potential source of donor material for revegetation of *Z. marina* in other areas both locally (e.g. 'Biomares' project (Cunha *et al.*, 2011)) and for other locations in Europe (e.g., 'CoastBusters' project, Belgium (Sterckx *et al.*, 2019)). As part of the 'Biomares' restoration project, the total estimated *Z. marina* distribution in the Ria Formosa was calculated at 5.01 ha in 2009 (Cunha, Assis & Serrão, 2009) and its potential suitability as a donor population was assessed. There is a concern that *Z. marina* may face local extinction in Portugal if measures are not taken to assure the protection of remaining meadows, and there have been attempts to revegetate areas that were previously known to have meadows on the Portuguese coast (Cunha, Assis & Serrão, 2013). *Z. marina* produces large numbers of seeds, but these have small dispersal ranges and are highly predated in natural conditions (Marbà *et al.*, 2004). Collection of seeds is relatively simple, but experimental propagation of seeds suffered large hurdles due to very low germination rates (Cabaço, Ferreira & Santos, 2010), so it was decided for past restoration activities that shoots from donor sites, rather than seed, would be required for restoration activities.

Zostera noltii is a small fine leaved intertidal species associated with muddy sediments in much of temperate Europe (Kuo & den Hartog, 2001). It is distributed from southern Norway to the European coastline of the Mediterranean (Borum & Greve, 2004). Globally it is not considered to be a threatened species due to its large range, but North Atlantic populations are currently declining (Short *et al.*, 2011). Due to its favored usage of the intertidal zone, it may be locally vulnerable in areas where there are aquaculture and wild harvest activities (Guimarães *et al.*, 2012), or by development and land reclamation activities (Reise, 2005), or in areas where erosion increases bank slope, reducing the area of favorable substrates.

Z. noltii is well established in the Ria Formosa, where it is intertidal. In 2012, it was observed covering 45 % of the intertidal zone, but it is potentially locally threatened by clam farming activities (Guimarães *et al.*, 2012). *Z. noltii* has also declined in some areas of Portugal, for example, the Mondego estuary experienced massive losses in the 1980s and 1990s related to anthropic eutrophication and bait collection disturbances (Martins *et al.*, 2005). *Z. noltii* has frequent flowering and large seed production, but low germination rates, germling survival, and seedling survival (Alexandre *et al.*, 2006) suggest this does not equate to high fertility. Clonal growth, via rhizome extension, is known to be an important mechanism within patches of seagrass, and *Z. noltii* has a rapid rhizome growth rate compared to other European seagrasses (Marbà *et al.*, 2004). There is also evidence that clonal fragments can be transported by currents and survive to establish elsewhere (Berković *et al.*, 2014). It is hoped that this species is also a suitable candidate for vegetative reproduction methods.

Cymodocea nodosa is a warm temperate subtidal species found in the Mediterranean and in the Atlantic but has its northern limit in Southern Portugal (Cunha, Assis & Serrão, 2013) where the population has very low genetic variability (Alberto, Mata, & Santos, 2001). It grows in both monospecific and mixed (with *Z. marina*) meadows in the Ria Formosa (Billingham *et al.*, 2003). Its global status is considered to be stable (Short *et al.*, 2011), but again local pressures can threaten specific populations, and local losses will impact the provision of valued ecosystem services.

1.3- Restoration and creation of seagrass meadows

Complex ecosystems are very difficult to create or restore, and marine ecosystems have the further challenge of being less accessible and more highly dynamic than terrestrial ecosystems, and impacted by unresolved stressors (Moberg & Ronnbacka, 2003). Historic success rates of published seagrass restoration projects worldwide is less than 38 %, actual success is likely much lower as project failures are less likely to be published (Bayraktarov *et al.*, 2016). In many published cases, success was defined simply by the presence of seagrass biomass during the monitoring period, and restoration of complex factors such as biodiversity values, resource provision, or nutrient and gas regulation services were not assessed (Bayraktarov *et al.*, 2016).

Possible methods for seagrass restoration are diverse and include seed, seedling, and vegetative transplants. Planting units range from single seeds up to frames or sod planting units of several meters. Larger unit sizes are currently considered better practice due to historic failures of smaller units (van Katwijk *et al.*, 2016) may help with anchoring against hydrological disturbance (Paling *et al.*, 2001; Paulo *et al.*, 2019). Use of sod units also reduces disturbance of the root mass during transplant, which likely reduces some stress to the plants. The rhizosphere microbiome of seagrass meadows is also very relevant to seagrass health (Ugarelli *et al.*, 2017). Bacteria involved in sulfur cycling and regulation are particularly important (Cúcio *et al.*, 2016), and transfer of this material with the seagrass may assist with adaptation to the new environment, where favorable sediment texture and biological systems may not already be present. Unit size may also be particularly important when planting transplants in to anoxic sediment conditions, as higher photosynthetic biomass may help transplants better adapt to the anoxic conditions (Leschen, Ford & Evans, 2010).

Usually during restoration plant-out, when planting single shoots or sprigs, they are anchored during transplant in some way (e.g. BMT Oceanica Pty. Ltd., 2013), as it is not expected that individual plants in unconsolidated sediment in marine environments will remain undisturbed by even daily hydrology, let alone storm events. Most published methods require some technique to secure the seagrass to the sediment; either metal or bamboo stakes, stones, weighted frames, or burial within *in situ* sediments; to prevent loss of the plants to currents. Sods and plugs maintain the existing root and rhizome structure (Kuo & den Hartog, 2006) from historic growth which is preserved during relocation. Even if this is not live material associated with the transferred shoots, this organic structure may provide support for the shoots and may assist to prevent disturbance and removal (Paling *et al.*, 2001) before new roots and rhizomes grow. This can eliminate the need to introduce plastic or metal materials to the restoration area and reduce plant out time. Despite many advantages, this method also has associated risks which highlight the importance of continuing to refine techniques, as discussed below.

1.4- Risks associated with use of donor vegetative material for restoration

Where a full or partial seagrass meadow would be destroyed by an allowed development in a coastal area, it is vitally important that the maximal amount of meadow material should be salvaged and re-located as mitigation for this loss (Lewis, 1987). Sod methods may be the most appropriate method in this case, as this will also provide refuge for associated epiphytes and minimize damage to the transferred material. But low success rates in translocation of material globally brings the legitimacy of taking material from healthy and non-threatened meadows completely into doubt (Cunha *et al.*, 2012). Instead, translocation of material should be used very sparingly as a restoration technique to ensure there is no net loss of seagrass area in the frequent case where transplantation fails (Wear, 2006).

Quantity, type, and collection methods of material for these activities should be carefully assessed. As the expansion of seagrass area is known to be very low (Cunha, Duarte & Krause-Jensen, 2004), bare areas created by sod removal are likely to infill very slowly, or not at all. The method should not be considered suitable in cases where we do not understand the long-term impact of this disturbance and in some areas, recovery is not assured. While patchiness of meadows does not imply lower value (Fonseca, Kenworthy & Thayer, 1998), changing structure and disturbance can be a threat to existing values. There is a growing body of knowledge regarding the impacts of bed fragmentation and edge effects on resident fauna of seagrass meadows, including commercially important species (Blandon & zu Ermgassen, 2014), the sediment microbiome (Ettinger *et al.*, 2017), and carbon storage capacity (Ricart *et al.*, 2015), but the importance of spatial parameters to seagrass meadows themselves is less well investigated. Removing material from donor meadows may introduce detrimental edge effects, such as increased herbivory (Statton *et al.*, 2015) and increasing exposure to increased turbidity and water velocity (Harris, 2012). Fragmentation of donor beds may create niches for fast growing species, increasing the competitive advantage of both local algae (Cullen-Unsworth & Unsworth, 2016) or invasive species, for example, *Caulerpa taxifolia* and *Codium fragile ssp. tomentosoides* (Williams, 2007), which may then outcompete seagrasses and form algae beds. Transporting of invasive species to new locations could also occur if transplanted units are not carefully checked.

To help seagrass plants withstand hydrodynamic forces, and to stabilize sediments, structural support of below-ground biomass is important for transplant success, even with sub-optimal functional capacity. Where rooting cannot be increased sufficiently to create short-term resilience, particularly over the first winter storm period after transplantation, an artificial substrate that mimics the structural complexity of root-mats may be a useful substitute and may assist to direct root growth to create an integrated root network. There is a risk that insertion into these textiles may damage plants or increase their exposure, leading to photosynthetic stress. As in terrestrial and aquatic restoration, textile products may be used during establishment of replanted meadows while plants have low root length/biomass. This can reduce the need for sod transplants by replicating structural conditions, if the shoots are not adversely impacted by this method of transplantation.

1.5- Where to restore

Seagrass re-vegetation has the further complexity in that larger seagrass species, such as *Z. marina,* strongly influence the biological and physical environment around them as ecosystem engineers, and thus are particularly vulnerable to ecological tipping points that prevent their survival or re-establishment in areas previously inhabited (Moksnes *et al.*, 2018). It is now considered that seagrass meadows are ecosystems which, due to internal feedbacks, can be quite resilient to changes in the environment, until a critical threshold is reached, after which there is rapid change (Suykerbuyk *et al.*, 2016). Rather than a gradual transitional alteration from healthy meadow to degraded, there may be trigger points which induce a complete system shift (Suding & Hobbs, 2009) to bare sand, or environments dominated by other producers.

This has very important implications for preservation and for restoration or creation of meadows (van Der Heide *et al.*, 2007). Where historic seagrass meadows have been completely removed, sites are often characterized by increased current velocities ad suspended sediment. It has additionally been demonstrated that seagrass meadows modify hydrodynamics structurally, which slows water velocities within them, leading to increased settling out of particles, increasing water clarity, and reducing bed sheer, preventing particle suspension (Reidenbach & Thomas, 2018). It has now been suggested that seagrass meadows may attenuate wave height above the meadow through drag induced friction (Koch *et al.*, 2006; Ondiviela *et al.*, 2014; Reidenbach & Thomas, 2018) with greater attenuation of wave height related to higher shoot density.

Correct selection of appropriate sites for revegetation are a primary concern for success, with water hydrodynamic velocities and sediment stability identified as the primary feedback characteristics that are both influenced by, and influence the survival of, seagrass meadows (Moksnes *et al.*, 2018). Additionally, individual seagrass plants appear to have low survival, with the requirement that these feedback mechanisms require a minimum number of plants or area of vegetated substrate. So, successful re-vegetation appears to require crossing what could be considered a minimum threshold for reintroduced individual shoots (van Katwijk *et al.*, 2016) , and the minimum unit size of a revegetation attempt should perhaps be considered to be a patch, rather than an individual, though the minimum patch size is not well established.

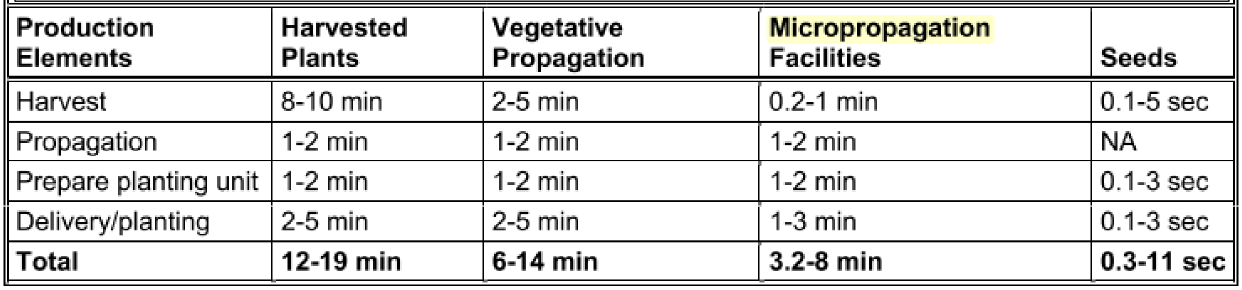
Assuming a site fits the physiochemical, hydrological, depth, and light availability conditions required, the success or failure of seagrass growth from natural recruitment, seeding, or shoot transplantation, will depend on the ability of these recruits to stabilize at the site and resist other natural and anthropic pressures on their continued survival, growth, and reproduction. Historic sites that had meadows, but are now non-vegetated sites may exist in a new environmental stable state characterised by an absence of seagrass, which needs to be accounted for in re-vegetation attempts (Van Der Heide *et al.*, 2007). Even in cases where the sources of seagrass loss have been removed or ameliorated, regeneration success is low in the medium to long term, and it has been suggested that this is due to plant out quantities being too small to overcome relevant tipping points, to restore positive feedback mechanisms, and to adequately spread risk (Suykerbuyk *et al.*, 2016; Paulo *et al.*, 2019). This highlights the need to ensure there are large quantities of material available for restoration efforts and presents the challenge of increasing the amount of material available without detriment to donor beds.

1.6- Propagation methods for seagrass donor material

Transplant material must come from a reproducible and viable sustainable source, so there is less disturbance of existing meadows. This source, be it seed, or clonal vegetative propagation should produce plants in large numbers, and with reasonable rapidity, good quality, and be cost effective. Even in wealthy regions with environmental rehabilitation budgets, the need to be cost effective is of primary importance when designing restoration/rehabilitation projects (Irving *et al.*, 2010). There is a growing body of literature on the high economic value of seagrass meadows (Cole & Moksnes, 2016), but cost effectiveness of methodologies will always remain an important factor in the scope of a restoration project. There are costs associated with each restoration method, and this is often a factor in whether projects can be begun at the scale required or can be continued, and whether they are widely supported (Floor, van Koppen & van Tatenhove 2018).

Time and labor requirements are a significant portion of the cost of restoration activities, and different methods have different costs. The time required to harvest large vegetative units is very large compared to other methods (Table 1.6.i) and has disadvantages as previously discussed. Seed methods have the lowest time requirement (Table 1.6.i), but low germination rates of seeds can lead to poor restoration outcomes, despite its relatively low cost and ease of methodology, and seedling planting historically perform the worst of any restoration method used (van Katwijk *et al.*, 2016). Seed injection methods (Orth *et al.*, 2009), embedding in bio-textiles, and broadcast distribution using buoy deployed seed head bags (Cullen-Unsworth & Unsworth, 2016) are methods employed try to increase this success. Vegetative propagation has the disadvantage of requiring lots of resources, as large tank areas and large volumes of seawater, as well as pumps and associated equipment, are needed to keep plants.

Table 1.6.i: Time Requirements for the production of submersed aquatic plants used in restoration projects



Production Elements	Harvested Plants	Vegetative Propagation	Micropropagation Facilities	Seeds
Harvest	8-10 min	2-5 min	0.2-1 min	0.1-5 sec
Propagation	1-2 min	1-2 min	1-2 min	NA
Prepare planting unit	1-2 min	1-2 min	1-2 min	0.1-3 sec
Delivery/planting	2-5 min	2-5 min	1-3 min	0.1-3 sec
Total	**12-19 min**	**6-14 min**	**3.2-8 min**	**0.3-11 sec**

Source: (Alistock & Shafer, 2006)

Plant tissue culture, specifically micropropagation, could offer an in-between solution, using vegetative production but at a much smaller biomass during multiplication of the new shoots allows a maximal number of plantlets to be created in a much smaller space. Plant tissue culture is a common propagation method in the fields of horticulture, agriculture, and biotechnology (Bhatia & Dahiya, 2015). It is an area of applied science that provides a broad platform for the aseptic culture of cells, tissues, organs, and their components under defined chemical and physical *in vitro* conditions. This science follows a basic concept in which the plant body or organ or any tissue can be dissected into smaller parts called “explants” and any explants can be further developed into a whole plant (Bhatia & Sharma, 2015). This concept led to the development of an effective technique called *in vitro* propagation. Costs involved in maintaining a lab and handling material can be reduced by having a simple but effective protocol and reducing the number of *in vitro* growth stages and can then be similar to vegetative production techniques and training of personnel can be simple and rapid.

1.7- *In vitro* micropropagation for clonal production of seagrass

Considering their serious decline, low recovery rates, and the need to stop "borrowing" from healthy meadows, there is an enormous demand for methods to successfully propagate *Zostera* spp. Micropropagation techniques, once protocols are established, can allow for rapid production of seagrass. Additional benefits of being able to select and screen donor shoots for their ability to survive in reduced light, have faster root growth, be less vulnerable to herbivory, or have better resistance to pollutants. *In vitro* propagation of *Z. marina and Z. noltii* may help to provide a rapid and effective method of cultivating donor shoots of these species from limited donor material (Alistock & Shafer, 2006). This can reduce collection effort required by restoration participants and limit donor bed disturbance. This is a clonal production method which has become increasingly useful in agricultural and ornamental crops due to rapid propagation times, no seasonal constraints, and small area requirements, compared to cutting or seedling planting (Loberant & Altman, 2010). *In vitro* material is also produced axenically, which allows them to be introduced to new environments free from detrimental organisms, such as *Labyrinthula zosterae* and *Phytophthora gemini* (Govers *et al.*, 2016).

Plantlets can be achieved either by micropropagation of differentiated tissue or by callus development and differentiation (Figure. 1.7.1). Either method may be suitable for seagrass production, but tissue production gives more easily predictable results and makes scaling production easier by increasing the number of axillary buds, embryonic buds on the leaf axil which can then develop in to new clonal shoots (Bhatia & Dahiya, 2015).

In vitro micropropagation classically follows a four-stage system:

1. Initiation- establishing a sterilized culture of the selected tissues in a vessel.
2. Multiplication-growth and division of selected clonal tissues.
3. Rooting- Root production and preparation for the ex vitro environment.
4. Acclimation- Transfer and adaptation to the natural environment

Plant growth *in vitro* is generally considered to be heterotrophic, as low concentration of carbon dioxide in the vessels generally limits photosynthetic activity (Kozai, 1991). Instead, sucrose is provided in the medium as an energy source.

Plants which commonly reproduce clonally are good candidates for micropropagation techniques. Many species of seagrass, despite producing seeds, rely on clonal growth both to maintain their population and expand their range (Short, *et al.*, 2007). *Z. noltii* shoots, for example, naturally reproduce by successive repetition of a growth unit, called a ramet, consisting of leaf, sheath, axillary shoot, node, roots and internode (Brun et al, 2006). These ramets are genetical identical to the parent plant but, importantly, physiologically independent, and able to survive and continue to reproduce once separated from the parent (Short *et al.*, 2007). Natural shoot organization is that the axillary shoot branches in the opposite direction to the previous branch. By increasing the rate of production of axillary shoots, micropropagation may be used to increase the clonal production rate of these plants, following which growth units can be again divided into new vessels and further reproduced. In terrestrial grasses, production can be up to tens of thousands of plantlets per year from very small donor biomass (Bhatia & Dahiya, 2015). The quantity of material required primarily determined by the success of sterilization techniques during initiation, and by the desire of the producer for increased probability of genetic variation, either by taking samples over a large spatial area, or using available past research on genetic variability in the donor site, or by genetic testing of collected material.

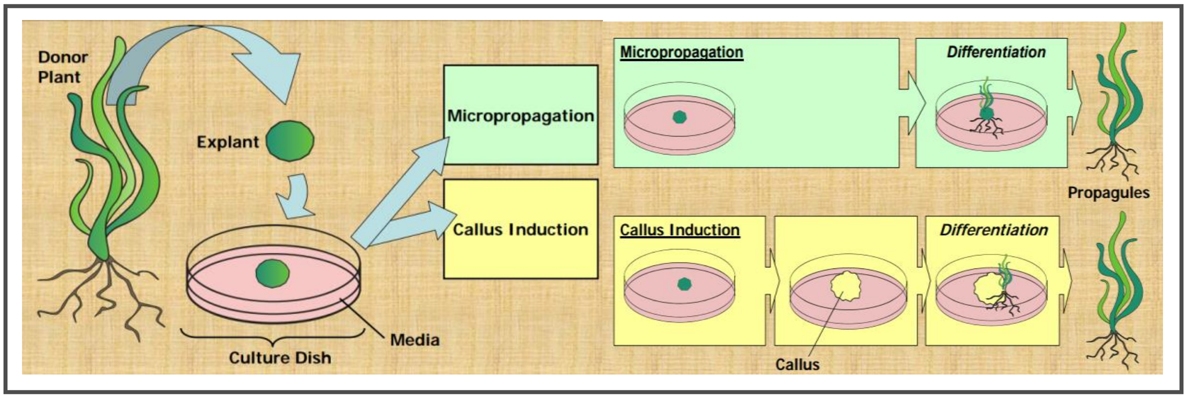


Figure 1.7.1: Simplified standard methods of in vitro *plant propagation, Kayti Tigani, 2007, Development of a SAV Tissue Culture Protocol for Restoration of Marine Habitats, https://www.inlandbays.org/wp-content/uploads/2010/12/0507STACTigani.pdf*

Clonal micropropagation techniques are relevant and useful so long as genetic diversity is considered (van Katwijk *et al.*, 2009). Seed germination rates of seagrasses are low in the donor area, the Ria Formosa, and clonal reproduction is likely locally important (Cabaço, Ferreira & Santos, 2010). In the donor area, Ria Formosa, *Z. marina,* has low genetic diversity and clonal growth is an important natural reproductive strategy in this area (Billingham *et al.*, 2003). Clonal reproduction is also considered important for *Z. noltii* in the donor area in addition to asexual reproduction, and reproductive strategy is impacted by disturbance (Alexandre, Santos, and Serrão, 2005).

For seagrass restoration purposes, bareroot plantlets, produced from stage-II material, may have advantages over stage-III products. These are easily handled and manipulated into planting units/substrates, leading to much more rapid plant-out, reducing labor requirements, and reducing potential desiccation time during manipulation. This would be a practical solution if root development occurs rapidly after plant-out. This could be in acclimatizing tanks (mesocosms or protected bays) or direct to the field restoration area. If roots can be rapidly established in this way, this would make the research and development process more rapid *in vitro*, reduce the need for establishing an *in vitro* stage-III protocol, and make the out-planting process more efficient.

Synthetic cytokinins, such as 6-benzylaminopurine (BAP), is used in plant micropropagation to induce callus development and increase cell replication, induce sprouting, and is added to MS medium to encourage shoot induction, increase shoot and node production, and increase branching (Koch & Durako, 1991). Studies of *Ruppia maritima* showed that the number of nodes and branches could be increased by up to 3 times with the addition of a variety of cytokinins (including BAP) compared to untreated samples, but increases were not dose-dependent for doses 5, 10 and 20 mg/L (Koch & Durako, 1991). Lower concentrations between 0.001mg/L and 1mg/L may demonstrate dose dependent interactions at these lower concentrations, as well as similar growth outcomes at similarly high concentrations.

The main disadvantage of *in vitro* techniques is the long research and development time required to develop a protocol to allow plants to grow, produce new shoots, and be divided in to explants in a way that promotes the health of the plant and its replication rate. Challenges to be overcome would include developing a decontamination technique which limits competitive organisms while maintaining viability of the target plant material, balancing media compositions to reduce changes in osmotic potential, allowing for gas exchange between vessels and the external environment to prevent imbalances, and choosing suitable growth hormones to promote rapid shoot development in the specific species. There may be a range of stress factors that impact plants both during their introduction to the *in vitro* environment and during *in vitro* to *ex vitro* planting out. Acclimation stress is most likely to be related to higher light availability in the field (Hazarika *et al.*, 2006), though this may be attenuated by the light refraction from the above water column.

During transfer of material to the transplant meadow, desiccation effects should be minimal if planted out quickly and while wet, compared to out-planting of terrestrial plants, as it is to a submerged *ex vitro* environment. To avoid osmotic stress, both while *in vitro* and when planted out, solute concentrations of the *in vitro* growth medium should be matched as well as possible to both the donor environments and to the plant out location (Wilson & Bennett, 2005). For seagrass, being used to submersion, hyperhydricity effects are not expected to be an issue, though the use of an agitator for aeration with liquid media trialed could assist to reduce the risk of hypoxic conditions that can induce it (Visser, de Klerk & Breeding, 2010). If plant exudates impact growth, ascorbic acid can be used 0.01 % to counter this (Ruiz-Carrera & Sánchez, 2008).

The scope of genetic variability and its importance for survivability must be considered, as diversity has been shown to increase resistance to disturbance (Hughes & Stachowicz, 2004) and reduced diversity can affect later population growth (Williams, 2001), leading to low long term success. It is understood that, for example, *Z. marina* in southern and southwestern Portugal (south of Lisbon) are genetically differentiated from Northern Portugal (Cunha *et al.*, 2014). It follows that southern sources are better donors for southern restoration, but in the absence of large northern donor beds, suitability of southern donors for northern (or international) restoration projects will need to be assessed by their success or failure in these environments. Any clonal propagation method should be aware of the risk of low genetic diversity when only a small number of clones are used to produce large quantities of material (Williams, 2001).

Disadvantages of clonal production methods such as low genetic variability could be overcome through broad collection regimes from varying donor beds (Fonseca, Kenworthy & Thayer, 1998), or can be harnessed if specific clones are assessed to be more suitable at revegetation sites or tolerant to stressors, for example, temperature related stress. Development of ecotypes in isolated populations can also exhibit different responses to salinity stress (e.g. Salo, Pederson & Boström, 2014), and this will also need to be accounted for when considering the applicability of collected material for restoration in systems with different physical constraints. Transplanted meadows with low genetic variability may help to overcome the limits to recruitment in bare sites. Once established, they may assist to create conditions that allow for outside recruitment, to increase genetic variability, and in the meanwhile providing habitat services and stabilization as required.

While this research requires time and many trials to perfect, protocols for micropropagation have been established for *Ruppia maritima* and used successfully in restoration activities (Bird, Jewett-Smith & Fonseca, 1994). There are also published protocols for *Halophila engelmannii* (Bird & Jewett-Smith, 1994) cell culture of *Halodule pinifolia* (Subhashini & Thangaradjou, 2014), tissue culture of *Heterzostera tasmanica*, and *Zostera muelleri* (Hamill & Sumby, 2002), *Posidonia oceanica* (Loquès, Caye & Meinesz, 1990), *Halophila ovalis, Ruppia megacarpa, Posidonia coriacea,* (Henry, 1998) and *Cymodocea nodosa* (García-Jiménez *et al.*, 2006). Currently there is no published *in vitro* micropropagation protocol for *Z. marina* or for *Z. noltii* and any work to investigate or overcome these methodological challenges may assist in the development of a viable protocol for future propagation.

1.8- Root development and transplantation of seagrass.

Root production is an important component of total seagrass production (Duarte *et al.*, 1998) and helps to provide stability to seagrass beds by decreasing physical removal of shoots, reducing sediment resuspension, and increasing sediment stability (Schwarz *et al.*, 2004; van Katwijk *et al.*, 2016). Restoration techniques are varied, but usually rely on transplanting donor material from existent meadows, either locally, or from other locations, which necessitates a method of removal for this material. Larger plug or sod type methods have often achieved better long term results than others that extract and transplant individual shoots or very small unit sizes to the new location (Paling *et al.*, 2001; Christensen *et al.*, 2004; Fishman *et al.*, 2004; Suykerbuyk *et al.*, 2016; van Katwijk *et al.*, 2016). These methods involve using coring units, shovels or machines to extract complete sections of meadow including shoots, sediment, and all other attached biota as a single unit to a particular depth, usually no more than a few centimetres for practicality. Units may be only several square centimetres in horizontal dimension up to several meters depending on the equipment used. It is yet unclear why sods type transplants are more successful, but one of the reasons suggested is that the root structure and function is maintained better than for separated and bare-rooted shoots. This may prevent rapid erosion by currents and waves due to the intermeshed root rhizome matrix, may maintain root function for gas and nutrient exchange, and also transfers the associated sediment characteristics and microbiome of the donor location. Some or all these factors may assist shoots to establish in the new location.

Adventitious roots are those arising from leaf, stem, rhizome, or old root tissue, rather than embryotic root development or simple branching of the primary root (Haissig, 1974a). Unlike in propagation via seeds, root development in micropropagated, cutting, or division propagated plantlets is adventitious. Roots account for up to 50 % of the total plant production depending on species and location and forming a dense web like structure in the sediments (Duarte *et al.*, 1998). Rapid growth of adventitious roots is important as it serves to stabilize plants within the sediments, reducing potential removal of transplanted shoots. Except for sod transplanting methods, many methods of seagrass restoration use shoots which may have reduced, divided, damaged, removed, or absent roots, so it is important to understand the impact of this.

Increasing the mass and speed of adventitious root growth for transplanted shoots may increase restoration success as this will help to stabilize shoots and allow functional roles such as gas and nutrient uptake to continue as normal in the new location. To investigate adventitious root growth in seagrasses and its possible impact on restoration success outcomes, several trials were conducted to try to assess health and growth parameters for seagrasses transplanted in to mesocosms under different conditions. In plant propagation, inducing growth of adventitious roots may be achieved simply by wounding the tissue in some plant species, as occurs when the cut is made (Steffens & Rasmussen, 2016). In other species, or for faster development, this may be enhanced by the application of synthetic auxins that can assist with stimulating development and differentiation of root cells (Haissig, 1974b). This method imitates natural production of growth hormones by the plant. Indole-3-butyric acid (IBA) is a plant growth modifier used to regulate and accelerate the formation of roots of young plants. Application of IBA is known to assist with adventitious root development in many terrestrial plant species, and is applied either within growth medium *in vitro*, or as a basal “dip” application to plants propagated by cuttings. It has been applied (in combination with 1-Naphthaleneacetic acid (NAA)) to seed grown plants of seagrass species *Posidonia oceanica* and produced strong results, with a 2-3 time reduction in time of root emergence and resulted in more than double the length of the roots grown compared to non-treated control plants (Balestri & Bertini, 2003). Other seagrasses, such as *Halophila decipiens* have been tested with application of IBA, and in many cases this inhibits new leaf growth and stem elongation, but changes to growth of roots was not recorded (Bird, Johnson & Jewett-Smith, 1998). For this species roots grew *in vitro* regardless of absence of or type of auxin treatment.

For some species of plants, adventitious root growth is already strong and does not require enhancement. Published results of application of auxins for root development in *Z. marina* and *Z. noltii* has not yet been assessed. Prior *in vitro* culture experimentation with *C.*

nodosa was not successful in achieving root development with any used treatments including IBA, but IBA was shown to reduce leaf elongation compared to control plants (García-Jiménez *et al.*, 2006).

1.9- Artificial substrates vs sods in restoration

Recent studies of survival and persistence of transplanted seagrasses have emphasized the need to create large areas of meadow as a key factor in restoration success. Restoration success or failure of seagrass meadows is highly influenced by the scope of the project and its ability to overcome the alternative alternative steady states for the location (Suding *et al.*, 2004) either algae dominated, or bare sediment dominated, or otherwise. This is because positive feedback mechanisms in natural meadows are likely an important factor in restoration success of new seagrass meadows (Suding & Hobbs, 2009). The importance of scale and spreading of risk has been identified as the highest determinant of success across all restoration regions and methods. The conceptual model of van Katwijk (2016) proposes a biphase stability for seagrass meadows with alternative “with seagrass” and “without seagrass” stable states, and that larger scale is the most useful factor for crossing required thresholds (or tipping points) to induce self-sustainable feedback mechanisms that will promote recovery, changing the area to “with seagrass” state.

Method of transplant was also related to success, with sod transplants considered to be the second most successful method (van Katwijk *et al.* 2016), following only transplantation of weighted rhizome fragments. Locally, there is some evidence of success in re-introduction of seagrasses including *Z. marina* using sod type transplant in an open ocean environment that historically had seagrass meadow in Portinho da Arrábida, Portugal, but early positive results were negatively impacted by significant storm events of the 2009/2010 winter (Cunha & Serrão, 2011). A combination of physical scouring of the bed, sediments burial of shoots, and months of low light due to heavy suspended sediment load, combined with fish herbivory pressure, severely impacted survival rates (Cunha *et al.*, 2014). More recent successes in maintaining persistent transplanted meadow in Portugal were achieved using sod transplants and were best related to the creation of large patches of transplants, rather than smaller planting units (Paulo *et al.*, 2019).

Other restoration projects have favored the use of textiles, such as those used in river restoration, to create planting units that anchor the transplants during the establishment period (Murray-Jones, 2008; Pickerell *et al.*, 2012). As in terrestrial and aquatic restoration, textile products may be used during establishment of replanted meadows while plants have low root length/biomass. This can reduce the need for sod transplants by replicating structural conditions.

Small donor populations likely restrict future ability to continue restoration studies and large projects highlighting the need both for appropriate transplantation methods, and for propagation of seagrasses, to ensure there is adequate material for restoration. A suitable propagation technique would assist in creating donor material for future efforts. Micropropagation, involving multiplication, rooting, and acclimating stages may help to provide this. The investigation of seagrass root adventitious root development after transplantation will assist restorers to make decisions about the type of transplant material and the handling method of transplants to ensure the best possible survival and health outcomes. Sod transplant units and textile-based substrate alternatives were compared in controlled environment to assess if they perform differently for survival and health indicators and the advantages discussed for the three target species.

1.10- Project goals

This thesis aimed to test novel approached designed to improve the success of seagrass transplants. The specific goals were to use the model species *Z. noltii, Z. marina*, and *C. nodosa* in three targeted investigations:

1. To investigate the suitability of simple, fast, and accessible *in vitro* micropropagation techniques for propagating transplant material of *Zostera marina*, and *Zostera noltii* specifically:

   i. To achieve operationally axenic cultures of Z. marina and Z. noltii. To establish these *in vitro* over a medium period of time (several months).
   ii. To begin to develop a protocol for multiplication of leaf, stem, and rhizome organs for the production of micropropagated donor shoots by investigating the effects of adding BAP to the growth media

2. To assess the growth rate of new adventitious roots on *Zostera marina*, *Zostera noltii, and Cymodocca nodosa*:
   i. To investigate if shoots can survive complete root damage during transplantation and the time required for recovery of adventitious roots (length and dry weight) to normal parameters after their removal.
   ii. To assess if the application of a common horticultural rooting growth hormone (auxin) IBA can be used to increase the growth rate of adventitious roots in seagrass species *Z. noltii, Z. marina* and *C. nodosa*.
3. To assess, for *Z. noltii, Z. marina* and *C. nodosa,* if textile-based transplant units can give similarly survival and health parameters compared to the currently favored sod transplant method, and to assess from these results which conditions may influence their success.

2- Methods

2.1- Material collection

Permitted material of *Z. marina* and *C. nodosa* were collected using SCUBA from a donor meadow in the Ria Formosa coastal lagoon near to Culatra Island, where material for transplant have previously been collected for restoration activities (Paulo *et al.*, 2019). Material was collected as sods (20 cm x 20 cm x 5-8 cm depth) in their natural sediment using plastic tubs and a shovel (Figure 2.1.1). These were transferred by boat in water filled tubs to the Ramalhete research center (taking approximately 30 minutes) and put immediately into large outdoor mesocosms where they were supplied with incoming coarse-filtered sea water at ambient temperature and salinity (Appendix 2). *Z. noltii* was collected from donor meadows in the "salina" ponds of the Ramalhete research station using the same shovels and tubs used for the other species and transferred by wheelbarrow (taking approximately 5 minutes) immediately to the outdoor mesocosms.

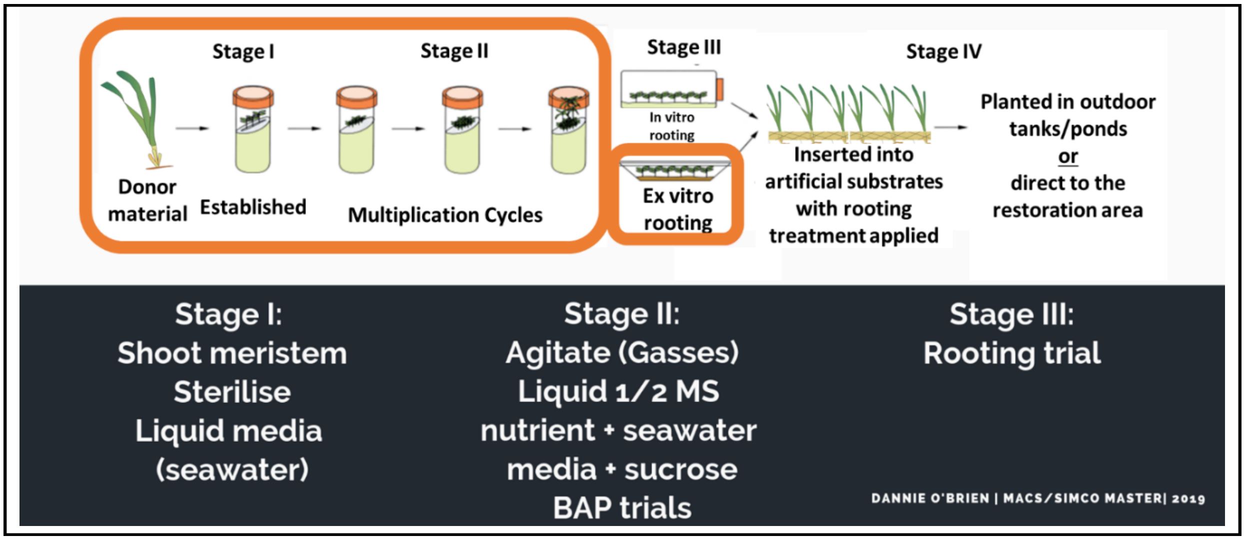
Figure 2.1.1: Left- Transfer of sods using shovels and trays from donor meadows. Photo: Diogo Paulo (Paulo *et al.*, 2019)*.*

Right- 20cm x 20cm x 7cm seagrass transplant sod unit. Photo: Author.

Almost all *Z. noltii* shoots were apical with only one or maximum two additional older shoots. Material used for all experiments was the apical shoot only, except in the case of the sod experiment where material was undisturbed and therefore mixed. Shoots collected were those that would have otherwise been removed by dredging maintenance activities at the research station. Material taken from the Ria Formosa was selected over a large area and removed in a maximum unit size of 20x 20cms by 5-8cm depth to avoid large disturbance of the donor bed. From these, some material was maintained as complete sod units. Others were divided into individual shoots and reserved loose sediment. This sediment was then combined for use as uniform substrate in the other mesocosms. This was done so that the donor material was more likely to be uniform across experimental treatments. Due to their "mono-meristematic leaf-replacing" growth habit (Short & Duarte, 2001a), individual terminal shoots were considered to be viable as planting units for the root development and textile substrate trials. Material selected for micropropagation trials were selected from healthy shoots including at least 2 cm of rhizome with at least 1 node, the leaf sheath, and some leaf material above the sheath (minimum 1cm).

2.2- *In vitro* micropropagation of leaf, stem, and rhizome.

Healthy apical shoots of *Z. marina* and *Z. noltii* were selected from the collected material for introduction to the *in vitro* environment to assess their response to stage 1 of the standard micropropagation protocol (Figure 2.2.1). As there is an existing published *in vitro* protocol for *C. nodosa*, this species was not tested for this investigation. Preference was given to apical tissue as these may carry lower microbiological loads due to their lower exposure- leading to easier sterilization- and are likely to be actively growing.



F*igure 2.2.1: Normal 4-phase protocol for micropropagation (source: http://irrecenvhort.ifas.ufl.edu) and the two target* in vitro *stages for this study, followed by ex vitro rooting into textile planting units, with establishment into mesocosms or directly to the field (orange outline).*

Growth media were made using sea water collected from above seagrass meadows in the ponds of Ramalhete at midday (February) and stored in dark glass bottles during transport. Murashige and Skoog Basal Salt Mixture (MS) (Murashige & Skoog, 1962; supplier Sigma- Aldrich; specifications in Appendix 1) was then added at half concentration (2.15 grams of powder per liter of liquid medium) following the suppliers' instructions. Food grade table sugar was used as a sucrose source and added at the concentrations desired (Table 2.2.i). The media was then autoclaved at 121°C and 15 PSI for 20 minutes. It was decided to use a completely liquid medium for plant initiations as this removes the added costs and complications of using gel-based media. Additionally, the vessels chosen, 5.0 mL Eppendorf Tube and 50 mL Falcon tubes are narrow, allowing the shoots to remain upright at all times. Fully liquid media allow the transfer of all nutrients, vitamins, and hormones to all parts of the plant tissue evenly. Hyperhydricity and water-logging are evidently not important risks of this technique as these are marine species that are used to constant immersion. The same seawater was used for all treatments. The final salts concentration of the media after MS salts had been added was 38 ppm, measured using an Atago® N-1 Hand-held Refractometer. MQuant ColorpHast® pH test strips indicated that the final pH of the media was 7.

For trial media with BAP, this was added after autoclaving under sterile conditions for the required concentrations using 6-benzylaminopurine solution 1 mg/ml (Sigma- Aldrich). IBA was added in the required concentrations from 1 mg / mL Indole-3-butyric acid stock solution made using by dissolving 100 mg of IBA powder (Sigma-Aldrich) in 2-3 mL of ethanol in a 100 mL volumetric flask and then filling to 100 mL with Millipore filtered water. Media were transferred to individual vessels under aseptic conditions in the fume hood within 30 cm of a Bunsen Burner flame.

Under aseptic conditions, inside a fume hood wiped with 70 % ethyl alcohol before use, the youngest, healthiest (no lesions or necrotic patches), and cleanest plant material was selected. Explants with an internode rhizome and leaf were used. All material was handled using forceps and scalpel sterilized by dipping in isopropyl alcohol and flamed for 1 minute using a Bunsen burner, then cooled for 3 minutes. Root tissue was removed from samples if present. Plant material was surface sterilized by agitating in experimental selected concentrations of sodium hypochlorite (NaClO) (diluted from 3 % concentration with autoclaved seawater to required concentrations) with 1 mL/L of Tween20 surfactant for specified times. (Table 2.2.i).

The material was then rinsed in sterile seawater as described in Table II.II.i to remove surface NaClO. Any bleach damaged or browned tissue was removed and the initiates were placed into sterilized Eppindorf tubes (5.0 mL) or Falcon tubes (50mL volume) and filled to 4 mL or 35 mL with autoclaved local seawater media (Figure 2.2.2) containing experimental combinations of ½ strength concentration MS (Murashige & Skoog, 1962) nutrient and vitamin medium and sucrose levels as described (Table II.II.i). An adaptive approach to trials was taken whereby procedures with positive results in earlier trials were used for later trials in order to obtain enough uniformly treated plants for trials using BAP growth factor.

Table 2.2.i: ***Initiation conditions for Z. noltii and Z. marina***

NaClO %	Sterilisation time (seconds)	Rinse time	Sugar concentration	Species	Replicates
0.5 %	60	180	20 g/L	*Z. noltii*	6
0.5 %	60	180	20 g/L	*Z. marina*	3
0.5 %	180	180	30 g/L	*Z. noltii*	4
0.5 %	60	180	30 g/L	*Z. marina*	6
0.5 %	180	180	30 g/L	*Z. marina*	25
0.5 %	180	180	20 g/L	*Z. marina*	17
0.5 %	180	60	20 g/L	*Z. noltii*	4
0.5 %	180	60	20 g/L	*Z. marina*	3
0.5 %	60	60	20 g/L	*Z. marina*	3
1.0 %	60	60	30 g/L	*Z. noltii*	4
1.0 %	60	180	30 g/L	*Z. marina*	5
1.0 %	60	180	20 g /L	*Z. noltii*	4
1.0 %	60	60	20 g /L	*Z. marina*	3

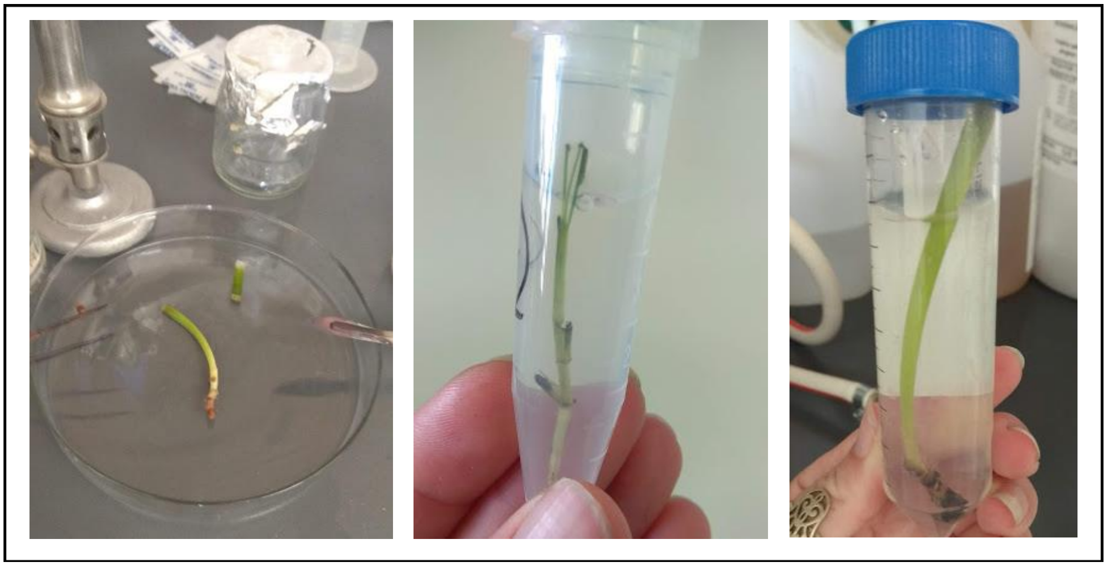


Figure 2.2.2: (L to R) Prepared and sterilized plant material; plant in 4 mL of liquid media in Eppindorf tube; plant in 35 mL of liquid media in Falcon tube. Photos: Author.

The initiates were then kept in a climate controlled plant growth chamber at 18°C under white light on a 12:12 light:dark cycle. Tubes were gently agitated by hand once every 24 hours to homogenize the media solution, incorporate gases in the liquid, and ensure the media at the surface layer of the plants was refreshed.

After 72 hours, the samples were visually assessed by being held in front of florescent lights and examined for contamination by bacteria or fungi colonies. Bacterial contamination was determined by visually cloudy medium. Fungal growth presence was determined using visible mycelial growth, cloudiness, or pellicle formation. Units without visible contamination were to be considered "functionally axenic" (Bird, Johnson & Jewett-Smith, 1998). If signs of contamination were identified the initiates were discarded as visible contamination is considered a potential competition or stress factor that would impact health indicators of the plant. Plants were then checked at regular intervals to assess survival.

Plants were transferred after an acclimation period of 10-14 days from initiation media to the BAP trial media to try to accelerate the production and development of new axillary buds. Mediums containing only cytokinin BAP were trialed at concentrations 0.001, 0.01, 0.1 and 1 mg/L. To investigate if shoot production could be induced through different cytokinin and auxin rations, trial combinations of BAP (cytokinin) : IBA (auxin) of 0:0, 0:0.1, 0:0.5, 1:0, 1:0.1, 1:0.5, 3:0, 3:0.1, and 3:0.5 mg/L were tested. Every 2 weeks plants were transferred to new vessels containing fresh growth media of the same type.

To test the effects of categorical variables contaminated vs uncontaminated (after 72 hours) and dead vs alive (at each time period) between experimental replicates, Pearson's Chi-squared tests were used (in Microsoft Excel) for factors NaClO concentration, sterilization time, rinse time, and sugar concentration in Microsoft Excel. Results of treatments were plotted using Excel.

Statistical hypothesis tested

H_0: Sterilization method (Sterilization time, sterilant concentration, rinse time, and sugar content of medium) makes no difference to the contamination rate or the survival of initiates.

H_0: BAP concentration has no impact on number of new shoots, shoot length, internodal space, leaf number, or callus production in the two test species (PERMANOVA).

2.3- Adventitious rooting trials

During transplantation, roots of seagrasses are frequently damaged or removed either accidently or deliberately. To assess the impact of root damage and the re-growth of adventitious roots of the species *Z. noltii, Z. marina,* and *C. nodosa*, shoots were selected from collected material and replanted into experimental mesocosms. Their growth monitored to assess both under and above ground growth metrics. Seagrass shoots receiving treatments had all their roots removed with a scalpel as close to the rhizome as possible (Figure 2.3.2). Control shoots were handled very carefully to maintain roots intact. Leaf length was controlled as all above ground biomass was cut to 30 cm starting length for *Z. marina* and *C. nodosa*, and to 15cm starting length for *Z. noltii*. This helped to increase uniformity in leaf mass among the samples and prevented browning and damage of the leaves by reducing the chance of them reaching the water surface in the mesocosms ensuring more true leaf elongation measurements.

Figure 2.3.1: Pre experiment preparation of Z. marina selected shoot by removing all root mass and cutting leaf length to 30cm long above ground. Bellow ground sections were between 5 and 7 cm and contained at least 2 nodes. Photos: Author.

The IBA hormone treatments were made using Indole-3-butyric acid powder (Sigma-Aldrich) made to 1 mg / mL stock solution by dissolving 100 mg in 2-3 mL of ethanol following the suppliers instructions in a 100 mL volumetric flask and then filling it to 100 mL with Millipore filtered water. Solutions were then made by diluting with autoclaved sterile sea water for 4 trial treatments 0, 3, 5, and 10 mg/L of IBA. These treatments were basally applied by soaking the bottom 5cm of each shoot in the rooting treatments for 10 minutes in the treatment solution in enclosed jars placed in the shade to preserve humidity and reduce desiccation of the leaves. The control shoots were soaked with their roots intact in 5cm of sterile seawater for 10 minutes in an enclosed jar.

The seagrass shoots were then planted individually and equally spaced into natural sediment (from the original collection site of the shoot material, separated from the shoots, mixed, and then distributed into the individual mesocosms to a depth of 8 cm. The mesocosms were round and 27 cm diameter by 45 cm deep. These were placed in large tanks supplied by coarse filtered seawater from the Ria Formosa. The imputed water was at ambient temperatures between 15 °C and 24°C and salinities ranging from 35 to 38. The shallow depth and sun exposure of the tanks allowed the water to heat. Mean and median water temperature in the mesocosms was 22°C but could be as high as 33°C for short periods of time of 3-4 hours (Appendix 2). The water depth was 8 – 10 cm above the rim of each experimental mesocosm unit to allow water exchange without introducing excessive disturbance from inflow or outflow pipes. The mesocosm surfaces were cleaned manually on a weekly basis to remove large concentrations of epiphytes and seagrass leaves were gently wiped of sediment and epiphyte build up. The tank set up was designed to remove algae growth on the water surface that could potentially shade plants. Shade was provided using shade cloth material over the tanks to reduce growth by micro and filamentous algae.

Eight replicate shoots were used per treatment (total shoots = 3 species x 5 treatments x 8 replicates = 40 shoots). Some trial shoots were lost during the experiment as they dislodged from the sediment and floated to the surface of the tanks. Because their correct treatment could not be identified these were eliminated from the experiment. This resulted in unequal replicates across samples, between 6 and 8 replicates per treatment. These were considered to be adequate sample sizes to assess variability between the treatments. The loss of these shoots from the treatment was noted for each mesocosm.

During the growth time the mesocosms were monitored daily and any death of shoots was recorded. After a 2 to 3 weeks period of growth (depending on species) the shoots were gently collected from the mesocosms and washed in sea water to remove all sediment. The root length of the longest root from the root tip to where it joins the rhizome was measured using a metal ruler to the nearest millimeter, where present. The roots were removed using a scalpel as close to the rhizome as possible and placed into individual paper packets. These were dried for 48 hours at 60°C in a drying oven and the dry weight of the root material was measured to the nearest 0.0001 gram. The number of nodes on the rhizome and leaves were counted at the start and end of the experimental period. All visible leaves were counted regardless of size. This was done to assess if any of the shoots were defoliating during the growth period, indicating bad health (leaf loss > new leaf growth in initial leaf number > end leaf number). There was some variability in the number of initial leaves and nodes on each shoot that should be accounted for when interpreting the final results. Each species was treated differently as their different growth characteristics give expected differences in these features.

A second experimental period using new seagrass shoots and following the previous method was then set up the following month. The additional factor of leaf elongation was added as the researcher had observed that there may be differences in leaf blade growth between the treatments. Leaf elongation of the newest central leaf (the most actively growing) was obtained by poking a hole through the leaf sheath with a pin (Figure 2.3.1), then after the experimental period by using a ruler to measure the distance between the hole scar on the sheath (which is expected to be in the original position) and the hole scar on the elongated central leaf, as per the methods described by Short & Duarte (2001). Leaf elongation for this study was only considered for the central leaf, it is not a sum of leaf elongation on all leaves of the shoot.

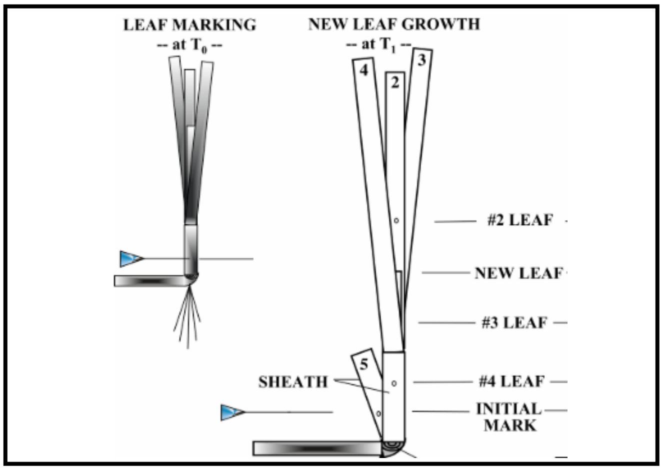


Figure 2.3.2: Creation of a pin scar in the leaf sheath. The difference between the sheath scar and the scar on the central (newest) leaf was measure using a ruler to calculate the elongation rate of the central leaf. Image source: Short and Duarte, 2001.

C. nodosa was first maintained for a growth period of 24 days for assessment. After assessment of the initial growth period this species was shown (by the growth metrics measured) to be much slower growing than the other 2 species and did not achieve similar root parameters to the control units. Because of this the second experiment was set up for a growth period 40 days to assess the growth parameters over that time.

Z. marina was fast growing and was first grown over a growth period of only 14 days. This was considered sufficient for many plants to reach similar parameters to the control units, so assessment was made at this earlier period as this shows the establishment of growth right after transplantation and treatment. The second experimental period was conducted with an extension of only 4 days to 18 days total to see if this assisted to demonstrate greater differences between the rooting treatments over this additional time period.

Z. noltii was grown over 21 days in the first growth period and the root length of some shoots reached control lengths so for the second time period this was extended by 4 days to 25 days to see if this demonstrated greater difference between test treatments.

To test if categorical indicators of health during the experiment ‘dead vs alive’ and ‘leaf loss vs gain’ were different between experimental replicates Pearson’s Chi-squared tests were used to test goodness of fit to expected values. It was then decided that despite losses due to absent replicates and reduced sample sizes for growth variables due to deaths, the reduced number of replicates could be used in an unbalanced design to analyse numeric growth parameters. For this the PERMANOVA+ add-on to Primer6 was used to conduct non-parametric permutational multivariate analysis of variance (PERMANOVA) for independent factors Treatment and Growth Time. This was considered to be the most robust method as such tests make no explicit distributions assumptions on the variables- allowing for the naturally high number of zeros and the common skewness of ecological parameters- but rather rank dissimilarities, and use permutations to obtain P-values for hypothesis testing. Results were only considered significant if PERMDISP tests showed that there was homogeneity of dispersion between samples. Results of treatments were plotted using R Studio (Version 1.1.423).

Statistical hypothesis tested

H_0: There is no significant difference in the death rate of shoots between treatments.

H_0: There is no significant difference in defoliation between treatments

*H_0: There is no significant difference among variables Total Leaf Elongation, Length of Longest Root, and Root Dry Weight, nor standardized variables Leaf Elongation per Day, Root Elongation of the Longest Root per day, and Increase in Root Dry Weight per Day for factors Treatment or Growth Time, or for the interaction Treatment * Growth Time*.

2.4- Artificial-substrate based planting units.

A fine weave and a coarse weave hessian textile product will be used to comparing their suitability as a structural support seagrass shoots with roots. Textiles pieces were cut to 20 cm x 20 cm square sections, and the inner 15 cm x 15 cm area marked as the planting area (Figure 2.4.1.A). The shoots will be inserted into the inner marked area of the textiles using the same technique as in Pickerell *et al.* (2012), the ‘tortilla’ method, to secure them (Figure 2.4.1. B & C).

Plants were selected randomly from stored material for each substrate to have even initial health and size. The below ground sections were prepared as per the rooting trials above by removing the roots. Total above ground length of the longest leaf was measured, and the plants were left at their whole length. Leaf number was counted for later assessment of loss or gain. Horizontal rhizome growth and number of nodes was not assessed for the sod units as this would disturb the rhizome and sediment in the sods, which is not desired in this case.

Sod sections were cut into 15cm x 15cm sections and were between 5cm and 8cm depth (Figure 2.4.1.D). The number of shoots per planting unit was between 11 shoots and 15 shoots (489 - 667 shoots per m^2) which is representative of local natural densities for species *Z. marina* and *C. nodosa*. *Z. noltii* usually grows in much higher densities than this locally and was between 60-70 shoots in the 15 x 15 cm sods collected; representing 2667 – 3111 shoots per m^2. It was decided for time and practicality reasons to thin this to 14 – 15 shoots per sod unit to match the same density as the other species. Each sod and textile unit was constructed in large trays with 15 - 20 cm of sea water to prevent the desiccation of the plants, sod units were given the same exposure to this environment to try to equalize initial conditions (Figure 2.4.1, E & F).

For each species there were 3 replicate units of each substrate treatment. Each planting unit was placed inside an individual mesocosm. These were situated within a larger tank with water exchange between the mesocosms. Textiles units were placed on top of 4-5 cm of natural sediment and the textile was additionally be covered in 1-2 cm of sediment. Test units were maintained as per the rooting trials above under natural water temperature and salinity conditions and under partly shaded light conditions as recorded using a HOBO® light/temperature data logger (Appendix 2). Mesocosms were rotated regularly to allow for any shading by external structures or influence of freshwater input pipes and outlets in the larger tanks containing the mesocosms. Shed leaves were removed from mesocosms and the water surface during the trials.

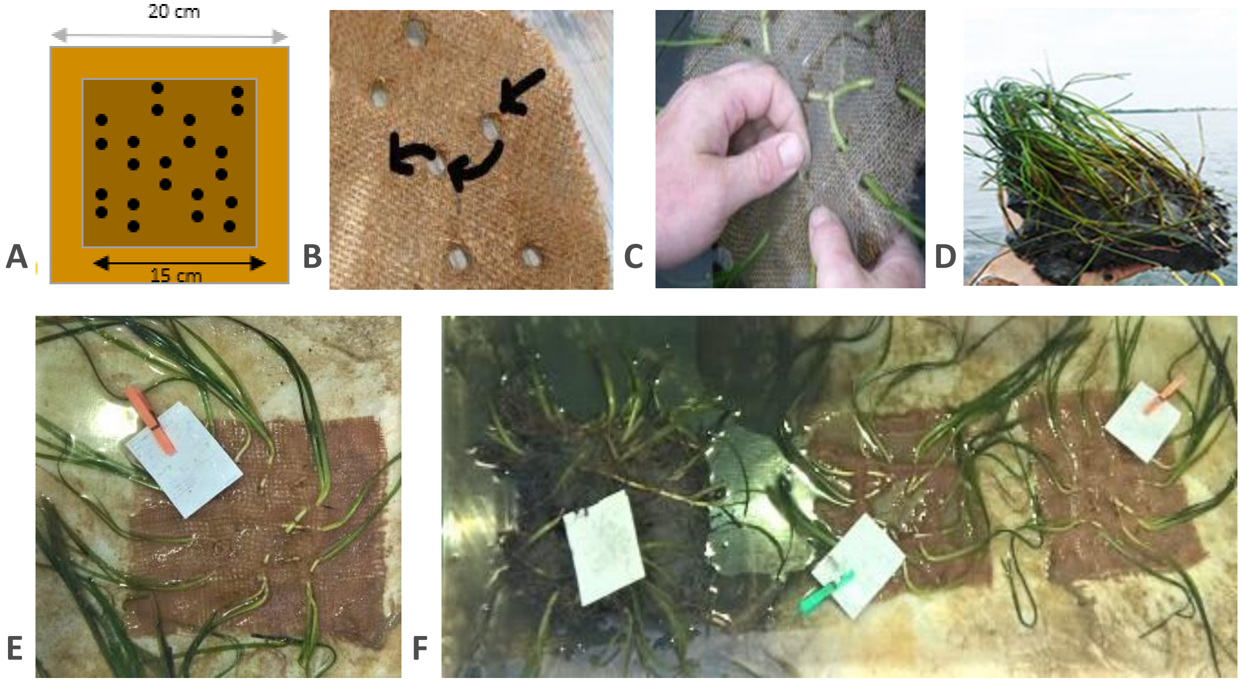


Figure 2.4.1: Methods for the creation of artificial-substrate based planting units

A) Plant unit textile size is 20 cm x 20 cm. Planting area is inside of 15 cm x 15 cm, to match sod unit. (Author).

B & C) Threading technique for "Tortilla method" using a fine-weave textile. (Pickerell, Cornell University).

D) Sod unit approximately 15 cm x 15 cm with 5-8 cm depth of sediment attached (Tampa Bay Watch).

E) Coarse weave textile unit ready to be transplanted. (Author).

F) Side by side comparison of (L to R) Sod, fine textile, and coarse textile planting units ready to start the growth trial. (Author).

At periods time periods T0 (1 day after transplant), T1, T2, and T3 (2 weeks apart for *Z. marina* and *C. nodosa*, and 1 week apart for *Z. noltii* due to their faster growth and leaf turnover rates), the listed parameters were recorded simultaneously.

- Number of dead replicates per treatment
- Number of leaves per shoot (3 randomly chosen replicate shoots) in each of the 3 replicate mesocosms for each of the 3 substrate treatments.
- Length of longest leaf (The same 3 replicate shoots as above) in each of 3 replicate mesocosms for each of the 3 substrate treatments.

- Relative photosynthetic efficiency parameters Effective quantum yield (ΔF/Fm’), Alpha (α), ETRmax and Ik using Pulse Amplitude Modulated (PAM) fluorometry for the same 3 replicate shoots as above.

Shoots were chosen haphazardly from a list of replicates within each mesocosm. If the chosen replicate was dead, a replacement replicate was chosen randomly to have 3 replicates for each mesocosm (9 replicates per substrate) for each time period, except for the last two time periods for *Z. noltii*, where intense grazing of the shoots in several mesocosms prevented the recording of accurate results in those test units.

The number of dead shoots was analysed using chi-squared tests to determine if there was an unequal death rate between the treatments from the start to the end of the experimental period and is used as an indicator of relative health. The number of leaves was counted including any visible leaf originating from the leaf sheath regardless of length. Leaf length was measured from the sediment surface to the end of the longest existent leaf (regardless of whether the leaf tip was intact or missing) to an accuracy of ±0.5 mm using a metal ruler. Both characteristics are common non-invasive (destructive) measures of comparing relative seagrass biomass. These were compared using Permutational multivariate analysis of variance (PERMANOVA) in Primer + PERMANOVA statistical program to assess differences for the independent factors: substrate, and time period. Root and rhizome parameters could not be assessed as it was highly desirable for the goals of the experiment to not disturb the bellow ground structures of the plants.

Rapid light curves, collected using Pulse Amplitude Modulated (PAM) fluorescence, are considered an increasingly important tool for assessing photosynthetic efficiency in seagrass ecophysiology studies. It was considered to be an optimal method for inclusion in this study as it is a widely used and not invasive indicator of relative photosynthesis (Ralph & Gademann, 2005). A Walz underwater pulse amplitude modulate fluorometer Diving-PAM (Heinz Walz GmbH, 1998) was used for this trial. All measurements were taken on clear sunny days under ambient light conditions between 12.30 pm and 03.30pm (at least 5 hours after sunrise) but light intensity varied a lot during the recording times (Standard deviation of Lux > mean Lux measured, Appendix 2). During the growth period light intensity in the middle of the day was usually up to 30,000 lx but on sampling days the shade cloth needed to be removed to allow access to the plants and this could result in light intensities of up to 125,000 lx.

Effective quantum yield tells us, relatively, many reaction centres can do photochemistry when are exposed to light. It is used as an indicator of stress, with lower values or declining values considered to be indicative of stress and reduced photosynthetic efficiency (Baker, 2008). It has previously been used to assess stress factor including low and high light adaptation (Ralph & Gademann, 2005), salinity stress (Salo *et al.*, 2014), stress from infection by pathogen *Labyrinthula zosterae* (Ralph & Short, 2002), and stress during harvest and transplantation for restoration (Paulo *et al.*, 2019). The other parameters are also related to either stress or photoadaptation and were compared to see if there was variation over transplant substrate or time since transplantation.

The sampling order of the mesocosms tested was randomized each sample period. Leaf clips were attached at 2 cm above the top of the leaf sheath of the second youngest leaf of all *Z. marina* and *C. nodosa* plants, or over the youngest 2 leaf blades 2 cm from the leaf base on shoots of *Z. noltii*. The clip was closed and were used to dark acclimate the leaf tissue for 5 minutes. The clip was then attached to the clip adaptor on the fiber optic cable of the Diving- PAM before opening the light shutter (Figure 2.4.2). The adaptor ensures a consistent distance of 3mm between the leaf tissue and the end of the fiber optic cable for all readings.

The WinControl-3 software (V. 3.29, Heinz Walz GmbH, 2018) was used to designate the RLC routine, and to visualize and export the acquired data. The start settings were individually chosen for each species (Appendix 3) using 8 incremental 10 second steps of irradiance from 0 to 285 µmol photon m–2 s–1 (*C. nodosa*), from 0 to 425 µmol photon m–2 s–1 (*Z. marina*), and from 0 to 594 µmol photon m–2 s–1 (*Z. noltii*). These were selected based on trials, and once decided were maintained for the whole experimental period. These aimed to be able to assess photoinhibition (a downward slope at the end of the curve) in the tissues, but in the field photoinhibition was not always reached. The settings were maintained throughout to ensure consistency.

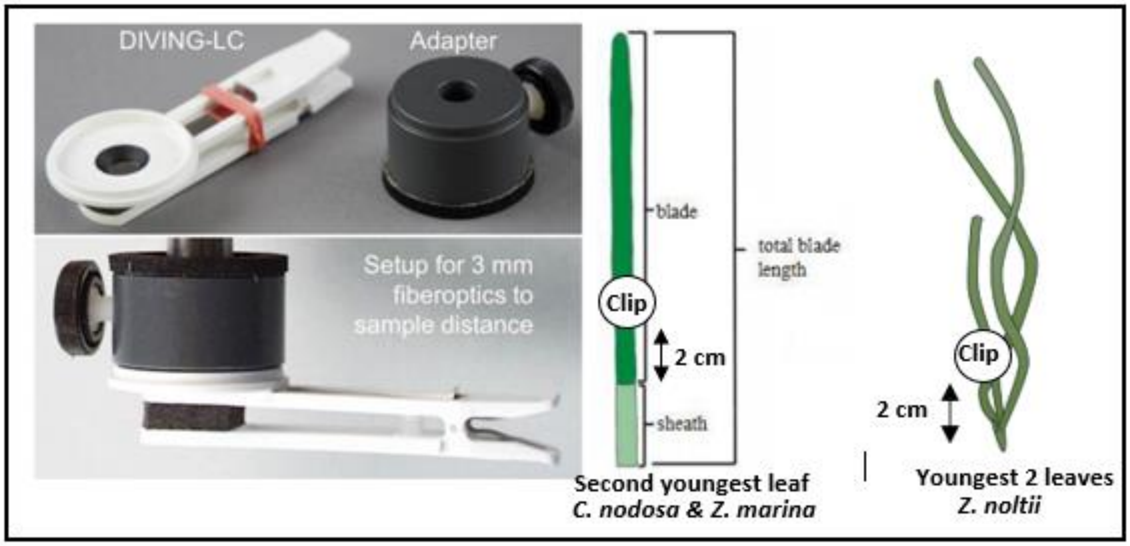


Figure 2.4.2: Left- Diving- PAM Leaf clip and adaptor set up. Source: Heinz Walz GmbH, 2018.
Right- Position of leaf clip on the three different seagrass species adapted to account for different growth forms and leaf blade characteristics.

The Walz Diving-PAM can integrate read photon flux densities to obtain photosynthetically active radiation (PAR) values. The integrated fluorometer measures the efficiency of photosystem II under light conditions (ΔF/Fm'), and then integrated software uses these parameters to calculate relative photosynthetic electron transport rate (ETR), which is an estimation of the number of electrons carried through photo-system II (PSII) during light-dependent oxygenic photosynthesis. The parameters α, ETRmax and Ik are extracted from rapid light curves (Figure 2.4.3) using the software program WinControl-3 using the non- decreasing function $ETR = ETR_m \cdot \tanh(\frac{\alpha \cdot PPFD}{ETR_m})$, which was chosen as photoinhibition was not reached for many of the curves and so β could not be calculated for the majority of cases. The parameter ΔF/Fm' represents the effective quantum yield of photosystem II (PSII) and is used to calculate the electron transport rate (ETR) for known PAR where ETR = ΔF/Fm' × 0.84 × 0.5 × PAR.

The parameters of the RLC will be used as an assessment of photosynthetic stress in this study but will also be considered in relation to other health indicators maximum leaf length and number of leaves on measured shoots. These parameters were then compared for differences for factors substrate, time period, and mesocosm (nested in substrate) using PERMANOVA in Primer+.

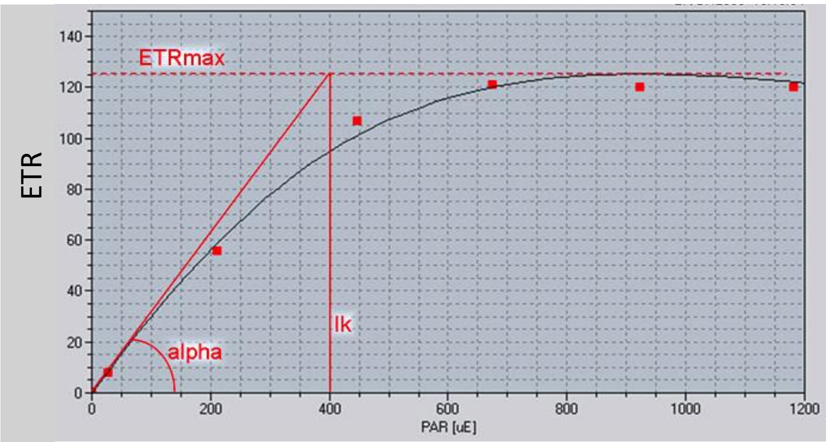


Figure 2.4.3: Generic Rapid light curve plotted using pre-programed values of PAR (x) and ETR (y). Alpha (α) represents the initial slope of the curve (light-limited) and represents the efficiency of light harvest. ETRmax is the position of maximum electron transport capacity, the highest value before plateau or decline of the curve. Ik is the minimum saturation irradiance, ie. where the maximum ETR would be without downward inflection in the curve, extrapolated from ETRmax and the slope of α. (Aquation, 2017)

Statistical hypothesis tested

H_0: There is no difference in survival rate (chi-squared analysis) or in the parameters number of leaves, length of longest leaf, ΔF/Fm', α, ETRmax and Ik (PERMANOVA analysis) between the sod transplant units and the artificial textile units.

3- Results

3.1- *In vitro* micropropagation of leaf, stem, and rhizome.

In vitro cultivation in ½ strength concentration MS (Murashige & Skoog, 1962) nutrient and vitamin medium with sucrose was able to maintain actively elongating shoots of both *Z. marina* and *Z. noltii* for up to 2 months, but the addition of BAP did not succeed in encouraging the growth of new shoots on the stem or rhizome.

For *Z. noltii*, 21 out of 22 (>95 %) sterilized ex plants were functionally axenic, regardless of NaClO treatment level (0.5 % or 1.0 %) or duration (60 seconds or 180 seconds). All shoots treated on either NaClO concentration for 60 seconds were alive after 72 hours, but for treatment with 0.5 % NaClO for 180 seconds, 50 % of shoots treated appeared dead (brown soft necrotic tissue impacting the whole sample) after 72 hours. The recommended treatment for *Z. noltii* is therefore 0.5 % NaClO, the lowest needed concentration, for 60 seconds only.

After 10 days, 16 (72 %) of *Z. noltii* ex plants were still alive (Figure 3.1.1) and these were transferred at 14 days to media containing 0.001, 0.01, 0.1 or 1 mg/L of BAP. After 19 days, eight plants (36 %) were still alive and showing signs of active growth (leaf elongation). Deaths were distributed uniformly across media. All living plants exhibited some elongation of leaf tissue from the apical growth point (between 3 and 5 mm), but none produced additional nodes or actively growing axillary buds. After 26 days there were only three plants alive and elongation had stopped. Of these, two plants continued to live for a total of 63 days, to the end of the experimental time, maintained by refreshing the growth media every 2 weeks. These did not produce new nodes or shoots during this time and did not continue to elongate.

For *Z. marina,* which was initiated on three separate occasions, all sterilized plants were functionally axenic and had living tissues after 72 hours (Appendix 4). The lowest successful decontaminant concentration and time from earlier trials was used for following initiations to prevent unnecessary damage to the plant tissues. Subsequent initiation treatment was done using 0.5 % NaClO with 1 mL/L of Tween20 surfactant for 60 seconds, then rinsing off the sterilant in sterile sea water for 60-180 seconds. This was a suitable treatment to obtain operationally axenic plants for micropropagation. If completely axenic plants are needed for future study, further treatment may be required using ethanol-based sterilization solutions in addition to NaClO.

Across the three different initiations, after eight days 46 (70 %) of plants were still alive and were actively elongating (Figure 3.1.1), with elongation of central leaf blade of up to 67 mm (8.375 mm/day). After 10 days, survival had declined to 31 plants (47 %) and a “fermentation” type smell and gas bubbles in the vessels suggested that decomposition of these lost plants had started to occur, perhaps in the presence of yeast contamination. After 14 days living plants from initiation one were transferred to media with BAP in concentrations 0.001, 0.01, 0.1 or 1 mg/L. After 22 days since initiation, 100 % (n=14) of these plants were dead regardless of treatment.

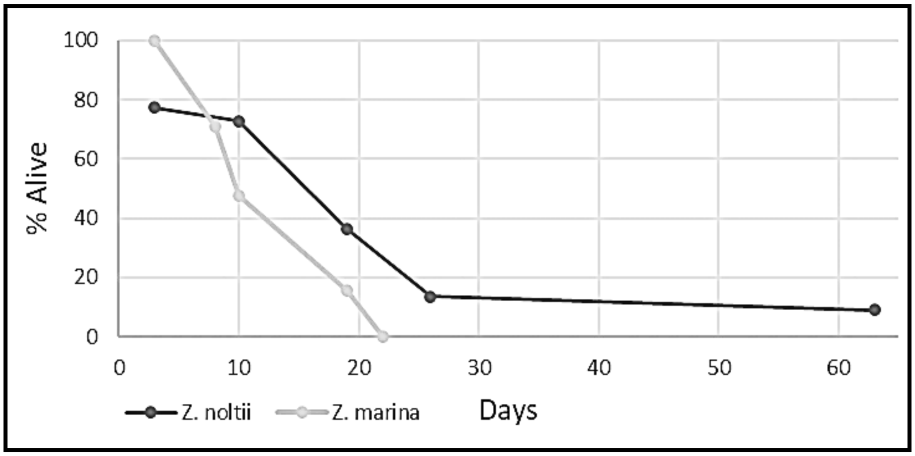


Figure 3.1.1: Percentage of initiated plants alive by time since their initiation for Z. noltii (n= 22, one initiation experiment) and Z. marina (n= 65, 3 initiation experiments)

From initiation two, plants were transferred at 14 days to media containing cytokinin: auxin combinations. After 18 days only 10 of the 35 shoots were still alive and deaths were spread evenly over treatments. Between day 10 and 18, central leaf blades continued to elongate up to 4.18 mm/ day on living shoots, but no new shoots formed from axillary buds. After 24 days 100 % (n=3) of shoots were dead, with a similar “fermentation” type smell and gas bubbles detected in the vessels.

For initiation three, only 3 of 16 plants were alive after 10 days. These were not transferred to experimental media due to lack of living plants for trials and by day 18 100 % of plants were dead.

3.2- Adventitious rooting trials

During the experimental period, some shoots were lost from the trials, as they were disturbed from the sediment, floated to the water surface, and could not be identified as belonging to a specific test mesocosm to be re-planted, so they were discarded. This led to slightly unequal replicates between treatments and necessitated a statistical approach that allows for unequal sample sizes. After losses, the minimum number of replicates for a treatment was nine. Growth data was non-normally distributed (Appendix 5) for many variables as there was a high frequency of zero values (no growth) that skewed the data.

Some shoots died during the growth period. The total death rate of 23 % for *C. nodosa* did not differ among treatments ($\chi^2(4, n = 82) = 2.05, P = 0.73$). Similarly, total death rates of 21 % for *Z. marina* (χ^2 (4, n = 71) = 6.47, P = 0.17) and 18 % for *Z. noltii* χ^2 (4, n = 80) = 6.50, P = 0.16) were less similar but not significantly different across treatments. Growth parameters for the remaining replicate plants were compared.

Leaf dynamics was assessed using the categories “start leaves>end leaves”, “start leaves = end leaves” and “start leaves < end leaves” showed no significant difference in loss, maintenance or gain of leaves among shoots in the treatments for any of the three species (for *Z. noltii* χ2 (8, n = 66) = 5.47, P = 0.71; for *Z. marina* χ2 (8, n = 63) = 69.91, P = 0.27 and for *C. nodosa* χ2 (4, n = 63) = 2.94, P = 0.94). Most shoots of all species increased leaf numbers during the test period (*Z. noltii* 79 %, *Z. marina* 86 %, *C. nodosa* 63 %).

Total root length and total dry weight were used as proxies for the ability of the three species to recover after all roots are damaged or removed. Z*. noltii root length* had significant variation across treatments ($P < 0.01$) for both the 21 and 25 day experimental periods. During the shorter 21 day experiment the 3, 5, and 10 mg/L treated shoots had only half to two-thirds the root length of the control ($P < 0.05$; Figure 3.2.1.A) but the 0 mg/L group had regrown roots to similar lengths as the control ($P = 0.19$). In the 25 day experiment, both the 0 and 10 mg/L groups had regrown roots to the control length ($P = 0.15$ & 0.29) but shoots treated with 3 and 5 mg/L had not ($P < 0.05$). Root dry weight appears to be less when treated with IBA at any concentration, but this was not significant ($P > 0.05$) after either experiment period (Figure 3.2.1.B).

Maximum root elongation rates for this experiment (represented by the length of the longest present root) varied among species due to their growth habit, and were in the order of 1 – 2 mm per day for Z. noltii, 2 – 4 mm per day for Z. marina, and <0.5 mm per day for C. nodosa (Figure 3.2.1). Daily growth rate was not considered for the control shoots as their root length and dry weight does not represent growth over the experimental period, only treatment groups were compared. Data from both experiments was combined as the rate is a standardized metric. Pairwise analysis of length revealed that IBA addition at any concentration reduced root elongation rates in *Z. noltii* by at least 30 % ($P < 0.01$; Figure 3.2.2.A) but dry weight increase showed a different pattern, with the 5 mg/L treated shoots having almost no daily increase compared to the other groups ($P < 0.02$; Figure 3.2.2.B).

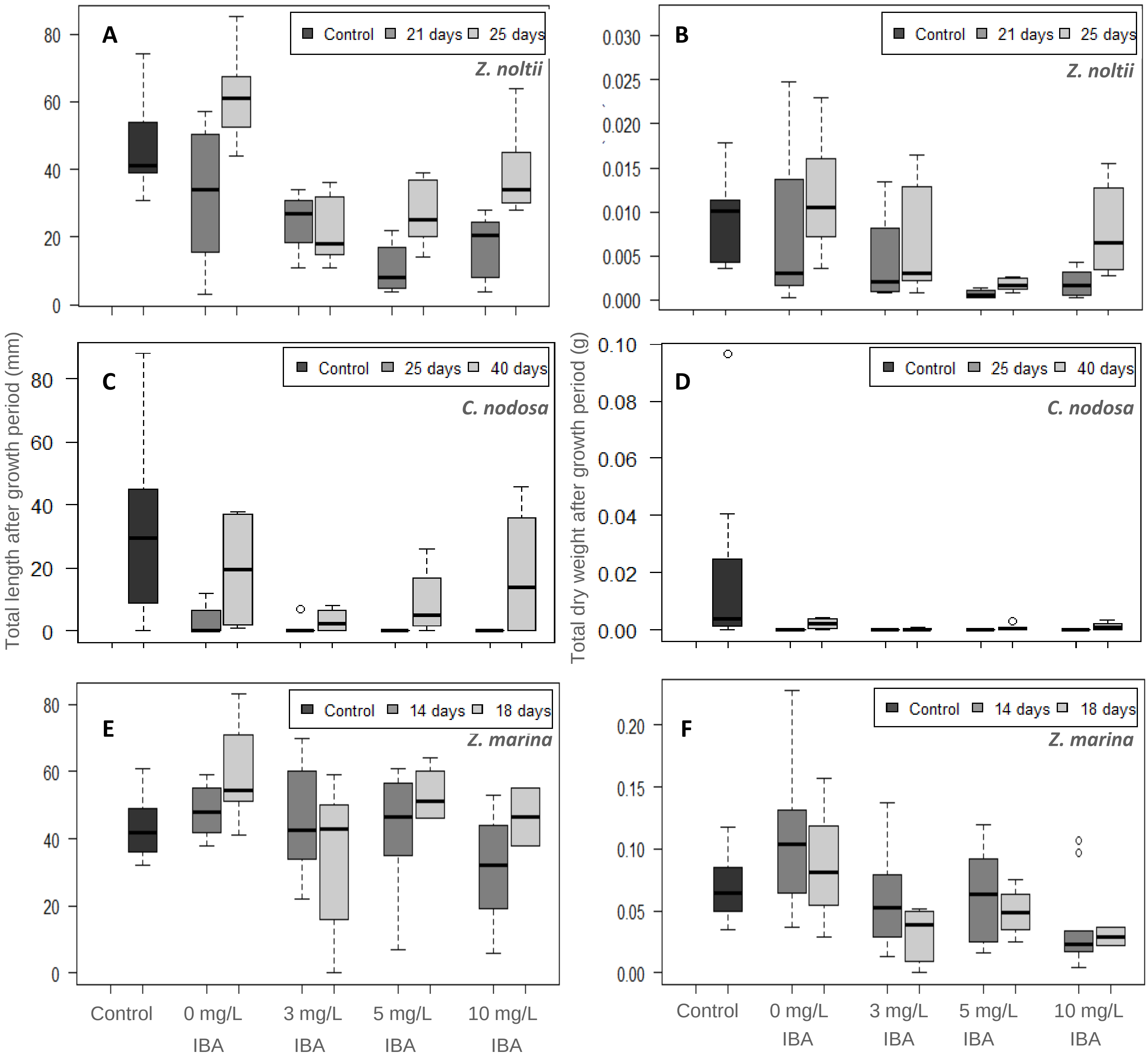


Figure 3.2.1: Adventitious root growth for specific growth periods. Growth period is species specific. A) Total length of the longest root of Z. noltii *with roots removed and with 4 growth factor IBA treatment concentrations compared to natural (control) root length. B) Total root dry weight of roots for the same.*

C) Total length of the longest root of C. nodosa *with roots removed and with 4 growth factor IBA treatment concentrations compared to natural (control) root length. D) Total root dry weight of roots for the same.*

E) Total length of the longest root of Z. marina *with roots removed and with 4 growth factor IBA treatment concentrations compared to natural (control) root length. F) Total root dry weight of roots for the same.*

Boxplots in this document: The bold middle line is the median, the box represents the middle 50 % of the data, with the edges at the 25th and 75th percentiles, whiskers represent 1.5 x the interquartile range and circles represent single points outside of this range.

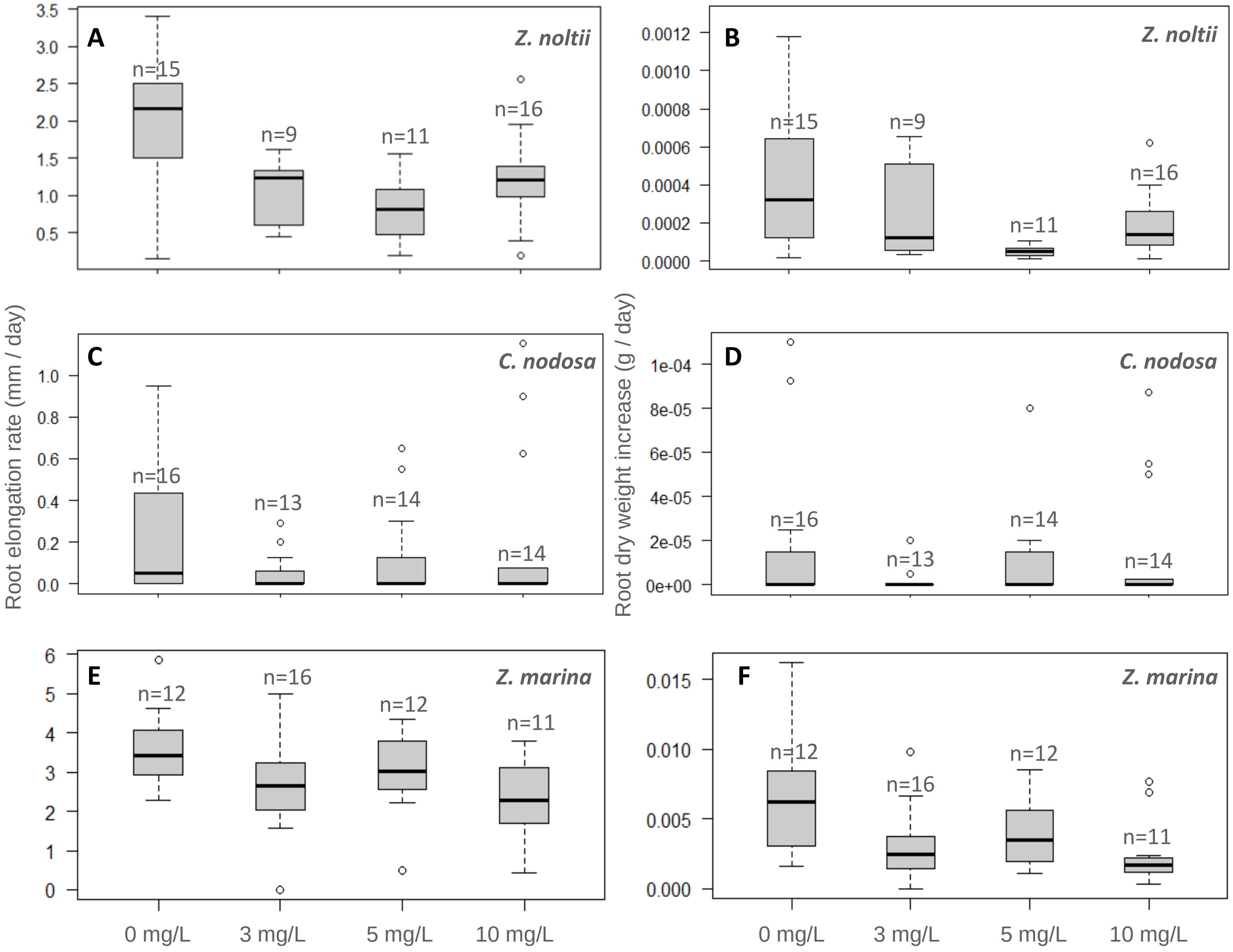


Figure 3.2.2: Daily adventitious root growth rate of shoots in both experiment periods.

A) Daily elongation of roots of Z. noltii *following root removal and treatment with 4 growth factor IBA concentrations. B) Daily dry weight increase of roots for the same.*

C) Daily elongation of roots of C. nodosa *following root removal and treatment with 4 growth factor IBA concentrations. D) Daily dry weight increase of roots for the same.*

E) Daily elongation of roots of Z. marina *following root removal and treatment with 4 growth factor IBA concentrations. F) Daily dry weight increase of roots for the same.*

N= the number of replicates included in the analysis.

All Boxplots in this document are as described in Figure 3.2.1.

Figure 3.2.3: Total root elongation (longest root) for shoots with zero new nodes (n=29), and one new node (n=8), across all treatments in C. nodosa *shoots. All Boxplots in this document are as described in Figure 3.2.1.*

C. nodosa showed much slower root growth than the two *Zostera spp*. Shoots had only 1 mm of new root growth (n=32) on average after 25 days (Figure 3.2.1.C) and were therefore very different to the control shoots for both length and dry weight (P < 0.01). In the 40 day experiment, thin roots had started to regrow on many of the shoots (Figure 3.2.1.C) and root length on shoots treated with 0 and 10 mg/L of IBA were no different to the control lengths (P > 0.13). Those with treatments of 3 and 5 mg/L had either no roots, or roots 50 % shorter on average than the control (P < 0.02). Differences in dry weight were not significant (P =0.08). Growth rates per day were also close to zero and not different between treatment groups (root elongation P = 0.43, dry weight increase P = 0.51; Figure 3.2.2.C & D). It was observed that any new roots on *C. nodosa* shoots infrequently grew from old node points (those roots had already been removed from), and that longer roots (mean length = 18 mm) were more likely to occur (P < 0.01), regardless of treatment, on shoots that had formed new node growth points. Shoots with no new node point had, on average, a longest root length of only 1 mm (Figure 3.2.3).

For *Z. marina* after both 14 and 18 day experiments, the root length of any of the treatments was identical to those of the control group (P = 0.17 & 0.12) representing normal root length in the collected material (Figure 3.2.1.E). Shoots with 0 to 10 mg/L IBA treatment obtained similar root dry weight to control shoots within 14 days (P > 0.13; Figure 3.2.1.F) but those treated at concentrations 3 and 5 mg/L had less (P < 0.05). In the 18 day experiment, treatment concentration 3 mg/L resulted in shoots with less root dry weight than the control and 0 mg/L treatment (P <0.03). Daily dry weight increase was less than half for IBA treated shoots than that of the 0 mg/L treatment (P = 0.02; Figure 3.2.2.F) and daily root elongation was less for the shoots on 3 and 10 mg/L treatments than for those on the 0 mg/L treatment (P < 0.01; Figure 3.2.2.E).

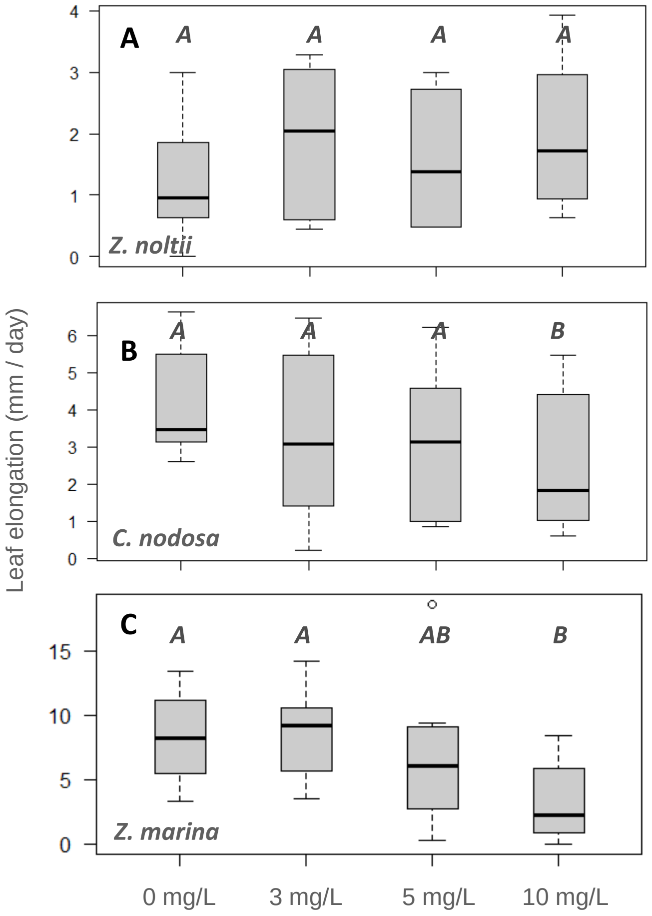


Figure 3.2.4: Daily leaf elongation rates of Z. noltii, C. nodosa*, and* Z. marina *for both experimental growth periods for the four IBA treatments. Different letters indicate significantly different groups within species. All Boxplots in this document are as described in Figure 3.2.1.*

Leaf elongation rates were in the order of 1-2 mm per day for *Z. noltii*, 2-4 mm per day for *C. nodosa*, and 3-10 mm per day for *Z. marina* (Figure 3.2.4). Growth rates were uniform across the treatment groups for *Z. noltii* (P = 0.80, Figure 3.2.4.A). For *C. nodosa*, there was a significant effect of the IBA treatment (P = 0.01) on leaf elongation with the 10 mg/L treatment group having less than half the leaf elongation than the 0 mg/L group (P = 0.01; Figure 3.2.4.B). There was also a significant effect of IBA concentration on leaf elongation in *Z. marina* (P = 0.01) and at high concentrations of 10 mg/L, leaf elongation was reduced by half compared to the treatment with 0 and 3 mg/L (P = 0.02; Figure 3.2.4.C).

Leaf number for *Z. noltii* increased significantly between the start and end of the experimental period (Table 3.2.i), *Z. marina* remained the same, and *C. nodoso* shoots had less leaves on average at the end of the experimental period, but not significanly so (Table 3.2.i).

Table 3.2.i: Pearson's Chi-squared analysis of differences in shoot leaf number between the start and end of the experimental period by species for all treatments. * designates significant P-values

Species	Average leaves (initial)	Average leaves (end)	χ^2	P- value
Z. noltii	3.1 (±0.9 SD)	3.9 (±1.6 SD)	18.34	0.00
C. nodosa	2.6 (±0.6 SD)	2.3 (±0.6 SD)	8.51	0.07
Z. marina	4.6 (±1.3 SD)	4.7 (±1.5 SD)	2.76	0.91

3.3- Artificial-substrate based planting units

Survival of shoots was not uniform across treatments (χ^2 $P < 0.03$) for any species. For *Z. marina* total death rate was 28 % over all treatments and was higher than could be expected in the fine artificial substrate, and lower than expected in the sod transplant treatment. For *C. nodosa* total death rate was 7 % consisting of 8 out of 11 shoots in a single sod treatment mesocosm dying. For *Z. noltii,* total death rate across all treatments was 23 %, and all in mesocosms impacted by herbivory. An unidentified herbivore removed all leaves from the shoots approximately 4-5 cm above the sediment surface. These shoots were observed to be damaged and it was observed that many later died during the experimental period.

Effective quantum yield (ΔF/Fm') for *Z. marina* and *C. nodosa* was affected by the factor time (*C. nodosa* $P < 0.01$; *Z. marina* $P < 0.01$) but not by substrate ($P > 0.05$). Average ΔF/Fm' in Z. *noltii* was uniform across time and substrate treatments ($P = 0.13$ & 0.29; Figure 3.3.1.A). *C. nodosa* shoots had the highest ΔF/Fm' in T0 compared to all later time periods ($P < 0.01$; Figure 3.3.1.B). For *Z. marina* there was large variability of ΔF/Fm' values across sample times. ΔF/Fm' values were similar ($P = 0.11$) at T0 and T2, but higher than at T1 and T3 ($P <0.05$; Figure 3.3.1.C)

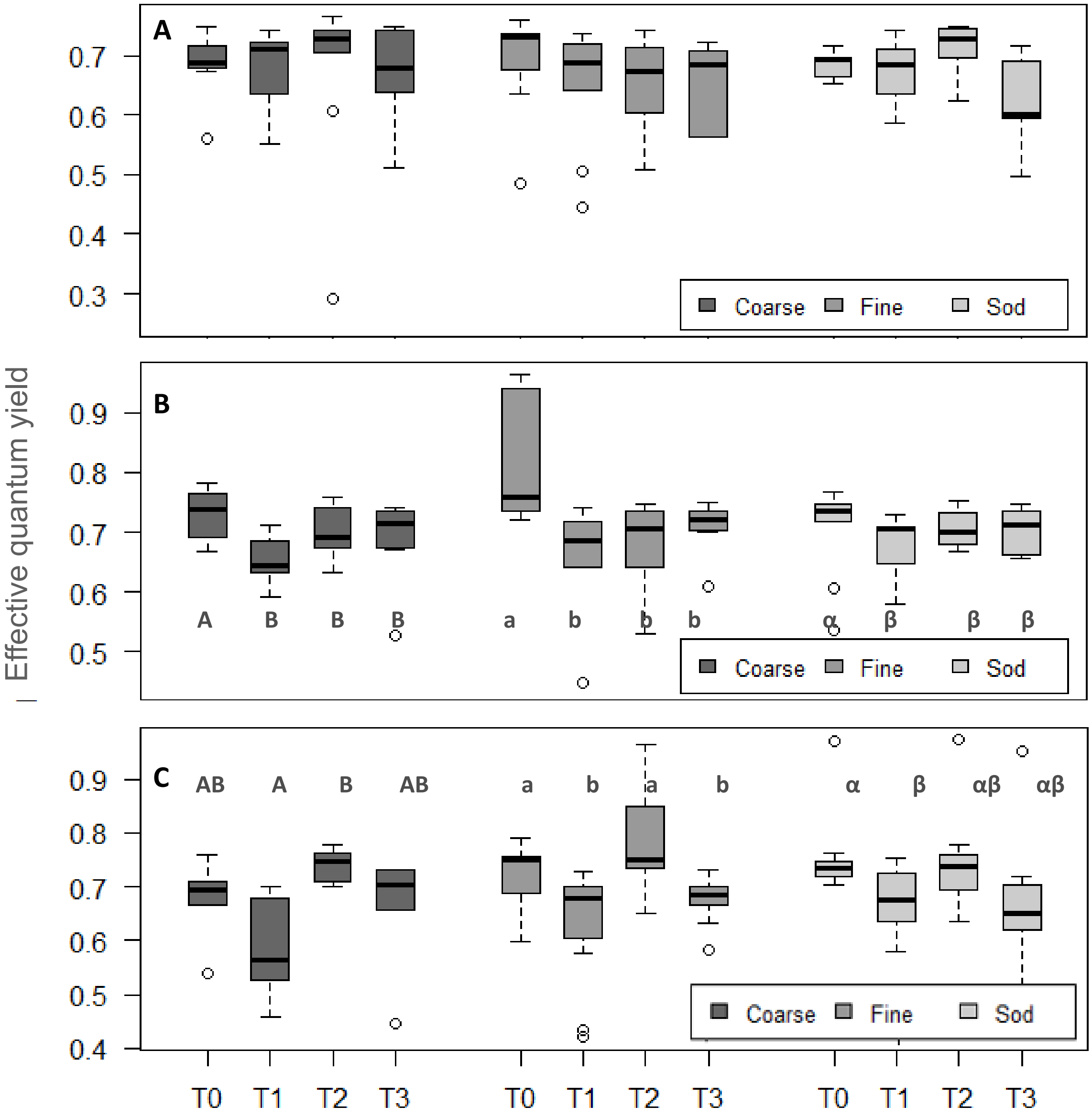


Figure 3.3.1: Effective quantum yield from RLCs measured by PAM fluorescence.

A) Z. noltii *effective quantum yield for shoots in treatment substrates (Coarse, fine and sod) over four measurement times (5 weeks).*

B) C. nodosa *effective quantum yield for shoots in treatment substrates (Coarse, fine and sod) over four measurement times (8 weeks).*

C) Z. marina *effective quantum yield for shoots in treatment substrates (Coarse, fine and sod) over four measurement times (5 weeks).*

For each species, temporal effects were also tested by pairwise analysis within substrate, and groups that do not share letters are significantly different. All Boxplots in this document are as described in Figure 3.2.1.

For the other growth and photosynthetic indicators of *Z. marina* there was no significant differences regardless of the substrate used (Table 3.3.i). Temporally, parameter RLC α was less in T1 than in both T2 and T3 ($P < 0.04$) and ETRmax was higher at T0 than at both T1 and T3 ($P < 0.04$; Figure 3.3.2) and T2 and T3 also varied ($P = 0.02$).

Table 3.3.i: Results of PERMANOVA analysis for growth and health indicators of *Z. marina* shoots in different substrates across 8 weeks (4 sampling periods), * indicates significant results. N=68-71.

Indicator	Factor	Pseudo-F	DF	P (perms)
Leaf length				
	Substrate	0.61	2	0.54
	Time	1.89	3	0.14
	Substrate x Time	1.66	6	0.14
RLC α				
	Substrate	0.20	2	0.82
	Time	3.80	3	0.01*
	Substrate x Time	0.25	6	0.96
RLC ETRmax				
	Substrate	0.32	2	0.72
	Time	3.43	3	0.02*
	Substrate x Time	0.46	6	0.84
RLC Ik				
	Substrate	0.06	2	0.94
	Time	2.69	3	0.05
	Substrate x Time	0.50	6	0.81

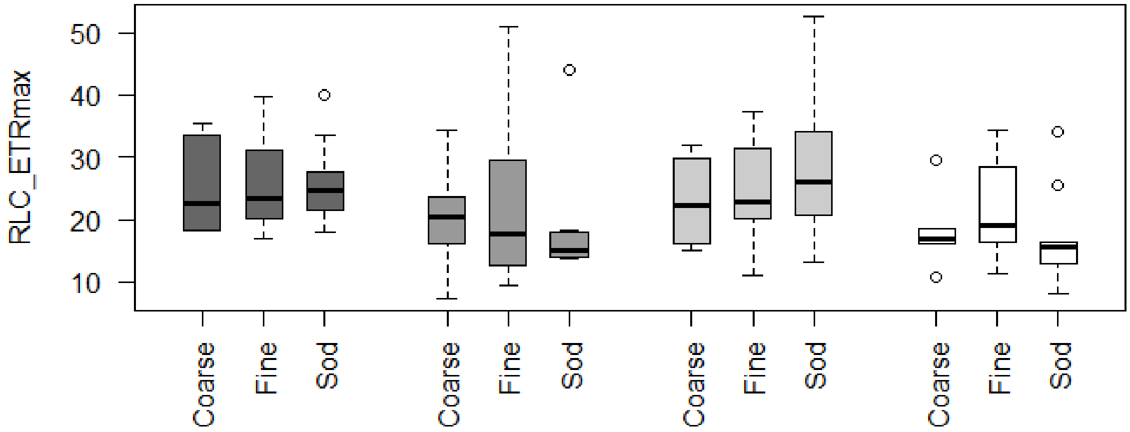


Figure 3.3.2: ETRmax values for Z. marina *shoots on three substrates (coarse, fine and sod) over the four measurement periods L to R: T0, T1, T2 and T3. All Boxplots in this document are as described in Figure 3.2.1.*

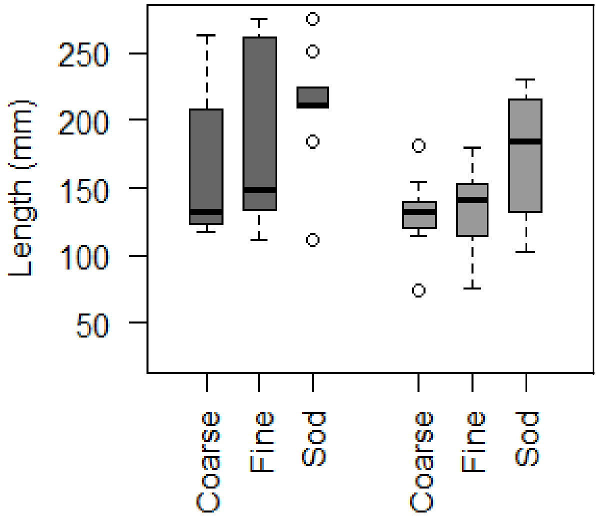


Figure 3.3.3: Leaf length of the longest leaf per shoot for T0 and T1 sample periods for Z. noltii *shoots in three different substrates (coarse, fine and sod). All Boxplots in this document are as described in Figure 3.2.1.*

Z. noltii suffered badly from herbivory (see images in Appendix 6) prior to sample periods T2 and T3 and many shoots were < 5cm long. The fine substrate samples were most affected, with almost all replicates damaged. For this reason, length and number of leaves were not analyzed for periods T2 and T3 for this species. In both T0 and T1, the coarse substrate shoots were significantly shorter than the sod substrate shoots (P< 0.01; Figure 3.3.3). This likely indicates that shoots were longer on average in the sod transplants than those selected for the fine substrate treatment and remained longer over the 2 weeks between T0 and T1 sampling.

Table 3.3.ii: Results of PERMANOVA analysis for growth and health indicators of *Z. noltii* shoots in different substrates across 5 weeks (4 sampling periods), * indicates significant results. N=68-70

Indicator	Factor	Pseudo-F	DF	P (perms)
RLC α				
	Substrate	0.61	2	0.70
	Time	0.93	3	0.48
	Substrate x Time	1.31	6	0.15
RLC ETRmax				
	Substrate	8.61	2	<0.01*
	Time	1.26	3	0.29
	Substrate x Time	1.13	6	0.35
RLC Ik				
	Substrate	5.99	2	<0.01*
	Time	0.57	3	0.64
	Substrate x Time	0.41	6	0.88

For parameters derived from the RLCs, *Z. noltii* shoots had significantly different ETRmax and Ik values depending on substrate (Table 3.3.ii). ETRmax and Ik for sod transplanted shoots was higher than for the artificial substrate based transplant treatments (Pairwise P < 0.01 for both variables; Figure 3.3.4.A). Values of α did not vary over substrate or time (Table 3.3.i).

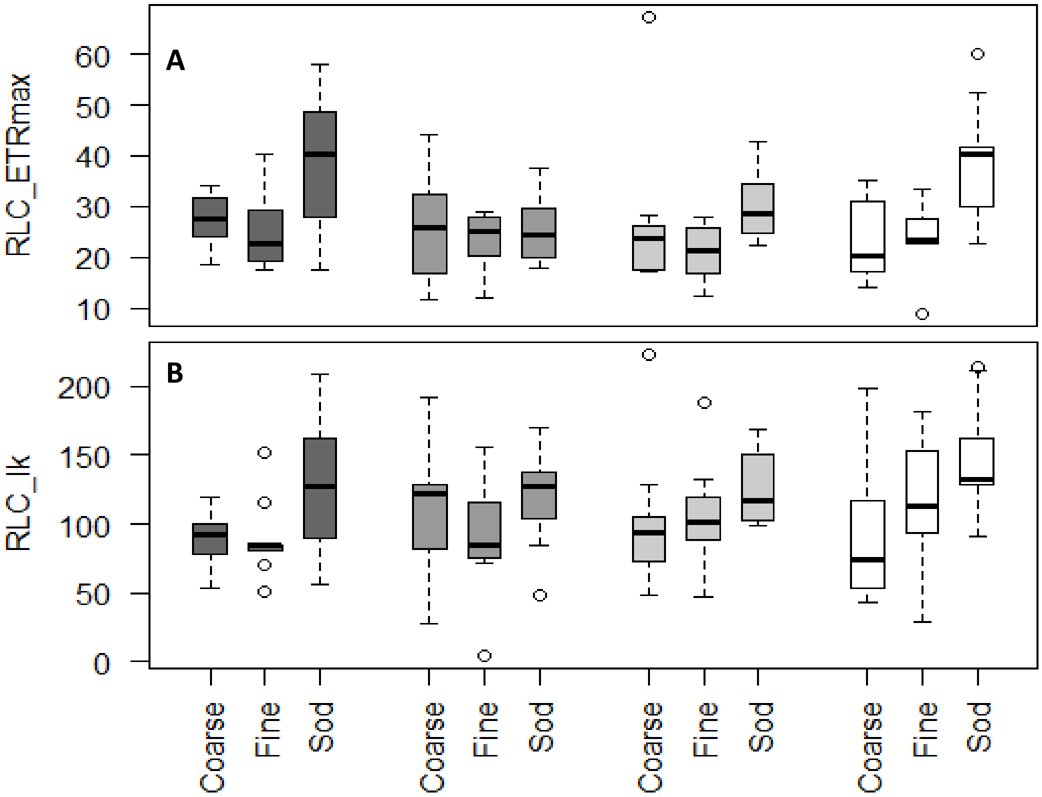


Figure 3.3.4: ETRmax and IK parameters from RLCs measured by PAM fluorescence.

A) ETRmax shoots of Z. noltii *on three substrates recorded at four different time periods for (L:R) T0, T1, T2 and T3 sample periods by transplant substrate.*

B) Ik parameter values for shoots of Z. noltii *on three substrates recorded at four different time periods for (L:R) T0, T1, T2 and T3 sample periods by transplant substrate.*

All Boxplots in this document are as described in Figure 3.2.1.

The leaf length of *C. nodosa* shoots was not different among treatments, but there were significant temporal differences (Table 3.3.iii) as shoots in T3 were 40 - 50 % longer than in earlier sample periods T0, T1 and T2 (P < 0.01; Figure 3.3.5).

ETRmax and Ik values showed temporal difference (Table 3.3.iii) but not related to substrate (Table 3.3.iii). For T3, shoots had lower ETRmax and Ik values than in all previous measurement times (P <0.01; Figure 3.3.6 & 3.3.7). Other factors did not appear to be different over time or among treatments (Table 3.3.iii).

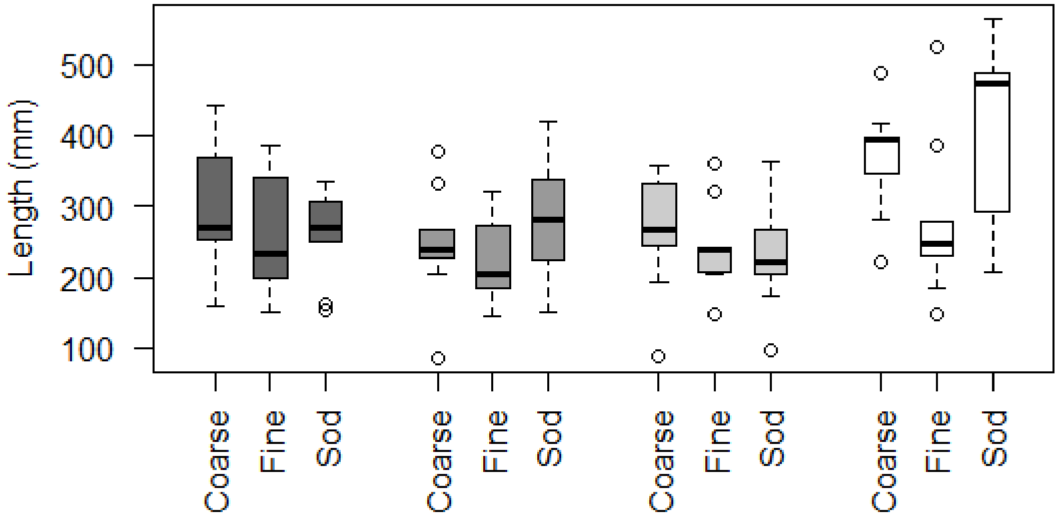


Figure 3.3.5: Length of the longest leaf of shoots of C. nodosa *on three substrates (coarse, fine and sod) over four measurement periods (From L to R: T0, T1, T2, T3) two weeks apart. All Boxplots in this document are as described in Figure 3.2.1.*

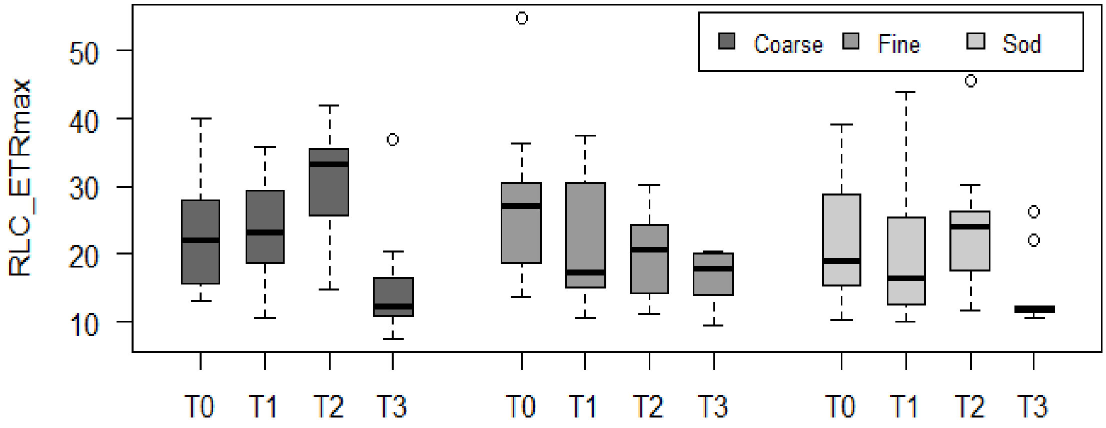


Figure 3.3.6: Maximum ETR values for shoots of C. nodosa *on three substrates (coarse, fine and sod). Recorded at four measurement periods 2 weeks apart. All Boxplots in this document are as described in Figure 3.2.1.*

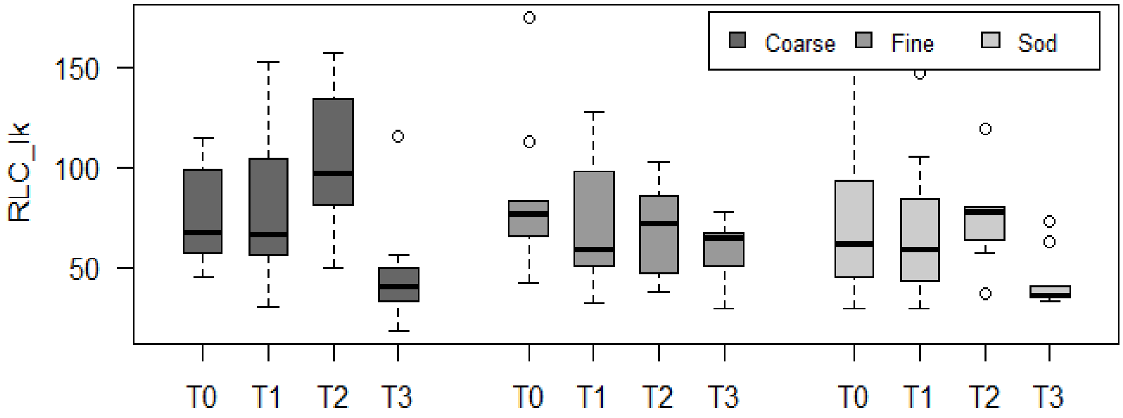


Figure 3.3.7: Ik parameter values for shoots of C. nodosa *on three substrates (coarse, fine and sod) recorded at four different time periods 2 weeks apart. All Boxplots in this document are as described in Figure 3.2.1.*

Table 3.3.iii: Results of PERMANOVA analysis for growth and health indicators of *C. nodosa* shoots in different substrates across 8 weeks (4 sampling periods), * indicates significant results. N = 68-70

Indicator	Factor	Pseudo-F	DF	P (perms)
Leaf length				
	Substrate	2.34	2	0.10
	Time	7.11	3	<0.01*
	Substrate x Time	0.07	6	0.99
Leaf Number				
	Substrate	2.25	2	0.11
	Time	0.41	3	0.75
	Substrate x Time	0.52	6	0.79
RLC α				
	Substrate	0.23	2	0.80
	Time	1.74	3	0.16
	Substrate x Time	1.05	6	0.40
RLC ETRmax				
	Substrate	0.96	2	0.39
	Time	6.12	3	<0.01*
	Substrate x Time	1.93	6	0.08
RLC Ik				
	Substrate	1.27	2	0.28
	Time	6.51	3	<0.01*
	Substrate x Time	2.19	6	0.05

4- Discussion and conclusions

4.1- *In vitro* micropropagation of leaf, stem, and rhizome.

Micropropagation is widely used in the rapid clonal propagation of terrestrial plants, and for many species has proven to be simple and cost-effective (Loberant & Altman, 2010). Seagrass micropropagation has many challenges- the removal of competitive or detrimental organisms (functionally axenic cultures); the need for a fully immersed environment; of providing suitable nutrition, particularly at the leaf boundary in liquid media; of maintaining osmotic balance; and of providing suitable light conditions.

Laboratory cultivation in tanks was first achieved for most seagrass genus in the 1980s (Lewis, 1990) but micropropagation of *in vitro* cultures has been much more difficult to achieve, with the main obstacles being the suitable sterilization of selected material and that many initiated cultures persisted only over short growth periods of one to two months (Moffler & Durako, 1984). This study has demonstrated that *Zostera* ssp. selected from southern Portugal can survive on a monophasic media of seawater for up to two months in small vessels, though survival rates for *in vitro* cultivated shoots dropped quickly after 10 days since initiation. This is consistent with past research where similar liquid media was used in larger tanks to maintain *Z. noltii* shoots over 60 days (Cabaço, Alexandre & Santos, 2006). Plants that survived past 25 days were also alive after 60 days, surviving multiple changes of media and physical handling. This suggests that the nutritional content of the media provided was adequate to sustain the survival of the shoots.

Z. marina had a much lower survival rate than *Z. noltii*, and no shoots survived past 22 days since initiation. This suggests that *Z. marina* deaths in the first 2 weeks may have been related to the initiation or decontamination procedure that caused a decline in the shoot health, leading to their eventual death. Damage of tissues during decontamination is a particular problem for seagrasses, as they have a much thinner protective cuticle compared to terrestrial plants (Kuo & den Hartog, 2006).

The decontamination procedures used were considered adequate to remove competitive organisms, which should be visually identifiable in the culture medium if successfully competing for space and resources (Bird, Johnson & Jewett-Smith, 1998). The possibility of - and the need for - truly axenic cultures for micropropagation is doubtful (Herman, 1990) and is based on outdated assumptions that all other organisms are detrimental to the growth or survival of the target plant. It has since been demonstrated that associated microbial communities are both specific (Cúcio *et al.*, 2016) and vital to the development of seagrasses (Tarquinio *et al.*, 2019).

The alternative explanation is that, despite a lack of visual indications, there was contamination by detrimental organisms in the growth media that lead to the observed shoot decline and death. This is supported by the continued elongation of shoots after initiation, suggesting that they were healthy enough to continue actively growing, rather than already undergoing senescence. The presence of a smell like that of fermenting vegetables may indicate the presence of yeast, a common contaminant in plant tissue culture (Leifert, Richie & Waites, 1991). Yeasts can be very detrimental to plant health as they are known to change media pH, compete for carbohydrate sources in the media, and can produce fermentation products such as ethanol and acetic acid which are both phytotoxic (Leifert, Richie & Waites, 1991). Further decontamination treatments, including using low concentrations of ethanol, or higher concentrations of NaClO, and indexing of culture medium may assist in reducing yeast contamination of the cultures and identify if yeasts have been eliminated (Leifert, Richie & Waites, 1991). Additionally, regular pH testing of the media could have helped to identify if changes were occurring that made the growing environment unfavorable to the seagrass shoots.

Living shoots in propagation failed to produce new shoots from axillary buds, nor did they increase the number of branches or nodes. The addition of cytokinin BAP in growth media at concentrations up to 3 mg/L did not induce growth or development of these organs either alone or in the presence of auxin IBA. These are vital organs to develop if the goal of clonal reproduction via micropropagation is to be achieved. The cytokinin BAP is known to increase the number of branches and nodes of many species (Loberant & Altman, 2010), including in seagrasses (Koch &

Durako, 1991; Bird *et al.*, 1996), but this did not occur for the two *Zostera* species tested. Using these results as a baseline, future studies could examine the effects of altering decontamination procedures to reduce yeast contamination. Alternative light and temperature regimes may also be trialed to determine if they have a positive impact on survival and growth rates of shoots. Reducing the amount of sucrose in the media may also have a positive effect, as this reduces the energy source available to competitive yeasts and bacteria (Kozai, 1991), and may be possible without compromising plant health.

Once healthy shoots have been established, there is a suite of alternative cytokinins that are commonly used in plant tissue culture that may increase the production of required branches and nodes (Koch & Durako, 1991). *Ruppia maritima,* for example, was cultivated to *ex vitro* stage using ½ strength MS media with artificial seawater and 10 g/L of sucrose and additional cytokinin 6-(γ,γ-Dimethylallylamino)purine (2iP) which is commonly used in tobacco and soy bean culture (Bird, Johnson & Jewett-Smith, 1998) but the rate of clonal replication from this treatment was not stated.

In conclusion, this study shows that *in vitro* micropropagation should be possible, but there are two main challenges to be overcome. First, we need further development of decontamination procedures that eliminate detrimental, but not necessarily all, non- target organisms, to increase the survival on *Z. marina* initiates. Secondly, that while the specific cytokinin BAP may increase the number of branches and nodes of some seagrasses the type of cytokinin appears to be species specific, and BAP is not useful for propagation of *Z. marina* and *Z. noltii.* Other common cytokinins should be tested to determine if these accelerate clonal growth.

4.2- Adventitious rooting trials

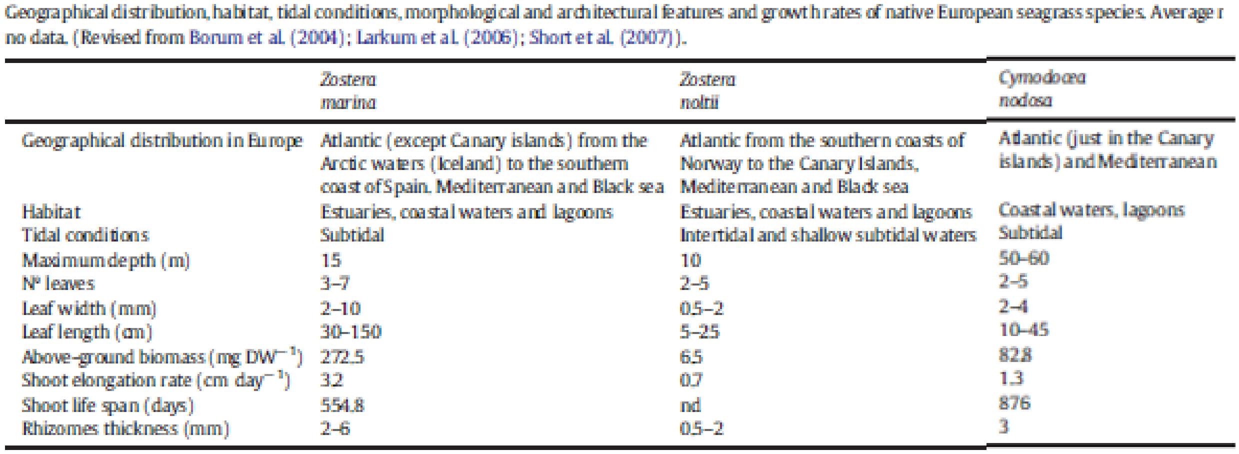
Reduced disturbance of roots and higher root mass are considered to be the great advantages of sod transplantation techniques. In this study, *Z. marina* and *Z. noltii* which had had their roots removed were able to regrow root length to the same as the control values within the experimental duration. Root development was very rapid and reached natural lengths in as few as 2 to 3 weeks in protected (mesocosm) growth conditions. Natural root length can differ a lot. For example, *Z. marina* roots can be up to 20 cm long (Borum & Greve, 2004), but the plants collected from the Ria Formosa in this sampling period had roots no longer than 9-10 cm. *Z. noltii* root length in the Ria Formosa is commonly between 30 and 55mm (Cabaço, Machás & Santos, 2009), and total root length in this study was consistent with this.

For the two *Zostera* species growth of adventitious roots originated from both new nodal growth points and from those where roots had previously been removed. In both *Zostera* species, some shoots without IBA treatment and roots removed achieved larger regrowth than the length and dry weight of the control samples. Rapid growth of adventitious roots is a common plant response to various stressors, such as nutrient or heavy metal stress, and particularly to wounding stress (De Klerk, Van der Krieken & De Jong, 1999; Steffens & Rasmussen, 2016). There are a range of wounding-related compounds that are produced by plants in response to physically cutting of tissues that have been shown to play a role in both defense and in root development, and enhance the response of plant tissues to other growth hormones (De Klerk, Van der Krieken & De Jong, 1999). It is unclear if this was a factor in this experiment, but it may have contributed to this larger growth in damaged, compared to undamaged, shoots.

For *C. nodosa*, roots on the control shoots were much shorter than the proposed maximum root length for *C. nodosa* of 35 cm (Borum & Greve, 2004). This may be a result of the collection method of the transplants, where only a maximum sod depth of 8-10 cm is collected, resulting in the removal of some of the roots during collection. More likely, the 3-9 cm roots represent the current natural root length in the area of collection. There was no obvious damage to the main root observed, and the values measured are consistent with those recorded in the Ria Formosa in past studies (Cunha & Duarte, 2007). After roots were removed in this study, root development was very slow. Many shoots did not re-grow any roots within 25 days. Additionally, roots were very rarely observed growing from nodes where roots had been damaged or removed. Roots only developed from new nodes on the rhizome, which is consistent with their normal growth habit of growing a single root per rhizome segment (Borum & Greve, 2004). When they grew, roots on the zero IBA and highest concentration IBA treatments were able to meet the naturally small lengths of the control group within 40 days. This slow rate of recovery is most likely because shoots need to first develop a new node on the rhizome, then produce roots from this new node.

The good news for restorers is that these two *Zostera* species can quickly re-establish root mass even if roots are damaged or completely removed during translocation. The shoots continued to be healthy with low death rates and strong growth of existing and new leaves. Leaf numbers did not decline after transplantation and were in the same range as reported in prior studies (Table 4.2.i). Leaf turn over and elongation in shoots with their roots removed matched those of the control group with intact roots. Leaf number increased for *Z. noltii* over the experimental period as the roots regrew. The number of leaves per shoot was on the lower end of the previously recorded range for *C. nodosa* (Table 4.2.i) but treatment groups but did not differ from control shoots. Daily leaf elongation rates for all species in this study appeared low compared to other reported figures, but these are difficult to compare; in this experiment leaf elongation was measured only for the newest leaf, whereas it is more common to record elongation as the sum for all leaves per shoot (Short & Duarte, 2001; Ondiviela *et al.*, 2014). Unlike terrestrial plants, seagrass species are able to uptake nitrogen through their leaves (as nitrate) as well as their roots (ammonium), and that this may be vital to their success (Stapel *et al.*, 1996;

Table 4.2.i: Geographical distribution, habitat, tidal conditions, morphological and architectural features and growth rates of native European seagrass species. (Ondiviela *et al.*, 2014)



Geographical distribution, habitat, tidal conditions, morphological and architectural features and growth rates of native European seagrass species. Average r
no data. (Revised from Borum et al. (2004); Larkum et al. (2006); Short et al. (2007)).

	Zostera marina	*Zostera noltii*	*Cymodocea nodosa*
Geographical distribution in Europe	Atlantic (except Canary islands) from the Arctic waters (Iceland) to the southern coast of Spain. Mediterranean and Black sea	Atlantic from the southern coasts of Norway to the Canary Islands, Mediterranean and Black sea	Atlantic (just in the Canary islands) and Mediterranean
Habitat	Estuaries, coastal waters and lagoons	Estuaries, coastal waters and lagoons	Coastal waters, lagoons
Tidal conditions	Subtidal	Intertidal and shallow subtidal waters	Subtidal
Maximum depth (m)	15	10	50–60
Nº leaves	3–7	2–5	2–5
Leaf width (mm)	2–10	0.5–2	2–4
Leaf length (cm)	30–150	5–25	10–45
Above-ground biomass (mg DW^{-1})	272.5	6.5	82.8
Shoot elongation rate (cm day^{-1})	3.2	0.7	1.3
Shoot life span (days)	554.8	nd	876
Rhizomes thickness (mm)	2–6	0.5–2	3

Vonk *et al.*, 2008; Papenbrock, 2012), particularly in the case where roots are damaged and likely have reduced function.

Treatments of auxin IBA are widely used in terrestrial horticulture to promote root initiation and development (Frick & Strader, 2018), but for the species tested, treatments of up to 10 mg/L did not increase the root development rate. Higher concentrations may inhibit *Zostera* root development compared to no IBA treatment, as root length and dry weights recorded were less than treatment with no IBA. Auxins, such as IBA, are known to induce leaf senescence in high concentrations (Yamaguchi *et al.*, 2010; Shamsi, *et al.*, 2019) and the application concentration of 10 mg/L appeared to inhibit the leaf elongation in shoots of *Z. marina* and *C. nodosa*.

During the cultivation periods, experimental transplanted shoots died, but death rates did not differ among treatments or from that of shoots planted with their roots left intact. It was likely that there were other factors (such as physical damage or other health factors) impacting the individual plants that died, and that deaths could not be attributed to the treatments administered. It appears that some losses should be expected regardless of method, and that root removal does not increase this rate in the short term.

These three species of seagrasses studied did not require intact root systems for their short-term survival. In the low energy mesocosm environment- while experiencing fluctuations in temperature, light, and salinity- survival and health of the transplanted shoots was high. They also appear to be able to simultaneously direct energy to both root and leaf growth. To do so this will likely impact rhizome-stored carbohydrate content (Pirc, 1989; Govers *et al.*, 2014) and this should be investigated in future work. If stored starches are an important energy source during post-transplant acclimation, selecting transplant times when plants have high levels may be an important factor influencing their transplant success.

Problems of root damage during harvest and transport is often a concern for restorers (Christensen, Almela & Diekmann, 2004), but this risk factor for *Zostera* species in suitably chosen restoration locations is likely not high. Based on the results of this study, restorers should expect that damage to or removal of roots during harvest and transplantation will not, by itself, impact on survival or growth outcomes in the first 1-2 months after transplanting. Additionally, regardless of root damage occurring, they should expect a mortality rate of ≈ 20 % of transplants of *Z. marina, Z. noltii,* and *C. nodosa* even in favorable and protected conditions. This rate may also vary by harvest site and donor population selection. What is likely lost is the physical/ structural benefits of an intact root system, which can assist in anchoring the shoots in the new location and prevent their physical removal, but this can be countered by other methods of anchoring.

Important for restorers dealing with *C. nodosa* transplants, roots are much slower to regrow, and establishment of new roots required the growth of a new node point for their production. In healthy plants and suitable conditions survival may still be high, but to give these shoots the greatest chance of establishment extra care should be taken to ensure that roots are not damaged during transplantation.

Assuming that the growth conditions in the receiving location are favorable, *Zostera* shoots with damaged roots can be expected to establish quickly after transplanting, and to regrow normal root length and weight, so long as they can be stabilized and physically anchored using any method for the initial growth period of up to one month. The transplanted shoots also appear to maintain survival and health outcomes regardless of the damage to (in this case, complete removal of) their roots. This information may assist restoration decision-makers considering what transplantation methods to use and whether to conduct transplantation activities near to times of disturbance, such as predicted periods of storms and high wave energies. IBA application as a treatment method is not considered to be a beneficial addition to restoration methodologies for these three species as it is an additional disturbance, has a financial and time cost to apply, does not increase post treatment root growth, and may inhibit leaf growth at higher concentrations.

4.3- Artificial-substrate based planting units

In aiming to compare sod and textile artificial substrate methods, this study intended to control and uniformize as many environmental conditions as possible to assist in identifying the role roots play in the success of seagrass transplantation. All species were expected to respond differently to the transplantation as they have different morphologies, life history, main reproductive strategy, range and habitat requirements (Borum & Greve, 2004; Ondiviela *et al.*, 2014). Shoots in textiles were transplanted onto the natural sediments they were collected from, preserving the sediment grain size, associated organic matter, and biota similar to the sods. Thus, differences between treatments in the earlier monitoring times should reflect disturbance or damage caused by the transplantation method - the individualising of shoots and the damage to root and rhizome- and later differences between treatments should reflect differences in adaptability and recovery among transplant methods.

Comparing sods to artificial substrates in the transplantation trials, death rates of shoots were difficult to relate to treatment. This was the case particularly for *Z. noltii*, where most deaths were associated with a damaging herbivory event 3 weeks into the experimental period, which impacted mesocosms with fine substrates more than other treatments. It was immediately noticed that the coarse weave mesh substrate would not be suitable for *Z. noltii* shoots in an environment with water movement. Shoots were dislodged very easily by water movement inside the mesocosm, even when placing equipment inside the mesocosms for measurements. The fine weave substrate and the sod transplants did not have this problem. This highlights the importance of considering morphological, as well as the usual time, equipment and cost factors, when selecting appropriate methodologies for transplantation projects.

Consistently lower ETRmax and Ik values for *Z. noltii* shoots in the artificial substrates suggests that sod transplant is a better method for this species. However, this result is difficult to support. As the prior experiment showed, transplantation and root damage did not seem to affect survival and health outcomes for this species. Additionally, we would expect stress to be highest closest to the transplantation time but there was no evidence that transplantation was the stressor, and no sign of recovery over time. Notably, *Z. noltii* suffered from uncontrolled factors during the experimental period, including dislocation, herbivory and high death rates. Leaf numbers could not be counted on many shoots due to herbivory, and shoot size was reduced to less than a quarter of the size of non-damaged shoots. In the case of *Z. noltii*, either fine mesh substrates or sods could be effective methods, and thus the selection process becomes more difficult.

Considering the other species*, C. nodosa* death rates were very low and also appear to be linked to a non-treatment-based source. Healthy green shoots died over one week period in a single (sod treatment) mesocosm. The three treatment types all adequately "anchored" the shoots during the whole experiment. Health and stress parameters were temporally variable but the different transplant treatments performed equally. All transplant methods tested produced successful transplants inside the mesocosm environment. A decline in ETRmax and Ik on the final measurement time, taken in isolation, would indicate a decline in health. Contrary to this, the shoots were observed to visually larger and healthier on this day, and total shoot length was as much as 10 cm longer than on the previous sampling period. These values may have been indicative of light or heat conditions, as the experimental period ended in late July, the start of summer, and when high water temperatures were recorded (Appendix 2). Transplant type did not appear to determine survival or health of transplants for this species, and can thus be selected based on other factors, such as time, financial cost, and risk.

Z. marina shoots were well anchored in the sod and both artificial substrates. Threading the shoots into the artificial substrates was easy and fast. The total death rate of 28 % was similar to that of the adventitious rooting experiment transplants, but the proportion of deaths was not uniform across the transplant methods. Higher mortality rates in the fine mesh substrate transplants suggest that there was damage to the shoots during insertion. This is supported by the timing of these deaths, which all occurred in the first 2 weeks after transplantation. The lower mortality rate in the sod transplants indicates that this method is very suitable for transplantation of *Z. marina,* but the coarse mesh substrate also performed well. Highest effective quantum yield was measured on the day of transplantation, for both this species and *C. nodosa*. This is unusual, as this should be the period of the highest stress, and is inconsistent with transplantation field studies (Paulo *et al.*, 2019). This parameter may be more representative of light conditions or may indicate an adaptation period and new lower productivity as shoots adjust to the new environment of the mesocosm, rather than indicating transplantation related stress. Care should be taken in using photosynthetic parameters as the main or only way of assessing transplant success, regardless of its relative ease and increasing popularity.

Historically, free planted single shoot transplants have high failure rates compared to sods (Suykerbuyk *et al.*, 2016; van Katwijk *et al.*, 2016) but the attachment of these to the textile substrate provides structural support in the form of a planting unit. This can then be weighed, to anchor the plants during establishment, a factor demonstrated to improve success in transplantation projects (van Katwijk *et al.*, 2016). For example, shoot density of *Z. noltii* in transplant units is not related restoration success (Suykerbuyk *et al.*, 2016) but patch density and larger spatial extent does (Suykerbuyk *et al.*, 2016; van Katwijk *et al.*, 2016). This fast growing species has very high horizontal rhizome elongation rates of up to 1.6 m/year (Cunha, Duarte & Krause-Jensen, 2004), allowing transplanted shoots to increase spatial coverage and densities after transplantation.

Z. noltii can grow naturally in very high natural densities as high as 5400 shoots per square meter (Curiel *et al.*, 1996). During material collection for this study, densities of up to 3200 shoots per square meter were recorded in the Ria Formosa. Since this is the case, there is a large amount of donor material available in a small area. If transplanted as sods, large areas of meadow must still be harvested to plant the large areas of meadow needed to spread risk and increase transplant success. These densities could be reduced. Densities of 530 to 660 per square meter were used in this experiment, consistent with the natural densities found in the harvested sods. If the shoots are inserted into textile substrates, rather than planted as sods, this would produce 4 to 6 times more spatial coverage with the same number of seagrass shoots. These densities should be tested and refined in the field for optimal success.

Despite *Z. noltii* being the most locally abundant of the species in this study (Cunha, Assis & Serrão, 2009) ongoing global and local threats necessitate the need to rapidly move away from sod harvesting for restoration projects. It is disruptive to donor meadows- which should be protected- and inefficient in many other ways. The other two species are either slower growing (Cunha, Duarte & Krause-Jensen, 2004), less abundant (Cunha, Assis & Serrão, 2009), or locally or globally threatened (Cunha, Assis & Serrão, 2013; Short *et al.*, 2011). Thus, the importance of using harvested material wisely is even more pressing. *C. nodosa* meadow density is often up to 1100 shoots but it has been shown that meadow coverage may be maintained even as shoot density has decreased (Tuya, *et al.*, 2014). Additionally, its horizontal growth rate can be very rapid, up to 2 meters per annum (Cunha, Duarte & Krause-Jensen, 2004), so the potential to plant in densities thinner than those in harvested sods, by using textile substrates, has great potential. By spreading shoots over larger areas while maintaining coverage, the required conditions for short term success may be met and the species naturally fast clonal reproduction can then increase densities in the medium term. This makes it another great test species for the creation of artificial substrate planting units. *Z. marina* is a popular target species for restoration efforts (Lee & Park, 2008; Cunha *et al.*, 2011; Paulo *et al.*, 2019), but it has the also the least abundant potential donor meadows (Cunha, Assis & Serrão, 2009) and the slowest vertical expansion (Cunha, Duarte & Krause-Jensen, 2004) of these tested species.

Artificial substrate units using harvested shoots should be developed and optimised only as an intermediary step to the development of planting units created from propagated seagrass shoots. Any propagation method developed will require labour, but the ethical question of continuing to threaten remnant meadows to create new ones needs to be rapidly addressed. Proper design and monitoring of artificial substrate planting units, created with harvested shoots, must be conducted to assess the labour and financial costs and feasibility of seagrass propagation.

Since root damage and transplant stress of the different methods has now been shown not to create variation between these methods, other characteristics of sod techniques - that the sediment grain size, nutrients, and microbiome associated with the shoots or sediments is also transferred- requires further investigation. Other studies have aimed to test below-ground integrity as a factor by planting bare-root shoots vs. sod transplants in the field (Suykerbuyk *et al.*, 2016), but by failing to consider that transplant target sites often have much different sediment characteristics to the donor site, and thus to the sods, they succeeded only in demonstrating that shoot anchoring of some type is vital. Now the ability of these species to survive and recover from root damage up to complete removal has been confirmed, this factor can also be eliminated as a possible advantage of sod methods. Additionally, I have demonstrated that the stress of inserting shoots into textiles, *per se*, does not lead to lower survival and health outcomes than using sod methods, so long as the appropriate mesh size is selected for the species morphology.

While performance may vary in the open environment, this experiment showed that where factors sediment type, associated organic and biological components, exposure, and donor population are controlled, transplanted shoots perform similarly well and recover well from transplantation stress in both artificial substrate planting units and in sod units. The next step in creating successful artificial planting units is understanding the contribution of these factors to transplant survival. This will help to establish if artificial planting units can become a suitable alternative to sod transplants in areas where sod type planting methods have been successful. Further investigation is needed to assess the feasibility of using artificial planting units rather than sod methods, which may not be achievable where inadequate donor beds are available or would be threatened by harvesting.

References

Alberto, F., Mata, L., & Santos, R. (2001). Genetic homogeneity in the seagrass *Cymodocea nodosa* at its Northern Atlantic limit revealed through RAPD. *Marine Ecology Progress Series*, *221*, 299–301. https://doi.org/10.3354/meps221299

Alexandre, A., Santos, R., & Serrão, E. A. (2005). Effects of clam harvesting on sexual reproduction of the seagrass *Zostera noltii*. *Marine Ecology Progress Series*, *298*, 115–122. https://doi.org/10.3354/meps298115

Alexandre, A., Cabaço, S., Santos, R., & Serrão, E. A. (2006). Timing and success of reproductive stages in the seagrass *Zostera noltii*. *Aquatic Botany*, *85*(3), 219–223. https://doi.org/10.1016/j.aquabot.2006.05.002

Alistock, S., & Shafer, D. (2006). *Applications and limitations of micropropagation for the production of underwater grasses*: *Submerged Aquatic Vegetation, ERDC/TN SAV-06-1* (6)

Aquation. (2017). *A Guide to Analysing Rapid Light Curves*. Retrieved March 8, 2019, from https://aquation.com.au/white-papers/a-guide-to-analyzing-rapid-light-curves/

Baker, N. R. (2008). Chlorophyll Fluorescence: A Probe of Photosynthesis *In Vivo*. *Annual Review of Plant Biology*, *59*, 89–113. https://doi.org/10.1146/annurev.arplant.59.032607.092759

Balestri, E., & Bertini, S. (2003). Growth and development of *Posidonia oceanica* seedlings treated with plant growth regulators: Possible implications for meadow restoration. *Aquatic Botany*, *76*(4), 291–297. https://doi.org/10.1016/S0304-3770(03)00074-3

Bayraktarov, E., Saunders, M. I., Abdullah, S., Mills, M., Beher, J., Possingham, H. P., Mumby, P. J., Lovelock, C. E. (2016). The cost and feasibility of marine coastal restoration. *Ecological Applications*, 26(4), 1055-1077. https://doi.org/10.1890/15-1077.1

Berković, B., Cabaço, S., Barrio, J. M., Santos, R., Serrão, E. A., & Alberto, F. (2014). Extending the life history of a clonal aquatic plant: Dispersal potential of sexual and asexual propagules of *Zostera noltii*. *Aquatic Botany*, *113*, 123–129. https://doi.org/10.1016/j.aquabot.2013.10.007

Bhatia, S., & Dahiya, R. (2015). Concepts and Techniques of Plant Tissue Culture Science. In *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*, 121–156. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802221-4/00004-2

Bhatia, S., & Sharma, K. (2015). Micropropagation. In *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*, 361–368. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802221-4.00011-X

Billingham, M. R., Reusch, T. B. H., Alberto, F., & Serrão, E. A. (2003). Is asexual reproduction more important at geographical limits? A genetic study of the seagrass *Zostera marina* in the Ria Formosa, Portugal. *Marine Ecology Progress Series*, *265*, 77–83. https://doi.org/10.1007/978-3-319-06419-2_84

Bird, K. T., & Jewett-Smith, J. (1994). Development of a medium and culture system for *in vitro* propagation of the seagrass *Halophila engelmannii*. *Canadian Journal of Botany*, *72*(10), 1503–1510. https://doi.org/10.1139/b94-185

Bird, K. T., Jewett-Smith, J., Fonseca, M. S. (1994). Use of *in vitro* propagated *Ruppia maritima* for Seagrass Meadow Restoration. *Education*, *10*(3), 732–737.

Bird, K. T., Brown, M. S., Henderson, T. T., O'Hara, C. E., & Robbie, J. M. (1996). Culture studies of R*uppia maritima* L . in bicarbonate- sucrose-based media. *Journal of Experimental Marine*

Biology and Ecology, *199*, 153–164.

Bird, K. T., Johnson, J. R., & Jewett-Smith, J. (1998). In vitro culture of the seagrass *Halophila decipiens*. *Aquatic Botany*, *60*(4), 377–387. https://doi.org/10.1016/S0304-3770(97)00093-4

Blandon, A., & zu Ermgassen, P. S. E. (2014). Quantitative estimate of commercial fish enhancement by seagrass habitat in southern Australia. *Estuarine, Coastal and Shelf Science*, *141*, 1–8. https://doi.org/10.1016/j.ecss.2014.01.009

BMT Oceanica Pty. Ltd. (2013). Transplanting *Posidonia* Seagrass in Temperate Western Australian Waters: A Practical 'How To' Guide. *BMT Oceanica Pty. Ltd.*, Document 42. Retrieved from http://www.bmtoceanica.com.au/media/4865945/Seagrass Transplantation Manual_Rev0_20131024.pdf

Borja, Á. (2005). The European water framework directive: A challenge for nearshore, coastal and continental shelf research. *Continental Shelf Research*, *25*(14), 1768–1783. https://doi.org/10.1016/j.csr.2005.05.004

Borum, J., & Greve, T. M. (2004). The four European seagrass species. *European Seagrasses : An Introduction to Monitoring and Management*, 1–7. Retrieved from http://www.seagrasses.org

Brun, F. G., Pérez-Pastor, A., Hernández, I., Vergara, J. J., & Pérez-Lloréns, J. L. (2006). Shoot organization in the seagrass *Zostera noltii*: implications for space occupation and plant architecture. *Helgoland Marine Research*, (60), 59–69. https://doi.org/10.1007/s10152-005-0017-0

Cabaço, S., Alexandre, A., & Santos, R. (2006). Survival and growth of the seagrass *Zostera noltii* in different culture media. In *Biologia Marina Mediterranea, Proceedings of the Mediterranean Seagrass Workshop*, 13, 24–28. Malta.

Cabaço, S., Machás, R., & Santos, R. (2009). Individual and population plasticity of the seagrass *Zostera noltii* along a vertical intertidal gradient. *Estuarine, Coastal and Shelf Science*, *82*, 301–308. https://doi.org/10.1016/j.ecss.2009.01.020

Cabaço, S., Ferreira, Ó., & Santos, R. (2010). Population dynamics of the seagrass *Cymodocea nodosa* in Ria Formosa lagoon following inlet artificial relocation. *Estuarine, Coastal and Shelf Science*, *87*(4), 510–516. https://doi.org/10.1016/j.ecss.2010.02.002

Christensen, P. B., Almela, E. D., & Diekmann, O. (2004). Can transplanting accelerate the recovery of seagrasses? In J. Borum, C. M. Duarte, D. Krause-Jensen, & T. M. Greve (Eds.), *European seagrasses: an introduction to monitoring and management,* 77–82.

Christianen, M. J. A., van Belzen, J., Herman, P. M. J., van Katwijk, M. M., Lamers, L. P. M., van Leent, P. J. M., & Bouma, T. J. (2013). Low-Canopy Seagrass Beds Still Provide Important Coastal Protection Services. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0062413

Cole, S. G., & Moksnes, P.-O. (2016). Valuing Multiple Eelgrass Ecosystem Services in Sweden: Fish Production and Uptake of Carbon and Nitrogen. *Frontiers in Marine Science*, *2*, 1–18. https://doi.org/10.3389/fmars.2015.00121

Cúcio, C., Engelen, A. H., Costa, R., & Muyzer, G. (2016). Rhizosphere microbiomes of European seagrasses are selected by the plant, but are not species specific. *Frontiers in Microbiology*, *7*:440, 1–15. https://doi.org/10.3389/fmicb.2016.00440

Cullen-Unsworth, L. C., Nordlund, L. M., Paddock, J., Baker, S., McKenzie, L. J., & Unsworth, R. K.

F. (2014). Seagrass meadows globally as a coupled social-ecological system: Implications for human wellbeing. *Marine Pollution Bulletin*, *83*(2), 387–397. https://doi.org/10.1016/j.marpolbul.2013.06.001

Cullen-Unsworth, L. C., & Unsworth, R. K. F. (2016). Strategies to enhance the resilience of the world's seagrass meadows. *Journal of Applied Ecology*, *53*(4), 967–972. https://doi.org/10.1111/1365-2664.12637

Cunha, B. A. H., Duarte, C. M., & Krause-Jensen, D. (2004). How long time does it take to recolonize seagrass beds? *European Seagrasses: An Introduction to Monitoring and Management*, 72–76.

Cunha, A. H., & Duarte, C. M. (2007). Biomass and leaf dynamics of *Cymodocea nodosa* in the Ria Formosa lagoon, South Portugal. *Botanica Marina*, *50*(April), 1–7. https://doi.org/10.1515/BOT.2007.001

Cunha, A. H., Assis, J., & Serrão, E. A. (2009). Estimation of available seagrass meadow area in Portugal for transplanting purposes. *Journal of Coastal Research*, Special Issue 56, 1100–1104.

Cunha, A. H., Erzini, K., Serrão, E. A., Gonçalves, E., Borges, R., Henriques, M., Henriques, V.,

Guerra, M. & Marbá, N. (2011). Restoration and Management of Biodiversity in the Marine Park Site Arrábida-Espichel- Final Report. Retrieved from http://ec.europa.eu/environment/life/project/Projects/index.cfm?fuseaction=home.showFile&rep=file&fil=LIFE06_NAT_P_000192_FTR_PT.pdf

Cunha, A. H., & Serrão, E. A. (2011). Tools for seagrass conservation and management in Portugal. *Ecologi@*, *3*, 23–36.

Cunha, A. H., Marbá, N. N., van Katwijk, M. M., Pickerell, C., Henriques, M., Bernard, G., Ferreira, M. A., Garcia, S., Garmendia, J. M. & Manent, P. (2012). Changing paradigms in seagrass restoration. *Restoration Ecology*, *20*(4), 427–430. https://doi.org/10.1111/j.1526-100X.2012.00878.x

Cunha, A. H., Assis, J. F., & Serrão, E. A. (2013). Seagrasses in Portugal: A most endangered marine habitat. *Aquatic Botany*, *104*, 193–203. https://doi.org/10.1016/j.aquabot.2014.02.007

Cunha, A. H., Erzini, K., Serrão, E. A., Gonçalves, E., Borges, R., Henriques, M., Guerra, M., Duarte, C. M., Marbá, N. N., & Fonseca, M. (2014). Biomares, a LIFE project to restore and manage the biodiversity of Prof. Luiz Saldanha Marine Park. *Journal of Coastal Conservation*, *18*(6), 643–655. https://doi.org/10.1007/s11852-014-0336-x

Curiel, D., Bellato, A., Rismondo, A., & Marzocchi, M. (1996). Sexual reproduction of Z*ostera noltii* Hornemann in the lagoon of Venice (Italy, North Adriatic). *Aquatic Botany*, *52*, 313–318.

De Klerk, G. D., Van der Krieken, W., & De Jong, J. C. (1999). Review the formation of adventitious roots: new concepts, new possibilities. In Vitro *Cellular and Developmental Biology - Plant*, *35*, 189–199.

Duarte, C. M., Merino, M., Agawin, N. S. R., Uri, J., Fortes, M. D., Gallegos, M. E., Marbá, N. & Hemminga, M. A. (1998). Root production and belowground seagrass biomass. *Marine Ecology Progress Series*, *171*, 97–108. https://doi.org/10.3354/meps171097

Duarte, C. M. (2002). The future of seagrass meadows. *Environmental Conservation*, *29*(2), 192–206. https://doi.org/10.1017/S0376892902000127

Duarte, C. M., Sintes, T., & Marbà, N. (2013). Assessing the CO2 capture potential of seagrass restoration projects. *Journal of Applied Ecology*, *50*(6), 1341–1349. https://doi.org/10.1111/1365-2664.12155

Duarte, C. M., & Krause-Jensen, D. (2017). Export from Seagrass Meadows Contributes to Marine Carbon Sequestration. *Frontiers in Marine Science*, *4*, 1–7. https://doi.org/10.3389/fmars.2017.00013

Ettinger, C. L., Voerman, S. E., Lang, J. M., Stachowicz, J. J., & Eisen, J. A. (2017). Microbial communities in sediment from *Zostera marina* patches, but not the *Z. marina* leaf or root microbiomes, vary in relation to distance from patch edge. *PeerJ*, *5*, e3246. https://doi.org/10.7717/peerj.3246

Fishman, J. R., Orth, R. J., Marion, S., & Bieri, J. (2004). A Comparative test of mechanized and manual transplanting of eelgrass , *Zostera marina* , in Chesapeake Bay. *Restoration Ecology*, *12*(2), 214–219. https://doi.org/10.1111/j.1061-2971.2004.00314.x

Floor, J. R., van Koppen, C. S. A., & van Tatenhove, J. P. M. (2018). Science, uncertainty and changing storylines in nature restoration: The case of seagrass restoration in the Dutch Wadden Sea. *Ocean and Coastal Management*, *157*, 227–236. https://doi.org/10.1016/j.ocecoaman.2018.02.016

Fonseca, M. S., Kenworthy, W. J., & Thayer, G. W. (1998). Guidelings for the Conservation and Restoration of Seagrasses in the United States and Adjacent Waters. In *NOAA Coastal Ocean program Decision Analysis Series Volume 12*. Retrieved from http://www.hillsborough.wateratlas.usf.edu/upload/documents/GuidelinesforConservationandRestorationinSeagrassinUS.pdf

Fourqurean, J. W., Manuel, S., Coates, K. A., Kenworthy, W. J., & Smith, S. R. (2010). Effects of excluding sea turtle herbivores from a seagrass bed: Overgrazing may have led to loss of seagrass meadows in Bermuda. *Marine Ecology Progress Series*, *419*, 223–232. https://doi.org/10.3354/meps08853

Frick, E. M., & Strader, L. C. (2018). Roles for IBA-derived auxin in plant development, *69*(2), 169–177. https://doi.org/10.1093/jxb/erx298

Ganassin, C., & Gibbs, P. J. (2008). A review of seagrass planting as a means of habitat compensation following loss of segrass meadow. *NSW Department of Primary Industries Fisheries Final Report,* Series No. 96, 1–43.

García-Jiménez, P., Navarro, E. P., Santana, C. H., Luque, Á., & Robaina, R. R. (2006). Anatomical and nutritional requirements for induction and sustained growth in vitro of *Cymodocea nodosa* (Ucria) Ascherson. *Aquatic Botany*, *84*(1), 79–84. https://doi.org/10.1016/j.aquabot.2005.07.006

Godet, L., Fournier, J., van Katwijk, M. M., Olivier, F., le Mao, P., & Retière, C. (2008). Before and after wasting disease in common eelgrass *Zostera marina* along the French Atlantic coasts: A general overview and first accurate mapping. *Diseases of Aquatic Organisms*, *79*(3), 249–255. https://doi.org/10.3354/dao01897

Govers, L. L., Suykerbuyk, W., Hoppenreijs, J. H. T., Giesen, K., Bouma, T. J., & van Katwijk, M. M. (2014). Rhizome starch as indicator for temperate seagrass winter survival. *Ecological Indicators*, *49*, 53–60. https://doi.org/10.1016/j.ecolind.2014.10.002

Govers, L. L., Man in 't Veld, W. A., Meffert, J. P., Bouma, T. J., van Rijswick, P. C. J., Heusinkveld, J. H. T., Orth, R. J., van Katwijk, M. M. & van der Heide, T. (2016). Marine *Phytophthora* species can hamper conservation and restoration of vegetated coastal ecosystems. *Proceedings of the Royal Society B: Biological Sciences*, *283*. https://doi.org/10.1098/rspb.2016.0812

Greve, T. M., & Binzer, T. (2004). Which factors regulate seagrass growth and distribution? *European Seagrasses: An Introduction to Monitoring and Management*, 19–23. https://doi.org/1545968311400091 [pii]\r10.1177/1545968311400091

Guimarães, M. H. M. E., Cunha, A. H., Nzinga, R. L., & Marques, J. F. (2012). The distribution of seagrass (*Zostera noltii*) in the Ria Formosa lagoon system and the implications of clam farming on its conservation. *Journal for Nature Conservation*, *20*(1), 30–40. https://doi.org/10.1016/j.jnc.2011.07.005

Haissig, B. E. (1974a). Influences of auxins and auxin synergists on adventitious root primordium initiation and development. *New Zealand Journal of Forestry Science*, *4*(2), 311–323.

Haissig, B. E. (1974b). Origins of adventitious roots. *New Zealand Journal of Forestry Science*, *4*(2), 299–310.

Hamill, J., & Sumby, J. (2002). *In vitro* culture of *Heterzostera tasmanica* and *Zostera muelleri*. Presented to the Western Port Sea Grass Seminar, Hastings Victoria, Australia, 29th November 2002.

Harris, P. T. (2012). Biogeography, Benthic Ecology, and Habitat Classification Schemes. In *Seafloor Geomorphology as Benthic Habitat*. P. T. Harris & E. K. Baker (Eds), 61-91. Elsevier Inc. https://doi.org/10.1016/B978-0-12-385140-6.00004-9

Hazarika, B. N., Teixeira da Silva, J. A., & Talukdar, A. (2006). Effective acclimatization of *in vitro* cultured plants: methods, physiology and genetics. *Floriculture, Ornamental and Plant Biotechnology Volume II*, 427–438.

Heinz Walz GmbH. (1998). Underwater Fluorometer Diving-PAM Submersible Photosynthesis Yield Analyzer Handbook of Operation. Effeltrich, Germany.

Hemminga, M. A. (1998). The root/rhizome system of seagrasses: an asset and a burden. *Journal of Sea Research*, *39*, 183–196. https://doi.org/10.1016/S1385-1101(98)00004-5

Hendriks, I. E., Olsen, Y. S., Ramajo, L., Basso, L., Steckbauer, A., Moore, T. S., Howard, J. & Duarte, C. M. (2014). Photosynthetic activity buffers ocean acidification in seagrass meadows. *Biogeosciences*, *11*(2), 333–346. https://doi.org/10.5194/bg-11-333-2014

Henry, M. G. (1998). The *in vitro* propagation of seagrasses *: Halophila ovalis, Ruppia megacarpa and Posidonia coriacea*. Honours Thesis. Retrieved from http://ro.ecu.edu.au/theses_hons/742

Herman, E. (1990). Non-axenic plant tissue culture: possibilities and opportunities. *Acta Horticulturae, International Symposium on* In Vitro *Culture and Horticultural Breeding 280*, 233–238.

Hughes, A. R., & Stachowicz, J. J. (2004). Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. *Proceedings of the National Academy of Sciences*, *101*(24), 8998–9002. https://doi.org/10.1271/bbb.64.767

Hughes, A. R., Williams, S. L., Duarte, C. M., Heck, K. L., & Waycott, M. (2009). Associations of concern: Declining seagrasses and threatened dependent species. *Frontiers in Ecology and the Environment*, *7*(5), 242–246. https://doi.org/10.1890/080041

Irving, A. D., Tanner, J. E., Seddon, S., Miller, D., Collings, G. J., Wear, R. J., Hoare, S. L. & Theil, M. J. (2010). Testing alternate ecological approaches to seagrass rehabilitation: Links to life-history traits. *Journal of Applied Ecology*, *47*(5), 1119–1127. https://doi.org/10.1111/j.1365-2664.2010.01852.x

James, R. K., Silva, R., Van Tussenbroek, B. I., Escudero-Castillo, M., Mariño-Tapia, I., Dijkstra, H. A., van Westen, R. M., Pietrzak, J. D., Candy, A. S., Katsman, C. A., van der Boog, C. G., Riva, R. E. M., Slobbe, C., Klees, R., Stapel, J., van der Heide, J., van Katwijk, M. M., Herman, P. M. J. & Bouma, T. J. (2019). Maintaining Tropical Beaches with Seagrass and Algae : A Promising Alternative to Engineering Solutions. *BioScience*, *69*(2), 136–142. https://doi.org/10.1093/biosci/biy154

Koch, E. W., & Durako, M. J. (1991). In vitro studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin effects on plant growth and development, *6*, 1980–1985.

Koch, E. W., Sanford, L. P., Chen, S., Shafer, D. J., & Smith, J. M. (2006). Waves in Seagrass Systems : Review and Technical Recommendations Engineer Research and Development Center Waves in Seagrass Systems : Review and Technical Recommendations. *US Army Corps of Engineers*. https://doi.org/ERDC TR-06-15

Kozai, T. (1991). Invited Review: Photoautotrophic micropropagation. *In Vitro Cellular Developmental Biology*, *27*, 47–51.

Kuo, J. & den Hartog, C. (2001). Seagrass taxonomy and identification key. In F T Short & R. G. Coles (Eds.), *Global Seagrass Research Methods*, 31–58. Elsevier.

Kuo, J. & den Hartog, C. (2006). Seagrass Morphology, Anatomy, and Ultrastructure. In A. W. D. Larkum, R. J. Orth, & C. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation*, 51–87.

Lamb, J. B., van de Water, J. A. J. M., Bourne, D. G., Altier, C., Hein, M. Y., Fiorenza, E. A., Abu, N., Jompa, J. & Harvell, C. D. (2017). Seagrass ecosystems reduce exposure to bacterial pathogens of humans, fishes, and invertebrates. *Science*, *355*(2), 731–733.

Lee, K., & Park, J. (2008). An effective transplanting technique using shells for restoration of Zostera marina habitats, *56*, 1015–1021. https://doi.org/10.1016/j.marpolbul.2008.02.010

Lefcheck, J. S., Marion, S. R., & Orth, R. J. (2017). Restored Eelgrass (*Zostera marina* L.) as a Refuge for Epifaunal Biodiversity in Mid-Western Atlantic Coastal Bays. *Estuaries and Coasts*, *40*(1), 200–212. https://doi.org/10.1007/s12237-016-0141-x

Leifert, C., Ritchie, J. Y., & Waites, W. M. (1991). Contaminants cultures of plant-tissue and cell cultures. *World Journal of Microbiology and Biotechnology*, *7*, 452–469.

Leschen, A. S., Ford, K. H., & Evans, N. T. (2010). Successful Eelgrass (*Zostera marina*) restoration in a formerly eutrophic estuary (Boston Harbor) supports the use of a multifaceted watershed approach to mitigating Eelgrass loss. *Estuaries and Coasts*, *33*(6), 1340–1354. https://doi.org/10.1007/s12237-010-9272-7

Lewis, R. R. (1987). The restoration and creation of seagrass meadows in the southeastern United States. In R. R. Durako, M.J., Phillips, R.C., Lewis (Ed.), *Proceedings of the Symposium on Subtropical Seagrasses of the Southeastern United States. Marine Resources Publication No. 42,* 153–173.

Lewis, R. R. (1990). Laboratory culture methods. In R. C. Phillips & P. Mcroy (Eds.), *Seagrass research methods*, 37–42. UNESCO.

Loberant, B., & Altman, A. (2010). Micropropagation of Plants. In Michael C Flickinger (Ed.), *Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseperation, and Cell Technology*, 35, 57–64.

Loquès, F., Caye, G., & Meinesz, A. (1990). Axenic culture of selected tissue of Posidonia oceanica. *Aquatic Botany*, *37*, 171–188.

Marbà, N., Duarte, C. M., Alexandre, A., & Cabaço, S. (2004). How do seagrasses grow and spread?, *European Seagrasses: An Introduction to Monitoring and Management*, 11–18. Retrieved from www.seagrasses.org

Martins, I., Neto, J. M., Fontes, M. G., Marques, J. C., & Pardal, M. A. (2005). Seasonal variation in short-term survival of *Zostera noltii* transplants in a declining meadow in Portugal. *Aquatic Botany*, *82*(2), 132–142. https://doi.org/10.1016/j.aquabot.2005.03.006

Moberg, F., & Ronnbacka, P. (2003). Ecosystem services of the tropical seascape: interactions, substitutions and restoration. *Ocean & Coastal Management*, *46*, 27–46. https://doi.org/10.1016/0032-3950(86)90312-6

Moffler, M. D., & Durako, M. J. (1984). Axenic culture of *Thalassia testudinum* banks *ex konig (hydrocharitaceae*). *American Journal of Botany*, *71*(10), 1455–1460. https://doi.org/10.1002/j.1537-2197.1984.tb12004.x

Moksnes, P.-O., Eriander, L., Infantes, E., & Holmer, M. (2018). Local regime shifts prevent natural recovery and restoration of lost Eelgrass beds along the Swedish west coast. *Estuaries and Coasts*, *41*, 1712–1731.

Muehlstein, L., Porter, D., & Short, F. T. (1991). *Labyrinthula zosterae* sp . *nov* ., The causative agent of wasting disease of eelgrass *Zostera marina*. *Mycologia*, *83*(2), 180–191. https://doi.org/10.2307/3759933

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*, 473–497.

Murray-Jones, S. (2008). Proceedings of the Second Seagrass Restoration Workshop. Adelaide. April 2008. Department for Environment and Heritage.

Nordlund, L. M., Koch, E. W., Barbier, E. B., & Creed, J. C. (2016). Seagrass ecosystem services and their variability across genera and geographical regions. *PLoS ONE*, *11*(10), 1–23. https://doi.org/10.1371/journal.pone.0163091

Nordlund, L. M., Jackson, E. L., Nakaoka, M., Samper-Villarreal, J., Beca-Carretero, P., & Creed, J. C. (2018). Seagrass ecosystem services – What's next? *Marine Pollution Bulletin*, *134*, 145–151. https://doi.org/10.1016/j.marpolbul.2017.09.014

Ondiviela, B., Losada, I. J., Lara, J. L., Maza, M., Galván, C., Bouma, T. J., & van Belzen, J. (2014). The role of seagrasses in coastal protection in a changing climate. *Coastal Engineering*, *87*, 158–168. https://doi.org/10.1016/j.coastaleng.2013.11.005

Orth, R. J., Carruthers, T. J. B., Dennison, W. C., Duarte, C. M., Fourqurean, J. W., Heck, K. L., Hughes, A. R., Kendrick, G. A., Kenworthy, W. J., Olyyarnik, S., Short, F. T., Waycott, M., & Williams, S. L. (2006). A global crisis for seagrass ecosystems. *BioScience*, *56*(12), 987. https://doi.org/10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2

Orth, R. J., Marion, S. R., Granger, S., & Traber, M. (2009). Evaluation of a mechanical seed planter for transplanting *Zostera marina* (Eelgrass) seeds. *Aquatic Botany*, *90*(2), 204–208. https://doi.org/10.1016/j.aquabot.2008.07.004

Paling, E. I., Van Keulen, M., Wheeler, K., Phillips, J., & Dyhrberg, R. (2001). Mechanical seagrass transplantation in Western Australia. *Ecological Engineering*, *16*(3), 331–339. https://doi.org/10.1016/S0925-8574(00)00119-1

Papenbrock, J. (2012). Highlights in Seagrasses' phylogeny , physiology, and metabolism: What makes them special? *ISRN Botany*, *2012*, 1–15. https://doi.org/10.5402/2012/103892

Park, R. S., Kim, J., Kang, C., An, S., Chung, K. I., Kim, H. J., & Lee, K. (2009). Current status and ecological roles of *Zostera marina* after recovery from large-scale reclamation in the Nakdong River estuary , Korea. *Estuarine , Coastal and Shelf Science*, *81*, 38–48. https://doi.org/10.1016/j.ecss.2008.10.003

Paulo, D., Cunha, A. H., Boavida, J., Serrão, E. A., Gonçalves, E. J., & Fonseca, M. (2019). Open Coast Seagrass Restoration . Can We Do It ? Large Scale Seagrass Transplants, *6*, 1–15. https://doi.org/10.3389/fmars.2019.00052

Pickerell, C., Schott, S., Manzo, K., Udelson, B., Krupski, N., & Barbour, K. (2012). The Tortilla Method: Development and testing of a new seagrass planting method. In *Restore America's Estuaries conference, Tampa, Florida*.

Pirc, H. (1989). Seasonal changes in soluble carbohydrates, starch, and energy content in Mediterranean seagrasses. *Marine Ecology*, *10*(2), 97–105.

Preen, A., Long, W. L., & Coles, R. (1995). Flood and cyclone related loss, and partial recovery, of more than 1000 Km2 of seagrass in Hervey Bay, Queensland, Australia. *Aquatic Botany*, *52*, 3–17. http://linkinghub.elsevier.com/retrieve/pii/030437709500491H

Ralph, P. J., & Short, F. T. (2002). Impact of the wasting disease pathogen, *Labyrinthula zosterae*, on the photobiology of eelgrass Zostera marina. *Marine Ecology Progress Series*, *226*(January), 265–271. https://doi.org/10.3354/meps226265

Ralph, P. J., & Gademann, R. (2005). Rapid light curves : A powerful tool to assess photosynthetic activity. *Aquatic Botany*, *82*, 222–237. https://doi.org/10.1016/j.aquabot.2005.02.006

Ralph, P. J., Tomasko, D., Moore, K., Seddon, S., & Macinnis-Ng, C. M. O. (2006). Human impacts on seagrasses: Eutrophication, sedimentation, and contamination. In A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation* (pp. 567–593). https://doi.org/10.1007/978-1-4020-2983-7

Reidenbach, M. A., & Thomas, E. L. (2018). Influence of the seagrass, *Zostera marina,* on wave attenuation and bed shear stress within a shallow coastal bay. *Frontiers in Marine Science*, *5*(October), 1–16. https://doi.org/10.3389/fmars.2018.00397

Reise, K. (2005). Coast of change: habitat loss and transformations in the Wadden Sea. *Helgoland Marine Research*, *59*, 9–21. https://doi.org/10.1007/s10152-004-0202-6

Ricart, A. M., York, P. H., Rasheed, M. A., Pérez, M., Romero, J., Bryant, C. V., & Macreadie, P. I. (2015). Variability of sedimentary organic carbon in patchy seagrass landscapes. *Marine Pollution Bulletin*, *100*(1), 476–482. https://doi.org/10.1016/j.marpolbul.2015.09.032

Rose, C. D., Sharp, W. C., Kenworthy, W. J., Hunt, J. H., Lyons, W. G., Prager, E. J., Valentine, J.F., Hall, M.O., Whitfield, P.E., & J.W. Fourqurean. (1999). Overgrazing of a large seagrass bed by the sea urchin *Lytechinus variegatus* in Outer Florida Bay. Marine Ecology Progress Series, 190, 211-222. https://doi.org/10.3354/meps190211

Ruiz-Carrera, V., & Sánchez, A. J. (2008). Development of a model for *in vitro* culture of *Vallisneria americana michx*. *U-Ciencia Publications*, *24*(3), 205–218.

Salo, T., Pedersen, M. F., & Boström, C. (2014). Population specific salinity tolerance in eelgrass (*Zostera marina*). *Journal of Experimental Marine Biology and Ecology*, *461*, 425–429. https://doi.org/10.1016/j.jembe.2014.09.010

Schwarz, A.-M., Matheson, F., & Mathieson, T. (2004). Coastal Ecosystems: The role of sediment in keeping seagrass beds healthy. *Water and Atmosphere*, *12*, 18–19.

Shamsi, I. H., Sagonda, T., Zhang, X., Zvobgo, G., & Joan, H. I. (2019). *The Role of Growth Regulators in Senescence*. In M. Sarwat & N. Tuteja (Eds), *Senescence Signalling and Control in Plants*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-813187-9.00006-8

Short, F. T., & Wyllie-Echeverria, S. (1996). Natural and human-induced disturbance of seagrasses. *Environmental Conservation*, *23*(1), 17–27.

Short, F. T., & Duarte, C. M. (2001). Methods for the measurement of seagrass growth and production. In F.T. Short & R. G. Coles (Eds.), *Global Seagrass Research Methods*, 155–182. https://doi.org/10.1016/B978-044450891-1/50009-8

Short, F. T., Carruthers, T., Dennison, W., & Waycott, M. (2007). Global seagrass distribution and diversity: A bioregional model. *Journal of Experimental Marine Biology and Ecology*, *350*, 3–20. https://doi.org/10.1016/j.jembe.2007.06.012

Short, F. T., Polidoro, B., Livingstone, S. R., Carpenter, K. E., Bandeira, S., Bujang, J. S., Calumpong, H. P., Carruthers, T. J.B., Coles, R. G., Dennison, W. C., Erftemeijer, P. L. A., Fortes, M. D., Freeman, A. S., Jagtap, T. G., Kamal, A. H. M., Kendrick, G. A., Kenworthy, J. W., La Nafie, Y. A., Nasution, I. M., Orth, R. J., Prathep, A., Sanciangco, J. C., van Tussenbroek, B., Vergara, S. G., Waycott, M., & Zieman, J. C. (2011). Extinction risk assessment of the world's seagrass species. *Biological Conservation*, *144*(7), 1961–1971. https://doi.org/10.1016/j.biocon.2011.04.010

Stapel, J., Aarts, T. L., Van Duynhoven, B. H. M., De Groot, J. D., Van Den Hoogen, P. H. W., & Hemminga, M. A. (1996). Nutrient uptake by leaves and roots of the seagrass *Thalassia hemprichii* in the Spermonde Archipelago , Indonesia. *Marine Ecology Progress Series*, *134*, 195–206.

Statton, J., Gustin-Craig, S., Kingsley, D. W., & Kendrick, G. A. (2015). Edge effects along a seagrass margin result in an increased grazing risk on *Posidonia australis* transplants. *PLoS ONE 10*, *10*. https://doi.org/10.1371/journal.pone.0137778

Steffens, B., & Rasmussen, A. (2016). The Physiology of Adventitious Roots. *Plant Physiology*, *170*(2), 603–617. https://doi.org/10.1104/pp.15.01360

Sterckx, T., Lemey, E., Huygens, M., Fordeyn, J., Groenendaal, B., Delbare, D., Vanagt, T., Pycke, B., Semeraro, A. & Mascart, T. (2019). Coastbusters : Investigation of ecosystem based coastal stabilisation solutions. In *Proceedings of the twenty-second world dredging congress, WODCON XXII, Shangai, CHINA, April 25-29, 2019*.

Subhashini, P., & Thangaradjou, R. T. (2014). Establishment of cell suspension culture protocol for a seagrass (*Halodule pinifolia*): Growth kinetics and histomorphological characterization. Aquatic Botany, 117, 33-40.

Suding, K. N., Gross, K. L., & Houseman, G. R. (2004). Alternative states and positive feedbacks in restoration ecology. *Trends in Ecology and Evolution*, *19*(1), 46–53. https://doi.org/10.1016/j.tree.2003.10.005

Suding, K. N., & Hobbs, R. J. (2009). Threshold models in restoration and conservation: a developing framework. *Trends in Ecology and Evolution*, *24*(5), 271–279. https://doi.org/10.1016/j.tree.2008.11.012

Suykerbuyk, W., Govers, L. L., Bouma, T. J., Giesen, W. B. J. T., de Jong, D. J., van de Voort, R., Giesen, K., Giesen, P. T. & van Katwijk, M. M. (2016). Unpredictability in seagrass restoration: analysing the role of positive feedback and environmental stress on *Zostera* *noltii* transplants. *Journal of Applied Ecology*, *53*(3), 774–784. https://doi.org/10.1111/1365-2664.12614

Tarquinio, F., Hyndes, G. A., Laverock, B., Koenders, A., & Christin Säwström. (2019). The seagrass holobiont: understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiology Letters*, *366*(6).

Tuya, F., Haroun, R., & Espino, F. (2014). Economic assessment of ecosystem services: Monetary value of seagrass meadows for coastal fisheries. *Ocean and Coastal Management*, *96*, 181–187. https://doi.org/10.1016/j.ocecoaman.2014.04.032

Tuya, F., Ribeiro-Leite, L., Arto-Cuesta, N., Coca, J., Haroun, R., & Espino, F. (2014). Decadal changes in the structure of *Cymodocea nodosa* seagrass meadows: Natural vs. human influences. *Estuarine, Coastal and Shelf Science*, *137*(1), 41–49. https://doi.org/10.1016/j.ecss.2013.11.026

Ugarelli, K., Chakrabarti, S., Laas, P., & Stingl, U. (2017). The seagrass holobiont and it's microbiome. *Microorganisms*, *5*(4), 81. https://doi.org/10.3390/microorganisms5040081

van Der Heide, T., van Nes, E. H., Geerling, G. W., Smolders, A. J. P., Bouma, T. J., & van Katwijk, M. M. (2007). Positive feedbacks in seagrass ecosystems: Implications for success in conservation and restoration. *Ecosystems*, *10*(8), 1311–1322. https://doi.org/10.1007/s10021-007-9099-7

van Katwijk, M. M., Bos, A. R., de Jonge, V. N., Hanssen, L. S. A. M., Hermus, D. C. R., & de Jong, D. J. (2009). Guidelines for seagrass restoration: Importance of habitat selection and donor population, spreading of risks, and ecosystem engineering effects. *Marine Pollution Bulletin*, *58*(2), 179–188. https://doi.org/10.1016/j.marpolbul.2008.09.028

van Katwijk, Marieke M., Thorhaug, A., Marbà, N., Orth, R. J., Duarte, C. M., Kendrick, G. A., Althuizen, I. H.J., Balestri, E., Bernard, G., Cambridge, M. L., Cunha, A., Durance, C., Giesen, W., Han, Q., Hosokawa, S., Kiswara, W., Komatsu, T., Lardicci, C., Lee, K. S., Meinesz, A., Nakaoka, M., O'Brien, K. R., Paling, E. I.,Pickerell, C., Ransijn, A. M.A., & Verduin, J. J. (2016). Global analysis of seagrass restoration: The importance of large-scale planting. *Journal of Applied Ecology*, *53*(2), 567–578. https://doi.org/10.1111/1365-2664.12562

Visser, R. G. F., Klerk, G. De, & Breeding, W. P. (2010). The hyperhydricity syndrome: Waterlogging of plant tissues as a major cause. *Propagation of Ornamental Plants*, *10*(4), 169–175.

Vonk, J. A., Middelburg, J. J., Stapel, J., & Bouma, T. J. (2008). Dissolved organic nitrogen uptake by seagrasses. *Limnology and Oceanography*, *53*(2), 542–548.

Walker, D. I., & McComb, A. J. (1992). Seagrass degradation in Australian coastal waters. *Marine Pollution Bulletin*, *25*(5–8), 191–195. https://doi.org/10.1016/0025-326X(92)90224-T

Wear, R. (2006). Recent advances in research into seagrass restoration. *Prepared for the Coastal Protection Branch, Department for Environment and Heritage*, (140), 21. http://www.sardi.sa.gov.au/_media/pdf/sardi_internet/our_org/publications/research_report_series/2006/No140_RD04-0038-4_Advances_research_seagrass_restoration.pdf

Williams, S. L. (2001). Reduced genetic diversity in eelgrass transplantations affects both population growth and individual fitness. *Ecological Applications*, *11*(5), 1472–1488.

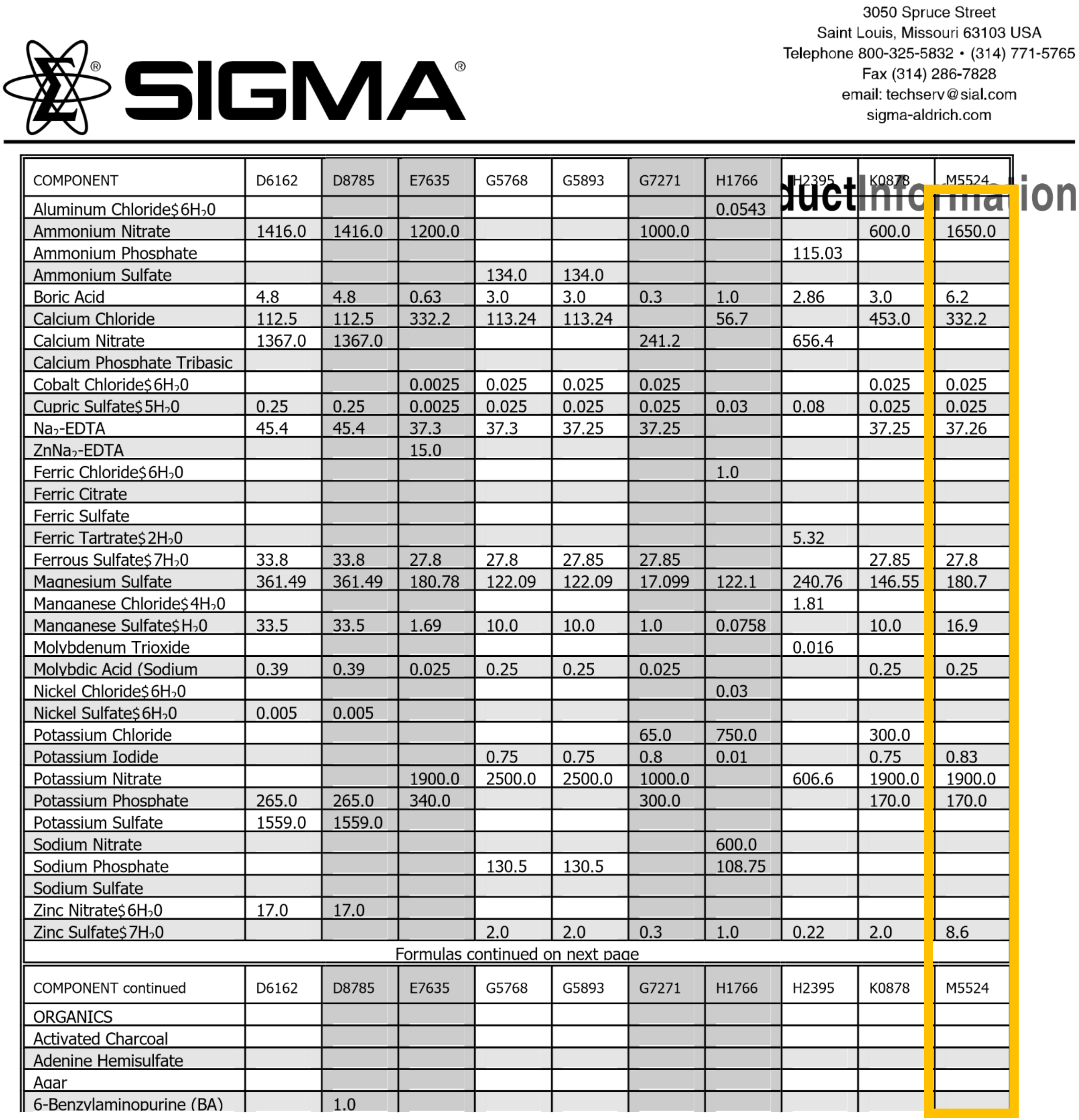
Williams, S. L. (2007). Introduced species in seagrass ecosystems: Status and concerns. *Journal of Experimental Marine Biology and Ecology*, *350*(1–2), 89–110. https://doi.org/10.1016/j.jembe.2007.05.032

Wilson, J. G., & Bennett, I. J. (2005). Under pressure: Does osmotic potential of the culture medium affect growth of seagrasses? In I. J. Bennett, E. Bunn, H. Clarke, & J. A. McComb (Eds.), *Contributing to a Sustainable Future; Proceedings of the Australian Branch of the IAPTC&B*, 21–24.

Yamaguchi, I., Cohen, J. D., Culler, A. H., Quint, M., Slovin, J. P., Nakajima, M., Yamaguchi, S., Sakakibara, H., Kuroha, T., Nobuhiro, H, Yakota, T., Ohta, H., Kobayashi, Y, Mori, H. & Sakagami, Y. (2010). Plant Hormones. In H.-W. (Ben) Liu & L. Mander (Eds.), *Comprehensive Natural Products II*, 4, 9–125.

Appendices

Appendix 1- Plant Cell Culture Basal Salt Mixtures and Media (mg /L) M&S media in orange.



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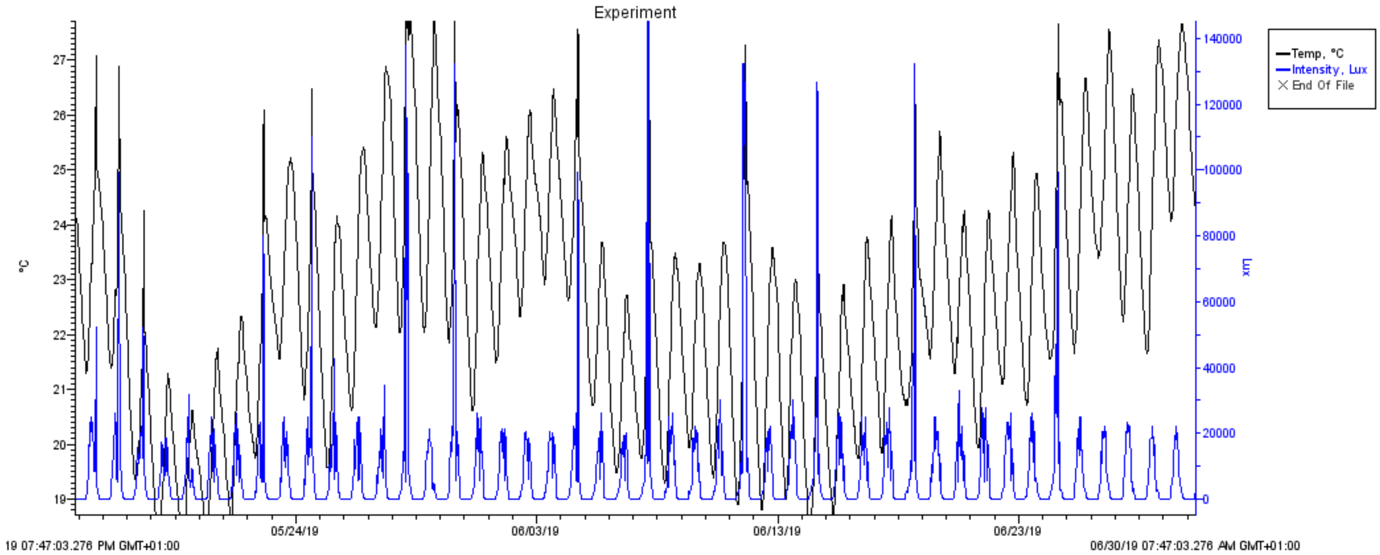
COMPONENT	D6162	D8785	E7635	G5768	G5893	G7271	H1766	H2395	K0878	M5524
Aluminum Chloride\$6H$_2$0							0.0543			
Ammonium Nitrate	1416.0	1416.0	1200.0			1000.0			600.0	1650.0
Ammonium Phosphate								115.03		
Ammonium Sulfate				134.0	134.0					
Boric Acid	4.8	4.8	0.63	3.0	3.0	0.3	1.0	2.86	3.0	6.2
Calcium Chloride	112.5	112.5	332.2	113.24	113.24		56.7		453.0	332.2
Calcium Nitrate	1367.0	1367.0				241.2		656.4		
Calcium Phosphate Tribasic										
Cobalt Chloride\$6H$_2$0			0.0025	0.025	0.025	0.025			0.025	0.025
Cupric Sulfate\$5H$_2$0	0.25	0.25	0.0025	0.025	0.025	0.025	0.03	0.08	0.025	0.025
Na$_2$-EDTA	45.4	45.4	37.3	37.3	37.25	37.25			37.25	37.26
ZnNa$_2$-EDTA			15.0							
Ferric Chloride\$6H$_2$0							1.0			
Ferric Citrate										
Ferric Sulfate										
Ferric Tartrate\$2H$_2$0								5.32		
Ferrous Sulfate\$7H$_2$0	33.8	33.8	27.8	27.8	27.85	27.85			27.85	27.8
Magnesium Sulfate	361.49	361.49	180.78	122.09	122.09	17.099	122.1	240.76	146.55	180.7
Manganese Chloride\$4H$_2$0								1.81		
Manganese Sulfate\$H$_2$0	33.5	33.5	1.69	10.0	10.0	1.0	0.0758		10.0	16.9
Molybdenum Trioxide								0.016		
Molybdic Acid (Sodium	0.39	0.39	0.025	0.25	0.25	0.025			0.25	0.25
Nickel Chloride\$6H$_2$0							0.03			
Nickel Sulfate\$6H$_2$0	0.005	0.005								
Potassium Chloride						65.0	750.0		300.0	
Potassium Iodide				0.75	0.75	0.8	0.01		0.75	0.83
Potassium Nitrate			1900.0	2500.0	2500.0	1000.0		606.6	1900.0	1900.0
Potassium Phosphate	265.0	265.0	340.0			300.0			170.0	170.0
Potassium Sulfate	1559.0	1559.0								
Sodium Nitrate							600.0			
Sodium Phosphate				130.5	130.5		108.75			
Sodium Sulfate										
Zinc Nitrate\$6H$_2$0	17.0	17.0								
Zinc Sulfate\$7H$_2$0				2.0	2.0	0.3	1.0	0.22	2.0	8.6

Formulas continued on next page

COMPONENT continued	D6162	D8785	E7635	G5768	G5893	G7271	H1766	H2395	K0878	M5524
ORGANICS										
Activated Charcoal										
Adenine Hemisulfate										
Agar										
6-Benzylaminopurine (BA)		1.0								

Per 4.3 grams of powder.

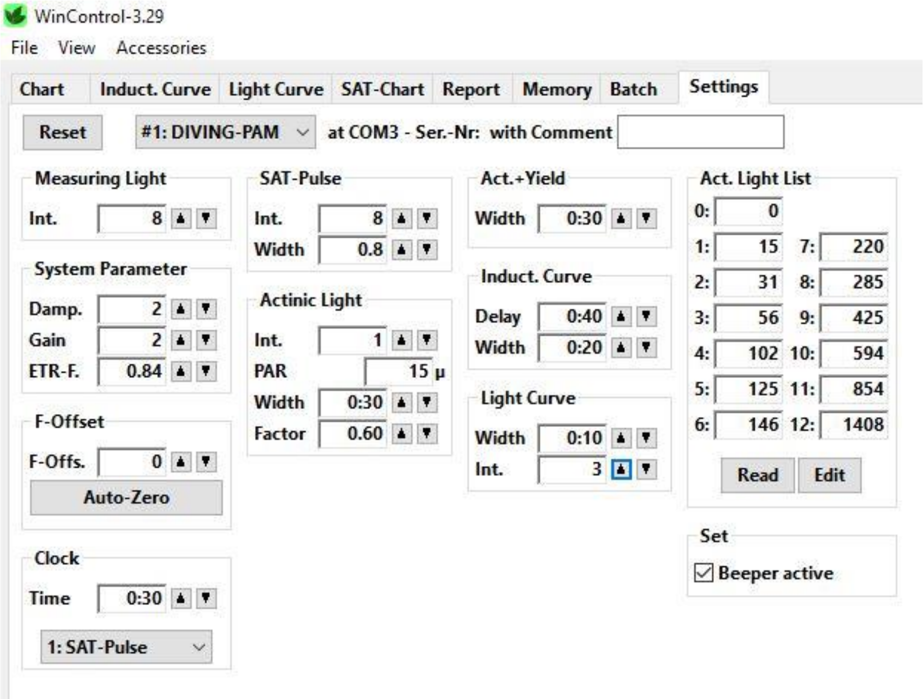
Appendix 2- Readout from HOBO Pendant Temperature/Light Data Logger data during the experimental period.



Plot Title:	Experiment					Plot Title:	Experiment2				
#	Date Time, GMT+01:00	Temp, °C	Intensity,	temp	Lux	#	Date Time, GMT+01:00	Temp, °C	Intensity,	Temp	Lux
1386	05/28/19 12:30:00 PM	24.835	14466.8			614	05/28/19 12:30:00 PM	25.902	99200.7		
1387	05/28/19 01:00:00 PM	27.468	132267.5			615	05/28/19 01:00:00 PM	26	93689.5		
1388	05/28/19 01:30:00 PM	27.764	137778.7			616	05/28/19 01:30:00 PM	26.488	99200.7		
1389	05/28/19 02:00:00 PM	27.468	5166.7			617	05/28/19 02:00:00 PM	27.272	93689.5		
1390	05/28/19 02:30:00 PM	28.456	115734.1			618	05/28/19 02:30:00 PM	27.665	85422.8		
1391	05/28/19 03:00:00 PM	28.853	104711.8	Mean	Mean	619	05/28/19 03:00:00 PM	27.961	79911.6	mean	Mean
1392	05/28/19 03:30:00 PM	29.152	93689.5	27.71371	86259.3	620	05/28/19 03:30:00 PM	27.567	68889.3	26.97929	88572.01
1722	6/04/2019 12:30	24.062	17911.2		50378.06	950	6/04/2019 12:30	23.677	4477.8	std dev:	10355.43
1723	6/04/2019 13:00	24.351	22044.6			951	6/04/2019 13:00	23.869	11711.2		
1724	6/04/2019 13:30	24.545	17222.3			952	6/04/2019 13:30	24.158	16533.4		
1725	6/04/2019 14:00	24.738	21355.7			953	6/04/2019 14:00	24.351	20666.8		
1726	6/04/2019 14:30	24.835	16533.4			954	6/04/2019 14:30	24.351	12400.1		
1727	6/04/2019 15:00	26.292	26178			955	6/04/2019 15:00	26.195	104711.8		
1728	6/04/2019 15:30	25.61	5511.1	24.919	18108.04	956	6/04/2019 15:30	26.585	93689.5	24.74086	37741.51
2058	6/11/2019 12:30	21.569	8266.7		6013.719	1286	6/11/2019 12:30	21.187	2669.5		39247.87
2059	6/11/2019 13:00	23.292	126756.4			1287	6/11/2019 13:00	21.569	2583.4		
2060	6/11/2019 13:30	24.545	132267.5			1288	6/11/2019 13:30	21.951	3444.5		
2061	6/11/2019 14:00	25.125	132267.5			1289	6/11/2019 14:00	22.333	2669.5		
2062	6/11/2019 14:30	25.222	126756.4			1290	6/11/2019 14:30	22.812	3100		
2063	6/11/2019 15:00	26.292	121245.2			1291	6/11/2019 15:00	24.641	85422.8		
2064	6/11/2019 15:30	26.39	93689.5	24.63357	105892.7	1292	6/11/2019 15:30	24.931	79911.6	22.77486	25685.9
2394	06/18/19 12:30:00 PM	21.378	11711.2		41708.69	1622	06/18/19 12:30:00 PM	21.378	4133.4		36069.3
2395	06/18/19 01:00:00 PM	21.473	8266.7			1623	06/18/19 01:00:00 PM	21.569	3444.5		
2396	06/18/19 01:30:00 PM	21.855	15844.5			1624	06/18/19 01:30:00 PM	21.76	9644.5		
2397	06/18/19 02:00:00 PM	22.142	24800.2			1625	06/18/19 02:00:00 PM	22.046	10677.8		
2398	06/18/19 02:30:00 PM	23.581	44089.2			1626	06/18/19 02:30:00 PM	23.292	38578		
2399	06/18/19 03:00:00 PM	24.062	42711.4			1627	06/18/19 03:00:00 PM	23.677	42711.4		
2400	06/18/19 03:30:00 PM	24.062	44089.2	22.65043	27358.91	1628	06/18/19 03:30:00 PM	23.677	31689.1	22.48557	20125.53
2682	06/24/19 12:30:00 PM	23.773	26178		14851.63	1910	06/24/19 12:30:00 PM	23.1	6200		15663.64
2683	06/24/19 01:00:00 PM	24.641	46844.8			1911	06/24/19 01:00:00 PM	23.773	17911.2		
2684	06/24/19 01:30:00 PM	24.545	42711.4			1912	06/24/19 01:30:00 PM	24.062	22044.6		
2685	06/24/19 02:00:00 PM	24.835	24800.2			1913	06/24/19 02:00:00 PM	24.835	60622.6		
2686	06/24/19 02:30:00 PM	26.488	93689.5			1914	06/24/19 02:30:00 PM	25.416	121245.2		
2687	06/24/19 03:00:00 PM	27.075	88178.4			1915	06/24/19 03:00:00 PM	24.835	5511.1		
2688	06/24/19 03:30:00 PM	27.665	99200.7	25.57457	60229	1916	06/24/19 03:30:00 PM	24.931	7577.8	24.42171	34444.64
3066	7/02/2019 12:30	28.159	132268		30048.15	2294	7/02/2019 12:30	26	2497.2		39647.51
3067	7/02/2019 13:00	28.555	93690			2295	7/02/2019 13:00	26.097	4305.6		
3068	7/02/2019 13:30	28.754	110223			2296	7/02/2019 13:30	26.488	4650		
3069	7/02/2019 14:00	28.953				2297	7/02/2019 14:00	27.665	55111.5		
3070	7/02/2019 14:30	29.252	148801			2298	7/02/2019 14:30	27.173	7233.4		
3071	7/02/2019 15:00	29.752	176356.7			2299	7/02/2019 15:00	28.258	79911.6		
3072	7/02/2019 15:30	29.652	187379	29.011	141452.8	2300	7/02/2019 15:30	27.961	4477.8	27.09171	22598.16
3402	7/09/2019 12:30	25.805	104711.8		33472.62	2631	7/09/2019 13:00	23.773	5338.9		29196.8
3403	7/09/2019 13:00	25.61	104711.8			2632	7/09/2019 13:30	24.062	5166.7		
3404	7/09/2019 13:30	25.708	42711.4			2633	7/09/2019 14:00	24.351	4650		
3405	7/09/2019 14:00	26	74400.5			2634	7/09/2019 14:30	24.931	4994.5		
3406	7/09/2019 14:30	27.272	115734.1			2635	7/09/2019 15:00	25.61	7233.4		
3407	7/09/2019 15:00	25.708	11022.3			2636	7/09/2019 15:30	25.805	8611.2		
3408	7/09/2019 15:30	28.06	99200.7	26.309	78927.51	2637	7/09/2019 16:00	25.902	7577.8	24.91914	6224.643
					35933.68	2966	07/16/19 12:30:00 PM	26.097	4650		1436.306
						2967	07/16/19 01:00:00 PM	26.488	5338.9		
						2968	07/16/19 01:30:00 PM	26.683	5166.7		
						2969	07/16/19 02:00:00 PM	27.961	82667.2		
						2970	07/16/19 02:30:00 PM	27.468	31689.1		
						2971	07/16/19 03:00:00 PM	28.456	88178.4		
						2972	07/16/19 03:30:00 PM	28.456	93689.5	27.37271	44482.83
											38944.99

Appendix 3- WinControl-3 settings for Heinz Walz GmbH Diving-PAM

Z. noltii



#1: DIVING-PAM at COM3 - Ser.-Nr: with Comment

Measuring Light
Int. 8

System Parameter
Damp. 2
Gain 2
ETR-F. 0.84

F-Offset
F-Offs. 0

Clock
Time 0:30
1: SAT-Pulse

SAT-Pulse
Int. 8
Width 0.8

Actinic Light
Int. 1
PAR 15 µ
Width 0:30
Factor 0.60

Act.+Yield
Width 0:30

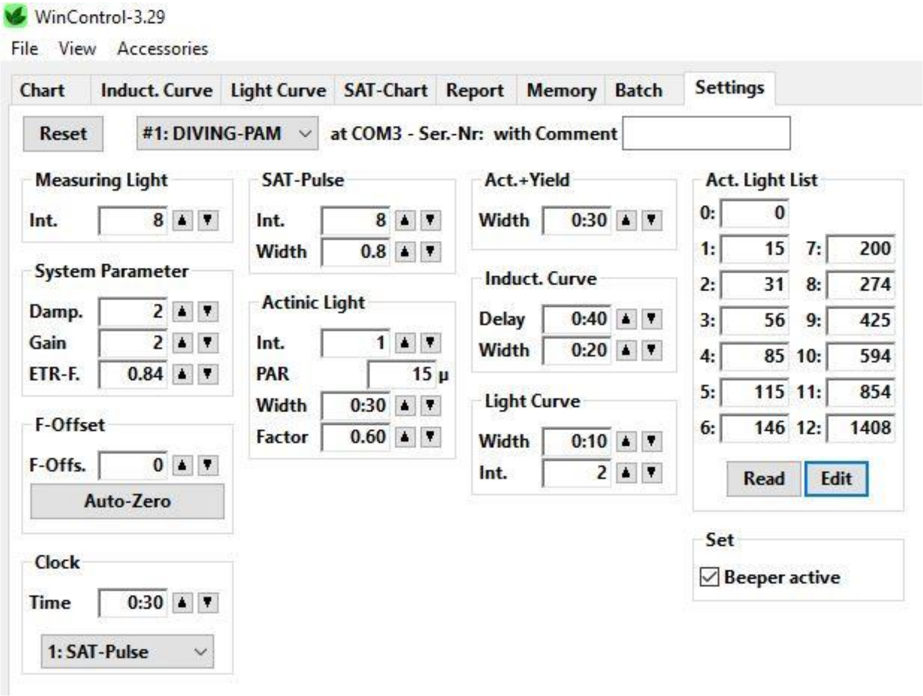
Induct. Curve
Delay 0:40
Width 0:20

Light Curve
Width 0:10
Int. 3

Act. Light List
0: 0
1: 15
2: 31
3: 56
4: 102
5: 125
6: 146
7: 220
8: 285
9: 425
10: 594
11: 854
12: 1408

Set
☑ Beeper active

Z. marina



#1: DIVING-PAM at COM3 - Ser.-Nr: with Comment

Measuring Light
Int. 8

System Parameter
Damp. 2
Gain 2
ETR-F. 0.84

F-Offset
F-Offs. 0

Clock
Time 0:30
1: SAT-Pulse

SAT-Pulse
Int. 8
Width 0.8

Actinic Light
Int. 1
PAR 15 µ
Width 0:30
Factor 0.60

Act.+Yield
Width 0:30

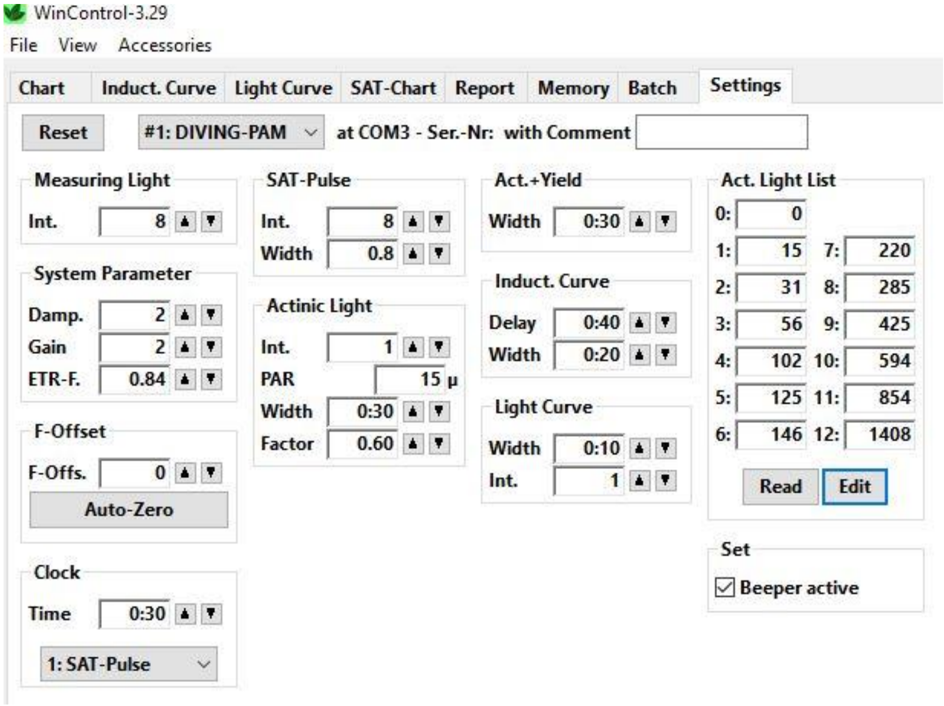
Induct. Curve
Delay 0:40
Width 0:20

Light Curve
Width 0:10
Int. 2

Act. Light List
0: 0
1: 15
2: 31
3: 56
4: 85
5: 115
6: 146
7: 200
8: 274
9: 425
10: 594
11: 854
12: 1408

Set
☑ Beeper active

C. nodosa



#1: DIVING-PAM at COM3 - Ser.-Nr: with Comment

Measuring Light
Int. 8

System Parameter
Damp. 2
Gain 2
ETR-F. 0.84

F-Offset
F-Offs. 0

Clock
Time 0:30
1: SAT-Pulse

SAT-Pulse
Int. 8
Width 0.8

Actinic Light
Int. 1
PAR 15 µ
Width 0:30
Factor 0.60

Act.+Yield
Width 0:30

Induct. Curve
Delay 0:40
Width 0:20

Light Curve
Width 0:10
Int. 1

Act. Light List

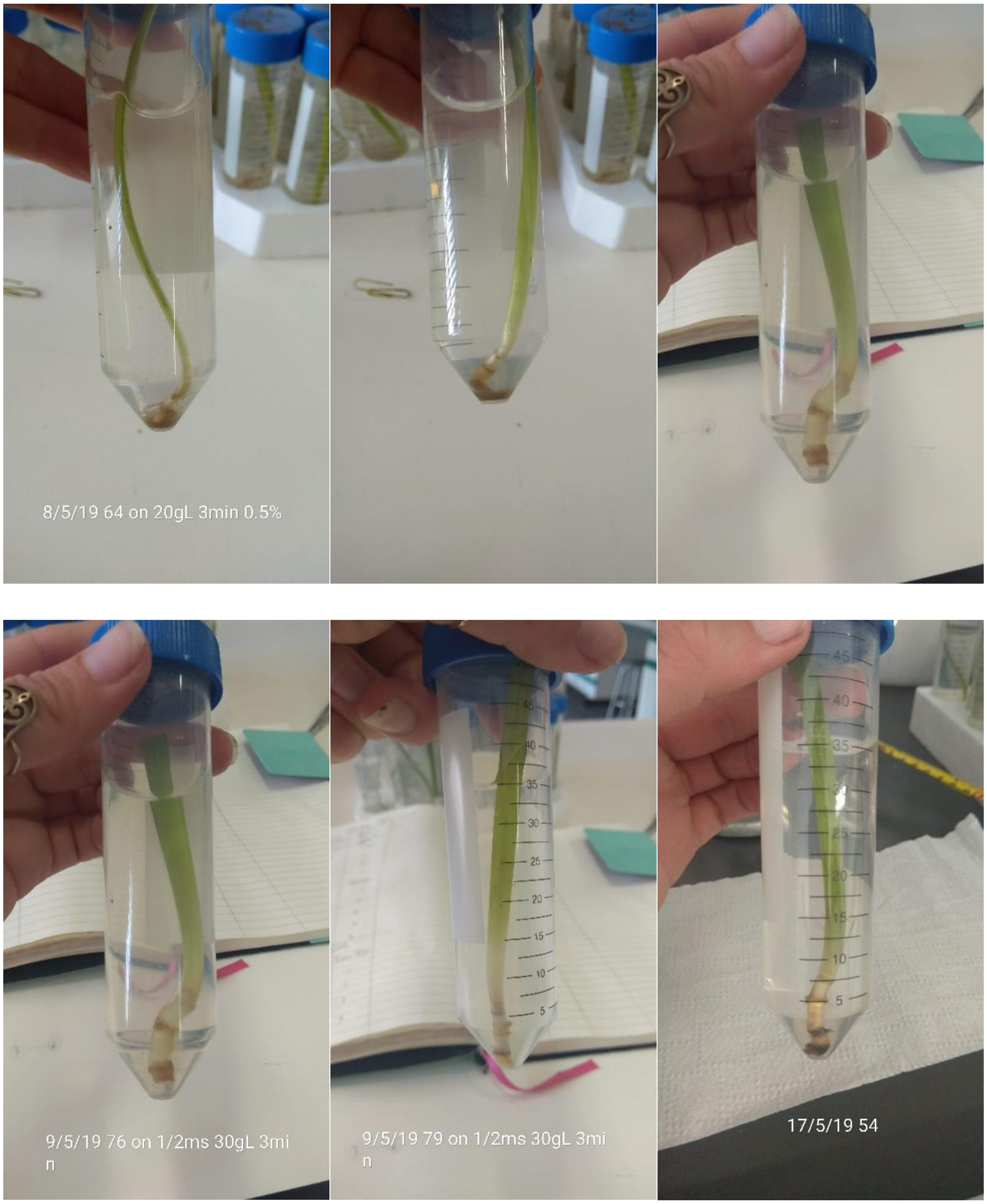
0:	0		
1:	15	7:	220
2:	31	8:	285
3:	56	9:	425
4:	102	10:	594
5:	125	11:	854
6:	146	12:	1408

Set
☑ Beeper active

Appendix 4- Photographs of initiations of *Z. noltii* and *Z. marina*

Left- Initiation cleaning and trimming of *Z. marina;* Center and Right- *Z. noltii* initiates visually examined and clean 72 hours after initiation.

Examples of visually clean shoots of *Z. marina* 72 hours after initiation



Appendix 5- Table of results of normality tests (Shapiro-Wilk test) for dependent variables for aventitious rooting trial experiments.

Species	Variable	W-value	P- Value	Significant (<0.05)
C. nodosa	Leaf elongation	0.96	0.05	N
	Longest root	0.66	<0.01	Y
	Root dry weight	0.28	<0.01	Y
	Leaf elong. rate	0.93	<0.01	Y
	Root growth rate	0.60	<0.01	Y
	Dry weight rate	0.50	<0.01	Y
Z. marina	Leaf elongation	0.95	0.04	Y
	Longest root	0.95	0.02	Y
	Root dry weight	0.90	<0.01	Y
	Leaf elong. rate	0.97	0.36	N
	Root growth rate	0.97	0.16	N
	Dry weight rate	0.87	<0.01	Y
Z. noltii	Leaf elongation	0.93	0.05	N
	Longest root	0.96	0.07	N
	Root dry weight	0.79	<0.01	Y
	Leaf elong. rate	0.92	0.05	N
	Root growth rate	0.96	0.05	N
	Dry weight rate	0.75	<0.01	Y

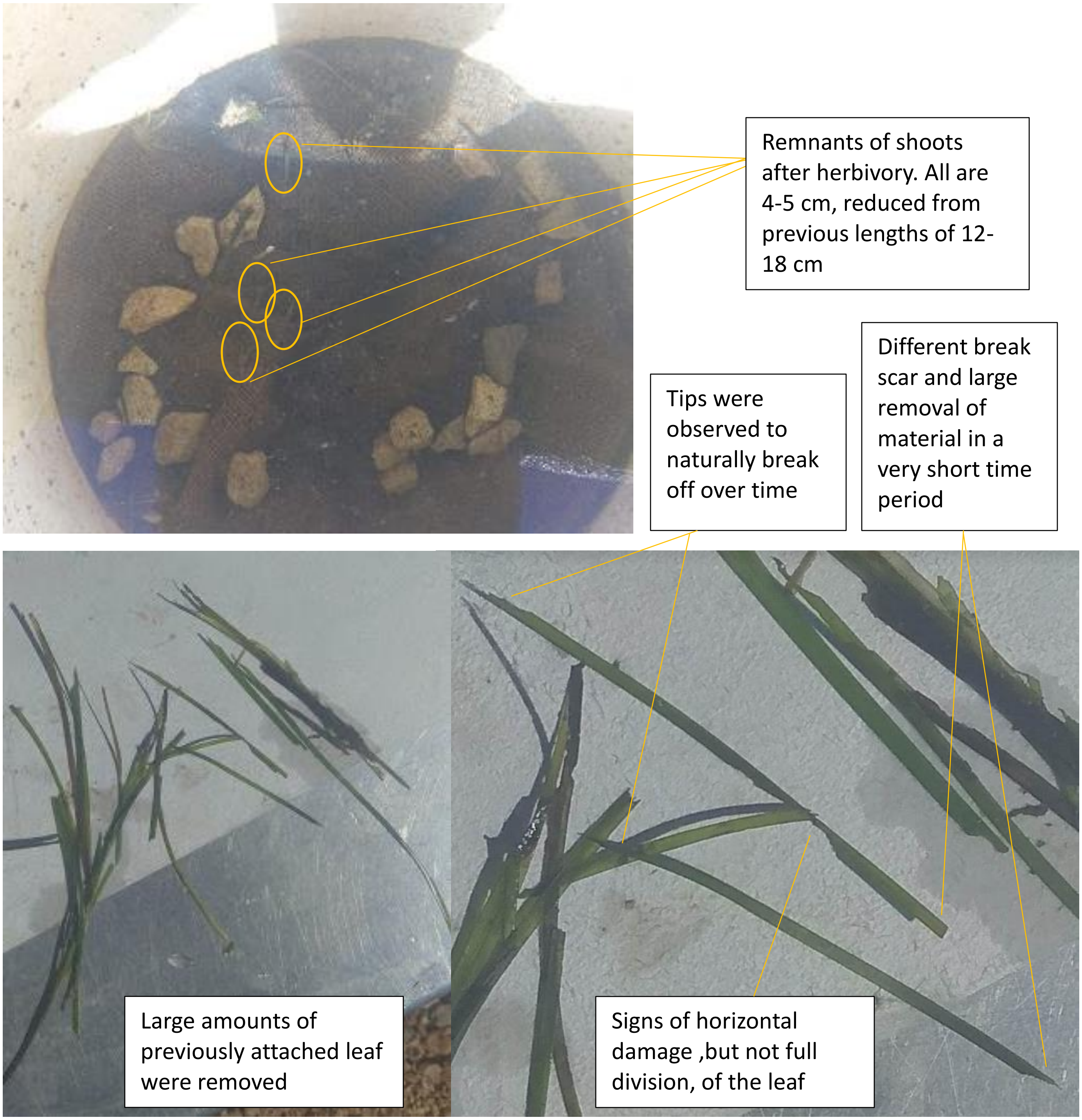
a- Treatment 3 mg/L had fewer initial nodes on average and the control treatment had more initial nodes on average than other treatment groups.

b- Treatment 10 mg/L has more initial nodes on average than other treatment groups.

c- Treatments 3 mg/L and 5 mg/L had fewer starting leaves on average than other treatment groups.

Appendix 6- Photographs of leaves of Z. noltii removed during the experimental period.

The leaves appear to have been damaged by an unidentified herbivore and were removed 4 – 5 cm from the sediment. This event seems to be related to the death of the plants affected, which in many cases did not recover.



Remnants of shoots after herbivory. All are 4-5 cm, reduced from previous lengths of 12-18 cm

Tips were observed to naturally break off over time

Different break scar and large removal of material in a very short time period

Large amounts of previously attached leaf were removed

Signs of horizontal damage ,but not full division, of the leaf

Further Graphs, PERMANOVA output, photographs and data not mentioned in-text have been provided as digital files only. Please see digital version for supplementary materials.