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Coastal mycology: boundary conditions for arbuscular mycorrhizal (AM) fungi in incipient sand dunes

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Coastal mycology: Boundary conditions for arbuscular mycorrhizal (AM) fungi in incipient sand dunes

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The University of Melbourne**

Abstract

Little is known about the relationship between the colonization by arbuscular mycorrhizal (AM) fungi in grasses in the ephemeral environment of incipient sand dunes. Yet, consideration of dune vegetation without including the soil biota in the substrate, means that a significant biological component of the dune system is ignored.

Ephemeral incipient dunes are an extreme and environmentally stressful ecosystem, encountering strong and salt-laden winds, sea water overwash, and low nutrients and these dunes are subject to a frequency and magnitude of storm action that foredunes are not. Incipient dunes represent a challenging opportunity in which to add to our understanding of coastal AM fungal tolerances, species richness and biogeography, in association with their equally resilient symbionts.

Arbuscular mycorrhizal fungi are ubiquitous in soil, and have an association with approximately 90% of terrestrial vascular plants, exchanging mineral nutrients and water for carbon compounds. These fungi are the most common mycorrhizal fungi in the sand dune ecosystem, and play a significant role in the establishment and proliferation of pioneer plants such as *Thinopyrum junceiforme* (Sea Wheatgrass), an exotic C₃ dune grass, and the native C₄ dune grass, *Spinifex sericeus* (Hairy Spinifex).

The unifying hypothesis was that AM fungi in incipient dunes have life history strategies that equip them for disturbance and sodium chloride levels not found in other contexts. A series of specific subordinate hypotheses, building one upon the other were posed to fill knowledge gaps on two mutualist plants and the biogeography of coastal arbuscular mycorrhizal (AM) fungi. The edaphic conditions the symbionts inhabit were analysed and compared, and differences were quantified of coastal AM fungal species, which it was hypothesized would differ across successional gradients. It was demonstrated that even in nutrient-poor ecosystems, substrate chemistry plays a significant role in influencing fungal communities. Furthermore, analyses confirmed differences in the presence of these fungal species between the incipient dune and foredune, the former of which was hypothesized would select for coastal fungi that are

better adapted to the disturbance regime. This highlighted that life history strategies have clearly favoured the most robust mutualists.

The hypothesis that coastal arbuscular mycorrhizal fungi are resilient to levels of sodium chloride far greater than those traditionally used in saline-tolerant studies was tested and confirmed, as it was for their host Sea Wheatgrass, whose production of roots was unaffected by saline irrigation. It was clearly demonstrated that Sea Wheatgrass exhibits an extraordinary phenomenon of resource allocation to roots rather than tillers when under stressful conditions. Such life history strategies allow this *r*-strategist to survive the hostile environment of incipient dunes.

Investigation of a further hypothesis illustrated that temporal dynamics of mycorrhizal colonization in dune grasses changes seasonally, as soil temperatures influence microbial populations. Furthermore, disturbance specialist arbuscular mycorrhizal fungi rapidly colonized plant remnants following storm scarping, supporting the hypothesis that these disturbance specialists would be found in higher proportions in plant roots post storms, than pre-storms.

The knowledge generated in this thesis provides a greater understanding of the robust subterranean ecology of coastal sand dunes, and highlights the biogeography of the coastal arbuscular mycorrhizal fungi found at the research site on the southern Australian coast. Furthermore, findings of this study challenge two points in previous studies concerning the veracity of Sea Wheatgrass in outcompeting Hairy Spinifex and of Sea Wheatgrass building incipient dunes with a greater resilience to storm events than those built by Hairy Spinifex. These findings were not substantiated by my study.

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Dedication

To my unborn child, who never saw the light of day. Mummy loves you darling.

'Would you know my name, if I saw you in Heaven?'

Eric Clapton

Declaration

This is to certify that:

1. The Thesis comprises only my original work towards the PhD except where indicated in the Preface.
2. Due acknowledgement has been made in the text to all other material used.
3. The Thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

A handwritten signature in black ink, appearing to read 'Lynda Michelle Lever', is written over a light gray rectangular background.

Lynda Michelle Lever, June 17, 2018

Preface

Chapter 2 is comprised of a substantially unchanged manuscript that has been published in *Plant Protection Quarterly, The Biology of Australian Weeds* 63, (2014) 29(4), 120 - 126. The authors of this manuscript are L.M. Hanlon (Lever), and M.B. Mesgaran. Lynda Michelle Hanlon (Lever) is the primary author, and contributed greater than 50% of the content. Lynda Michelle Hanlon (Lever) wrote the initial draft of the paper and performed subsequent editing in response to the comments of the editors.

Chapter 3 is comprised of a substantially unchanged manuscript that has been published in the *Journal of Coastal Research*, (2016), 1 (57) (SI 75), 283 - 287. The authors of this manuscript are L.M. Hanlon (Lever), L.K. Abbott, and D.M. Kennedy. Lynda Michelle Hanlon (Lever) is the primary author and contributed greater than 50% of the content. Lynda Michelle Hanlon (Lever) wrote the initial draft of the paper and performed subsequent editing in response to the comments of co-authors.

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One does not arrive at the end of PhD research without having travelled many roads to get there. Along those roads, I have had the honour of encountering many splendid people who have walked part way, or all of the way with me, offering encouragement, support and sage advice. Lyn, you feature prominently along that walk, and I am so very grateful for your friendship and guidance, and offer my deep respect in return. I am indebted to you for your financial support of the molecular analyses in my research. I am deeply appreciative of other academics who gave me the courage to believe that what I was undertaking really was worthwhile. In particular, Professor Richard Koske, The University of Rhode Island, who said my *'hypotheses were feasible and the research needed on so many levels'*, and Professor Patrick Hesp, Flinders University, South Australia, whose opening words at my first international conference, *'there I was alone and unloved on the dunes(!)'* really struck a chord, and gave me the courage to face the disbelievers of coastal mycology. Assistant Professor Mohsen Mesgaran, The University of California, Davis, is a true scholar and gentleman, and I am delighted he co-authored my first publication. I am indebted to Dr. Alex Idnurm at The University of Melbourne for generously allowing me to use the facilities of the Botany Mycology laboratory. I am also very thankful to Dr. Bede Mickan for his statistical wizardry. Thanks also to Chandra Jayasuriya for her cartography of my research site and its environs, and to Gerry Fahey for EndNote help.

I was very fortunate to have shared my PhD journey with a fabulous cohort of talented, funny, caring, and often loud (!) RHD peers, many of whom have 'beaten me to the bonnet' – Dr. Ben Iaquinto, Dr. Fabio Delai, Dr. Thu Ba Huynh, and particularly Dr. Dora Carias Vega, Dr. Marcella Chaves Agudelo, and Dr. Paula Satizábal (Paulisita), whose constant positivity and warmth I continue to value so much; thanks for the laughs. In particular, Paula's generosity of spirit has been a great source of

encouragement during the writing-up stages of my research. The rest of us are coming up behind you and will be there soon, so thanks too, to Arturo Gonzalez-Rodriguez (Speedy), Chengjun Li (CJ), Abdullah Al Baky (Baky-Boy), and Runjie Yuan (Reggie). What an incredible mix of cultures, topics, and disciplines we all encompassed, yet we still supported and encouraged each other when things were a bit bumpy, and celebrated each other's victories when things were smooth. I am all the richer for having shared my time with you.

My research would never have occurred if not for the generosity of scholarships and bursaries I was awarded, and I am indebted to the organizations listed below, for the funds that supported me through my studies, and those that enabled me to purchase laboratory and field equipment, run field trips, and to attend conferences and seminars:

- The Australian Postgraduate Award, The University of Melbourne
- The Elizabeth Ann Crespin Scholarship, The University of Melbourne
- The Victorian Environmental Assessment Council, Bill Borthwick Scholarship
- The Dawson Bursary (awarded twice), The University of Melbourne
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I am so very grateful for the assistance I received from Mr. Warren Chapman, Manager, Natural Resource Planning, and the team of people at the Barwon Coast Committee of Management, who allowed my research at Thirteenth Beach. No matter how many buckets of sand I needed to get up those very long sets of stairs from the beach, Warren and the team were available. I am so thankful to Warren for keeping me in tune with the vagaries and mischief of Mother Nature at the research site in between my field trips. Warren's benign words '*there has been an event*' now conjure up scenes of decimated incipient dunes, and the exposed and amazing Bridgewater Formation running up to and under what remained of my research site!

Family and bloodlines have an immense input to the journey and completion of a PhD, and I am sincerely thankful to my husband, David William Hanlon, for his unfailing support of my work, and for his belief in me. For his tolerance of my tantrums and moody silences I am in awe, and for sharing in my sky-high elation and joy, I am

delighted; I wonder how he will cope with an even-tempered ‘me’ again! The gritty determination of my Irish great, great, great Grandmother, Margaret (Mary) Tanner, to persevere and succeed in challenging times, has certainly come down the bloodline to me. However, it is the love and unceasing belief in my abilities by my parents, Edward Charles, and Elizabeth Valma (Val), nee Kirk, that have nurtured my soul and being throughout my life. This Doctoral Thesis is taken under my maiden name, in honour of my late parents.

‘If it’s not worth going after, it’s not worth getting.’

ECL

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List of Abbreviations

Abbreviation/acronym	Full name
AFP	Air-filled porosity
AHD	Australian Height Datum
AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
AMSL	Above mean sea level
BP	Before present
C	Carbon
CaCO ₃	Calcium carbonate
CEC	Cation exchange capacity
DEM	Digital Elevation Model
DEPI	Department of Environment and Primary Industries
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dS/m	DeciSiemens per metre
EAL	Environmental Analysis Laboratory
EC	Electrical conductivity
EC _e	Electrical conductivity of a saturated extract
EVC	Ecological Vegetation Class
FDA	Fluorescein diacetate
GBIF	Global Biodiversity Information Facility
GPS	Global Positioning System

Abbreviation/acronym	Full name
ICBN	International Code of Botanical Nomenclature
INVAM	International Culture Collection of (Vesicular) Mycorrhizal Fungi
KOH	Potassium hydroxide
LHS	Life history strategies
LOI	Loss on ignition
MHWS	Mean high water spring
mM	Millimolar
Mol	Molar
MSL	Mean sea level
NaCl	Sodium chloride
NMDS	Non-metric dimensional scaling
NSW	New South Wales
ORCID	Open Researcher and Contributor ID
OTU	Operational taxonomic unit
P	Phosphorus
pH	Power of hydrogen
ppm	Parts per million
PCR	Polymerase chain reaction
QIIME	Quantitative Insights into Microbial Ecology
RHD	Research Higher Degree
RIRDC	Rural Industries and Development Corporation
RLC	Root length colonized
RNA	Ribonucleic acid

Abbreviation/acronym	Full name
rpm	Revolutions per minute
SSU	Small sub-unit
TOC	Total organic carbon
$\mu\text{S}/\text{cm}$	MicroSiemens per centimetre
VAM	Vesicular arbuscular mycorrhizal (fungi)
WRE	Water retention efficiency

Chapter 1

Introduction and Literature Review

1.1 Introduction

This research on coastal mycology on the southern Australian coast examines the life history strategies of arbuscular mycorrhizal (AM) fungi and their associated host plants, living on the environmentally stressful ecosystem of an incipient coastal foredune (hereafter referred to as an incipient dune). The dune is vegetated by pioneer grasses, namely the native *Spinifex sericeus* (Hairy Spinifex), and the exotic *Thinopyrum junceiforme* (Sea Wheatgrass) in Victoria. However, little is known about the relationship between dune plants and AM fungi in these ephemeral, disturbed environments (Koske et al. 2004).

AM fungi are obligate symbionts which cannot be cultured axenically (Johri et al. 2015; Vandenkoornhuyse et al. 2003). The research site experienced two major storm events during this study, allowing a unique opportunity to observe changes in mycorrhizal colonization in pioneer plants, on a scarped incipient dune.

Coastal dunes are an ecosystem arising from the interplay and interaction between physical and biological processes (Durán & Moore 2013). Incipient dunes are formed by pioneer vegetation which traps sand within it (Hesp 1983). Where sand depositional rates are high, plant growth is encouraged (Hesp 1988), and some species such as *Ammophila arenaria* (Marram Grass) grow vigorously when sand accretes rapidly around them (Gadgil 2006; Huiskes 1979; Maun & Lapierre 1984). Further, different species of dune plants can form different dune morphologies (Hesp 1989; Heyligers 1985; Hilton et al. 2006).

Early work on plant roots and mycorrhizal fungi in sand dunes established the importance of these mutualists in stabilizing sand dunes (Forster 1979; Sutton & Sheppard 1976). Importantly, AM fungi can influence plant community composition (van der Heijden, Bardgett & van Straalen 2008), and promote seedling recruitment in nutrient-poor ecosystems such as dunes, through the supply of soil nutrients via extensive hyphal networks (van der Heijden 2004).

The Australian coast is approximately 29,900 km in length, of which 49% consists of sandy beaches (Short 2006). Early studies of Australian beaches documented the

impact of major storm events and their associated erosion of dunes (Andrews 1912), although it was not until the 1970s that systematic, long-term studies of beaches were undertaken (Thom 1974). It is now accepted that a fundamental part of beach adjustment during storms is that of dune erosion (Pye & Blott 2008), and that the response of incipient dunes is determined, for example, by beach morphology and the intensity and frequency of storm events (van Puijenbroek et al. 2017).

Coastal foredunes lie parallel to the beaches on which they form (McLachlan & Brown 2006) and where there is an abundance of fine sand (Maun 2009). Although dunes occur in all latitudes and climates (Martinez, Psuty & Lubke 2004), the majority are found on windward coasts (Maun 2009) where their development is the result of onshore winds blowing sand particles above the line of the swash, where vegetation traps the sand (Bitton & Hesp 2013; Carter, Hesp & Nordstrom 1990). Dunes can occur on both eroding and prograding shorelines (Doody 2013). On prograding shores, incipient dunes form closest to the swash when pioneer plants trap wind-blown sand (Hesp 2002; Hilton & Konlechner 2011), or when other obstructions such as wrack halt the movement of sand (Jackson & Nordstrom 2013; Kennedy & Woods 2012). Without perennial vegetation, or on erosional beaches for example, such incipient dunes will not survive the next high tide or storm surge (Hesp 2002), or the sand will be transported inland by the wind, or returned to the sea.

As incipient dunes stabilize, their topography becomes more complex, and where there is a high sediment supply, foredunes can develop with new incipient dunes in front of them (Hesp 1988).

The critical role of plants in the initiation and development of dunes has been recognized since the late 19th century (Cowles 1899a). Additionally, vegetation is critical in moderating beach response during high energy events through buffering the hinterland from overwash, and slowing the transfer of sand from the upper to the lower shoreface (Fernandez et al. 2016).

Following high energy events, the life history strategies of dune vegetation and its regrowth and persistence through, for example, rhizomatous fragments, is of utmost importance in halting the movement of wind-blown sand (Hesp 1991; Maun 2009), and initiating new incipient dunes (Hesp 2002; Hilton & Konlechner 2011). However,

a significant number of pioneer and other dune-building plants cannot grow without a symbiotic relationship with AM fungi (Koske et al. 2004), and so the presence of the fungus is of paramount importance in the formation of incipient dunes. Furthermore, soil micro-organisms together with vegetation play a pivotal role in sand stabilization (Alarcón & Cuenca 2005). For example, it has been long-established that the sticky fungal exudates of AM fungi bind sand grains into aggregates (Sutton & Sheppard 1976) which improve soil structure, thereby influencing plant succession (Koske, Sutton & Sheppard 1975; Nicolson 1960; Six & Paustian 2014).

Other studies have focussed on dune vegetation communities and dynamics, such as the effects of successional flora changes on sand dune morphology (Heyligers 2006; Lubke 2004; Pegman & Rapson 2005), or the relationship between geomorphic processes and vegetation on dune-barrier systems (Hilton et al. 2006; Stallins 2003). However, the subterranean microbial biomass associated with dune vegetation was not taken into consideration in such studies. Yet, consideration of dune vegetation without including the soil biota in the substrate means that a significant biological component of the dune system is ignored.

1.1.1 Spatial and temporal scales in plants and dune morphology

Vegetation responds to coastal processes on two scales – the short-term scale of seconds to days, and the long-term scale, from years to millennia (Feagin et al. 2015) (Figure 1.1). In the short-term, disturbances such as overwash or storms are rapid and may up-root or scour vegetation, whilst processes that are long-term such as sea-level rise can alter sediment transport through, for example, changes to topographic elevation (Feagin et al. 2015). For incipient dunes to establish during quiescent and disturbance-free periods, the role of vegetation is vital (Balke, Herman & Bouma 2014) in this ephemeral environment, where long-term plant colonization is prevented (Koske et al. 2004).

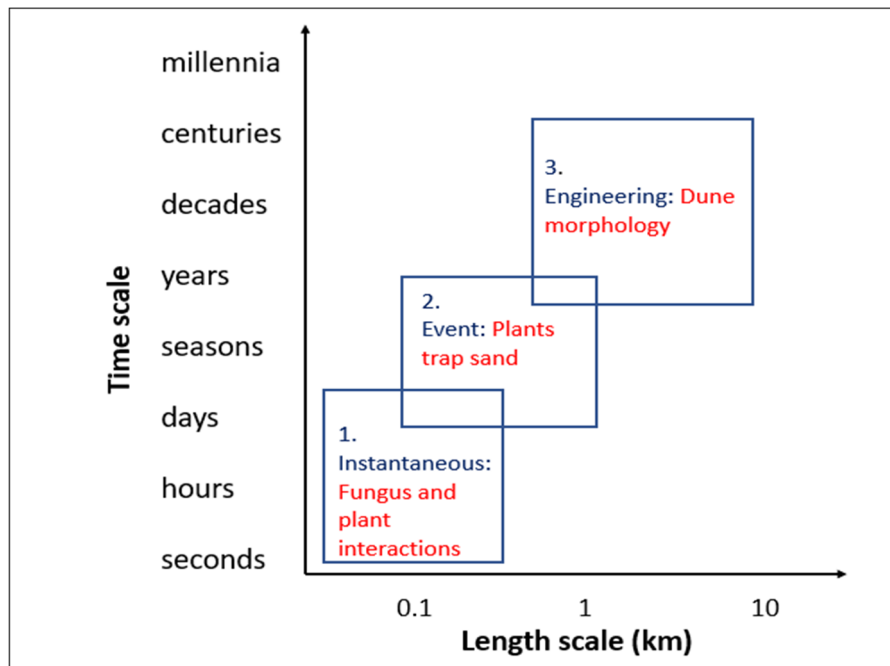


Figure 1.1: A conceptual model of the temporal and spatial scales which link symbiotic vegetation and geomorphic processes in sand dunes. (1) The fungi and plant interact, (2) sand grains adhere to sticky fungal and root exudates; plant aerial biomass traps aeolian-transported sand, (3) mature vegetation traps further aeolian-transported sand, building and stabilizing the dune over time.

Coastal dune vegetation needs to be adaptable, and to respond to the availability of AM fungi, which are of paramount importance to the establishment of new plants (Koske et al. 2004). Hyphal networks that are linked to established plants are key to the colonization of seedlings in perennial vegetation habitats such as grasses on sand dunes, as demonstrated in the seminal work of Nicolson (1960). Furthermore, microbial interactions that occur within seconds on a geomorphic scale, impact upon dune geomorphology over time (Figure 1.1). As symbiotic pioneer vegetation establishes, root and fungal exudates bind sand grains (Koske et al. 2004) thereby increasing the wind-resistance of individual grains of up to 1 – 2 mm in diameter to aeolian processes (McLachlan & Brown 2006). Vegetation that engineers, or modulates geomorphic processes by trapping aeolian-transported sand grains around and within them, may then stabilize dunes over time (Corenblit et al. 2015) (Figure 1.1).

In the dynamic biogeomorphic ecosystem of the dunes, there are strong geomorphology-plant feedbacks (Balke, Herman & Bouma 2014), which are greatly

influenced by the mutualistic symbiosis between AM fungi and incipient dune vegetation (Koske & Polson 1984). Variable environmental conditions, or stochastic variability, are a confronting but natural phenomenon for biota in sand dunes (Balke, Herman & Bouma 2014). Such conditions include scarping of incipient dunes and loss of pioneer vegetation (Bitton & Hesp 2013). However, where catastrophic events may decimate some species, they also open niches for more opportunistic species whose life history strategies enable them to reproduce quickly and take advantage of the changed conditions (Chagnon et al. 2013). For example, AM fungal species that reproduce quickly are more able to re-colonize scarped plant fragments than are those AM fungi whose propagules take longer to colonize a host (Hart & Reader 2002).

Much of what we know about AM fungi and dune vegetation is from the early studies mid- to late last century (Koske & Polson 1984; Nicolson 1959), or early in the 2000s (Alarcón & Cuenca 2005). However, little is yet known about the biogeography of AM fungi (Chen et al. 2017), or their tolerance to sea water flooding (Hanley et al. 2017) combined with severe disturbance.

1.1.2 Research objectives and aims

This research will make direct contributions to the field of coastal mycology, which lacks specific consideration of the relationship between AM fungi, sand dune grasses, and ephemeral incipient dunes. Based on gaps in the current knowledge about coastal AM fungi, the main hypothesis is that AM fungi in incipient sand dunes have life history strategies that equip them for disturbance and sodium chloride levels not found in other contexts. In order to address this overarching hypothesis, a series of objectives was undertaken to elucidate the presence, abundance, and tolerances of this ancient group of biotrophs.

The unifying objectives are:

- To determine the boundary conditions under which coastal AM fungi are found in association with symbiotic dune C₃ and C₄ grasses on ephemeral incipient dunes on Thirteenth Beach, on the southern coast of Victoria, Australia (Chapter 2).

- To describe the biology, reproduction, growth, development and habitat of the exotic C_3 Sea Wheatgrass, which is found in association with the native C_4 Hairy Spinifex (Chapter 3).
- To investigate changes in seasonal abundance of AM fungi in, and between, the roots of the exotic C_3 Sea Wheatgrass, and the native C_4 Hairy Spinifex, and to ascertain the response of symbionts to storm damage (Chapter 4).
- To quantify mycorrhizal response to increasing and extreme levels of sodium chloride irrigation, pre-and post-colonization of host plant roots (Chapter 5).
- To investigate the presence of infective AM fungal propagules in the foredune, and their veracity in colonizing a host from the incipient dune (Chapter 6).
- To investigate and assess AM fungal richness across dune gradients from the incipient dune and the foredune, thereby establishing the biogeography of AM fungi at the study location Chapter 6).

1.1.3 Methodology

The topics, questions and hypotheses presented in this study are interwoven, and evidence from several areas of inquiry have been collected to test the veracity of hypotheses posed. The inter-relatedness of some of the topics necessarily creates overlaps between them. Research for this Thesis was based upon field-collected data for sand and plant matter collections at Thirteenth Beach, Victoria, laboratory work on plant-fungal material, and glasshouse experiments with crops and fungal inoculum.

1.1.4 Botanical nomenclature

In following the protocol of the International Code of Botanical Nomenclature (ICBN) for plant names (Greuter et al. 1999), the first mention of a plant species in any Chapter will be given in scientific botanical nomenclature, and thereafter be referred to by its common binomial name. Although there is no universally accepted style for capitalization of common names, it is generally accepted that both parts of the binomial be capitalized (Spencer & Lumley 1991). All images of plants and all tables and figures will use botanical nomenclature.

1.1.5 Thesis structure

The evidence for hypotheses posed is covered in a sequence of connecting Chapters, each dealing with one aspect of the research (Figure 1.2), and data collection or analysis from a particular section of the dune (Figure 1.3). Published manuscripts are integrated into the Thesis, and lay the foundation for the experiments, field work and laboratory work undertaken.

Chapter Two introduces the geomorphology of the study site, with digital elevation model cross-sections of the dune system preceding the storms that severely scarped the incipient dune following the first season's data collection. Edaphic and chemical analyses of the sand are discussed in relation to nutrient limitations for plants. Addenda to the published manuscript includes the geology of the local area to explain the underlying formations that were exposed by the removal of sand due to severe storms, and subsequent sediments that were deposited by wave action.

Chapter Three discusses the exotic C₃ dune plant, Sea Wheatgrass. The complex taxonomy of Sea Wheatgrass is examined, with emphasis on morphological differences between the genera *Thinopyrum* and *Elymus*, both of which Sea Wheatgrass has been classified under. Reproduction, biology and dispersal mechanisms are addressed, as are the history of the grass in Australia and its distribution. The native habitat of Sea Wheatgrass is described, and the nature of its growth and development are provided. The association of Sea Wheatgrass under one of its heterotypic synonyms (*Agropyron junceiforme*) is briefly introduced. Addenda to the published manuscript introduces the native C₄ dune grass, Hairy Spinifex, including taxonomy and nomenclature, biology, distribution and mycorrhizal associations.

Chapter Four discusses the data from of two years of seasonal field work (summers and winters), investigating the changes in relative abundance of mycorrhizas in Hairy Spinifex and Sea Wheatgrass. The storm which severely scarped the study site following the collection of the first summer's harvest presented a unique opportunity to investigate mycotrophic response to, and changes due to, disturbance and loss of substrate. The percentage of leaf matter of dune grasses was measured within non-contiguous 1m² quadrats across the incipient dune over the course of two years, and changes to these percentages as a result of the storms were studied. In addition,

changes to the enzyme concentrations of microbial decomposers in the substrate were measured seasonally, through fluorescein diacetate hydrolysis concentrations.

Chapter Five discusses two experiments. The first experiment details a bioassay to determine the presence of infective AM fungal propagules in the incipient dune sand, and at what week of plant growth AM colonization began. The second experiment entails using increasing and extreme levels of sodium chloride irrigation on propagules of Sea Wheatgrass grown in incipient dune sand under ambient glasshouse conditions. The experiment was run over several harvests with five levels of sodium chloride ranging from zero to that of twice the concentration of sodium chloride found in sea water (0 ppm - 70,000 ppm/109.40 dS/m). Additionally, the application of saline irrigation was imposed over two differing time regimes – from the day the plant propagules were planted (pre-AM fungal colonization), and from week five when the presence of infective AM fungal propagules had been established by a bioassay. Results are discussed in relation to changes in the proportions of roots colonized over harvests (time), and levels of sodium chloride. The response to the increasing levels of sodium chloride over time on enzyme concentrations of microbial decomposers in the sand was also investigated, using fluorescein diacetate hydrolysis.

Chapter six encompasses three experiments across the dune gradient to extrapolate the life history strategies and niche differentiation of AM fungi between the stable foredune and disturbed incipient dune. These data will elucidate differences that select for AM fungi across the dune ecosystems, and show how fungal species are distributed. The first experiment undertakes a bioassay to assess the presence of infective AM fungal propagules in the foredune, with chemical and edaphic analyses of the sand elucidating differences that may select for AM fungal species between the ecosystem gradients of incipient dune and the foredune. The second experiment investigates the efficacy of infective AM fungal propagules from the foredune in colonizing a host plant from the incipient dune, by enumerating changes to the proportion of roots colonized by mycorrhizas over time, through several destructive harvests. The third experiment undertakes an assessment of AM fungal species and richness across the ecosystems and gradients of the dune at Thirteenth Beach, in relation to differential responses to edaphic conditions and disturbance, which select for either *r*- or *K*-

strategists. Analyses are discussed in relation to AM fungal species composition between gradients and vegetation types on the foredune and incipient dune.

Chapter Seven provides a synthesis and implications of the research findings in this study for coastal mycology, and includes suggestions for future studies.

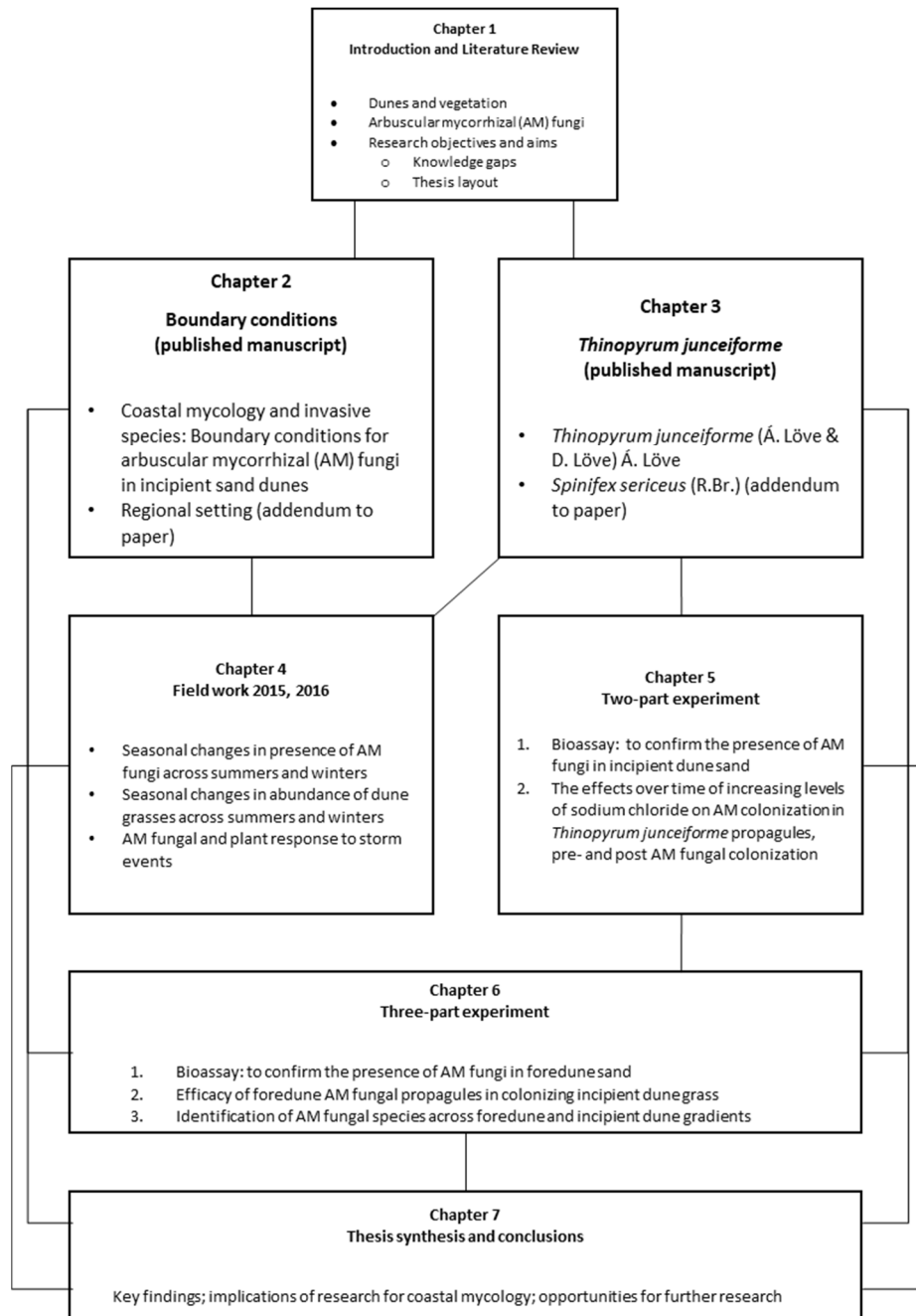


Figure 1.2: Overview of Chapters in this Thesis and how they inter-relate

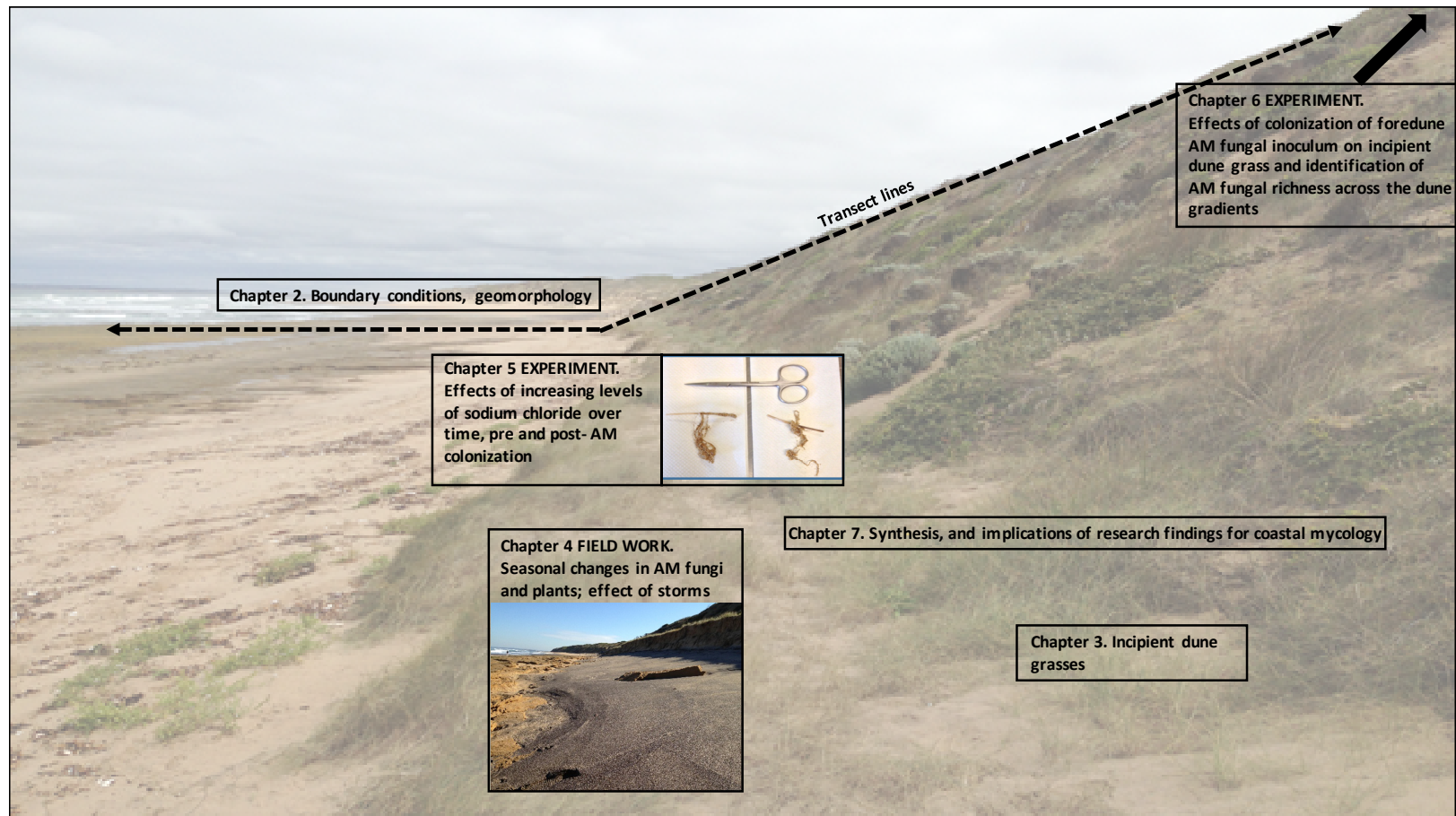


Figure 1.3: Photograph of the study site illustrating the conceptual map of this body of work, showing areas on the dunes and beach where studies have taken place, or samples were taken for experiments and laboratory work

1.2 Literature Review

1.2.1 Dunes and vegetation

Plants on coastal dunes encounter unique and extreme abiotic stresses such as salt-laden winds, intermittent burial by sand, and low nutrients (Maun 2004). The harshest conditions in the dune ecosystem are to be found at the upper limit of the swash (Hesp 1991). The incipient dunes are ephemeral in nature, and vegetated by pioneer plant species which trap wind-blown sand. However, these dunes are subject to a magnitude and frequency of wave and storm action that foredunes generally are not. Such regular disturbances deposit organic nutrients such as wrack (Jackson & Nordstrom 2013; Kennedy & Woods 2012) that aid floral and microbial growth.

Plants living on incipient dunes have specific adaptations in order to survive the conditions associated with proximity to mean high water spring (MHWS) (Hesp 1991). Such adaptations include tolerance to salt spray, for example in *Cakile* spp. (Sea Rocket) (Boyd & Barbour 1986; Hesp 1991), sand burial, for example in Hairy Spinifex (Bergin 2011) and Marram Grass (Gadgil 2002), and marine inundation, for example in Sea Wheatgrass (Hanlon & Mesgaran 2014) (Chapter 2). There are numerous mechanisms in plants that allow for such physiological tolerances including leaf roll (Pavlik 1983), timing of germination (Lee & Ignaciuk 1985), sodium chloride accumulation (Rozema 1982), and tenacious, deeply rooted rhizomes. Such rhizomes maintain aerodynamic roughness (Hesp 1988), and often persist to re-colonize the incipient dune following storm events even when tillers have been destroyed. Vegetative re-growth in *Poaceae* plants such as Sea Wheatgrass for example, is from nodes, or tiller buds, and it is from these nodes that the highly fibrous root system of grasses forms and tillers are produced (Yamaji & Ma 2014).

Plant growth is encouraged in some dune plant species where the rates of aeolian-deposited sand are the greatest (Hesp 1988). Plants that can withstand periodic episodes of burial, a phenomenon that governs their ecology in the harsh dune environment (Maun 1998, 2004) have been shown to reallocate resources from roots to shoots (Maun 1998; Shi et al. 2004), and to show stimulated growth responses when

uncovered after burial (Gilbert & Ripley 2010). However, in contradiction to resource shifts from subterranean to aerial organs, the nodes of Sea Wheatgrass preferentially allocate resources to roots, of which prodigious amounts are formed (Hanlon, Abbott & Kennedy 2016; Hanlon & Mesgaran 2014).

The sand-trapping plant species of the dunes stabilize the sand through their network of roots and their symbiosis with AM fungi (Azevedo de Assis et al. 2016). The hyphae of AM fungi enmesh sand grains to form delicate aggregates (Forster & Nicolson 1981a; Sutton & Sheppard 1976), and sticky fungal polysaccharides adhere sand grains to plant roots (Feagin et al. 2015; Rillig 2004) thus altering the edaphic properties of the dune (Feagin et al. 2015). Such properties include the input and breakdown of organic matter from leaf abscission or root exudates (Feagin et al. 2015; Pluske, Murphy & Sheppard 2017). Once stabilized, the colonization of burial-sensitive plants is facilitated and vegetation will thereby influence the long-term dynamics of the dune (Tsoar 2005), providing that such vegetation is not removed through anthropogenic activities or extreme weather events, for example (Tsoar 2005).

For any organism, the dunes are a harsh ecosystem in which to live, with extremes of soil moisture, high temperatures, abrasion and desiccation from salt-laden winds, and overwash on the incipient dunes and in the swash (Ievinsh 2006; Maun 2009). Moreover, this dynamic niche also encounters nutritional deficiencies and high light intensities of which only a few plant species are tolerant (Martinez, Vazquez & Sanchez 2001).

1.2.2 Dominant dune grasses in Victoria

The coastal dunes of Australia are vegetated with both exotic and native plants, and in south eastern Australia of the 148 common coastal beach plants, 24% are recognized as exotic species (Carolin & Clarke 1991). The three main dune-building grasses in Victoria comprise two exotic species, Marram Grass and Sea Wheatgrass, and the native Hairy Spinifex (Cousens et al. 2012). All of these grasses have the potential to modify dune morphology (Cousens et al. 2012), as they are all capable of trapping sand thereby limiting its movement (Hilton et al. 2006).

1.2.3 Arbuscular mycorrhizal (AM) fungi and psammophilic plants

Table 1.1: A synopsis of global studies on the role of arbuscular mycorrhizal (AM) fungi in the proliferation of psammophilic and pioneer plants, and stabilization of sand dunes.

COUNTRY, SOURCE	MAIN FINDINGS
Australia, Koske (1975)	Endogone spores were abundant in NSW sand dunes, with greatest abundance in stabilized dunes.
Australia, Logan et al. (1989)	Colonization of coastal sand dune plants in NSW enhanced nutritional uptake and sand aggregation.
England and Scotland, Nicolson (1959)	Mycorrhizal colonization in sand dune plants allowed the transfer of nutrients from sand to plants, and plant to fungus, thus enhancing plant survival.
England, Nicolson (1960)	Mycorrhizas influenced the role of grasses in dune succession due to beneficial effects of colonization on host plants.
Iceland, Greipsson et al. (2002)	Mycorrhizal colonization in dune grasses was vital in polar region dunes for dune stabilization, dune reclamation, and plant succession.
Scotland, Nicolson and Johnston (1979)	Mycorrhizas improved the growth of Marram Grass and Sea Wheatgrass (then called <i>Agropyron junceiforme</i>), in embryo dunes.
USA, Koske and Polson (1984)	American dune grasses were dependent upon mycorrhizal colonization (then called VA fungi), which significantly enhanced plant growth, leading to dune stabilization by mycorrhizal plant roots.
USA, Rose (1988)	Mycorrhizas (then called VAM fungi), were essential for plant nutritional uptake, survival of pioneer dune plants, and sand aggregation, correlating with plant succession on the dunes.
USA, Sylvia (1989)	Mycorrhizas were vital for the establishment of dune plants, particularly on re-nourished dunes and for transplanted nursery-propagated beach plants.
Venezuela, Alarcón and Cuenca (2005)	Mycorrhizas were ecologically important for vegetation in all areas of coastal dunes.

AM fungi were first found in the coastal dune system in the early, and seminal, studies of Nicolson (1960) and have since been found in association with psammophilic and pioneer plants in a number of countries (Table 1.1). The fungi are fundamental to the survival and proliferation of pioneer plants in the dunes (Koske et al. 2004), where the uptake of nutrients, especially phosphorus and nitrogen (Mensah et al. 2015), and water, are greatly enhanced by AM fungal symbiosis. For example, *Ammophila*

breviligulata (American Dune Grass) has been shown to have up 19% more leaves per tiller when colonized by AM fungi, particularly under a regime of reduced irrigation (Emery, Bell-Dereske & Rudgers 2015) suggesting that some species of AM fungi, like coastal plants, are specifically adapted to the harsh environment of the dunes (Yamato et al. 2012).

Grasses in fresh water lacustrine sand dunes, such as on the Great Lakes of Northern America, have also long been known to have mycorrhizal associations, with fungal external mycelium indicated in sand aggregation and dune stabilization (Koske, Sutton & Sheppard 1975). The edaphics of lacustrine dunes have similarly harsh environmental conditions for symbionts as those of coastal dunes, with low levels of nutrients, water retention and organic matter (Pan et al. 2016).

1.2.4 Arbuscular mycorrhizal (AM) fungi

‘Mycorrhiza’ is from the Greek, *mykís*, *mykes* for fungus, and *rhiza*, for root (Alexopoulos, Mims & Blackwell 1996). AM fungi are primitive, obligate biotrophs and are associated with the first plants on land (Redecker 2002; Rillig 2004). The fungi are in the fossil records of the Ordovician Period of the Paleozoic Era, 460-455 Ma (Redecker 2002; Rillig 2004). Fossilized fungal spores and hyphae of Glomales-like fungus were found in what was presumed to be a shallow marine setting, in what is now known as Wisconsin (Redecker, Kodner & Graham 2000). Together plants and fungi have co-evolved, and AM fungi now form mutualistic associations with more than 90% of terrestrial vascular plants (Schnepf, Roose & Schweiger 2008; Young 2015), where they are ubiquitous in soil (Smith & Read 2008) and integral to the functioning of ecosystems throughout the world (Remy et al. 1994). As they have existed unchanged morphologically for > 400 million years, they may be regarded as living fossils (Parniske 2008).

AM fungi are endophytes in the monophyletic phylum of Glomeromycota, order Glomerales (Schüßler, Schwarzott & Walker 2001), and are in the genera *Glomus*, *Funnelformis*, *Septoglomus* and *Rhizophagus* (Redecker et al. 2013). The fungi are described as primitive due to a lack of sexual reproduction (Harrison 2005), being associated with a wide diversity of plant species, and there are relatively few species (Morton 1990). The hyphae of AM fungi are generally coenocytic and aseptate, whereby hundreds of

nuclei share the same cytoplasm, with individual spores also sharing the coenocytic trait (Parniske 2008).

Arbuscular mycorrhizas form a tripartite relationship between plant roots, the edaphic environment, and mutualistic fungi, and these must be considered together in any study of AM fungus-plant interactions (Brundrett & Abbott 2002). Additionally, mycorrhizas differ from other plant-fungus interactions, in that AM fungi are intimately associated with the exchange of nutrients and photosynthates between living cells (Nehls et al. 2001; Pfeffer, Bago & Shachar-Hill 2001). Between 20% (Gamper, Hartwig & Leuchtmann 2005; Parniske 2008) and up to 30% (Drigo et al. 2010) of plant photosynthates are consumed by AM fungi at ambient atmospheric carbon dioxide, equating to an estimated five billion tonnes of carbon per annum (Parniske 2008). It is mycorrhizas rather than plant roots that uptake and transfer to plants immobile nutrients such as phosphorus in soils (Smith & Read 2008) and also nitrogen and other inorganic nutrients (Schüßler, Schwarzott & Walker 2001). Further, greater amounts of water than the plant roots alone can access, are transferred to plants via the extensive network of fungal hyphae in the soil (Smith & Read 2008). In addition, the fungi are a key component of carbon sequestration in soils via the transfer of photosynthates from the host plant to AM fungal hyphae, albeit the turnover of extraradical hyphae is swift (Zhu & Miller 2003).

It has been demonstrated that some AM fungal spores can germinate in response to chemical signals from the roots of host plants, with the fungal hyphae growing through the soil towards the host's roots (Wright 2005). When the hyphae encounter a plant root, an appressorium is formed which penetrates the root, and enters the cortex (Wright 2005). The hyphae are branched, and extend both inside (intra-radical), and outside (extra-radical) the plant cell, producing vesicles and loops or coils, and arbuscules which are the sites where mineral nutrients and water are exchanged for carbon compounds between the fungus and the plant (Koide & Mosse 2004). Extra-radical hyphae and spores are also formed (Brundrett & Abbott 2002), and it is the extra-radical phase in particular that impacts upon soil structural stability (Harrison 2005), by enmeshing and entangling soil particles, or sand grains, to form aggregates (Feagin et al. 2015; Forster & Nicolson 1981a; Rillig 2004; Rose 1988; Six & Paustian 2014).

AM fungi cannot be cultured axenically, requiring that they be propagated via host plants (Helgason & Fitter 2009; Morton 1990; Zhu et al. 2016). However, visual identification of mycorrhizas in plant roots through microscopy techniques is arduous and imperfect (Brundrett & Ashwath 2013). Identification is complicated by the relative abundance of structures such as spores, hyphae and colonized root length that change over sampling times, and may not identify accurately the fungus at species level (Merryweather & Fitter 1998). Furthermore, morphological traits are easily misidentified by the presence of lineages that stain weakly, such as *Glomus occultum* (syn. *Paraglomus occultum*) (Morton & Redecker 2001), or by the difficulty in removing darkly-stained phenolic compounds without disintegrating the samples (Brundrett et al. 1996).

Molecular techniques also have their limitations in the identification of AM fungi in plant roots, such as when only a small amount of DNA has amplified or when an inappropriate choice of target rRNA marker region is employed, thereby presenting the inability to differentiate between closely related AM fungal species (Kohout et al. 2014). Furthermore, nested PCR amplicons may bias significantly the relative abundance of mycorrhizas in plant roots (Shi et al. 2012). The debate over the taxonomy of AM fungi in *Glomeromycota* has also generated confusion among biologists who study the symbiont, with the phylum undergoing several changes during the last decade (Redecker et al. 2013). The current classification is based on molecular-phylogenetic evidence, which incorporates fungal morphological characteristics where they are accordant (Redecker et al. 2013). The consensus of taxonomic nomenclature is accepted as phylum Glomeromycota, family *Glomeraceae*, genera *Funneliformis*, *Glomus*, *Rhizophagus* and *Septoglomus* (Redecker et al. 2013).

AM fungi are the dominant group of soil organisms involved in promotion of aggregation of sand grains (McLachlan & Brown 2006), which unlike clay particles lack the electrostatic attraction forces of cations and anions (Ashman & Puri 2002). The mycelial network of AM hyphae that envelopes sand particles confers some wind resistance upon those particles, by increasing substantially the weight of single grains as they adhere to form aggregates with other grains (McLachlan & Brown 2006). The adhesion of sand grains into micro-aggregates, and then to larger aggregates occurs during active symbiosis (Clough & Sutton 1978). On the face of the incipient dune when plant roots are exposed, sand grains can sometimes be observed covering the

roots and adhering to glue-like glycoproteins from AM fungi. Additionally, the sticky polysaccharides in fungal exudates (Rillig 2004) that enmesh and adhere sand grains around and over plant roots, create tassel-like root and sand formations. Over days, seasons and perhaps years in the case of perennial plants, the plant root mass grows, with fungal exudates entangling more sand grains. Together with plant roots AM fungi which are sometimes referred to as ecosystem engineers, (Cameron 2010; Manaut et al. 2015) help to build foredunes.

1.2.5 Arbuscular mycorrhizal (AM) fungi and C₃ and C₄ grasses

Perennial grasses are classified as being C₃ or C₄, which refers to the different metabolic pathways that the plants use to capture carbon dioxide during photosynthesis (Brüggemann et al. 2011; Sage, Sage & Kocacinar 2012). Grasses with C₃ metabolism are more primitive than are C₄ grasses, although the first stage of carbon fixation in both types of grass involves a 3-carbon molecule, albeit in C₄ grasses, a 4-carbon molecule is initially produced, which then enters the C₃ (Calvin) cycle (Taiz & Zeiger 2006). C₃, or cool-season grasses, are adapted to cooler weather and have a preference for higher precipitation, whereas the warm-season C₄ grasses prefer regions of lower precipitation and higher temperatures than do C₃ species (Pau, Edwards & Still 2013). It is not uncommon for wild species of both grass types to co-exist in coastal settings. For example, C₄ native Hairy Spinifex and the exotic C₃s, Sea Wheatgrass and Marram Grass are found in coastal dunes. Although Marram propagules imbibed in sea water have been found to germinate when planted in sand and irrigated with fresh H₂O (Konlechner & Hilton 2009), seedlings and mature plants of Marram cannot survive direct contact with sea water (Huiskes 1979; Sykes & Wilson 1988). Therefore Marram colonizes the lower areas of dunes only when the risk of inundation by sea water has passed (Huiskes 1979; Sykes & Wilson 1988).

Studies are contradictory regarding AM fungal colonization in grasses. For example, Brundrett et al. (1996) argue that grasses in general tend to be facultative in their association with AM fungi, and that C₃ grasses are facultatively mycorrhizal in disturbed and low nutrient environments – an apt description of the incipient dune ecosystem. To the contrary, Hetrick et al. (1990) and Ramos-Zapata et al. (2011)

contend that C₃ grasses are ‘weakly’ mycorrhizal, albeit no percentage of colonization is given, and that C₄ grasses are obligate mycotrophs. Other studies have shown that the roots of most psammophilic plants are mycorrhizal (Camprubi et al. 2012).

Contradictions regarding mycorrhizal abundance may be related to how abundance is assessed. The modified grid line intersect method (Giovannetti & Mosse 1980; Newman 1966; Tennant 1975) is commonly used. This methodology entails plant roots which have been cleared of their cell contents and stained to highlight AM fungal structures, being cut to a uniform length and dispersed randomly in a gridded 9 cm Petri dish. By scanning along the grid lines using a dissecting microscope, sections of plant root that intersect the grids are enumerated as colonized or not. (Giovannetti & Mosse 1980; Newman 1966; Tennant 1975). The percentage colonization (% RLC) of root length by mycorrhizas and the total colonized root length (cm) (Newman 1966; Tennant 1975) are used to estimate the length of mycorrhizal root (Abbott & Robson 1978). Other methods entail enumerating a minimum of 100 intersections to assess a sample, or using a visual estimate to within 5% to 10% of the degree of mycorrhizal colonization in plant root samples (Giovannetti & Mosse 1980). The latter method is open to subjective counts unless some sort of calibration is used, but is regarded as sufficient if precision is not required (Brundrett et al. 1996). A further method employs a magnified intersection method whereby only arbuscules are enumerated as they are a universal feature of AM fungi (McGonigle et al. 1990). Arbuscules are finely branched hyphal structures that develop within the cell wall of a plant’s cortical cell, creating large surface areas of membrane-to-membrane contact between plant and fungus, across which nutrients and photosynthates are exchanged (Fitter 2005).

Relative abundance may also be constrained by enumeration of mycorrhizas in samples taken from the natural environment, compared to those grown in pot cultures with introduced inoculum. Where Ramos-Zapata et al. (2011) assessed seasonal mycorrhizal abundance in the sand dune plants of Yucatan Mexico, pot cultures of psammophilic plants inoculated with mycorrhiza from a bait crop were used by Camprubi et al. (2012).

1.2.5.1 AM fungal symbiosis with Sea Wheatgrass and Hairy Spinifex

There are no studies that specifically address interactions between AM fungi and Sea Wheatgrass and the combined roles of plant and fungi on the ephemeral environment of maritime incipient sand dunes, apart from those of Forster (1979), and Forster and Nicolson (1981b), at Tentsmuir in Scotland. In these early, pivotal studies, Sea Wheatgrass was addressed under the heterotypic synonym of *Agropyron junceiforme*.

There is a paucity of knowledge regarding the mycorrhizal status of the native dune grass, Hairy Spinifex. Notwithstanding this, the colonized root length in Hairy Spinifex by AM fungi equated to 55% in an early study conducted on the mycorrhizal status of 41 sand dune species on the coastal dunes of New South Wales (Logan, Clarke & Allaway 1989). The root length of Hairy Spinifex (stolons were not explicitly mentioned), extended > 2 m beyond the plant and had internal and external hyphae, as well as vesicles, although no coils or arbuscules were observed (Logan, Clarke & Allaway 1989).

1.2.6 Knowledge gaps

Understanding the tolerances and processes that drive the relative abundance of a species is a central aim of ecology (Dumbrell et al. 2010; Lekberg et al. 2012), yet little work has been undertaken on the colonization of coastal sand dune plants by AM fungi (Johansen et al. 2015; Koske et al. 2004) and bears further investigation.

Despite AM fungi being cosmopolitan, their diversity and distribution at regional scales is less well understood than that of animals and plants (Martiny et al. 2006) and there remains a substantial gap in knowledge of fungal biogeography (Chen et al. 2017; Öpik et al. 2013). In Australia this is particularly so (Öpik et al. 2013), where there have been very few studies of coastal AM fungi, and none on the southern coastline. Additionally, knowledge of the temporal dynamics of AM fungi, and changes of fungal species within or between hosts is lacking, with few studies of dune vegetation acknowledging the vital importance of AM fungi to the survival of psammophilic plants (Koske & Polson 1984; Roy-Bolduc et al. 2016). This is especially pertinent where plant and AM fungal communities live on the ephemeral incipient dunes, where saline overwash is

common, and storm scarping can decimate vegetation. In this regard, there is a need to know how coastal AM fungi respond to such disturbance.

In disturbed and harsh ecosystems such as incipient dunes, fitness and phenotypic variation select for the most tolerant of species (Helgason & Fitter 2009), and denote the paramount importance of AM fungi in the substrate. Moreover, mechanisms that result in differences between AM fungal communities across coastal ecosystem salt gradients remain largely unknown (Lekberg et al. 2007), albeit distributions are likely to be affected by environmental parameters such as disturbance, soil moisture and availability of nutrients (Rillig et al. 2002).

Knowledge gaps are addressed across several Chapters with each Chapter discussing subordinate hypotheses. Chapters build one upon the other in order to substantiate the unifying hypothesis

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Chapter 2

**Coastal mycology and
invasive species:
Boundary conditions for
arbuscular mycorrhizal
(AM) fungi in incipient
sand dunes**

This chapter is from the following publication:

Hanlon, L.M., Abbott, L.K., and Kennedy, D.M. (2016) ‘Coastal mycology and invasive species: Boundary conditions for Arbuscular Mycorrhizal (AM) fungi in incipient sand dunes’, *The Journal of Coastal Research*, SI 75, 283-287.

I was responsible for the planning, preparation, writing, execution, and interpretation of data in this work, for publication. The co-authors contributed valuable advice, and jointly agreed upon the research objectives.

Data and research on the boundary conditions for arbuscular mycorrhizal (AM) fungi in incipient sand dunes in this Chapter are published in *The Journal of Coastal Research*, SI 75, 283-287. Addenda to the publication are sections 2.1, 2.3.1, 2.3.2, 2.5.1 and 2.7.

2.1 Abstract

Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil, and are associated with some 90% of terrestrial vascular plants, aiding plants to access water and nutrients the plant roots alone cannot, in exchange for photosynthates from their host. AM fungi were first found in dune systems in the 1960s, and many of the described species form symbiotic associations with psammophilic plants including dune grasses. The ephemeral environment of incipient sand dunes prevents long-term colonization by plants. Little research has been undertaken to examine the contribution of AM fungi to plant survival in the disturbed environment of incipient sand dunes, or what role, if any, they play in exotic plant species outcompeting native species. A first step to understanding these roles is to examine the edaphic and biological conditions of incipient dunes. The findings quantify the boundary conditions that surround and support AM fungi and their host plant roots in incipient sand dunes on the southern coast of Victoria, and include the chemical and geomorphological characterizations of the dunes studied. It was found that the nutrient levels (total organic carbon, phosphorus, and nitrogen) were low in contrast to the higher levels of nitrogen found on the Atlantic coast, and pH levels such that aluminium would be toxic for the majority of plants, whilst iron was limited. Additionally, it was found that the incipient

dune sand was not saline, and that chemical characteristics between the toe and the crest of the incipient dune did not differ greatly.

2.2 Introduction

Dune erosion is a fundamental part of beach adjustment during storms (Pye & Blott 2008), and the role of vegetation in releasing sand into the littoral system is critical. Incipient dunes form closest to the swash, when pioneer vegetation (Hilton & Konlechner 2011), or obstructions such as wrack trap windblown sand (Kennedy & Woods 2012). Furthermore, the critical role of plants in the initiation and development of dunes has long been recognized (e.g. Cowles 1899a).

In Victoria, there are three main dune grass species, *Ammophila arenaria* (Marram Grass), *Thinopyrum junceiforme* (Sea Wheatgrass) (Figure 2.1), and *Spinifex sericeus* (Hairy Spinifex) (Cousens et al. 2012), but only the latter is native. Marram Grass is mainly found in the foredunes, and Hairy Spinifex and Sea Wheatgrass dominate the incipient dunes (Cousens et al. 2012). Sea Wheatgrass is an erect, rhizomatous, perennial grass, growing to 0.5 m in height, and is endemic across a wide range of the European coasts (Chapter 3). It has spread rapidly along Victoria's coast since it was first recorded in 1933.



Figure 2.1: *Thinopyrum junceiforme* on the incipient dune at the base of the foredune on Thirteenth Beach, Victoria, 2015.

Grasses are known to have mutualistic associations, or symbioses, with arbuscular mycorrhizal (AM) fungi (Ramos-Zapata et al. 2011). However, little is known about the symbioses of AM fungi and vegetation in incipient dunes, despite a substantial variety of AM fungi being found in sand dunes (Koske et al. 2004). AM fungi are obligate biotrophs, forming symbioses with some 90% of terrestrial vascular plants (Young 2015). They can substantially enhance their host's ability to uptake water and nutrients (Koske et al. 2004) thereby aiding rapid establishment of plants in disturbed ecosystems. Additionally, glue-like polysaccharides in AM fungal mycelium bind sand grains together (Koske et al. 2004) (Figure 2.2).

Incipient dunes present a challenging environment in which to study AM fungi, which are hypothesized to have a role in the rapid spread of Sea Wheatgrass. Little is known about the physical and chemical environment of incipient sand dunes in relation to the vegetation and fungal flora it supports.



Figure 2.2: Root system of a 12-week old *Thinopyrum junceiforme* plant, grown from a 5 cm node in a pot in natural beach sand. Note the grains of sand adhering to the root hairs, forming a 'mycorrhizal necklace' of sand aggregates.

The aim of this study was to define boundary conditions on a section of the southern Victorian coast where AM fungi are found in association with Hairy Spinifex and Sea Wheatgrass. Results from field research on the geomorphology of the research site are

presented. Also included are the edaphics, or chemical and biological conditions in the sand, that surround and support AM fungi and their host plant roots. Total organic carbon, and labile carbon percentages of the sand are quantified, as are the nitrogen and extractable phosphorus percentages. Additionally, the pH, EC, water content, mean grain size and calcium carbonate content of the sand of the incipient dune are reported.

2.3 Background

The research site is approximately 370 m long, situated between The Hole and The Corner at the eastern end of Thirteenth Beach, a 4.5 km stretch along the 7 km length of coast from Black Rocks to Barwon Heads (Figure 2.3). The beach comprises an aeolianite cliff dating from 90,000 years BP which is now buried by modern sand (Alsop 1983).

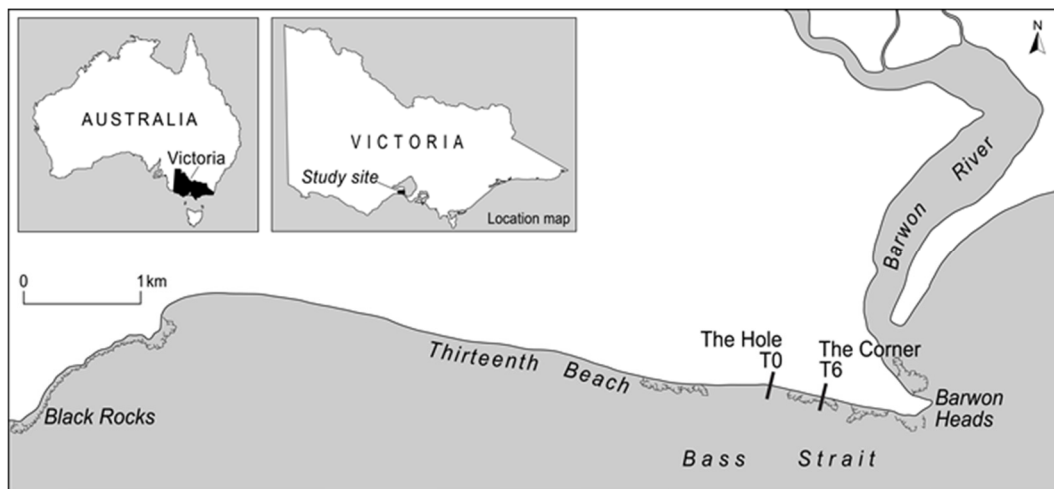


Figure 2.3: Map of Thirteenth Beach, Barwon Heads, showing research site between The Hole and The Corner. (Cartographer Chandra Jayasuriya).

The site faces Bass Strait and experiences waves averaging 1.2 - 1.5 m (Water Technology Pty. Ltd. 2004). The height of the foredune is 9.37 m above mean sea level (MSL). The height of the incipient dune toe where it meets the beach is consistent along its length, being 5 m above MSL at either end of the site, and 4 m above MSL in the middle. The width of the incipient dune ranges from 5 - 9.5 m.

Annual mean minimum and maximum temperatures for March (when the data were collected) are 13.5° C and 24.7° C respectively (Bureau of Meteorology 2015a), with a mean annual rainfall of 549.2 mm (Bureau of Meteorology 2015b). Annual wind roses show due westerly winds of 21 km/h at 9.00 am and due southerly winds of 23 km/h at 3.00 pm, with a mean annual wind direction of due south (Bureau of Meteorology 2015c). Sand movement is greatest in summer months, and least during winter months (Alsop 1973).

2.3.1 Geology of Barwon Heads

Barwon Heads, which sits in the Connewarre Lowlands, is on the southern coast of the Bellarine Peninsula, approximately 100 km from Melbourne. The surficial geology is Quaternary (Holocene to Pleistocene) aeolian dune and beach sands (Jolly & Dodd 1997). During the Pleistocene, basalt lava from volcanic eruptions at Mount Duneed flowed south and west across the Bellarine Peninsula, which has been partially or completely submerged by sea levels several times throughout the last 20 million years (Rosengren 2009). The sedimentary fill of the Torquay Basin, a depression which formed during the rifting of Australia and Antarctica (Holdgate et al. 2003), includes Newer Volcanics basalt (Holdgate, Gallagher & Wallace 2002). This outcrop unit is near the study area, at Black Rocks and at the base of The Bluff, on which aeolian calcarenite unconformably overlies (Bird 1993) (Figure 2.4). The Holocene dunes of Thirteenth Beach are backed by Pleistocene dune calcarenite (Bird 1993), of the Bridgewater Formation (Lipar & Webb 2015; Muller, Dahlhaus & Miner 2006).

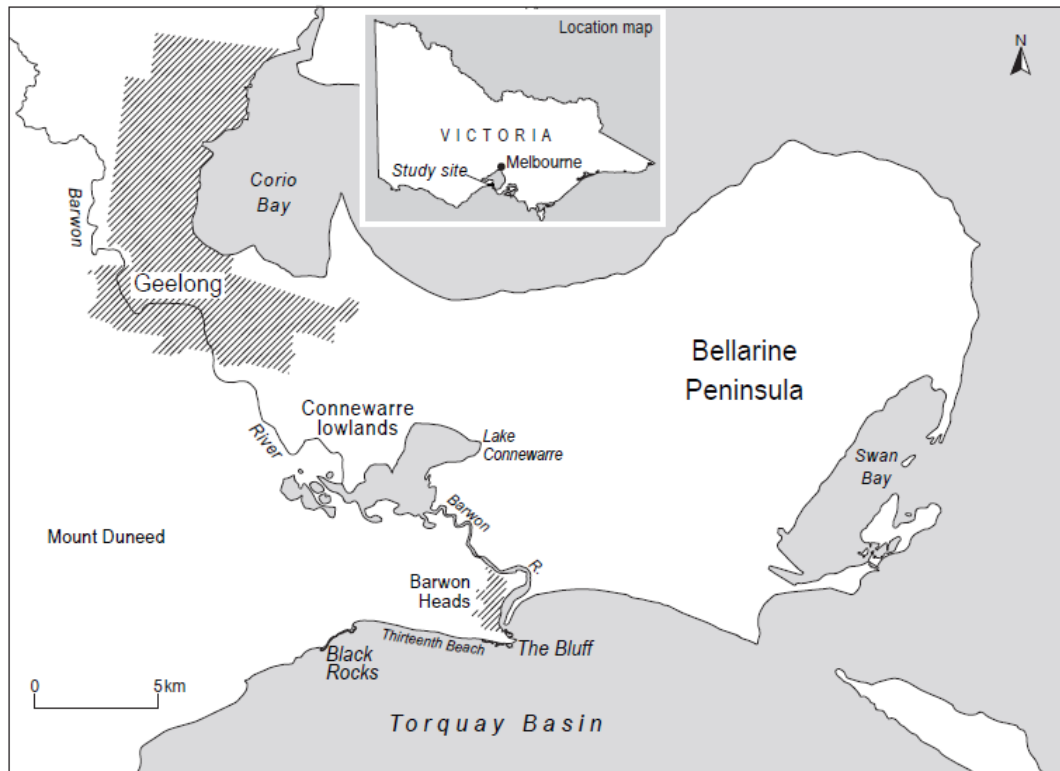


Figure 2.4: Map of the geological environs of Thirteenth Beach, Barwon Heads. The Bridgewater Formation runs along much of the coastline, with its rocky platform submerged by sand, and high tides. (Cartographer Chandra Jayasuriya).

At Thirteenth Beach, the Bridgewater Formation is exposed at low tide as shore platforms. Following storm events that severely scarped the site in May 2015, sand loss revealed sections of the Formation beneath the pre-storm beach surface, and continuation of the platform to the base of the dune (Figures 2.5 and 2.6). Wave action in a second storm event in 2015 deposited a thick layer of basaltic sediments at the base of the scarped incipient dune (Figure 2.7).



Figure 2.5: Section of exposed Bridgewater Formation platform at Thirteenth Beach, following storms in May 2015. Marker (arrowed) is 30 cm high.



Figure 2.6: Storm-exposed section in May 2015, of underlying Bridgewater Formation which continued to the base of the incipient dune at Thirteenth Beach. Arrow points to incipient dune scarp.



Figure 2.7: Thick layer of basaltic sand deposited at research site following a second storm event in 2015. Arrow indicates incipient dune scarp.

2.3.2 Site history and dynamics

Thirteenth Beach Road which sits above and runs parallel to the research site, was built in 1936, however it was buried by sand drifts (Alsop 1975, 1983). In the 1960s, beach re-nourishment with grading to an aerodynamic shape, was undertaken in the areas worst affected by blowouts (Alsop 1975, 1983). Slat fencing was installed to trap wind-blown sand in less affected areas, and Marram Grass was planted to stabilize the trapped sand (Alsop 1983). Between The Hole and The Corner at Thirteenth Beach, Marram Grass no longer remains. The beach at the research site is moderately sloping, with a single transverse bar and many rips running approximately 250 m apart along its length (Short 2005). Thirteenth Beach is a wave-dominated micro-tidal beach (< 2 m).

2.4 Methods

A Trimble R6 GNSS Surveying System with a vertical accuracy of ± 3 cm, was used to survey the site. The digital elevation model (DEM) comprised 13,191 data points, and was conducted over two days in November 2014, prior to the storms that severely scarped the site in May and June 2015. Cross-sections of the seven transects surveyed were generated, to illustrate the morphology of the incipient dune.

Soil moisture readings were taken at 26 locations along the crest and the toe of the incipient dune using a MEA ThetaProbe, HH2 Moisture Meter. In the 24 hours prior to taking moisture readings, 0.6 mm of rainfall occurred (Bureau of Meteorology 2015b). Sand samples were taken from the top 10 cm of the dune/beach surface, which is where most microbial activity and nutrient circulation takes place (Queensland Government and G.R.D.C. 2015). Samples were collected by hand at 10 locations along the dune crest and toe. The samples were placed in individual plastic bags within a cool bag, for transport back to the laboratory.

In the laboratory, samples were stored in a refrigerator at 4°C. Particle size was analysed with a Beckman Coulter LS 13 320 Laser Diffraction Particle Size Analyzer. Air-dried, homogenized samples were analysed for pH and electrical conductivity (EC) in a 1:5 soil/0.01 M calcium chloride (pHCa) solution at 22.3°C. The EC of the

saturation extract (ECe) was calculated by multiplying the EC by the soil multiplier factor for sand (13) (State Government of Victoria 2015). Oven-dried moisture content was calculated by homogenizing 10 20g samples from the crest and toe of the incipient dune, and drying at 80°C for 48 hours. The carbonate content of the dune sand was determined through loss on ignition (LOI) (Kennedy & Woods 2013). Five 3g sub-samples of sand from across the crest of the incipient dune were finely ground, dried at 105°C and then heated for 24 hours at 400°C, followed by 1000°C for one hour. Chemical analyses were conducted by Environmental Analysis Laboratory (EAL), whereby samples were dried at 60°C for 48 hours prior to crushing and analysis. Total nitrogen (N) and total phosphorus (P) were measured as the two main elements transferred from the fungi to their symbionts (Smith & Smith 2011), from three locations across the dune face. Total carbon and labile carbon were also measured. EAL in-house protocols included extractable phosphorus in a 1:3 nitric/hydrochloride acid digest, total organic carbon percentage (TOC) (LECO CNS2000 Analyser), labile carbon (C) percentage using the protocol of Blair et al. (1995); and total nitrogen percentage (N) (LECO TruMAC CNS Analyser) using the protocol of Rayment and Lyons (2011).

2.5 Results

2.5.1 Digital elevation model and cross-sections of the research site

Dune morphology was characterized by a DEM of the research site (Figure 2.8). The incipient varies in elevation from 8.23-8.31 m (Table 2.1), and has a gentle slope, measured from the break in slope where it meets the foredune, to where the incipient toe meets the beach (Figure 2.9). The slope varies from 0.33° at the western end (T0), to 0.40° at the eastern end (T6), with slopes between 0.18-0.24° through the remaining transects. Cross-sections of the seven transects down the dune face, from the highest point of the foredune (9.37 m AMSL) to the lowest point taken on the intertidal shore platform (-0.38 AMSL) at low tide, are shown in Figure 2.9. The incipient dune (boxed areas in Figure 2.9), is the main area in which dune grasses and AM fungi were studied

for this Thesis. Further work on AM fungi was undertaken on the top of the foredune (Chapter 6).

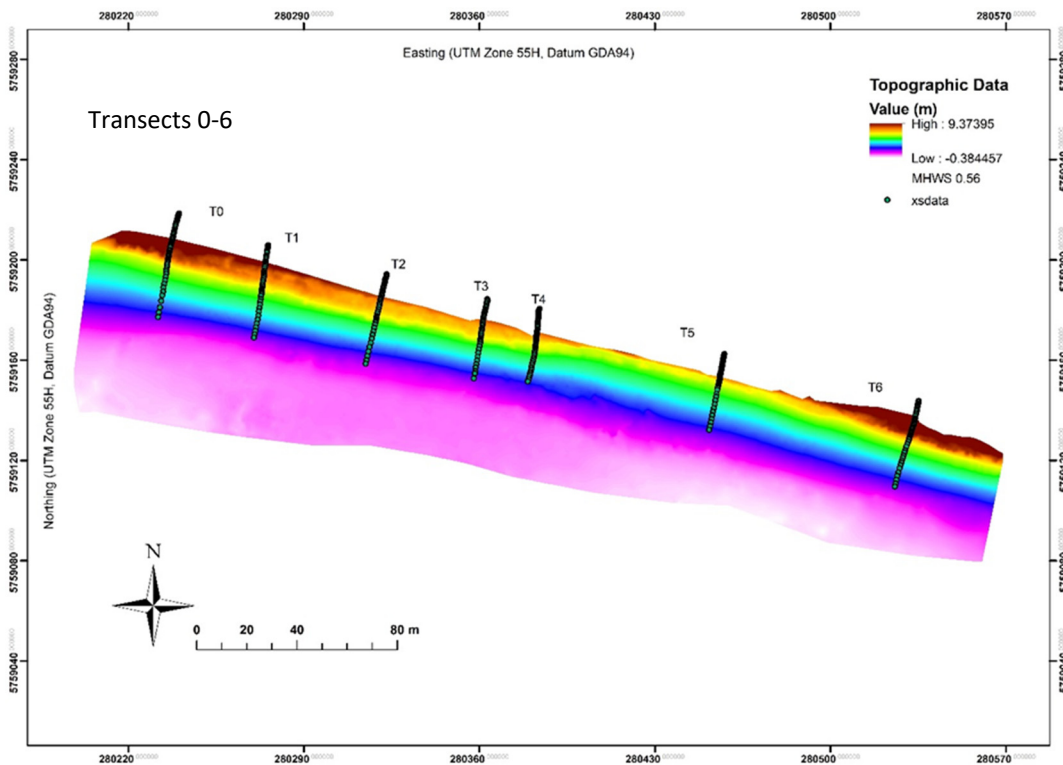


Figure 2.8: DEM of Thirteenth Beach between The Hole (T0, western end) and The Corner (T6, eastern end). Lines represent transects.

Table 2.1: Elevations (m) (AHD) of the foredune and incipient dune along seven perpendicular transects.

Transect	Highest point on foredune	Start of rocky platform	Incipient dune
T0	14.27	1.39	8.31 – 4.69
T1	13.03	1.22	5.85 – 4.17
T2	12.99	0.91	5.75 – 3.82
T3	12.78	1.29	5.48 – 3.75
T4	12.29	1.7	5.25 – 3.92
T5	13.16	1.49	4.64 – 3.95
T6	13.38	1.18	8.23 – 4.82

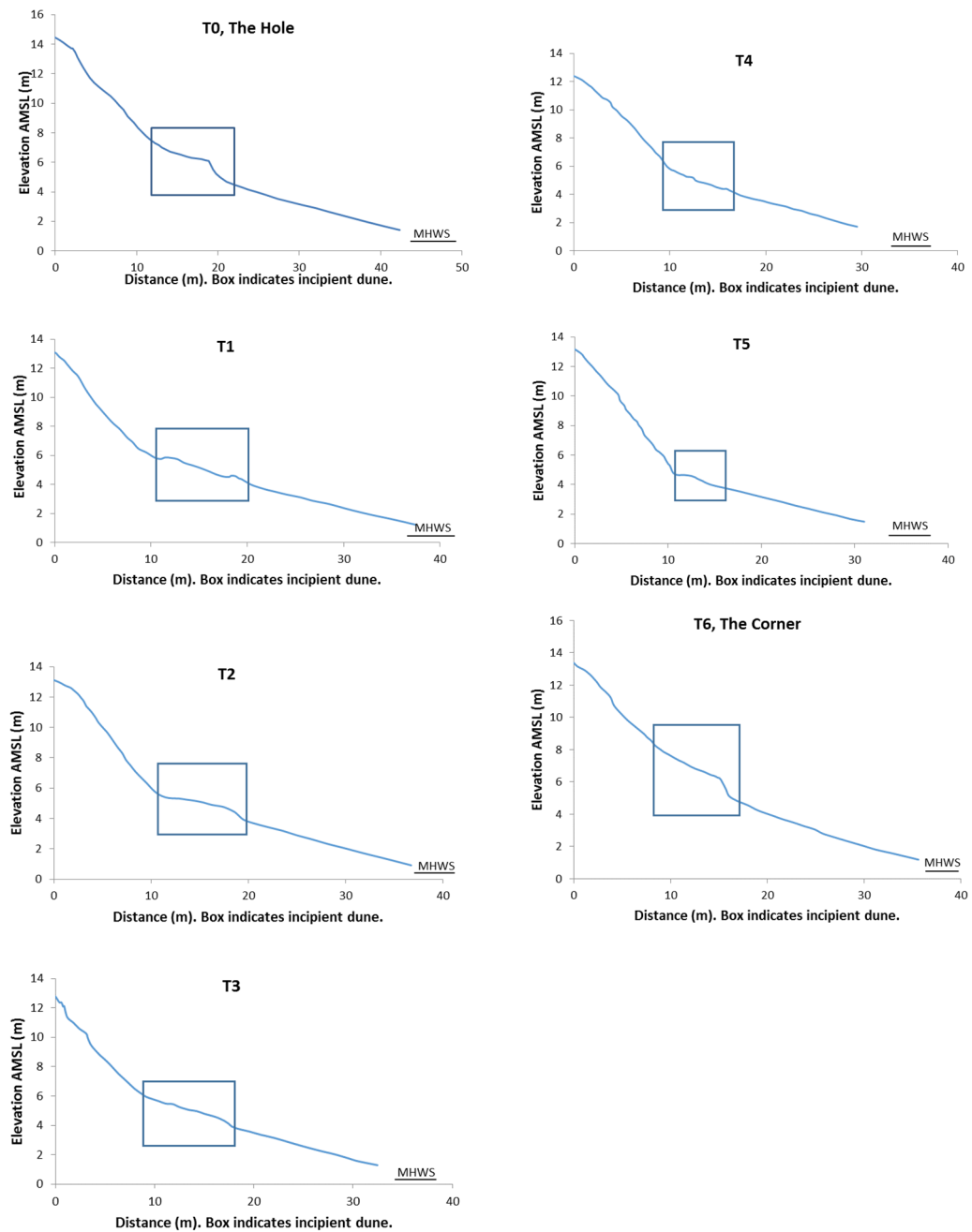


Figure 2.9: Cross-sections of the seven transects at Thirteenth Beach, between The Hole and The Corner, from the top of the foredune to the start of the rocky platform. Boxed area is the incipient dune.

2.5.2 Grain size and chemical characteristics

The dunes are composed of medium, moderately well-sorted, fine grade sand, with a mean grain size of 1.46 Ø for the toe of the dune and 1.45 Ø for the crest (Table 2.2). The average calcium carbonate (CaCO_3) content is 15% (Table 2.2). The crest of the incipient dune is pH_{Ca} 6.09 and the toe pH_{Ca} 6.06, making iron concentrations less than optimum, and aluminium concentrations toxic for the majority of plants. However, $\text{EC}_{1:5\text{Ca}}$ indicated that the sand is not saline (Table 2.2). Overall, edaphic conditions between the incipient dune crest and the toe did not differ greatly except in relation to volumetric soil water content (%), with the toe being more saturated due to its proximity to the high tide mark. This agrees with global estimates of typical dune volumetric soil water ranges of 1.5-6.0% (van der Valk 1974).

Table 2.2: Edaphic conditions (0 - 10 cm), Thirteenth Beach incipient dune crest, and toe.

Procedure	(n)	Crest	Toe
$\text{pH}_{1:5\text{Ca}}$	6	6.09	6.06
$\text{EC}_{1:5\text{Ca}}$ (dS/m)	6	0.003	0.003
EC_e (*13)	6	0.04	0.04
Volumetric soil water (%)	26	3.00	5.18
Oven dried moisture (%)	10	0.04	0.04
Mean grain size (Ø)	6	1.45	1.46
CaCO_3 content (%)	5	15.00	

All the elements measured at the research site are low (Table 2.3), which is characteristic of dune sands (Maun 2009) including low total organic carbon (average 2.15%) and limited phosphorus (272 - 340 ppm). Results for labile carbon (< 0.02%) are lower, for example, than those on the east coast of Scotland (Wall, Skene & Neilson 2002).

Table 2.3: Chemical analysis (0 - 10 cm), Thirteenth Beach incipient dune crest.

Location	Acid extractable P (ppm)	TOC (%)	Labile C (%)	Total N (%)
The Hole	340	2.30	<0.02	0.021
Mid-Way	272	2.12	<0.02	0.019
The Corner	287	2.04	<0.02	0.021

2.6 Discussion

Literature on the nutritional status of Australian dune sands is lacking, but as with other beach systems, it is assumed there would be substantial spatial variation (Perumal & Maun 2006). For example, Welsh dune soils were found to contain as little as 0.006 - 0.008% nitrogen, but had ‘appreciable’ amounts of phosphorus and potassium (Hassouna & Wareing 1964). By comparison, the surface soils of New Zealand range from 0.09 - 0.87% total soil nitrogen (Rayment & Lyons 2011). Nonetheless, dune soils are generally poor in the macronutrients nitrogen, phosphorus and potassium (Hawke & Maun 1988; Hazelton & Murphy 2007). They are also alkaline due to calcium carbonate sourced from marine environments, which may cause nutrient deficiencies, albeit reduce the toxicity of sodium chloride (McLachlan & Brown 2006).

The availability of the majority of nutrients is reduced at $\text{pH}_{\text{Ca}} 3.5 - 4$, with the exception of iron which becomes limited at $\text{pH}_{\text{Ca}} 6.5 - 7.0$ additionally, exchangeable aluminium becomes toxic at $\text{pH}_{\text{Ca}} > 4.7$ (Hazelton & Murphy 2007). The pH_{Ca} of the sand (Table 3) in a sand:silt:clay soil would allow all of the major nutrients to be available for plants, however as sand lacks cation exchange capacity (CEC) due to its lack of electrostatic charge and buffering capacity (Ashman & Puri 2002), it tends to be nutrient poor (Maun 2009). Nonetheless, particles of organic matter between the sand grains have a variable electrostatic charge which allows some exchange of nutrients, depending upon the chemicals in the soil solution (Ashman & Puri 2002). Furthermore, the deposition of wrack from wave action would periodically raise nutrient levels (Zhang 1996). Notwithstanding this, the iron and aluminium levels

would not suit the majority of plants. For example, the low levels of iron would induce chlorosis and loss of leaves (Mengel 1994), and the levels of aluminium would inhibit root and shoot growth (Delhaize & Ryan 1995).

The two main plants that occupy the incipient dune at Thirteenth Beach are Hairy Spinifex and Sea Wheatgrass. Literature on the aluminium tolerance of these plants is lacking, most likely as they are wild species that are generally not cultivated. However, cultivated wheat is tolerant to aluminium at pH_{Ca} 4.0 - 4.5 (Hazelton & Murphy 2007), and as Sea Wheatgrass is a wild relative of wheat, aluminium levels would not be a limiting factor in its growth. Additionally, although acidic soils can impair the functioning of most microbial processes such as the breakdown and cycling of organic matter from which plants access nutrients and carbon (Gazey & Ryan 2014), mycorrhiza have shown enhanced metal sorption capacity compared to other micro-organisms (Joner, Briones & Leyval 2000). For example, mycorrhizal plants successfully inhabit many environments where soil acidity results in elevated levels of metals, such as in mine spoils and other heavy-metal contaminated sites (Göhre & Paszkowski 2006).

Coastal dunes contain little or no clay or silt, therefore salts are easily leached down the profile, and thus the sand at Thirteenth Beach is not saline (Table 2.2), in agreement with previous literature on coastal sand salinity levels. For example, Barbour et al. (1976a) found that along the leading edge of vegetation of 34 beaches on the Pacific Coast in the USA, the concentration of soluble salt at a depth of 10 cm, was 0.008 - 0.280%.

The average calcium carbonate of the sand is 15% (Table 2.2), thus it is not the dominant component at Thirteenth Beach.

The periodic deposition of organic matter (Koop & Griffiths 1982), and rainfall and nutrients in sea spray (Maun 2009), aid in supplying nutrients to flora on the dunes. However, the lack of woody plants and shrubs along the incipient dune, as well as low levels of animal remains, are reflected in the low total organic carbon of the research site. Total organic carbon in soils is comprised of the decomposed remains of animal and plant residues, root exudates, living and dead micro-organisms, and soil biota (Batjes 2014; Pluske, Murphy & Sheppard 2017). Notwithstanding this, few beach

systems have been researched sufficiently to provide nutrient budgets (McLachlan & Brown 2006), and the literature is largely from the Northern Hemisphere (e.g. Atkinson 1973; Koske & Halvorson 1981; Wall, Skene & Neilson 2002; Willis 1963; Willis et al. 1959; Willis & Yemm 1961). Thus, comparisons of my results have no Southern Hemisphere equivalents, however, the labile carbon levels ($< 0.02\%$) at Thirteenth Beach were lower than those of the most recent literature (Wall, Skene & Neilson 2002), which showed carbon levels of $0.1 - 0.6\%$ in the foredune at Tentsmuir Point, Scotland.

In general, pioneer plants require low quantities of nitrogen and phosphorus, and the limited level of phosphorus at the site ($272 - 340$ ppm) is offset by pioneer plants gaining access to it via by the extra-radical mycelium of AM fungi. Mycelia work like an extension to the plant roots, growing beyond the phosphorus depletion zone around the roots to take up phosphorus, as well as some trace elements from the edaphic environment (Koske et al. 2004). There is no literature on the status of nitrogen in Southern Hemisphere beach sands, however the nitrogen percentage at Thirteenth Beach (average 0.020%) is higher than on Northern Hemisphere beaches. For example, on the Atlantic Coast of Maine and New Hampshire USA, N is $0.004 - 0.012\%$ nitrogen by weight (Croker, Hager & Scott 1975), and on the coast of Rhode Island it is 0.0008% (Koske & Halvorson 1981).

The toe of the incipient dune sits above mean high water spring (MHWS) but is not protected from storm surges and is therefore subject to scarping and plant loss. Although gradual or minor disturbances do not necessarily lead to decreases in AM hyphal abundance (Abbott & Robson 1991), hyphal networks in soil are easily disrupted by rapid environmental changes such as storm events (Chapter 4). Such events prevent long-term plant colonization (Koske et al. 2004), and as a result, the contribution of AM fungi to plant survival in incipient dunes has been little studied.

2.7 Summary

This Chapter has defined the boundary conditions under which AM fungi are found in association with the exotic sand dune grass, Sea Wheatgrass, and the native sand dune grass, Hairy Spinifex, at Thirteenth Beach, Victoria. The geomorphology of the

dune was characterized by a DEM prior to storm events that severely scarped the site; such storms illustrating the challenging and dynamic nature inherent on the incipient dunes that plants and soil biota inhabit. The nutrients measured in the natural beach sand are low for most plant requirements, however pioneer plants require low quantities of nitrogen and phosphorus, surviving through adaptations to resource stresses, and through their symbioses with AM fungi and other soil microbes (Cockcroft & McLachlan 1993). The data from the research will enable the prediction of the how close to mean high water spring (MHWS) Sea Wheatgrass, Hairy Spinifex and AM fungi are likely to be found.

Chapter 3

Dominant sand dune grasses at Thirteenth Beach, Victoria

Thinopyrum junceiforme (Á. Löve & D. Löve) Á. Löve, and *Spinifex sericeus* (R.Br.)

This chapter is from the following publication:

Hanlon, L.M. and Mesgaran, M.B. (2014) ‘*Thinopyrum junceiforme* (Á. Löve & D. Löve) Á. Löve, *Plant Protection Quarterly, The Biology of Australian Weeds* 63. 29 (4), 120-126.

I was responsible for all of the planning, preparation, writing and execution of this work for publication. The co-author contributed data on the morphology and vegetative reproduction of *Thinopyrum junceiforme*, and the compilation of Figures 3.3, 3.6 and 3.7. Addenda to the publication are sections 3.1, 3.2, 3.12, 3.12.1, 3.13, 3.14, 3.15, 3.16, 3.17, and Table 3.1.

3.1 Abstract

There are primarily two grasses which occupy the ephemeral, incipient dunes of Victoria, on the southern coast of Australia, the exotic C₃ *Thinopyrum junceiforme* (Sea Wheatgrass), and the native C₄ *Spinifex sericeus* (Hairy Spinifex). The first herbarium record for Sea Wheatgrass in Australia is from a specimen collected at Ricketts Point, Victoria in 1933. Propagules of the plant are presumed to have come from cargo or ballast from the sailing vessels which plied between Europe and southern Australia from the 1830s to 1950. Hydrochory is the most likely vector for Sea Wheatgrass, which is now distributed along the coast of Victoria to Henley Beach in Adelaide, and on the northern coast of Tasmania. The complex taxonomy of Sea Wheatgrass is described in this work, including its nomenclature, biology, reproduction and dispersal mechanisms. As addenda to the published manuscript, the nomenclature, distribution, biology and distribution of Hairy Spinifex is introduced. Mycorrhizal associations with the two grasses are also discussed, placing the mutualists in context with the boundary conditions of Thirteenth Beach, Barwon Heads, Victoria.

3.2 Introduction

This Chapter introduces the exotic, invasive sand dune grass, Sea Wheatgrass, which I studied in association with the obligate fungus, arbuscular mycorrhizal (AM) fungi. The literature generalizes that Victoria's sand dunes are dominated by three grass species – the native C₄ warm-season grass Hairy Spinifex, and the exotic and invasive C₃ cool-season grasses Sea Wheatgrass and *Ammophila arenaria* (Marram Grass) (Cousens et al. 2012). All of these species are capable of trapping sand, thereby potentially limiting its movement (Heyligers 1985; Hilton et al. 2006) and modifying dune morphology (Cousens et al. 2012). Sea Wheatgrass is found on the same part of the sand dune system as the native dune grass, Hairy Spinifex. Marram Grass is not found as close to the swash as Sea Wheatgrass or Hairy Spinifex as it cannot tolerate direct contact with sea water (Huiskes 1979; Sykes & Wilson 1988). The literature proposes that Sea Wheatgrass grows lower on the swash (Cousens et al. 2012; Hilton, Harvey & James 2007), and forms more resilient incipient dunes than those formed by native flora such as Hairy Spinifex (Heyligers 2006, 2009; Hilton et al. 2006). However, to date there is no scientific evidence to substantiate this claim. Additionally, it is contended that Sea Wheatgrass is displacing indigenous sand dune flora such as Hairy Spinifex (Cousens et al. 2012; Hilton et al. 2006; Hilton, Harvey & James 2007).

This Chapter focuses on the biology, native range, climatic requirements and other aspects of Sea Wheatgrass and its presence within the Australian coastal environment. It also investigates the taxonomy, nomenclature, biology and pathogens of Hairy Spinifex, whose mutualistic association with AM fungi is discussed in this Thesis, together with that of Sea Wheatgrass.

3.3 *Thinopyrum junceiforme* - botanical name

The genus name *Thinopyrum* is derived from the Greek '*thino*; *this*' for shore, and '*pyros*', for wheat (Löve 1984), and the species name *junceiforme* is derived from the Latin '*juncus*' for rush-like (Stearn 2004) and '*formis*; *forme*' for resembling, shaped-like (Gledhill 2002).

3.3.1 Common names

The common name for *Thinopyrum junceiforme* (Figure 3.1) is Sea Wheatgrass. It is also referred to as Sand Couch-grass, Sea Couch (Hilton et al. 2006) and Russian Wheatgrass (United States Department of Agriculture n.d.).



Figure 3.1: An incipient dune with a swale between it and the lee of the foredune. *Thinopyrum junceiforme* is growing in the swale and across the incipient dune. Note the small clump of *Spinifex sericeus* (circled) in the foreground

3.4 Taxonomy

The genus *Thinopyrum* comprises tetraploid plants ($2n = 4x = 28$) (Jauhar & Peterson 2001; Löve 1980). It is a member of the tribe *Triticeae* (family *Poaceae*), with a number of the species being resistant to drought, salinity and disease (Niento-Lopez, Casanova & Soler 2000). There are three species complexes within the genus *Thinopyrum*: *T. elongatum* (Tall Wheatgrass) (Host) D.R. Dewey, *T. intermedium* (Intermediate Wheatgrass) (Host) Barkworth & Dewey and *T. junceum* (Russian Wheatgrass) (L.) Á. Löve. The taxa in *T. junceum* have been categorized variously as species or subspecies in different genera such as *Triticum*, *Agropyron*, *Elymus*, *Elytrigia* or *Thinopyrum* (Moustakas, Symeonidis & Coucoli 1986). There are many intergeneric hybrids which

complicate the taxonomy of Wheatgrasses (Refoufi & Esnault 2006). *Thinopyrum junceiforme* falls within the complex of *T. junceum*, and is found around the Atlantic and Baltic coasts (Niento-Lopez, Soler & Garcia 2003). Nomenclature is complicated (Table 3.1), with the complex *T. junceum* also being denominated as *Agropyron junceum* (L.) P. Beauv., *A. junceum* ssp. *mediterraneum* Simonet & Guin., *Elytrigia juncea* (L.) Nevski, and *Elymus farctus* (Viv.) Runemark ex Melderis (Niento-Lopez, Soler & Garcia 2003). Tetraploid *Leymus* spp. have also been confused with *Thinopyrum*, as both are perennial, wild relatives of wheat (Merker & Lantai 1997; Zhang & Dvorak 1991). *Leymus* occurs naturally in North America and Eurasia (Zhang & Dvorak 1991), and lacks the J genome of *Thinopyrum* (Wang & Jensen 1994). *Thinopyrum junceiforme* has also been denominated as *Agropyron junceiforme* Á. Love & D. Love, *A. junceum* ssp. *Boreoatlanticum* Simonet & Guin., *Elytrigia junceiformis* Á. Love & D. Love, and, like *T. junceum*, has also been known as *Elymus farctus* (Niento-Lopez, Soler & Garcia 2003). There is a clear morphological differentiation between the genera *Thinopyrum* and *Elymus*, with the former having rudimentary awns or lacking them altogether, whereas *Elymus* has wider laminae, and higher spike densities than *Thinopyrum* (Löve 1984; Niento-Lopez, Casanova & Soler 2000). The visual identification point for *Thinopyrum junceiforme* is a short, hirsute ligule that can be observed by pulling back the leaf blade

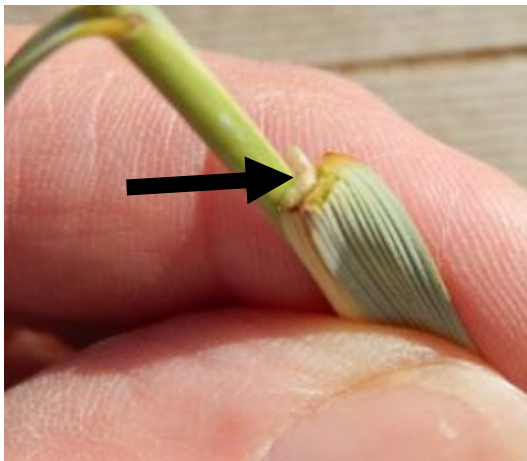


Figure 3.2: The short, hirsute ligule (arrowed) is a key identification characteristic for *Thinopyrum junceiforme*.

at the collar region (Figure 3.2). The currently accepted name for the species is *Elytrigia juncea* ssp. *boreoatlantica* (Simonet & Guin.) Hyl. (Valdes et al. 2009). However, in this review it is referred to by one of its heterotypic synonyms, *Thinopyrum junceiforme*, which is the name used in the most recent Australian and New Zealand literature (Heyligers 2006; Hilton et al. 2006; Hilton, Harvey & James 2007).

Table 3.1: Heterotypic synonyms for *Thinopyrum junceiforme* (in bold), based on GBIF, and the Catalogue of Life.

Binomial	Binomial
<i>Agropyrum farctum</i>	<i>A. hackelii</i>
<i>A. junceiforme</i>	<i>A. junceum</i>
<i>A. junceum bessarabicum</i>	<i>A. junceum mediterraneum</i>
<i>A. junceum sartorii</i>	<i>A. rechingeri</i>
<i>A. sartorii</i>	<i>A. bessarabicum</i>
<i>Braconotia juncea</i>	<i>Bromus truncates</i>
<i>Elymus rechingeri</i>	<i>E. farctus sartorii</i>
<i>E. farctus striatulus</i>	<i>E. junceiformis</i>
<i>E. multinodus</i>	<i>E. striatulus</i>
<i>Elytrigia bessarabica</i>	<i>E. farcta</i>
<i>E. junceae</i>	<i>E. juncea bessarabica</i>
<i>E. juncea boreoatlantica</i>	<i>E. juncea sartorii</i>
<i>E. junceum subsp. boreoatlantica</i>	<i>E. junceiformis</i>
<i>E. mediterranea</i>	<i>E. rechingeri</i>
<i>E. sartorii</i>	<i>E. striatula</i>
<i>Festuca juncea</i>	<i>Frumentum junceum</i>
<i>Thinopyrum bessarabicum</i>	<i>T. junceiforme</i>
<i>Thinopyrum junceum</i>	<i>T. junceum mediterraneum</i>
<i>T. runemarkii</i>	<i>T. sartorii</i>
<i>Triticum farctum</i>	<i>T. glaucum</i>
<i>T. junceum</i>	<i>T. litoreum</i>
<i>T. sartorii</i>	

3.5 History

The coastal dune flora of Australia changed rapidly from the late 1800s, following the introduction of South African, American and European plants such as *Ammophila arenaria* (L.) Link (Marram Grass), *Cakile maritima* Scop. (European Sea Rocket), *Cakile edentula* (Bigelow) Hook. (Sea Rocket), and *Chrysanthemoides monilifera* subsp. *rotundata* (L.) Norl. (Bitou Bush) (Hilton et al. 2006). Prior to that, Australia had only a few species that were capable of colonizing the area between the swash and foredune such

as *Atriplex billardi* (Moq.) Hook.f. (Glistening Saltbush) and *Spinifex sericeus* R.Br. (Hairy Spinifex) (Hilton et al. 2006). Sea Wheatgrass now exerts a great influence over the establishment of both incipient dunes and foredunes, adversely affecting Hairy Spinifex colonies (Heyligers 1985; Hilton et al. 2006). Furthermore, in the foredunes, Marram has displaced *Austrofestuca littoralis* (Labill.) E.B. Alexeev (Beach Fescue), and created steep, unstable dunes (Heyligers 1985; Hilton et al. 2006).

Sea Wheatgrass was first recorded in Australian herbarium records in 1933, with a specimen collected from Ricketts Point, Victoria (MEL 0626849A). It may well have arrived much earlier, in ballast or cargo from the Windjammers, or sailing vessels, which plied between Europe and southern Australia from the 1830s through to 1950 (South Australia Maritime Museum n.d.). Australian herbarium records show that the first Sea Wheatgrass collections from other states were from Rocky Cape, Tasmania in 1948 (HO77017) and the Long Beach sand dunes, South Australia in 1983 (AD98409214).

3.6 Distribution

Sea Wheatgrass originates from the western European, Mediterranean, Atlantic and Baltic coasts (Figure 1.3) (Heyligers 2006; Hilton, Harvey & James 2007; Niento-Lopez, Casanova & Soler 2000), where it plays a major role in dune establishment (Heyligers 2006) as a pioneer species in embryo and incipient sand dunes (Harris & Davy 1986b; Hilton, Harvey & James 2007). In active foredunes of northwest Europe, Sea Wheatgrass is one of the few plants that can withstand periodic, temporary sand burial (Doody 2013), thereby creating dunes that are rarely higher than 1 m in elevation (Hilton et al. 2006). The plant has naturalized in Oregon and California on the west coast of the United States of America, where it is considered a native plant (United States Department of Agriculture n.d.). In Britain, Sea Wheatgrass is regarded as a primary dune colonizer, where it develops dense swards on pre-established foredunes (Harris & Davy 1986b). Sea Wheatgrass is also found in southern New Zealand (Hilton et al. 2006).

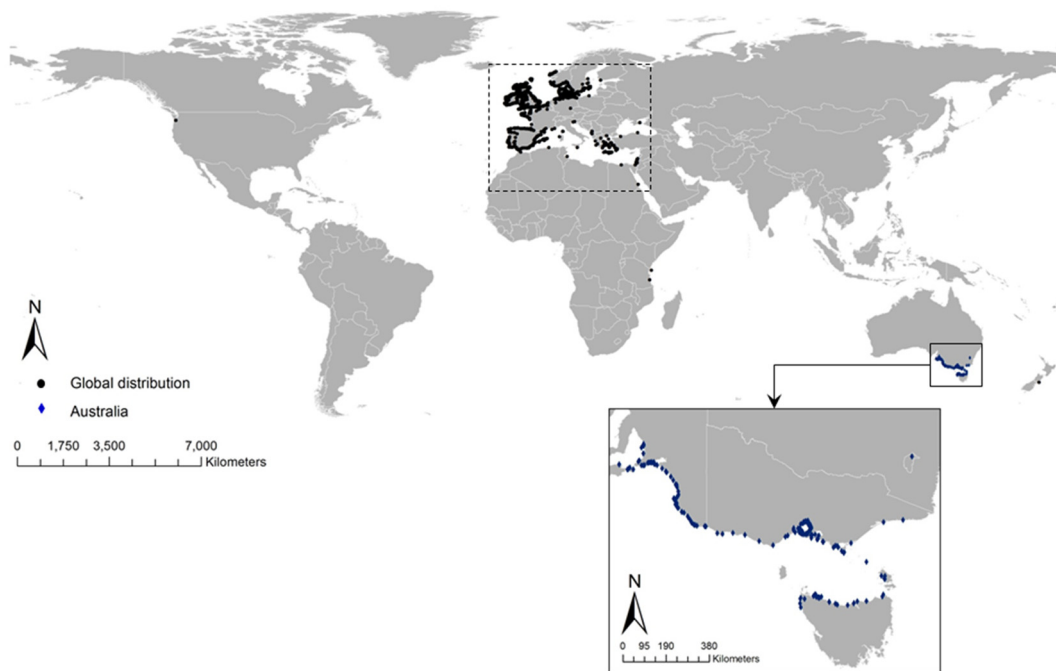


Figure 3.3: Global distribution of *Thinopyrum junceiforme*, with native range in dotted box (GBIF 2014) and Australian distribution in the solid-line box (Australia's Virtual Herbarium n.d. -a).

In Australia, Sea Wheatgrass is found on the foredunes and incipient dunes of the southern coast of the mainland, from Henley Beach, Adelaide (34.92°S , 138.49°E) along the coast and inland to Naracoorte, on the South Australian border with Victoria (38.05°S , 140.94°E), and on the northern coast of Tasmania (Figure 3). In Victoria, it appears from the mouth of the Anglesea River (38.41°S , 144.18°E), around Port Phillip and Westernport Bays, including Phillip Island, and down to Wilsons Promontory National Park (39.06°S , 146.41°E) (Figure 1.3). Records do not indicate that it grows along the eastern coast of the mainland, except at Cape Conran Coastal Park, East Gippsland (37.79°S , 148.74°E). In Tasmania, Sea Wheatgrass is recorded from the mouth of Bottle Creek on the north-west coast (41.1°S , 144.66°E) across to Cape Portland on the north-east coast (40.75°S , 147.96°E) and it is also found on Flinders Island (40.00°S , 148.11°E) (Figure 1.3). The specimens recorded in the Australian Capital Territory are related to four samples collected from The Netherlands between 1962 and 1987, and grown in experimental plots (Australia's Virtual Herbarium n.d. -b).

3.7 Habitat

Sea Wheatgrass is endemic across a wide latitudinal range in the Northern Hemisphere, extending from Finland (64°N, 26°E), to Spain's Cadiz region (36.5°N, 6.28°W) (Hilton et al. 2006). As with many coastal species, the grass occupies a niche of high temperatures, high salinity, desiccation and abrasion from winds, and extremes of soil moisture content (Hesp 1991; Ievinsh 2006; Maun 2009). Additional factors in this dynamic niche include high light intensity and nutritional deficiencies (Harris & Davy 1986b; Hesp 1991; Martinez, Vazquez & Sanchez 2001). Harris and Davy (1986b) propose that in their natural habitat, *Elymus farctus* (*Thinopyrum junceiforme*) tillers need vernalization in order to flower. Field observations from research in Australia note that the production of inflorescences is limited, and this may be because Australian coastal average minimum winter temperatures are not as low as those in Britain (M.B. Mesgaran unpublished data).

3.7.1 Substratum

Sand dune soils are generally poor in the macronutrients, nitrogen (N), phosphorus (P) and potassium (K) (Hawke & Maun 1988; Zhang 1996). In Victoria, the coastal sediments are carbonate-dominated west of Wilsons Promontory and silica dominated eastward (Bird 1993). Literature on the nutritional status of Australian dune sands is lacking (refer Chapter 2), however Sea Wheatgrass needs to tolerate both low, and fluctuating, levels of nutrients, and also needs to be tolerant of a wide range of conditions from inundation by sea water and mobile sand. In Australia, the plant grows lower on the beach, and closer to the swash than any native species (Hilton et al. 2006); this could be because no other plants can survive repeated seawater inundation unless they are halophytes. Sea Wheatgrass is one of three species in the genus *Thinopyrum* that are salt tolerant, along with Tall Wheatgrass and *T. bessarabicum* (Savul. & Rayss) Á. Löve, and this tolerance is controlled by multiple genes on several chromosomes (Wang et al. 2003). *Thinopyrum bessarabicum*, for instance, has been found to withstand hydroponic solutions of 350 mM sodium chloride for prolonged periods (Gorham et al. 1985), whereas halophytes grow in concentrations of 400 mM sodium chloride, or higher (Flowers 2004).

3.7.2 Plant associations

Both native and exotic plants grow in association with Sea Wheatgrass on incipient dunes and foredunes in Victoria (Figure 3.1). On the incipient dunes, such plants may include native Hairy Spinifex, and the exotics *Cakile* spp. (Sea Rockets) and *Euphorbia paralias* L. (Sea Spurge). On the foredunes, the vegetation is more varied and includes the native plants *Atriplex cinerea* Poiret X *A. paludosa* (Coast Saltbush), and *Ficinia nodosa* (Rottb.) Goetgh., Muasya & D.A. Simpson (Knobby Clubrush), with *Lepidosperma gladiatum* Labill. (Coast Sword-sedge) growing in the swale in the lee of incipient dunes. On open sites on the foredunes, hardy succulents such as native and naturalized *Carpobrotus* spp. (Pigface), and native plants *Rhagodia baccata* (Labill.) Moq. (Seaberry Saltbush) and *Tetragonia tetragonioides* (Pall.) Kuntze (Sea Spinach), manage the inhospitable environment remarkably well, as does the native *Geranium solanderi* var. *solanderi* Carolin (Native Geranium). Exotic weed species such as *Fumaria* spp. (Fumitory), *Oxalis* spp. (Soursob) and *Allium triquetrum* L. (Angled Onion) are just a few of the smaller flowering plants, also found in association with Sea Wheatgrass on the foredunes.

3.8 Growth and development

Sea Wheatgrass is a rhizomatous, perennial grass, growing to approximately 50 cm in height, but under favourable conditions it can grow as tall as 80 cm. Plants can grow from a single node and produce as many as 20 – 100 tillers. The blue-green leaves of Sea Wheatgrass are glabrous below and finely pubescent above, usually 30 cm in length, but they can grow up to 50 cm, with the widest part of the blade varying from 3 mm to 8 mm (average 5 mm) (M.B. Mesgaran unpublished data). Most populations from South Australia have wider leaves than those of Victorian or Tasmanian populations (M.B. Mesgaran unpublished data). Spike lengths are about 15 cm, with each spike bearing 10 spikelets. Sea Wheatgrass is a C₃ cool season grass, but other data describing its physiology or biochemistry are lacking. Notwithstanding this, temporary burial is a common occurrence in plants of sandy environments, and many plants such as Sea Wheatgrass survive such burial, with the short-term suspension of physiological activity such as photosynthetic capacity, which is quickly reinstated once

uncovered (Harris & Davy 1988; Perumal & Maun 2006). This is due to newly-emerged leaves from previously buried plants having an increased chlorophyll content (mg g^{-1} fresh weight), and a higher energy content in subterranean organs (Perumal & Maun 2006; Yuan, Maun & Hopkins 1993). Young plants of Sea Wheatgrass lack the axillary meristems and energy reserves required to grow new shoots when buried, and they re-allocate their resources from non-photosynthetic organs to maintain the photosynthetic ones, until the plant is uncovered by winds or storms (Harris & Davy 1988). It has been shown that multi-node rhizome fragments have more success in emergence, and from greater depths, than do single-node fragments of Sea Wheatgrass (Harris & Davy 1987).

In Britain, Sea Wheatgrass requires vernalization in order to flower; however, flowering usually occurs in the second year of growth, and Harris and Davy (1986b) found that such flowering was limited due to the proximity of plants to wave disturbance along the swash, as well as grazing by rabbits. It is hypothesized that as



Figure 3.4: Flowering spike of *Thinopyrum junceiforme* (Source: M.B. Mesgaran).

the winters in Australia are not as cold as those in Europe, flowering is limited, appearing in December, January, and occasionally in February (Figure 3.4) (M.B. Mesgaran unpublished data), although the flowers do not persist for long on the stems (Rudman 2003). The seasonal variation in the ability of Sea Wheatgrass rhizome buds to produce adventitious shoots and roots under glasshouse conditions was found to be strongly correlated to nitrogen as a limiting factor, with carbohydrate reserves also implicated (Harris & Davy

1986a). Additionally, dormancy was found to show a peak in late winter and early spring, with a sharp decline in late spring early summer (Harris & Davy 1986a). This variation was inversely related to the growth rate of the parent plants at time of harvesting (Harris & Davy 1986a).

3.8.1 Mycorrhizas

Approximately half of the 150 described species of arbuscular mycorrhizal (AM) fungi are found in sand dunes (Koske et al. 2004). In particular, *Glomus intraradices* N.C. Schenck & G.S. Sm. a species of AM fungi, was shown to be highly tolerant of harsh conditions such as aridity and salinity, reducing the concentration of sodium in the shoots of plants in saline environments (Yamato et al. 2012). However, there is no literature specifically on the involvement of AM fungi with Sea Wheatgrass, although grasses in general tend to be facultatively mycorrhizal (Brundrett et al. 1996), or weakly mycorrhizal (Ramos-Zapata et al. 2011) in their association with AM fungi. The early work of Forster (1979) which focussed on aggregation of sand by plants and microbes, asserted that microbial abundance associated with Sea Wheatgrass (under the heterotypic synonym of *Agropyron junceiforme*) was less in winter when annual species were dying, and many perennial species were dying back.

3.9 Reproduction, dispersal, physiology of seeds and germination

Sea Wheatgrass can reproduce both sexually and asexually (Löve 1984), but production of flowering tillers in Britain (Harris & Davy 1986b) and mature seeds in Australia (M.B. Mesgaran unpublished data) is low. In Australia, the main route of propagation is by rhizome growth and fragmentation. The plant has cells with four sets of chromosomes (Jauhar & Peterson 2001; Merker & Lantai 1997), with the genome of J1J1J2J2 (Colmer, Flowers & Munns 2006), and is a self-crosser, although it is capable of cross-fertilization (Moustakas, Symeonidis & Coucoli 1986). Refoufi and Esnault (2006) contend that it has little genetic diversity, as assessed by isozymes and RAPD markers.

Hydrochory, or the passive dispersal of propagules by water, is the most likely vector for the dispersal of Sea Wheatgrass caryopses along the coastline of southern Australia. Drift bottle programmes by Olsen and Shepherd (2006) demonstrated that surface water flows along the South Australian coast, east through Bass Strait, then south east past the west coast of Tasmania in winter, reversing the direction in summer. Thus, currents could be responsible for the transportation of plant fragments to the shores of Tasmania from the mainland. Heyligers (2007) noted that dispersal patterns of four other introduced species to Australian beaches follow the circulation of ocean currents around Australia, and such currents may have been responsible for the dispersal of propagules to the South Australian coast. Although no research has been conducted on the dispersal mechanisms of Sea Wheatgrass, it is likely to be via such fragments being scarped, or torn, from eroding sand dunes during storms (Hilton et al. 2006; Hilton, Harvey & James 2007). For example, following a severe storm on the Norfolk (UK) coast in 1978, Sea Wheatgrass (under the heterotypic synonym *Elymus farctus*), rapidly re-colonized the swash, and both seeds and fragments of rhizomes were of equal importance in establishing new clumps, and in producing similar tiller densities (Harris & Davy 1986b). Similar recruitment was observed in the 1960s at Shallow Inlet, Wilsons Promontory where a sand spit developed after the previous spit was washed out in 1901. The new spit initially lacked vegetation but in the 1960s, Sea Wheatgrass was found to have colonized it (Heyligers 2006). Clumps of Sea Wheatgrass rhizomes have also been observed at the mouth of the Glenelg River at Nelson in Victoria, where there was no actively growing parent plant on the beach, suggesting that the rhizomes might have been dispersed by hydrochory (M.B. Mesgaran unpublished data).

Woodell (1985) suggested that seed germination patterns of coastal plants can be separated into three categories and that their response to germination in saline conditions correlated with their habitat. He found that the greatest germination response of Sea Wheatgrass seeds ($n=180$) was in freshwater (53%), followed by 18% in half-strength sea water, 5% in full-strength seawater, and the complete inhibition (0%) in one and a half strength sea water (sodium chloride concentration 600 mM) (Woodell 1985). Seeds from all treatments recovered sufficiently when transferred to distilled water for some germination to occur (Woodell 1985). Even though the seeds germinated under controlled conditions, it is likely that under natural conditions, Sea

Wheatgrass seeds in the swash may be stimulated to germinate following precipitation. That Sea Wheatgrass seeds do not germinate in full-strength sea water may also aid in its dispersal by keeping seeds dormant and the embryo surviving on the endosperm within the seed coat until the seed reaches land.

In Australia, reproduction is largely vegetative from new shoots off nodes along the highly meristematic rhizomes, which are produced in great lengths (Figure 3.5). Under glasshouse conditions, each 5 cm node of Sea wheatgrass produced up to 30 m rhizome length in the course of one season (M.B. Mesgaran unpublished data). The length of most internodes was approximately 7 cm, and skewed toward lengthier internodes (Figure 3.6). On land, single-node Sea Wheatgrass rhizome fragments can emerge from depths of up to 17 cm. Multi-node fragments can emerge from greater depths (> 17 cm), producing more emergent shoots and more quickly than single node shoots, especially in late winter to early spring (Harris & Davy 1986a).



Figure 3.5: Rhizomes from one *Thinopyrum junceiforme* plant harvested in the field.

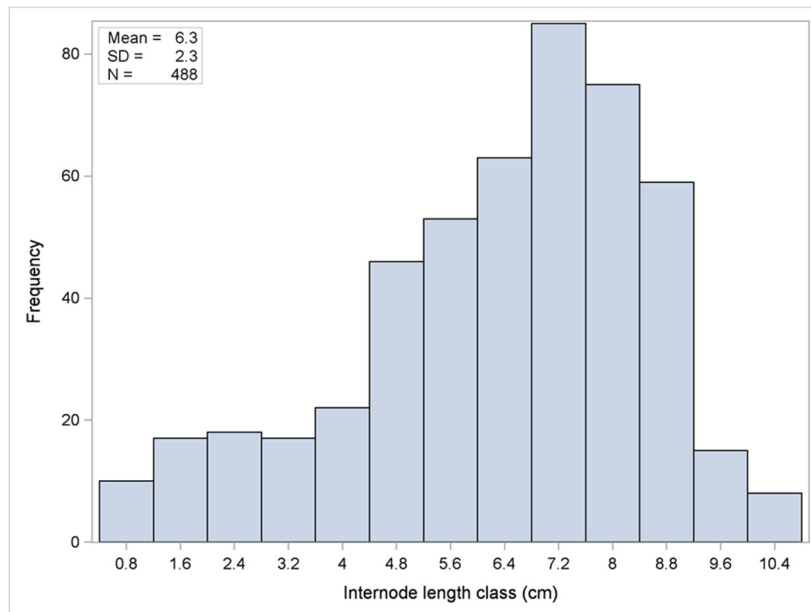


Figure 3.6: Rhizome internode length from one *Thinopyrum junceiforme* plant grown under glasshouse conditions, in the course of one season. The rhizome was 30 m long, with internodes approximately 7 cm apart, skewed towards lengthier internodes (M.B. Mesgaran unpublished data).

3.10 Population dynamics

Population dynamics data for Sea Wheatgrass in Australia are not available. The maximum life-span of the species is unknown, as are mortality rates between life stages, apart from the rate of regeneration after dispersal by water, or burial by sand, discussed above. Research is required on population dynamics to enable the development of weed management strategies, rather than ad hoc herbicide applications. Such strategies could be used by all beach management authorities.

3.11 Importance

In Victoria, anecdotal evidence suggests that Sea Wheatgrass is impacting upon the rookery of *Eudyptula minor* J.R. Forster (Fairy Penguin) on Phillip Island's Summerlands beach, by creating steep-fronted incipient dunes that are too high for the birds to climb in order to access their burrows (P. Dann personal communication

2013). Furthermore, on Phillip Island and beaches in Victoria such as those on the Barwon Coast and Geelong, the endangered *Thinornis rubricollis* Gmelin (Hooded Plover), which prefers a nest scrape with little vegetation on which to lay its eggs, is likely to have its nesting sites encroached upon by Sea Wheatgrass (Cousens et al. 2012). Steep fronted incipient dunes that are thickly vegetated with Sea Wheatgrass are also found along the South Australian coast, such as at Normanville (Figure 3.7). Hilton et al.(2006) proposed that the sand-binding ability of Sea Wheatgrass makes it more resilient to erosive processes in comparison to native flora. Indeed, Sea Wheatgrass is one of four exotic species that were noted by Heyligers (1985) as being more efficient than native species at trapping sand and building dunes where otherwise dunes would not have formed. Such dunes have the potential to limit sediment movement, thereby changing the ecosystems and geomorphology of the coastlines on which they appear. Thus, it is of concern that Sea Wheatgrass can rapidly colonize the swash and incipient dunes after propagules are washed ashore following storms. One example where Sea Wheatgrass has spread with great rapidity is along the Younghusband Peninsula in South Australia, where James (2012) reports that the plant has spread at approximately 18 ha per year, outcompeting native species and altering ecosystems.



Figure 3.7: Steep-fronted dune vegetated by *Thinopyrum junceiforme* in Normanville, South Australia. (Source: M.B. Mesgaran).

Groves et al. (2003) list Sea Wheatgrass as a weed species, albeit not specifically a coastal weed, as there is no formal listing or category for coastal weed species. Cousens et al. (2012) note that Sea Wheatgrass is one of the dominant exotic species on the coast displacing native vegetation. However, many coastal managers, including state government co-ordinators, are unaware of the existence of Sea Wheatgrass, let alone its potentially adverse impacts (R. Cousens personal communication 2014), which may explain the lack of specific legislation related to this species.

3.12 *Spinifex sericeus* (R.Br.) (Hairy Spinifex)

Hairy spinifex is a native sand dune grass in Queensland, New South Wales, Victoria, Tasmania and South Australia (DEPI 2014), and is found in association with Sea Wheatgrass at the research site at Thirteenth Beach, Barwon Heads, Victoria.

Hairy Spinifex is an erect, stoloniferous, perennial C₄ warm-season grass growing to 50 cm high, with grey to silvery-green loosely rolled to flat pubescent leaf blades 10 - 40 cm long (Lamp, Forbes & Cade 2001). The branching stolons with internodes from 10 - 38 cm long, can extend for as far as 20 metres down and across incipient dune faces, and into the swash area of the beach (Bergin 2011) (Figure 3.8). The nodes of the plant can produce adventitious roots from which discrete plants grow, and this form of clonal, vegetative reproduction is the predominant form of breeding in Hairy Spinifex (Whalley, Chivers & Waters 2013). The plant is tolerant of extremes of light intensity, salt spray, dry periods and high winds (Hesp 1991), with germination of caryopses being optimum when temperatures alternate between the extremes of 10 - 25° C and 20 - 35° C (Harty & McDonald 1972). Furthermore, Hairy Spinifex can withstand burial by sand, with aggressive new shoot growth emerging to the new sand surface, thus building dunes (Bergin 2011).



Figure 3.8: Colony of *Spinifex sericeus* on the incipient dune at Thirteenth Beach, Barwon Heads, Victoria, in February 2015 prior to storms scarping the site in May and June 2015 and May 2016. New plants are emerging in the swash, from sand-buried stolons.

3.12.1 Distribution of Hairy Spinifex

Hairy Spinifex colonizes coastal sands in Victoria, where it is one of the most important native plants which binds and stabilizes sand dunes (Carolin & Clarke 1991). It colonizes sand dunes in Tasmania, where it is sparse on siliceous beaches, but greater in abundance on calcium carbonate sands in the north of the state (Reid et al. 1999). Hairy Spinifex also grows from the Adelaide region of South Australia (Webster 1987), along the eastern seaboard through to tropical Queensland, and is the main species used in New South Wales and Queensland for revegetation of foredunes (Carolin & Clarke 1991). Furthermore, the plant is to be found in New Caledonia and New Zealand (Carolin & Clarke 1991; Lamp, Forbes & Cade 2001). A similar plant, *Spinifex hirsutus* (Beach Spinifex), occurs from the Eyre Peninsula in South Australia, across the Nullabor, through to Western Australia, where it flowers from November through to January (Jacobs, Whalley & Wheeler 2008; Webster 1987). This species has stouter stems and broader leaves than those of Hairy Spinifex (Lamp, Forbes & Cade 2001).

3.13 Taxonomy and nomenclature of Hairy Spinifex

Spinifex is derived from the Latin *spina*, *spinae*, for thorn-maker or spine, which refers to the sharp leaves of the Asian species first described (Jacobs, Whalley & Wheeler 2008; Quattrocchi 2000; Robinson 1991), and *sericeus* from the Latin for silky, with glossy, straight, closely-pressed hairs (Stearn 2004). In south-east Australia, it flowers from November to January, however in Queensland, flowering occurs from June to August (Webster 1987). Although Hairy Spinifex has been known as *S. hirsutus* (Lamp, Forbes & Cade 2001), the latter is now confined to the species which grows on the southern coast of Western Australia, and the South Australian coast. Morphologically, Hairy Spinifex and Beach Spinifex are similar, except that Beach Spinifex has stolons and internodes > 9 mm diameter (Jacobs, Whalley & Wheeler 2008; Webster 1987), whilst the stolons and internodes of Hairy Spinifex are < 9 mm (Webster 1987).

There is contradictory evidence as to whether Hairy Spinifex is stoloniferous, or both stoloniferous and rhizomatous, as rhizomes and stolons intergrade and it is rare to find both occurring on a single species, as they do in *Cynodon dactylon* (Couch Grass) (Duigan 1991). Perhaps the confusion has arisen as a consequence of finding sand-buried stolons that had generated new plants from nodes. Indeed it is known that Hairy Spinifex continues to grow under partial burial by sand (Jacobs, Whalley & Wheeler 2008), as do other dune grasses such as Sea Wheatgrass.

There are between three to five species within the genus *Spinifex* (Curtis & Morris 1994; Jessop, Dashorst & James 2006), however, only four species are named. These are *S. sericeus* and *S. hirsutus*, as previously discussed, *S. longifolius* (Long-leaved Spinifex) which is native to Western Australia, the Northern Territory, New Guinea and Indonesia (Ripley & Rowland 2004), and *S. littoreus* which is found in India and Asia (Webster 1987). However, 'Spinifex' when used as a common name, should not be confused with the grasses that grow in inland Australia, which belong to the genera *Plectrachne* and *Triodia*, and which were accidentally named 'Spinifex' following a mistranslation from the journal of a French explorer (Jacobs, Whalley & Wheeler 2008). Additionally, these mis-named grasses have a significantly different growth

habit from *Spinifex* sp., forming dense, rounded tussocks with resinous stems, and flower spikes up to 183 cm (6') in height (Willis 1970).

3.14 The biology of Hairy Spinifex

Hairy Spinifex is dioecious, with male reproductive organs in one individual, and female reproductive organs in another; however the plant may be propagated both vegetatively and sexually (Maze & Whalley 1992). Each female inflorescence comprises 40 - 160 spikelets, with less than half the spikelets (0 - 43%) on each seed head being fertile, with plants varying in fertility between years and locations (Bergin 2011a). The spherical female flowers (Figure 3.9) which are approximately 25 cm in diameter, disarticulate at their base at maturity (Connor 1984), and can often be seen rolling along the beach in windy weather. The flowers may become lodged in other vegetation, or be buried by sand, allowing caryopses to drop and generate new plants (Carolin & Clarke 1991; Connor 1984). Further, if blown into water, they can be dispersed by longshore currents (Heyligers 1998). Viable seed formation is correlated with the proximity of female plants to male plants (< 2 m) (Bergin 2011). Dioecism is not uncommon in grasses, occurring in approximately 20 genera, four of which are found in Australia – *Spinifex*, *Zygochloa*, *Pseudochaetochloa* and *Distichlis* (Connor & Jacobs 1991). In all of these genera except the latter, the sex ratio approximates or equals 1M:1F (Connor 1984). Hairy Spinifex male inflorescences (Figure 3.10) have a greater number of florets than do the females, suggesting that dispersal by pollen is favoured over the seed dispersal which is used by the female flowers (Connor 1984; Maze & Whalley 1990). The male flowers consist of up to 15 spikelets on a single raceme, are approximately 10 - 20 cm long, and terminate in a 1 cm bristle (Connor 1984); unlike the female inflorescences, they persist on the plant for many months. The maximum growth of Hairy Spinifex occurs during spring and summer (Bergin 2011). Discrete plants can grow from internodes where there is sufficient growing space away from the parent stolon (Bergin 2011; Hesp 1989).



Figure 3.9: *Spinifex sericeus* female inflorescence at Thirteenth Beach, 2014, prior to storm events that scarped the site.



Figure 3.10: Colony of male *Spinifex sericeus* plants at Thirteenth Beach, 2014, prior to storms that scarped the site.

The upright shoots of Hairy Spinifex reduce wind velocity, causing sand deposition, and often burial of plant leaves and stems; this then promotes aggressive shoot emergence and re-establishment of the plant on the surface of the sand, thereby aiding in the building of a dune (Bergin 2011). Hairy Spinifex forms low, hummocky dunes (Hesp 2002) (Figure 3.11), albeit it cannot accrete more sand than 30 cm per annum (Ranwell 1972), rendering it incapable of building high dunes, such as those formed by *Ammophila arenaria* (Marram Grass) (Hesp 2002).



Figure 3.11: Low incipient dune formed by *Spinifex sericeus* on Phillip Island, Victoria, 2013.

3.15 Pathogens

Male and female inflorescences can become infected with *Ustilago spinificis* F.Ludw. (Spinifex Smut) (Bergin 2011). The smut is a native Australian species and one of 64% of smuts in Australia that are parasitic exclusively on Poaceae species (Shivas 2010). Other Spinifex smut host species are *S. longifolius* R.Br., *S. hirsutus* Labill. and *Zygochloa paradoxa* (R.Br.) S.T.Blake (Shivas et al. 2013). Distribution of the pathogen is throughout Victoria, Tasmania, South Australia, Queensland and the Northern Territory (Kirby 1988). The spores germinate readily, with Sori of basidiospores being hidden by the floral envelopes, transforming them into an agglutinated and powdery spore mass (Shivas et al. 2013). Infected female spikelets have swollen appendage 1.5 - 4 cm above the spine base, the seeds of which are likely to be unviable (Kirby 1988).

3.16 Mycorrhizal associations

The work of Logan et al. (1989) on the mycorrhizal status of 41 sand dune plant species on the coastal dunes of New South Wales, established that roots of 36 species of plants contained AM fungi, and that Hairy Spinifex had some 55% of its root length colonized by these fungi. The root length (stolons were not explicitly mentioned) of Hairy Spinifex extended 2 m+ beyond the plant, and had both external and internal hyphae, as well as vesicles, but no coils or arbuscules. Spores of AM fungi have also been found in association with Hairy Spinifex and other sand dune plants (Koske 1975). Other studies of AM fungi in sand dunes demonstrated that AM hyphal polysaccharides were associated with Hairy Spinifex, and covered the plant roots with fine, sticky filaments, which bound sand particles to them (Carolin & Clarke 1991) (Figure 3.12).

The age of plants may impact upon the production of fungal exudates (Nicolson 1960). The age of samples taken from the research site at Thirteenth Beach, Victoria, is unknown, however Hairy Spinifex stolons were removed from the actively growing apical end of plants, albeit attached to older parent plants, from the youngest part of the dune system (the incipient dune). Furthermore, co-existing grass species have discernible AM associations (Vandenkoornhuyse et al. 2003), leading to a host

preference in the fungus (Vandenkoornhuyse et al. 2003), and this also could result in differing levels of exudates across grass species.



Figure 3.12: Long, coarse roots of *Spinifex sericeus* at Thirteenth Beach, February 2016, with few root hairs and sand particles adhering to fungal exudates.

Studies of AM fungi from environmental samples such as Hairy Spinifex, are challenging due to the obligate nature of the fungus, which cannot be cultured axenically (e.g. Johri et al. 2015; Vandenkoornhuyse et al. 2003). There is also a paucity of literature regarding plant root phenology, and plant root exudates in sand dune grasses and their symbionts. Although scant, the literature regarding Hairy Spinifex and symbiotic associations does confirm the presence of AM fungal structures in the plant's roots.

3.17 Summary

This chapter has introduced the exotic sand dune grass Sea Wheatgrass, and the native sand dune grass Hairy Spinifex, which are found in association with each other on the incipient dunes of Thirteenth Beach, Barwon Heads. The taxonomy, nomenclature,

biology and distribution ranges of the grasses have been addressed, as has their mutualistic symbioses with AM fungi. Root hair differences between Sea Wheatgrass and Hairy Spinifex in relation to fungal exudates and sand aggregation have been addressed. Together with the published manuscript on Sea Wheatgrass (Hanlon & Mesgaran 2014), Hairy Spinifex has been placed in context with the boundary conditions discussed in Chapter 2, and the field work undertaken (Chapter 4) of this Thesis.

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Chapter 4

Seasonal changes in abundance of arbuscular mycorrhizal (AM) fungi in C_3 and C_4 grasses on an incipient sand dune, and response to storm disturbance

4.1 Abstract

The seasonal changes in abundance of mycorrhizas in the roots of incipient dune grasses were studied over two consecutive summers and winters, during which time severe storm events scarped the study site at Thirteenth Beach on the southern coast of Victoria. Mutualistic pioneer plants aid the recovery from scarping and erosion by stabilizing the dune through revegetation. The colonized root length (cm) in the dune grasses post-storms was greater than pre-storms, which relates to the capability of opportunistic *r*-strategists to colonize rapidly ruderal habitats such as incipient dunes. Such life history strategies enable the survival of mutualists in unpredictable and unstable environments. The percentage of leaf matter, or aerial biomass, of the grasses within non-contiguous 1 m² quadrats across the incipient dune was also measured seasonally, as other studies had observed the exotic C₃ to displace the native C₄. At all sampling times during the study period, the native C₄ grass had a higher percentage of tillers than that of the exotic species, contradicting the findings of previous studies at other sites.

4.2 Introduction

Sand dunes are dynamic aeolian systems (Foster & Tilman 2000), which are globally widespread (Martinez, Psuty & Lubke 2004). Dunes are a sand-sharing environment, from which sediment is taken by erosion, thereby re-shaping the nearshore environment under storm conditions for example, and to which sand is returned under ambient conditions (Woodroffe 2012). The development of coastal dunes coincides with wave-dominated coasts where there is a sufficient supply of fine to medium, well-sorted sediment (2Ø - 1Ø or 1/4 - 1/2 mm; 3Ø - 2Ø or 1/8 - 1/4 mm, respectively) (Woodroffe 2012) available for entrainment inland by ambient on-shore winds (Martinez, Psuty & Lubke 2004; Woodroffe 2012). Where onshore winds dominate, broad dune-fields may develop (Martinez, Psuty & Lubke 2004). Furthermore, coastal dynamics such as disturbance (Forey et al. 2008), wind direction and velocity (Bagnold 1954; Sherman & Nordstrom 1994), sediment availability (Carter, Hesp & Nordstrom 1990) and vegetation cover (Gadgil 2006; Heyligers 1985; Hilton et al. 2006; Kim 2005), can impact upon the development of active, or stabilized dunes (Martinez,

Psuty & Lubke 2004). The scarping and erosion of dunes by waves is cyclical, and it is now recognized that dune erosion is a fundamental part of beach adjustment during storms (Pye & Blott 2008). Furthermore, the role of vegetation in releasing sand into the littoral system is critical in beach/storm response, as is the presence of plant roots which assist in maintaining the integrity of dune slumps resulting from storms (Pye & Blott 2008). Recovery from scarping and erosion is achieved when the dune becomes stable through revegetation (Woodroffe 2012).

Incipient dunes are a unique ecosystem at the interface between terrestrial and aqueous environments (Martinez, Psuty & Lubke 2004; Maun 2009), accreting and eroding with wind and waves (Carter, Hesp & Nordstrom 1990). On accretional beaches when pioneer vegetation traps wind-blown sand, incipient dunes are formed close to the swash (Hesp 1988; Hilton & Konlechner 2011). Other obstructions such as driftwood or wrack also halt the movement of sand across the beach and contribute to the initiation of incipient dunes (Hesp 1988; Kennedy & Woods 2012).

As early as the 1800s, Cowles (1899b) described sand dunes as a 'restless maze', which undergo a continuum of accretion and erosion (Psuty 2004), over annual, daily and seasonal scales (Maun 2009). Stochastic processes, such as seasonal changes and episodic erosion events from wave action and gales (Dumbrell et al. 2010; Ranasinghe 2016) also affect the three-way partnership of arbuscular mycorrhizal (AM) fungi, the edaphic environment, and plant roots. Arbuscular mycorrhizas are ubiquitous in soils, including coastal sands, and their symbioses are associated with some 80-90% of terrestrial vascular plants (Smith & Read 2008). In particular, the fungus improves plant nutrition by increasing water (Augé 2001) and nutrient uptake (Mensah et al. 2015) and assists the establishment and survival of plant seedlings by improving stress tolerance (D'Souza & Rodrigues 2013; Koske et al. 2004). AM fungi are also regarded as ecosystem engineers, forming stable aggregates in soils as varied as semiarid soils with differing levels of calcium carbonate (Kohler et al. 2017), and the deeply weathered Ferralsols of the humid tropics (Demenois et al. 2017).

In his seminal work, Nicolson (1960) quantified the seasonal abundance of AM fungi in the roots of *Poaceae* species, including *Thinopyrum junceiforme* (under the heterotypic synonym *Agropyron junceiforme*) (Sea Wheatgrass), on the incipient dunes, foredunes and backdunes at Gibraltar Point, Lincolnshire, England. Fungal abundance was studied

in relation to soil pH, zones in the dune system, the degree of dune stability, and the quantity and type of wrack and organic matter present (Nicolson 1960). For example, the roots of *Festuca rubra* var. *arenaria* (Red Fescue) in the stable dunes had a greater abundance of AM fungal colonization than did the roots of *Ammophila arenaria* (Marram Grass), and colonization rates in the roots of Sea Wheatgrass increased substantially as the dunes became more stable (Nicolson 1960).

The temporal dynamics of AM fungi have been quantified in a number of natural environments, for example, temperate grasslands and woodlands (Bennett et al. 2013; Dumbrell et al. 2011), as well as mangroves (D'Souza & Rodrigues 2013). In these settings, distinct, diverse assemblages of AM fungal taxa were associated with variables between winter and summer. Such seasonal changes in fungal abundance may be as a result of differences in temperatures and sunshine hours (Dumbrell et al. 2011), which would also affect the relative abundance of other soil biota. For example, warming of the soil stimulates soil microbial activity, and it is well-established that soil drying-rewetting events such as summer storms after a period of drought, can alter edaphic microbial community structures (Insam 1990; Lund & Goksøyr 1980; Lundquist et al. 1999).

Changes in AM fungal species composition have also been observed seasonally. For example, in a Swedish semi-natural grassland, two significantly different communities of AM fungi were associated between that of *Prunella vulgaris* (Common Self-heal) and *Antennaria dioica* (Stoloniferous Pussytoes) during the growing season (Santos-González, Finlay & Tehler 2007). Common Self-heal had a richer AM fungal community than did Stoloniferous Pussytoes, hosting 19 different communities of AM fungi, of which four sequences were rare (Santos-González, Finlay & Tehler 2007). Further, it was hypothesized that the low abundance of one particular Glomeromycotan fungus (*Glo8*) during September and October (Nordic Autumn), was related to seasonal fluctuations in the mineralization of nitrogen (Santos-González, Finlay & Tehler 2007), which in turn is affected by soil temperatures and moisture exerting an influence upon soil biota (Fierer & Schimel 2002).

In the study of Dumbrell et al. (2011) in a British site comprising a mosaic of wood, heath and grassland, AM fungal community structure and composition revealed significantly different and distinct assemblages in summer and winter, which were

associated with resource limitations and low soil temperatures in the cooler periods. At the Thirteenth Beach study site, long-term seasonal means show the winter minimum and maximum temperatures to be 5.9° C and 13.9° C respectively, whilst summer minimum and maximum means are 10.9° C - 24.8°C, respectively, however no data on sunshine hours are available (Bureau of Meteorology 2017). It is to be expected that given the temperature differences between the cooler and warmer seasons, the southern Australian site will also demonstrate temporal dynamics. However how, and to what extent, AM fungal abundance or their assemblages differ between the grass species studied, has not been reported to date.

Table 4.1: Arbuscular mycorrhizal studies on Australian coasts

Authors	State	Coastal area studied in association with AM fungi
Jehne and Thompson (1981)	Queensland	Coastal dunes in Cooloolo
Koske (1975)	New South Wales	Foredune, first dune ridge and back dune soils of mid and southern coast
Logan et al. (1989)	New South Wales	Northern beaches
Peterson et al. (1985)	Queensland	Coral cays with humus substrates, on Heron Island

AM fungi have profound effects on community dynamics and plant survival (Liu et al. 2015), for example through the cycling of phosphorus, carbon and nitrogen (Fitter 2005), providing stress tolerance to drought (Li et al. 2014), and increasing resistance to pathogens by stimulating the plant's immune system (Cameron et al. 2013). Despite such profound effects, little is known about mycorrhizas and their mutualist partners in natural ecosystems such as sand dunes, in which mycorrhizas are likely to play a key role in community dynamics (de León et al. 2016b). Further, the seasonal dynamics of AM fungi in pioneer plants on incipient dunes remain largely unknown. There is still much to be uncovered regarding the biogeography of these fungi (Liu et al. 2015; Öpik et al. 2013), particularly in Australasia (Davison et al. 2015a; Öpik et al. 2010; Öpik et al. 2013), and in Australia, where there have been few studies of coastal AM fungi, with none to date occurring on the southern temperate coastline. Indeed, of the studies undertaken in Australia, two were in Queensland on the north-eastern coast, and two in New South Wales, on the eastern coast, with all four studies either in the foredunes or coral cays (Table 4.1).

Koske and Polson (1984) noted that little of the published data dealing with sand dune vegetation acknowledged the paramount importance of AM fungi to the survival of psammophilic plant species, particularly those of the disturbed incipient dunes. They argue that low level of phosphorus in dune sands that is available for plant uptake, compounded by the low mobility of phosphorus in soils in general, is offset by a symbiotic relationship between host plant and fungus (Koske & Polson 1984). Furthermore, in an experiment using *Ammophila breviligulata* (American Dunegrass) Koske and Polson (1984) found that 80% of the plants grown in sand without viable AM fungal spores had died after three months, whereas mycorrhizal plants suffered only a 22% loss. Additionally, Roy-Bolduc et al. (2016) argue that the ecological importance of AM fungal communities is rarely included in vegetation studies. However, the vigour and influence of vegetation on the establishment of sand dunes has been well addressed by geomorphologists (Hesp 1981; Hilton et al. 2006). For example, Hilton et al. (2006) document the role of sand-binding, rhizomatous grasses in the formation of incipient dunes, arguing that they form dunes which are resilient to disturbance. Further, Hilton et al. (2007) propose that such grasses impede the development of transgressive dunes and coastal barriers, thereby changing the geomorphology of the coast, and leading to environmental implications such as loss of native species and habitat. As a first step towards dealing with such implications, it is important to understand the biotic and abiotic interactions between plants, soil, and soil microbes, including AM fungi, as they predominate many ecological functions, and are important in explaining how ecosystems respond to change (van der Putten et al. 2013).

Changes in an ecosystem select for the most resistant species (Chagnon et al. 2013). For example, the number of mycorrhizal propagules in plant roots that survive in disturbed soils, determines consequent levels of AM formation (Jasper, Abbott & Robson 1991). In addition, fungal species differ in how long, and whether, they can survive in dead root fragments (Tommerup & Abbott 1981), such as those scarped off sand dunes. Such fungal species include those in the family *Glomeraceae*, including *Glomus fasciculatum* and *G. monosporum*, (formerly, *Glomus fasciculatus*, and *G. monosporus* respectively), and *Scutellospora calospora* (formerly *Gigaspora calospora*) in the family *Gigasporaceae*. It has been demonstrated that the latter three fungal species can grow new hyphae from the broken ends of senescent hyphae, within the cortex of dead

plant roots, in pot cultures of *Trifolium subterraneum* (Subterranean Clover) (Tommerup & Abbott 1981).

Fungi in the family *Glomeraceae*, of which *Glomus* is one of four genera including *Funnelformis*, *Rhizophagus* and *Septoglomus* (INVAM 2017), have *r*-selection traits (Sýkorová et al. 2007; van der Heyde et al. 2017). The *r*/*K* selection theory in ecology relates to an organism's combination of functional traits which influence their growth, survival and reproduction, and their life history strategies, whereby there are trade-offs between the organism and the environment (Chagnon et al. 2013). Trade-offs may also be associated with an organism's investment in reproduction and growth, for example, an organism cannot be simultaneously quick at reproducing, and fast at colonizing a niche (Grime 1977; Hart & Reader 2002; Pianka 1970). *Glomeraceae*, for instance, have been found to have low soil colonization (extraradical), but high root colonization (intraradical), illustrating a different life history strategy for colonizing than that of *Gigasporaceae* which has high extraradical, but low intraradical colonization, of its host (Hart & Reader 2002).

AM fungal *r*-strategists are capable of rapid colonization in ruderal habitats (Sýkorová et al. 2007). The life history strategies and *r*-selection traits of certain genera of AM fungi may, therefore, enable survival and tolerance of unpredictable or unstable environments such as incipient sand dunes. Similar traits would also apply to host plants like Sea Wheatgrass and Hairy Spinifex which colonize unstable dunes, and traits related to asexual reproduction in plants have been found to be relevant in habitats of soil disturbance (McIntyre, Lavorel & Tremont 1995).

The *r*-strategy is also associated with marine-dispersed, rhizomatous plant fragments. These fragments are more robust and grow faster than do pioneer species grown from seed (Hilton & Konlechner 2011; Maun 2009). This is likely to be due to the presence of spores of AM fungi already in the sand and in the plant roots (Cordoba et al. 2001; Gemma, Koske & Habte 2002; Logan, Clarke & Allaway 1989). Alexander et al. (1992) argued to the contrary, that hyphal and root fragments are more important in re-colonizing disturbed sites than are spores. Successful colonization will however, depend upon the stage of AM fungal development. For example, *Acaulospora laevis* hyphae are effective only when young in colonizing plant roots, and the spores have

a dormancy period (Abbott & Robson 1981b). Further, their hyphae lose the ability to colonize a host in disturbed soils (Jasper, Abbott & Robson 1989a, 1989b).

Recent studies have shown that AM fungi have been found in a large range of disturbance types, such as intensively tilled agricultural fields, where the *Glomeraceae* fungus *Funneliformis mosseae* (formerly *Glomus mosseae*), differed significantly in spore densities in tilled compared to non-tilled soils (Wetzel et al. 2014). Furthermore, it is hypothesized that AM fungal diversity may increase initially in response to disturbance in ploughed fields, before settling into equilibrium in the longer term (Peyret-Guzzon et al. 2016). Although tilled agricultural soils differ to sand which is moved off and onshore, the movement of both types of substrate nonetheless creates disturbance to soil biota, and may alter community structure (Forey et al. 2008; Peyret-Guzzon et al. 2016).

It has been proposed that the response to disturbance by AM fungal species depends upon the context (McIntyre, Lavorel & Tremont 1995), such as chemical inputs, soil disruption and agricultural practices (van der Heyde et al. 2017), and there is a body of work which addresses fungal responses to disturbance from the early study of Miller (1979), to more recent studies (e.g. Hart et al. 2016; Pereira et al. 2014). Miller (1979) for example, found that the majority of plants in mine-spoil deposits that had been left undisturbed since being re-vegetated were mycorrhizal, whereas the plants in disturbed sites were not. Pereira et al. (2014) found that land use in remnant forests, rather than soil physical and chemical properties, affected AM fungal community composition. Contradicting this, Helgason and Fitter (2009) contended that selection traits in AM fungi may be influenced by soil chemical and physical properties such as pH, EC_e and oxygen, independently of the symbiont. In another study of disturbance in forests, Hart et al. (2016) found the taxa of AM fungal species in logged and unlogged areas did not differ, however the composition of the taxa did differ in relative abundance. Conversely, the study by Lekberg et al. (2012) found that disturbance events did not alter community compositions, rather, local stochasticity specialists, resilient to unpredictable environmental events, became the dominant species.

Notwithstanding the body of work on AM fungal response to disturbance (Aytok et al. 2013; De Souza et al. 2013; Forey et al. 2008; Soteras et al. 2015; van der Heyde et al. 2017), the tolerance of AM fungi to disturbance, together with salinity, as

components of their boundary conditions in the natural environment of beaches, has received scant attention. However, as the extensive mycelia of AM fungi are likely to be strongly influenced by environmental selection, and the fungi have sufficient fitness deviation and phenotypic variation for selection to occur (Helgason & Fitter 2009), it is highly likely that specialists have evolved to tolerate a saline, disturbed ecosystem. For example, it has been demonstrated that coastal endophytes from native grasses conferred salt tolerance to rice (Rodriguez et al. 2008).

In the context of perpetually disturbed, coastal environments where host plants are often subject to saline inundation from waves (Figure 4.1) and can be torn away in storm surges, for example, the fitness and phenotypic variation argument proposed by Helgason and Fitter (2009) is strong. Such fitness denotes the importance of AM fungi in the substrate, and their role in the quick re-establishment of plants. This re-establishment can occur through washed-up plant fragments, or surviving remnants on the eroded dune face, and on slumps. This complex and dynamic environment provides the boundary conditions (Chapter 2) in which communities of AM fungi and



Figure 4.1: Incipient dune at Thirteenth Beach, flattened by wave overwash, February 2016.

their *Poaceae* hosts persist in highly disrupted coastal incipient sand dunes. However, studying the organism presents challenges given that as obligate symbionts, they cannot be grown without a host plant (Helgason & Fitter 2009; Zhu et al. 2016).

In this study, seasonal changes in abundance of AM fungi in the roots of two sand dune grasses that commonly occupy the ephemeral and disturbed incipient dunes of Victoria, Australia, have been presented, taking into account the storms that scarped the site in May and June 2015, during

the research period. The hypotheses are that,

- There should be a greater abundance of AM fungi in the sand dune plant roots in the warmer months than in the colder months, due to soil temperatures exerting an influence upon AM fungal populations.
- The storm events would result in a decrease in AM fungal colonization due to a reduced number of plants, and substrate loss offshore, however, fungal colonies should quickly re-establish in plants due to populations of surviving disturbance-specialist AM fungal species in the sand.

Additionally, the seasonal changes in enzyme activity of the major microbial decomposers in the substrate surrounding the roots of the grasses on the incipient dune were investigated.

4.3 Materials and methods

4.3.1 Study site

Details of the study site (Figure 4.2) are discussed in Chapter 2, and include the geomorphology and soil chemical characteristics of the incipient dune. The two main plant species that occupy the incipient dune are the exotic C₃ Sea Wheatgrass, and the native C₄ Hairy Spinifex (Chapter 3).



Figure 4.2: The study site at Thirteenth Beach, Victoria, looking eastward from the steps at The Hole (foreground), to the steps at The Corner (white arrow), following the storms in May 2015. The site is approximately 370 m long. (Photograph W. Chapman). Yellow arrow points to top of incipient dune.

4.3.2 Assessment of plant leaf matter within 1 m² non-contiguous quadrats

Nine 20 m transects were laid out horizontally along the approximately 370 m length of Thirteenth Beach, between the western end (The Hole) and the eastern end (The Corner), with approximately 20 m in between each transect. Within each transect, 10 1 m² quadrats were laid randomly, and non-contiguously (Brockhoff & Allaway 1989). Percentage cover of living leaf matter of both grasses was assessed via calibrated visual estimation using laminated templates equivalent to 5%, 10%, 20% and 50% of 1 m². This method was chosen as it was quick, non-destructive and inexpensive, and the calibration templates eliminated observer bias (Catchpole & Wheeler 1992).

4.3.3 Collection of samples

4.3.3.1 Plant roots for assessment of AM fungal abundance

The incipient dune was divided into three segments of approximately the same length, running horizontally along the dune. Within the segments, six root samples each of Sea Wheatgrass and Hairy Spinifex were collected (n=36) in February and August (summer and winter respectively), in 2015 and 2016. A hand trowel was used to excavate sand from around the base of selected plants to collect their roots. Rhizomes or stolons were not collected. Roots were harvested from 10 cm depth by digging gently below tillers, and tracing the roots back to the parent plant to ensure they were attached to the species being collected. Care was taken to ensure as many fine lateral roots or root hairs, where they existed, were collected with the plants, in order to prevent bias in mycorrhizal measurements (Brundrett et al. 1996). Plant selection was based on healthy tillers (Brockhoff & Allaway 1989) within each segment. Plants were placed into labelled plastic bags within a cool bag for transport back to the laboratory, where they were stored in a refrigerator for no more than 48 hours before processing.

4.3.3.2 Sand collection for assessment of fungal and bacterial enzymes

As AM fungi are not the only microbes in the dune substrate, fluorescein diacetate analysis was employed to measure how bacterial enzymes changed seasonally, adding a further dimension to the little studied subterranean ecosystem of maritime dunes.

At each seasonal harvest, three replicates of sand were collected from under the grasses on the toe and the crest of the incipient dune, in each of the three segments along the incipient dune (n=18). A light-weight aluminium auger fitted with plastic liners was used for sand collection. The auger enabled sand cores from 0 - 10 cm depth to be removed without grains from the surface caving in, thereby contaminating samples. A modified trowel was used to slide under the bottom of the submerged auger to loosen it from the surrounding sand, whilst sealing off the open end of the plastic insert. A control sample was taken from sand without plants growing on or near it. Bags were sealed, labelled and placed in a cool bag for transport back to the laboratory, as above.

4.3.4 Treatment of samples

4.3.4.1 Plant tillers and roots

Plant aerial parts were separated from root matter, sand adhering to the roots was gently washed off, and roots were blotted on absorbent paper. Tillers and roots were weighed for fresh weight. Roots were cut into approximately 1 - 2 cm lengths, placed in small vials of 10% potassium hydroxide to clear the cells of cytoplasm, and left to clear at room temperature for up to three days. Following clearing, a random sub-sample of 0.2 g of roots per vial was placed in a 125 µm stainless steel mesh sieve, and washed with distilled water. Where fresh root masses were less than 0.2 g, the entire root mass was used. Using the modified protocol of Abbott and Robson (1981), sub-samples were placed in clean vials and stained for four hours at room temperature in 0.05% trypan blue in 1:1:1 lactic acid, glycerol and distilled water (lactoglycerol). Roots were de-stained and stored in 1:1:1 lactoglycerol until ready to score for mycorrhizal fungi. A modified gridline intersect method (Tennant 1975) was used to enumerate

mycorrhizas in an 8.5 cm-diameter petri dish marked into 1 cm² grids, under an Olympus SZ11 stereoscope.

4.3.4.2 Sand

Mean grain size of the incipient dune toe is 1.46 Ø, and 1.45 Ø on the crest (Chapter 2). Fluorescein diacetate analysis of the substrate was employed at each field harvest in summer and winter 2015 and 2016. Fluorescein diacetate analysis measures total enzyme activity in soils and environmental samples, particularly of the main microbial decomposers (Adam & Duncan 2001). Free and membrane-bound enzymes hydrolyze fluorescein diacetate to release a coloured end-product that absorbs strongly at 490 nanometres (nm), and which can be read by a spectrophotometer or plate reader (Adam & Duncan 2001). Values for fluorescein diacetate are obtained by using a calibration curve which relates fluorescein concentrations to optical density (Taylor et al. 2002).

Three replicates of sand from 0 - 10 cm depth on the toe, and the crest, of the incipient dune from each of the three segments along the dune, were homogenized, sieved through a 4 mm mesh sieve to remove coarse organic matter (Abbott & Robson 1981), and left to air dry. The modified protocol of Schnürer and Rosswall (1982), using 1:2 chloroform:methanol rather than acetone (University of Toledo 2004), was used to measure total enzyme activity in the sand. Sub-samples of 2 g of sand were placed into sterile 50 mL falcon tubes, to which 20 mL sodium phosphate buffer (60mM pH 7.6) and 0.1 mL fluorescein diacetate stock solution (4.6 mM) were added. Control 1 had sand but no fluorescein diacetate stock solution, Control 2 had fluorescein diacetate stock solution, but no buffer. The falcon tubes were placed in an incubating shaker at 24° C at 150 rpm for one hour. Following incubation, the tubes were removed to a fume hood where the fluorescein diacetate reaction was terminated by adding 20 mL 2:1 chloroform:methanol to each sample. Samples were then centrifuged at 2000 rpm for three minutes. Values for hydrolysis were attained using a modified method of Adam and Duncan (2001) with 20 µL aliquots in a CellStar Microplate (Greiner Bio-One GmbH). The aliquots were read in a multi-node plate reader (Perkin Elmer EnSpire) at 490 nanometres (nm), which generated a calibration curve relating to the fluorescein concentration, or enzymatic activity, of the samples.

4.4 Statistical analyses

One-way analyses of variance (ANOVA) in IBM SPSS Statistics v.24, was used to assess plant aerial biomass, colonized root length, and percentage colonization of Sea Wheatgrass and Hairy Spinifex, across years, and seasons. The fresh weights of plant tillers and roots were calculated. Additionally, fluorescein diacetate analyses were calculated from the concentrations generated by the plate reader.

4.5 Results

4.5.1 Fresh weights, plant tillers and roots

The weight of fresh plant tillers harvested from the field from individual Hairy Spinifex and Sea Wheatgrass plants (n=18) were higher for Hairy Spinifex than they were for Sea Wheatgrass across seasons and years, with pre-storm tiller weights being higher in both grass species than they were post-storm (Table 4.2). Roots from both plant species were taken at 10 cm depth (n=18), and although seasonal fresh root weights for Sea Wheatgrass were erratic, they were higher in the winters than they were in the summers (Table 4.2). The fresh root weights of Hairy Spinifex remained relatively constant throughout seasons and years (Table 4.2).

Table 4.2: Fresh tiller weights (g) and fresh root weights (g) (n=18), for field sampled *Thinopyrum junceiforme* and *Spinifex sericeus* plants from Thirteenth Beach, Victoria in summer (S) and winter (W) 2015 and 2016

Plant organ	<i>Thinopyrum junceiforme</i> weight (g)				<i>Spinifex sericeus</i> weight (g)			
	2015		2016		2015		2016	
	S	W	S	W	S	W	S	W
Tillers, fresh weight	3.00	0.65	0.85	0.53	14.86	3.95	9.22	8.48
Roots, fresh weight	0.15	0.25	0.11	0.44	0.56	0.62	0.46	0.41

4.5.2 Plant leaf matter

There was a highly significant difference between the combined grass species' leaf matter assessed within non-contiguous 1 m² quadrats across the incipient dune, during summer and winter 2015 and 2016 ($P < 0.05$) (Table 4.3).

Table 4.3: Section of one-way ANOVA showing significant differences between the combined dune grass species' leaf matter assessed in non-contiguous 1 m² quadrats on the incipient dune. Significant outcome in bold.

Variable	df	F	Sig.
Year and species	3	.65	.586
Species	1	32.50	.000
Year, Season and Species	3	1.98	.126

Hairy Spinifex had a greater percentage of leaf matter across the incipient dune, than that of Sea Wheatgrass during 2015 and 2016, with means reflecting this, 95% CI (Table 4.4). The percentage of leaf matter of Hairy Spinifex measured within quadrats increased in winter 2015 and summer 2016 (sampled in August and February, respectively), following storms that scarped the site in May and June, 2015. Between the sampling intervals, the storms caused sand and plant loss (Figure 4.3), and subsequent slumps on the incipient dune (Figure 4.4). The percentage of leaf matter measured within quadrats decreased slightly in winter 2016, but remained higher in abundance than prior to the storms (Figure 4.5). The percentage of Sea Wheatgrass tillers measured within 1 m² quadrats also increased in winter 2015 following the storms, but subsequently decreased over the next two seasons (summer and winter 2016), to less leaf matter per quadrat than measured in the first harvest in summer 2015 (Figure 4.6).

Table 4.4: Summary of data showing higher percentage of *Spinifex sericeus* leaf matter in 1m² quadrats across the incipient dune at Thirteenth Beach, compared to that of *Thinopyrum junceiforme* leaf matter in 1m² quadrats across the incipient dune, in summer and winter 2015 and 2016, 95% CI.

Season and year	Species	% leaf matter	95% confidence interval	
			Lower	Upper
Summer 2015	<i>Spinifex sericeus</i>	22.66	11.92	33.40
	<i>Thinopyrum junceiforme</i>	12.66	1.93	23.40
Winter 2015	<i>Spinifex sericeus</i>	30.00	19.26	40.74
	<i>Thinopyrum junceiforme</i>	14.78	4.03	25.52
Summer 2016	<i>Spinifex sericeus</i>	40.67	30.00	51.40
	<i>Thinopyrum junceiforme</i>	9.22	-1.52	20.00
Winter 2016	<i>Spinifex sericeus</i>	37.79	27.03	48.52
	<i>Thinopyrum junceiforme</i>	7.80	-2.97	18.52



Figure 4.3: Storm scarp at Thirteenth Beach, May 2015, looking eastward towards The Corner. Rhizomes and stolons of *Thinopyrum junceiforme* and *Spinifex sericeus* respectively, trail down the scarp face of the incipient dune from which plants and sand were torn away by wave action during storms (Photograph W. Chapman). Arrow points to top of incipient dune.



Figure 4.4: Slump on incipient dune at Thirteenth Beach, resulting from storms in 2015. New *Spinifex sericeus* growth is shown, with older and newer stolons trailing to beach surface, where new plant colonies will form. Marker (circled) is 30 cm high. Arrow indicates top of incipient dune.

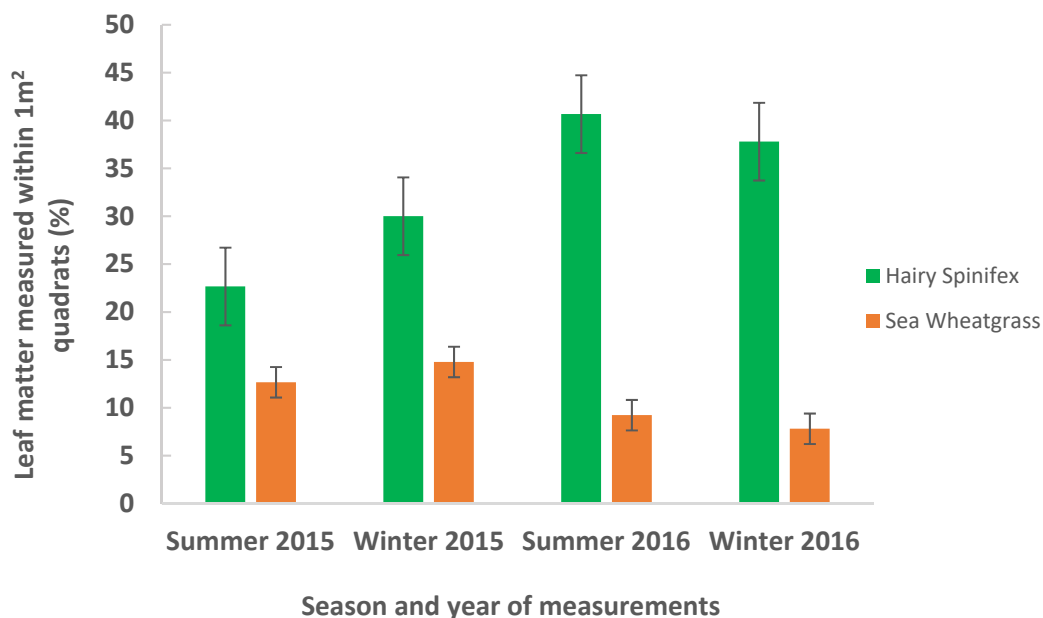


Figure 4.5: Percentage of leaf matter measured within 1m² non-contiguous quadrats in nine 20 m transects across the incipient dune at Thirteenth Beach over summer and winter 2015 and 2016, illustrating the higher percentage of tillers per quadrat each season in *Spinifex sericeus* (Hairy Spinifex). Bars show standard error (SE).

4.5.3 AM fungal abundance in plant roots

4.5.3.1 Colonized root length (cm)

In summer 2015, following eleven years of relative stability since the previous storm events (W. Chapman, pers. comm.), the colonized root length (cm) of the combined grasses were considerably lower (.197, CI 95%), than for the subsequent three post-storm harvests at .677, .800 and .235, CI 95%, respectively (Table 4.5).

Table 4.5: Section of one-way ANOVA showing AM fungal colonized root length (cm) (CRL cm) in incipient sand dune grasses over seasons and years at Thirteenth Beach. The lower colonized root length (cm) before severe storms at the site is shown in bold.

Season and year	CRL (cm)	95% confidence interval	
		Lower	Upper
Summer 2015	.197	-.031	.425
Winter 2015	.677	.405	.950
Summer 2016	.800	.528	1.073
Winter 2016	.235	.781	.508

In summer 2015, colonized root length (cm) of Hairy Spinifex and Sea Wheatgrass were 0.18 cm and 0.17 cm respectively, and in winter 2015 these had risen to 0.85 cm and 0.47 cm for Hairy Spinifex and Sea Wheatgrass, respectively (Figure 4.6). In summer 2016, the colonized root length (cm) of Hairy Spinifex was 0.99 cm, and in Sea Wheatgrass it was 0.6 cm, decreasing in winter 2016 in both plant species to 0.39 cm and 0.61 cm for Hairy Spinifex and Sea Wheatgrass, respectively (Figure 4.6).

Table 4.6: Results of one-way ANOVA showing the relationship between seasons and years, in colonized root length of sand dune grasses in non-contiguous 1 m² quadrats on the incipient dune at Thirteenth Beach. Significant outcome in bold.

Variable	df	F	Sig.
Transect	8	.469	.876
Species	1	.805	.371
Season and year	3	3.907	.010
Species, season and year	3	2.027	.113

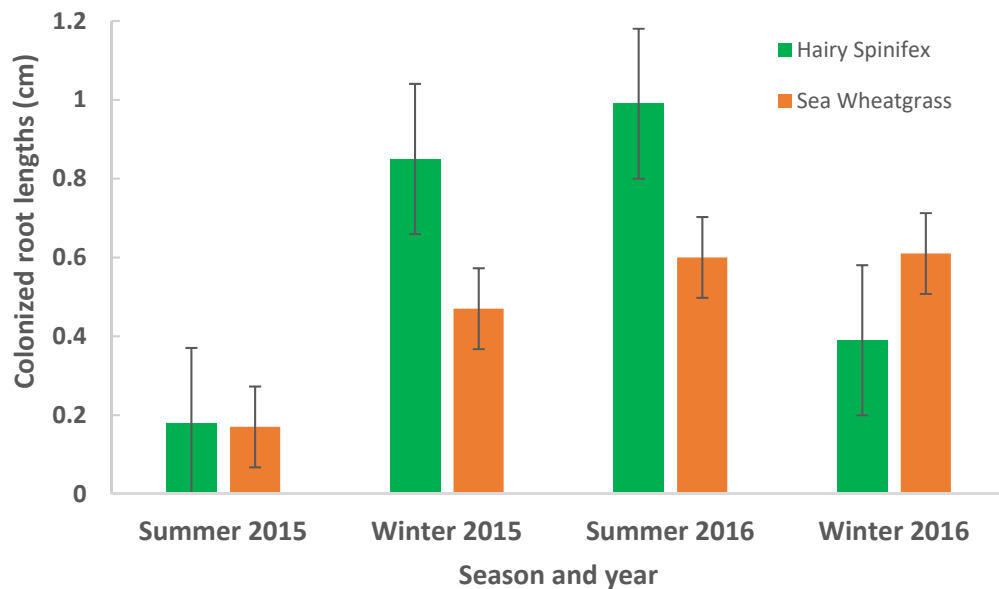


Figure 4.6: AM fungal colonized root length (cm) in incipient dune grasses at Thirteenth Beach, illustrating the generally greater colonization in *Spinifex sericeus* (Hairy Spinifex) roots than in *Thinopyrum junceiforme* (Sea Wheatgrass) roots. Colonization was lower in both grass species in summer 2015, prior to severe scarping of the incipient dune during May and June, 2015. Bars show standard error (SE)

4.5.3.2 Percentage mycorrhizal colonization (% RLC)

There was no significant difference in percentage colonization (% RLC) of the grass species, between seasons (two times) and between years (two years).

4.5.4 Assessment of microbial decomposer concentrations in sand

The total enzyme concentrations of microbial decomposers were measured by fluorescein diacetate hydrolysis concentrations in 2 µg air-dried sand. The highest level of enzyme concentrations was recorded in summer 2015 on the toe and the crest of the incipient dune, and encompassed the three sampling transects along the 370 m length of the research site - the eastern end of the site (The Corner), mid-way along the dune, and the western end of the site (The Hole) (Figure 4.7). The enzyme concentrations were severely reduced on the toe and crest of the incipient dune along the three transects in winter 2015 and summer 2016 (Figure 4.7), following the storms that scarped the site May and June 2015. However, the concentrations rose slightly on the toe, but not on the crest of the incipient dune in summer 2016 (Figure 4.7). In winter 2016, there was a sharp rise in the enzyme concentrations on the incipient dune toe at the eastern end of the dune (The Corner), and there was a trend towards increasing concentrations in comparison to the two previous seasons on the toe and crest of the incipient dune mid-way, and at the western end of the research site (The Hole) (Figure 4.7). However, the total enzyme activity had not recovered to equal that prior to the storms in May and June 2015.

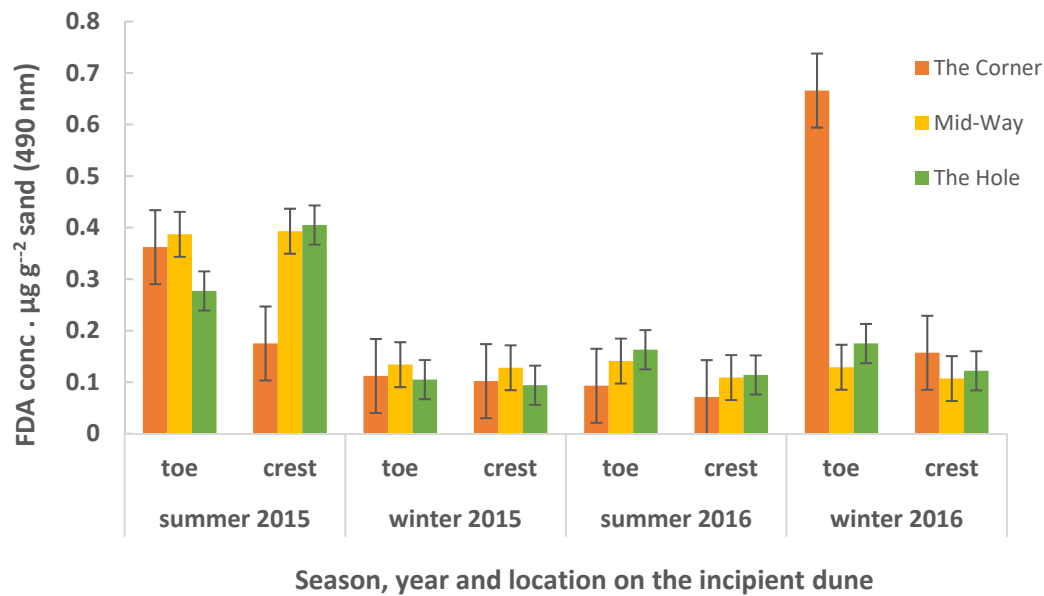


Figure 4.7: Enzyme levels in sand measured by fluorescein diacetate hydrolysis concentrations (µg) released in 2 g air-dried sand read at a wavelength of 490 nanometres (nm), in sand samples collected over two summer and two winter harvests (2015, 2016) at Thirteenth Beach. Sand was collected from three transects along the incipient dune at the research site, on the toe and the crest of the incipient dune. Orange bars represent the eastern end of the site (The Corner), yellow bars represent mid-way along the dune, and green bars represent the western end of the site (The Hole). Bars show standard error (SE).

4.6 Discussion

In this study it was hypothesized that there would be seasonal changes in abundance of AM fungi in the roots of the psammophilic grasses at Thirteenth Beach, which occupy the disturbed environment of the incipient dune. It was further hypothesized that the storms that occurred at the site during the research period would result in a decrease in plant root colonization due to substrate loss offshore, but that AM fungal colonies would quickly re-establish in plants due to populations of surviving AM fungal propagules in the sand.

Clearly, the hypothesis that the storm would result in a decrease in AM fungal colonization of plant roots due to reduced numbers of host plants, and substrate loss offshore, was not substantiated. The interaction of disturbance from storms, and populations of AM fungi that had colonized plants throughout the 11 years of quiescence prior to the storms, cannot be ignored in explaining the differences

between mycorrhizas pre-and post-storms, from one summer to the next (summer 2015 and summer 2016). Different life history diversification strategies were likely at work (e.g. Chagnon et al. 2013), ensuring the survival of coastal AM fungi at the Thirteenth Beach. Additionally, the asymptote of fungal abundance that was reached by the summer of 2016 before the colonized root length (cm) began decreasing to a new level of abundance, could be in parallel to the dune morphology adjusting along its continuum of responses to environmental conditions, albeit coastal systems show great variance in their response to changing conditions and dynamics (Davidson-Arnott 2010). For example, coastal morphology is subject to tidal and aeolian dynamics at a temporal scale from seconds to minutes, or to rocky cliff erosive forces over hundreds of years (Davidson-Arnott 2010). For sand dunes, the mobile substrate needs to become stable and resistant to aeolian transport via wrack, for example, which stops the movement of sand (Kennedy & Woods 2012), or pioneer seedlings (Hesp 1989), before the autogenic factors including vegetation and AM fungi, become the driving forces in the continuum of scarping, accretion and dynamic equilibrium.

The first summer's data collection (2015) took place during a period that had not endured severe storms or scarping for some 11 years (W. Chapman, pers. comm.). This period of comparative stability and accretionary dune morphology (Psuty 2004) may have allowed for a relatively low proportion of, but likely specialized, *K*-strategist species of AM fungi to reach equilibrium, whereby temperatures, and mild agitation (Soteras et al. 2015), may not have had a significant effect upon populations. In ecology, the *r/K* selection theory refers to the combination of functional traits, or the morphological or physiological attributes of a phenotype, which influence their reproduction, growth, survival and life history strategies (Chagnon et al. 2013). One life history strategy trait of AM fungi is the rate and extent of their colonizing ability (Hart & Reader 2002), whereby *K*-strategists have long-lived, low, but stable populations, and a slow growth-rate (Smith & Smith 2012b). On the other hand, *r*-strategists are typically short-lived, with high and rapid rates of reproduction, and take advantage of temporary habitats (Smith & Smith 2012b). AM fungi in the family *Acaulosporaceae*, for example, colonize faster than do AM fungi in the family *Gigasporaceae* (Hart & Reader 2002), typical of the *r-K* selection theory.

In substantiating the hypothesis regarding quick re-colonization of plant roots by surviving AM fungal propagules in the sand, I propose that the rapid- recolonization demonstrated in the data was not re-establishment of the prior *K*-strategist species, but rather competitive *r*-strategists (Sýkorová et al. 2007), whose life history strategies enabled them to take advantage of the new, albeit unstable niche that was created by the storm. Such life history strategies enable survival in unstable and unpredictable environments, and denote the vital role of AM fungi in the substrate as they rapidly colonize plant fragments or surviving remnants on slumps and embryo dunes. Further, the increases in colonized root length (cm) following the storms favour the *r*-selection traits of genera such as *Glomus* (Sýkorová et al. 2007; van der Heyde et al. 2017), that are likely to be identified in the ephemeral, disturbed setting of incipient dunes. For example, it may be that there is a positive growth response in AM fungal communities in disturbed sites, wherein *r*-strategist fungi are stimulated to sporulate rapidly (Hart & Reader 2002), to colonize remaining host plants, in order to overcome, for example, the devastating effects of substrate removal.

Within the autogenic dynamics of the dune, the summer 2016 sampling showed greater colonized root length than in winter 2015. I propose that warming soil temperatures were responsible in furthering the rapid colonization of plant roots, as demonstrated by Helgason et al. (1999), by the AM fungal *r*-strategists in the incipient dune ecosystem. These findings are in agreement with Heinemeyer et al. (2003), whereby there was greater root length colonization by AM fungi in grasses in summer than in cooler seasons, and also with Fitter et al. (2000), whereby mean root length colonized in *Plantago lanceolata* (Ribwort Plantain) by *Glomus mosseae*, were greater at 20°C than they were at 12°C. Furthermore, in an earlier study by Baon et al. (1994), the colonization of *Hordeum vulgare* (Barley Grass) by *Glomus intraradices* was reduced significantly as soil temperatures decreased, resulting in colonization at 15°C, but not at 10°C. Additionally, a drop in the soil temperature from 20°C to 15°C resulted in reduced plant root growth that was more pronounced in mycorrhizal, than in non-mycorrhizal plants (Baon, Smith & Alston 1994). These data add weight to the findings of the second summer's field data (2016) in this Chapter, whereby there was a significant interaction between season and year.

Explanations for sporadic or erratic colonization events are scant in the literature (Smith & Read 2008). However, I suggest that the increases in colonized root length (cm) following the storm events were due to disturbance specialists emerging. Such specialists may be aggressively competitive (Jansa, Smith & Smith 2008; Smith & Read 2008) for symbiotic partners in a disrupted and fractured niche, before declining in relative abundance as the dune stabilizes and other indigenous populations of AM fungi increase. Although the species of AM fungi at the research site were not analysed seasonally, it is logical to assume that long-term *K*-strategists were the primary colonizers of the dune plants before the storms, with their ecological trade-off being the inability to withstand severe disturbance. Such trade-offs with a habitat or ecological niche have long been recognized, whereby evolution moulds life history strategies, based on the genetic variability of an organism (Southwood 1988). In other words, the ability or otherwise of an organism to survive and grow under quiescent times would not select for a life history strategy to cope with stress (McGill et al. 2006). Further studies are needed to elucidate the within-species changes of community composition and function (Evans & Wallenstein 2014) and traits (McGill et al. 2006) in AM fungi that may result as a consequence of severe disturbance.

That Hairy Spinifex had a statistically significant higher leaf matter within quadrats than that of Sea Wheatgrass, contrasts with data in South Australia from two areas along the Youngusband Peninsula (Hilton et al. 2006), whereby Sea Wheatgrass was observed to displace native sand dune grasses such as Hairy Spinifex. Dissimilarities in the geomorphology and underlying geology between the research site of this study, and those of the South Australian sites, may be attributable to the inverse quantities observed of the leaf matter of one grass species, over the other. Whereas the characteristics of this study's site were those of geologically constrained site, as demonstrated by the exposure of the Bridgewater Formation post-storms, the South Australian sites were on eroding, and quasi-stable sand dunes (Hilton et al. 2006). Further, other processes, both subterranean and those of waves, for example, may differ from one site to the other (Kennedy 2014). Another possible explanation for differences between relative leaf matter percentages is that of the stages of vegetation establishment, whereby established vegetation with strong rhizomes, or stolons, may account for a greater mass of one plant species over the other (Keijzers, De Groot & Riksen 2015). Although fresh tiller weights for individual plants of both species were

substantially lower in the three harvests that followed the storms, Hairy Spinifex had consistently a higher fresh tiller weight per plant than did Sea Wheatgrass, throughout the summer and winter of 2015 and 2016.

Seasonal changes in concentration of microbial decomposers in the substrate surrounding the roots of the grasses on the incipient dune were also investigated. Individual species were not identified but rather an estimation of their overall activity, using fluorescein diacetate hydrolysis (Green, Stott & Diack 2006; Taylor et al. 2002). There was no apparent trend in concentrations across the sampling times of two summers and two winters, in agreement with Chen et al. (2005), who argued that seasonal abundance in the biomass pool in sand dunes is inconsistent. Notwithstanding this, the data support the negative effects of the storms on the microbial decomposers at Thirteenth Beach, whereby their concentrations were on average two to three times higher in the harvest before the storms, than in the three harvests following the storms. One notable exception was that of the substrate collected at the eastern end of the site (The Corner) in winter 2016, whereby a large spike in fluorescein was recorded. The spike in fluorescein is inexplicable, but may be the result of sand collection where animal urine was relatively fresh, as the area is often favoured by owners walking their dogs.

In general, there were higher concentrations of decomposers in the sand on the toe of the incipient dune than on the crest of the dune. This would be due to the detritus, wrack and other nutritional inputs being deposited along the toe during high tides (Kennedy & Woods 2012). Additionally, daily and seasonal fluctuations in temperature and moisture affect the abundance of soil microbial biota, and plant growth is also an important factor which regulates the wide range of edaphic microbes in natural ecosystems (Cornwell et al. 2008; Yao, Bowman & Shi 2011), including beaches.

4.7 Summary

Sand dunes are environmentally stressful habitats for plants and fungi for many reasons, including the effects of salt spray, substrate removal, low nutrients and frequent tidal inundation (Maun 2004). Such habitats represent an opportunity to

increase our knowledge of the biogeography and abundance of AM fungal species tolerant to these conditions, both seasonally and in response to storms. This study found that there was a highly significant difference over two years between the aerial abundance of leaf matter of Hairy Spinifex on the incipient dune of Thirteenth Beach compared to that of Sea Wheatgrass. Furthermore, season and year had a significant effect upon increases in colonized root length (cm) in the two grasses. Collection data from the three harvests following the storm events, illustrate clearly an increase in mycorrhizas in the grasses, due to root fragments being colonized rapidly by ruderal specialist AM fungi. Concentrations of decomposers in the substrate were also affected by storm, and were on average two to three times higher pre-storm events than in the post-storm harvests.

To my knowledge, this is the first body of work that has addressed the presence and seasonal abundance of AM fungi, and other soil biota, in the disturbed ephemeral environment of incipient sand dunes. This study increases our understanding of the subterranean coastal ecosystem of incipient dunes, and biogeographical locations of AM fungal populations, demonstrating the robust nature of these ecosystems in relation to storm-induced disturbance in ephemeral environments.

Chapter 5

The effects of increasing levels of sodium chloride on arbuscular mycorrhizal (AM) fungal colonization in *Thinopyrum junceiforme* (Sea Wheatgrass), an incipient sand dune grass

5.1 Abstract

The effects of extreme and increasing levels of sodium chloride (17,500 ppm - 70,000 ppm/1.7% - 7.0% salinity) on mycorrhizas in roots of a coastal incipient dune grass were identified. In a glasshouse experiment, saline irrigation was introduced either on the first day of plant propagule planting before mycorrhizas had colonized plant roots (early), or from week five of plant growth after the presence of mycorrhizas in plant roots had been confirmed (late). In the early application of saline irrigation, there were decreases in colonization of roots by mycorrhizal fungi at 1.7% salinity, however in all other levels the initial effects of salinity in decreasing colonization of roots, were followed by the formation of mycorrhizas. For the late application of saline irrigation, mycorrhizal responses were erratic across sodium chloride levels with no sustained effect. Plant mortalities were similar between the early and late application of saline irrigation, with 32% and 34% recorded respectively. No plants produced tillers throughout the experiment, but all plants produced roots. This demonstrated that life history strategies of resource allocation from the nodes of *Thinopyrum junceiforme* (Sea Wheatgrass) select for roots preferentially to shoots in this plant, at least during the early stages of establishment. This would enable the plant to establish and survive the extreme conditions of the incipient dune. Furthermore, salinity did not delay the colonization of roots by coastal AM fungi, thereby demonstrating their tolerance to levels of salinity well above those normally imposed in saline-tolerant experiments.

5.2 Introduction

Thinopyrum junceiforme (Sea Wheatgrass) is an invasive, exotic dune grass in southern Australia, which colonizes foredunes and incipient dunes (Hanlon & Mesgaran 2014; Heyligers 1985; Hilton et al. 2006). Due to its location at the upper limit of non-storm wave wash, the ecosystem of incipient dunes presents a challenging environment for plant growth. Incipient dune sand, however, is not saline despite its proximity to the sea (e.g. Barbour, de Jong & Johnson 1976b; Hanlon, Abbott & Kennedy 2016).

Soils that are saline are conventionally classified as having electrical conductivity (EC) values > 4 dS/m. Further, soil texture, for example sand, must be taken into account in any saturated extract suspensions (EC_e) in which salinity is examined (Hazelton & Murphy 2007). Additionally, sand has low cation exchange capacity (CEC), or the ability to hold and exchange nutrients (Maun 2009), due to its lack of electrostatic charge and buffering capacity (Ashman & Puri 2002). Buffering capacity allows for a soil solution to adjust to changes in ionic composition; the lower the cation exchange capacity, the lower the buffering capacity of a soil (Ashman & Puri 2002). In well-buffered soils, nutrients are released and exchanged, and this exchange allows for the concentration of nutrients to be maintained in the soil solution, regardless of leaching and plant absorption (Ashman & Puri 2002).

Due to the low cation exchange capacity and buffering capacity of sand, sodium chloride (NaCl) anions and other dissolved nutrients in sea water, flush quickly down through the large interstitial pore spaces surrounding sand grains (Maun 2009). This flushing of dissolved nutrients prevents water-logging in the upper 0 - 10 cm of sand, where the majority of soil biota are found (Carson 2017; van Leeuwen et al. 2017). Additionally, the leaching rate is more rapid and the nutrient concentrations lower where there is vigorous water circulation from tides and waves, such as on high energy beaches (McLachlan & Brown 2006). Wave and capillary action, as well as precipitation and evaporation, can vary greatly in time and space (Nolet et al. 2014). Nonetheless, when incipient dunes are inundated by spring tides or storm surges, salinity in the sand matrix can increase temporarily by several fold (Tsang & Maun 1999), as the pores fill and the sand becomes saturated (Nolet et al. 2014).

The tolerance to sea water by Sea Wheatgrass is controlled on several chromosomes by multiple genes (Wang et al. 2003) (Chapter 3), yet the effects of salinity on the plant's fungal symbionts are not well understood, and are sometimes contradictory (Bencherif et al. 2015; Juniper & Abbott 2006; Wang et al. 2011). For example, Juniper and Abbott (2006) found that germination and hyphal growth in pot-grown plants, was inhibited by increasing concentrations of sodium chloride when applied over time, albeit that this may have related to fungal spore size and species, and host plant species.

Notwithstanding this, some species of AM fungi have been shown to alleviate the antagonistic effect of sodium chloride in plants such as *Zea mays* (Maize) (Estrada et al.

2013b), *Cajanus cajan* (Pigeon Pea) (Garg & Pandey 2015), and *Cicer arietinum* (Chickpea) (Garg & Baher 2013), by enhancing osmotic adjustment, or increasing proline biosynthesis (Evelin, Kapoor & Giri 2009; Talaat & Shawky 2014). Proline is an essential osmo-protectant, which is synthesized by plants under environmental stresses, such as salinity (Garg & Baher 2013). However, in pot cultures, and in the natural environment, the concentration of salts may differ greatly over relatively small areas (Juniper & Abbott 2004). Further, local or environmental stochasticity, or random variations in temperatures or moisture, for example (Smith & Smith 2012a), can directly influence host and symbiont populations (Dumbrell et al. 2010).

Wang et al. (2011) demonstrated that saline flooding of mangrove species such as *Heritiera littoralis* (Red Mangrove), *Acanthus ilicifolius* (Holy Mangrove) and *Acrostichum aureum* (Mangrove Fern) had a highly significant effect upon colonization and species richness. In their study at the Yellow River Delta, China, Wang et al. (2011) examined nine mangrove root and nine rhizosphere soils sampled from across tidal gradients, for the presence of mycorrhizas. EC measurements ranged from 1.95 dS/m - 4.24 dS/m, indicating slightly saline water (Hazelton & Murphy 2007). Molecular sequencing identified 23 phylotypes of which 22 were *Glomeraceae*, and one *Acaulosporaceae* (Wang et al. 2011). There were no significant differences in regard to soil properties and tidal elevations, however host species and duration of flooding significantly affected the AM fungal community structure (Wang et al. 2011). That is, the longer the flooding event, the less mycorrhizal richness, due to the anaerobic conditions in the submerged sections of pneumatophores (Wang et al. 2011). Additionally, it was found that moderate flooding did not inhibit AM fungal colonization, nor did the host species have any significant effects upon the diversity of phylotypes (Wang et al. 2011). However, intensive flooding did result in decreased colonization and species richness (Wang et al. 2011).

Conversely, in mixed vegetation within Indian mangroves at the Ganges River estuary, EC levels were highly saline (Hazelton & Murphy 2007) across successional gradients, ranging from 7.4 dS/m - 16 dS/m, and the major plant nutrients, nitrogen, phosphorus and potassium, were very low (Sengupta & Chaudhuri 2002). In this ecosystem, AM fungal colonization varied with host species, and was negatively correlated to

increasing salinity levels. Seven *Glomeraceae* phylotypes dominated the plant roots across the estuary's mixed successional stages (Sengupta & Chaudhuri 2002).

It has been demonstrated that in natural, saline ecosystems, wild plants can be strongly mycorrhizal, with high percentage colonization (% RLC) by AM fungus (Wilde et al. 2009; Yamato, Ikeda & Iwase 2008). Furthermore, a great diversity of AM fungi have been found in plant hosts in saline habitats (Estrada et al. 2013a). For example, on the Tabriz Plain, Iran, (Aliasgharzadeh et al. 2001), in saline soils and salt marshes in Europe (Carvalho, Correia & Martins-Loucao 2004; Wilde et al. 2009) and in mangroves in China (Wang et al. 2011) (Table 5.1).

Table 5.1: Plants from naturally occurring saline environments associated with arbuscular mycorrhizal (AM) fungi.

SOURCE	LOCATION	PLANT SPECIES
Aliasgharzadeh (2001)	Tabriz Plain, Iran	<i>Allium cepa</i> (Bulb Onion), <i>Medicago sativa</i> (Sand Lucern), <i>Triticum aestivum</i> (Wheat), <i>Hordeum vulgare</i> (Barley), <i>Salicornia</i> spp. (glasswort), <i>Salsola</i> spp. (Saltwort)
Carvalho et al. (2004)	salt marsh, Portugal	<i>Aster tripolium</i> (Salt Aster), <i>Inula crithmoides</i> (Golden Samphire), <i>Puccinellia maritima</i> (Saltmarsh Grass)
Wilde et al. (2009)	salt marshes, Europe	<i>Aster tripolium</i> (Salt Aster), <i>Puccinellia</i> spp. (saltmarsh grasses), <i>Salicornia europaea</i> (Sea Asparagus)
Wang et al. (2011)	mangrove, China	<i>Heritiera littoralis</i> (Red Mangrove), <i>Acanthus ilicifolius</i> (Holly Mangrove), <i>Acrostichum aureum</i> (Mangrove Fern)

Although the above studies in the natural environment substantiate the tolerance of some AM fungal species to salinity, there have been few experimental studies that have employed levels of sodium chloride equivalent to those in natural sea water, in order to observe AM fungal contribution to the survival of wild psammophilic plants.

The study of Camprubi et al. (2012) is one notable exception in the use of seawater irrigation on psammophiles and their symbionts. Dilutions of 10%, 25%, 50% and full-strength sea water, were employed to flood a suite of nine psammophile species grown from surface sterilized seeds, in autoclaved beach sand inoculated with *Glomus*

intraradices, now referred to as *Rhizophagus intraradices*. Either one, or two applications of 100% sea water killed all non-mycorrhizal control plants, and of the other treatments on non-mycorrhizal plants, mortalities increased as sodium chloride levels and irrigation applications increased (Camprubi et al. 2012). Flooding mycorrhizal plants with 10% and 25% sodium chloride dilutions did not result in plant mortalities, and three mycorrhizal host plants survived 100% sea water irrigation (Camprubi et al. 2012). However, in between the days plants were irrigated with saline solutions plants were watered with fresh water (Camprubi et al. 2012), which would have allowed remaining salts to be flushed through the sand.

Much is still to be elucidated on the effects saline inundation on wild coastal plants such as psammophiles, and their fungal symbionts, as our understanding of their tolerance to salt stress and how it affects survival in their harsh environment is limited (Hanley et al. 2017; Klironomos & Kendrick 1993). An early study by Koske et al. (1996) tested the germination of *Gigaspora gigantea* spores by immersing them in full strength sea water for periods of up to 20 days. However, in other studies, full-strength sea water, or sodium chloride concentrations equivalent to those in sea water, have not been used when assessing the response of AM fungi and coastal plants to salinity. For example, Asghari et al. (2005) used one-quarter-strength sea water (12 dS/m, or 125 mM) on the coastal plant *Atriplex nummularia* (Salt Bush), and Yamato et al. (2008) used approximately one-half-strength sea water (200 mM) on spores collected from under three coastal plant species.

Another important aspect to enhancing our understanding of the effect of sodium chloride on AM fungi, would be to investigate the life history stage at which AM fungal colonization takes place. For example, the study by McMillan (1998) argues that fungal germination is delayed by salinity, and that this is more conspicuous in early stages of colonization rather than in the later stages. Subsequently, it was suggested that propagule size at the introduction of sodium chloride may influence susceptibility to salinity (Juniper & Abbott 2006).

One of the central aims of ecology is to understand what processes or tolerances drive the relative abundance of species (Dumbrell et al. 2010; Lekberg et al. 2012). That a considerable variety of dune-building plant species are unable to grow without a symbiotic relationship (Koske et al. 2004), is of vital ecological significance in

unpredictable and disturbed ecosystems (Maun 2009) such as incipient dunes. Further, the ecophysiological behaviour of AM fungal species under saline stress is not well understood (Kaya et al. 2009).

This study sought to elucidate changes to the percentage colonization (% RLC) of coastal AM fungi in the roots of psammophilic grass propagules harvested from the incipient dune, and subjected to various levels of sodium chloride irrigation, over several harvests. Changes in the percentage colonization (% RLC) of AM fungi in plant roots were observed over several harvests under the imposition of varying concentrations of sodium chloride, up to and including twice that of sea water. Additionally, as AM fungi are not the only microbes in the edaphic environment, changes in the concentration of microbial decomposers in the sand in which the plants were grown were observed over the same harvests and salinity treatments. It was hypothesized that,

- Plant propagules and fungi would be tolerant to levels of salinity above those traditionally imposed in saline-tolerant experiments, due to the ecosystem in which plant and fungus were found. Further, it was queried whether there would be the production of tillers from nodes on the host plant, under sustained saline irrigation.
- Salinity imposed pre-colonization (early application) would detrimentally affect mycorrhizal colonization of plants compared to those in which sodium chloride was imposed post-colonization (late application).

5.3 Materials and methods

5.3.1 Bioassay

An initial bioassay was carried out to estimate the inoculum potential of beach sand collected from 0 - 10 cm depth immediately bedside Sea Wheatgrass and Hairy Spinifex plants growing on the incipient dune at Thirteenth Beach, Barwon Heads. The bioassay used a bait crop of *Allium porrum* cv. 'Musselburgh' (Garden Leek) as they grow rapidly and their cells are cleared easily of cytoplasm (Brundrett, Ashwath & Jasper 1996).

5.3.1.1 Bioassay experiment design

A bioassay was conducted using sand taken from immediately beside a number of Sea Wheatgrass and Hairy Spinifex plants growing on the incipient dune at Thirteenth Beach. Seeds of Garden Leek were sown into the untreated sand and grown for two weeks. There were three destructive harvests over time of four replicate pots of sand collected adjacent to each plant species. Roots of the Garden Leeks growing in the sand were assessed for mycorrhizal colonization (Juniper & Abbott 2006) to estimate the inoculum potential of the AM fungi present.

5.3.1.2 Collection and preparation of incipient dune sand for bioassay

Sand was collected to a depth of 10 cm from immediately beside healthy Sea Wheatgrass and Hairy Spinifex grasses, in three approximately equally-spaced transects 60 m long, with 60 m in between each transect running horizontally east-west, along the crest of the incipient dune. A light-weight aluminium auger with an internal diameter of 7.5 cm, lined with plastic inserts was used to collect the sand. An auger was used rather than hand digging, in order to prevent surface sand grains from caving in, thereby dominating the samples. A modified trowel was used to loosen the auger from the surrounding sand, and slipped under the base of the auger to seal the open end of the plastic liner. Liners were then tied at both ends, labelled with vegetation type, and stored in a chilled container for transport back to the nursery cool room, where they were stored for no longer than 48 hours at 4° C before being processed. All sand samples from immediately beside Sea Wheatgrass were then amalgamated and sieved through a 1 cm aperture mesh in order to remove detritus such as seaweed, and larger particulate matter. The same procedure was undertaken for sand collected from immediately beside Hairy Spinifex. Edaphic and chemical analyses of the sand, and volumetric soil water (%) are reported in Chapter 2.

Sand was placed into 950 mL plastic pots which had been lined with two layers of shade cloth, to prevent sand migrating through the drainage holes. Prior to planting the bait (bioassay) crop, the moisture content (%) of the sand was re-checked using a Theta Probe with Moisture Meter (Delta-T Devices, Cambridge, England). The moisture meter was used throughout the bioassay on random pots, to ensure that

overhead irrigation did not exceed field capacity. Irrigation was by an overhead system, calculated at 95 mL twice per day for one minute each session.

5.3.1.3 Preparation and planting of bait crop

Seeds were imbibed in hot water and left for 24 hours before being dibbled into 950 mL pots of sand at the rate of three seeds per pot, at no more than 1 cm depth. There were 12 replicates per sand location. Pots were labelled to indicate harvest number, and from which grass species the sand had been collected immediately next to. Seeds were gently watered in with Charlie Carp (N:P:K 9:2:6).

5.3.1.4 Assessment of inoculum

Seedlings emerged 10 days after planting. The first harvest was four days later at week two, subsequent harvests were at weeks 4.5 and 6.5. Mycorrhizas were not observed in the Garden Leek roots at the first destructive harvest, however by the second harvest there were mycorrhizas present. The extent of colonization had increased by the final destructive harvest, confirming the presence of infective AM fungal propagules in the incipient dune sand from directly beside Hairy Spinifex and Sea Wheatgrass plants.

5.3.1.5 Harvest procedures of bioassay plant roots

At each harvest, plants were removed gently from the sand, washed in tap water, and blotted on absorbent paper before roots were removed from aerial parts, and placed in 10% potassium hydroxide until cleared of host cytoplasm (Phillips & Hayman 1970). The modified protocol of Phillips and Hayman (1970) was followed, whereby roots were rinsed in distilled water before being stained in 0.05% trypan blue for four hours. Excess stain was removed in lactoglycerol (1:1:1 lactic acid:glycerol:distilled water), and roots stored in the solution until scored under a dissecting microscope.

5.3.2 Pre-experiment Sea Wheatgrass propagation trial

The presence of infective AM fungal propagules was confirmed; however, it had not been established whether wild Sea Wheatgrass propagules would grow under nursery conditions. Rhizome cuttings no further than 30 cm from the apical meristem of wild

Sea Wheatgrass plants were harvested from the incipient dune. Rhizome buds closer to the apical meristem are more likely to produce adventitious roots than are those towards the distal end (Harris & Davy 1986a; Ranwell 1972). Propagules were stored in plastic zip-lock bags in a chilled Esky for transport to a 4° C cool room, where they were stored for no longer than 48 hours before being planted. A modified protocol of Pavlik (1983) was used to cut rhizomes into uniform lengths using a five centimetre template. Each five-centimetre section had a central bud, but no tillers.

Edaphic and chemical analyses of the sand are reported in Chapter 2. Field capacity (FC w/v%) was also tested. In order to optimize propagation success, Sea Wheatgrass cuttings were grown with production horticulture techniques, using air-filled porosity (AFP) (Handreck & Black 2002) and water retention efficiency (WRE) (Creswell 2002), as a guide to watering regimes in natural beach sand. To my knowledge, this technique has not been used before to propagate wild plants from sand dunes.

5.3.3 Sodium chloride (NaCl) experiment design and treatments

The experiment was a randomized block design with two times for commencement of saline irrigation: early application, from the day of planting propagules and pre-colonization, and late application from week five, post-colonization, as established by an initial destructive harvest across replicate blocks (Maun & Tsang 1999). There were five treatments of sodium chloride (Table 5.3), five harvests and five replicates. Harvests began at week five of plant growth, with subsequent destructive harvests taking place at fortnightly intervals thereafter, at weeks seven, nine, 11 and 13 of plant growth. Plant deaths were recorded across treatments. Changes over time and salt treatments were also assessed in microbial decomposer concentrations, using fluorescein diacetate levels in the sand (Adam & Duncan 2001; Taylor et al. 2002). Membrane-bound and free enzymes hydrolyze fluorescein diacetate to release a coloured end-product which can be read at 490 nanometres (nm) in a spectrophotometer or plate reader (Adam & Duncan 2001; Green, Stott & Diack 2006; Taylor et al. 2002).

5.3.3.1 Sand procedures for salt (NaCl) experiment

Beach sand was collected and treated in the same manner as for the bioassay (Section 5.3.1). Three 500 g pots with two layers of pre-wetted Chux® inserted to prevent the loss of sand through drainage holes, were filled with sand and watered in increments of 50 mL until field capacity was reached. The pots were left to drain for two days. Moisture retention was measured with a Theta Probe with Moisture Meter (Delta-T Devices, Cambridge, England) to monitor volumetric soil water changes over seven consecutive days. Together with water retention efficiency results, irrigation was calculated at 60 mL per pot every four days to avoid watering to field capacity. Air-filled porosity was calculated at 0.15%. The standard air-filled porosity recommended for plants growing in potting media is > 13% (Anonymous 2016; Gibbs 2010). The low level of air-filled porosity in the incipient dune sand indicated that plant roots and AM fungi would encounter sub-optimum levels of oxygen for some time following irrigation. Irrigation would thus imitate tide-induced low oxygen particularly near the surface of the sand (Dye 1980).

5.3.3.2 Plant propagules

Rhizome cuttings were taken from plants in the field, as the major method of colonization of incipient dunes by pioneer plants such as Sea Wheatgrass, is by vegetative regeneration of rhizomatous fragments (Koske et al. 1996). Furthermore, both the fungus and host plant are found in association with each other in the incipient dune system (Nicolson 1960). Rhizome cuttings were made in accordance with the pre-experiment protocol for Sea Wheatgrass propagules.

Single rhizome segments were planted on a 45° angle to just above the bud, into 500 mL biodegradable jiffy pots filled with natural incipient dune sand, weighed to a consistent weight across replicates. To avoid migration of sand or fungal propagules out of the pots, the pots did not have drainage holes.

5.3.3.3 Glasshouse specifications

The experiment was established and maintained on wire benches in a glasshouse, with automatic evaporative coolers which switched on and off at 25.5° C and 23° C

respectively. Automatic shade screens operated to cover the roof and windows when light levels reached 800 W/m².

5.3.3.4 Irrigation

Sea water consists of 92 natural elements (Brown et al. 1989). Of these elements, there are six principal anions, and five principal cations, comprising 99.9% of the total dissolved constituents (Brown et al. 1989) (Table 5.2). The highest concentrations of chemicals are that of Na⁺ (sodium) and Cl⁻ (chloride), which together account for approximately 3.5%, or 35,000 ppt of dissolved salts (Brown et al. 1989; Maun 2009) (Table 5.2).

Table 5.2: Principal chemical elements in sea water in parts per thousand by weight (after Brown et al. 1989).

Ion	Concentration ‰ (ppt)
Chloride, Cl ⁻	18.980
Sulphate, SO ₄ ²⁻	2.649
Bicarbonate, HCO ₃ ⁻	0.140
Bromide, Br ⁻	0.065
Borate, H ₂ BO ₃ ⁻	0.026
Fluoride, F ⁻	0.001
Sodium, Na ⁺	10.556
Magnesium, Mg ²⁺	1.272
Calcium, Ca ²⁺	0.400
Potassium, K ⁺	0.380
Strontium, Sr ²⁺	0.013

Salinity, or the concentration of dissolved salts, is reported in various units across studies, such as g/L (grams of sodium chloride per litre of water), ppm (parts per million), ‰ ppt (parts per thousand by weight), or molar concentration (mol). For this study, 35 g/L of pharmacy grade sodium chloride, equal to that which is found dissolved in sea water, and equivalents to half, one and a half, and twice that of the

sodium chloride contained in sea water, were used in the manual irrigation of propagules. Table 5.3 illustrates the range of sodium chloride levels used in this study, and other units to which they convert. The level of salt indicates the step-wise increases across the range of treatments, from 0 sodium chloride (using distilled water only), to half the concentration of sodium chloride found in sea water (0.5), and so forth up to and including 2.0, or twice the concentration of sodium chloride found in sea water (Table 5.3).

Table 5.3: Treatment levels of salinity used in this study from 0 (using distilled water only), to 2.0 (twice the concentration of sodium chloride (NaCl) found in sea water), and corresponding equivalent units used in other studies.

Level of salt	g/L NaCl in H_2O	ppm NaCl	mM NaCl	mol NaCl	EC $\mu S/cm$ (approx)	dS/m (approx)	% salinity
0	0	0	0	0	0	0	0
0.5	14.63	17,500	250	0.25	27,350	27.35	1.70
1.0	29.25	35,000	500	0.50	54,700	54.70	3.50
1.5	43.88	52,500	750	0.75	82,050	82.00	5.20
2.0	58.50	70,000	1000	1.00	109,400	109.40	7.00

Deionized water was used to ensure no ions other than pharmacy grade sodium chloride were present in the dissolved solutions used for irrigation. To test that the highest level of sodium chloride solute (70,000 ppm/1000 mM) did not saturate the distilled water, 58.5 g/L sodium chloride, equivalent to 1000 mM, were dissolved in 1 L distilled water prior to the commencement of the experiment. Pots were manually watered with 60 mL of their treatment on day one, then every four days for five weeks, with salinized water (early timing) or distilled water (late timing). Thereafter, all plants were manually irrigated with 60 mL of saline treatment every four days. Solutions were mixed on the day of use. Pots irrigated with distilled water only were irrigated with 60 mL on day one and every four days thereafter.

5.3.4 Plant root procedures at harvest

Plants were gently removed from their pots to ensure no roots broke off. Across sodium chloride treatment levels and timing (early or late application of sodium chloride irrigation), root masses had formed aggregates. Roots were cut from propagules and washed gently to remove sand particles or aggregates adhering to them. Excess water was removed by placing the roots on absorbent paper, then total root masses were weighed, and subsamples of 0.02 g per propagule were cut into 1 - 2 cm segments. Roots were stained using the same protocol as in Section 4.3.4.1.

5.3.5 Sand procedures at harvest

At each harvest, sand from the top 5 cm of each treatment was homogenized, and a 0.02 g subsample taken to measure changes in enzyme concentrations in the sand surrounding the roots of the plant propagules. The same protocol as that used in Section 5.4.4.2 was used to measure enzyme concentrations.

5.4 Statistical analyses

A three-way analysis of variance (ANOVA) in IBM SPSS Statistics v.24, was used to determine the main effect of sodium chloride treatments, irrigation timing pre- or post-colonization (early and late application of irrigation, respectively), and harvests on percentage colonization (% RLC) of Sea Wheatgrass roots. Where plant root masses were smaller than 0.02 g, they were treated as plant deaths, and not accounted for in the ANOVA analysis. Plant deaths were also calculated.

5.5 Results

5.5.1 Pre-experiment Sea Wheatgrass propagation trial

Sea Wheatgrass propagules did not produce tillers during the experiment, yet prodigious amounts of roots were produced (Figure 5.1).



Figure 5.1: Root mass produced from a 5 cm *Thinopyrum junceiforme* rhizome cutting taken from Thirteenth Beach, November 2014, and grown in a natural beach sand in ambient conditions, in a 3 Lt pot for 12 weeks. Ruler is 30 cm long, arrow and scale show intact rhizome cutting, with arrow pointing to node. No aerial parts were produced.

5.5.2 Formation of aggregates

Across the early and late timing of irrigation (pre-colonization and post-colonization respectively), aggregates had formed around the roots of all plants irrigated with distilled water or sodium chloride levels (e.g. Figures 5.2-5.4). The largest of the aggregates was from harvest one, at week five, and weighed 9.38 g. The plant had been treated with late application of 5.3% sodium chloride, or one and a half times that found in sea water. In some pots, aggregates formed delicate, flat shapes around tangled, fine roots (e.g. Figure 5.2) or larger masses (e.g. Figure 5.3). Other aggregate formations included small clumps of sand grains adhering to the sticky fungal exudates on roots (e.g. Figure 5.4). The aggregates were easily disrupted when rinsed in water, however some remained tightly adhering to the plant roots until the roots were subjected to clearing in potassium hydroxide.



Figure 5.2: *Thinopyrum junceiforme* propagule from harvest two, week seven, with aggregates around roots. The plant had been treated late application of 3.5% (35,000 ppm) sodium chloride irrigation, equivalent to the dissolved sodium chloride found in full-strength sea water.



Figure 5.3: *Thinopyrum junceiforme* propagule from harvest five, week 13. The plant had been treated with early application 5.2% (52,500 ppm) sodium chloride irrigation, equivalent to the dissolved sodium chloride found in one and a half times that of sea water.



Figure 5.4: *Thinopyrum junceiforme* propagule from harvest five, week 13, with small aggregates adhering to roots. The plant had been irrigated with distilled water.

5.5.3 Plant deaths and sodium chloride levels

The number of Sea Wheatgrass propagule deaths related to either pre- or post-colonization (early or late application of sodium chloride irrigation, respectively), and salt levels, did not differ greatly across the sum of harvests. However, plant mortalities increased over time (harvests) in early and late applications of sodium chloride irrigation, but not necessarily over increasing saline levels. Plants from the early application of saline irrigation suffered 32% mortality across salt levels and harvests, whereas those in the late application of saline irrigation suffered 34% mortality overall.

The highest number of plant deaths in the early application of saline irrigation, were in harvest three, week nine, at 35,000 ppm sodium chloride irrigation (H3, W9, green bar, Figure 5.5), equivalent to that of full-strength sea water (3.5% salinity), and harvest five, week 13, at 70,000 ppm sodium chloride (H5, W13, red bar, Figure 5.5), equivalent to twice that of sea water strength (7.0% salinity). Plant deaths in weeks nine and 13 were 80%, in their respective levels of sodium chloride treatments.

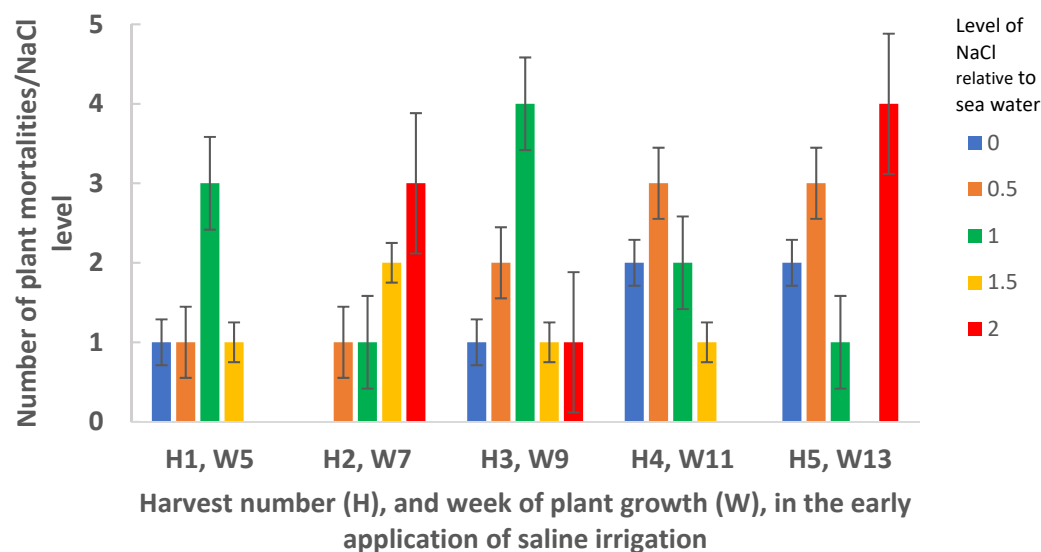


Figure 5.5: Plant mortalities across harvests (H) 1-5, weeks (W) 5-13 respectively, and sodium chloride (NaCl) levels relative to those found in sea water, from 0 to twice the concentration (0 ppm – 70,000 ppm). Saline irrigation was imposed from the first day of planting *Thinopyrum junceiforme* propagules, pre-colonization (early application) in natural incipient dune sand from Thirteenth Beach. Bars show standard error (SE).

Over the five harvests, the greatest cumulative plant losses in the early application of sodium chloride irrigation were at 3.5% sodium chloride (35,000 ppm), equivalent to that of full-strength sea-water (green bars, Figure 5.5), with 50% plant deaths overall. The lowest mortality rate of 25% cumulative plant losses over the five harvests in the early application of saline irrigation, was in plants receiving 5.2% sodium chloride (52,500 ppm), equivalent to one and a half times the salinity of sea water (yellow bars, Figure 5.5).

Late application of sodium chloride irrigation commenced once the presence of mycorrhizas was confirmed in the roots of Sea Wheatgrass propagules, in a destructive harvest at week five. In the late application of sodium chloride irrigation, plant deaths were greatest in harvests four and five, weeks 11 and 13 respectively (Figure 5.6). In harvest four at week 11, Sea Wheatgrass mortalities were highest at 3.5% sodium chloride (H4, W11, green bar), or equivalent to full-strength sea water (35,000 ppm) (Figure 5.6). In harvest five at week 13, the greatest plant losses were at 5.2% sodium chloride (H5, W13, yellow bar), equivalent to one and a half times sea water strength (52,500 ppm) (Figure 5.6). Plant losses at weeks 11 and 13 were 80%, in their respective levels of sodium chloride treatments.

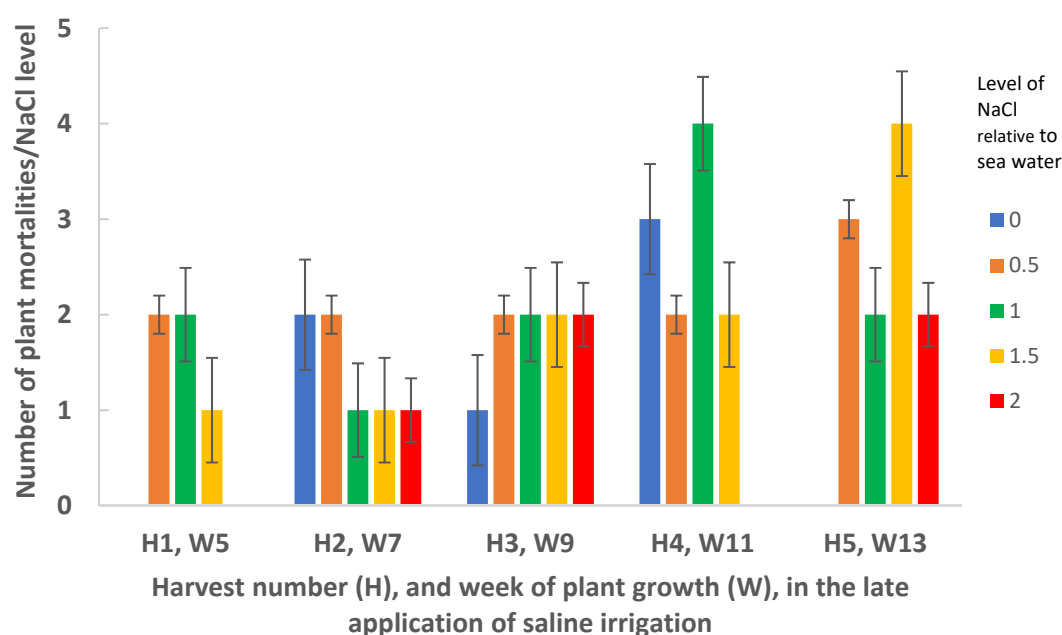


Figure 5.6: Plant mortalities across harvests (H) 1-5, weeks (W) 5-13 respectively, and sodium chloride levels relative to those found in sea water, from 0 to twice the concentration (0 ppm – 70,000 ppm). Saline irrigation commenced in week five (late application), post-colonization as established by a destructive harvest of *Thinopyrum junceiforme* propagules planted in natural incipient dune sand from Thirteenth Beach. Bars show standard error (SE).

Over the five harvests, the greatest cumulative plant losses in the late application of sodium chloride irrigation, were at 1.7% sodium chloride (orange bars, Figure 5.6) and 3.5% sodium chloride (green bars, Figure 5.7), equivalent to half-strength sea water (17,500 ppm) and full-strength sea water (35,000 ppm), with 25% plant deaths each. The lowest cumulative mortality rate in the late application of sodium chloride irrigation was at 7.0% sodium chloride (red bars, Figure 5.6), equivalent to that of twice sea water strength, with 25% plant deaths.

5.5.4 Effects of harvest (weeks post-propagation) on percentage colonization (% RLC) in Sea Wheatgrass roots

In the test of between-subjects effects, there was a significant interaction between harvests, and irrigation timing, sodium chloride levels and percentage colonization. This resulted in a highly significant difference of 0.002 ($P < 0.05$) in percentage colonization (% RLC) of Sea Wheatgrass roots related to harvest, averaged across

sodium chloride levels, and the early or late application (pre- or post-colonization), of sodium chloride irrigation (Table 5.4).

Table 5.4: Results of three-way ANOVA showing significant differences in mycorrhizal percentage colonization (% RLC) in *Thinopyrum junceiforme* roots, related to harvest, irrigation timing (pre- or post-colonization), and levels of sodium chloride (NaCl) irrigation imposed. Significant outcome in bold.

Variable	df	F	Sig.
Harvest	4	4.584	.002
Irrigation timing	1	1.327	.251
NaCl (%) level	4	.810	.520

The highly significant differences ($P < 0.05$) between harvests, averaged across salt treatments and the early or late application (pre- or post-colonization) of sodium chloride treatments, for percentage colonisation (% RLC) of Sea Wheatgrass roots, was at the 0.5 level of sodium chloride irrigation (17,5000 ppm) (Table 5.5). Differences in percentage colonization (% RLC) between harvest one (week five) and all other harvests except harvest three (week nine), show harvest one had 14.6% greater percentage colonization (% RLC) than harvest two (week seven) (Table 5.5, 5.6). There was 12.3% higher percentage colonization (% RLC) in harvest one than harvest four (week 11), and 14.3% greater percentage colonization (% RLC) in harvest one than harvest five (week 13), across salt treatments and application times (Tables 5.5, 5.6).

Table 5.5: Section of Tukey analysis from three-way ANOVA, showing percentage colonization (% RLC) differences between harvest one (week 5) and harvests two, three, four and five (weeks seven, nine, 11 and 13 respectively), in *Thinopyrum junceiforme* irrigated with sodium chloride (NaCl) equivalent to that found in half-strength sea water (0.5, 17,500 ppm). Significant outcomes in bold.

Variable	Harvest (H) and week (W)	Harvest (H) and week (W)	% RLC difference	Sig	95% confidence interval	
					Upper	Lower
% RLC, 0.5% NaCl	H1, W5	H2, W7	14.6	.01	1.98	26.26
		H3, W7	2.8	.97	-9.74	15.31
		H4, W11	12.3	.04	.23	25.50
		H5, W13	14.3	.03	.82	26.57

Table 5.6: Results from three-way ANOVA showing percentage colonization (% RLC) differences between harvests and timing (early or late application) of sodium chloride irrigation at half-strength sea water (0.5, 17,500 ppm), in mycorrhizal colonization of *Thinopyrum junceiforme*.

Harvest (H) and week (W)	% RLC	95% confidence interval	
		Upper	Lower
H1, W5	67	61	73
H2, W7	54	46	59
H3, W9	64	58	71
H4, W11	55	48	62
H5, W13	53	46	60

5.5.5 Assessment of microbial decomposer concentrations in sand

The early application of sodium chloride irrigation treatments commenced on day one, week one of plant propagation in beach sand, whereas the later application commenced at the beginning of week five of plant growth. The commencement timing of these treatments produced differences in the enzyme levels in the sand, as assessed by fluorescein diacetate hydrolysis.

In the early application of sodium chloride treatments, there was a trend towards increasing enzyme activity across all levels of saline irrigation in comparison to harvest one, week five, at harvest two, week seven. The exception was in the pots irrigated with distilled water; in these pots, the enzyme levels decreased at harvest two in comparison to harvest one (Figure 5.7). At harvests three and four, weeks nine and 11 respectively, there was a decrease in enzyme levels across all sodium chloride treatments, with harvest four showing the least enzyme activity during the experiment (Figure 5.7). By harvest five, week 13, enzyme activity as measured by fluorescein diacetate concentrations ($\mu\text{g g}^{-2}$ sand), had increased across all sodium chloride treatments, and the pots irrigated with distilled water, to above those measured in all previous harvests (Figure 5.7).

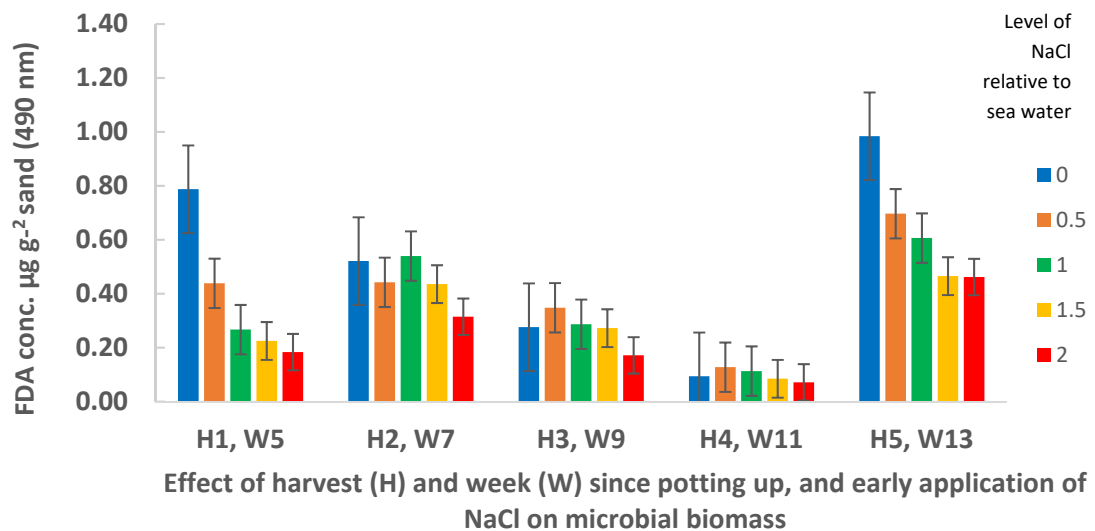


Figure 5.7: Enzyme levels in sand, measured by concentration of fluorescein diacetate released in air-dried sand ($\mu\text{g g}^{-2}$ sand) from Thirteenth Beach, and measured at 490 nanometres across harvests and treatments of sodium chloride irrigation applied from day one (early application) of propagation. Bars show standard error (SE).

In response to the later application of sodium chloride irrigation, which was introduced at week five of plant growth, the sand enzymatic levels were initially similar across sodium chloride treatments at harvest one, week five, including those in the pots which were irrigated with distilled water (Figure 5.8). Thereafter, there was an increase in enzyme concentrations across all sodium chloride treatments, and the distilled water

pots, at harvests two and three, weeks seven and nine respectively. Fluorescein diacetate concentrations ($\mu\text{g g}^{-2}$ sand) decreased in harvests four and five, weeks 11 and 13 respectively, to below those of harvest one, week five, across all sodium chloride treatments and the distilled water treatment (Figure 5.8).

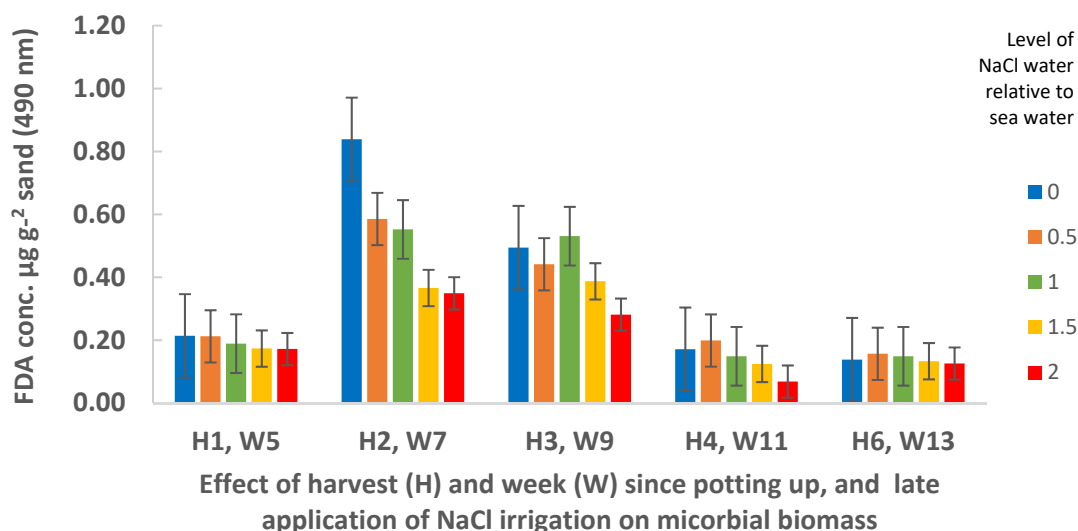


Figure 5.8: Enzyme levels in sand, measured by concentration of fluorescein diacetate released ($\mu\text{g g}^{-2}$ sand) from Thirteenth Beach, and measured at 490 nanometres across harvests and treatments of sodium chloride irrigation applied from week five (late application), of plant growth. Bars show standard error (SE).

5.6 Discussion

This study addressed the effects of increasing levels of sodium chloride irrigation over time upon coastal AM fungi, and their mutualist psammophile host from the incipient dune ecosystem. The levels of sodium chloride irrigation imposed on symbionts were well above those that have been used in other studies (e.g. Asghari et al. 2005; Camprubi et al. 2012). Furthermore, levels of sodium chloride irrigation were imposed over two time regimes, early and late - from the day the propagules were planted, or pre-colonization (early), and from week five when mycorrhizal colonization in plant roots had been established (late). Timing of colonization was established by a bioassay prior to the sodium chloride experiment.

Studies that investigate changes to mycorrhizal colonization over time, in conjunction with increasing sodium chloride levels are rare. One exception is that of Juniper and Abbott (2006), who demonstrated that reduced colonization by AM fungi in the presence of sodium chloride was dependent upon the stage of colonization.

My study did not support the hypothesis that colonization would be delayed by the introduction of sodium chloride irrigation from day one, or pre-colonization (early application). Rather, there was no effect upon mycorrhizal colonization of Sea Wheatgrass assessed as percentage colonization (% RLC) related to early or late application of saline irrigation. Life history strategies of the coastal fungi are likely to be responsible for their tolerance to salinity pre-colonization, as AM fungi occur naturally in saline soils, such as those found around sandy lakes in northern Iran (Barin et al. 2013), where, for example, mycorrhizas colonize *Medicago sativa* (Sand Lucern), and *Allium cepa* (Bulb Onion). In Sand Lucern, it was found that mycorrhizas were positively correlated with sodium chloride concentrations, and the EC of the soil, however for Bulb Onions, where the abundance of mycorrhizas was less than in Sand Lucern, differences were related to soil chemistry, such as phosphorus levels (Barin et al. 2013). It is apparent therefore, that in my study sodium chloride did not inhibit colonization by mycorrhizas as the mutualists are from an environment subject to salinity by marine overwash. This outcome stresses the importance of using realistic levels of sodium chloride when testing resilience to salinity on plants and AM fungus from a naturally saline environment – be that soil or marine overwash.

Harvest, or the length of time over which sodium chloride levels were imposed, did result in a significant difference in percentage colonization (% RLC) between pre- and post-colonized plant roots, although effects of the sodium chloride levels were erratic. Such erratic responses may be attributable to the life history strategy of fungal species, or the ability of the fungi to adjust to (Juniper & Abbott 2006), or adapt to (Lambert, Cole & Baker 1980) changes in their environment over time, rather than any stochastic events (Dickie et al. 2015). For example, the response to which sodium chloride inhibits mycorrhizal colonization by *Scutellospora calospora* and *Gigaspora decipiens* differs between the fungi, and it is argued that this is related to the efficacy of the fungi in adjusting to changes in their environment (Juniper & Abbott 2006). Further, differences in AM fungal colonization under saline conditions may be more limiting at

particular life history stages of the fungi than at other times, for example the size of the propagules when sodium chloride is introduced (Juniper & Abbott 2006). Life history strategies are strongly indicated in the work of this Chapter, in the changing abundance of mycorrhizas in increasing sodium chloride levels over time. These changes demonstrate a range of tolerances to salinity in the dynamic ecosystem of coastal AM fungi, where sea water flooding is not an uncommon event.

Coastal and estuarine host plants are often subjected to sea water flooding, and AM fungi have been shown to mitigate the antagonistic effects of salts in mutualists, by, for example, osmotic adjustment or increasing the synthesis of proline in plants (Evelin, Kapoor & Giri 2009; Talaat & Shawky 2014).

Notable studies on the alleviation of salt stress mediated by AM fungi in mangrove plants (Sengupta & Chaudhuri 2002; Wang et al. 2011), highlighted the salinity of mangrove sediments. Differences between mangrove sediments and sands are the cation exchange capacity and buffering capacities of the two substrates. The cation exchange capacity in mangrove sediments (Ferreira et al. 2007; Liu et al. 2017), allows for a buffering capacity to withstand changes in the ionic composition of the substrate (Ashman & Puri 2002). Conversely, sand has very little cation exchange capacity and buffering capacity (Ashman & Puri 2002; Maun 2009), therefore it lacks electrostatic charges to which cations and anions adhere and exchange (Ashman & Puri 2002). As a consequence, the two substrates offer very different micro-environments and nutrient holding capacities for plants and fungus, particularly around the rhizosphere (Liu et al. 2017). Notwithstanding this, in order to discriminate better the effects of salinity levels on AM fungal abundance in plant roots, greater understanding would come from observing changes over time on the abundance of AM fungi, rather than focussing on colonization at one point in time or one level of salinity (Sengupta & Chaudhuri 2002; Wang et al. 2011).

This study had investigated whether the sustained salinity levels imposed over time would produce photosynthesizing tillers from the nodes on the host plant propagules. That tillers were not produced from the nodes during this study, but roots were, demonstrates the ability of Sea Wheatgrass to survive under sub-optimum growing conditions. This outcome is most likely a survival mechanism, or plasticity, in response to the dynamic and highly variable environment with resource limitations (Qu, Zhao

& Zhou 2014) in which the plant lives. Roots are also the site of mycorrhizal colonization and the co-incidence of plant survival without tillers, but in the presence of mycorrhizal colonization, cannot be ignored.

The phenomenon of roots but no tillers being produced from nodes is supported by other studies of wild coastal plants (Harris & Davy 1988; Maun 2004; Pavlik 1983), albeit the lack of aerial parts on an actively growing plant is unusual in agriculture and horticulture. Nonetheless, this study found that the wild relative of cultivated wheat, Sea Wheatgrass, also forms adventitious roots from nodes, without necessarily producing aerial parts from them, confirming the early findings of Sykes and Wilson (1988).

The production of adventitious roots from nodes is not uncommon in plants exposed to regular sand accretion where burial is a strong selective force, and under which plants have developed morphological, physiological and/or genetic adaptations in order to survive (Maun 1998). Hairy Spinifex, for example, is known to have a greater subterranean biomass than above-ground biomass, and is capable of altering resource allocation as a response to events such as sand burial (Maze & Whalley 1990). Further, much of the below-ground biomass of Hairy Spinifex is composed of buried stolons and culms, which aid in stabilizing the dunes (Maze & Whalley 1990).

The survival and growth of Sea Wheatgrass roots in my study differs to the findings of Harris and Davy (1987), whereby Sea Wheatgrass roots were compromised in favour of photosynthetic organs. In their study of the response to sand burial of Sea Wheatgrass (then referred to under the heterotypic name of *Elymus farctus*), Harris and Davy (1987), found that when buried for one week, the existing leaves of Sea Wheatgrass were preferentially maintained at the expense of developing leaves, roots and stems, albeit all plants buried for two weeks senesced. However, the Harris and Davy study (1987) used seedlings that had already produced tillers, whereas my experiments used node cuttings on rhizomes, to which no tillers were attached, or produced.

It has also been demonstrated that *Elymus mollis*, (American Dune Grass), not a taxonomic synonym for Sea Wheatgrass, but nonetheless a wild member of the tribe Triticeae, allocated more dry matter to the growth of roots from rhizome cuttings than

to live blades, under both high and low nitrogen regimes (Pavlik 1983). Conversely, Marram Grass was found to produce twice as much live blade area than that of American Dune Grass, at the expense of root growth (Pavlik 1983). Thus, not all dune grasses allocate resources in the same manner, with some species such as Sea Wheatgrass and American Dune Grass being more ‘root-oriented’ than others such as Marram Grass, which is more ‘shoot-oriented’.

Discrete plants may emerge eventually from nodes along the rhizomes of Sea Wheatgrass, depending upon the distance from the parent plant and providing there is sufficient space for them to grow. Similar growth patterns have been observed in Hairy Spinifex (Bergin 2011). Moreover, new aerial clumps are likely to form where there is sufficient space not to compete for the limited nutrient resources of the dunes, and when conditions are favourable to expend the energy required for tiller production.

Recent studies have addressed the vital importance of the node as a distribution hub for mineral nutrients, and preferential allocation of such resources to developing plant organs, especially in graminaceous plants such as *Oryza sativa* (Paddy Rice) (Yamaji & Ma 2014). Although the mechanism for preferential distribution of minerals has not yet been elucidated, it is hypothesized that each element requires a different transport mechanism (Yamaji & Ma 2014). Further, several intervascular transporters in the nodes of *Hordeum vulgare* (Barley Grass) and Paddy Rice which have been identified, may act as transporters of minerals (Yamaji & Ma 2014).

In *Poaceae* plants, the nodes are connected to other nodes via highly organized and complex vascular systems (Nelson 2000); it is from nodes that aerial parts are connected, and fibrous roots are generated. (Yamaji & Ma 2014). That the nodes in Sea Wheatgrass are distribution centres for the preferential allocation of mineral nutrients (Yamaji & Ma 2014) has been illustrated by the Sea Wheatgrass propagules in my study, and the root masses they grew at the expense of tiller production.

Of interest also to this study was whether the enzyme levels in the sand as assessed by fluorescein diacetate hydrolysis, would decrease as salinity levels increased, and whether timing would be a factor in any changes. It was demonstrated in the early and late application of sodium chloride irrigation that as salinity levels increased, the enzymatic activity in the sand decreased. However, it did not disappear completely,

and there were residual enzymes in both treatments and all sodium chloride levels at the conclusion of the study. While the species responsible for the enzyme concentration were not identified, it is apparent these communities were not as resilient as were those of AM fungi and Sea Wheatgrass in the incipient dunes. The organisms that may have represented the largest edaphic microbial population could have been bacteria, which are prokaryotes with a differing anaerobic metabolic pathway to eukaryotes, such as that of fungi and microalgae (Bourke et al. 2017). Prokaryotes are unable to sustain the anoxic conditions typical of a flooding event, for example prolonged tidal inundation. Conversely, the highest concentrations of bacteria have been found during low tides (Koop & Griffiths 1982), and the bacterial biomass in beach sand has been positively correlated with tidal salinity (Costódio, Kuroshima & Barreiros 2006), albeit not at levels above 35,000 ppm.

Further studies that would be of benefit could investigate and identify the mechanisms involved in salt tolerance and survival of mutualists from the wild, under natural conditions. There is a prodigious body of work on the beneficial effects to plants from AM fungi in sodium chloride-affected environments, however, studies that deal specifically with the effect of salts on AM fungi, as opposed to the effect of AM fungi on plant growth under saline conditions, are scant (e.g. Estaun 1989; Hirrel 1981; Juniper & Abbott 1993; McMillen, Juniper & Abbott 1998), and very little of the literature is recent (e.g. Juniper & Abbott 2006).

My experiment demonstrates unequivocally the hardy and resilient nature of the AM species found in association with psammophiles at the most stressful part of the beach-dune interface, where the ecologically strong fitness criteria addressed by Maun (2004) clearly extends to include salinity levels not usually tackled in experiments. Nonetheless, visual enumeration of mycorrhizas inside plant roots is painstaking and imperfect, and technical difficulties limit quantification, which may otherwise enhance our knowledge of the ecology and biology of AM fungi (Brundrett & Ashwath 2013).

Data from this study illustrate unambiguously the ability of coastal AM fungi to survive multiple levels of sodium chloride well above those imposed in other studies (e.g. Al-Karaki 2006; Cantrell & Linderman 2001; Daei et al. 2009; Juniper & Abbott 2004), by employing concentrations of 1.7%, 3.5%, 5.2% and 7.0% sodium chloride. This study contributes to the body of work which attests to the enhanced survival of symbiotic

psammophilic plants (e.g. Alarcón & Cuenca 2005; Botnen et al. 2015; Jehne & Thompson 1981; Koske et al. 1996). Further, the present study complements that of

- i. Nicolson (1960), who reported on the distribution of AM colonization across grass species in sand dunes.
- ii. Sylvia and Burks (1988), who illustrated that *Uniola paniculata* (Sea Oats) inoculated with selected species of AM fungi were better adapted to the environmental extremes of the sand dunes than non-inoculated plants, and
- iii. Koske et al. (1996), who tested the viability of AM fungal spores from sand dunes, in full-strength sea water.

The colonization success of the incipient coastal dune fungi reported here, adds to the body of knowledge that demonstrates species of AM fungi differ in their capacities to endure an extensive range of adverse chemical and physical edaphic conditions. Such conditions include, for example, disturbance (Abbott & Robson 1991), soil contamination (Entry et al. 2002), desert salinity (Aliasgharzadeh et al. 2001) and heavy metals (Gohre & Paszowski 2006). It is abundantly clear that there are variations in AM fungal communities between plant species, locations, seasons, and land uses, which indicate strongly the existence of ecological differences between AM fungal species (Abbott & Robson 1982a; Husband et al. 2002).

Finally, it is of paramount importance to enhance our knowledge on the role of AM fungal symbiosis in coastal sand dune plants such as Sea Wheatgrass. This is because there are plant-fungus implications for sand dune stabilization, restoration and conservation (Gemma & Koske 1997), particularly in light of the position of dunes as a buffer between land and sea (Charbonneau et al. 2017; Condon & Barr 1968; Nordstrom & Jackson 2013).

5.7 Conclusions

In this Chapter, effects on AM fungal percentage colonization (% RLC) in a coastal dune grass irrigated with extreme and increasing levels of sodium chloride over time (17,500 ppm - 70,000 ppm), have been identified. The effects on the growth of Sea

Wheatgrass roots and tillers were examined, and discussed in relation to resource allocation from plant nodes to plant roots. Changes in the enzyme concentrations of microbial decomposers as sodium chloride treatments increased over time were also examined. I believe this to be the first work where sodium chloride levels equivalent to, and above those that are dissolved in natural sea water, have been used on AM fungal symbionts and their host plants. These data add to the body of work on coastal mycology, by demonstrating the tolerance to extreme and increasing levels of sodium chloride under which the fungus can survive. What would be of further benefit, would be to run the experiments again but ceasing sodium chloride irrigation at week six for example, and replacing it with distilled water to observe mycorrhizal responses such as changes to percentage colonization (% RLC). Sea Wheatgrass could also be examined to see whether tillers would be produced if sodium chloride irrigation were replaced with distilled water at week six.

Chapter 6

Arbuscular mycorrhizal (AM) fungi across sand dune gradients, and response of an incipient dune grass to AM fungal inoculum from the foredune

6.1 Abstract

Coastal AM fungi on incipient sand dunes survive in extreme environments that are regularly eroded and replaced. Both host plant and fungus need to respond to their environment whereby life history strategies select for the most robust of mutualists. In a three-part experiment a bioassay was used to assess the viability of AM fungi in the stable foredune, before assessing the vigour of the fungal inoculum in colonizing a host from the disturbed incipient dune. The introduced inoculum from the foredune successfully colonized the incipient dune host plant, with the extent of colonization increasing over time in the bioassay. Species richness of AM fungi were then examined across the two gradients to elucidate any differences which may affect functional attributes of the coastal AM fungi between the foredune and incipient dune. Sixteen AM fungal species were identified in the sand and plant roots across the dune gradient, with differences in edaphic conditions between the two ecosystems demonstrating that the fungi exhibit selectivity and are not distributed randomly. Consequently, some AM fungal species found in the foredune sand were not found in the incipient dune sand, however *Glomus* spp. dominated both foredune and incipient dune niches.

6.2 Introduction

The inoculum potential of arbuscular mycorrhizal (AM) fungi relates to the vigour and abundance of the fungal propagules when introduced to a susceptible host (Liu & Luo 1994). Fungal community structures are not static, and can be affected by biotic stochastic events such as disturbance through faunal grazing (Chagnon et al. 2013; Fujiwara & Takada 2017) or abiotic events such as drought (Symanczik et al. 2015). Changes to symbiotic hosts such as defoliation may also influence an AM fungal community, due to fluctuations in plant-supplied carbon (Chagnon et al. 2013; Ijdo et al. 2010). Additionally, production of phytochemicals that stimulate symbiosis may be altered by the prior presence of an AM fungal symbiont (Juniper, Abbott & Jayasundara 1997).

Soil pH can also influence the relative abundance of AM fungi in plant roots (Johnson et al. 2010; Wang et al. 1993). For example, inoculation experiments which examined the response of transferred fungal inoculum to an environment with a different soil pH from where the fungi had been harvested, found less arbuscules were produced by the fungi than when grown in their indigenous soil (Johnson et al. 2010). Furthermore, it has been observed that a change in pH can result in a complete change in AM fungal species (Wang et al. 1993), such as in acidic soils with elevated levels of aluminium, which are found in some 30 - 40% of arable land globally (Kelly, Morton & Cumming 2005).

The nutrient uptake by AM fungal species may change when transferred to a soil with a different pH, such as with *Glomus* isolates from acidic soils which differ in their nutrient acquisition when grown in alkaline soils (Clark & Zeto 1996). Additionally, some AM fungal species are more abundant in acidic soils than are others, such as *Acaulospora* spp. and *Gigaspora* spp. which are more common in acid soils than are *Glomus* spp. (Clark 1997). *Glomus* spp. for example were found in soil pH > 5.5, but not in soil pH 4.5, in long-term experimental plots at Rothamsted (Wang et al. 1993).

Knowledge of the temporal and spatial distribution of AM fungi and their influence on plant communities, is poorly documented (van der Heijden et al. 2006; Vandenkoornhuyse et al. 2003), and the mechanisms responsible for creating differences in AM communities in general, remain elusive (Lekberg et al. 2007). That AM fungi vary widely in their ability to co-colonize the same plant, is also an interaction not well understood (Thonar et al. 2014). This may be complicated by the fact that symbiotic plants can host many fungal species simultaneously, which may or may not be apparent in the subsampled segment (Davison et al. 2015a).

AM fungi also differ in the degree in which they can colonize host roots (Schwartz et al. 2006). For example, *Glomaceae* can colonize a host within three weeks, compared to *Gigasporaceae* or *Acaulosporaceae* which can take up to eight weeks (Hart & Reader 2002), suggesting differences in life history strategies or between *r*-strategists and *K*-strategists. A life history strategy describes how an organism acquires and invests resources into growth and reproduction (Chagnon et al. 2013), or how it accomplishes the requirements of its life-cycle (Hart & Reader 2002). Further, in ecology the *r*-*K* selection theory relates to an organism's combination of functional traits which

influence its growth, survival and reproduction (Chagnon et al. 2013). For example, an organism cannot be simultaneously quick at reproducing and fast at colonizing a niche (Grime 1977; Pianka 1970); it is either one or the other, and is a product of the organism's evolutionary past (de Souza et al. 2005). However differences in life history strategies of AM fungal populations are not well understood (Ijdo et al. 2010), partly because the fungus are obligate biotrophs, which cannot be cultured axenically (Field et al. 2014; Pressel et al. 2016). Nonetheless, it is presumed that the life history strategies of AM fungal populations in disturbed soils would select for *r*-strategists (Ijdo et al. 2010). On the incipient dune, this is likely too, with AM fungal species such as *Glomaceae* capable of colonizing hosts quickly (Hart & Reader 2002), via propagules in plant fragments scaped off the dunes during storms or through tidal incursions. Conversely, it is proposed that the stable conditions of the foredune, which are above the incursions of overwash, would select for *K*-strategists.

Despite AM fungi being ubiquitous in soils, their distribution and diversity at regional scales have received far less attention, and are less understood, than are plants and animals (Martiny et al. 2006). Yet like these communities, the significance of microbial communities across landscapes is becoming increasingly important (Barberán et al. 2014), however there remains a significant gap in our knowledge of fungal biogeography (Chen et al. 2017). Furthermore, there are very few studies of coastal mutualists over environmental gradients (Yamato et al. 2012). It has been shown however, that through sand dune successional stages as firstly phosphorus then nitrogen become less limiting, the nutritional pressures across dune gradients select for different types of AM fungi (Read 1989), with some species better adapted to coastal conditions than are others (Yamato et al. 2012). For example, in vegetation on sand dunes in Tottori Prefecture Japan, *Glomus* spp. and *Diversispora* spp. were found to be the dominant AM fungal species 30 m from the shoreline (Yamato et al. 2012).

Identification of AM fungal species through microscopy is complicated by the relative abundance of structures such as hyphae, spores, and colonized sections of roots that change over field sampling times (Abbott 1982a). Further, the fungi exhibit great phenotypic plasticity (Hart, Reader & Klironomos 2001), such as width of hyphae, hyphal entry points, and size of internal structures (Abbott 1982b). Thus,

morphological identification can be problematic and may not give accurate identification at the species level (Merryweather & Fitter 1998).

6.2.1 Identification of AM fungal species

Visual identification through microscopy of AM fungi inside the roots of host plants, is imperfect and arduous (Brundrett & Ashwath 2013) with the dominance and relative diversity of AM fungal species being affected strongly by sample sizes and detection procedures (Shi et al. 2012). For example, colonization of mycorrhizas in plant roots is generally recorded as percentage colonization (% RLC) (Smith & Read 2008), but estimates of the colonized root length (cm) are also used (Tawaraya, Imai & Wagatsuma 1999). Furthermore, morphological traits can be easily misidentified if not subjected to comparative analyses (Redecker et al. 2013), and identification can be complicated further by the presence of darkly pigmented roots whose phenolic compounds are difficult to remove without the samples disintegrating (Brundrett et al. 1996). Moreover some lineages stain weakly or not at all, such as *Glomus occultum* (syn. *Paraglomus occultum*) and *Acaulospora trappei* (now known as *Archaeospora trappei*) (Morton & Redecker 2001).

The isolation and extraction of DNA and RNA nucleic acids coupled with polymerase chain reaction (PCR) amplification of target genes, enables identification of organisms such as AM fungi that cannot be cultured axenically, and has revolutionized microbial ecology (Smith & Osborn 2009). PCR of target genes is now used widely for studying and identifying AM fungal communities (Gorzelak et al. 2012) and potentially any AM fungus in any root sample can be identified, without the need for clearing, staining or microscopy (Redecker, Hijri & Wiemken 2003). However, molecular methods also have their limitations, for example, the difficulty in sequencing when only a small amount of DNA has amplified. Furthermore, the choice of the target region for amplification is critical for community analysis, as the ability to differentiate between closely related AM fungal species is determined by the target region (Kohout et al. 2014) and there is extensive genetic variability in the fungus (Gorzelak et al. 2012). Nevertheless, recent molecular studies have shown taxonomically diverse fungal communities across successional and edaphic stages of sand dunes, with mutualists

following a gradient from pioneer species through to stable vegetation (Roy-Bolduc et al. 2015; Roy-Bolduc et al. 2016).

The studies in this Chapter sought to investigate the efficacy of transferred AM fungal inoculum in sand from the stable foredune, in colonizing an exotic grass from the incipient dune where disturbance is commonplace. In addition, the studies in this Chapter also sought to elucidate factors that select for different AM fungal species and functional traits across the dune gradients and ecosystems of incipient dune and foredune at the research site of Thirteenth Beach. This will help to elucidate where the fungi are distributed. Identifying the microbial biogeography of coastal sites is of paramount importance in allowing more informed decisions to be made, for example, on the restoration and conservation of coastal sand dunes, or ecosystem remediation (Feagin et al. 2015). However, the diversity of AM species in sand dunes is little known (Johansen et al. 2015), particularly in the Southern Hemisphere where no molecular work on AM fungi in Australian sand dunes is represented in the literature. Thus, the studies in this Chapter also sought to identify coastal AM fungal species at Thirteenth Beach, on the southern coast of Victoria.

The hypotheses were that,

- Infective AM fungal propagules from the foredune would colonize a bait crop of *Allium porrum* cv. ‘Musselburgh’ (Garden Leek) seedlings, due to their common use as a bait crop.
- Infective AM fungal propagules from the foredune would colonize host plant roots from the incipient dune, but colonization may be delayed due to differences in soil chemical and edaphic conditions across the environmental gradients and ecosystems of the foredune and the incipient dune.
- AM fungal species and richness in the stable foredune would be greater than and different to AM fungal species and richness in the disturbed incipient dune, due to differences in soil chemistry and life history strategies of incipient dune AM fungi, selecting for robustness at the expense of abundance.

6.3 Materials and methods

6.3.1 Experiment 1. Bioassay of foredune inoculum potential, and experiment design

A preliminary bioassay was conducted to ascertain the AM fungal inoculum potential of foredune sand collected from 0 - 10 cm depth immediately beside mixed foredune dune vegetation at Thirteenth Beach, Barwon Heads. The foredune vegetation is classified under the Ecological Vegetation Class (EVC) 1 (Bellarine Catchment Network 2010). *Allium porrum* cv. 'Musselburgh' (Garden Leek) were used as the bait crop in the bioassay as they grow rapidly and their root cells are cleared easily of cytoplasm (Brundrett et al. 1996).

Sand was collected from three approximately equally-spaced transects 60 m long, with 60 m in between each transect, running horizontally east-west along the top of the foredune. A light-weight aluminium with an internal diameter of 7.5 cm and fitted with removable plastic inserts was used to extract the sand. A modified trowel was slipped under the base of the auger when samples were collected to prevent loss of substrate. Liners were then removed and tied at each end, labelled and stored in ziplock bags in a chilled Esky for transport to a cool room, where they were stored at 4°C for no more than 48 hours. The sand was homogenized then passed through a 1 mm mesh to remove larger particulate matter such as leaves and small stones.

The volumetric soil water (%) of the foredune sand was tested in 950 mL pots, to which two layers of shade cloth were hot-glued to prevent the sand from migrating through the drainage holes. Moisture content (%) of the sand was double-checked with a Theta Probe with Moisture Meter (Delta-T Devices, Cambridge, England) prior to seeds being planted, and throughout the bioassay. This ensured overhead irrigation would not exceed field capacity. Irrigation was calculated at 95 mL per one-minute session, twice per day.

Twelve replicate pots of sand were potted up and planted with three Garden Leek seeds per pot. Pots were laid out in a random block design in a shade house at ambient temperatures of 20°C to 25°C. Three destructive harvests of four pots each took place

over time post-germination, when plant roots were assessed for the presence of mycorrhizas (Juniper & Abbott 2006).

6.3.1.1 Preparation and planting of bait crop

Garden Leek seeds were imbibed in hot water and left overnight, then dibbled into 950 mL pots of sand, no more than 1 cm below the surface, at the rate of three seeds per pot, with 12 replicates. Immediately after planting, each pot was watered with Charlie Carp (N:P:K 9:2:6).

6.3.1.2 Assessment of inoculum

Seedlings emerged 10 days after planting. Destructive harvests took place at weeks 2, 4.5 and 6.5 post-germination (Juniper & Abbott 2006). Mycorrhizas were not observed in the plant roots at the first harvest. At week 4.5 post-germination mycorrhizas were observed in the plant roots, with the presence of mycorrhizas increasing by week 6.5 post-germination.

6.3.1.3 Harvest procedures of bioassay plant roots

Plants were removed gently from the sand and washed in tap water before roots were removed. A modified protocol of Phillips and Hayman (1970) was followed, whereby roots were placed in 10% potassium hydroxide until cleared of host cytoplasm, rinsed gently in distilled water, then stained in 0.05% trypan blue for four hours. Excess stain was removed in 1:1:1 lactic acid, glycerol and distilled water (lactoglycerol), and roots stored in this solution until enumerated under a dissecting microscope.

6.3.2 Experiment 2. Efficacy of foredune AM fungal inoculum in colonizing an incipient dune grass

Edaphic and chemical analyses of the foredune sand were undertaken to compare conditions to that of the incipient dune sand, from where plant propagules were harvested. Field capacity was assessed using the protocol in Chapter 5, and production horticulture techniques were used to inform irrigation requirements, using water-retention efficiency (WRE) (Creswell 2002), and air-filled porosity (AFP) (Handreck &

Black 2002) protocols. These were calculated at 52% and 0.28% respectively. To my knowledge, this technique has not been used before in the propagation of wild beach plants.

Rhizome cuttings of *Thinopyrum junceiforme* (Sea Wheatgrass) from the incipient dune were used as host plants for AM fungal inoculum from the foredune sand. The grain size of the sand from these two gradients did not differ greatly (Chapter 2). Cuttings were taken from the actively growing ends of plant rhizomes growing on the incipient dune. The plant propagules selected did not have roots or tillers in which indigenous AM fungal propagules may already be lodged, but they did have healthy nodes from which tillers and roots are produced.

6.3.2.1 Experiment design

The experiment was a two-factor block design. There were five destructive harvests over time, with plants being irrigated with distilled water. There were five replicate blocks each containing 11 plants. An initial destructive harvest of plants was undertaken across replicate blocks (e.g. Tsang & Maun 1999), between weeks four and five, to confirm the presence of mycorrhizas. Thereafter subsequent harvests were undertaken at fortnightly intervals. At harvest roots were treated with the modified protocol of Phillips and Hayman (1970) and mycorrhizas enumerated under a dissecting microscope.

6.3.2.2 Sand collection and preparation

The same protocol as that used for sand collection for the bioassay of the foredune inoculum potential (Experiment 1) was used for Experiment 2. Sand was placed into 500 mL biodegradable jiffy pots, weighed to a standard weight across replicates. There were no drainage holes in the jiffy pots to avoid outward migration of sand and AM fungal propagules.

6.3.2.3 Collection and preparation of plant propagules

Rhizome cuttings of Sea Wheatgrass were harvested from the same transects used in Experiment 1. Cuttings no further than 30 cm from the apical meristem were used, as rhizome buds closest to the apical meristem are more likely to produce adventitious

roots than are those at the distal end (Harris & Davy 1986a; Ranwell 1972). Propagules were stored and transported in the same manner as the sand in Experiment 1.

A five-centimetre template was used to cut the propagules to uniform lengths, each of which had a central plump node. Each rhizome cutting was planted at a 45° angle in the sand to just above the node. The experiment was conducted under the same glasshouse conditions as those reported in Chapter 5.

6.3.2.4 Irrigation

Using the results from point 6.2.1.3, irrigation was calculated at 60 mL every four days, to avoid over-watering. Pots were manually watered with distilled water.

6.3.2.5 Plant root procedures at harvest

The same protocol as that used in the study on increasing levels of sodium chloride on AM fungal symbiosis in an incipient dune grass (Chapter 5), was used to process the roots of Sea Wheatgrass harvested from the incipient dune which were grown in sand from the foredune.

6.3.3 Experiment 3. AM fungal species and richness on the foredune and the incipient dune

Field samples of the roots of wild plants and beach sand were collected from transects along the length of the foredune, and the incipient dune over two days in early spring, 2016. It was hypothesized that AM fungal richness in plants and sand from the stable foredune would be greater than that of plants and sand from the disturbed incipient dune. Such differences were hypothesized to be due to differences in the edaphic chemistry of the two ecosystems, and life history strategies of incipient dune AM fungi selecting for robustness at the expense of abundance.

6.3.3.1 Experiment design

Eight 20 m transects, with 20 m in between each transect, were laid east-west horizontally to Bass Strait, on the incipient dune and the foredune. This number of

transects allowed for a thorough investigation into spatial heterogeneity of AM fungal species across the two ecosystem gradients of foredune and incipient dune. The average difference in elevation from the top of the foredune to the break in slope of the incipient dune is 6.89 AMSL (Figure 6.1). A global positioning system (GPS) was employed to ensure both sets of transects were in the same position relative to each other, so that comparisons of fungal species in the pairs of transects of foredune and incipient dune could be investigated. Magnetic north was used to align the transect sets rather than grid north, which would have set the foredune transects approximately 11° more westerly, relative to the incipient dune transects. Within each transect, a 3 m long sub-section was used to harvest five representative samples each of plant roots and sand. A total of 24 subsamples of plant roots, and 16 subsamples of sand were collected. The plant roots consisted of eight from the foredune mixed vegetation, eight from Hairy Spinifex roots from the incipient dune, and eight from Sea Wheatgrass roots from the incipient dune. There were 16 subsamples of sand, with eight each from the incipient dune and the foredune.



Figure 6.1: Collection areas for inoculum and propagules at Thirteenth Beach, showing differences in elevations. Orange line represents the top of the foredune from where sand was collected, yellow line shows the incipient dune from where plant propagules were collected. The average difference in elevation between the two collection sites is 6.89 m AMSL.

6.3.3.2 Sand and plant roots collection and preparation

Sand was collected under the same protocol as that used in Experiment 1 (bioassay) and Experiment 2 (efficacy of foredune inoculum in colonizing an incipient dune

grass). Samples were bulked for each 3 m sub-section, and bags labelled to indicate which transect the sand samples had been collected from.

Samples of plant roots were similarly treated, with the foredune mixed vegetation being bulked, but Hairy Spinifex and Sea Wheatgrass roots from the incipient dune being kept separately from each other. Only the finer plant roots were collected. On the incipient dune, roots were traced back to the parent plant to ensure the correct species were harvested.

Sand and plant root matter were transported in an insulated industrial Esky with dry ice to ensure the molecular integrity of the samples was maintained for transport to the laboratory, where they were stored in a freezer until ready to process.

6.3.3.3 Procedure for assessment of AM fungal species and richness

Sub-samples weighing 0.5 g were taken from the bulked, homogenized samples of sand and plant root matter collected from the incipient dune and foredune at Thirteenth Beach for assessment of fungal diversity. The biodiversity of the AM fungal community within plant roots and substrate from the incipient dune and foredune were surveyed using Next-Generation sequencing on the Illumina mi-seq platform, at the Australian Genome Research Facility (AGRF).

Total DNA was extracted with in-extraction bead beating, with a PowerSoil® DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. The sand was not pre-crushed. Root samples were freeze-dried and crushed. 0.5 g from each plant and sand sample were used for PCR barcoding and PicoGreen® fluorometry. Samples were repeated with a dilution to ensure they were not affected by PCR inhibition.

Genomic DNA from plant roots and dune sand, and polymerase chain reaction (PCR) amplifications with the custom primer pair AMV4.5NF - AMDGR were conducted, to amplify 18S ribosomal RNA gene fragments (rRNA) (Zhu et al. 2016). This primer pair has been used to describe accurately AMF community richness (Lumini et al. 2010). Amplicon read length was 250 bp paired ends.

The PCR cycling procedures of Lumini et al. (2010) were used, whereby there was initial denaturing at 94°C (three minutes), followed by 35 cycles of denaturing at 94°C (45 seconds), annealing at 60°C (45 seconds) extension at 72°C (60 seconds), and a final extension at 72°C (seven minutes), then a ramp of 4°C (one second). Equi-molar pooling of all samples, including those that did not produce a visible band on the agarose gel (23/40), but which had trace amounts of DNA as indicated by fluorometry, were then undertaken. The decision to equi-molar pool all samples was based on the sensitivity of the Illumina MiSeq® sequencing platform being able to detect low and trace amounts of DNA amplicons that Sanger sequencing cannot (Shokralla et al. 2015).

Paired-ends reads using PEAR, version 0.9.5 (Zhang et al. 2014) were assembled by aligning the forward and reverse reads. The Primers were identified and trimmed, with trimmed sequences processed using UPARSE software and Quantitative Insights into Microbial Ecology (QIIME) version 1.8 (Caporaso et al. 2010) and USEARCH, version 8.0.1623 (Edgar 2010; Edgar et al. 2011). Using USEARCH tools sequences were quality filtered and sorted by abundance, and any full-length duplicate sequences, singletons or unique reads were discarded. The remaining sequences were clustered into Operational Taxonomic Units (OTUs) based on a minimum identity of 97%. Using QIIME, fungal taxonomy was assigned with the UNITE database, version 7 (Kõljalg et al. 2005). Additionally, OTU sequences were mega-blasted with SILVA (SILVA database 2017), however this data bank did not adequately identify AM fungal species. MaarjAM (MaarjAM database 2017) which is specific to Glomeromycota, was subsequently used to re-run all sequences.

6.4 Statistical analyses

A one-way analysis of variance (ANOVA) in IBM SPSS Statistics v.24, was used to assess the differences over time in AM fungal richness in colonized root lengths (cm) and percentage colonization (% RLC) in Sea Wheatgrass propagules from the incipient dune, grown in sand from the top of the foredune. To distinguish between the relative abundance of OTUs, location and plant species, of the AM fungal community similarity at the OTU level, a non-metric multi-dimensional scaling (NMDS) based on

Bray-Curtis dissimilarity, and a permutational analysis of variance (Permanova), were run in the *vegan* package 2.0-10 of R v.1.0.

6.5 Results

6.5.1 Experiment 1 results. Bioassay

The colonized root length (cm) and the percentage colonization (% RLC) of AM fungi, increased over time from harvest one to harvest three in the bait crops of Garden Leeks grown in sand from immediately beside Sea Wheatgrass and Hairy Spinifex from the incipient dune, and in sand from immediately beside the mixed vegetation of the foredune sand (Table 6.1).

Table 6.1: Colonized root lengths (cm) (CRL cm) and percentage colonization (% RLC) of mycorrhizas in *Allium porrum* cv. ‘Musselburgh’ (Garden Leek) grown in natural beach sand collected from immediately beside three vegetation types on sand dunes at Thirteenth Beach.

Harvest	Week	Foredune sand		Incipient dune sand			
		Mixed vegetation		<i>Thinopyrum junceiforme</i>		<i>Spinifex sericeus</i>	
		CRL cm	% RLC	CRL cm	% RLC	CRL cm	% RLC
1	2	0.02	10	0.01	8	0.01	8
2	4.5	0.04	15	0.03	13	0.03	13
3	6.5	0.19	26	0.06	13	0.13	15

The percentage colonization (% RLC) of AM fungi increased over time (harvests) in all bait plants, with the inoculum potential being greater in sand collected from immediately beside mixed vegetation on the foredune, than from sand that had been collected from immediately beside plants on the incipient dune (Figure 6.2)

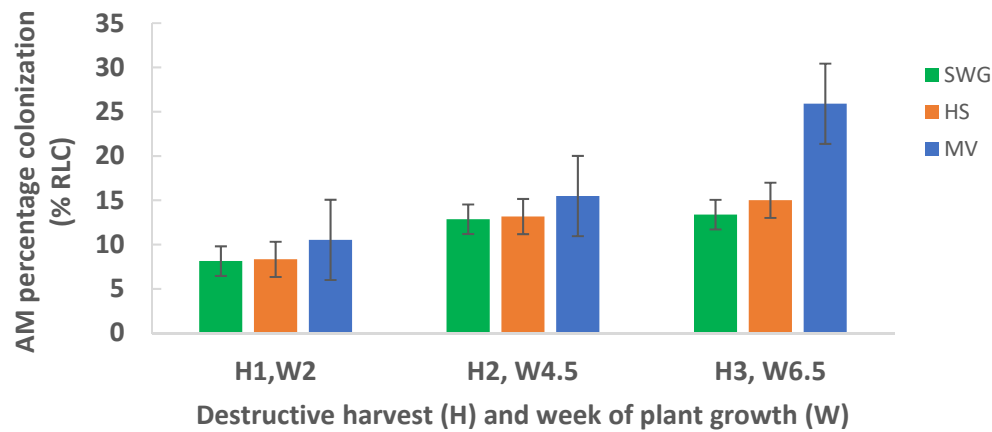


Figure 6.2: AM fungal percentage colonization (%RLC) in *Allium porrum* bioassay bait crops, over three destructive harvests, using sand from immediately beside *Thinopyrum junceiforme* (Sea Wheatgrass SWG) and *Spinifex sericeus* (Hairy Spinifex HS) from the incipient dune, and from immediately beside mixed vegetation (MV) on the foredune at Thirteenth Beach. H represents the destructive harvest and number; W and number represents the week of plant growth. Bars show standard error (SE).

6.5.2 Experiment 2 Results. AM fungal inoculum from the foredune at Thirteenth Beach

6.5.2.1 Analyses of differences between sand edaphic conditions in foredune and incipient dune

The foredune is composed of medium, moderately well-sorted sand, as described by the Udden-Wenworth size classification for sediment grains (Leeder 1982), with a mean grain size of 1.63 Ø (Table 6.2). The average calcium carbonate (CaCO_3) content is 2.12% (Table 6.2). At pH 6.6, the sand is slightly acidic but not saline with an EC_e (*13) of 0.04 (Table 6.2). The volumetric soil water content of 1.45%, agrees with that of global ranges for sand dunes (Bar (Kutiel) et al. 2016; van der Valk 1974).

The incipient dune is composed of medium, moderately well-sorted, fine grade sand, with a mean grain size of 1.45 Ø, with an average calcium carbonate content of 15% (Table 6.2). The pH is acidic at pH 6.07; the sand is not saline with an EC_e (*13) of 0.04 (Table 6.2). The volumetric soil water content (%) of the incipient dune is 4.09

(Table 6.2), agreeing with global estimates for soil ranges in dune soils of 1.5 - 6% (van der Valk 1974).

Table 6.2: Edaphic conditions (0-10cm), Thirteenth Beach foredune, compared to the incipient dune. Foredune sampled November 2016, incipient dune sampled in March 2015 (prior to storm events).

Procedure	(n)	Foredune	Incipient dune
pH _{1:5Ca}	6	6.6	6.07
EC _{1:5Ca} (dS/m)	6	.003	.003
EC _e (*13)	6	.040	.040
Volumetric soil water (%)	26	1.45	4.09
Oven dried moisture (%)	10	.23	.04
Mean grain size (Ø)	6	1.63	1.45
CaCO ₃ content (%)	5	2.12	15.00

All elements measured in the incipient dune and foredune ecosystems at Thirteenth Beach are low, which is typical of dune sands (Kachi & Hirose 1983; Maun 2009) (Table 6.3). Measured nutrients in the foredune sand show acid extractable phosphorus at 335 ppm, total organic carbon (TOC) of 1.1%, labile carbon 0.14%, and total nitrogen 0.03% (Table 6.3). In the incipient dune sand, measured nutrients are acid extractable phosphorus at 300 ppm, total organic carbon at 2.15%, labile carbon measured < 0.02% and total nitrogen measured equalled 0.02% (Table 6.3).

Table 6.3: Chemical analysis (0-10 cm), Thirteenth Beach foredune, compared to the incipient dune.

Procedure	(n)	Foredune	Incipient dune
Acid extractable P (ppm)	3	335	300
TOC (%)	3	1.10	2.15
Labile C (%)	3	0.14	< 0.02
Total N (%)	3	0.03	0.02

6.5.2.2 Colonized root length (cm)

Colonized root length (cm) increased rapidly from harvest one to harvest three (weeks five, seven, and nine respectively), when an asymptote was reached before there was a substantial decline in colonized root length (cm) at harvests four and five (weeks 11 and 13, respectively) (Figure 6.3). There was a large outlier in the means of colonized root length (cm) in week nine (Figure 6.4, Table 6.4), which when removed (Table 6.5), decreasing plants to $n=9$ from $n=10$, reduced by half the mean for all harvests, giving a significant outcome of 0.43 ($P < 0.05$). The reason for this outlier cannot be substantiated and may be the result of environmental stochasticity.

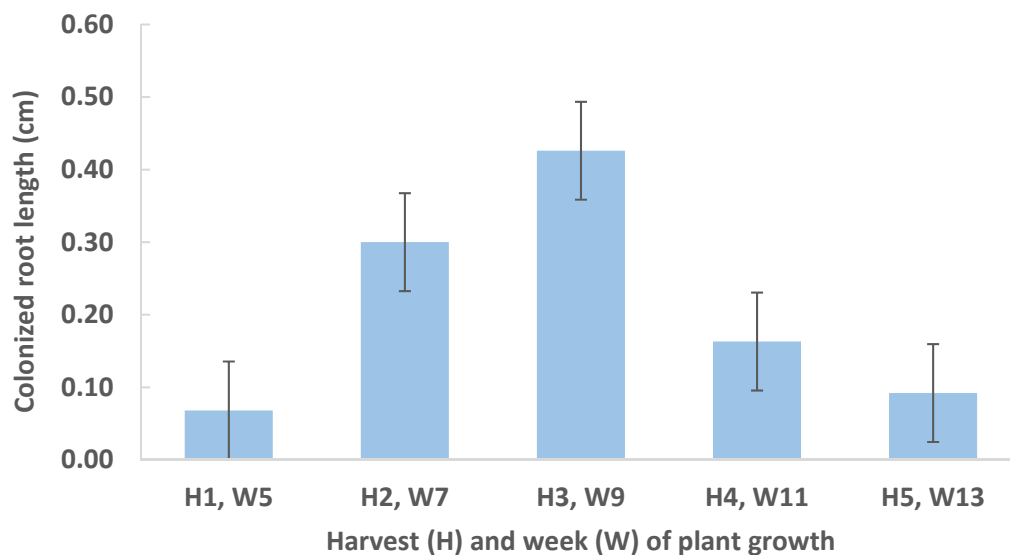


Figure 6.3: Colonized root length (cm) over five harvests (weeks five, seven, nine, 11 and 13 respectively), in *Thinopyrum junceiforme* grown with inoculum from the foredune at Thirteenth Beach, with outlier removed from harvest three, week nine (H3, W9). Bars show standard error (SE).

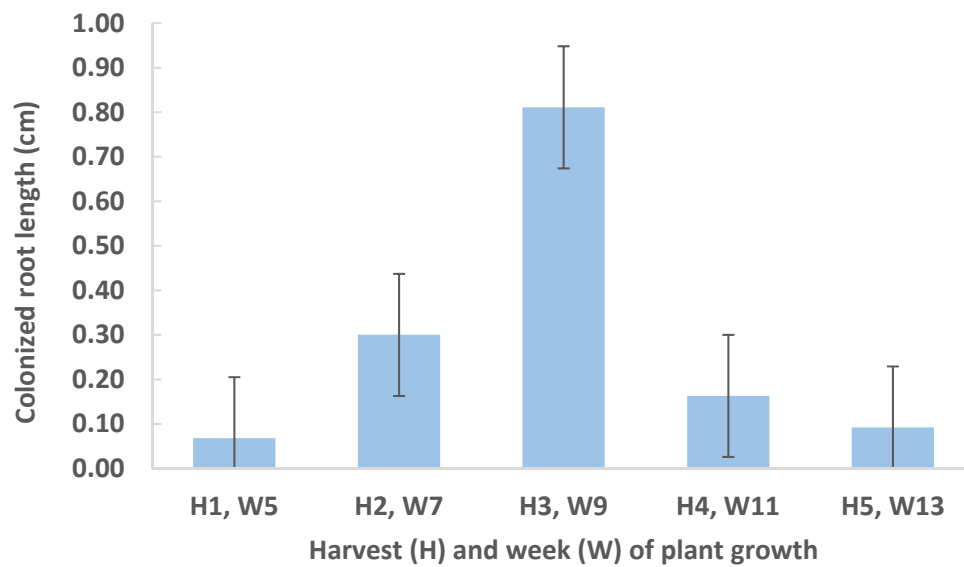


Figure 6.4: Colonized root length (cm) over five harvests (weeks five, seven, nine, 11 and 13 respectively), in *Thinopyrum junceiforme* grown with inoculum from the foredune at Thirteenth Beach, showing outlier in harvest three, week 9 (H3, W9). Bars show standard error (SE).

Table 6.4: Section of one-way ANOVA showing effect at harvest three, week nine, with outlier shown in bold, in colonized root length (cm) (CRL cm) of *Thinopyrum junceiforme* from the incipient dune grown in transferred inoculum from the foredune at Thirteenth Beach.

Week	CRL (cm)	95% confidence interval	
		Upper	Lower
5	.068	-.322	.458
7	.300	-.090	.690
9	.811	.421	1.201
11	.163	-.227	.553
13	.092	-.298	.482

Table 6.5: Section of one-way ANOVA showing effect at harvest three, week nine (in bold) with outlier removed in colonized root length (cm) (CRL cm) of *Thinopyrum junceiforme* from the incipient dune grown in transferred inoculum from the foredune at Thirteenth Beach.

Week	CRL (cm)	95% confidence interval	
		Upper	Lower
5	.068	-.112	.248
7	.300	.120	.480
9	.426	.236	.617
11	.163	-.017	.343
13	.092	-.088	.272

6.5.2.3 AM fungal percentage colonization (% RLC)

Percentage colonization (% RLC) increased rapidly from harvest one to harvest three (weeks five, seven and nine respectively), when an asymptote was reached before a substantial decline in percentage colonization (% RLC) at harvests four and five (weeks 11 and 13 respectively) (Figure 6.5). There were no significant differences at the $P < 0.05$ level for percentage colonization (% RLC). Despite these results, roots had begun to grow by harvest one, week five, with sand aggregates forming around sticky fungal exudates (Figure 6.6).

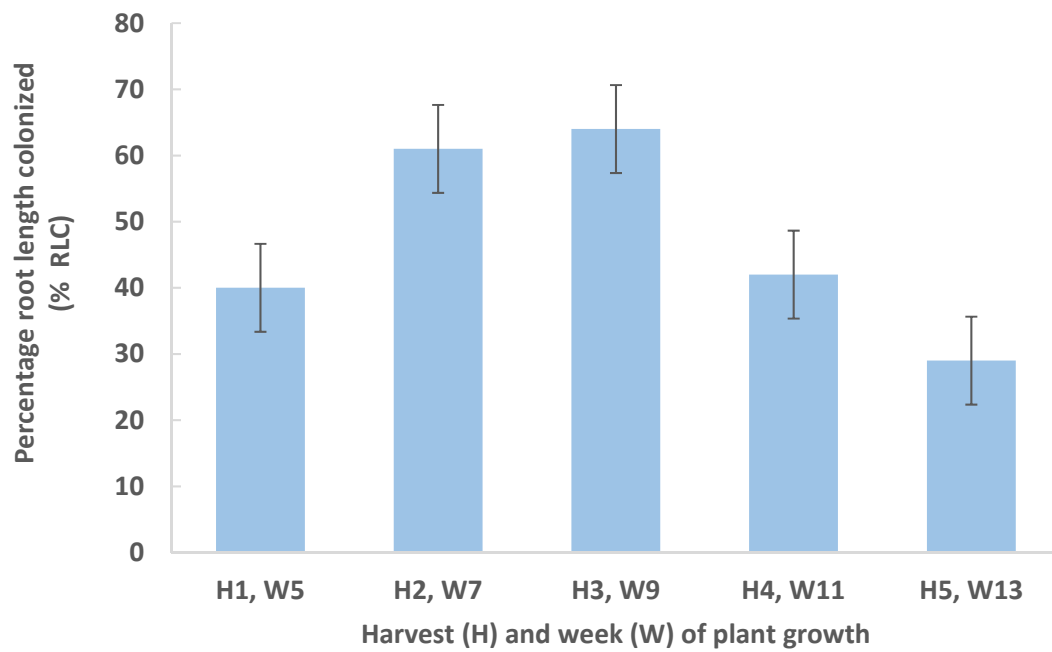


Figure 6.5: Percentage colonization (% RLC) over five harvests, in *Thinopyrum junceiforme* grown with inoculum from the foredune at Thirteenth Beach. Bars show standard error (SE).



Figure 6.6: *Thinopyrum junceiforme* propagules from harvest one, week five (H1, W5), showing root mass at five weeks, and aggregates that had formed around fungal exudates. Note the lack of tillers.

6.5.3 Experiment 3 results. AM fungal species richness

When the DNA results from the samples were blasted through the MaarjAM data base, a total of 451 OTUs (operational taxonomic units) were assigned, which represented the absolute abundance of all Eukaryote species amplified by AMV4.5NF - AMDGR. This number included singletons and multiple occurrences of species, which had been identified in incipient dune sand, foredune sand, and the roots of Sea Wheatgrass, Hairy Spinifex and mixed vegetation.

Ninety-one AM fungal OTUs were identified by MaarjAM blast, occurring 693 times in the dune sand and plant roots. The OTUs were then identified to each sample and location on the incipient dune and foredune from which they had been taken (Figure 6.7). Data from the eight transects were bulked, so that all eight samples of plant roots, or sand, were combined into one series of OTUs for that sample (Figure 6.7). This

gave an indication of overall species richness in relation to location on the dune, and host species, before duplicate OTUs and singletons were removed.

The greatest OTU richness was found in the roots of Hairy Spinifex and the mixed foredune vegetation, with each having a total of 57 OTUs, followed by Sea Wheatgrass with 56 OTUs (Figure 6.7). The foredune sand had 53 OTUs, and the lowest abundance was in the incipient sand with 30 OTUs (Figure 6.7).

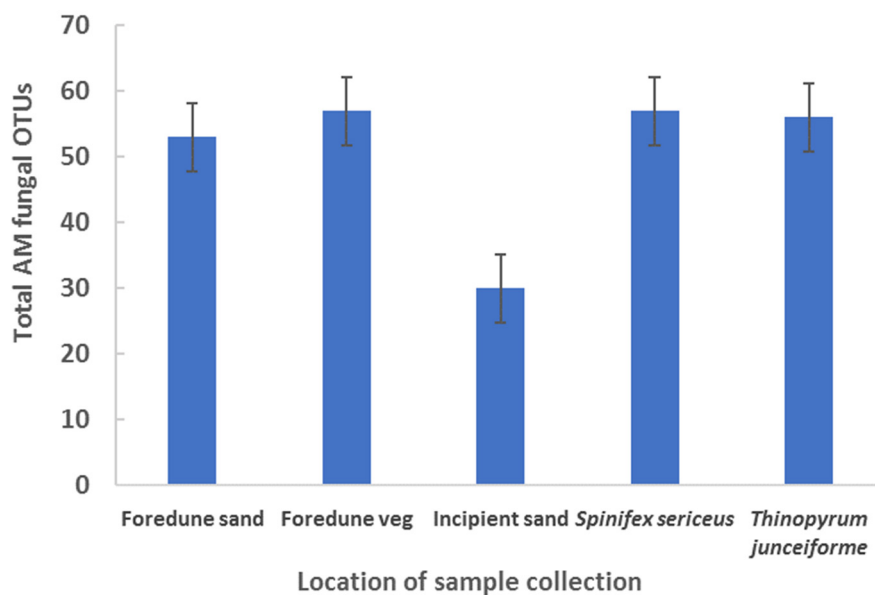


Figure 6.7: Total AM fungal OTUs from homogenized samples in each transect at Thirteenth Beach, September 2016. *Spinifex sericeus* and *Thinopyrum junceiforme* roots were sampled from the eight transects on the incipient dune, and foredune vegetation was sampled from the eight transects on the foredune. Sands were sampled from the homogenized transects across the foredune, and the incipient dune. Error bars show standard error (SE) where n=8.

Glomus spp. had the greatest abundance, with 42 OTUs identified. These were combined into one group. That some species are apportioned multiple OTUs is because there is not an overall cut-off level for identification that suits all taxa (Botnen et al. 2015).

A representative stacked figure was compiled, including singletons, to show the relative abundance of AM fungal OTUs, which are indicated by bands of different colours (Figure 6.8). The largest band shows the bulked *Glomus* spp. OTUs (Figure 6.8). The greatest AM fungal species richness was in the foredune sand with 53 OTUs represented, including 28 *Glomus* spp., whereas the incipient dune sand had 30 OTUs, of which 17 were *Glomus* spp. (Figure 6.8). The mixed vegetation roots from plants on the foredune had 57 OTUs, with 33 being bulked *Glomus* spp. (Figure 6.8). Sea Wheatgrass roots had 56 OTUs, of which 32 were bulked *Glomus* spp., while Hairy Spinifex had the lowest AM fungal diversity, with 40 of the 57 OTUs being the bulked *Glomus* spp. (Figure 6.8).

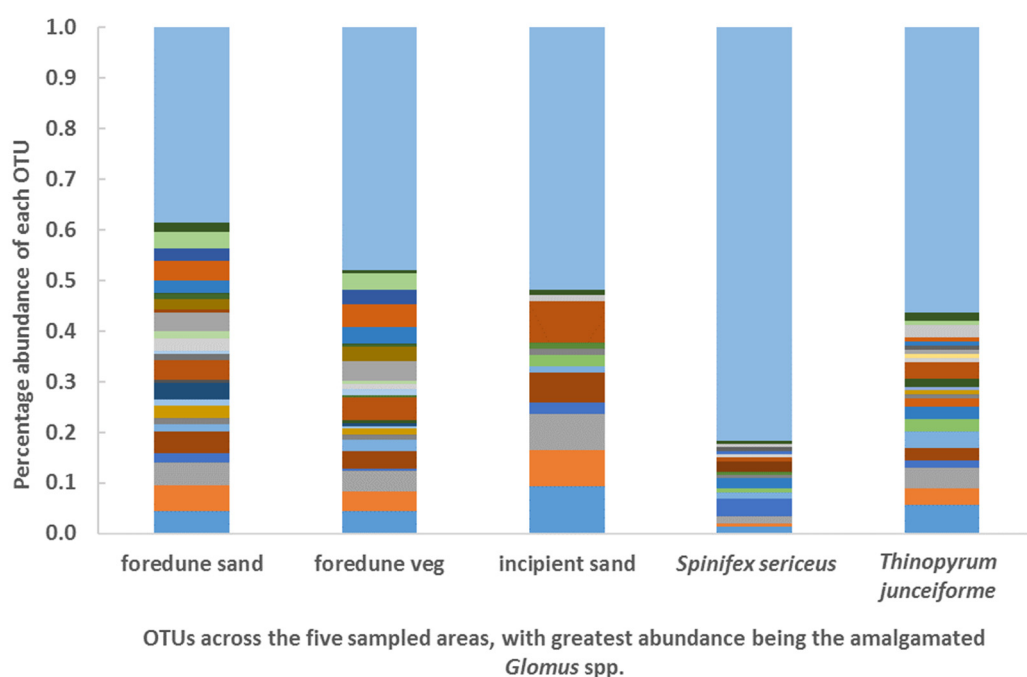


Figure 6.8: Relative abundance of AM fungal OTUs from each combined sample location at Thirteenth Beach, September 2016. Each combined sample is from eight transects horizontally along the incipient dune, or foredune. The varying band widths and colours indicate relative abundance of each AM fungal species represented in the sample. The greatest abundance is that of the bulked *Glomus* spp., of various OTU numbers.

One OTU may be found in multiple samples, thus when duplicate OTUs, and singletons were removed, a total of 73 OTUs, across 16 AM fungal species remained across the research site (Table 6.6). It was necessary to remove singletons as they are

likely to be the result of errors in sequencing (Botnen et al. 2015), and may therefore overestimate AM fungal species richness (Kunin et al. 2010).

Table 6.6: The 16 coastal AM fungal species identified by molecular analysis in dune sand, and roots of sand dune plants, collected from Thirteenth Beach in September 2016, and the number of times their OTUs occur within the dune system.

AM fungal species	Occurrence
<i>Archaeospora schenckii</i>	3
<i>Claroideoglossum</i> spp.	148
<i>Claroideoglossum torrecillas</i>	59
<i>Diversispora</i> spp.	13
<i>Diversispora</i> sp. 8479	6
<i>Diversispora</i> MO-GC1	3
<i>Diversispora torrecillas</i>	7
<i>Glomus</i> spp.	369
<i>Glomus alguacil</i> 09b	13
<i>Glomus goomaral</i>	13
<i>Glomus yamato</i> 08	6
<i>Glomus yamato</i> 09	2
<i>Scutellospora</i> spp.	9
<i>Scutellospora calospora</i>	2
<i>Scutellospora</i> (Early-31)	16
<i>Scutellospora</i> (Early-32)	6
	675

There was a highly significant difference between DNA differentiating AM fungal communities from the dune sands taken from immediately beside vegetation, to the DNA of AM fungal communities from the roots of mixed vegetation, Hairy Spinifex and Sea Wheatgrass (0.001, 95% CI), at the OTU level (Figure 6.9). At the 97% OTU similarity level, there was a significant difference in the community level of AM fungi in the foredune sand, and the roots of the mixed vegetation in the foredune, visualized by the proximity of symbols representing these communities in the foredune sand and the roots of mixed vegetation (Figure 6.9). The greatest disparity between AM fungal

community similarity levels was in the roots of Hairy Spinifex and Sea Wheatgrass from the incipient dune, and the AM fungal foredune communities, visualized by the distance between symbols representing these communities (Figure 6.9). The combined frequencies of detected OTUs from the incipient dune and foredune sands totalled 240, whereas the combined frequencies of OTUs in the roots of mixed vegetation, Hairy Spinifex and Sea Wheatgrass totalled 435 (Table 6.8). In general, however, the highest frequency of OTUs were shared by the sand immediately beside mixed vegetation on the foredune, and the foredune sand (Table 6.7).

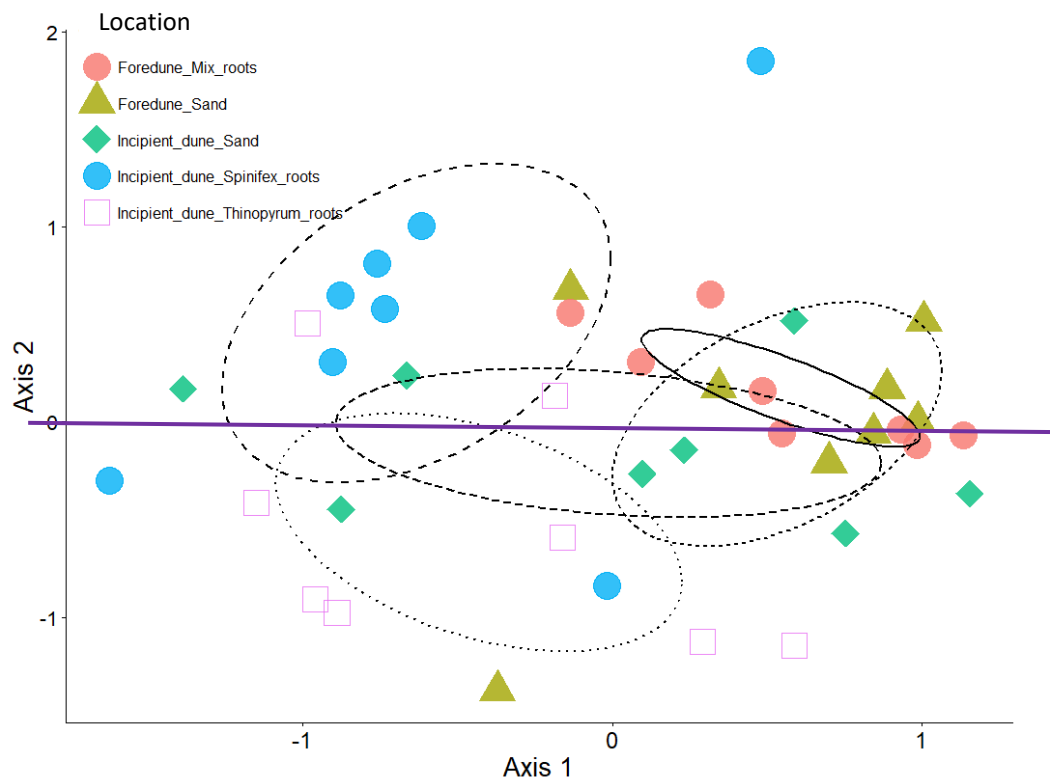


Figure 6.9: Non-metric multi-dimensional scaling (NMDS) analysis of differences between AM fungal communities, at operational taxonomic unit (OTU) level, across the sampled sand and plant roots from incipient dune and foredune communities at Thirteenth Beach, September 2016. Dispersion ellipses were calculated based on sample location (sand, roots of mixed foredune vegetation, and roots of *Thinopyrum junceiforme* and *Spinifex sericeus* from the incipient dune) (95% CI), 2D stress = 0.22.

Table 6.7: Location in the dunes where the fungal OTUs occur.

Location	OTU occurrence
Foredune vegetation	173
Foredune sand	155
<i>Spinifex sericeus</i>	140
<i>Thinopyrum junceiforme</i>	122
Incipient dune sand	85
	675

The number of OTUs varied between transect points, except in transect six, where Hairy Spinifex and Sea Wheatgrass both had 24 OTUs (Table 6.8), albeit not necessarily the same species of AM fungi (Appendix III). Additionally, the spatial heterogeneity displayed by OTU richness changed in abundance over distances of approximately 40 m between the collection points (Figure 6.10, A - E).

Table 6.8: OTU abundance along transects and sample types, from Thirteenth Beach, September 2016.

Sample type	Transects						
	1	2	3	4	5	7	8
Foredune sand	9	15	18	28	19	29	17
Foredune vegetation	14	25	20	25	20	17	25
Incipient dune sand	7	11	7	10	10	14	18
<i>Spinifex sericeus</i>	6	3	8	21	29	26	23
<i>Thinopyrum junceiforme</i>	15	2	16	2	5	36	22

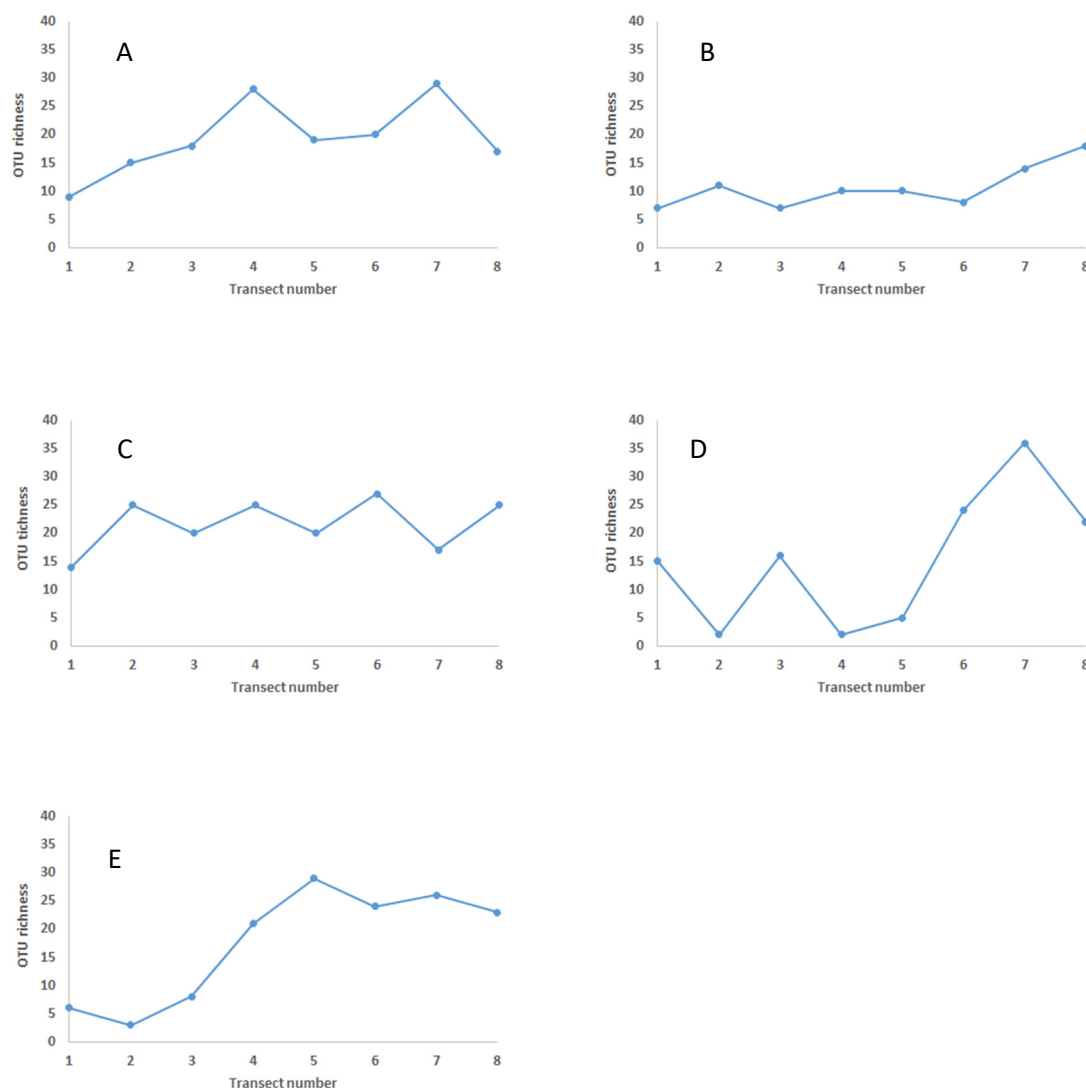


Figure 6.10: A - E Relative abundance of OTUs across transects in foredune sand (A), incipient dune sand (B), roots of foredune vegetation (C), roots of *Thinopyrum junceiforme* (D), and roots of *Spinifex sericeus* (E), from samples at Thirteenth Beach, September 2016.

Collection points for samples were along a 3 m section approximately in the middle of each transect, and approximately 40 m apart from one sampling point to the next. Many of the bulked samples examined for DNA had the same AM fungal species as each other (Appendix III). The greatest difference in fungal species richness was between the foredune sand, and that of the incipient dune sand and Hairy Spinifex, whereby the foredune sand had 16 different AM fungal species to those found in the incipient dune sand, and in the roots of Hairy Spinifex (Table 6.9). The least difference in AM fungal species richness was between that of the incipient dune sand and the

roots of Sea Wheatgrass, whereby the incipient dune sand had only one different AM fungal species to that identified in the roots of Sea Wheatgrass (Table 6.10). Although the assemblages of AM fungal communities differed across the five collection types (foredune and incipient dune sands, and roots of the three vegetation types), the majority of the shared OTUs were from the bulked *Glomus* spp. (Figure 6.8, Appendix IV).

Table 6.9: Numbers of differing AM fungal species identified by molecular analysis, between the combinations of the incipient dune and foredune sand, and the roots of *Thinopyrum junceiforme*, *Spinifex sericeus* and the foredune mixed vegetation, at Thirteenth Beach, September 2016. Numbers include the bulked *Glomus* spp.

	Dune sand location		Plant roots		
	Foredune	Incipient dune	<i>Thinopyrum junceiforme</i>	<i>Spinifex sericeus</i>	Mixed vegetation
Foredune sand		16	10	16	4
Incipient dune sand	3		1	3	4
<i>Thinopyrum junceiforme</i>	9	12		11	8
<i>Spinifex sericeus</i>	8	7	4		8
Mixed vegetation	4	15	7	14	

The greatest range of OTUs across transects was found in Sea Wheatgrass roots, where there were two OTUs in transects two and four, and 36 OTUs in transect seven (Figure 6.11). The Hairy Spinifex transects showed a range of OTUs from three in transect two, to 29 in transect five (Figure 6.11). In the foredune sand, OTUs ranged from nine in transect one, to 29 in transect seven, while the foredune mixed vegetation ranged from 14 OTUs in transect one to 27 OTUs in transect six. The smallest range of OTUs was in the incipient dune sand, with seven OTUs in transects one and three, and 18 in transect eight (Figure 6.11).

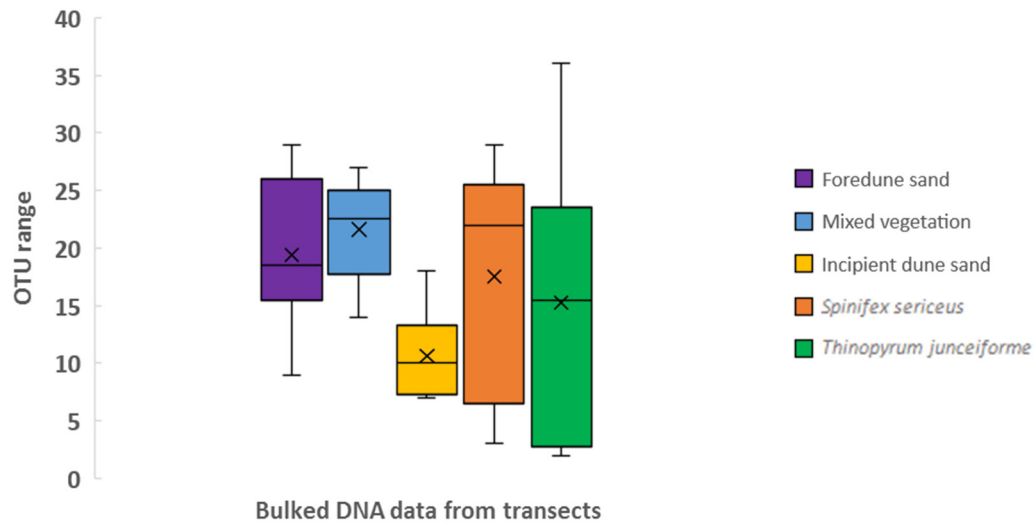


Figure 6.11: Spread of OTUs across bulked samples within transects at Thirteenth Beach, September 2016. Horizontal line represents median of data, perpendicular line represents the range of OTUs, X represents the mean.

The fungal species occurring most frequently were the 42 bulked *Glomus* spp., followed by *Claroideoglomus* spp., and *Claroideoglomus torrecillas*. *Claroideoglomus* spp. accounted for 148 occurrences, over 11 different OTUs (Table 6.10). Of the total 73 OTUs assigned by Maarj-AM blast, with singletons removed, the combined 42 OTUs assigned to *Glomus* spp., accounted for 369 occurrences across the dune system (Table 6.11, Appendix IV). *Claroideoglomus torrecillas* accounted for 59 occurrences over two OTUs (Table 6.12). Altogether, there were 675 occurrences of AM fungi in the foredune sand and incipient dune sand from immediately beside vegetation, and in the roots of Hairy Spinifex and Sea Wheatgrass on the incipient dune, and in the roots of the mixed vegetation on the foredune (Appendix III).

Table 6.10: *Claroideoglomus* spp. of various OTU numbers, as identified by molecular analysis, location and occurrence in the dune system at Thirteenth Beach, September 2016.

OTU	AM fungal species	foredune sand	foredune vegetation	incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	Total
2	<i>Claroideoglomus</i> sp.	8	7	6	1	4	26
15	<i>Claroideoglomus</i> sp.	7	6	5		3	21
278	<i>Claroideoglomus</i> sp.	6	8	7	1	4	26
307	<i>Claroideoglomus</i> sp.	1	2				3
352	<i>Claroideoglomus</i> sp.	6	7			1	14
365	<i>Claroideoglomus</i> sp.	3	5				8
371	<i>Claroideoglomus</i> sp.	2	1				3
395	<i>Claroideoglomus</i> sp.	4	6			1	11
397	<i>Claroideoglomus</i> sp.	6	8			1	15
411	<i>Claroideoglomus</i> sp.	4	5				9
425	<i>Claroideoglomus</i> sp.	5	6			1	12
		52	61	18	2	15	148

Table 6.11: *Glomus* spp. abundance, identified by molecular analysis, and location and occurrence in the dune system at Thirteenth Beach, September 2016.

OTU	AM fungal species	foredune sand	foredune vegetation	incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	Total
Refer Appendix IV	<i>Glomus</i> spp. x 42	60	81	44	114	70	369

Table 6.12: *Claroideoglomus torrecillas* OTUs as identified by molecular analysis, location and occurrence in the dune system at Thirteenth Beach, September 2016.

OTU	AM fungal species	foredune sand	foredune vegetation	incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	Total
1	<i>Claroideoglomus torrecillas</i>	7	8	8	2	7	32
3	<i>Claroideoglomus torrecillas</i>	7	7	6	2	5	27
		14	15	14	4	12	59

There were 361 non-AM fungi phyla OTUs, which were removed from the blast results. They comprised Ascomycota (3), Basidiomycota (18), Blastocladiomycota (1), Chytridiomycota (74), Cryptomycota (11), uncultured Eukaryota (39), unassigned phyla (212), and Zygomycota (3).

6.6 Discussion

The hypothesis for experiment one, that mycorrhizas would establish in the bait crop of Garden Leeks grown in sand from immediately beside the three vegetation types (mixed foredune vegetation, and the two incipient dune grasses) was confirmed by the presence of mycorrhizas in the roots of the bait crop. Mycorrhizas also increased over time (harvests) in the roots of the bait crop of Garden Leeks. The hypothesis for experiment two that AM fungal inoculum in sand from the foredune would colonize an incipient dune grass was also confirmed, but was not substantiated by any delays in colonization associated with differences in soil chemical and edaphic conditions. The hypothesis for experiment three was demonstrated clearly with rich and varied AM fungal species being identified across the dune gradient, with spatial heterogeneity along distances of only 40 m. Differing species were found between the gradients of foredune and incipient dune, however *Glomus* spp. was the most cosmopolitan AM fungal species across the dune gradients. The greatest number of AM fungal OTUs was in the roots of foredune vegetation, with the least OTUs in the incipient dune sand. Furthermore, there were distinctly different community similarity levels of AM fungi in the grasses of the incipient dune compared to those in the roots of the foredune vegetation and foredune sand.

6.6.1 Experiment 1 discussion. Bioassay

The hypothesis for experiment one was substantiated by mycorrhizas establishing in the bait crops of Garden Leeks grown in sand from immediately beside the three vegetation types (mixed foredune vegetation, and the two incipient dune grasses).

Across all destructive harvests, mycorrhizal presence was greater in the Garden Leeks grown in the foredune sand from immediately beside mixed vegetation, to that in the roots of Garden Leeks grown in incipient dune sand from immediately beside the dune

grasses. This confirms the greater inoculum potential of the foredune sand AM fungi to that of the incipient dune sand AM fungi. The presence of mycorrhizas increased rapidly from week two, post-germination, to week 6.5 in both foredune sand and incipient dune sand. By the final destructive harvest, the percentage colonization (% RLC) of mycorrhizas in the Garden Leeks grown in foredune sand from immediately beside mixed vegetation was 12.50% greater than that of plants grown in incipient dune sand from immediately beside Sea Wheatgrass. The inoculum from foredune sand immediately beside mixed vegetation was 10.90% greater than in incipient dune sand collected from immediately beside Hairy Spinifex.

The inoculum potential of the foredune sand agrees with that demonstrated in the seminal studies of Nicolson (1960), whereby the greatest proportion of mycorrhizas were found in the stable sand dunes, and not the incipient dunes (named pioneer embryo dunes), at Gibraltar Point, Lincolnshire. Additionally, the stable foredunes at the research site host greater plant diversity than the incipient dunes, and higher fungal richness has also been found associated with greater plant diversity in Norwegian sand dunes (Botnen et al. 2015).

The fertility of the foredune sand, compared to that of the incipient dune sand clearly influenced the increases in mycorrhizas over time in the bioassay. The level of labile, or active carbon, from living organisms and new residues (Pluske, Murphy & Sheppard 2017) in the foredune sand was greater than that of the incipient dune sand, thereby providing a readily available energy and nutrient source for micro-organisms (Kögel-Knabner 2017). Furthermore, higher intensities of mycorrhizas in roots have been associated with increased carbon (Smith & Read 2008), and up to 30% of plant-derived carbon is utilized by AM hyphae in the soil, albeit communities differ among host plants (Husband et al. 2002; Vandenkoornhuyse et al. 2003).

The total organic carbon in the foredune sand, that is, the soil organic carbon derived from root exudates and the decomposition of soil biota and plants (Pluske, Murphy & Sheppard 2017), was almost half that found in the incipient dune sand. At first this seems counter-intuitive, however tides and storms periodically deposit rapidly degradable wrack and other organic detritus which support microbial populations (Del Vecchio et al. 2017), whereas the main input on the foredune is from the leaf matter

of coastal vegetation. The vegetation on the foredune is largely native coastal dune scrub, Ecological Vegetation Classification (EVC) 1 (Bellarine Catchment Network 2010). The leaves of many of these species are leathery or waxy, with recalcitrant lignins that are negatively correlated to biotic degradation that would otherwise reduce them more quickly to labile sources of carbon (Austin & Ballaré 2010). Rapid decay of litter is also favoured by well-aerated substrate, hot, moist conditions (Pluske, Murphy & Sheppard 2017), and photodegradation from sunlight (Austin & Ballaré 2010). Ample solar radiation is delivered to the foredune, as there are no large trees creating shade, however, the foredune sand has very low volumetric soil water and air-filled porosity. Thus, the main source of organic input on the foredune is from living organisms such as plant root exudates, bacterial excretions, soil fauna, and fungal hyphae (Hoyle, Murphy & Sheppard 2017). Indeed, plant root exudates were found to explain a large proportion of fungal community variation in a study on Îles-de-la-Madeleine in the Gulf of St. Lawrence, in Québec, Canada by Roy-Bolduc *et al.* (2015).

Living organisms in the substrate are also influenced by substrate texture, and pH (Yanardağ *et al.* 2017). At Thirteenth Beach, the pH of the incipient dune sand is acidic, however the foredune is close to, or may be considered, as neutral. (Hazelton & Murphy 2007). Furthermore, as pH is a logarithmic function of the hydrogen ions in a solution, for each change of 1 unit of pH, the hydrogen ion activity differs by a magnitude of 10 (Weil & Brady 2016; White 2006). Thus, the foredune sand at Thirteenth Beach is approximately 5 times less acidic than that of the incipient dune sand. As $\text{pH}_{\text{Ca}} < 6.5\text{-}7.0$ pH limits the availability of iron, the pH 6.6 of the foredune would select for different AM fungal species composition and abundance than in that of the incipient dune sand. This conclusion concurs with that of Roy-Bolduc *et al.* (2016) who found that the pH differences across successional gradients in a Canadian dune system supported taxonomically distinct AM fungal communities. Additionally, it has been established that within coastal species of AM fungi, *Glomus* spp. were of different, albeit similar, phylogenies depending on how close to the sea they were found (Yamato, Ikeda & Iwase 2008). Furthermore, proximity to the ocean conferred salt stress alleviation on host plants by coastal AM fungi (Yamato, Ikeda & Iwase 2008).

Despite the levels of nitrogen and phosphorus in the foredune sand being marginally greater than that of the incipient dune, they are still considered to be low, which is

typical of dune sands (Maun 2009). Other edaphic conditions, such as the calcium carbonate of the foredune substrate being nearly 13% less than that of the incipient sand, suggest that differences in relative abundance, and species, of AM fungi are to be expected over the edaphic gradients of the dune system. For example, *Glomus* spp. were found to be the most abundant AM fungus in the youngest part of a dune chronosequence in Quebec, Canada, while Ascomycota, an ectomycorrhizal fungal species, was dominant in the swales (Roy-Bolduc et al. 2015).

6.6.2 Experiment 2 discussion. AM fungal inoculum from the foredune at Thirteenth Beach

The hypothesis for experiment two that AM fungal inoculum in the foredune sand would colonize the roots of an incipient dune grass was confirmed, but any delays in colonization associated with differences in soil chemical and edaphic conditions were not substantiated. Mycorrhizas had established in the node cuttings of Sea Wheatgrass by the first harvest, with colonized root length (cm) increasing from the first harvest at week five to harvest three at week nine. However, there was an abrupt decrease in colonized root length (cm) in weeks 11 and 13. This may be explained as a stress-tolerant strategy, which some AM fungal species demonstrate due to lowered or fluctuating supplies of carbon from the host plant (Chagnon et al. 2013). For example, shading has been shown to reduce colonized root length of AM fungi (Heinemeyer et al. 2003). Furthermore, as the exchange of carbohydrates for soil nutrients such as phosphorus is central to AM symbiosis (Bücking & Shachar-Hill 2005), it is reasonable to propose that plant photosynthesis would indirectly affect the growth of mycorrhizas (Heinemeyer et al. 2003).

No tillers were produced in the Sea Wheatgrass cuttings therefore there were no photosynthesizing organs to supply carbon in exchange for soil nutrients and water. Yet the lack of aerial parts on actively growing sand dunes grasses is not an uncommon phenomenon (Chapter 5), and most likely a life history strategy in response to the dynamic and disturbed niche of incipient dunes (Qu, Zhao & Zhou 2014). However, all the nodes produced roots, demonstrating that resource allocation in Sea Wheatgrass under stressful conditions is to roots rather than shoots (Harris & Davy 1988; Maun

2004). Moreover, aggregates were forming on and around the roots due to sand grains adhering to fungal exudates. Such aggregates are formed when AM fungi and host plants are in active symbiosis (Clough & Sutton 1978).

I argue that the physiology of Sea Wheatgrass, whereby the nodes are distribution hubs for mineral nutrients (Yamaji & Ma 2014) (Chapter 5), are also responsible for reducing the supply of carbon to the symbiont at a point where further allocation would be detrimental to plant survival. It follows that a loss of carbon supply would lead to a reduction in colonized root length (cm), as a stress-tolerant mechanism of the fungus (Chagnon et al. 2013). Differences in tolerance to carbon fluctuations are likely to be a strong selective force in AM fungi with contrasting life history strategies to their host (Ijdo et al. 2010). Moreover, it has been demonstrated that the proportion of carbon flow to the fungus is related to the amount of phosphorus the fungus gives its host (Kiers et al. 2011). This reciprocal arrangement may allow the symbionts to detect a variation of resources in those supplied by their mutualists, and to adjust or change the bi-directional allocation of resources (Kiers et al. 2011). Thus, although the foredune AM fungal inoculum was compatible in regard to colonizing the incipient dune grass, the symbionts may not have been compatible at the functional level (Ravnskov & Jakobsen 1995) of nutrient exchange, resulting in individual survival mechanisms taking over. Further studies could elucidate and identify the mechanisms involved in bi-directional survival strategies. Furthermore, little is yet known about the longevity of introduced AM fungi (Tawaraya, Hirose & Wagatsuma 2012); it may well be that the host symbiont could not sustain its mutualist.

In ecology, the life history strategy concept strives to extrapolate how organisms use and invest their resources in stable or unstable environments (Chagnon et al. 2013), thus positioning the lifeforms along an r - K continuum. At one end of the spectrum is the K -strategist whose life history strategies in AM fungi have selected for stable plant ecosystems (de Souza et al. 2005), where fungal mycelium is generally not disrupted by storms and scarping. At the other end are the r -strategists of ruderal or disturbed environments, whereby opportunistic behaviour and fast reproduction take advantage of fleeting conditions (Ijdo et al. 2010). Differences in life history strategies between r and K fungal strategists have been demonstrated in defoliation experiments (Ijdo et al. 2010). For example, the generation of carbon storage structures differed from the

phylogenically different *Glomeraceae* *r*-strategist *Glomus intraradices*, to that of the *Gigasporaceae* *K*-strategist, *Dentiscutata reticulata* (Ijdo et al. 2010). Notwithstanding the low levels of mycorrhizal colonization of *D. reticulata*, the levels did not differ between plants with leaves and those which had been defoliated (Ijdo et al. 2010). This indicates high levels of mycorrhizal colonization are not a life history strategy of *D. reticulata* (Ijdo et al. 2010), and also that carbon was not limiting to the lifespan of the fungus in the short term. Further, it is known that different proportions of carbon are used by different AM fungal species (Pearson & Jakobsen 1993). However, what cannot be ignored is the plant's response to lack of tillers, as plant, soil and fungus all work together; it is a tripartite relationship.

The rapid decline in percent colonization (% RLC) and root length colonized (cm) by AM fungi by harvest four (week 11), also supports the concept that over time, as the substrate settled and compacted, the aeration of the sand may not have been optimal for the survival of the inoculum (Gaur & Adholeya 2000). Sand, like soil, behaves differently when constrained by the boundaries of a pot, and that is one reason why pot plants are grown in potting mixes, or soil-less media, which may or may not have a small component of their mix made up of sandy loam to aid in aeration (Handreck & Black 2002). Compaction alters the movement of air and water, and can change pore size distribution, with rain drops, or irrigation, being able to cause compaction on a minute scale, particularly on a bare surface (Leeper & Uren 1993). Further, the air-filled porosity of the foredune sand had been calculated at 0.28%, using a production horticulture protocol for the propagation of plants in potting media (Handreck & Black 2002), in which air-filled porosity > 13% is recommended (Gibbs 2010). The sub-optimum levels of oxygen following irrigation, exacerbated by the micro-compaction on the sand surface, could have negatively impacted fungal metabolic activity over time, thereby dramatically reducing fungal colonization.

In contrast to the dramatic reduction in fungal colonized root length (cm) in harvests four and five (weeks 11 and 13 respectively), the rapid increase in colonized root length (cm) and percentage colonization (% RLC) over time from harvest one to harvest three (weeks five to nine), may have been the result of an aggressive AM fungal colonizer in the inoculum (Vierheilig et al. 2000) from the foredune. *K*-strategists generally favour stable environments (2010), where competition is high (de Souza et al. 2005), and

allocate their resources to growth. Such aggressive colonization has been demonstrated by instances where one fungus can reduce the ability of another fungus to colonize plant roots (Vierheilig et al. 2000). For example, when *Funneliformis mosseae* (formerly *Glomus mosseae*) or *G. caledonium* were present in an inocula, the *G.* strain E3 was unable to colonize plant roots (Hepper et al. 1988). It has also been found that fungal species differ in their symbiotic capabilities when co-inoculated (Hepper et al. 1988; Pearson, Abbott & Jasper 1993).

The plant propagules in this study did not have any roots or leaves in which indigenous fungal propagules may have been secreted, furthermore the roots were not treated to destroy any remnant indigenous fungal propagules, therefore their presence cannot be ruled out. If any indigenous fungal propagules were present in the plant fragments, it may be that the new edaphic environment affected detrimentally their relative abundance, thereby presenting no competition towards the inoculum from the foredune sand. For example, differences in pH can affect strongly the diversity of AM fungi (Dickie et al. 2013). In an early study by Hepper et al. (1988), the competitive ability of AM fungi was found to be dependent upon the pH and nutrient status of the soil, albeit such work was under taken in a soil/sterilized sand mixture which had been amended with nitrogen and magnesium.

6.6.3 Experiment 3 discussion. AM fungal species richness

It was hypothesized that AM fungal richness would be greater in the stable foredune compared to the disturbed incipient dune, due to differences in soil chemistry and life history strategies in incipient dune AM fungi selecting for robustness over abundance. This was affirmed by the greater number of fungal OTUs in the foredune sand and vegetation than that of the incipient dune sand and its vegetation. Further, such differences selected for dissimilar AM fungal species to those found in the incipient dune sand, and in the roots of mutualist dune grasses.

The factors which determine fungal community structures and activities in different soils are not yet fully understood (Jansa, Oberholzer & Egli 2009). However, the distinct vegetation zones and contrasting abiotic conditions between the two gradients which hosted differing species and abundance of AM fungi is supported by the work

of Davison et al. (2015a). In a study of 1014 root samples from vegetation collected world-wide, Davison et al. (2015b) found that indigenous fungal communities were influenced by environmental conditions, which differed spatially between locales. Moreover, it has been demonstrated that the soil chemical environment, including pH, were indicated strongly in the composition of some fungal communities as ecosystems became more complex along chronosequences (Lekberg et al. 2011), such as the gradient from the incipient dune to the foredune.

The perpetually disturbed incipient dune sand had the least fungal OTUs, confirming the hypothesis that disturbance affects relative abundance, albeit does not destroy those species with life history strategies adapted to such conditions. Further, it was clearly shown that the richer abundance of plant species, and the stability of the foredune selected for a greater relative abundance of fungal OTUs than the grasses of the incipient dune. This is in keeping with the *K*-strategists whose life history strategies select for stable ecosystems, where mycelium can be produced in abundance (de Souza et al. 2005), and with AM fungal diversity increasing over successional time. For example, Johnson et al. (1991) found that fungal diversity was correlated with increasing pH, carbon, nitrogen, and water over successional time. However, discounting anthropogenic interference, any temporal variability of these edaphic conditions would likely be stochastic, highlighting the importance of seasonal collections in order to analyse naturally occurring trends of plant and fungal populations (Augé 2001).

Of the two ecological gradients at Thirteenth Beach, the foredune has a higher pH, and greater amounts of labile carbon and total nitrogen in comparison to the incipient dune. The foredune also has much lower volumetric soil water (%) due to its distance from daily incursions of water from waves. However, where Johnson et al. (1991) found that fungal species did not increase over gradients, rather one species became dominant, this was not the case over successional gradients at Thirteenth Beach. Although the bulked *K*-strategist *Glomus* spp. in the foredune sand and the roots of the mixed vegetation on the foredune were abundant, they were not dominant. However, the intimate relationship between soil properties, fungal species richness and abundance, and variety of vegetation cannot be overlooked: Here the intimate

relationship between soil, plant and fungus is clearly influenced by the stability of the foredune, as measured by OTUs.

Empirical data regarding the dispersal and vectors of Glomeromycotan fungi are limited (de León et al. 2016a) however dispersal of spores by wind is not considered to be significant (Egan, Li & Klironomos 2014). Over short distances, vectors are believed to be largely invertebrates (Egan, Li & Klironomos 2014), whilst over longer distances vectors include mammals, with native bush rats consuming and defecating the largest variety of fungal taxa (Vernes & Dunn 2009). Furthermore, hyphal fragments and spores do not migrate downwards from the foredune to the incipient dune, as a result of rainfall, for example (Koske et al. 2004). Thus, different AM fungal species were expected to be found between the two ecosystems due to the distance from sea-water incursion, differences in edaphic conditions and differences in host species. Moreover, both fungus and host are affected by the biotic and abiotic factors in their environment, with fungal taxa responding to soil pH, moisture content and chemistry, for example (Fitter 2005). Additionally, the environment inside host plant roots is unlikely to be the same across species, therefore niche differentiation is highly likely (Fitter 2005). The fungi are not distributed at random among their mutualists, but exhibit strong selectivity (Fitter 2005). For example, on the incipient dune, AM fungi are specifically adapted to the dynamics of their ecosystem (Yamato et al. 2012), with disturbance *r*-specialists in scarped plant fragments or surviving plant remnants quickly re-establishing communities following storms. Contemporary studies have also shown that AM fungal *r*-strategists are fast colonizers of ruderal and early successional ecosystems (de León et al. 2016a).

Disturbance to the incipient dune sand from storm scarping did not lead to an environment lacking in AM fungi. Instead, there were 14 AM fungal species (30 before the *Glomus* spp. were bulked), in agreement with Lekberg et al. (2012), who found that OTUs and AM fungal richness were not significantly different due to disturbance, in a semi-natural grassland. Great species richness has also been found in re-vegetated mine sites (De Souza et al. 2013), albeit the soils had been remediated from between four to 16 years previously, and had likely settled into homeostasis.

As with coastal plants, coastal AM fungi are specifically adapted to their dynamic environment (Yamato et al. 2012), and this particularly applies to those species

occupying the incipient dune. For example, it has been demonstrated that some *Glomeraceae* are disturbance-tolerant *r*-strategists (Ijdo et al. 2010), whose hyphal biomass is concentrated inside the roots of host plants (Maherali & Klironomos 2007). Further, *Glomeraceae* colonization of *Trifolium subterraneum* (Subterranean Clover) was not compromised by disturbance when grown in soil from an agricultural annual pasture (Jasper, Abbott & Robson 1991). However, *Gigasporaceae* have been found to show disturbance tolerance by allocating greater resources to extra-radical hyphae than to other root-borne mycorrhizas (Maherali & Klironomos 2007). Yet, despite these differing life history strategies which potentially enable both AM fungal families to co-exist in the same niche, there were no *Gigasporaceae* found at the study site, suggesting they are not as disturbance tolerant as are *Glomeraceae* in a coastal setting. Thus, the assumption that disturbance events drive the relative abundance of AM fungal species must be taken in the context of where such fungi are found. If natural disturbance is a common occurrence, the fungal communities should show resilience, and a high degree of tolerance towards it (van der Heyde et al. 2017).

There is evidence of differential response to disturbance in the species richness of AM fungi, and that such responses depend upon the nature of disturbance (van der Heyde et al. 2017). AM fungal richness studies have been undertaken in relation to, e.g., mechanical disturbance from tillage (Alguacil et al. 2008), forestry (Soteras et al. 2015), organic amendments (del Mar Montiel-Rozas et al. 2016), and heavy metals (Del Val, Barea & Azcón-Agular 1999). However, the coastal niche of AM fungi, and their species richness in the Southern Hemisphere, has received scant attention, although molecular studies have been undertaken to establish the presence and diversity of mycorrhizas in Northern Hemisphere coastal dunes (Botnen et al. 2015; Rodríguez-Echeverría & Freitas 2006), and those of the North-Eastern Hemisphere in Tottori, Japan (Yamato et al. 2012). One recent exception is the work of Johansen et al. (2015), with molecular studies of AM fungi in a coastal dune on the north island of New Zealand, from which more appropriate parallels may be drawn and built upon by the work of this Thesis.

In the study of Johansen et al. (2015), plant root samples collected from the dune were used to sequence molecular data; however, although sand was collected from which to extract fungal spores, the substrate did not undergo molecular examination, which

might otherwise have revealed further OTUs and fungal richness. Notwithstanding this, pyrosequencing of the SSU rRNA gene established 22 OTUs, from 12 samples along a 90 m transect, 6 m behind a frontal dune crest (Johansen et al. 2015). The location was presumably out of the way of disturbance from tides, and more likely to host *K*-strategists, than ruderals. Heterogeneity of taxons was observed over sections of 30 m in length, in agreement with Mummey and Rillig (2008), and Davison et al. (2012), who have found AM fungal community composition can change in as little as 30 cm to as much as 50 m, respectively. This localized scaling of communities also agrees with the analyses undertaken for this Chapter, whereby communities changed over distances of 40 m, in both the disturbance-prone incipient dune, and the stable foredune ecosystems.

The New Zealand study site of Johansen et al. (2015) was small in comparison to the study reported in this Chapter, but did focus on *Spinifex sericeus*, which is also a native coastal plant in that country. The work identified seven genera, across Diversisporales and Glomerales, and demonstrated the diversity of AM fungi that can be found in a single dune plant species (Johansen et al. 2015). Life history strategies were not addressed in explaining the diversity of AM fungal species across the site, albeit, it was hypothesized that some of the spores in the dune sand may have originated from the thickly vegetated hills that surround the beach (Johansen et al. 2015). However, I hypothesize that as the forest floor offers a very different ecosystem to that of a sand dune, life history strategies would select for different species of AM fungus due to differences in soil edaphics and host plant physiologies between the forest and dune.

The global biogeography of AM fungi is largely unknown (Davison et al. 2015a), yet building an atlas of fungal biogeography, using the life history strategies of symbionts in association with the environment in which they are found, could potentially predict more accurately where, and what, AM fungal species are likely to found in natural environments. In enhancing our understanding of microbial biogeography, this information would also be of enormous benefit in ecosystem restoration.

The present study aids in building a picture of diversity patterns, specifically in a southern Australian coastal dune setting. However, both the New Zealand study, and that of this Chapter, would have benefitted from molecular sequencing of samples

over a period of different seasons, to compare any changes to community composition over time.

6.7 Conclusions

In this first analysis of AM fungal biogeography on the incipient dunes of Southern Australia, it has been demonstrated that environmental gradients select for different species of AM fungi, and that community structures show spatial heterogeneity along distances of only 40 m. Sixteen AM fungal species were identified by molecular analyses, occurring 675 times across the incipient dune and the foredune. The greatest frequency of fungal species was that of the bulked *Glomus* spp., (42 OTUs), followed by that of *Claroideoglomus* spp. (11 OTUs). As with the seminal work of Nicolson (1960) at Gibraltar Point, Lincolnshire, the greatest abundance of AM fungi in this study at Thirteenth Beach was found in the stable foredunes.

The dunes are a complicated biological system, full of variability and challenges for biota and researcher alike. However, life history strategies and niche differentiation of AM fungi between the incipient dune and the foredune exhibit strong selectivity, with *r*-strategists quickly colonizing the ephemeral incipient dune after storm events, giving way to *K*-strategists as the dune settles into homeostasis during longer periods of relative stability. *K*-strategists also occupy the stable foredune, where their species richness is apparent due to their distance above the reach of overwash and scarping.

Chapter 7

Thesis synthesis and conclusions

7.1 Introduction

This Thesis investigated coastal mycology in Australia, where there has been no work undertaken on ephemeral incipient dunes. Earlier work in Australia was conducted either in foredunes of New South Wales (Koske 1975; Logan, Clarke & Allaway 1989) or in foredunes and coral cays of Queensland (Jehne & Thompson 1981; Peterson, Ashford & Allaway 1985).

I have demonstrated that life history strategies and strong selective forces in AM fungi and *Thinopyrum junceiforme* (Sea Wheatgrass), equip the mutualists to tolerate salinity levels and disturbance that symbionts in other ecosystems are unlikely to endure. This argument reinforces the necessity to examine the entire relationship between AM fungi, their mutualist host plants, and the substrate of the dunes, in any ecological study of dune biota. Moreover, the addition to mycorrhizal databases of AM fungal species and richness found on the disturbed and transient incipient dunes at Thirteenth Beach on the southern coast of Victoria, will add to the biogeographical atlas of AM fungi.

7.2 Overview of findings

Chapter 2 established the dune morphology of the research site prior to severe storms which scarped the incipient dune. The research site at Thirteenth Beach is perched on underlying rock and is therefore dynamic.

Key findings in Chapter 2 were the chemical and edaphic conditions of the incipient dune. These analyses allowed the subterranean boundary conditions under which coastal AM fungi are found in association with symbiotic C₃ and C₄ dune grasses to be determined as a starting point to defining the requirements and tolerances of AM fungi on disturbed, ephemeral incipient dunes.

Chapter 3 placed the exotic Sea Wheatgrass and the native *Spinifex sericeus* (Hairy Spinifex) grasses in context with the boundary conditions established in Chapter 2, and laid the foundations for the field and laboratory studies which followed. The taxonomy, biology, reproductive and dispersal modalities, and nomenclature of the

grasses were reviewed. Complex heterotypic synonyms associated with Sea Wheatgrass were identified. That Sea Wheatgrass is classified under the botanical nomenclature of *Thinopyrum junceiforme* in the most recent Australian and New Zealand literature remains at odds with the currently accepted name of *Elytrigia junceum* subsp. *boreoatlantica*. The table of synonyms should aid in future studies of the grass by being a quick cross-reference of the botanical binomials under which other studies may be found. Similarly, the description of morphological differences between the genera *Thinopyrum* and *Elymus*, both of which Sea Wheatgrass has been classified under, will make identification of the grass easier. The differences in root hair morphology between the exotic and native grasses in relation to AM fungal exudates and sand aggregation on roots was highlighted. Studies regarding colonization of Hairy Spinifex by AM fungi are scant, therefore the findings in Chapter 3 which confirm the presence of AM fungi in the native grass, increase our current knowledge of host plant species.

Chapter 4 documented the dynamics in seasonal abundance of AM fungi in the roots of the exotic and native dune grasses over two summers and winters, and the seasonal changes in aerial biomass of the grasses. Scarping of the incipient dune created conditions under which to investigate mycotrophic responses to disturbance and loss of substrate and mutualists. This Chapter demonstrated for the first time the profound vigour of coastal AM fungi, whereby disturbance and life history strategies have selected for the most resistant *r*-strategist species, enabling their survival and tolerance of the unstable and unpredictable environment of incipient dunes. The significantly lower abundance of AM fungi pre-storm compared to post-storm, clearly denotes the ecological fitness of AM fungi to this niche, and their vital importance in the fast re-establishment of symbiosis in surviving or washed-up plant fragments on scarped dunes and slumps. This then leads to the formation of new dunes as sand accretes around mutualistic pioneer vegetation.

A further key finding in Chapter 4 was the resilience and abundance of Hairy Spinifex compared to that of Sea Wheatgrass, whereby the native grass had greater aerial biomass both pre-and post-storms to that of the exotic grass. Previously, it had been indicated that Sea Wheatgrass formed more resilient incipient dunes than those that are formed by native flora such as Hairy Spinifex. However, there was no quantitative scientific evidence to substantiate these claims, and no hypotheses as to mechanisms

that may be driving any invasiveness of Sea Wheatgrass on Australian shores. Although symbionts can influence the success of plant invasions, through for example, positive feedback loops that increase the supply of nutrients to one plant species over another, thereby helping to create larger root masses for further colonization sites, this was not the case during the course of my study. Indeed, seasonal abundance, and aerial and subterranean biomasses of Hairy Spinifex, pre-and post-storms, were consistently higher than those of Sea Wheatgrass. Furthermore, it was clearly demonstrated that Hairy Spinifex is as tolerant of frequent sea water inundations as is Sea Wheatgrass, as both species grow in association with each other occupying the same niche and conditions on the incipient dune, with both species colonizing the swash in quiescent periods.

Chapter 5. The key finding in this Chapter is that the germination of coastal AM fungi is not delayed by sodium chloride. Experiments for this Chapter employed the novel approach of imposing levels of sodium chloride irrigation up to and including twice that found dissolved in sea water (70,000 ppm/109.40 dS/m). This approach enabled the study of AM fungal colonization under salinity levels equivalent to (and above) those encountered in the natural environment. Life history strategies are indicated strongly to be the reason for changes in abundance of AM fungi in plant propagules overtime. These findings contribute to the body of work that attests to the enhanced survival of psammophilic plants by their mutualistic association with coastal AM fungi, whereby AM fungi mitigate the antagonistic effects of salts.

A further key finding in Chapter 5 demonstrated the tolerance to extreme and increasing levels of sodium chloride by the survival of Sea Wheatgrass propagules. Furthermore, the survival of plant propagules without the growth of any aerial biomass throughout the experiment, supports the phenomenon of resource allocation to roots in Sea Wheatgrass rather than to tiller production. This finding affirms that not all dune grasses allocate resources in the same manner when facing challenging conditions to their survival.

Chapter 6 investigated the niche differentiation and life history strategies of AM fungi between the disturbed incipient dune and stable foredune, thereby elucidating the differences that select for AM fungi across dune gradients. The first experiment examined the presence of infective AM fungal propagules in the foredune sand; the

second experiment examined the efficacy of the foredune AM fungi in colonizing an incipient dune host, and changes to the extent of colonization by AM fungi over time. The third experiment was an assessment of AM fungal species richness and diversity across the ecosystems and gradients of the dune. In using AM fungal propagules in the sand from the foredune to grow an incipient dune plant, an important finding established colonization was not impaired by differences in ecosystems from where the mutualists were harvested. Furthermore, although tillers were not produced by the host plant during the course of the experiment, roots were, around which aggregates formed on sticky fungal exudates.

A pivotal finding in this work is the identification of rich and varied AM fungal community structures, which showed spatial heterogeneity along distances of only 40 m. Across the incipient dune and foredune sand, and the roots of mixed vegetation and that of Hairy Spinifex and Sea Wheatgrass, 675 OTUs were detected, representing 16 different AM fungal species. Furthermore, the fungal communities in the roots of the foredune vegetation differed from, and were greater than, those in the roots of the incipient dune grasses. These findings confirm that differences in edaphic conditions across dune gradients show niche differentiation which selects for coastal AM fungal species that are adapted eminently to the challenges of the ecosystems in which they are found.

A precis of these Chapters and their main findings is shown in Table 7.1.

Table 7.1: Field work, laboratory analyses, glasshouse experiments, and main findings of the work undertaken in this study.

Chapter	Key objectives	Work undertaken	Main findings
Two	To investigate and describe the boundary conditions under which AM fungi are found in association with symbiotic dune grasses on the incipient dunes of Thirteenth Beach, on the southern coast of Victoria, Australia.	Background to the climate, mean high water spring (MHWS) tidal levels, and dune dimensions in relation to mean sea level (MSL) at the site Geology of the peninsular in which the site is located Site history and dynamics Digital elevation model (DEM) of the dune; cross-sections Particle size analysis of the dune sediment EC, pH_{Ca}, volumetric soil water %, oven dried moisture %, CaCO₃ %, TOC %, Labile C %, Total N % analyses Chapter published, and work presented at The International Coastal Symposium, March 2016	Dune morphology (pre-storms) represents a snap-shot in time which could be used by the Barwon Coast Committee of Management (the managers of the site), to observe patterns of sand accretion and vegetation re-establishment over time. Edaphic and chemical conditions of the sand at the site established that CaCO₃ is not the dominant component of the sand Nutrients are low, however pioneer plants would be assisted in uptake of nutrients by colonization by AM fungi The nutritional data are the first to establish the N content of a Southern Hemisphere coastal sand.
Three	To investigate and describe the biology, reproduction, growth, development and habitat of the exotic C ₃ dune grass, <i>Thinopyrum junceiforme</i> (Sea Wheatgrass) which is found in association with the native C ₄ sand dune grass, <i>Spinifex sericeus</i> (Hairy Spinifex)	Botanical and common names of the species, taxonomy History and distribution of the species in Australia Habitat, substratum and plant association Growth and development Population dynamics Mycorrhizal associations <i>Spinifex sericeus</i> described under the same topics as those for <i>Thinopyrum junceiforme</i>, including pathogens Chapter published on <i>Thinopyrum junceiforme</i>	Confirmation of AM fungi in the roots of two grasses increases our current knowledge of host species Aggregates formed around fungal exudates on both grass species roots, influenced by differences in root hair morphology

Chapter	Key objectives	Work undertaken	Main findings
Four	To examine and quantify through field and laboratory work over two summers and two winters (2015, 2016), the seasonal changes in the extent of colonization by AM fungi in the roots of the two dune grasses being studied, and to quantify changes in the extent of colonization by AM fungi and response to storm damage, of AM fungi and plants following the storm events of 2015.	Seasonally harvested field root samples treated with potassium hydroxide (KOH) to clear plant cells of cytoplasm and stained with Trypan Blue (standard protocol) to ascertain proportions of roots colonized Seasonal aerial biomass of <i>Thinopyrum junceiforme</i> and <i>Spinifex sericeus</i> enumerated, and fresh weights of tillers and roots recorded Seasonal concentrations of enzymes produced by microbial decomposers in the incipient dune, and in response to storm damage.	The profound vigour and resilience to disturbance of coastal AM fungi unambiguously demonstrated The greater aerial abundance, and resilience to scarping, of <i>Spinifex sericeus</i> compared to the exotic <i>Thinopyrum junceiforme</i> demonstrates that the latter does not displace former species at the research site. Both grasses are as tolerant to saline overwash as each other, and occupy the same niche and conditions on the incipient dune (and swash, in quiescent periods).
Five	To observe and quantify changes to mycorrhizas in response to increasing and extreme levels of NaCl, pre- and post-colonization as established by a bioassay using <i>Allium porrum</i> (Garden Leek)	Glasshouse bioassay with three destructive harvests, post-germination of bait crop, to ascertain when AM fungal colonization took place NaCl irrigation imposed on Sea Wheatgrass propagules over time 5 x NaCl levels x 5 harvests x 2 commencements (pre- or post-colonization) 5 replicates Standard protocols employed to assess the presence, proportion and length of mycorrhizas in plant roots at harvest Assessment of effect NaCl upon microbial decomposer concentrations in sand using fluorescein diacetate (FDA)	Coastal AM fungal infection of host plants was not impaired by NaCl Life history strategies (LHS) are strongly indicated in changes to proportions of AM fungi over time The tolerance of coastal AM fungi to extreme and increasing levels of NaCl unambiguously demonstrated AM fungi contribute to the enhanced survival of mutualistic psammophilic plants Resource allocation in <i>Thinopyrum junceiforme</i> is to roots, rather than tiller production, in challenging conditions
Six	To investigate the effects of coastal AM fungal colonization across dune gradients, with fungal propagules from the foredune and a host plant from the incipient dune, and to investigate and assess AM fungal	Glasshouse bioassay to ascertain presence of infective AM fungal propagules in foredune sand	The plants produced roots but not tillers, around which aggregates formed on sticky fungal exudates

Chapter	Key objectives	Work undertaken	Main findings
	richness across dune gradients to establish the biogeography at the study site	5 x harvests x 5 replicates, standard protocols used to assess extent of AM fungal colonization Molecular analysis of sand and plant roots from the foredune and incipient dune Assessment and compilation of AM fungal diversity and species richness across dune gradients	The node is a central hub in <i>Thinopyrum junceiforme</i> allocating resources to roots than to shoots in challenging conditions Fungal community structures are rich and varied across the dune gradient, with spatial heterogeneity along distances of only 40 m Fungal communities differed between the foredune and the incipient dune, illustrating adaptations to edaphic conditions across the gradients of the ecosystems

7.3 Contributions to knowledge

The relationship between AM fungi and grasses in the highly disturbed and transient ecosystem of incipient dunes has been brought to light, with the identification of tenacious and hardy AM fungal species that are well-adapted to this harsh environment. This knowledge contributes to understanding the robust nature of the disturbance-specialists in the subterranean biome of coastal sand dunes. The addition of the species identified to mycorrhizal databases, will add to the biogeographical atlas of AM fungal species richness. Furthermore, in employing the application of sodium chloride levels well above those normally imposed in saline-tolerant experiments, it is hoped that those experiments undertaken in future on coastal vegetation bear more realistic sodium chloride levels in mind, to reflect more closely the actual salinity conditions plants endure.

7.4 Areas for further research

Coastal mycology would benefit greatly by further studies of AM species richness at other sites along the Australian coastline, and in other climatic zones. Differences in boundary conditions and coastal AM fungi found within them would add to the biogeographical atlas, and build upon the picture of the disturbance specialists that dominate incipient dunes. If undertaken along several contiguous stretches of coastline, this may elucidate fungal biogeography on a regional scale. Furthermore, research on changes in seasonal abundance and species richness may bring to light new species, or identify which *r-K* strategists specialize during times of disturbance and relative stability.

Research comparing the coastal AM fungi associated with vegetation on actively prograding and actively eroding coasts would prove invaluable in ascertaining any differences between fungal communities in these two ecotypes. Boundary conditions on prograding systems with a positive sediment budget for example, may bring to light changes in coastal AM fungal species as a chronosequence of younger to older dunes is built by pioneer, and then climax vegetation. On eroding beaches, it would be

worthwhile to identify which coastal AM fungi are at the forefront of substrate and host plant loss. Of paramount importance is ensuring that any research on coastal dunes and coastal AM fungi includes the edaphic and chemical conditions the fungi are found in, and identification of their symbiotic hosts.

In running the glasshouse experiment again, sodium chloride treatments should cease after a given period, and be replaced by irrigation with water, in order to ascertain when, and if Sea Wheatgrass could produce tillers. This may elucidate further life history strategies and survival and, or, recovery mechanisms of the grass following stressful environmental conditions. If tillers grew, and hence carbohydrates produced, molecular analyses could be undertaken to examine any changes in coastal AM fungi, and any changes in their extent of colonization.

7.5 Concluding remarks

The study site is just one section along the coastline of Australia, and is restrained by the underlying rocky platform. However, it cannot be stated strongly enough that on any coastal beach, where there is perennial, and pioneer vegetation, coastal AM fungi are likely to be found in association with it. The fungi are ubiquitous in soils, including sand, and are associated with up to 90% of terrestrial vascular plants including those found on beaches. Underlying geology has no bearing on the abundance of AM fungi, albeit soil properties do, as was clearly demonstrated across the successional gradients of the study site for this Thesis.

The importance of coastal AM fungi to the establishment and survival of vegetation on the ephemeral and perpetually disturbed incipient dunes is of great importance. I propose that without the fungi, there would be few, or no plants, and without mutualistic plants, there would be no dunes. Sand would indeed be simply individual grains, blowing in the wind. Thus, the geomorphic role of mutualists in the building of coastal dunes cannot be overstressed.

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Appendix I

Coastal Mycology and Invasive Species: Boundary Conditions for Arbuscular Mycorrhizal (AM) Fungi in Incipient Sand Dunes



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ABSTRACT

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Arbuscular mycorrhizal (AM) fungi are ubiquitous in soil, and are associated with some 90% of terrestrial vascular plants, aiding plants to access water and nutrients the plant roots alone cannot, in exchange for photosynthates from their host. AM fungi were first found in the dune system in the 1960s, and many of the described species have been found in dune ecosystems, where they form symbiotic associations with psammophilic plants including dune grasses. The ephemeral environment of incipient sand dunes prevents long-term colonization by plants, and little research has been undertaken to examine the contribution of AM fungi to plant survival in the disturbed environment of incipient sand dunes, or what role, if any, they play in exotic plant species outcompeting native species. A first step to understanding these roles is to examine the edaphic and biological conditions of incipient dunes. Our findings quantify the boundary conditions that surround and support AM fungi and their host plant roots in incipient sand dunes on the southern coast of Victoria, and include the chemical and geomorphological characterizations of the dunes studied. We found the nutrient levels (TOC, P, and N) to be low, in contrast to the higher levels of N found on the Atlantic coast, and pH levels such that Al would be toxic for the majority of plants, whilst Fe is limited. Additionally, we found that the incipient dune sand was not saline, and that chemical characteristics between the toe and the crest of the incipient dune did not differ greatly.

ADDITIONAL INDEX WORDS: *Ephemeral environments, soil microbes, beach sand nutrients.*

INTRODUCTION

Dune erosion is a fundamental part of beach adjustment during storms (Pye and Blott, 2008) and the role of vegetation in releasing sand into the littoral system is critical. Incipient dunes form closest to the swash, when pioneer vegetation (Hilton and Konlechner, 2011), or obstructions such as wrack trap windblown sand (Kennedy and Woods, 2012). Furthermore, the critical role of plants in the initiation and development of dunes has long been recognized (*e.g.* Cowles 1899).

In Victoria, there are three main dune grass species, *Ammophila arenaria* (marram grass), *Thinopyrum junceiforme* (sea wheatgrass) (Figure 1), and *Spinifex sericeus* (hairy spinifex) (Cousens *et al.*, 2012), but only the latter is native. Marram is mainly found in the foredunes, and spinifex and sea wheatgrass dominate the incipient dunes (Cousens *et al.*, 2012). Sea wheatgrass is an erect, rhizomatous, perennial grass, growing to 0.5 m in height, and is endemic across a wide range of the European coasts (Hanlon and Mesgaran, 2014). It has spread rapidly along Victoria's coast since it was first recorded in 1933.



Figure 1. *Thinopyrum junceiforme* on the incipient dune at the base of the foredune on Thirteenth Beach, Victoria (Source: L.M. Hanlon).

Grasses are known to have mutualistic associations, or symbioses, with arbuscular mycorrhizal (AM) fungi (Ramos-Zapata *et al.*, 2011). However, little is known about the

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symbioses of AM fungi and vegetation in incipient dunes, despite a substantial variety of AM fungi being found in sand dunes (Koske *et al.*, 2004). AM fungi are obligate biotrophs, forming symbioses with some 90% of terrestrial vascular plants (Young, 2015). They can substantially enhance their host's ability to uptake water and nutrients (Koske *et al.*, 2004) thereby aiding rapid establishment of plants in disturbed ecosystems. Additionally, glue-like polysaccharides in AM fungal mycelium bind sand grains together (Koske *et al.*, 2004) (Figure 2).

Incipient dunes present a challenging environment in which to study AM fungi, which we hypothesize have a role in the rapid spread of sea wheatgrass. Little is known about the physical and chemical environment of incipient sand dunes in relation to the vegetation and fungal flora it supports.



Figure 2. Root system of a 12-week old sea wheatgrass plant, grown from a 5 cm node in a pot in natural beach sand. Note the grains of sand adhering to the root hairs, forming a 'mycorrhizal necklace' of sand aggregates (Source: L.M. Hanlon).

The aim of this study is to define boundary conditions on a section of the southern Victorian coast where AM fungi are found in association with hairy spinifex and sea wheatgrass. We present results from field research on the geomorphology of the research site, and the edaphic, or chemical and biological conditions in the sand that surround and support AM fungi and their host plant roots. We quantify the total carbon, total organic carbon (TOC), and labile carbon percentages of the sand, and the nitrogen and extractable phosphorus percentages. Additionally, we report on the pH, EC, water content, mean grain size and calcium carbonate content of the sand of the incipient dune.

Background

The research site is approximately 370 m long, situated between The Hole and The Corner at the eastern end of Thirteenth Beach, a 4.5 km stretch along the 7 km length of coast from Black Rocks to Barwon Heads (Figure 3). The beach comprises an aeolinite cliff dating from 90,000 year B.P. which is now submerged by modern sand (Alsop, 1983).

The site faces Bass Strait and experiences waves averaging 1.2-1.5 m (Water Technology, 2004). The height of the foredune is 9.37 m above mean sea level (MSL). The height of the incipient dune toe where it meets the beach is consistent along its length, being 5 m above MSL at either end of the site, and 4

m above MSL in the middle. The width of the incipient dune ranges from 5-9.5 m.

Annual mean minimum and maximum temperatures for March (when the data were collected) are 13.5°C and 24.7°C respectively (Bureau of Meteorology, 2015), with a mean annual rainfall of 549.2 mm (Bureau of Meteorology, 2015). Annual wind roses show due westerly winds of 21 km/h at 9.00 am and due southerly winds of 23 km/h at 3.00 pm, with a mean annual wind direction of due south (Bureau of Meteorology, 2015). Sand movement is greatest in summer months, and least during winter months (Alsop, 1973).

METHODS

A Trimble R6 GNSS Surveying System was used to survey the site, with a vertical accuracy of ± 3 cm. Soil moisture readings were taken at 26 locations along the crest and the toe of the incipient dune using a MEA ThetaProbe, HH2 Moisture Meter. In the 24 hours prior to taking moisture readings, 0.6 mm of rainfall occurred (Bureau of Meteorology, 2015). Sand samples were taken from the top 10 cm of the dune/beach surface, which is where most microbial activity and nutrient circulation takes place (Queensland Government and G.R.D.C., 2015). Samples were collected by hand at 10 locations along the dune crest and toe. The samples were placed in individual plastic bags within a cool bag, for transport back to the laboratory.

In the laboratory, samples were stored in a refrigerator at 4°C. Particle size was analysed with a Beckman Coulter LS 13 320 Laser Diffraction Particle Size Analyzer. Air-dried, homogenized samples were analysed for pH and electrical conductivity (EC) in a 1:5 soil/0.01 M CaCl₂ (pH_{Ca}) solution at 22.3°C. The EC of the saturation extract (EC_e) was calculated by multiplying the EC by the soil multiplier factor for sand (13) (State Government of Victoria, 2015). Oven-dried moisture content was calculated by homogenizing 10 20g samples from the crest and toe of the incipient dune, and drying at 80°C for 48 hours. The carbonate content of the dune sand was determined through LOI (Kennedy and Woods, 2013). Five 3g sub-samples of sand from across the crest of the incipient dune were finely ground, dried at 105°C and then heated for 24 hours at 400°C, followed by 1000°C for one hour. Chemical analyses were conducted by Environmental Analysis Laboratory (EAL), whereby samples were dried at 60°C for 48 hours prior to crushing and analysis. Total N and total P were measured as the two main elements transferred from the fungi to their symbionts (Smith and Smith, 2011), from three locations across the dune face. Total carbon and labile carbon were also measured. EAL in-house protocols included extractable P in a 1:3 nitric/HCl digest, total organic carbon percentage (TOC) (LECO CNS2000 Analyser), labile C percentage using the protocol of Blair *et al.* (1995); and total N percentage (LECO TruMAC CNS Analyser) using the protocol of Rayment and Lyons (2011).



Figure 3. Map of Thirteenth Beach, Barwon Heads, showing research site between The Hole and The Corner (Cartographer: C. Jayasuriya).

RESULTS

Dune morphology was characterized by a DEM of the research site. The incipient varies in elevation from 8.23–8.31 m (Table 1), and in slope from 0.33° at the western end (T0) to 0.40° at the eastern end (T6), with slopes between 0.18–0.24° through the remaining transects.

Table 1. Elevations (m) (AHD) of the foredune and incipient dune along seven perpendicular transects.

Transect	Top	Bottom	Incipient
T0	14.27	1.39	8.31 – 4.69
T1	13.03	1.22	5.85 – 4.17
T2	12.99	0.91	5.75 – 3.82
T3	12.78	1.29	5.48 – 3.75
T4	12.29	1.70	5.25 – 3.92
T5	13.16	1.49	4.64 – 3.95
T6	13.38	1.18	8.23 – 4.82

The dunes are composed of medium, moderately well-sorted, fine grade sand, with a mean grain size of 1.46 Ø for the toe of the dune and 1.451 Ø for the crest (Table 2). The average CaCO_3 content is 15% (Table 2). The crest of the incipient dune is pH_{Ca} 6.09 and the toe pH_{Ca} 6.06, making Fe concentrations less than optimum, and Al concentrations toxic for the majority of plants. However, $\text{EC}_{1:5\text{Ca}}$ indicates that the sand is not saline (Table 2). Overall, edaphic conditions between the incipient dune crest and the toe did not differ greatly except in relation to volumetric soil water content (%), with the toe being more saturated due to its proximity to the high tide mark. This agrees with global estimates of typical dune volumetric soil water ranges of 1.5–6.0% (Van der Valk, 1974).

Table 2. Edaphic conditions (0–10 cm), Thirteenth Beach incipient dune crest and toe.

Procedure	(n)	crest	toe
$\text{pH}_{1:5\text{Ca}}$	6	6.09	6.06
$\text{EC}_{1:5\text{Ca}}$ (dS/m)	6	0.003	0.003
EC_e (*13)	6	0.040	0.040
Volumetric soil water (%)	26	3.00	5.18
Oven dried moisture (%)	10	0.04	0.04
Mean grain size (Ø)	2	1.451	1.456
CaCO_3 content (%)	5	15.0	

All the elements measured at the research site are low (Table 3), which is characteristic of dune sands (Maun 2009) including low TOC (average 2.15%) and limited P (272–340 ppm). Results for labile carbon (<0.02%) are lower, for example, than those on the east coast of Scotland (Wall *et al.*, 2002).

Table 3. Chemical analysis (0–10 cm), Thirteenth Beach incipient dune crest.

Location	acid extractable P (ppm)	TOC (%)	labile C (%)	total N (%)
The Hole	340	2.30	<0.02	0.021
Mid-way	272	2.12	<0.02	0.019
The Corner	287	2.04	<0.02	0.021

DISCUSSION

Literature on the nutritional status of Australian dune sands is lacking, but as with other beach systems, it is assumed there would be substantial spatial variation (Perumal and Maun, 2006). For example, Welsh dune soils were found to contain as little as 0.006–0.008% N, but had ‘appreciable’ amounts of P and K (Hassouna and Wareing, 1964). By comparison, the surface soils of New Zealand range from 0.09–0.87% TSN (Rayment and Lyons, 2011). Nonetheless, dune soils are generally poor in the macronutrients N, P and K (Hawke and Maun, 1988). They are also alkaline due to CaCO_3 sourced from marine environments, which may cause nutrient deficiencies, albeit reduce the toxicity of sodium chloride (McLachlan and Brown, 2006).

The availability of the majority of nutrients is reduced at pH_{Ca} 3.5–4, with the exception of Fe which becomes limited at pH_{Ca} 6.5–7.0 additionally, exchangeable Al becomes toxic at pH_{Ca} >4.7 (Hazelton and Murphy, 2007). The pH_{Ca} of the sand (Table 3) in a sand:silt:clay soil would allow all of the major nutrients to be available for plants, however as sand lacks cation exchange capacity (CEC) due to its lack of electrostatic charge and buffering capacity (Ashman and Puri, 2002), it tends to be nutrient poor (Maun, 2009). Nonetheless, particles of OM between the sand grains have a variable electrostatic charge which allows some exchange of nutrients, depending upon the chemicals in the soil solution (Ashman and Puri, 2002). Furthermore, the deposition of wrack from wave action would

periodically raise nutrient levels (Kennedy and Woods, 2013). Notwithstanding this, the Fe and Al levels would not suit the majority of plants.

The two main plants that occupy the incipient dune at Thirteenth Beach are hairy spinifex and sea wheatgrass. Literature on the Al tolerance of these plants is lacking, most likely as they are wild species that are generally not cultivated. However, if the plants are surviving the concentration of Al, they are likely to be tolerant to it. Wheat is tolerant to Al at pH_{Ca} 4.0–4.5 (Hazelton and Murphy, 2007), and as sea wheatgrass is a wild relative of wheat, Al levels would not be a limiting factor in its growth. Additionally, although acidic soils can impair the functioning of most microbial processes such as the breakdown and cycling of organic matter from which plants access nutrients and carbon (Gazey and Ryan, 2014), mycorrhiza have shown enhanced metal sorption capacity compared to other micro-organisms (Joner *et al.*, 2000). For example, mycorrhizal plants successfully inhabit many environments where soil acidity results in elevated levels of metals, such as in mine spoils and other heavy-metal contaminated sites (Göhre and Paszkowski, 2006).

Coastal dunes contain little or no clay or silt, therefore salts are easily leached down the profile, and thus the sand at Thirteenth Beach is not saline (Table 3), in agreement with previous literature on coastal sand salinity levels. For example, Barbour *et al.* (1976) found that along the leading edge of vegetation of 34 beaches on the Pacific Coast in the USA, the concentration of soluble salt at a depth of 10 cm, was 0.008–0.280%.

The average CaCO₃ of the sand is 15% (Table 3), and although rates of 10 to 20% CaCO₃ are regarded as high by Nordstrom *et al.* (1990), it is not the dominant component at Thirteenth Beach.

Although few beach systems have been researched sufficiently to provide nutrient budgets (McLachlan and Brown, 2006), the periodic deposition of OM (Kennedy and Woods, 2013), and rainfall and nutrients in sea spray (Maun 2009) aid in supplying nutrients. Total organic carbon (TOC) is largely the result of plant decay and the low levels of TOC at the site are reflected in the lack of woody plants and shrubs along the incipient dune. Labile carbon levels (<0.02%) are lower than those of Wall *et al.* (2002) who found levels of 0.1–0.6% in the beach and foredune at Tentsmuir Point, on the east coast of Scotland.

In general, pioneer plants require low quantities of N and P, and the limited level of phosphorus at the site (272–340 ppm) is offset by pioneer plants gaining access to P via by the extra-radical mycelium of AM fungi. Mycelia work like an extension to the plant roots, growing beyond the phosphorus depletion zone around the roots to take up P, as well as some trace elements from the edaphic environment (Koske *et al.*, 2004). The N percentage at Thirteenth Beach (average 0.020%) is higher than on beaches along the Atlantic Coast of Maine and New Hampshire USA for example, where levels are 0.004–0.012% N by weight (Croker *et al.*, 1975).

The toe of the incipient dune sits above MHWS but is not protected from storm surges and is therefore subject to scarping and plant loss. Although gradual or minor disturbances do not necessarily lead to decreases in AM hyphal abundance (Abbott and Robson, 1991), hyphal networks in soil are easily disrupted by rapid environmental changes such as storm events. Such

events prevent long-term plant colonization (Koske *et al.*, 2004), and as a result, the contribution of AM fungi to plant survival in incipient dunes has been little studied.

CONCLUSIONS

This study has sought to define the boundary conditions under which AM fungi are found in association with the invasive, exotic sea wheatgrass and the native hairy spinifex. The nutrients measured are low for most plant requirements, however pioneer plants require low quantities of N and P, surviving through adaptations to resource stresses, and through their symbioses with AM fungi and other soil microbes (Cockcroft and McLachlan, 1993). The data from our research will enable us to predict the how close to MHWS sea wheatgrass, hairy spinifex and AM fungi are likely to be found.

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Appendix II

The Biology of Australian Weeds

63. *Thinopyrum junceiforme* (Á. Löve & D. Löve) Á. Löve

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Name

Botanical name

The genus name *Thinopyrum* is derived from the Greek 'thino; this' for shore, and 'pyros', for wheat (Löve 1984), and the species name *junceiforme* is derived from the Latin 'junceus' for rush-like (Stearn 2004) and 'formis; forme' for resembling, shaped-like (Gledhill 2002).

Common names

The common name for *Thinopyrum junceiforme* (Figure 1) is sea wheatgrass. It is also referred to as sand couch-grass, sea couch (Hilton et al. 2006), and Russian wheatgrass (United States Department of Agriculture n.d.).

Taxonomy

The genus *Thinopyrum* comprises tetraploid plants ($2n = 4x = 28$) (Löve 1980, Jauhar and Peterson 2001). It is a member of the tribe of *Triticeae* (family *Poaceae*), with a number of the species being resistant to drought, salinity and disease (Niento-Lopez et al. 2000). There are three species complexes within the genus *Thinopyrum*: *T. elongatum* (tall wheatgrass) (Host) D.R. Dewey, *T. intermedium* (intermediate wheatgrass) (Host) Barkworth & Dewey and *T. junceum* (Russian wheatgrass) (L.) Á. Löve. The taxa in *T. junceum* have been categorized variously as species or subspecies in different genera such as *Triticum*, *Agropyron*, *Elymus*, *Elytrigia* or *Thinopyrum* (Moustakas et al. 1986). There are many intergeneric hybrids which complicate the taxonomy of wheatgrasses (Refoufi and Esnault 2006).

Thinopyrum junceiforme falls within the complex of *T. junceum*, and is found around the Atlantic and Baltic coasts (Niento-Lopez et al. 2003). Nomenclature is complicated, with the complex *T. junceum* also being denominated as *Agropyron junceum* (L.) P. Beauv., *A. junceum* ssp. *mediterraneum* Simonet & Guin., *Elytrigia juncea* (L.) Nevski, and *Elymus farctus* (Viv.) Runemark ex Melderis (Niento-Lopez et al. 2003). Tetraploid *Leymus* spp. have also been confused with *Thinopyrum*, as both

are perennial, wild relatives of wheat (Zhang and Dvorak 1991, Merker and Lantai 1997). *Leymus* occurs naturally in North America and Eurasia (Zhang and Dvorak 1991), and lacks the *J* genome of *Thinopyrum* (Wang and Jensen 1994). *Thinopyrum junceiforme* has also been denominated as *Agropyron junceiforme* (Á. Löve & D. Löve), *A. junceum* ssp. *boreoatlanticum* Simonet & Guin., *Elytrigia junceiformis* Á. Löve & D. Löve, and, like *T. junceum*, has also been known as *Elymus farctus* (Niento-Lopez et al. 2003). There is a clear morphological differentiation between the genera *Thinopyrum* and *Elymus*, with the former having rudimentary awns or lacking them altogether, whereas *Elymus* has wider laminae, and higher spike densities than *Thinopyrum* (Löve 1984, Niento-Lopez et al. 2000). The visual identification point for *Thinopyrum junceiforme* is a short, hirsute ligule that can be observed by pulling back the leaf blade at the collar region (Figure 2).



Figure 1. An incipient dune with a swale between it and the lee of the foredune. *Thinopyrum junceiforme* is growing in the swale and across the incipient dune note the small clump of *Spinifex sericeus* (circled) in the foreground (Photo: L.M. Hanlon).

The currently accepted name for the species is *Elytrigia juncea* ssp. *boreoatlantica* (Simonet & Guin.) Hyl. (Valdes et al. 2009). However in this review, it is referred to by one of its heterotypic synonyms, *Thinopyrum junceiforme*, which is the name used in the most recent Australian and New Zealand literature, for example Heyligers (2006), Hilton et al. (2006) and Hilton et al. (2007).

History

The coastal dune flora of Australia changed rapidly from the late 1800s, following the introduction of South African, American and European plants such as *Ammophila arenaria* (L.) Link (marram grass), *Cakile maritima* Scop. (European sea rocket), *Cakile edentula* (Bigelow) Hook. (sea rocket), and *Chrysanthemoides monilifera* subsp. *rotundata* (L.) Norl. (bitou bush) (Hilton et al. 2006). Prior to that, Australia had only a few species that were capable of colonizing the area between the swash and foredune such as *Atriplex billardiensis* (Moq.) Hook.f. (glistening saltbush) and *Spinifex sericeus* R.Br. (hairy spinifex) (Hilton et al. 2006). Sea wheatgrass now exerts a great influence over the establishment of both incipient dunes and foredunes, adversely affecting hairy spinifex colonies (Heyligers 1985, Hilton et al. 2006). Furthermore, in the foredunes, marram grass has displaced *Austrofestuca littoralis* (Labill.) E.B. Alexeev (beach fescue), and created

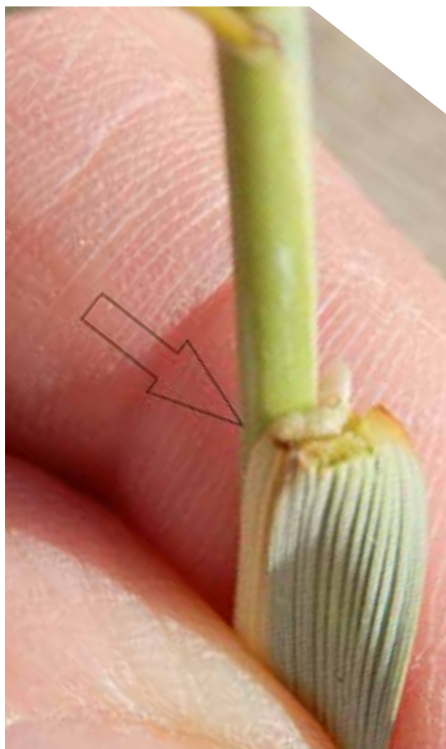


Figure 2. The short, hirsute ligule (arrowed) is a key identification characteristic for *Thinopyrum junceiforme* (Photo: L.M. Hanlon).

steep, unstable dunes (Heyligers 1985, Hilton *et al.* 2006).

Sea wheatgrass was first recorded in Australian herbarium records in 1933, with a specimen collected from Ricketts Point, Victoria (MEL 0626849A). It may well have arrived much earlier, in ballast or cargo from the Windjammers, or sailing vessels, which plied between Europe and southern Australia from the 1830s through to 1950 (South Australia Maritime Museum n.d.). Australian herbarium records show that the first sea wheatgrass collections from other states were from Rocky Cape, Tasmania in 1948 (HO77017) and the Long Beach sand dunes, South Australia in 1983 (AD98409214).

Distribution

World

Sea wheatgrass originates from the western European, Mediterranean, Atlantic and Baltic coasts (Figure 3) (Nieto-Lopez *et al.* 2000, Heyligers 2006, Hilton *et al.* 2007), where it plays a major role in dune establishment (Heyligers 2006) as a pioneer species in embryo and incipient sand dunes (Harris and Davy 1986a, Hilton *et al.* 2007). In active foredunes of northwest Europe, sea wheatgrass is one of the few plants that can withstand periodic, temporary sand burial (Doody 2013), thereby creating dunes that are rarely higher than 1 m in elevation (Hilton *et al.* 2006). The plant

has naturalized in Oregon and California on the west coast of the United States of America, where it is considered a native plant (United States Department of Agriculture n.d.). In Britain, sea wheatgrass is regarded as a primary dune colonizer, where it develops dense swards on pre-established foredunes (Harris and Davy 1986a). Sea wheatgrass is also found in southern New Zealand (Hilton *et al.* 2006).

Australia

In Australia, sea wheatgrass is found on the foredunes and incipient dunes of the southern coast of the mainland, from Henley Beach, Adelaide (34.92°S, 138.49°E) along the coast and inland to Naracoorte, on the South Australian border with Victoria (38.05°S, 140.94°E), and on the northern coast of Tasmania (Figure 3). In Victoria, it appears from the mouth of the Anglesea River (38.41°S, 144.18°E), around Port Phillip and Westernport Bays, including Phillip Island, and down to Wilsons Promontory National Park (39.06°S, 146.41°E) (Figure 3). Records do not indicate that it grows along the eastern coast of the mainland, except at Cape Conran Coastal Park, East Gippsland (37.79°S, 148.74°E). In Tasmania, sea wheatgrass is recorded from the mouth of Bottle Creek on the north west coast (41.1°S, 144.66°E) across to Cape Portland on the north east coast (40.75°S, 147.96°E) and it is also found on Flinders Island (40.00°S, 148.11°E) (Figure 3). The specimens recorded in the Australian Capital Territory are related to four samples collected from The Netherlands between 1962 and 1987, and grown in experimental plots (Australia's Virtual Herbarium –b).

Habitat

Climatic requirements

Sea wheatgrass is endemic across a wide latitudinal range in the Northern Hemisphere, extending from Finland (64°N, 26°E), to Spain's Cadiz region (36.5°N, 6.28°W) (Hilton *et al.* 2006). As with many coastal species, the grass occupies a niche of high temperatures, high salinity, desiccation and abrasion from winds, and extremes of soil moisture content (Hesp 1991, Levinsh 2006, Maun 2009). Additional factors in this dynamic niche include high light intensity and nutritional deficiencies (Hesp 1991, Martinez *et al.* 2001). Harris and Davy (1986a) argue that in their natural habitat, *Elymus farctus* (*Thinopyrum junceiforme*) tillers need vernalization in order to flower. Field observations from research in Australia note that the production of inflorescences is limited, and this may

be because Australian coastal winter temperatures are not as low as those in Britain (M.B. Mesgaran unpublished data).

Substratum

Sand dune soils are generally poor in the macronutrients, nitrogen (N), phosphorus (P) and potassium (K) (Hawke and Maun 1988, Zhang 1996). In Victoria, the coastal sediments are carbonate-dominated west of Wilsons Promontory and silicon oxide-dominated eastward (Bird 1993). Literature on the nutritional status of Australian dune sands is lacking. However, in a coastal dune system on the west coast of the South Island of New Zealand, Sykes and Wilson (1991) found that the alkaline sand had low P levels, ranging between 4.8 $\mu\text{m mL}^{-1}$ to 6.6 $\mu\text{m mL}^{-1}$, but N levels were not included as they were <0.05%. As with other beach systems, there would be substantial variations in soil nutrients (Hawke and Maun 1988, Zhang 1996, Perumal and Maun 2006), where for example, the deposition of wrack, or organic matter, on beaches from wave action would periodically raise nutrient levels (Zhang 1996). Therefore, sea wheatgrass needs to tolerate both low, and fluctuating, levels of nutrients, and also needs to be tolerant of a wide range of conditions from inundation by sea water and mobile sand. In Australia, the plant grows lower on the beach, and closer to the swash than any native species (Hilton *et al.* 2006); this could be because no other plants can survive repeated seawater inundation unless they are halophytes.

Sea wheatgrass is one of three species in the genus *Thinopyrum* that are salt tolerant, along with tall wheatgrass and *T. bessarabicum* (Savul. & Rayss) Å. Love, and this tolerance is controlled by multiple genes on several chromosomes (Wang *et al.* 2003). *Thinopyrum bessarabicum*, for instance, has been found to withstand hydroponic solutions of 350 mM NaCl for prolonged periods (Gorham *et al.* 1985), whereas halophytes grow in concentrations of 400 mM NaCl, or higher (Flowers 2004).

Plant associations

Both native and exotic plants grow in association with sea wheatgrass on incipient dunes and foredunes in Victoria (L.M. Hanlon personal observations). On the incipient dunes, such plants may include native hairy spinifex, and the exotics *Cakile* spp. (sea rockets) and *Euphorbia paralias* L. (sea spurge). On the foredunes, the vegetation is more varied and includes the native plants *Atriplex cinerea* Poiret X *A. paludosa* (coast saltbush), and

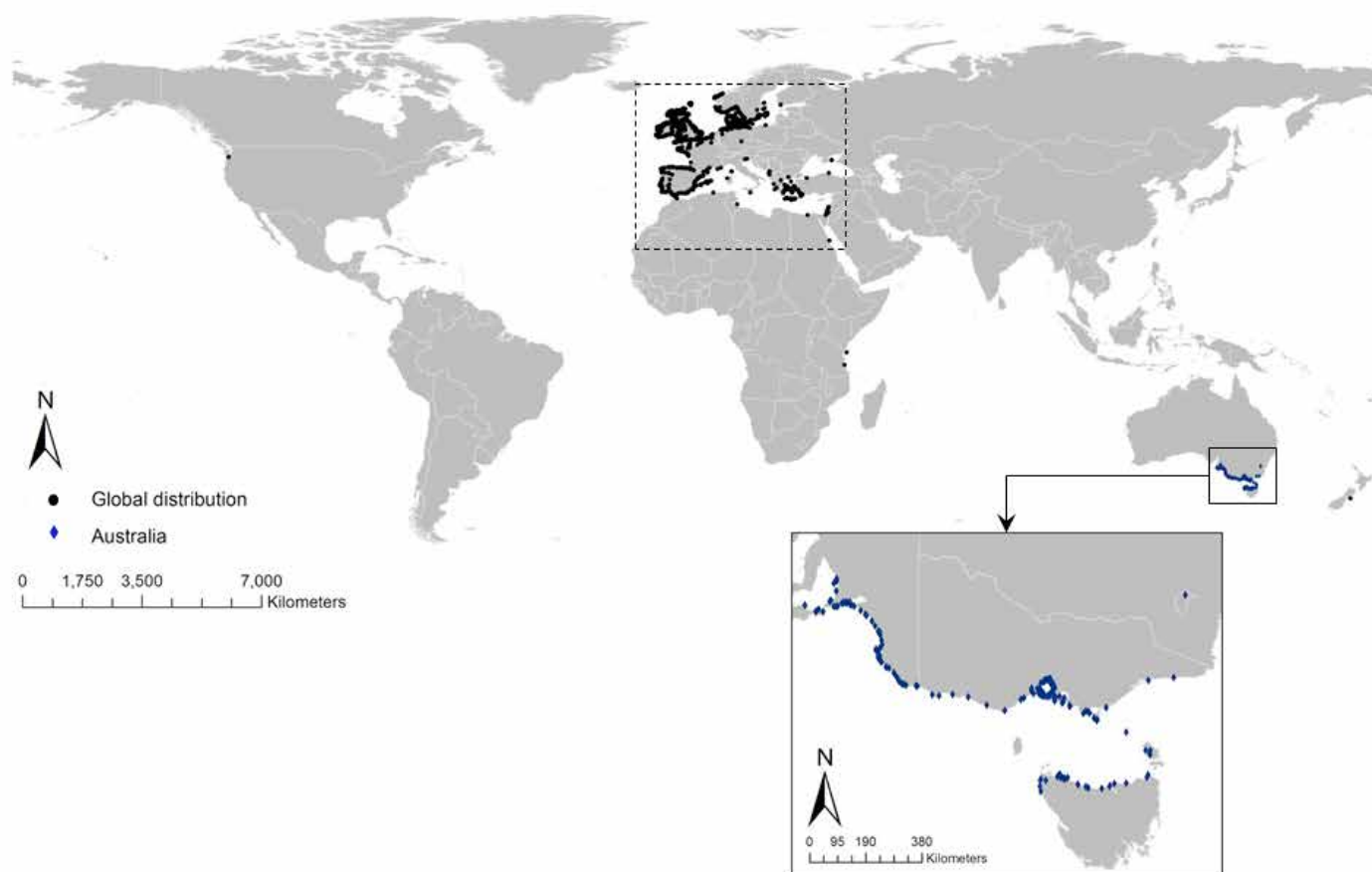


Figure 3. Global distribution of *Thinopyrum junceiforme*, with implied native range in dotted box (GBIF n.d.) and Australian distribution in the solid-line box (Australia's Virtual Herbarium n.d. –a).

Ficinia nodosa (Rottb.) Goetgh., Muasya & D.A. Simpson (knobby club rush), with *Lepidosperma gladiatum* Labill. (coast sword-sedge) growing in the swale in the lee of incipient dunes. On open sites on the foredunes, hardy succulents such as native and naturalized *Carpobrotus* spp. (pigface), and native plants *Rhagodia baccata* (Labill.) Moq. (seaberry saltbush) and *Tetragonia tetragonioides* (Pall.) Kuntze (sea spinach), manage the inhospitable environment remarkably well, as does the native *Geranium solanderi* var. *solanderi* Carolin (native geranium). Exotic weed species native as *Fumaria* spp. (fumitory), *Oxalis* spp. (soursob) and *Allium triquetrum* L. (angled onion) are just a few of the smaller flowering plants, also found in association with sea wheatgrass on the foredunes.

Growth and development

Morphology and physiology

Sea wheatgrass is a rhizomatous, perennial grass, growing to approximately 50 cm in height, but under favourable conditions it can grow as tall as 80 cm. Plants can grow from a single node and produce as many as 20–100 tillers. The blue-green leaves of sea wheatgrass are glabrous

below and finely pubescent above, usually 30 cm in length, but they can grow up to 50 cm, with the widest part of the blade varying from 3 mm to 8 mm (average 5 mm) (M.B. Mesgaran unpublished data). Most populations from South Australia have wider leaves than those of Victoria or Tasmanian populations (M.B. Mesgaran unpublished data). Spike lengths are about 15 cm, with each spike bearing 10 spikelets. Sea wheatgrass is a C3 cool-season grass, but other data describing its physiology or biochemistry are lacking. Notwithstanding this, temporary burial is a common occurrence in plants of sandy environments, and many plants such as sea wheatgrass survive such burial, with the short-term suspension of physiological activity such as photosynthetic capacity, which is quickly reinstated once uncovered (Harris and Davy 1988, Perumal and Maun 2006). This is due to newly-emerged leaves from previously buried plants having an increased chlorophyll content (mg g^{-1} fresh weight), and a higher energy content in subterranean organs (Yuan et al. 1993, Perumal and Maun 2006).

Young plants of sea wheatgrass lack the axillary meristems and energy reserves required to grow new shoots when buried, and they re-allocate their resources from

non-photosynthetic organs to maintain the photosynthetic ones, until the plant is uncovered by winds or storms (Harris and Davy 1988). It has been shown that multi-node rhizome fragments have more success in emergence, and from greater depths, than do single-node fragments of sea wheatgrass (Harris and Davy 1986b).

Phenology

In Britain, sea wheatgrass requires vernalization in order to flower; however flowering usually occurs in the second year of growth, and Harris and Davy (1986a) found that such flowering was limited due to the proximity of plants to wave disturbance along the swash, as well as grazing by rabbits. It is hypothesized that as the winters in Australia are not as cold as those in Europe, flowering is limited, appearing in December, January, and occasionally in February (Figure 4) (M.B. Mesgaran unpublished data), although the flowers do not persist for long on the stems (Rudman 2003).

The seasonal variation in the ability of sea wheatgrass rhizome buds to produce adventitious shoots and roots under glasshouse conditions was found to be strongly correlated to nitrogen as a limiting factor, with carbohydrate

reserves also implicated (Harris and Davy 1986b). Additionally, dormancy was found to show a peak in late winter and early spring, with a sharp decline in late spring-early summer (Harris and Davy 1986b). This variation was inversely related to the growth rate of the parent plants at time of harvesting (Harris and Davy 1986b).

Mycorrhiza

Approximately half of the 150 described species of arbuscular mycorrhizal (AM) fungi are found in sand dunes (Koske et al. 2004). In particular, *Glomus intraradices* N.C. Schenck & G.S. Sm. a species of AM fungi, was shown to be highly tolerant of harsh conditions such as aridity and salinity, reducing the concentration of sodium in the shoots of plants in saline environments (Yamato et al. 2012). However, there is no literature specifically on the involvement of AM fungi with sea wheatgrass, although grasses in general tend to be facultatively mycorrhizal (Brundrett

et al. 1996), or weakly mycorrhizal (Ramos-Zapata et al. 2011) in their association with AM fungi. The early work of Forster (1979) which focussed on aggregation of sand by plants and microbes, asserted that microbial abundance associated with sea wheatgrass (under the heterotypic synonym of *Agropyron junceiforme*) was less in winter when annual species were dying, and many perennial species were dying back.

Reproduction

Floral biology

Sea wheatgrass can reproduce both sexually and asexually (Löve 1984), but production of flowering tillers in Britain (Harris and Davy 1986a) and mature seeds in Australia (M.B. Mesgaran unpublished data) is low. In Australia, the main route of propagation is by rhizome growth and fragmentation. The plant has cells with four sets of chromosomes (Merker and Lantai 1997, Jauhar and Peterson 2001),

with the genome of $J_1J_1J_2J_2$ (Colmer et al. 2006), and is a self-crosser, although it is capable of cross-fertilization (Moustakas et al. 1986). Refoufi and Esnault (2006) contend that it has little genetic diversity, as assessed by isozymes and RAPD markers.

Dispersal

Hydrochory, or the passive dispersal of propagules by water, is the most likely vector for the dispersal of sea wheatgrass caryopses along the coastline of southern Australia. Drift bottle programmes by Olsen and Shepherd (2006) demonstrated that surface water flows along the South Australian coast, east through Bass Strait, then south east past the west coast of Tasmania in winter, reversing the direction in summer. Thus, currents could be responsible for the transportation of plant fragments to the shores of Tasmania from the mainland. Heyligers (2007) noted that dispersal patterns of four other introduced species to Australian beaches follow the circulation of ocean currents around Australia, and such currents may have been responsible for the dispersal of propagules to the South Australian coast. Although no research has been conducted on the dispersal mechanisms of sea wheatgrass, it is likely to be via such fragments being scarpred, or torn, from eroding sand dunes during storms (Hilton et al. 2006, Hilton et al. 2007). For example, following a severe storm on the Norfolk (UK) coast in 1978, sea wheatgrass (under the heterotypic synonym *Elymus farctus*), rapidly re-colonized the swash, and both seeds and fragments of rhizomes were of equal importance in establishing new clumps, and in producing similar tiller densities (Harris and Davy 1986a). Similar recruitment was observed in the 1960s at Shallow Inlet, Wilsons Promontory where a sand spit developed after the previous spit was washed out in 1901. The new spit initially lacked vegetation but in the 1960s, sea wheatgrass was found to have colonized it (Heyligers 2006). Clumps of sea wheatgrass rhizomes have also been observed at the mouth of the Glenelg River at Nelson in Victoria, where there was no actively growing parent plant on the beach, suggesting that the rhizomes might have been dispersed by hydrochory (M.B. Mesgaran unpublished data).

Physiology of seeds and germination

Woodell (1985) suggested that seed germination patterns of coastal plants can be separated into three categories and that their response to germination in saline conditions correlated with their habitat. He found that the greatest



Figure 4. Flowering spike of *Thinopyrum junceiforme* (Photo: M.B. Mesgaran).

germination response of sea wheatgrass seeds ($n=180$) was in freshwater (53%), followed by 18% in half-strength sea water, 5% in full-strength seawater, and the complete inhibition (0%) in one and a half strength sea water (sodium chloride (NaCl) concentration 600 mM) (Woodell 1985). Seeds from all treatments recovered sufficiently when transferred to distilled water for some germination to occur (Woodell 1985). Even though the seeds germinated under controlled conditions, it is likely that under natural conditions, sea wheatgrass seeds in the swash may be stimulated to germinate following precipitation. That sea wheatgrass seeds do not germinate in full-strength sea water may also aid in its dispersal by keeping seeds dormant and the embryo surviving on the endosperm within the seed coat until the seed reaches land.

Vegetative reproduction

In Australia, reproduction is largely vegetative from new shoots off nodes along the highly meristematic rhizomes, which are produced in great lengths (Figure 5). Under glasshouse conditions, each 5 cm node of sea wheatgrass produced up to 30 m rhizome length in the course of one season (M.B. Mesgaran unpublished data). The length of most internodes was approximately 7 cm, and skewed toward lengthier internodes (Figure 6).

On land, single-node sea wheatgrass rhizome fragments can emerge from depths of up to 17 cm. Multi-node fragments can emerge from greater depths (>17 cm), producing more emergent shoots and more quickly than single-node shoots, especially in late winter to early spring (Harris and Davy 1986b).

Population dynamics

Population dynamics data for sea wheatgrass in Australia are not available. The maximum life-span of the species is unknown, as are mortality rates between life stages, apart from the rate of regeneration after dispersal by water, or burial by sand, discussed above. Research is required on population dynamics to enable the development of weed management strategies, rather than *ad hoc* herbicide applications. Such strategies could be used by all beach management authorities.

Importance

Detrimental

In Victoria, anecdotal evidence suggests that sea wheatgrass is impacting upon the rookery of *Eudyptula minor* J.R. Forster (fairy penguin) on Phillip Island's Summerlands beach, by creating



Figure 5. Length of rhizomes from one *Thinopyrum junceiforme* plant harvested in the field (Photo: L.M. Hanlon).

steep-fronted incipient dunes that are too high for the birds to climb in order to access their burrows (P. Dann personal communication 2013). Furthermore, on Phillip Island and beaches in Victoria such as those on the Barwon Coast and Geelong, the endangered *Thinornis rubricollis* Gmelin (hooded plover), which prefers a nest scrape with little vegetation

on which to lay its eggs, is likely to have its nesting sites encroached upon by sea wheatgrass (Cousens *et al.* 2012). Steep-fronted incipient dunes that are thickly vegetated with sea wheatgrass are also found along the South Australian coast, such as at Normanville (Figure 7).

Hilton *et al.* (2006) proposed that the sand-binding ability of sea wheatgrass

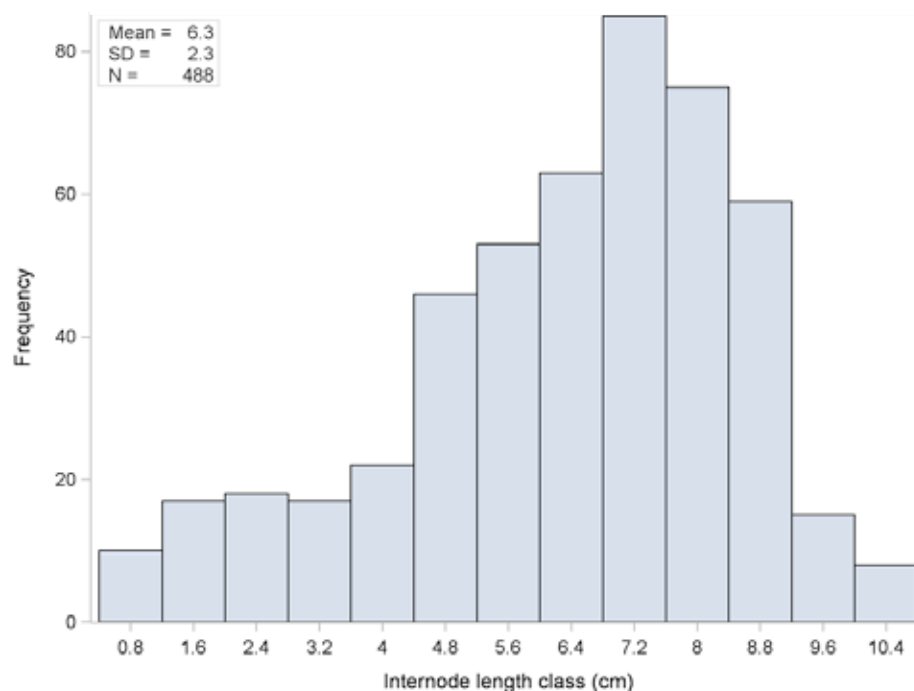


Figure 6. Rhizome internode length from one *Thinopyrum junceiforme* plant grown under glasshouse conditions, in the course of one season. The rhizome was 30 m long, with internodes approximately 7 cm apart, skewed towards lengthier internodes (M.B. Mesgaran unpublished data).

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Figure 7. Steep-fronted incipient sand dune vegetated by *Thinopyrum junceiforme* in Normanville, South Australia (Photo: M.B. Mesgaran).

makes it more resilient to erosive processes in comparison to native flora. Indeed, sea wheatgrass is one of four exotic species that were noted by Heyligers (1985) as being more efficient than native species at trapping sand and building dunes where otherwise dunes would not have formed. Such dunes have the potential to limit sediment movement, thereby changing the ecosystems and geomorphology of the coastlines on which they appear. Thus it is of concern that sea wheatgrass can rapidly colonize the swash and incipient dunes after propagules are washed ashore following storms. One example where sea wheatgrass has spread with great rapidity is along the Younghusband Peninsula in South Australia, where James (2012) reports that the plant has spread at approximately 18 ha per year, outcompeting native species and altering ecosystems.

Beneficial

Sea wheatgrass is a potential gene source for salt tolerance in wheat (Wang *et al.* 2003), which has been investigated in Triticeae in general, as well as in *T. bessarabicum* (Gorham *et al.* 1985, Gorham *et al.* 1986). Sea wheatgrass has also been investigated as a potential gene source for scab resistance in wheat (Jauhar and Peterson 2001).

Legislation

Groves *et al.* (2003) list sea wheatgrass as a weed species, albeit not specifically a coastal weed, as there is no formal listing or category for coastal weed species. Cousens *et al.* (2012) note that

sea wheatgrass is one of the dominant exotic species on the coast displacing native vegetation. However, many coastal managers, including state government co-ordinators, are unaware of the existence of sea wheatgrass, let alone its potentially adverse impacts (R. Cousens personal communication 2014), which may explain the lack of specific legislation related to this species.

Weed management

Herbicides

Phillip Island Nature Parks (PINP) in Victoria, has used Verdict™ 520, Fusilade™, Starane™, glyphosate and metsulphuron-methyl at the recommended rate for similar plants, as well as lower rates (J. Fallow personal communication 2014). Staff at PINP report that the lower rates of herbicides were more efficient than the recommended rate, as it is believed that lower rates allow for translocation of the herbicide throughout the plant, rather than killing the aerial parts alone via one larger application.

The Barwon Coast Foreshore Committee of Management, Victoria, has also used Verdict™ 520. The application rate was 50 mL of Verdict 520 per 100 L of water, plus 500 mL of Uptake™ spray oil added to the product, as an adjuvant to improve the spreading and wetting qualities of the herbicide (W. Chapman personal communication 2014). It has been reported that this treatment is making an impact on sea wheatgrass, with minor off-target damage to native grasses (W. Chapman personal communication 2014). Additionally, it has been found that two applications, 2–4 weeks

apart, as suggested by the manufacturer of Verdict™ 520 for use on other invasive grasses, were not required for the suppression of sea wheatgrass (W. Chapman personal communication 2014).

Natural enemies

On Phillip Island, and around the Barwon Coast, *Theba pisana* (Müller) (sandhill snail) has been observed by the authors to graze on sea wheatgrass, causing damage to leaves. However this invertebrate is not specific to sea wheatgrass or coastal areas, and is also found in land crops in Australia (Fox 2011).

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Appendix III

Total OTU and species richness in plant roots and sand from the incipient dune and foredune at Thirteenth Beach, Victoria, between The Hole and The Corner, September 2016. Singletons have been removed.

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
1	<i>Claroideoglossus torrecillas</i>	7	8	8	2	7	32
2	<i>Claroideoglossus</i> sp.	8	7	6	1	4	26
3	<i>Claroideoglossus torrecillas</i>	7	7	6	2	5	27
6	<i>Glomus</i> sp.	6	4	4	5	6	25
9	<i>Glomus goomara</i>	3	1	2	5	2	13
10	<i>Glomus</i> sp.	4	4	3	6	5	22
14	<i>Glomus</i> sp.	6	5	8	6	4	29
15	<i>Claroideoglossus</i> sp.	7	6	5		3	21
16	<i>Glomus</i> sp.	2	5	3	6	4	20
19	<i>Glomus</i> sp.	1	1		1	3	6
20	<i>Glomus</i> sp.	1	2		2	4	9
21	<i>Glomus</i> sp.	2	1	2	5	1	11
24	<i>Scutellospora</i> (Early-31)	2	4	1	2	4	13
26	<i>Glomus</i> sp.	2	8	3	1	2	16

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
32	<i>Glomus</i> sp.	1	1	2	4	1	9
33	<i>Glomus</i> sp.	1		1	1	2	5
35	<i>Glomus</i> sp.	2	3	1			6
37	<i>Scutellospora</i> (Early-39)			2	1	3	6
50	<i>Glomus yamato</i> o8				3	3	6
59	<i>Scutellospora</i> sp.					2	2
66	<i>Scutellospora</i> sp.	2	2	1	1	1	7
73	<i>Diversispora torrecillas</i>	4	2			1	7
78	<i>Glomus yamato</i> o9			1	1		2
87	<i>Archaeospora schenckii</i>	2	1				3
98	<i>Glomus</i> sp.	5	4	4		1	14
117	<i>Glomus</i> sp.				2		2
142	<i>Glomus</i> sp.		2				2
154	<i>Glomus</i> sp.		1		4	2	7
166	<i>Diversispora</i> sp. 8479	5	1				6
167	<i>Diversispora</i> sp.				3		3
211	<i>Glomus</i> sp.				3		3
227	<i>Scutellospora</i> (Early-31)		1			2	3
229	<i>Glomus</i> sp.	2	1		4	1	8
251	<i>Glomus</i> sp.	1	2				3
253	<i>Glomus</i> sp.				1	1	2

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
268	<i>Glomus</i> sp.		1	1	4	4	10
269	<i>Glomus</i> sp.			2	3	1	6
278	<i>Claroideoglomus</i> sp.	6	8	7	1	4	26
298	<i>Diversispora</i> sp.	2					2
300	<i>Glomus</i> sp.	2	3		5	2	12
301	<i>Glomus</i> sp.	1			3		4
307	<i>Claroideoglomus</i> sp.	1	2				3
313	<i>Glomus</i> sp.		2				2
315	<i>Diversispora</i> sp.	4	2		1	1	8
336	<i>Diversispora</i> MO-GC1	2	1				3
351	<i>Glomus</i> sp.				1	2	3
352	<i>Claroideoglomus</i> sp.	6	7			1	14
355	<i>Glomus</i> sp.	1	4				5
364	<i>Scutellospora calospora</i>				1	1	2
365	<i>Claroideoglomus</i> sp.	3	5				8
371	<i>Claroideoglomus</i> sp.	2	1				3
372	<i>Glomus</i> sp.					2	2
376	<i>Glomus</i> sp.	2	5	3	5	2	17
377	<i>Glomus</i> sp.	2	1		4	2	9
379	<i>Glomus</i> sp.	1		1	1	1	4
388	<i>Glomus</i> sp.	2	4	1	5	2	14

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
395	<i>Claroideoglomus</i> sp.	4	6			1	11
397	<i>Claroideoglomus</i> sp.	6	8			1	15
398	<i>Glomus</i> sp.				1	2	3
399	<i>Glomus</i> sp.	2	3		5	1	11
411	<i>Claroideoglomus</i> sp.	4	5				9
415	<i>Glomus</i> sp.	3	4	1	5	1	14
417	<i>Glomus</i> sp.	4	6	3	5	4	22
420	<i>Glomus alguacil</i> o9b			1	1	3	5
422	<i>Glomus</i> sp.	2	2	1	3	2	10
423	<i>Glomus</i> sp.				1	1	2
425	<i>Claroideoglomus</i> sp.	5	6			1	12
428	<i>Glomus</i> sp.				2		2
431	<i>Glomus</i> sp.	1			2		3
434	<i>Glomus</i> sp.				3	2	5
438	<i>Glomus</i> sp.	1	1		5	1	8
439	<i>Glomus</i> sp.		1			1	2
440	<i>Glomus alguacil</i> o9b	3	1	1	1	2	8
73		155	173	85	140	122	675

Appendix IV

Glomus spp. OTUs, and location in the dune system.

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
6	<i>Glomus</i> sp.	6	4	4	5	6	25
10	<i>Glomus</i> sp.	4	4	3	6	5	22
14	<i>Glomus</i> sp.	6	5	8	6	4	29
16	<i>Glomus</i> sp.	2	5	3	6	4	20
19	<i>Glomus</i> sp.	1	1		1	3	6
20	<i>Glomus</i> sp.	1	2		2	4	9
21	<i>Glomus</i> sp.	2	1	2	5	1	11
26	<i>Glomus</i> sp.	2	8	3	1	2	16
32	<i>Glomus</i> sp.	1	1	2	4	1	9
33	<i>Glomus</i> sp.	1		1	1	2	5
35	<i>Glomus</i> sp.	2	3	1			6
98	<i>Glomus</i> sp.	5	4	4		1	14
117	<i>Glomus</i> sp.				2		2
142	<i>Glomus</i> sp.		2				2
154	<i>Glomus</i> sp.		1		4	2	7
211	<i>Glomus</i> sp.				3		3
229	<i>Glomus</i> sp.	2	1		4	1	8
251	<i>Glomus</i> sp.	1	2				3
253	<i>Glomus</i> sp.				1	1	2
268	<i>Glomus</i> sp.		1	1	4	4	10
269	<i>Glomus</i> sp.			2	3	1	6
300	<i>Glomus</i> sp.	2	3		5	2	12
301	<i>Glomus</i> sp.	1			3		4
313	<i>Glomus</i> sp.		2				2
351	<i>Glomus</i> sp.				1	2	3
355	<i>Glomus</i> sp.	1	4				5
372	<i>Glomus</i> sp.					2	2
376	<i>Glomus</i> sp.	2	5	3	5	2	17
377	<i>Glomus</i> sp.	2	1		4	2	9
379	<i>Glomus</i> sp.	1		1	1	1	4
388	<i>Glomus</i> sp.	2	4	1	5	2	14

OTU	SPECIES	Foredune sand	Foredune veg	Incipient sand	<i>Spinifex sericeus</i>	<i>Thinopyrum junceiforme</i>	TOTAL
398	<i>Glomus</i> sp.				1	2	3
399	<i>Glomus</i> sp.	2	3		5	1	11
415	<i>Glomus</i> sp.	3	4	1	5	1	14
417	<i>Glomus</i> sp.	4	6	3	5	4	22
422	<i>Glomus</i> sp.	2	2	1	3	2	10
423	<i>Glomus</i> sp.				1	1	2
428	<i>Glomus</i> sp.				2		2
431	<i>Glomus</i> sp.	1			2		3
434	<i>Glomus</i> sp.				3	2	5
438	<i>Glomus</i> sp.	1	1		5	1	8
439	<i>Glomus</i> sp.		1			1	2
42		60	81	44	114	70	369

Appendix V

Coastal Plants of the Bellarine Peninsula, ECV 1, Coastal Dune Scrub/Coastal Dune Grassland Mosaic (Bellarine Catchment Network 2010)

Botanical nomenclature	Common name	Flowering time
<i>Acacia sophorae</i>	Coast Wattle	July – October
<i>Actites megalocarpa</i>	Dune Thistle	September – July
<i>Alyxia buxifolia</i>	Sea Box	October – February
<i>Apium prostratum</i>	Sea Celery	September – February
<i>Atriplex cinerea</i>	Coast Saltbush	September – March
<i>Austrostipa stipoides</i>	Prickly Spear-grass	October – March
<i>Carpobrotus rosii</i>	Pig Face	August – January
<i>Clematis microphylla</i> var. <i>microphylla</i>	Small-leaf Clematis	July – November
<i>Correa alba</i>	White Correa	Throughout the year
<i>Dianella brevicaulis</i>	Coast Flax-lily	August – May
<i>Isolepsis nodosa</i>	Knobby Club-rush	Throughout the year
<i>Lepidosperma gladiatum</i>	Coast Sword-sedge	September – March
<i>Leptospermum laevigatum</i>	Coast Tea-tree	August – October
<i>Leucophyta brownii</i>	Cushion Bush	September - December
<i>Leucopogon parviflorus</i>	Coast Beard-heath	July – November
<i>Muehlenbeckia adpressa</i>	Climbing lignum	September -December
<i>Myoporum insulare</i>	Native Juniper	October – November
<i>Olearia axillaris</i>	Coast Daisy-bush	February - April
<i>Olearia glutinosa</i>	Sticky Daisy-bush	February – April
<i>Poa poiformis</i> var. <i>poiformis</i>	Coast Tussock-bush	September – January
<i>Pultenaea tenuifolia</i>	Slender Bush-pea	September – March

Botanical nomenclature	Common name	Flowering time
<i>Senecio pinnatifolius</i>	Dune Groundsel	September – March
<i>Spinifex sericeus</i>	Hairy Spinifex	September – January
<i>Swainsona lessertifolia</i>	Coast Swainson-pea	June – October
<i>Tetragonia implexicoma</i>	Bower Spinach	August – February
<i>Zygophyllum billardierei</i>	Coast Twin-leaf	Throughout the year