Evaluating the functional role of betacyanin in salinity tolerance of Horokaka (*Disphyma australe*)

By

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Green-leafed *Disphyma australe* growing on the rocks in Te Kopahou Reserve, Wellington.

Abstract

Yield loss in agriculture due saline soils is a growing problem in arid and semi-arid regions as traditional crop species are inherently sensitive to salinity in the root zone. In the face of diminishing fresh water resources it is necessary to explore the traits which allow naturally salt tolerant species to exploit high saline environments. In the hope of transferring these traits via genetic modification to traditional crop species, or utilising these species as niche crops in their own right. While a majority of plants appear green, red pigmented plants are commonly associated with marginal environments. In these leaves anthocyanins or less commonly betalains are responsible for leaf reddening. The betalains are small class of tyrosine derived chromo alkaloids found in the core *Caryophyllales* and in some Basidiomycetes. There are two structural groups: the red/violet betacyanins and the yellow/orange betaxanthins. Due to this distribution pattern, betalain pigments are thought to function in salinity stress tolerance. However, minimal research has been conducted to support this salinity tolerance hypothesis due to a lack of an appropriate model species.

Horokaka (*Disphyma australe*) exhibits colour dimorphism among populations, green and red morphs grow contiguously in coastal environments where the frequency of red morphs positively correlates with increased substrate salinity. Betacyanins have previously been implicated in serving a photo protective for *D. australe.* In dimorphic populations *D. australe* along the south Wellington coastline, the red morph has been shown to be more tolerant to the combination of high light and salinity, as measured by higher CO2 assimilation rates, reduced inhibition of PSII and enhanced water use efficiency relative to the green morph. In these studies, betacyanin production in the red morphs was shown to depend on duel exposure to both salinity and high light, however the green morph was unable to produce betacyanin under the same conditions (Jain & Gould, 2015). This easy manipulation of leaf colour by salinity and high light offers a system to study whether betacyanin pigments aid salinity tolerance. I aimed further investigate the photo protective hypothesis of betalain using *D. australe*, and how this may influence distribution patterns by focusing on three areas: the capacity for new root growth along a salinity gradient, germination capacity under saline conditions, and ion content in the roots at low, moderate and high NaCl concentrations.

Shoots with no roots and a minimum of two mature leaf pairs were cut from green and red morphs of *D.asutrale* growing in the greenhouse facilities at Victoria University of Wellington. The shoots were grown hydroponically in 10% Hoaglands solution supplemented or not with (50, 100, or 150 mM) NaCl. To test the light screening capacity of leaf betacyanin a red filter was secured of half the green shoots, the cuttings were grown for 5 weeks under a controlled 16h light/ 8h dark photoperiod. Final weights of the shoot and roots, along with tissue water content of the shoots and roots were obtained to establish the relative capacity for new root growth when subjected to increasing salinity. Seeds were germinated *in vitro* in the presence of increasing NaCl concentrations (0, 100, 200, 300, and 400 mM NaCl), and subject to recovery tests after stress. The germination percentages and velocity were determined to establish te relative tolerance and competitiveness of the two *D. australe* morphs. Salt treatments were also applied to plants with an established root system, by 14-day treatment with increasing NaCl concentrations (0, 200, 400, 800 mM). The tissue water content of the shoots and ion contents (Na^+ and K^+) in the roots were determined in the control and the stressed plants of the two colour morphs. The different germination behaviour of the two morphs and capacity for root development appears to contribute to their distribution along a salinity gradient. Despite some differences under the control treatment, the concentrations of the two ions (Na+ and K+) were similar in the two morphs, not explaining differences in salinity tolerance, except for the increase of K+ in the roots of the green morph in the absence of NaCl. This specific response may be relevant for distribution patterns in D. australe.

The ecological implications of these findings, which can contribute to vegetation distribution of *D. australe* in coastal environments, and the relevance of betacyanin accumulation in salinity tolerance for halophytes, and potential application for improved crop vigour are discussed.

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1 Thesis background

1.1 The salinization of cultivated land

Set against a backdrop of finite natural resources, increases in global agricultural productivity are important to ensure sufficient availability of food and raw materials for a growing population (Qaim, 2011). Occupying 38% of the earth's terrestrial surface (Foley et al., 2011), agriculture is integral to human survival. If well-managed, it can contribute to the maintenance of water quality, erosion control, biological pest control, pollination and economic development. Modern agriculture is characterized by being highly mechanized and specialized (Foley et al., 2011; FAO, 2018). However, there are several features that are rendering cultivated land vulnerable to the effects of high salinity in the root zone of conventional crop species, such as the weathering of parent rock material and irrigation with poor quality water (Deinlein et al., 2014; Panta et al., 2014; Munns & Gilliham, 2015; Byrt et al., 2018a). High soil salinity, also referred to as soil salinization, is having detrimental impacts on agricultural production worldwide through the accumulation of soluble salts to toxic levels in plant tissues. This salinization inhibits growth, reducing the productivity of crop plants (Läuchli & Grattan, 2011). Together with climate change, salinization of the earth's soil is perpetuating an interlaced human and environmental challenge (Foley et al., 2011). Diverting resources away from the human food chain, for purposes such as biofuel production and fodder for livestock, further impose threats to food security, driving the need to close yield gaps. Additionally, agriculture is now a dominant force behind many environmental threats, including climate change, biodiversity loss and degradation of land and freshwater (Foley et al., 2011). With a need to protect forests and other areas, we can no longer support the expansion of agriculture through land clearing. While improved agricultural management will help to prevent further degradation, future research must focus on closing yield gaps to achieve optimal production levels through integrating changes to agricultural practices by way of addressing how we farm and what we farm alongside embracing genetic modification technologies.

1.1.1 Soil salinization

Chlorides and sulfates of sodium, calcium, and magnesium, are the predominant soluble salts found in soil and water (Munns & Tester, 2008; White *et al.*, 2014). Of these, sodium chloride (NaCl) is the most soluble and abundant salt released (Munns & Tester, 2008; Panta *et al.*,

2014; Almeida *et al.*, 2017; Alharby *et al.*, 2018). Soils are classified as saline when the concentration of soluble salts in soil and water exceeds an electrical conductivity of soil extract (ECe) of 4 dS/m^{-1} at 25ºC (Läuchli & Grattan, 2011; Wu, 2018), this is approximately 100 mM NaCl (Muchate *et al.*, 2016). Excess salinity contributes to the degradation of soils, leading to hard setting, suppressed hydraulic conductivity, restricted air, and water movement, run-off, and erosion. These effects manifest themselves in vegetation through reduced water availability and oxygen content, impaired root penetration and seedling emergence (Jesus *et al.*, 2015).

1.1.2 Causes of salinization

Researchers distinguish between primary and secondary causes of salinity (Panta *et al.*, 2014). Primary salinity arises through natural causes such as rainfall, salt from the ocean, and the weathering of parent rocks. Secondary salinity is soil salinization owing to and intensified by human behaviour and poor management; such as land clearing and irrigation practices (Panta *et al.*, 2014; Almeida *et al.*, 2017). Land clearing involves the removal of deeply rooted vegetation, which causes an accumulation of water that would have otherwise been utilized by vegetation, and the subsequent rising of the water table; this process is known as dryland salinization. With irrigation, the accumulation of salts in arable soils may be attributed to the use of poor quality irrigation water (Läuchli & Grattan, 2011; Deinlein *et al.*, 2014). Current agricultural systems rely heavily on groundwater for irrigation. Consequently, irrigation systems are prone to salinization owing to the presence of dissolved mineral salts in water supplies (Läuchli & Grattan, 2011); about half the existing irrigation systems in the world are under the influence of either salinization, alkalization or waterlogging (Munns & Gilliham, 2015). Urbanization and industrialization are driving the demand for freshwater for energy production, mineral extraction, and domestic use. Thus, this increasing scarcity of freshwater is promoting irrigation with poor quality water (saline and sodic water), enhancing the risk of secondary salinization (Scudiero *et al.*, 2016). Of the 230 million ha of irrigated land 20% (45 million ha) is impacted by secondary salinity (Munns & Tester, 2008; Daliakopoulos *et al.*, 2016). Soil salinity is a threat to arid and semi-arid regions these include but are not limited to Pakistan, India, Argentina, China, USA, and Australia (Daliakopoulos *et al.*, 2016). However, Developing nations will be disproportionately impacted by salinization as they rely on agricultural production for economic growth and possess fewer resources to counter the effects of yield loss due to salinization (Qaim, 2011). Salinization will also indirectly affect developed

nations that rely on external production for food supply and consumption (Foley *et al.*, 2011; FAO, 2018).

1.1.3 Risk to global food security

While, not all agricultural regions are equally at risk of soil salinization, the continued salinization of agricultural land poses a significant threat to global food security. Today, one third of food crops is grown on irrigated land (Munns & Tester, 2008; Yang & Guo, 2018). Three quarters of the world's food is derived from just 12 plants and five animal species and just under half the daily calorie intake globally comes from three of these plants: wheat, rice and maize (FAO, 2018). The global population is predicted to reach 9.8 billion by 2050. Based on current levels of consumption, global food production will need to increase up to 70-100 % by this time to match this growth (Panta *et al.*, 2014). As the world population grows, so too will demand for crops for biofuels, medicines and the human food chain, which includes food for both direct consumption such as fruits and vegetables, and food for livestock. Limited food availability already affects more than 800 million people, and aside from producing crops for food, we rely on these resources for livestock, biofuels and medicinal purposes (Foley *et al.*, 2011; Shabala, 2013; Grigore & Toma, 2017). The ongoing salinization of arable land requires that urgent solutions be found to increase the salinity tolerance in traditional crops, and to exploit genetic diversity. Improvements in agricultural practices and crops have seen a steady rise in yields over the past decades. However, the rate has since been declining.

1.2 What are the possible solutions?

1.2.1 Changing where we farm

We have sought to extend the productive ranges of major crops and in doing so, have under-utilized non-traditional crops, that is, local varieties of fruits, vegetables and grains. The future of agriculture relies on encouraging the production of traditional crop species and embracing regionally diverse crop production. (FAO, 2018). This utilization of non-traditional crop species would strengthen traditional knowledge systems among indigenous communities (Qaim, 2011; Shabala, 2013). Further, there is a disassociation between food production and urban populations which needs to be addressed (Foley *et al.*, 2011)The primary objective of this movement is to see communities and individuals producing their own food and integrating the process of growing food into people's everyday lives. Thereby, causing a shift in cultural

practices by addressing the disconnect between urban populations and food production (Foley *et al.*, 2011; FAO, 2018).

1.2.2 Changing what we farm

Among traditional crop plants there are species and varieties which are naturally more robust to high saline environments. The seed crop quinoa has been cultivated in the Andes for atleast 7000 years. The interest is this crop is increasing all over the world, owing to its stress tolerance, it has varieties which are able to cope with salinity levels of (EC) 40 dS/m. It also boasts exceptional nutritional quality (Adolf *et al.*, 2013a). As with quinoa, barley (*Hordeum vulgare*) is a salt tolerant cereal crop (Shabala *et al.*, 2010). An additional method of changing what crops we farm lies in the cultivation of halophytic species, such as Salicornia as a vegetable crops in their own right (Rozema & Schat, 2013). Saline agriculture is the cultivation of crops irrigated with brackish and saline water, it can facilitate the adaptation to increasing salinization and decreasing availability of fresh water. This shift toward the domestication of native halophytes, and expansion of highly tolerant crop species would further diversify crop production (Almeida *et al.*, 2017) and mitigate against increased soil salinization.

1.2.3 Genetic modification

Genetic engineering for GM crops is the manual introduction of genes into a plants genetic material (Lemaux, 2008; Schiemann *et al.*, 2019). A variety of methods are available to identify and introduce desirable traits in plants (Schiemann *et al.*, 2019), these techniques aim to increase crop resilience and yield, income, nutrition and food security (FAO, 2018). They encompass conventional breeding methods and genetic engineering to a growing amount of modern bio-techniques such as genome editing, including synthetic biology and gene drives (Schiemann *et al.*, 2019). The genetic modification of crops faces backlash relating to the potential consequences for the environment and human health risks (Lemaux, 2008). However, genetic engineering provides an opportunity to increase crop resilience, yield, income, nutrition, food security and sustainability (Schiemann *et al.*, 2019). Regulatory frameworks already exist globally, and the risk assessment process of GM plants follows an internationally coordinated and multi-step approach to identify hazards and the need to implement risk management measures (Schiemann *et al.*, 2019). These frameworks are employed to protect the health of humans and the environment from unwanted pests and diseases. At present this

technology has shown to be beneficial to both farmers and consumers, producing economic gains as well as positive effects for the environment and human health (Qaim, 2011). In 2008 GM soybeans made up 53% of the global GM crop area. Further, this technology accounted for 70% of global soybean production (Qaim, 2011). GM maize is the second most dominant crop. Accounting for 30% of GM crop area globally and 24% of total maize production in 2008 (Qaim, 2011). Additional commercially available GM crops include cotton, canola, alfalfa, sugar beet, papaya and squash (Lemaux, 2008; Qaim, 2011). In addition to improving drought tolerance, GM technologies could alleviate fungal, bacterial and viral stressors in major cereal, root and tuber crops (Qaim, 2011). Further, GM technologies lead to bio fortification that is an enhanced nutrient content. A well-known GM bio fortified crop is Golden Rice, developed for 'medical application' to alleviate vitamin A deficiency (Wesseler & Zilberman, 2017). GM crops benefit all scales of agriculture (Qaim, 2011). In developed nations GM crops have been adopted by large scale farms hoping to improve large scale production (Lemaux, 2008). In developing countries, small scale farmers are embracing GM technology to battle disease and pests which they would otherwise not have the means to do (Lemaux, 2008).

1.3 Understanding plant structure and function

1.3.1 Root systems: Structure and function

The root system encompasses the below ground biomass of all vascular plants. While Roots are important drivers of many ecosystem processes (Bardgett *et al.*, 2014) such as preventing the erosion of loose soils (Bardgett *et al.*, 2014; Bellini *et al.*, 2014). The primary function of roots is to take up nutrients, especially nitrogen (N) and phosphorous (P) and water from the soil, roots provide anchorage and secure the plant to the substrate (Bardgett *et al.*, 2014; Bellini *et al.*, 2014). Roots grow from the root apical meristem, and have three distinct zones (Fig. 1.1). This growth is initiated during embryogenesis, in dicotyledonous plants the root is the first structure to protrude through the seed coat. Post germination, root systems continue to expand, even under adverse conditions, although the mechnisms of this phenomenom are unknown (Munns & Tester, 2008). Plants have evolved a wide range of strategies to capture resources and respond to changes in their availability on space and time, enabling plants to cope with changing environmental conditions, especially soil and nutrient supply (Bardgett *et al.*, 2014). Root systems exhibit a high level of morphological diversity, and innovations in root traits through time have had an important role in the colonization of diverse habitats. Root

form and function is highly organized across species and biomes, although diversity in morphologial traits is greatest in the tropics where species diversity is highest. Although root structure varyies significantly between species and environments, from deeply penetrating to shallow and horizontally extending roots (Munns, 2002; Bojorquez-Quintal *et al.*, 2014). There are two primary and contrasting root forms: (1) thick roots which deepend on symbiosis with mycorrihizal fungi for soil resources and (2) thin roots which enable plants to leverage photosynthetic carbon for soil exploration (Ma *et al.*, 2018). In addition to their structural functions, roots are the first line of defence in high saline environments where they generate hormonal signals when they sense stress.

Figure 1.1 Schematic cross section of a eudicot root segment. Image source: unknown.

1.3.2 Uptake and transport of water and minerals.

In higher plants, roots acquire water, minerals and salt from the surrounding environment and transport them to the shoot. Water and solutes that enter a plant cannot be transported to the shoots without first entering the xylem stream (Flowers & Colmer, 2015). Water, minerals and salt are acquired from the surrounding environment via root hairs and radially transported to the endodermis. This uptake of water and associated minerals and subsequent radial transport can occur through three different routes (Fig. 1.2): the apoplastic pathway (through the cell wall), the symplastic pathway (through plasmodesmata between cells) and a coupled transcellular route (involving polarized influx and efflux carriers); this final route has been documented in literature, at present it is not well established (Barberon & Geldner, 2014; Byrt

et al., 2018). Under optimal conditions, the apoplastic pathway facilitates the flow and diffusion of water and nutrients through free spaces and cell walls. The rate of diffusion depends upon the ionic gradient between the external solution and the apoplastic free space. While the apoplastic pathway is a passive flow, it typically accounts for <1% of net uptake. This flow of nutrients through extracellular space is blocked by the casparian strip, consequently water and nutrients must be transported through the endodermis. Conversely, the symplastic pathway is credited as the primary route for water and nutrient flow from the soil to the xylem. Plasmodesmata channels in cell walls provides a continuity between cells, the size and frequency of these channels in cell walls determines the rate of flow via the symplastic pathway. In pea roots, increased symplastic transport during osmotic stress corresponds with changes in plasmodestmata dimensions. The uptake of water and nutrients into the symplastic pathway occurs via the plasma membrane which acts as a selective surface at the root-soil interface (Barberon & Geldner, 2014). Under high salinity, the increased concentration of Na^+ in the soil establishes an electrochemical gradient that supports the passive transport of Na^+ from the soil into the root (Keisham *et al.*, 2018; Isayenkov & Maathuis, 2019)

Figure 1.2 Radial transport of nutrients in roots. (A.) schematic figure of the three different pathways involved in the net uptake and flow of water and nutrients from soil to the endodermis: the apoplastic pathway (blue), the symplastic pathway (in grey) and coupled trans-cellular pathway (in red) comprising influx (yellow) and efflux (purple) carriers that transport nutrients from one cell to the other. (B) Magnification of the flow of nutrients through the apoplastic pathway restricted at the endodermis. Image source: Barberon & Geldner (2014)

1.4 Salt stress in plants

Plant stress has been defined as a condition in which a plant is unable to express its full genetic potential for sustained growth, development, and reproduction (Läuchli & Grattan, 2011). Salinity poses two major threats to plant growth (Table 1.1). Ions outside the root cause osmotic stress, while ions that enter the plant cause ionic stress (Galvan-Ampudia & Testerink, 2011). If elevated levels of Na^+ and Cl^- cannot be efficiently sequestered in vacuoles, expelled through the shoot, or diluted for growth the resulting accumulation of these ions in the cytosol causes a significant reduction in photosynthetic activity, followed by the eventual senescence of plant tissues (Shabala, 2000). These two principal effects of salinity manifest in two distinct phases through time, impairing the growth, development and nutritional balance of plants (Munns & Tester, 2008).

Table 1.1 Primary effects of salinity stress on plants. These two effects occur in distinct phases through time and a visibly distinct. Table modified from Munns and Tester (2008).

1.4.1 Osmotic stress

Osmotic stress is an immediate response to elevated concentrations of NaCl in the root zone (Munns & Tester, 2008). This initial phase of salt stress is characterized by reduced water potential in the root zone as the concentration of soluble salts in the soil increases (Almeida *et al.*, 2017). Although osmotic stress is independent of the accumulation of salt in the shoots (ionic stress), prolonged exposure to NaCl in the root zone causes ions to accumulate within the leaves, thus inducing ionic stress (Munns & Tester, 2008). In general, the osmotic effect of salt outside the roots is detrimental to plant growth and causes a reduction of the increase in dry matter compared to a control and/or standard growth in a non-saline environment (Munns & Tester, 2008; Isayenkov & Maathuis, 2019). Primarily, the rate at which leaves expand and new leaves emerge is significantly reduced, lateral buds develop slowly and fewer branches

form. Shoot growth is typically more sensitive than root growth under osmotic stress. However, at present there is a gap surrounding this phenomenon (Munns & Tester, 2008).

1.4.2 Ion toxicity

Ion toxicity, unlike osmotic stress, develops over time. It is due to a combination of ion accumulation in the shoot and an inability to tolerate the ions that have accumulated in older tissues which are no longer expanding (Munns & Tester, 2008). Ions do not accumulate in new shoots, instead these ions are diluted and used for growth. This accumulation in older tissues causes increased senescence of plant tissues. When older leaves die at a rate greater than which new leaves are produced the photosynthetic capacity of the plant is reduced to a point in which it can no longer supply carbohydrates for growth. This delayed onset of stress is only detrimental at high salinities, or in salt sensitive species. Under high saline conditions, Na influx is facilitated by pathways that generally function for K^+ influx. As the ionic radii of Na^+ and K^+ are similar in their hydrated forms, discrimination between the two ions is difficult (Keisham *et al.*, 2018). As a consequence of this plants suffer from K^+ deficiency which is detrimental to continued development, as K^+ is required for various enzymatic reactions and osmotic adjustment (Jaime-Pérez *et al.*, 2017).

1.4.3 Implications for agriculture

Many commercially grown crop species are sensitive to salt concentrations in the root zone above 4 dS/m^{-1} (Flowers & Colmer, 2015; Almeida *et al.*, 2017; Byrt *et al.*, 2018a). A defining feature of stress is the redistribution of energy resources away from growth to stress defence (Munns & Gilliham, 2015). As such crop plants experience a significant reduction in the growth, productivity (yield) and germination of plants (Wu, 2018). A selection of globally important crop plants and their associated salinity thresholds can be found in Table 1.2.

Table 1.2 Salinity threshold of a variety of globally important crops. The ECe value represents the salinity level at which significant yield decreases have been reported.

1.5 Defence systems

Salinity tolerance is a complex phenomenon (Flowers & Colmer, 2008). In all saline environments, roots are the first line of defence (Wu *et al.*, 2018). Any dissolved solutes that enter the transpiration stream from the environment must be accommodated, re-circulated or excreted (A *et al.*, 2010). Salt tolerance is not simply tolerance against toxicity of Na^+, but requires an adaptation to its secondary effects, such as water deficiency. Salt responses universally include inter-and intracellular Na^+ transport together with stress signalling pathways (Hasegawa, 2013). These behaviours allow for the continued gain of dry matter at low water potentials in high saline environments. This tolerance to salinity relies largely on the controlled mineral uptake, the compartmentalization of Na^+, K^+ and Cl^-, and the synthesis of compatible solutes (Wu *et al.*, 2015; Moir-Barnetson *et al.*, 2016). In general, the primary traits that facilitate mechanisms of salinity tolerance fall into three distinct categories; osmotic tolerance, Na^+ exclusion and tissue tolerance. The latter two collectively function to alleviate ionic stress (Munns & Tester, 2008). The relative importance of these three mechanisms varies with plant species, length of exposure and local environmental conditions.

1.5.1 Osmotic adjustment

Plants adjust osmotically through the accumulation and sequestering of inorganic ions are in vacuoles (generally Na^+ and Cl^-), while in the cytoplasm organic solutes are accumulated to prevent adverse effects on metabolism (Flowers *et al.*, 2015). The ability to compartmentalize internally high concentrations of Na^+ in the vacuole coordinates with osmotic adjustment capabilities (Munns & Tester, 2008). The response to osmotic stress is rapid, initiating reduced stomatal conductance to preserve water by employing long distance signalling mechanisms from the root to the shoot (Isayenkov & Maathuis, 2019). This signalling response is non-discriminatory, responding to a variety of salts. Sufficient osmotic adjustment allows for the continued movement of water and moisture into the cells, synthesis and accumulation of essential compounds (Akcin *et al.*, 2017). Decreasing stomatal aperture is a response mechanism induced by osmotic stress. This reduced stomatal conductance has been shown to correspond with the onset of salinity stress in Barley (Shabala *et al.*, 2010).

1.5.2 Tissue tolerance

Tissue tolerance encompasses the ability to absorb and compartmentalize accumulated ions (Munns & Tester, 2008; Keisham *et al.*, 2018). The compartmentalization of Na^+ and Cl^- within cell vacuoles (at the cellular and intracellular levels) in conjunction with intentional distribution of ions to organs where they will cause the least damage reduces cytosolic Na^+ concentrations. The SOS (Salt Overly Sensitive) signalling pathway, consisting of SOS1, SOS2 and SOS3 (Fig. 1.3) is a key regulator to maintain a low cytoplasmic Na^+ concentration in plant cells (Cheng *et al.*, 2018). The SOS pathway facilitates the movement of excess Na^+ out of root cells for transport to the shoot and into vacuoles. When toxic ions are continually sequestered into vacuoles, organic solutes that are compatible with the cells metabolic activities must be accumulated within the cytosol and organelles to balance osmotic pressure (Munns *et al.*, 2016; Yang & Guo, 2018). Compatible solutes or osmo-protectants are of low molecular mass and well soluble compounds that are typically non-toxic at high cellular concentrations (Sozharajan & Natarajan, 2016). Compatible osmolytes may also reduce water loss and enhance cell turgor and cell expansion. Common osmolytes include proline, sucrose, polyols, mannitol, glucose, fructose and sucrose (Yang & Guo, 2018) (Sozharajan and Natarajan, 2016). Proline accumulation induced by NaCl has been shown to correlate with salinity induced growth inhibition. Further, in response to abiotic stress glycine betaine has been shown to serve as an effective compatible osmolyte and scavenger of Reactive Oxygen Species (ROS) (Sozharajan and Natarajan, 2016). The accumulation of these compatible solutes also occurs in response to drought stress and low temperature.

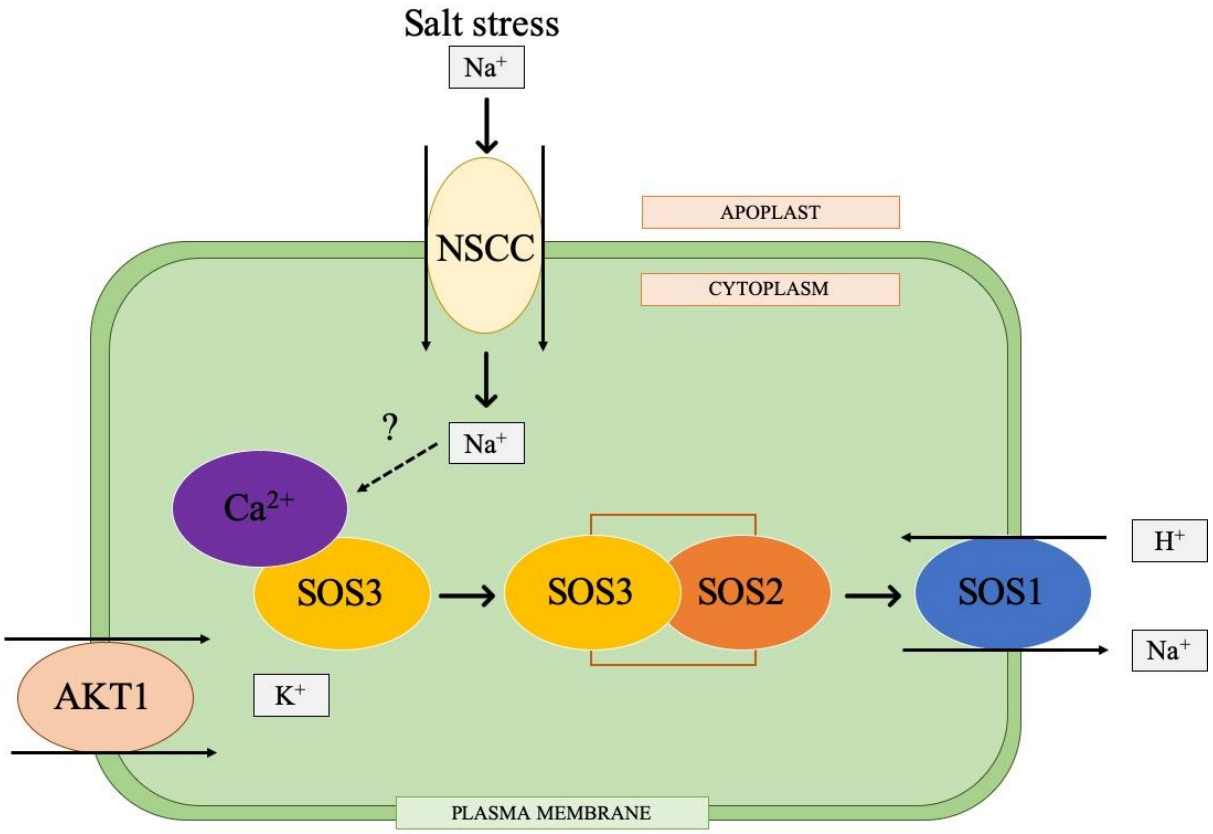


Figure 1.3 Simplified overview of the SOS pathway in a plant cell following Na^+ influx modified from (Keisham *et al.*, 2018). Na^+ enters through channels such as NSCCs and transporters such as HAK5 and HKTs. The SOS pathway is triggered by salt induced increase in cytosolic concentration of Ca^{2+} sensed by SOS3. An unidentified sensory mechanism activated the Ca^{2+} signalling cascade. SOS3 is a Ca^{2+}sensor protein, once activated SOS3 travels to and interacts with SOS2, activating its protein kinase and forming the SOS2-SOS3 complex. SOS2 then phosphorylates SOS1 which codes the Na^+/H^+ antiporter and facilitates the movement of Na^+ out of the cell. As a response to salt stress Na^+ may also be compartmentalized inside vacuoles by NHX (Na^+/H^+ exchanger). H^+ pumps are pivotal to salt tolerance as they function heavily in ionic and osmotic homeostasis.

1.5.3 Ca^{2+} signalling

Ca^{2+} functions as a versatile secondary messenger in general defence mechanisms and facilitates the transport of Na^+ out cells (He *et al.*, 2018) Ca_2 acts directly, or indirectly through other signalling molecules, as a primary facilitator for the regulatory network (He *et al.*, 2018). The Ca^{2+} signal is initiated by the sharp influx of Ca^{2+} through Ca^{2+} channels, such as the 'Plasma membrane cyclic nucleotide-gated channels (CNGCs) and the Two pore channel

(TPC1) (Fig. 1.3). Prior to stimulation by external stimuli, Ca^{2+} is stored in reservoirs primarily the apoplast and the vacuole (He *et al.*, 2018). Excess Ca^{2+} is quenched out of the cell by Ca^{2+} / H^{+} exchangers. Additionally, the role of Ca^{2+} in salt tolerance mechanism is significant as Ca^{2+} is a Na^{+} antagonist and is inherently link to salt detoxification processes. Ca^{2+}regulates ion transporters to maintain a high K^{+}/Na^{+} ratio in the cytosol, by activating the SOS pathway (Fig. 1.3) to promote Na^{+} efflux, together with blocking NSCCs to reduce continued Na^{+} influx (He *et al.*, 2018). Additionally, studies have implicated Ca^{2+} signalling with the enhanced development and secretion rates of salt through epidermal glands (Ding *et al.*, 2010). Further, a study on *Arabidopsis thaliana* demonstrated the importance of Ca^{2+} signalling for ABA-mediated seed germination. ABA sustains dormancy through the inhibition of germination (Rajjou *et al.*, 2012).

1.5.4 Na^{+} secretion – Salt glands and salt bladders

While many of the defence traits described above are ubiquitous among plants, a group of plants, the recretohalophytes, have developed unique and highly effective structures to combat excess salinity and survive in adverse environments. These structures are salt bladders and salt glands. Under high salinity these structures serve as additional storage organs and directly secrete ions out the cell, thereby mitigating the elevation of cytosolic Na^{+} and improving tissue tolerance (Yuan *et al.*, 2016; Wu, 2018). Salt glands have been documented in 14 families, where they appear to have originated independently at least 12 times (Dissanayake & Larkin, 2017). In some species salt glands have been shown to secrete both Na^{+} and K^{+} in some species, however they have greater selectivity for Na^{+} over K^{+} (Wu, 2018). When *Avicennia marina*, a mangrove species found in Australasia, was exposed to increasing salinity the number of salt glands in the leaves and rates of secretion per gland increased (Wu, 2018). Calcium signalling in the cells has been shown to aid this process (Ding *et al.*, 2010). Further, salt bladders in *Mesembryanthemum crystallinum*, a common ice plant, were shown to aid salt tolerance by maintaining ion content within photosynthetically active tissues.

1.6 Naturally tolerant plants

1.6.1 Glycophytes and halophytes

Among plants there exists a continuum of tolerance from the absence of stress to severe stress (Läuchli & Grattan, 2011; Flowers *et al.*, 2015). While there is some debate about where the

limit is drawn in defining salt tolerant species, most plant species have been grouped as either halophytes (salt tolerant) or glycophytes (salt sensitive) (Flowers & Colmer, 2015). Halophytes are widely distributed across plant families, having evolved independently in many lineages(Bennett *et al.*, 2013). However, they account for less than 0.2% of all plant species (Flowers & Colmer, 2015). Halophytes are plants that survive to reproduce in environments where salt concentrations are around 200 mM NaCl, although some halophytes have been shown to grow and reproduce concentrations exceeding this concentration (Flowers & Colmer, 2008; Moir-Barnetson *et al.*, 2016). Coastal halophytes show varying responses to substrate salinity (Flowers & Colmer, 2008; Munns & Tester, 2008). *Mesembryanthemum crystallinum* is a facultative halophyte (Cosentino *et al.,* 2010). Facultative halophytes grow optimally in the absence of salt but tolerates high concentrations of salt in the environment. Comparatively, *Avecennia marina* is an obligates halophyte (Nguyen *et al.,* 2015). Unlike facultative halophytes, obligate halophytes require saline conditions for growth and development. For many halophytes low to moderate concentrations of Na^+ are commonly found to be benign, or even beneficial, stimulating growth in many plants species where they are K^+ deprived, additionally plants will optimise excess to ions to maintain turgor pressure. However, for glycophytes excess toxic ions will disrupt metabolic functioning and cause early senescence (Bose *et al.*, 2017; Wu, 2018).

1.6.2 Ion transport and accumulation

A general response to salinity of plants refers to the regulation of ionic transport and ion homeostasis. Glycophytes cope with salinity by mostly by limiting the transport of toxic ions to the shoots. They have been shown to accumulate in the roots, through retention in vacuoles or phloem redistribution from the shoots back to roots. The accumulation of Na^+ in the leaves inhibits protein synthesis, enzymatic activity and photosynthesis (Bojorquez-Quintal *et al.*, 2014). Halophyte species on the contrary, accumulate toxic ions in their leaves, where they are maintained at low cytosolic concentrations by compartmentalization in vacuoles.

Figure 1.4 Different strategies of halophytes (top) and glycophytes (bottom) in response to salt stress. The thickness of the lines represents the proposed contributions of the strategy to overall salinity tolerance as described by Wu (2018). Image source: (Wu ,2018)

1.6.3 Research interest

Halophytes are considered a valuable resource for agriculture, given the near limitless supply of salt water available. They are a potential source of salt tolerant genes and a valuable source of knowledge for mechanisms underlying this complex trait, additionally they can be utilised as niche crops in their own right (Shabala, 2013; Panta *et al.*, 2014; Flowers & Colmer, 2015; Bose *et al.*, 2017). Increasing the salt tolerance of crops will also allow the more effective use of poor quality irrigation water (Munns & James, 2003) which is currently a limiting factor to plant growth (Akcin *et al.*, 2017). Although salinity tolerances amongst halophytes are

consistently high, overall tolerance to salinity is indicated to be species specific. As there is considerable variation among species growth rates, photosynthetic efficiency and osmotic adjustment in saline environments (Madawala *et al.*, 2014; Moir-Barnetson *et al.*, 2016). There is a concerted research effort to understand the molecular genetic mechanism by which halophytes can thrive in highly saline environments, in the hope that these same mechanisms might be transferred via GM into traditional crop species. However, research in this area has been limited by the lack of a suitable salt-tolerant genetic model to identify specific traits (Stepien & Johnson, 2008).

1.7 Plant pigments

Plants typically appear green as a consequence of their dependency on blue and red light for photosynthesis (Menzies, 2013). However, red-leafed plants are commonly associated with arid and/or semi-arid environments, where plants experience pro-longed exposure to a range of abiotic stressors, including UV radiation, light and temperature fluctuations, heavy metal accumulation, and increased salinity. In these environments, abiotic stress has been shown to induce or upregulate foliar pigmentation in plants. Red pigments are widely distributed in the plant kingdom, in these leaves anthocyanins or less commonly betalains are responsible for leaf reddening (Nakashima *et al.*, 2011; Miguel, 2018). It is generally accepted that these pigments afford adaptive advantages when exposed to environmental stressors, although the exact mechanisms are unclear (Wang *et al.*, 2007; Jain & Gould, 2015). Plant pigments have been shown to be instrumental in reducing photo inhibition through their involvement in metabolic processes such as ROS scavenging (Jain *et al.*, 2015). While, much has been documented about the functional roles of anthocyanins, the flavonoid pigments that appear red to blue depending on vacuolar pH (Wang *et al.*, 2007; Jain & Gould, 2015). Far less is known about the roles of a smaller group of pigments, the betalains (Jain *et al.*, 2015; Jain, 2016).

1.8 Betalains

Betalains are mutually exclusive with anthocyanins across the plant kingdom (Chung *et al.*, 2015; Jain & Gould, 2015; Osbourn, 2017). The chemistry, biosynthesis and molecular genetics of these pigments are not well understood and many questions have been raised as to the mystery of their evolutionary history, their functional significance and possible roles in stress tolerance (Nakatsuka *et al.*, 2013; Chung *et al.*, 2015; Osbourn, 2017). Betalain pigments

are nitrogenous secondary metabolites found in the order Caryophyllales – which includes cacti, carnations, amaranths, ice plants, beets and many carnivorous species. With exception of two families: Caryophyllaceae and Molluginaceae (Jain & Gould, 2015; Osbourn, 2017). Additionally, they are found in some basidiomycetes (Jain *et al.*, 2015). They are thought to have evolved relatively recently as an adaptation to the adverse conditions found in arid-and semi-arid environments (Calcott, 2014; Osbourn, 2017). Betalain synthesis maybe observed at different stages in plant growth or upregulated when exposed to biotic or abiotic stress (Miguel, 2018). These pigments can be produced in a variety of plant tissues; the seeds (*Chenopodium quinoa willd*) (Panuccio *et al.*, 2014), fruits (*Opuntia ficus-indica*) (García-Cayuela *et al.*, 2019), flowers, (*Mirabilis jalapa)* (Brockington *et al.*, 2015) (*Bougainvillea spectabilis)* (Calcott, 2014); leaves, *Suaeda salsa* (Wang *et al.*, 2007), stems and/or roots *Beta vulgaris* (Miguel, 2018). In stems and leaves red pigments absorb green and yellow light where they are postulated to serve a photoprotective function reducing light stress on chloroplasts (Jain & Gould, 2015). There are two classes of these chromo alkaloids: the yellow/ orange betaxanthins (λmax = 470 nm) and the red/violet betacyanins (λmax = 536 nm) (Wang *et al.*, 2007; Jain *et al.*, 2015). Betacyanins share chemical and physical properties with anthocyanin, they have a similar absorption spectra in the visible spectrum and they are both potent anti-oxidants (Jain *et al.*, 2015).

1.8.1 Biosynthesis

Although exact pathway for betalain biosynthesis has not being completely elaborated, current research suggests the pathway shown in Fig. 1.2 (Nakatsuka *et al.*, 2013; Chung *et al.*, 2015; Miguel, 2018). Betalains are biosynthesized from the amino acid tyrosine by several enzymatic and spontaneous chemical steps. Both betalain groups have a common core structure betalamic acid. Betaxanthin pigments are formed when betalamic acid condenses with amino acids and/or other amines. In contrast, betacyanin is produced when betalamic acid links to cyclo-dopa (Miguel, 2018).

Figure 1.5 Simplified schematic of betalain biosynthesis in *Beta vulgaris*. Image source: Miguel, 2018

1.8.2 Current applications

Betalain pigments are widely used as additives for food, drugs and cosmetic products because of their natural colorant properties, pH stability, absence of toxicity and anti-radical activities (Wang *et al.*, 2007). These characteristics signal that there is much more to learn about these pigments. In recent years there has been a wide body of literature form which aims to clarify the factors regulating betalain accumulation (Nakatsuka *et al.*, 2013). The synthesis of betalain in response to high salinity provides promising avenue for the development of salt resistant crops. At present, many studies have compared tolerance between cultivars species such as Quinoa (Adolf *et al.*, 2013), *Limonium* spp. (Al Hassan *et al.*, 2017), *Portulaca* spp. (Muchate

et al., 2016; Sdouga *et al.*, 2019) and *Amaranthus* spp. (Nakashima *et al.*, 2015). However, continued research in this area has be challenged by the lack of an appropriate model to formally quantify betalain accumulation in response to abiotic stress. This model will preferentially be a typical halophyte possessing betacyanic and acyanic colour morphs.

1.9 Horokaka *(Disphyma australe)*

Disphyma australe belongs to the family Aizoaceae in the order Caryophyllales (Winter *et al.*, 2018). It is a dicotyledonous succulent halophyte, native to New Zealand where it is found in coastal habitats. *D. australe* is an ideal model to investigate whether betacyanin contributes to overall salinity tolerance as it exhibits colour dimorphism among natural populations (Fig. 1.5). Red morphs produce betacyanin in their stems, leaves and flowers (Fig. 1.6), while green morphs synthesise betacyanin only in their flowers (Fig. 1.7). As with *Suaeda salsa*, a betalain producing plant common in China (Wang *et al.*, 2007), the red-leafed (betacyanic) morphs of *D. australe* are green in optimal conditions and upregulate red pigmentation when exposed to excess salt and white light (Wang *et al.*, 2007; Jain *et al.*, 2015). Consequently, both morphs appear green in the absence of stress (Fig. 1.8). Stress induced betacyanin accumulation in *D. australe* occurs at the nodes first and extends into the leaves, given that the betacyanin precursors, tyrosine and L-DOPA are readily available (Jain *et al.*, 2015). The ability of *D. australe* to regulate betacyanin production in the vegetative tissues of red morphs but not green morphs provide a model to investigate the functional role of betacyanin accumulation as a stress response mechanism. Comparisons between to morphs minimizes variation in physiological traits except for betacyanin production, which would otherwise be present. Additionally, *D. australe* can easily be propagated from nodal cuttings, allowing for the production of clonal colonies.

Figure 1.6 Naturally occurring dimorphic population of *Disphyma australe.*

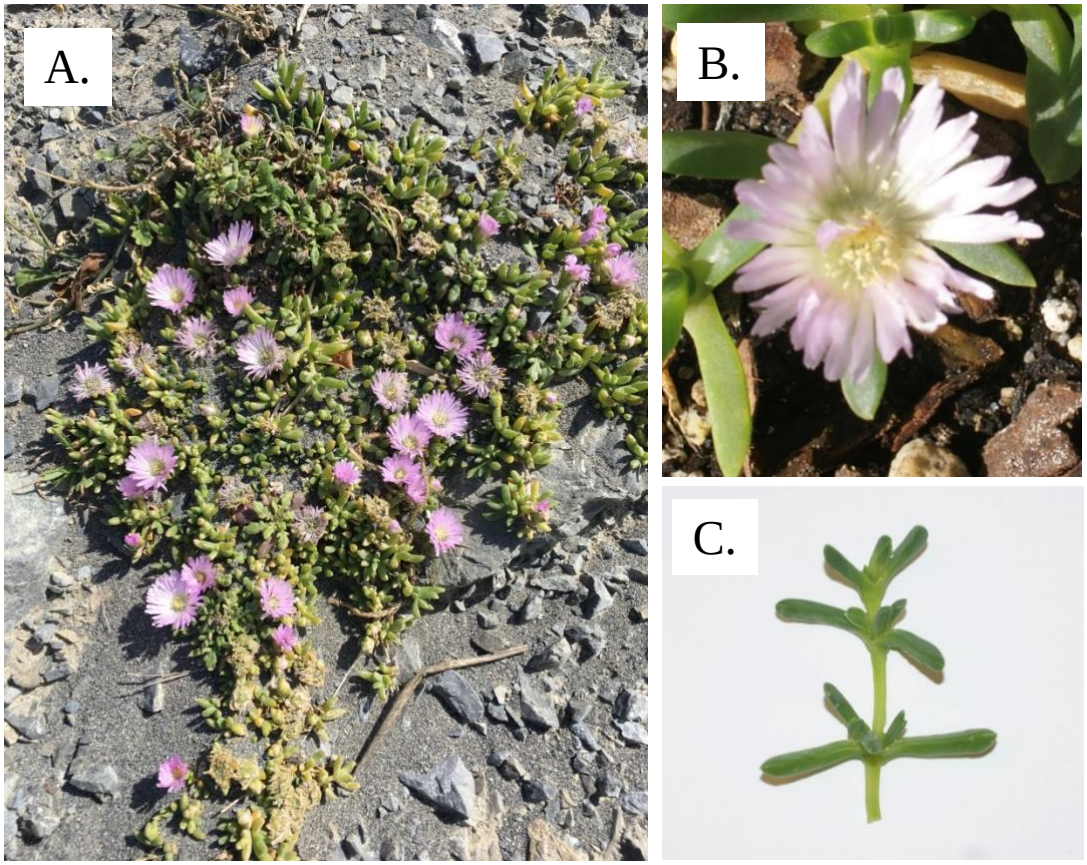


Figure 1.7 (A.) flowering D. australe plant growing in Te Kopahou Reserve, (B.) flower of a green morph (C.) shoot cutting of a green morph

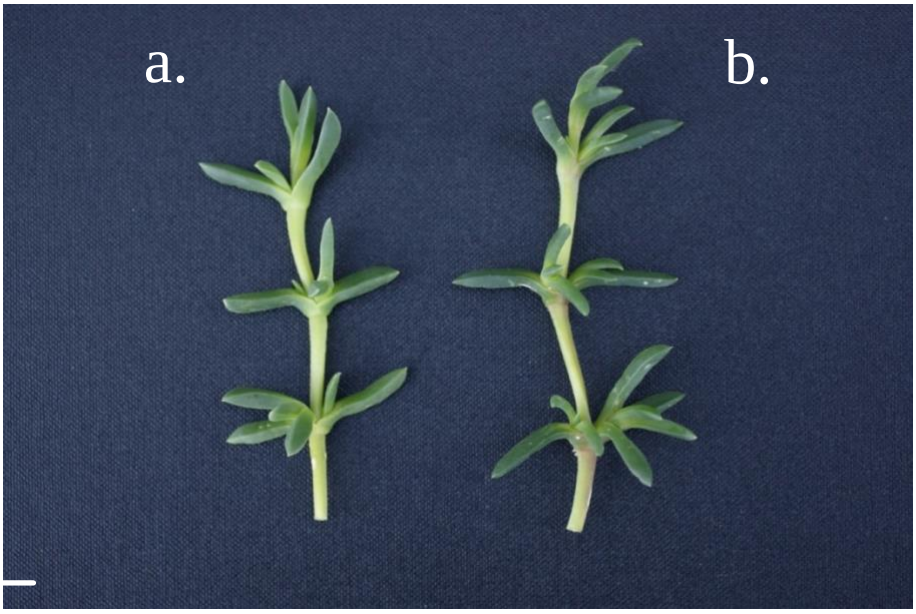


Figure 1.8 Top: shoot cuttings of green (a.) and red (b.) D. australe morphs taken from plants grown under non-saline and low light conditions in the green house facilities at Victoria University of Wellington. Scale bar represents 10 mm.

1.9.1 Previous research on betacyanin synthesis in Disphyma australe

Betalain pigmentation primarily occurs in the epidermis where they absorb green/yellow wavebands of light reducing photo inhibition and photo oxidative damage when leaves are exposed to excess white light (Jain *et al.*, 2015). This duel exposure to salinity and high light has been shown to contribute to betalain biosynthesis in the shoots of red *D. australe* morphs (Wang *et al.*, 2007; Jain *et al.*, 2015). Jain (2016) found that in a population of *D. australe* growing on the Wellington south coast (Table 1.3), red morphs are more abundant closer to the high tide line. Comparatively, the green leafed morphs are more frequent as the distance from the high tide line increases and subsequently the substrate salinity decreases (Jain, 2016). This suggest a differential capacity to tolerate high salinity in the root zone. Earlier studies have demonstrated that *D. australe* has an enhanced tolerance to salinity compared with other halophyte species, being able to maintain growth and photosynthetic capacity when grown in 100% seawater (Madawala *et al.*, 2014). However, this study does not discriminate between green and red morphs. Among the postulated functions of leaf betacyanin, several studies evidence the protective function against photo inhibition produced by high light and salinity. Recent studies have sought to distinguish the optical properties of betacyanin as a photo

protectant from physiological effects which could promote its role as tolerance mechanism (Jain & Gould, 2015; Jain *et al.*, 2015). These authors produced a series of studies which reported a greater capacity of red morphs to tolerate salinity stress. This was shown through maintain CO_2 fixation, water use efficiency and photosynthetic capacity. In addition, salt treated red morphs exhibited a decreased chlorophyll a:b ratio, in which chlorophyll b content increased and chlorophyll a content decreased (Jain, 2016). Further evidencing the enhanced salt tolerance of red morphs compared to green morphs.

Table 1.3 Distribution of *Disphyma australe* morphs in relation to substrate salinity. Table modified from Jain (2016).

1.9.2 Research Aims

The intersection between the function of betacyanins in stress tolerance and the natural ability of halophytes to tolerate adverse environments is considered in this thesis, which primarily focuses on the distribution of *D. australe* morphs along a salinity gradient. This study hopes to determine how betacyanin accumulation aids salinity tolerance and therefore determines the distribution of *D. australe* along a salinity gradient, by focusing on three key areas; root development, germination and ion content in the root systems.

Thus, I aim to

- Separate the light screening effect of epidermal betacyanin from other physiological traits which may enhance salinity tolerance.
- Investigate the relative capacity for root development and continued root growth under increasing salinities.
- Establish relative germination capacities of green and red morphs at increasing salinities.
- Determine the ability to maintain ion homeostasis under increased salinities.
- Evaluate the overall competitive ability of green morphs compared to red morphs, and how this may influence distribution.

To this end I will measure the growth of root biomass under increasing NaCl concentrations, compare the germination capacity and recovery rates of seeds from green and red plants at increasing NaCl concentrations and evaluate the capacity for green and red morphs to maintain K^+/Na^+ and homeostasis in the roots of *Disphyma australe* seedlings treated with low (200 mM), medium (400 mM) and high (800 mM) concentrations on NaCl.

1.9.3 Plant collection

All plant material for this study was collected from Te Kopahou Reserve and and grown in the green house facilities at Victoria University of Wellington. Te Kopahou Reserve is a 600 hectare conservation site extending inland from Wellingtons south coast between -41.362016, 174.716629 and -41.348616, 174.750761 (Fig. 1.8). It is characterised by extensive south facing dunes where dimorphic communities can easily be collected from the track Plant

material was identified visually from morphological characteristics, such as leaf length and width, shape of leaf margins and apices, and flower, stem and leaf colour described by Jain (2016).

Figure 1.9 Aerial map of the South Coast, the red box indicates the area of shore line where plant material was collected from.

2 Root growth along a salinity gradient

2.1 Abstract

Betacyanin have been postulated as serving multiple functions in photoprotection, this is conistent with the location of betacyanin in the the epidermis. Epidermal betacyanins are red, they like anthocyanins, absord green/yellow wavebands of light. This may ameliorate excitation pressure on chloroplasts, reducing the susceptibility for photoinhibition and photooxidative damage when leaves are exposed to excess light. Testing for the photoprotective role of betacyanins is challening. As plants employ a wide range of mechanisms to mitigate the adverse effects of abiotic stress, it is difficult to separate from these traits the contribution of red pigments. To examine betacyanin function on biomass accumulation in *Disphyma australe* I simultaneously subjected shoots cuttings from green- and red-leafed morphs to saturated light and increasing NaCl concentrations. Further, to separate the light screening function of epidermal betacyanin from additional physiological traits, I secured a red filter with comparative absorption spectra to betacyanin between the light source and green seedlings. Consequently, this study presents finidings on the capacity for root development at increasing salinity levels between three colour groups: green, green morph without a light screening red filter; filter, green morphs with a light screening red filter; and red, red morph.

Between colours significant differences were only detected between Filter and green, and green and red at 0 mM NaCl. This data provides further evidence as to the spatial distribution of *D. australe* along a salinity, suggesting that green morphs are able to exploit optiomal conditions (0 mM NaCl) for enhanced growth. Where as red *D.austale* morphs exhbitied steady growth at all NaCl concentrations. The results of this study support the photoprotection role of betacyanin accumulation in shoots as a function of increased salinity tolerance. Further, they indicate that the presence of betacyanin affords a greater physiological tolerance, and may contribute to the relative distribution of green and red morphs along a salinity gradient.

The available literature indicates that abiotic stress effects root development, however the results are variable, and at present no studies have compared the capacity for root development in betacyanic and acyanic plants.

2.2 Introduction

Disphyma australe grows naturally in coastal environments (Madawala *et al.*, 2014; Jain, 2016), where green- and red-leafed morphs were shown to be distributed along a salinity gradient (Jain, 2016). In coastal environments the distribution of species is reliant on their relative capacity to tolerate spatial and temporal variations in airborne and substrate salinity (Sucre & Suárez, 2011; Madawala *et al.*, 2014). The continued development of roots underpins the continued growth of a plant and is a fundamental component of plant survival (Motte *et al.*, 2019). As roots primarily function to take up water and nutrients from the soil (Bardgett *et al.*, 2014; Bellini *et al.*, 2014; Steffens & Rasmussen, 2016). Further, roots are ecologically important as they stabilize soil in shifting environments (Bellini *et al.*, 2014). Both green- and red-leafed morphs of *Disphya australe* grow adventitious roots from the nodes as the shoot grows along the surface of the soil (Fig. 2.1)

Figure 2.1 Red Disphyma australe stem cutting with adventitious roots growing from the nodes. Scale bar represents 10 mm.

In coastal environments plants are inherently at risk of photinhibition which has been shown to contribute towards a salinity dependent reduction in growth. However, photoinhibition on overall plant productivity is difficult to quantify (Nakashima *et al.*, 2011). To prevent photo inhibition a range photo protective mechanisms as used by the plant to both suppress the photo damage to PSII and minimize the inhibition of repair systems (Takahashi *et al.*, 2010) . Studies have shown that the onset of photo inhibition is strongly correlated with the absorption of excessive light and that subsequent photo damage is proportional to the intensity of light

absorbed (Takahashi *et al.*, 2010). This suggests that shielding chloroplasts from saturated light is an effective manner of photo protection (Nakashima *et al.*, 2011). Betacyanins have previously been shown to protect chloroplasts from high light when compromised by salinity stress (Jain, 2016). In the previously described dimorphic populations of green- and red-leafed *Disphyma australe* along the south Wellington coastline. The red morph was shown to be more tolerant to the combination of high light and salinity, as measured by higher CO2 assimilation rates, reduced inhibition of PSII and enhanced water use efficiency relative to the green morph (Jain *et al.*, 2015).

While there is evidence that roots recover well after exposure to salinity (Munns, 2002), and studies which document salt specific effects of salinity on root elongation (Munns, 2002; Shabala *et al.*, 2015; Zhan *et al.*, 2015; Byrt *et al.*, 2018), there is little research to establish the capacity for plants to initiated root development and growth under increasing saline conditions. Further, nothing has been documented about the relative capacities of betacyanic and acyaninc plants to establish root biomass in saline environments. Thus, I hoped to compare the capacitity for adventitious root development between green- and red-leafed *Disphyma australe* morphs along a salinity gradient. This study hopes to ascertain if the presence of betacyanin in the shoots and its associated photoprotectant properties was the principle factor determining continued root growth, overall plant productivity and therefore influencing spatial distribution along a salinity gradient. I hypotheisised that the presence of betacyanin will influence root developement, leading to an increased or maintained biomass accumulation under increasing salinity concentrations.

Addiotionally, I aimed to separate the light screening capacity of leaf betacyanin from additional physiological traits which may influence distribution along a salinity gradient. By securing a red filter with a comparative absorption spectra to betacyanin between the light source and green seedlings. I hypothesised that the red filter will shield the green morphs from saturated light, and facilitate root development under increasing saline environments.

By simultaneously photo stressing the seedlings and subjecting them to increasing NaCl in the growth medium I aimed to:

1. Establish the relative capacity for new root growth and competitiveness of the two morphs when subjected to increasing saline conditions.
2. Test the light screening effect of epidermal betacyanin on seedling establishment under high light and saline conditions.

To this end I measured mean above and below ground biomass accumulation, the average ratio of root : to total biomass of the three colour groups (filter, green and red) under increasing salinity concentrations (0, 50, 100, 150 mM NaCl) and the Tissue water content of the shoots and roots.

2.3 Materials and methods

2.3.1 Plant material

Shoot cuttings, lacking roots and with a minimum of two mature leaf pairs (Fig. 2.2 A) were cut from each of the red plants, eight cuttings were from each of the green plants. The plants, previously collected from Te Kopahou Reserve, were growing in the green house facilities at Victoria University of Wellington.

2.3.2 Nutrient solution

Cuttings were grown hydroponically in indiviadual pots with 50 mL of growth medium. The growth medium was 10% full strength hoaglands solutions (Hoagland's No. 2 Basal Salt Mixture; Sigma Aldrich) supplemented or not with either 50, 100 or 150 mM NaCl (Sigma Aldrich). The upper limit of the study was set at 150 mM as pilot study results indicated no adventitious root growth at concentrations ≥ 200 mM NaCl. The solution was changed every second day to maintain salinity and volume.

2.3.3 Study conditions

For each of the colour groups there were five replicates, each replicate represented cuttings from an individual plant, minimizing variations in physiological traits plants (Fig 2.2 B). To replicate the effect of the photoprotectant properties of epidermal betacyanin half of green cuttings were grown under a supergel filter (Rosco Supergel 346 Tropical Magenta;

Kenderdine electrical ltd). Rosco supergel absorbs 540 nm, replicating the aborption maxima of betacyanins (536 nm). The plants were grown in the green house with a 16 h light /8h dark cycle, the average light intensity was 1100 μmol^{-1} throughout the study.

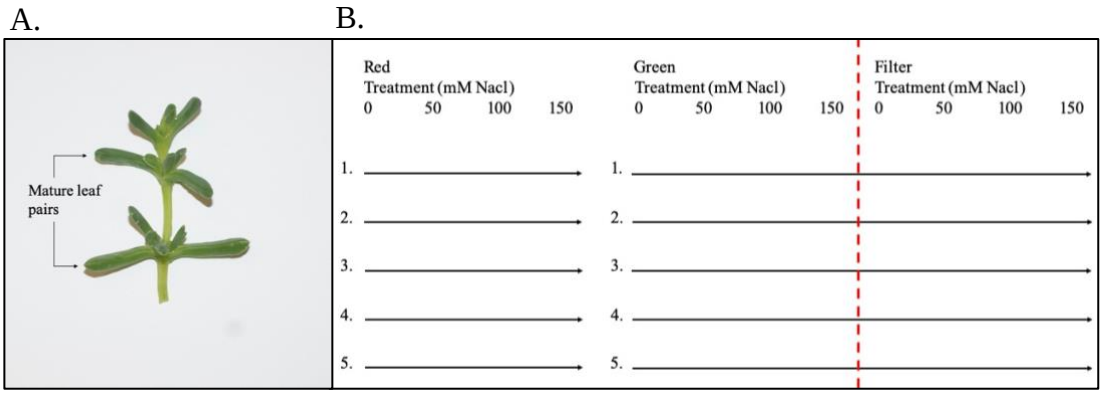


Figure 2.2 (A.) *D. australe* shoot cutting with two mature pairs labelled. (B.) Simplified schematic of the study set up, each arrow represents a clonal line (n=5). Each clonal line was tested at each controlled NaCl treatment. The same clonal lines were used in the green and filter groups to minimize variation in physiological traits. Colour groups: Red, betacyanic morph; Green, acyanic morph; Filter, acyanic morph grown under red filter. The red dashed line represents the placement of the red filter, separating the green and filter groups.

2.3.4 Plant growth parameters

After 5 weeks of growth in these conditions, the fresh and dry weights of root and shoot systems were determined for each plant. Dry weight was determined by freeze drying the plants to a constant weight (48h – 72hr). From those measurements I calculated the root weight ratio (RWR), tissue water content percentage (TWC%) and the change in shoot biomass (Δshoot biomass).

2.3.5 Statistics

Statistical analysis was carried out in IBM SPSS Statistics 25. Data normality was assessed with the Shapiro Wilk test ($p > 0.05$). Two-way anova was used to test for differences between treatment and colour. Followed by planned LSD post hoc with Bonferroni correction for

multiple comparisons. Data which was log transformed to obtain normality are indicated in the results. Where normality could not be met ($p < 0.05$), significance was tested the non-parametric Kruskal-Wallis H test, followed with planned pairwise comparisons using Dunn's procedure with a Bonferroni correction for multiple comparisons.

2.4 Results

2.4.1 Effect of increasing salinity on below ground biomass accumulation

The results demonstrate a salinity dependent reduction in root development in both the green morph with the filter (Filter) and green morph with out the filter (Green) (Fi.g 2.3). In the green group a significant reduction was detected at concetrations ≥ 50 mM NaCl. However, the filter group demonstrated a significant reduction at 150 mM NaCl. This finding supports the light screening capacity of epidermal betacyancin to alleviate photo inhibition when chloroplasts are subjected to salinity stress.

The red morph (Red) maintained root development as salinity concentrations increased ($p = 0.501$). This result both supports my hypothesis stated in section 2.2, that that the presence of betacyanin will influence root developement, leading to an increased or maintained biomass accumulation under increasing salinity concentrations. Further, this finding supports previous finidings which implicate epidermal betacyanins as an effective photo protectant. Additonally, when NaCl was absent from the growth medium, the root DW of the filter group did not differ significnatly from the red group. However, both the filter and red groups differed significantly from the green morphs without the filter. Consequently, epidermal betacyanins are shown to reduce the effects of saturating light on *Disphyma asutrale*. Examples of root growth are shown in Fig. 2.4

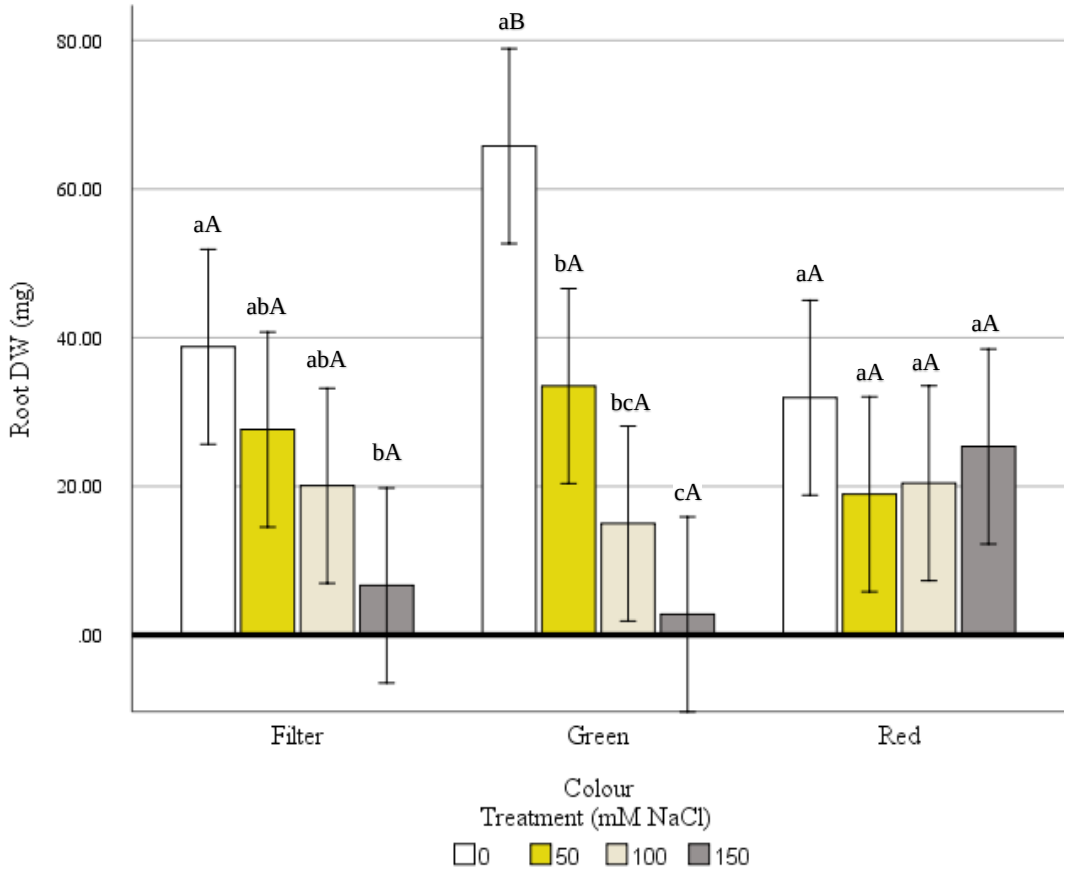
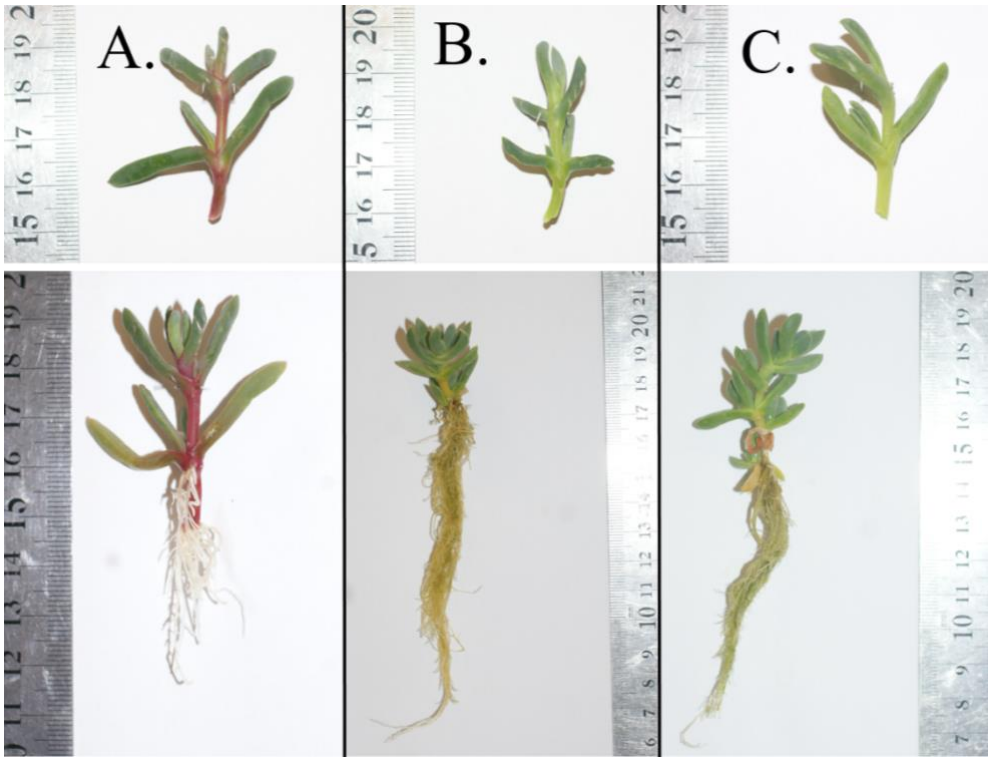
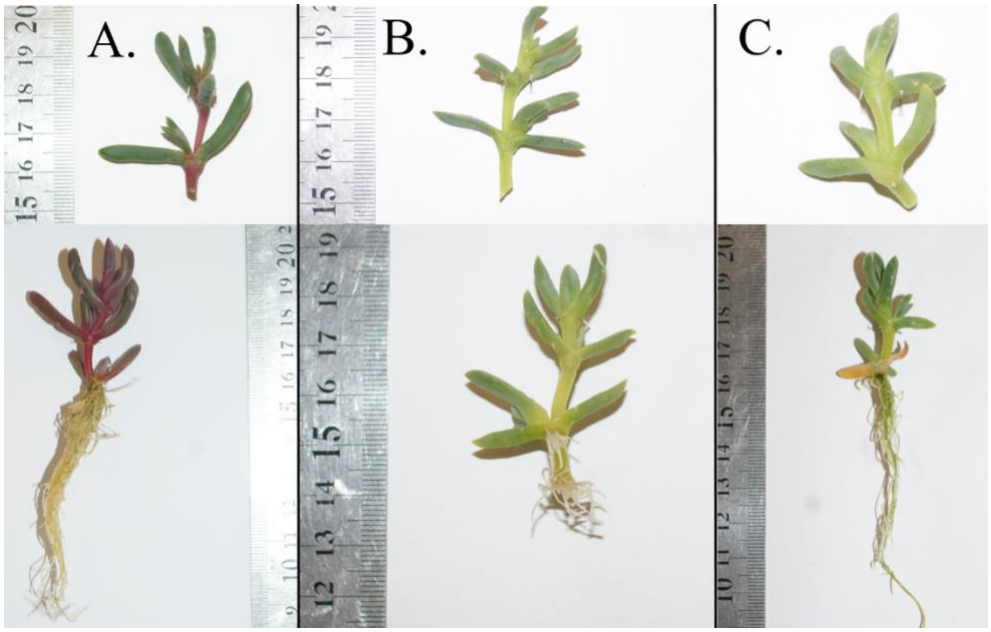


Figure 2.3 Root DW (mg) accumulation between the three colour groups at the indicated NaCl concentrations. The values presented represent means with SE (n=5). Different lower case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According to the LSD post hoc with Bonferroni correction ($p < 0.05$).

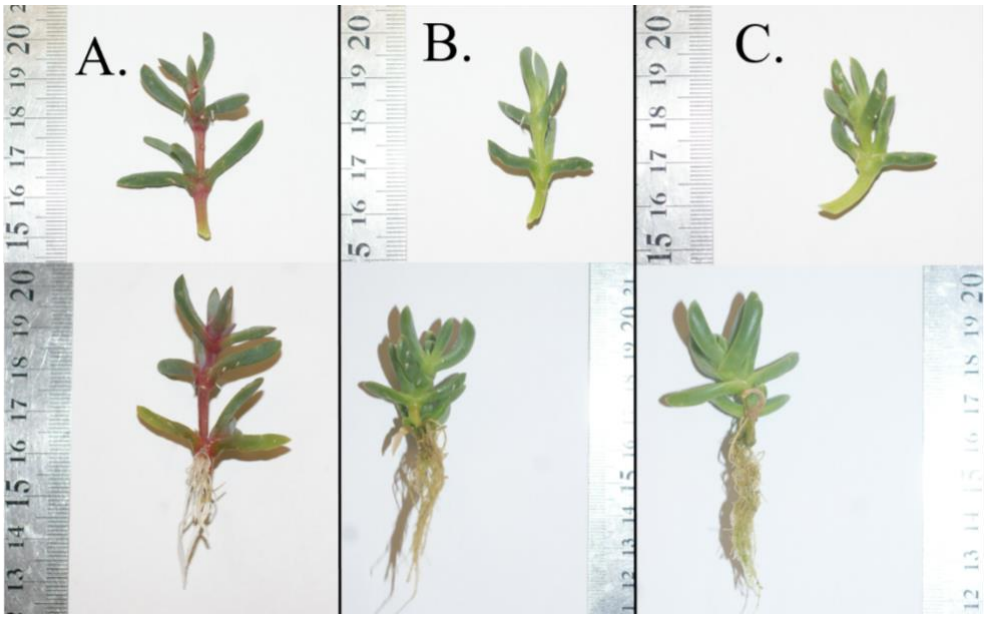
Control (0 mM NaCl)



50 mM NaCl



100 mM NaCl



150 mM NaCl

Figure 2.4 Examples of root growth between the three colour groups: A. Red, B. Green and C. Filter, at the indicated treatment levels.

2.4.2 Effect of increasing salinity on above ground biomass

The final DW of the shoots differed only for the red group at 150 mM NaCl ($p = 0.018$). However, at the treatment level the final shoot DW of the red group at 150 mM did significantly differ from the other two groups. Ultimately, the results presented here demonstrate that regardless of colour, shoot weight is subject salinity induced inhibition. This finding is further supported in (Fig. 2.5) which reports the total change in shoot fresh weight over the course of the study.

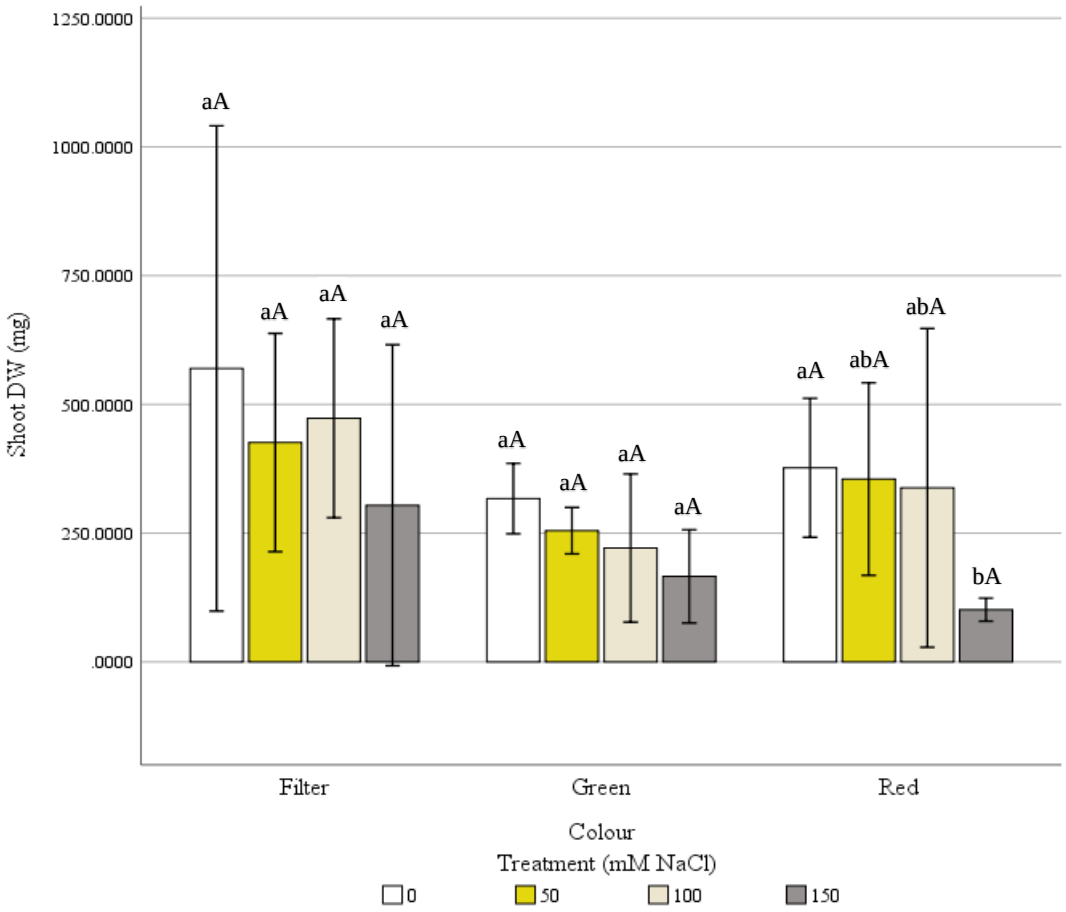


Figure 2.5 Shoot DW (mg) between the three groups at the indicated NaCl concentrations. The values presented are means with SE (n=5). Different lower case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According to the LSD post hoc with Bonferroni correction ($p < 0.05$).

Over the course of the study, treatment level had a statistically significant effect on shoot growth in the three colour groups; Filter ($p = 0.007$), Green ($p = 0.012$) and Red ($p = 0.002$) (Fig. 2.6). In which all three groups experienced stimulate growth at when NaCl was present in the growth medium at a low concentration (50 mM NaCl). Overall, the results demonstrate a salinity dependent reduction in shoot growth regardless of colour group. Unlike the results reported in Fig. 2.3 on root bio mass accumulation, the green-leafed morphs appears to preferentially accumulate biomass in their shoots when subjected to increased salinity in the

growth medium. However, all three colour groups experienced a significant reduction in shoot growth at ≥ 100 mM NaCl. Subsequently, this salinity dependent inhibition of shoot growth was greatest for the red group. Although, at 150 mM NaCl the results between the colour groups were not significantly different.

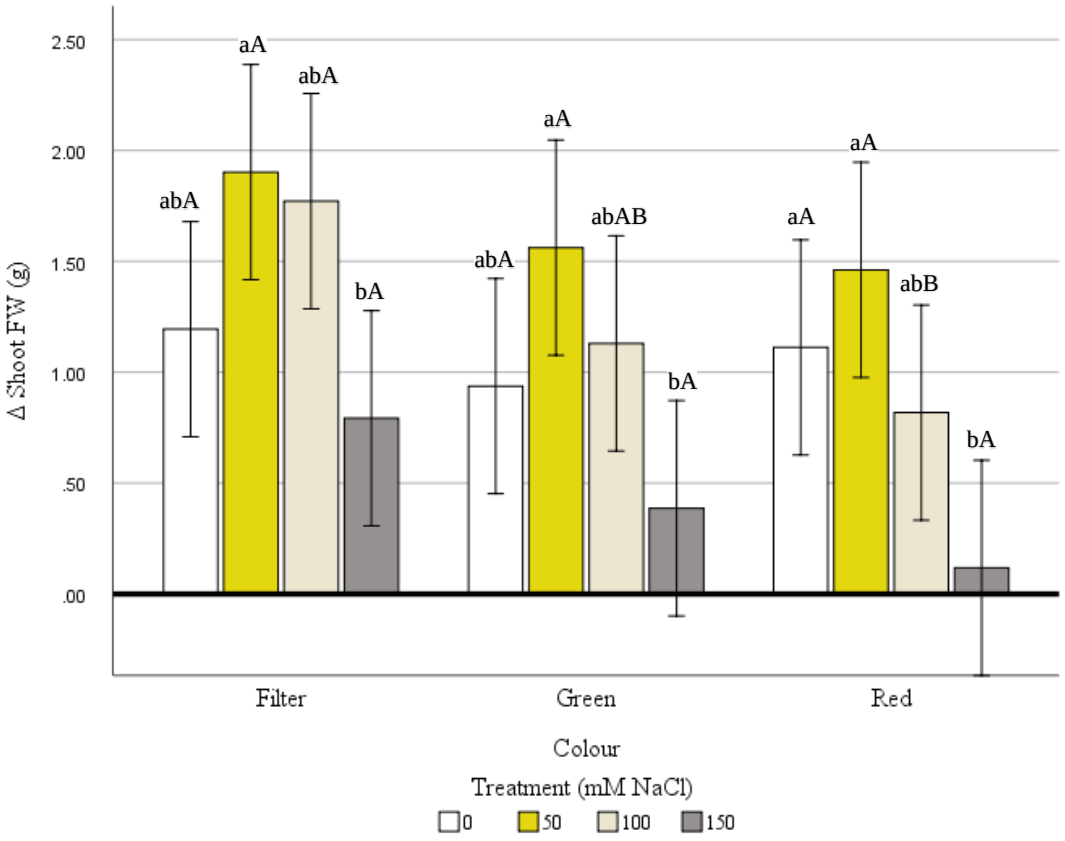


Figure 2.6 ΔShoot FW (g) between the three groups at the indicated NaCl concentrations. The values presented are means with SE (n=5). Different lower case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According to the LSD post hoc with Bonferroni correction ($p < 0.05$).

2.4.3 Effect of increasing salinity on Root Weight Ratio

At increasing salinity on the ratio of root : to total biomass was significant only in the green group (Fig. 2.6), which demonstrated a salinity dependent reduction in RWR between 0 mM and 150 mM ($p = 0.002$). Implying that epidermal betacyanin does effectively filter saturated

light and influence on the capacity to maintain RWR at increasing NaCl, as no significant difference was detected in the filter ($p = 0.077$) and red ($p = 0.193$) colour groups.

Between the treatment groups only the 0 mM and 150 mM NaCl ($p = 0.033$) yielded a significant difference in RWR across the three colour groups. Suggesting that when NaCl is present at moderate concentrations the ratio of root to total biomass remains unaffected.

Additionally, colour did influence the ratio of root to total biomass at each treatment level. In the red group RWR was greatest at 150 mM NaCl, indicating a competitive advantage over the green morphs under increasing salinity levels.

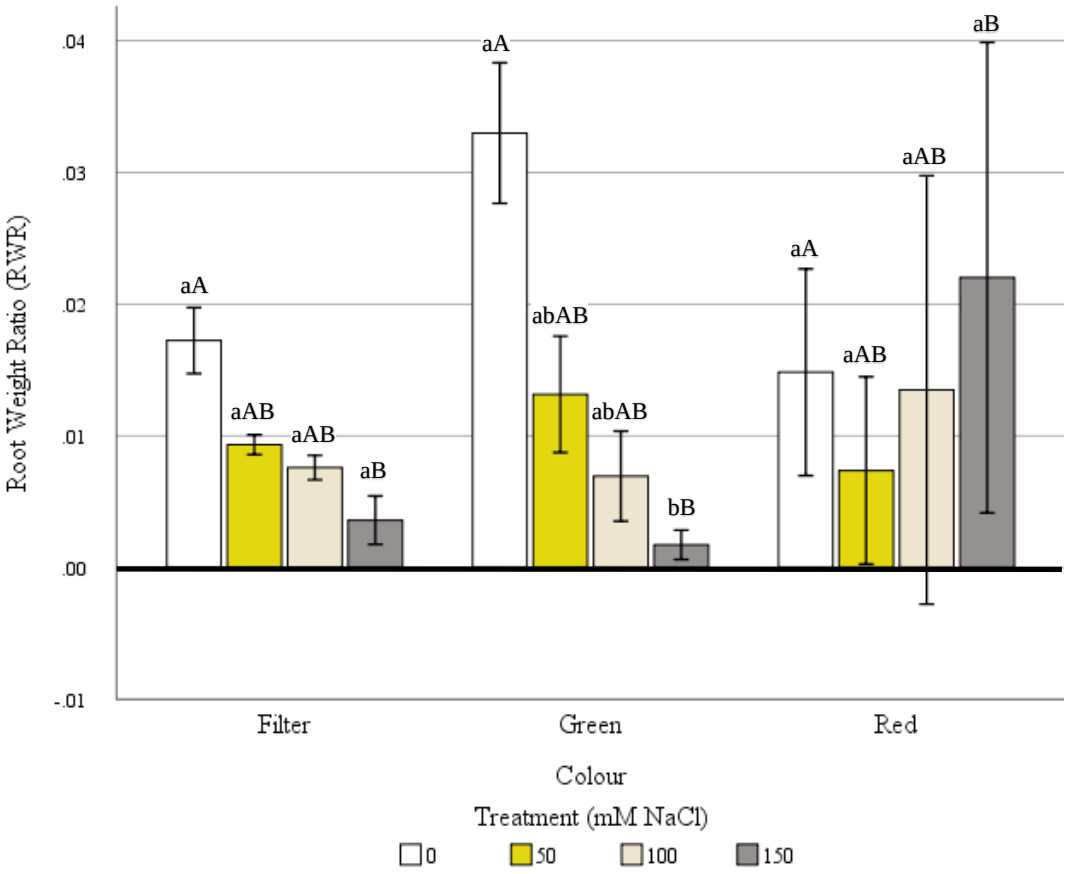


Figure 2.7 Ratios of root : total dry biomass for the three colour groups at the indicated NaCl (mM) concentrations. The values presented are means with SE (n=5). Different lower case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences within colour groups for each treatment. According to the Dunn's procedure with Bonferroni correction ($p < 0.05$).

2.4.4 Effect of increased salinity on TWC

TWC of the above and below ground biomass are shown in table 2.1. For the filter and green colour groups, TWC% was highest at 50 mM NaCl in the shoots. In the red group TWC% in the shoots was highest at 150 mM NaCl. The results further support the epidermal betacyanin alleviate photo inhibition on chloroplasts subject to salinity both physiologically through metabolic process, and by filterting saturated light.

However in both the shoots and roots of the green group with the filter (filter) and green group with out the filter (green), the TWC% of the filter was consistently lower. This implies that filter maybe adversely affecting the photo synthetic capacity of this group. These findings are discussed further below.

Table 2.1 Tissue Water content percentage (TWC %) of the shoots and roots at the indication NaCl treatments. The values presented are means with SE (n=5).

Tissue	Treatment (mM NaCl)	TWC%		
		Filter	Green	Red
Shoots	0	75.8 ± 9.0	83.2 ± 2.4	82.5 ± 3.5
	50	85.4 ± 3.6	89.9 ± 0.4	87.0 ± 2.2
	100	82.5 ± 2.7	89.5 ± 3.0	84.1 ± 4.0
	150	84.4 ± 5.5	88.2 ± 2.2	90.3 ± 1.0
Roots	0	92.1 ± 1.3	93.2 ± 1.5	78.3 ± 11.7
	50	91.1 ± 1.2	92.8 ± 0.7	91.6 ± 1.4
	100	79.9 ± 8.8	85.2 ± 8.0	90.8 ± 1.2
	150	43.3 ± 29.8	74.6 ± 18.8	75.2 ± 18.7

2.5 Discussion

In this study I evaluated the effect of salinity and photo stress on root development in green- and red-leafed morphs of *Disphyma australe.* Further, I aimed to separate the light screening capacity of leaf betacyanin from additional physiological functions of betacyanin accumulation. In earlier studies on dimorphic populations of green- and red-leafed *D. australe* along the south Wellington coastline. Betacyanin production in the red morphs was shown to depend on duel exposure to both salinity and high light, the green morph is unable to produce betacyanin under the same conditions (Jain & Gould, 2015). Additionally, the red morph was shown to be more tolerant to the combination of high light and salinity, as measured by higher CO2 assimilation rates, reduced inhibition of PSII and enhanced water use efficiency relative to the green morph. Photo damage to PSII has long been attributed to excess light absorped by chloroplasts, therefore this damage was assumued to be an unavoidable consequence of photosynthesis. However, studies have demonstrated the the selective filtering of light, both artificially and by epidermal pigments, could reduce photoinhibition with little detriment to photosynthetic carbon fixation and overall plant growth (Takahashi et al., 2010). How this function may aid development and growth of roots in coastal environments has not previously been evaluated. Overall, the findings presented in this study conclude that epidermal betacynin aids root growth and seedling establishment, and may contribute to the distribution of green- and red-leafed morphs of *Disphyma australe* along a salinity gradient.

This study focused on understanding the capacity of root biomass accumulation under increased salinity concentrations when betacyanin is present in the vegetative tissues. Both morphs performed relatively well under increased salinity concentrations, as they were able to develop roots under increased salinity and saturated light, and showed a strong resistance to tissue dehydration in the shoots. Previous studies confirmed this observation noting *D. australe's* capacity for osmotic adjustment under high salinity (Neales & Sharkey, 1981). However, the results revealed a potential trade-off between high salt tolerance and reduced growth rate in low saline conditions. As only the green morph exhibited stimulated root growth when NaCl was absent from the growth medium. The red morph showed comparatively slow root growth in low saline environments and in the absence of NaCl entirely, relative to the green morph. *Amaranthus cruentus L.,* a betacyanin producing dicot, was evaluated for the effects of water stress on leaves with and without betacyanin accumulation under high light.

The researchers described evidence for photoinhibition when subjected to water stress, such as: reduced relative water content and decreased Chl content. This effect was reported to be greater in leaves where betacyanin was present. The authors postulated that the reduced Chl content reported in water stressed-betacyanic leaves may facilitate the degree of light attenuation by increasing the relative abundance of betacyanin to Chl (Nakashima *et al.*, 2011). Implying that the betacyanin producing plants may experience a trade-off in which they accumulate betacyanin in the epidermis at the cost of Chl abundance. As sustained Chl content is fundamental for photosynthesis and subsequent carbon fixation for growth (Bose *et al.*, 2017). This may offer an explaination for the reduced growth rate observed in the red morph when NaCl was absent from the growth medium (Fig. 2.3). As in these studies betacyanin synthesis appeared to be preferentially induced by high light over high salinity (Fig. 2.8).

Futhermore, the results of this study showed that in the red morph, increasing salinity did not adversely affect root biomass accumulation. A finding which supported the hypothesis that red morphs will experience sustained growth under increasing salinities. In some circumstances, patterns of biomass allocation provides a measure of community dominance, as it indicates a plants capacity to tolerate the stressor. There is a generalized assumption that root growth is less affected by salt stress than shoot growth (Munns & Tester, 2008; Munns & Ternaaat, 1986). Although some studies show verying results (Madawala *et al.*, 2014). In concert with sustained root growth under increased salinity, the final shoot DW of the red morphs was significanlty reduced at 150mM NaCl. These results support the assumption, that root growth is enhanced by salt stress. However, this may come at the expense of sustained shoot growth, facilitated by a reduction in Chl content.

All three co,oour groups experinced a salinity dependent reduction in shoot growth at NaCl concentrations > 50 mM NaCl over the course of the study. Flowers & Colmer (2008) postulated why growth declines at high salinities, they determined that although unclear, there are several possibilities. Such as: reduced C fixation and altered photosynthesis and respiration rates due to shifting biomass allocation between leaf, stem and root. Another possibility is that growth may decline as a result of reduced turgor, which is dependent on high concentrations of ions in the apoplast. Further possibilities relate to osmotic adjustment, such as an inability to effeciently accumualte and/or distribute nutrients or synthesize sufficient organic solutes or

the engrgetic demands of ion compartmentation. The energetic demands of general halophyte processes may be substantial, energy would be consumed in ion transport to regulate net uptake and compartmentaion of Na^+ and Cl^-, and in the synthesis of compatible solutes. The extent of these demands are unknown and if the tonoplast membranes in halophytes are proficient at preventing Na^+ and Cl^- leakage (Flowers & Colmer, 2008)

The green colour group displayed a salinity dependent reduction in root biomass. Conversely, where a filter was applied increased salinity also had a significant effect of root DW (mg) accumulation. These results indicate that the ability to accumulate roots under increased salinity is not solely afforded by the photoprotective role of betacyanin presence. From this we can ascertain that the continued dry weight accumulation under adverse conditions is independent from photosynthesis. Nakashima *et al* (2011) reported that leaf betacyanin provided photoprotection atleast in part by attenuating excess light in stressed leaves of *Amaranthus cruentus*. Indicating lower photoinhibition susceptibility in betacyanin producing morphs. Plant growth is a balance between above and below ground biomass accumulation. A higher RWR indicates that plants are diverting energy to below ground biomass accumulation. Energy not ustilised here could be diverted toward shoot biomass accumulation or utilised for repair systems (such as the repair of photosystem II). In the filter and red groups colour has not effect on RWR across the salinity gradient. In the green group a siginificant difference was detected between 0 mM and 150 mM NaCl. This further supports that the presence of betacyanin reduces photoinhibition. Consequently plants are able to utilise energy for sustained growth, rather than diverting this energy for photosystem repair.

In this study, only the green morph allocated significantly less biomass to the roots; ≥ 50mM NaCl in the green group and > 100 mM NaCl in the filter group. At these same values the growth in shoot weight for the green and filter groups was the greatest (Fig. 2.5). Madawala *et al.*, (2014) evaluated the Root Weight Ratio of four ice plant taxa; *C. edulis, C. chilensis, D. australe,* and *C. edulis* x *D. australe* hybrid, grown at increasing salinities (0%, 50% and 100% seawater). The researchers reported that all four ice plant taxa allocated proportionately less biomass to the roots when under higher salinity (50% and 100% seawater). While, it is commonly reported that plants respond to environmental perturbations by allocating biomass among organs to enhance resource capture (Madawala *et al.*, 2014). A general pattern for

biomass allocation between root and shoots under salinity stress is yet to be established (Munns & Termaat, 1986; Munns & Tester, 2008; Madawala *et al.*, 2014)

I evaluated the capacity of red, green and green morphs with a light screening red filter to develop roots in increasing salinities in which light stress was applied. I aimed to separate the photoprotective function of epidermal betacyanins from the possible physiological traits they may afford such as effective ROS scavenging. While the results of this study supported previous findings that betacyanin alleviates the adverse effects of photo inhibition, such as ROS mediated leaf senescence under high salinity. They demonstrate that epidermal betacyanin effectively filters excess light. There by supporting findings or earlier studies (Jain *et al.*, 2015). The light screening effect of betacyanin was evident in green morph with the red filter applied (filter). As this group expereinced an inhibited capacity develop roots in the absence of NaCl, as shown by the final DW of the root system that was significantly different from the green morph without the filter (green), but did not differ from the red morph (Fig. 2.3). Further, the ratio of root : to total dry biomass. However, the explaination given above in which Chl abundance is reduced through the accumulation of epidermal betacyanin, another explaination is needed. It has been speculated that betacyanin pigmentation may hinder photosynthetic light acquisition utlised for carbon fixation (Nakashima *et al.*, 2011).

3 *In vitro* Germination Assay

3.1 Abstract

In saline environments, responses of plants during germination and early seedling stages is curicila for the establishment of the species (Llanes et al., 2005). While there are conflicting reports as to the relative senesitivity of germination to salt stress, it is well established that salt stress decreases growth in most plants, including halophytes. Where, germination responses of halophytes to environmental constraints determine their distribution in saline environments (Sosa *et al.,* 2005). The seed germination of potential of *Disphyma australe* is not known. The aim of this experiment was to determine repsonses green and red *Disphyma austrle* morphs during germination. Seeds were germinated *in vitro* in the presence of increasing NaCl concentrations (0, 100, 200, 300, and 400 mM NaCl), and subjected to recovery tests after stress. The germination percentages and velocity were determined to establish te relative tolerance and competitiveness of the two *D. australe* morphs.

The threshold salinity for a significant reduction in germination did not significantly differ between the green and red colour morphs. The different germination behaviour of the two morphs appears to contribute to their distribution along a salinity gradient. Although it did not affect seed viability, as seeds from both the green and red morphs seeds exhitied high germination rates in the recovery tests after treatment with 300 mM and 400 mM NaCl. Although germination in saline enviornments does not necessarily correlate with salt tolerance at later stages of development, it is a suitable to establish salinity tolerance in saline environment, and has yet to be evaluated for *Disphyma australe*

3.2 Introduction

The distinct formation of vegetative zones is a common feature in coastal plants communities, where the distribution of plants is attributle to both abiotic factors, such as light, temperature and salinity, and biotic factors such as competition (Carter & Ungar, 2003). Seed germination is a critical stage in the life cycles of higher plants as success of seed germination is a determing feature for the continuation of a plant species in a given environment (Donohue *et al.,* 2010; Rajjou *et al.,* 2012; Al Hassan *et al.,* 2017). As sessile organisms, plants cannot leave the place where their seeds germinate. Therefore, seeds must able to sense that their surrounding environment is appropriate for seedling survival and establishment. Seeds determine when to

germinate through the perception of environmental cues such as temperature, light, water, oxygen availability and soil salinty into endogenous developmental programs (Jung & Park, 2011). Survival in saline conditions is largely dependent on on the capacities for seeds to germinate as mature plants are typically more resistant and adaptable (Adolf *et al.*, 2013; Al Hassan *et al.*, 2017). Germination is vulnerable to injury, disease and sensitive to salt stress the plants that overcome this bottleneck shape plant communities (Cheng *et al.*, 2018). As some halophytes show optimal growth at 200 mM NaCl, Rozema & Schat (2013) speculated that germination rates in non-saline conditions will be inhibited. However, Carter & Ungar (2003) demonstrated that seed gerimination of halophytes is stimulated when soil slainity is reduced through seasonal events, such as rainfall. The high Na^+ concentrations of saline soils reduces soil water potential (Cheng *et al.*, 2018) inhibiting water uptake and inducing osmotic stress. That inturn suppresses embryo development. Compared to those of glycophytes, halophytes typically cope with higher salinities in the soil solution during the germination phase (Adolf et al., 2013). However, this is not always the case and several studies have reported arrested germination under increasing salinites. As such, for red morphs to frequently inhabit environments with a higher salinity than green morphs, they must be able to germinate in high saline environments. However, earlier studies evaluating the germination responses of *Atriplex prostrata* and *Salicornia europaea* to environmental constraints demonstrated that zonation within an inland marsh is not determined at the germination stage of development (Soso *et al.*, 2005; Carter & Ungar, 2003).

In addition to the range of NaCl concentrations which promote and/or inhibit germination, the capacity of seeds to germinate after exposure to excess NaCl is notable (Al Hassan et al., 2017). The ability of seeds to germinate after being maintained under high salinity conditions and transferred to distilled water is termed “recovery” (Al Hassan et al., 2017). Seeds are typically located near the surface where NaCl accumulates in salinized locations (Carter & Ungar, 2003).This accumulation of NaCl at the surface of salinized soil changes over time through the continuos evaporation of ground water and/or seasonal patterns of rainfall which is the primary determinant of freshwater availability in saline environments. Thus, for successful establishment of plants is saline environments seeds must remain viable at high salinities, in a salinity imposed ‘secondary dormancy’ to germinate when salinity decreases (Llanes *et al.*, 2005). Further, imposed dormancy may facilitate a priming effect in which seeds ‘pre-treated’ with NaCl germinate at greater rates than do the non-primed controls. Several studies have

addressed recovery of seed germination in halophyts taxa (Adolf *et al.*, 2013; Al Hassan *et al.*, 2017; Borsai *et al.*, 2017). The authors postulating that prior exposure to salinity stimulates the initial defense mechanisms required for salinity tolerance such as osmotic adjustment and the activation of anti-oxidant defense systems.

In *Disphyma australe*, vegetative shoots of the red morphs have been shown to be physiologically more tolerant to salt stress than are their green shoot counter-parts (Jain, 2016). However, at present no studies comparing the germination and recovery capacity of the two morphs have been carried out. This investigation explored how variation in morphology influenced germination capacity under increasing salinities. I compared the germination and recovery rates of seed from red and green morphs at different concentrations of NaCl relative to those in distilled water. I hypothesise that the capacity to produce betacyanin will allow the seeds from red morphs of *Disphyma* australe to germinate at a higher salinity. By allowing *D. australe* to overcome salinity and associated ionic stress during the germination phase, the presence of betacyanin may influence the distribution pattern among natural populations.

3.3 Materials and methods

3.3.1 Collection of the seed stock

Seed pods were collected from natural populations of *D. australe* growing in Te Kopahou reserve during January and February of 2019. Collection followed the Wellington City Councils eco-sourcing guidelines. The pods were taken back to Victoria University of Wellington where they were bisected and the seeds were collected into 15 mL Falcon tubes and stored in the dark at 4ºC prior to germination. Although the colour morphs were kept separate, seeds originating from different plantswere pooled together for the following assay.

3.3.2 Germination analysis

Germination plates were examined with a dissecting microscope (Zeiss Stemi 305 compact Greenough stereo microscope), fitted with a camera (Zeiss Axiocam 105 color).

3.3.3 Seed viability test

Seed viability was assessed with the tetrazolium (TZ) test using a modified methods of Patil & Dadlani (1958). 100 Red and 100 Green D. australe seeds were imbibed for 180 mins in distilled water. Once imbibed, the seeds were dissected to expose the embryo (Fig. 2.1), submerged in 0.5% 2, 3, 5-Triphenyltetrazolium chloride (Sigma-Aldrich), and incubated in a warming cupboard (40ºC) for 1 hr.

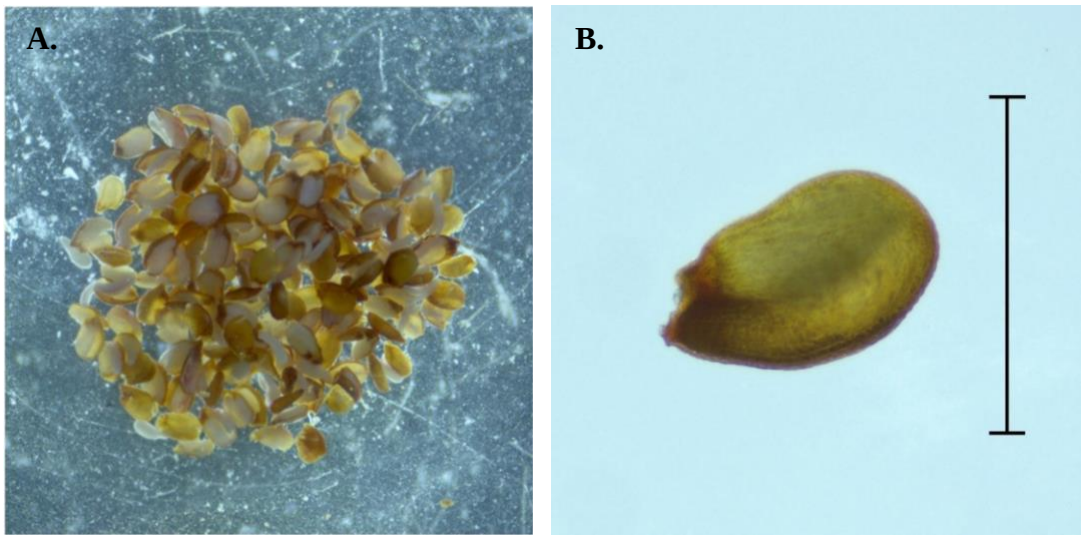


Figure 3.1 (A.) Bisected *Disphyma australe* seeds submerged in 0.5% 2, 3, 5-Triphenyltetrazolium chloride. (B.) Bisected *Disphyma australe* seed. Scale bar represents 1mm.

3.3.4 Seed sterilization

Seeds were sterilized following a modified version of the methods detailed in Zhong et al (2016). The seeds were held at 4ºC in the dark for 72hr to break dormancy prior to sterilization. Five replicates of 100 randomly-selected seeds were used from pooled stocks taken from red and green morphs. The seeds were collected in 1.5 ml eppendorf tubes to which 500µL of 70% (v/v) ethanol was added. The tubes were vigorously inverted for 1min (Zhong *et al.*, 2016), and then the ethanol was pipetted off, and the seeds resuspended and rinsed five times in distilled water within a sterile environment.

3.3.5 Germination protocol

Sterilized seeds were sown onto 90 mm qualitative filter paper (LabServ) in 90 x 1.5 mm pertridishes (Citotest) containing 3000µL of autoclaved distilled water supplemented with NaCl. There were four concetrations of NaCl (100, 200, 300 and 400 mM) and a control of distilled water. Five replicates of 100 sterile seeds of each red and green morph were used per treatment. The dishes were sealed with parafilm and placed in a culture room (22 ºC with a 12 h light/12 h dark photoperiod). Germination rates were recorded every every week for four weeks. A seed was considered as germinated at radicle protrusion through the seed coat (Fig. 3.2).

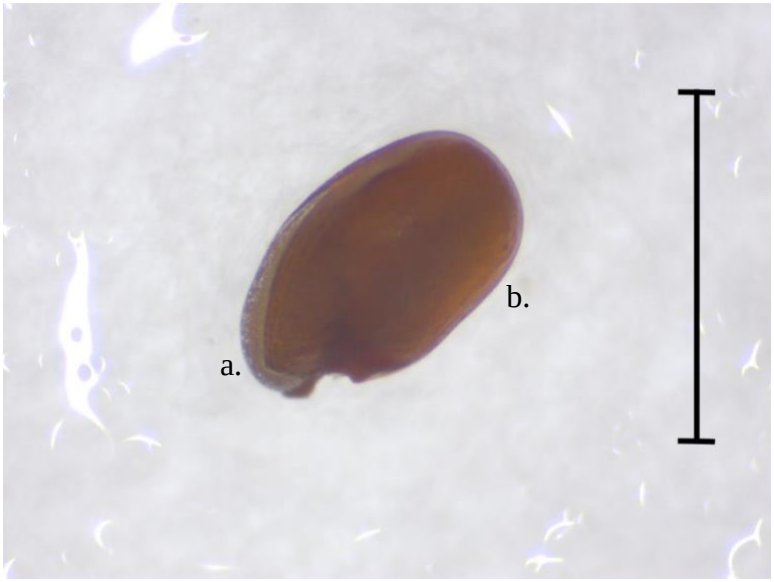


Figure 3.2 Germinated Disphyma australe seed. The radicle (a.) is visible as it protrudes through the seed coat (b.). The scale bar shown represents 1 mm.

3.3.6 Mean germination time

Mean germination time (MGT) was calculated as:

$$MGT = \Sigma (D \times n) / \Sigma n$$

Where D is the days from the beginning of the germination test, n is the number of seeds newly germinated on the day (Borsai *et al.*, 2017).

3.3.7 Recovery tests

Seeds that did not germinate in the previous test in the presence of NaCl were used in recovery tests. Seeds were briefly rinsed in distilled water and resown on filter paper in Pertri dishes (Citotest) containing distilled water and maintained under the same temperature and light regimes as described above. Final germination percentages were recorded after 7 days. The seed germination percentages were arcsine transformed and differences were assessed with the non-parametric Kruskall Wallis-H test and planned post hoc with Bonferroni correction. Differences were considered significant at $p \leq 0.05$.

3.3.8 Statistics

Statistical analysis was carried out in IBM SPSS Statistics 25. The data was arcsine transformed and significant differences between each treatment was tested by a one-way ANOVA with planned Tukey's Post hoc to determine statistically significant differences ($p \leq 0.05$). Normality was assessed with the Shapiro-Wilk test for normality ($p \geq 0.05$). Homogeneity of variances was assessed by the Levene's test ($p \geq 0.05$). Where the assumption of normality could not be met with the Shapiro-Wilk test ($p \geq 0.05$) the non – parametric Kruskal-Wallis H test was employed to determine differences significant differences ($p < 0.05$)

3.4 Results

3.4.1 Initial germination assay

Seeds from both morphs reached near 100% germination when sown on distilled water (Fig. 3.3) Increasing salinity in the germination medium significantl reduced final germination rates in seeds from both the green and red morphs. With 100 mM naCl germination rates for the red morphs were approximately two-fold greater than those of the green morphs. At NaCl concentrations of 200 mM and above, germination rates in both morphs were similarly low.

The means for final germination were consistently higher in the red morphs (Fig. 3.5) across all four treatment levels, indicating a greater tolerance to salinity stress during the germination phse, and in the control, the difference between red at green at each treatment level were not statistically significant ($P = 0.172$).

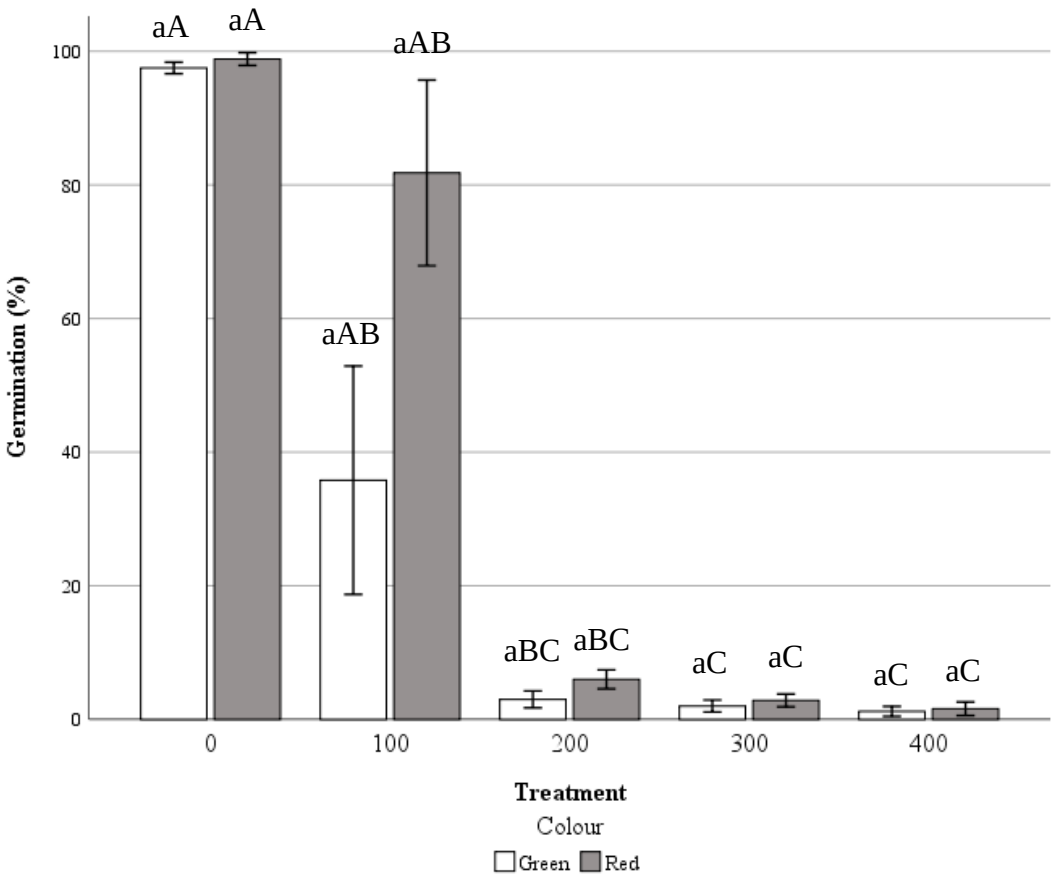


Figure 3.3 Final germination percentage of each colour at the treatment level indicated. Values presented are means ± SE (n=5). Different lower case letters above the bars indicate significant differences between colours at each treatment level. Difference capital letters above the bars indicate significant difference between treatments within each colour ($p \leq 0.05$).

Additonally, seedlings germination under 100 mM NaCl produced pigmentation in their vegetative tissues (Fig. 3.4). This pigmentation was not visible in seedlings germinated in distilled water (0 mM NaCl). While germination could be determined at higher salinities (200, 300 and 400 mM NaCl), the seedlings did not possess well developed shoots. This can be attributed to salinity dependent inhibition of germination at these salinities (Fig. 3.5)

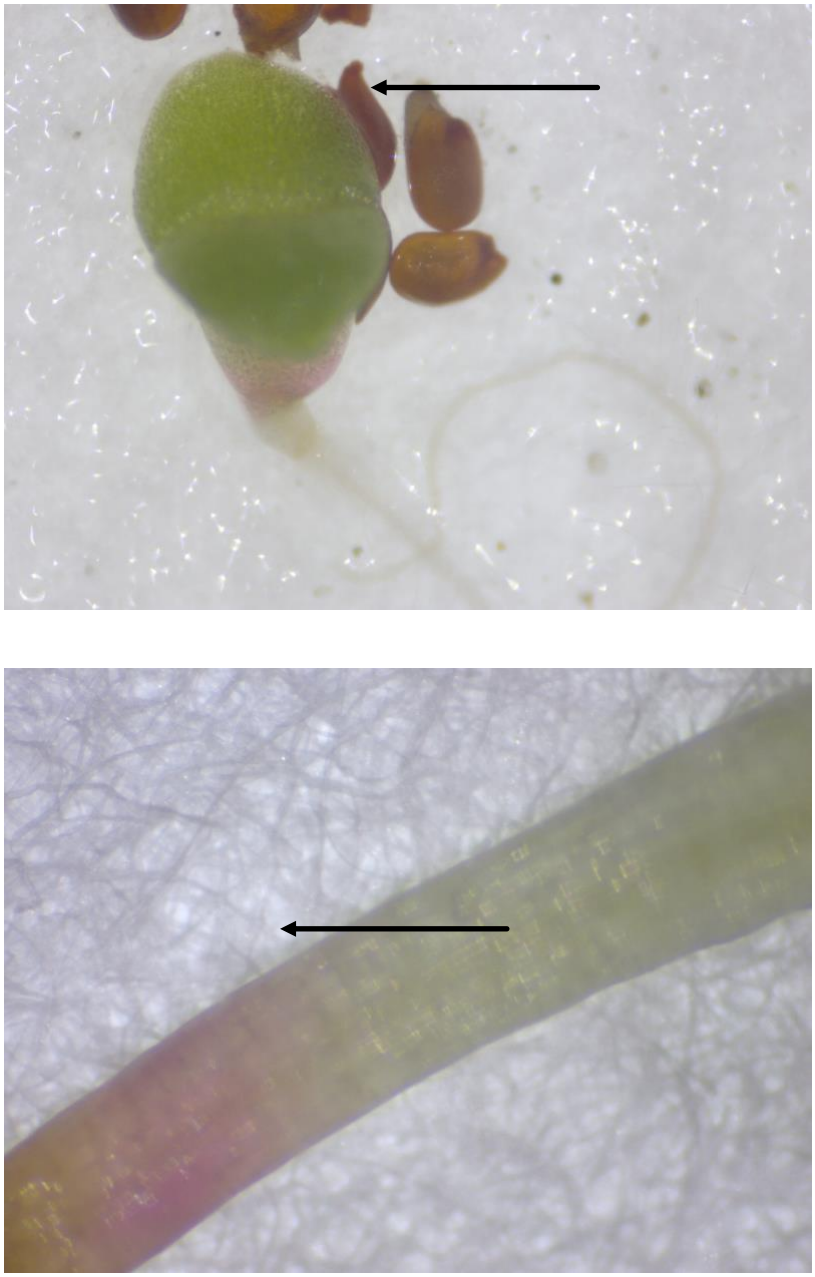
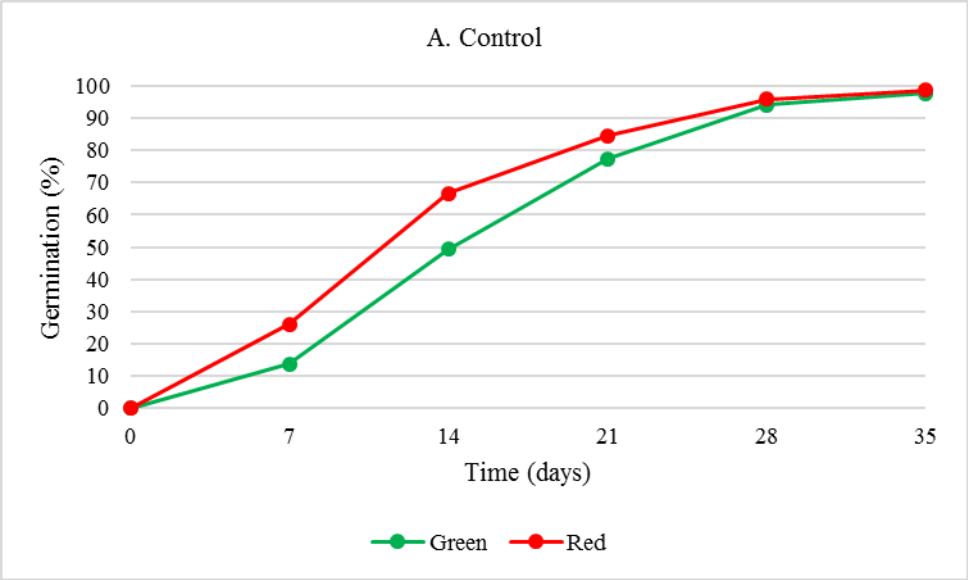
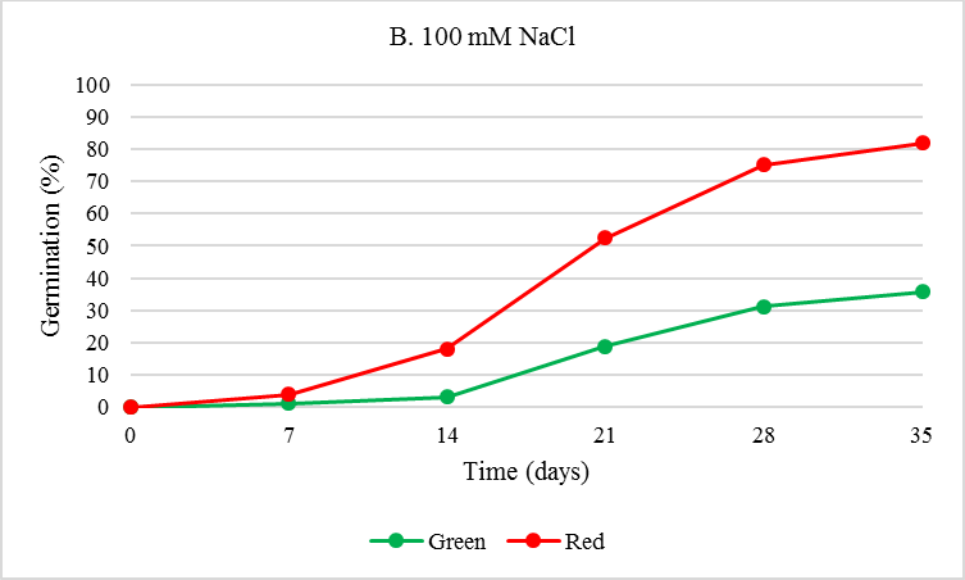
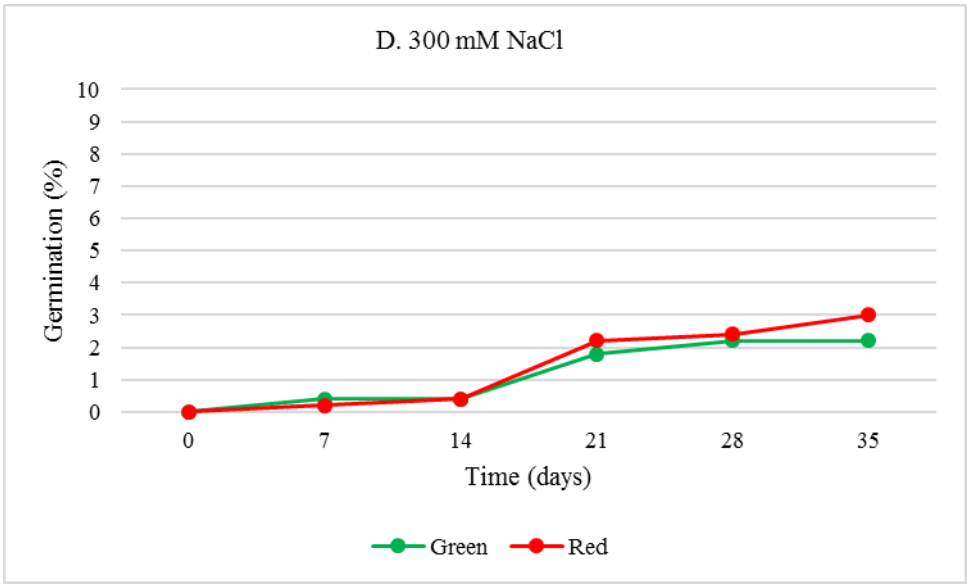
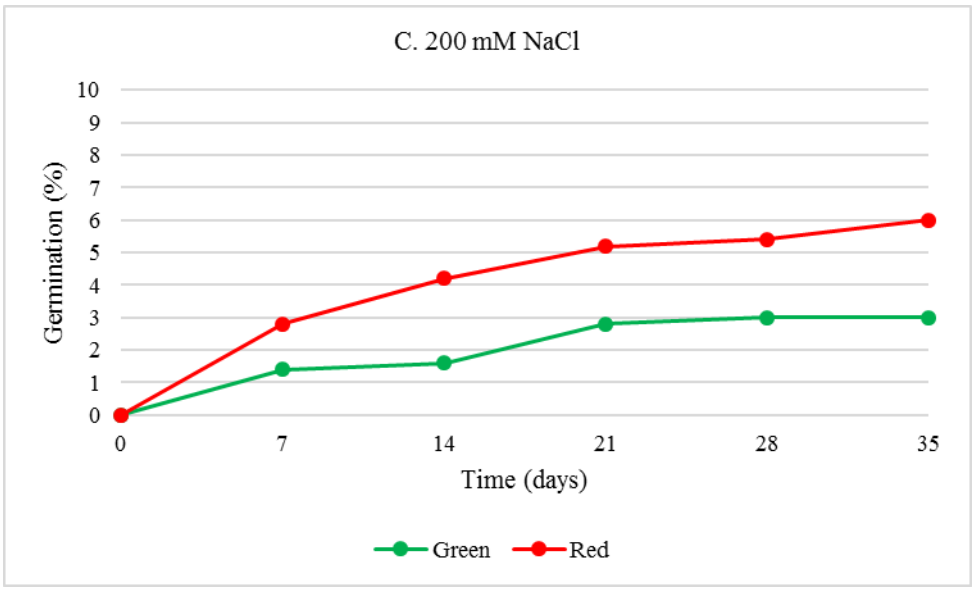


Figure 3.4 Examples of seedlings germinated in 100 mM NaCl. Arrows point to pigmentation at the base of the stem.


A. Control
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
Time (days)
Green
Red


B. 100 mM NaCl
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
0
7
14
21
28
35
Time (days)
Green
Red


D. 300 mM NaCl
Germination (%)
10
9
8
7
6
5
4
3
2
1
0
0
7
14
21
28
35
Time (days)
Green
Red


C. 200 mM NaCl
Germination (%)
10
9
8
7
6
5
4
3
2
1
0
0
7
14
21
28
35
Time (days)
Green
Red

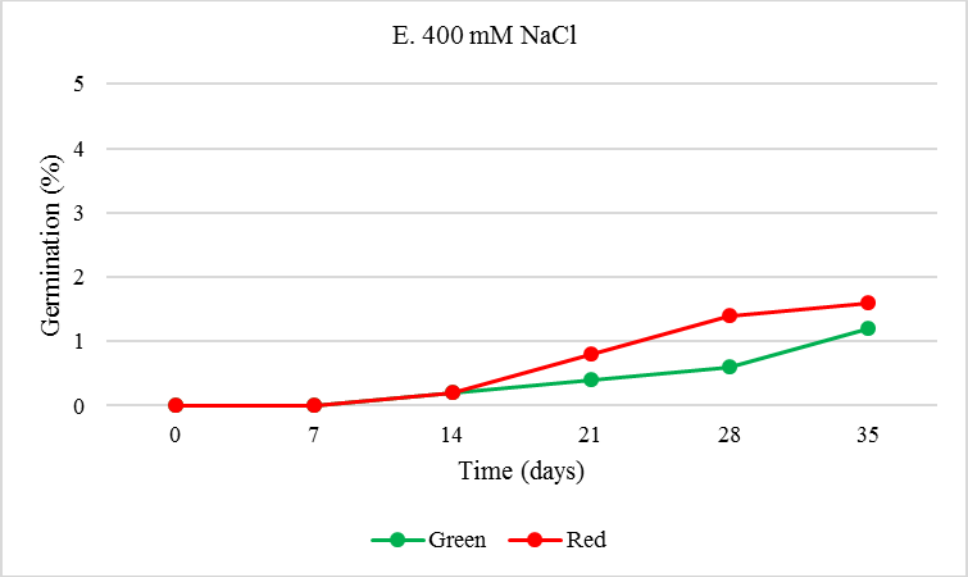


Figure 3.5 Change in germination percentage over the course of the study period at the control and each treatment level A. Control (0 mM NaCl), B. 100 mM NaCl, C. 200 mM NaCl D. 300 mM NaCl and E. 400 mM NaCl. The values shown are means (n=5).

3.4.2 Mean germination time

Lower values of MGT indicate faster rates at which a population of seeds has germinated (Panuccio *et al.*, 2014). Increasing salinity concentrations resulted in longer MGTs in both green and red morphs (Table 3.1). MGT values were consistently lower in the red group. In MGT between the colour groups. Red and Green did not differ significantly in MGT at each treatment level ($p = 0.61$). A significant difference was detected between treatment levels ($p = 0.002$). At high salinity (400 mM NaCl) MGT was significantly different to the control ($p = 0.018$)

Table 3.1 Mean germination time (MGT) of seeds during the initial germination tests at the indicated treatment level. Different lower case letters beside the values indicate significant differences between colours at each treatment level. Difference capital letters beside the values indicate significant difference between treatments within each colour ($p \leq 0.05$).

MGT		
Treatment (mM NaCl)	Seed germination	
	Green	Red
0	5.03 ± 0.16$_{aA}$	4.00 ± 0.17 $_{aA}$
100	9.61 ± 0.44 $_{aAB}$	8.21 ± 0.71 $_{aAB}$
200	9.22 ± 2.84 $_{aAB}$	3.68 ± 0.11 $_{aAB}$
300	14.28 ± 3.45 $_{aAB}$	11.22 ± 3.35 $_{aAB}$
400	25.20 ± 6.86 $_{aB}$	15.40 ± 6.02 $_{aB}$

3.4.3 Recovery rates

Significant differences were detected within both green and red colour groups ($p = 0.002$ and $p \leq 0.0005$ respectively) in which salt pre treatments stimulated recovery at concentrations ≥ 300 mM NaCl (Fig. 3.6). However, at concentrations ≥ 300 mM NaCl no significance difference was detected in green ($p = 0.845$) or red ($p = 0.853$). Conversely, at treatments ≤ 200 mM NaCl no significant differences were in green ($p = 0.993$) and red ($p = 0.992$). Within treatments the only significant difference detected was within 200 mM NaCl ($p = 0.040$). In which the red morph exhibited a greater recovery percentage than the green morph.

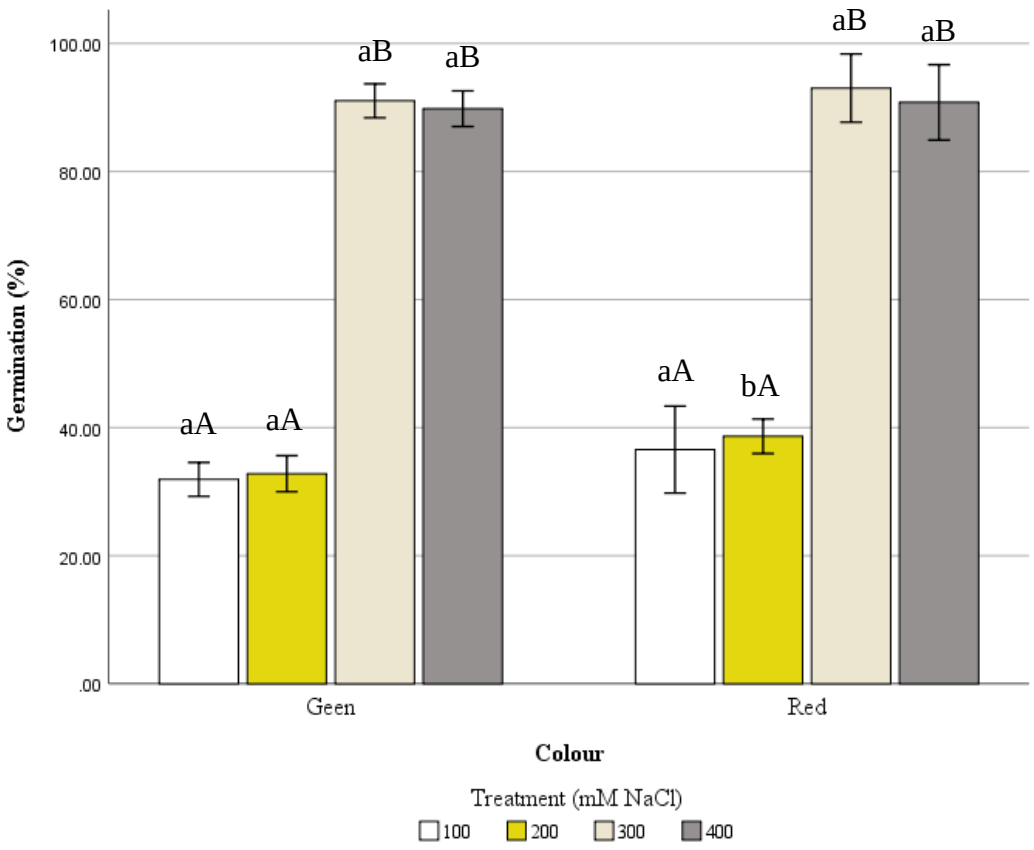


Figure 3.6 Germination recovery rates in green and red morphs of *D. australe* previously exposed to NaCl at the treatment levels indicated (mM NaCl) indicated. Values presented are means ± SE. Different lower case letters above the bars indicate significant differences between colours at each treatment level. Difference capital letters above the bars indicate significant difference between treatments within each colour ($p \leq 0.05$).

3.5 Discussion

Differences in germination capacity may contribute to the distribution of green and red *Disphyma australe* along a salinity gradient in natural populaitons along the south Wellington coast. Seed survival in the soil contributes to population persistence and community diversity (Rajjou et al., 2012). In which seeds that germinate under a particular condition must also possess the necessary physiologcal traits to survive and reproduce in that environment with respect to light, temperature, ion concentrations, or geographic location (Donohue et al., 2012). High tolerance to ion-toxicity during the germination phase is advantageous in salt affected areas (Adolf et al., 2013). Seeds from the red morphs of *D. australe* consistently display a enhanced tolerance in elevated Na^+ in the germination medium. However, both morphs

displayed a similar capacity to recover from pre-treatment with NaCl and germinate in distilled water. Taken together, the results of this study contribute to our understanding the differential distribution of green and red morphs.

Excess NaCl in the growth medium reduces water potential and induces a water deficit, further the accumulation of excess Na^+ and Cl^- inhibits cll growth and division. Cheng et al (2018) report the inhibition of germination in the presence on NaCl corresponds to the regulation of the phyto hormone ABA (Fig. 3.7). ABA is considered to be the primary regulator of seed germination preventing the transition from dormancy to germination in response to unfavourable conditions (Rajjou *et al.*, 2012; Cheng *et al.*, 2018). Elevated NaCl concentrations inhibited seed germination in both *D.australe* morphs. The inhibitory effect of NaCl on seed germination became more evident with increasing NaCl concentrations. Several studies have established that the development of a germinating embryo into a seedling is arrested under exposured to high concentrations of NaCl (Rajjou, L et al., 2012(Ghoulam *et al.*, 2002a; Adolf *et al.*, 2013; Panuccio *et al.*, 2014; Al Hassan *et al.*, 2017; Borsai *et al.*, 2017)).

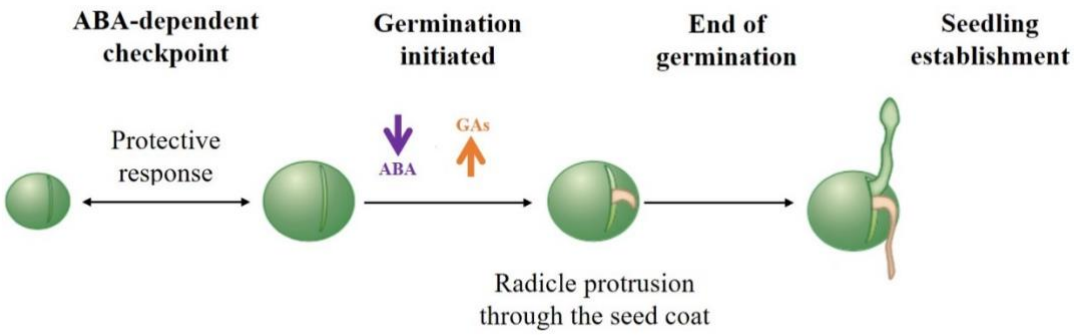


Figure 3.7 Standard progression of germination from a mature dormant seed to seedling establishment. Abbreviations: ABA, abscisic acid; Ga, gibberellin. Image modified from (Rajjou *et al.*, 2012).

The green and red morphs of *D. australe* showed different levels of tolerance at the seed germination stage (Fig. 3.5). The green morph was shown to be the most sensitive, as

significant reductions in germination percentages were observed in the in presence on 100 mM NaCl. The red morph appeared to the most salt tolerant, as NaCl inhibition of germination capacity, although present, was not as severe. However at each treatment level the differences between each morph was not significant. Salt concentrations inhibitory for seed germination have also been determined for other betacyanic species, however these studies could only compare germination rates between cultivators. Studies on Mediterranean endemic *Liminium* taxt revealed an inhibition of germination for salt sensitive species at concentraions higher than 50 mM NaCl. For example in *Limonium girardianum* and *Limonium santapolense,* where germination was reduced in the presence of 50 mM and 100 mM NaCl repsectively. Some *Liminium* taxa were shown to be tolerant during the seed germination phase, as it is the case for *Limonium virgatum* in which germination was optimum in the presence of NaCl (Al Hassan *et al.*, 2017). These findings have similarly been repoted for Beet root (*Beta vulgaris*), a glycophytic member of the Chenopodiacaea family, which has been shown to be sensitive to elevated salinity at the germination phase (Ghoulam *et al.,* 2002). Quinoa *(Chenopodium quinoa Willd*) is a betalainic halophyte capable of survivng in soils with an ECe of 40 dS/m (Adolf *et al.,* 2013b; Panuccio *et al.,* 2014) and its most tolerant varieties are able to germinate in salinities as high as 400 mM NaCl. However, genotypes of Quinoa growing in low saline areas are reported to have lower germination rates at high salinities (> 200 mM NaCl) (Adolf *et al.,* 2013).

As some halophytes show optimal growth at 200 mM NaCl, Rozema & Schat (2013) posited that they will experience reduced growth and low germination rates in non-saline conditions. Positing that growth and germination will be stimulated at 150-300 mM NaCl (Rozema & Schat, 2013). However, the results presented here did not reflect this as both green and red morphs experienced arrested development with higher salinities. Inhibited germination under high salinity has been observed in many halophytes. Carter & Ungar (2003) studied the salinity dependent distribution of *A. prostrata* and *S. europaea* across three zonal communities. Their data showed the germination in these two halophytes was stimulated when increased percipitation reduced substrate salinity. This results was demonstrated for both the heteromorphic seed types studied. Additionally, a study on six *Portulaca* accessions, a vegetable crop in the family Portulacaceae of the order Caryophyllales, found that salt stress affected germination in a manner similar to osmotic stress reducing final germination percentages across all taxa (Borsai et al., 2017). Like *D. australe*, *Portulaca* is betalainic

halophyte. At the highest concentration the vaviety *P. grandiflora* had a final germination percentage of 79%. The most salt sensitive taxon had a final germination percentage below 3% (Borsai et a., 2017). Earlier studies on *P.oleracea*, a species shown to be relatively resistant to drought or salinity stress recorded that seed germination was reduced by 50% at a NaCl concentration of 100 mM (Sdouga *et al.*, 2019). Both these studies showed that seed germination in *Portulaca* taxa in a concentration-dependent manner.

In coastal environments fresh water availability is primarily determined by seasonal rainfall (Sucre & Suárez, 2011). Subjecting seeds to NaCl in the priming treatments applied osmotic stress to them. Priming many improve seed vigour in two ways; 1. Initiating germination related process and 2. Allowing the growth arrested seeds to reinforce their capacity to accumulate adaptive defense responses useful to withstand environmental stress during seedling establishment (Rajjou, L et al., 2012). Early repair mechanisms are the most probable explanation for the beneficial effects of priming treatments. Betacyanin is a potent antioxidant, with proven ROS scavenging capabilities, and is likely to be a contributing factor to the enhance germinataion capability expressed by the red morphs. Recvoery after pro-longed exposure was reported in *Limonium* species (Al Hassan *et al.*, 2017). The recvoery response was most pronounced in *L. virgatum*, which showed a priming effect > 200 mM NaCl. A similar effect was shown in these results for *D. australe.*

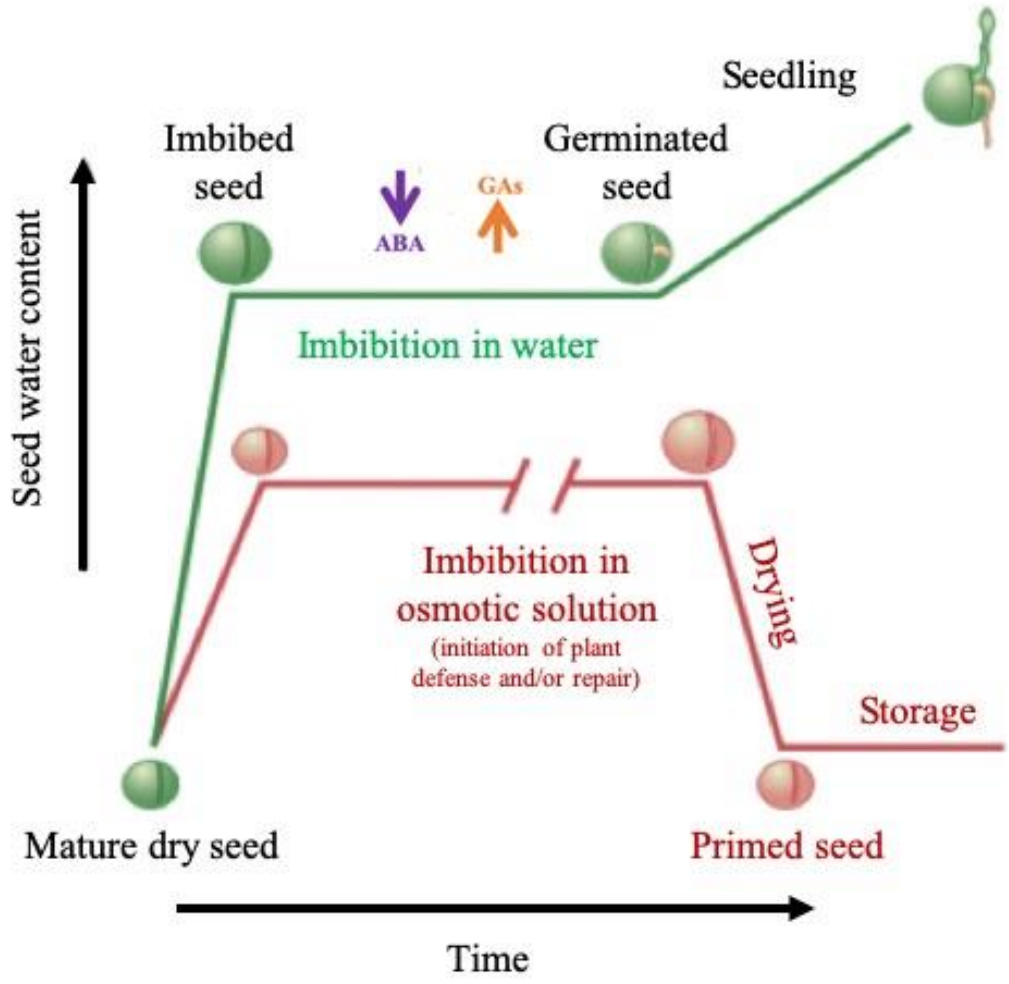


Figure 3.8 Process of seed priming (pre-treatment) compared to regular germination. Seed priming is the partial hydration of seeds to a point where the germination processes have begun but are not completed. Primed seeds can be dried before storage. Abbreviations: ABA, abscisic acid; Ga, gibberellin. Image modified from (Rajjou *et al.*, 2012).

The role of metabolism in seed development and germination is a growing area of interest where studies on Arabidopsis showed that the transition from dormancy to germination resulted in the accumulation of sugars, amino acids and shikimate-derived metabolites. As betacyanin is shikimate dervied this presents interesting area of further investigation, given the accumulation of betacyanin in the tissues of seedlings grown under saline conditions (100 mM NaCl) (Fig. 3.5) Additonally, further investigations into the environmental tolerance limits at

the seedling stage of developent for these species are needed to ascertain whether plant zonation is established at that stage of development in coastal environments.

4 Ion content in the root systems

4.1 Abstract

Disphyma australe is a dicotyledonous halophyte with a proven capacity to tolerate high saline environments. Maintaining K^+/Na^+ homeostasis in vegetative tissues when subjected to salt stress is a primary defence mechanism required for plant survival in adverse conditions. It is well established that halophytes have an enhanced capacity to accommodate, recirculate and exclude excess Na^+ when present in the environment, to suppress Na^+ concentrations in the cytosol of cells. While it is commonly accepted that low levels of substrate salinity are preferential for halophyte existence, the upper limit of salinity exposure is variable among plants, and not well established. Further, it is not known if betacyanin aids the maintenance of K^+/Na^+ homeostasis and if this affords a greater salinity tolerance to the plants which possess this unique pigment.

Thus, I aimed to establish if the capacity to maintain K^+/Na^+ homeostasis in the roots of *D. australe* significantly differed between green and red morphs across increased NaCl treatments. Salt treatments were applied to young plants, by 14 day treatment with aqueos NaCl concentrations (0, 200, 400, 800 mM). The tissue water content of the shoots and ion contents (Na^+ and K^+) in the roots were determined in the control and the stressed plants of the two colour morphs. Despite some differences under the control treatment, the concentrations of the two ions (Na^+ and K^+) were similar in the two morphs, not explaining differences in salinity tolerance, except for the increase of K^+ in the roots of the green morph when irrigated with distilled water (0 mM NaCl). Overall the results indicate that although increased substrate salinity inhibited K content in the roots of *D. australe*, this effect was irrespective of colour. This result was further supported by the tissue water content % which demonstrated that the greatest, and only significant difference in TWC between treatment levels was detected at 800 mM NaCl. When the ratio K^+/Na^+ was assessed irrespective of total K and Na content, the greatest observable difference between red and green occurred at the control (0mM NaCl). Providing evidence for a trade-off between capacity to tolerate high saline environments, and the ability to succeed when NaCl is absent from the environment.

4.2 Introduction

All halophytes display a common need to regulate cellular ion concentrations as they adjust to decreasing external water potential (Flowers & Colmer, 2008). In coastal habitats, halophyte species exhibit distribution patterns that correspond with gradients in soil salinity. Ions such as Na^+ and Cl^- are considered toxic at high concentrations and it is generally assumed that Na^+ and Cl^- are compartmentalized, predominantly in vacuoles so that concentrations within the cell are maintained within tolerable limits (Flowers & Colmer, 2008; Cosentino *et al.*, 2010; Munns *et al.*, 2016). Although evidence for the compartmentalization of Na^+ into vacuoles is limited, a few studies have shown that when exposed to excess NaCl the accumulation of Na^+ into vacuoles is suppressed in root cells and activated in the cells of the leaves (Flowers & Colmer, 2008).

The root system is the first site of detection and the first line of defense against excess Na^+ in cells (Bojorquez-Quintal et al., 2014). The capacity for enhanced tolerance through Na^+ uptake and distribution has been reported previously in *Disphyma australe* (Jain, 2016) and other betalain halophytes such as *Portulaca* spp. (Borsai *et al.*, 2017; Sdouga *et al.*, 2019) and *Sesuvium portulacastrum* (Muchate *et al.*, 2016). The assumption we can draw from these, and other studies, is that halophytes will preferentially accumulate toxic ions in the shoot where they are accommodated in vacuoles or diluted for growth. The previous study undertaken on *D. australe* only tested for Na^+, further this study only reported findings for the control group (0 mM NaCl) and one treatment level (200 mM NaCl). For halophytes a low level of salinity in the growth medium is often favoured, and plants will use these ions as a cheap osmoticum to maintain turgor pressure and dilute excess ions for growth (Munns & Tester, 2008). Further, the relative concentrations on both Na^+ and K^+ are important for understanding tolerance in salt stressed plants, as excess Na^+ in the tissues is associated with K^+ deficiency (Al Hassan *et al.*, 2017). Consequently, I aim to expand on these findings by comparing the relative accumulation of both Na^+ and K^+ at low (200 mM) medium (400 mM) and high (800 mM) NaCl concentrations.

I hypothesise that:

1. Concentrations of Na^+ in the roots of *D. australe* will not differ significantly between the green and red morphs at each treatment level.

2. K^+ content in the roots of green morphs will decrease in correspondence with increased substrate salinity.

To this end, I will quantify Na^+ and K^+ content at the control and three treatments levels in the root systems of the green and red morphs. Additionally, I will calculate the fresh weight and dry weight of the shoot system compared with the non-stressed controls.

4.3 Materials and methods

4.3.1 Plant material

Four Shoot cuttings with a minimum of two mature leaf pairs and lacking roots were cut from 10 red and 10 green *D. australe* morphs growing in the green house facilites at Victoria University of Wellington. The cuttings were rinsed with distilled water and transplanted in pots filled with a 2:1 potting mix to sand mixture.

4.3.2 Growth conditions

The pots were left to eastablish for 6 weeks during February and March 2019. The plants were moved indoors at watered with distilled water for 1 week prior to treatment. Treatments were initiated by watering the plants every second day with 30 mL of aqueous NaCl solutions at 200, 400, or 800 mM final concentrations, or with distilled water for the non-treated controls. The saline solutions were allowed to drain freely from the pots, though no run-offs were observed during watering.After 14 d the plants were destructively harvested at the root and shoot systems were separated for further analysis.

4.3.3 Sample preparation

Total root FW could not be confidently measured, as the root system of *D. australe* plants included a mass of thick roots which broke when the plants were uprooted. The root fraction recovered from the plants was thoroughly cleaned with a paint brush, freeze dried until a constant weight (48-72 h) and stored at -80ºC until being processed. The fresh weight (FW) of the shoot systems were collected directly after harvesting. Dry weight

was determined by drying the plants in a contherm oven at 60° to a constant weight (48h – 72hr).

4.3.4 Calculations

From these measurements I calculated the tissue water content (TWC) (Muchate *et al.*, 2016a):

$$TWC = (FW\text{-}DW/FW) \times 100$$

4.3.5 Determination of ion content in the root system

Ion contents in roots were deterimined according to the "Sample Preparation Procedure for Spectrochemical Determination of Total Recoverable Elements" (2013) in aquous extracts obtained by gently the heating the samples (25 mg of dried, ground plant material) in 2 mL of HNO_3 and 5 mL HCl for 30 min at a reflux temperature of 95ºC. Once cooled, the samples were diluted to a final volume of 50 mL in a volumateric flask. Concentrations of sodium (Na) and Potassium (K) were quantified with an Atomic Absorption Spectrometer (AAS) (iCE 3000 Series AAS, ThermoFisher Scientific). Standards for sodium and potassium were prepared with NaCl and KCl respectively (Fig. 4.1).

4.3.6 Statistics

Statistical analysis was carried out in IBM SPSS Statistics. Where the assumptions of normallity and homogeneity of variances could be met, the data was assessed with a two-way ANOVA and planned LSD post hoc with Bonferroni correction. When the assumption of normality could not be met with the Shapiro wilk test for nomrality ($p > 0.05$), data was analysed using the non-parametric Kruskal-Wallis H test to test for statistically significant differences in the samples ($p < 0.05$). These tests were followed with planned pairwise comparisons using the Dunn's procedure with Bonferroni correction for multiple comaprisons.

4.4 Results

Rather than reporting the concentrations of the Na^+ and K^+ , the following results report the content of Na and K present in the root systems at the indicated treatment level, because AAS detects and quantifies the concentration on atoms present in an aqueous solution.

4.4.1 Effect of increasing NaCl on Na content in the root system

Both green and red morphs of *D. australe* experienced a salinity dependent increase of Na content in their roots with respect to the control (Fig. 4.1). At each treatment level the concentration of Na in the root systems of the green and red morphs did not differ significantly ($p = 0.741$). This results supports the first hypothesis stated in section 4.2. Within colour groups the Na content differed significantly between treatments for both green ($p < 0.0005$) and red ($P < 0.0005$).

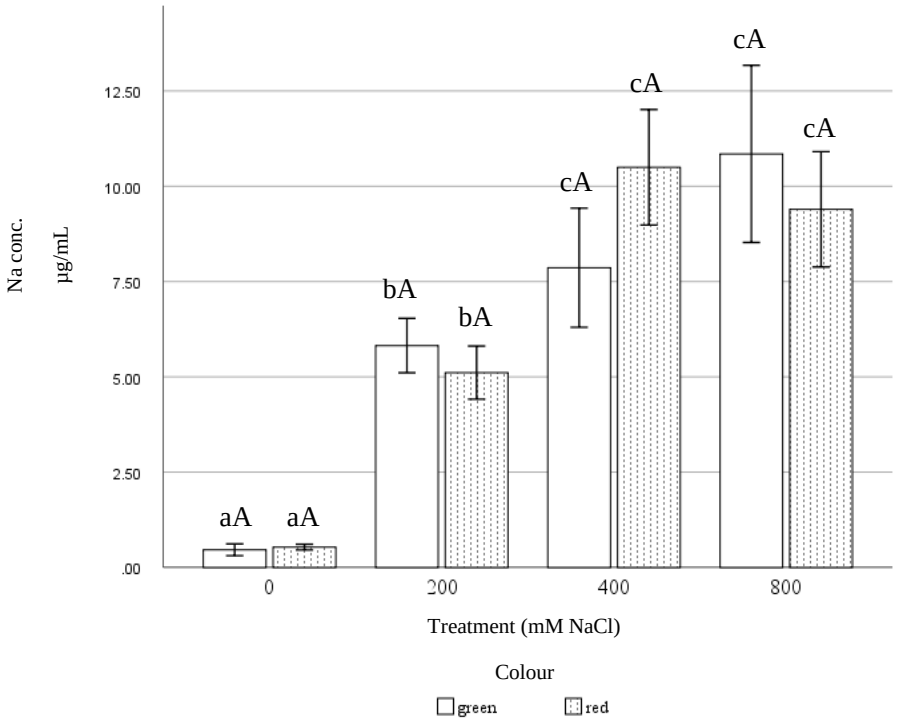


Figure 4.1 Root Na content between green and red morphs at the indicated NaCl concentrations. The values presented represent means with SE (n=10). Different lower

case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According planned Dunn's post hoc with Bonferroni correction ($p < 0.05$).

4.4.2 Effect of increasing NaCl on K content in the root system

Increased substrate salinity had a negative impact on K levels in the green and red morphs of *D. australe* at concentrations >200 mM NaCl (Fig. 4.2). At all three treatment levels K content was observed to be greatest in the red morphs. However, As with Na content, the K content in the root sytems did not differ signincantly between green and red at each treatment level ($p = 0.191$). This result did not support hypothesis 2 as stated in section 4.2. However, within in colour group increased NaCl in the growth meadium had siginificant impact on K content in the roots of the green and red morphs. For both morphs the K content at low (200 mM) and medium (400 mM) treatment levels was not significant ($p > 0.05$). Both colour groups displayed a similar trend in which the K content for the control group and high NaCl treatment were not signifincatly different. Suggesing that both the absence of NaCl and excess NaCl in the growth medium has a similar effect on K content.

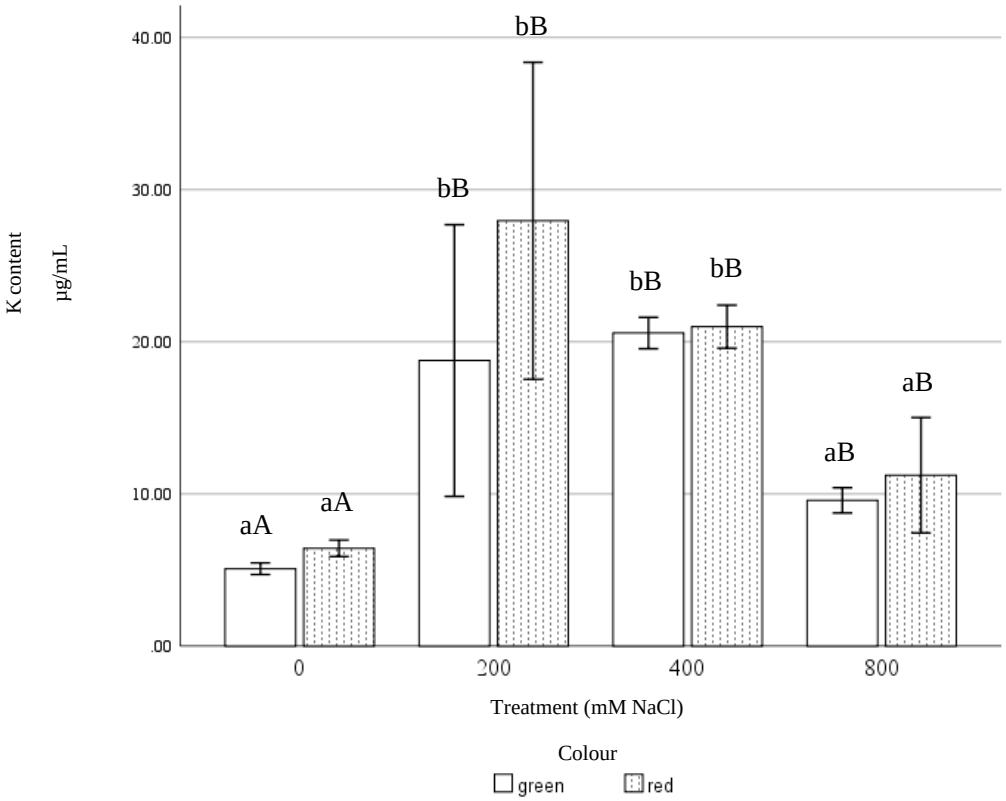


Figure 4.2 Root K content between green and red morphs at the indicated NaCl concentrations. The values presented represent means with SE (n=10). Different lower case letters above the bars indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According planned Dunn's post hoc with Bonferroni correction ($p < 0.05$).

4.4.3 Effect of increasing NaCl on K^+/Na^+

Overall, increased NaCl negatively impacted the ratio of K^+/Na^+ in the roots of *D. australe* (Fig. 4.3). Interestingly, when NaCl was absent entirely, the green morph sustained a higher ratio. However, when NaCl was present at low, moderate and high concentrations the red morph consistently maintained a higher ratio.

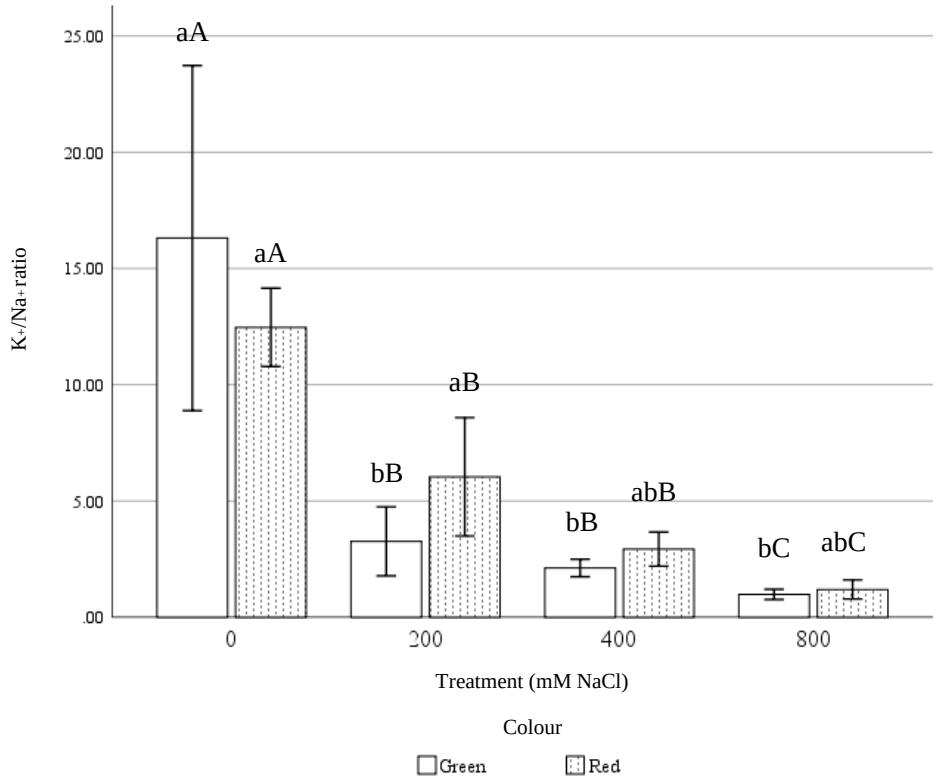


Figure 4.3 K^+/Na^+ ratio in the roots of green and red morphs of *D. australe* at low (200 mM NaCl), moderate (400 mM NaCl) and high (800 mM NaCl) salinity. The values displayed are means ± SE (n=10).

4.4.4 Effect of salinity on Tissue water content

Table 4.1. The results demonstrate that a high (800 mM) concentration of NaCl had a deleterious effect on TWC in both the red and green and morph. This difference was significant for both colour groups ($p < 0.0005$). Further, this effect was more pronounced than the absence of NaCl entirely. In which either colour displayed a significant difference in TWC between the control (0 mM) and low (200 mM) or moderate (400 mM) substrate salinity. Between colour groups at each treatment level, the only significant difference between green and red detected at 400 mM NaCl ($p = 0.040$). As the p-value is close to 0.05, the difference is not strong.

Table 4.1 TWC % accumulation between red and green at the indicated NaCl concentrations. The values presented represent means with SE (n=10). Different lower case letters indicate significant differences between treatments for each colour group and the different capital letters indicate significant differences between colour groups for each treatment. According to the LSD post hoc with Bonferroni correction ($p < 0.05$).

Treatment (mM NaCl)	TWC %	
	Green	Red
0	$93.37 \pm 0.43_{aA}$	$94.17 \pm 0.39_{aA}$
200	$93.41 \pm 0.26_{aA}$	$94.02 \pm 0.26_{aA}$
400	$94.53 \pm 0.17_{aA}$	$93.66 \pm 0.21_{aB}$
800	$90.96 \pm 0.39_{bA}$	$90.96 \pm 031_{bA}$

4.5 Discussion

I hypothesised that differences in salinity tolerance might explain variations in distribtion patterns between the green- and red leafed betacynin. The data did not indicate a correlation between ion content in the root system and salinity tolerance in the two morphs.

4.5.1 Na content corresponds with substrate salinity

One of the main differences in the general repsonses to salintiy of halophytes refers to the regulation of ionic transport (Al Hassan *et al.*, 2017). In which dicotyledonous halophytes, such as *Disphyma australe* will transport toxic ions (Na^+ and Cl^-) to the leaves for storage. The content on Na in the roots corresponded with increased substrate salinity, this concentration dependent increase of ion content in root systems was shown in *Limonium* sp. (Al Hassan *et al.*, 2017). The continued uptake of ions can be considered as a strategy to maintian plant growth under salt stress conditions, using these inorganic solutes as cheap osmoticums for turgor, and diluting them for growth (Fig. 4.4) (Munns & Tester, 2008). At low (200 mM), moderate (400 mM) and high (800 mM) concetrations of NaCl, the green and red morphs did not differ significantly in Na content. Both morphs expereinced a significant increase in Na content and concentrations ≥400 mM NaCl. the Although, unclear the following mechanism was postulated by Moir-Barnetson *et al* (2016), to explain th maximum slainity tolerance observed in *Tecticornia*, as shown by the relative growth rates (RGRs) of shoots and relative tissue ion concentration. The researchers positied that elevated root ion content at increased external NaCl may indiciate a a down regulation of net ion flux. The induction of such a feed back mechanism may arise in plants if the cells of the shoot tissues reach maximum capacity for Na^+ and Cl^- vacuolar sequestration. In the absence of a downregulation of net ion fluxes, the continued delivery of ions to the shoots would cause a build up of ions to toxic levels in the cytoplasm or apoplast.

Figure 4.4 Example of a stem cutting from a red morph grown under saline conditions (200 mM NaCl). Betacyanin pigmentation is visible at the base of the plant in the older tissues, this pigmentation fades toward the top of the stem, indicating sustained growth when exposed to abiotic stress.

Sodium uptake in the roots of *Disphyma australe* corresponded to increased NaCl treatments. Jain (2016) undertook a comparative study on Na^+ content in the roots, leaves and stems on *Disphyma australe* after 5 weeks of exposure to 200 mM NaCl. The results cited that when exposed to increased NaCl both green and red *D. australe* morphs will preferentially store Na^+ in the leaves (Jain, 2016). It is well established that dicotyledonous salt-tolerant species accumulate toxic ions in their leaves where they are maintained at low cytosolic concentrations by compartmentalization in vacuoles (Borsai *et al.*, 2017). While the exact physiological mechanism through which betacyanin synthesis aids this process has not been fully elucidated, similar findings were reported for *Mesembryanthemum crystallinum (M.crystallinum),* in which tonoplast activity in increased with the addition of NaCl in the leaves (but not roots) of mature plants upon exposure to salinity (Cosentino *et al.*, 2010). Like *D.asutrale, M.crystallinum* is a betacyanic succulent halophyte in the family Aizoaceae. Although further investigation is required to know if this same behaviour occurs in betacyanic morphs of *D.asutrale.*

These findings, along with similar studies are reported in Table 4.2. Overall these findings suggest that irrespective of salinity halophytes will preferentially transport Na^+ to the shoots for storage and excretion. This trait has been shown in halophytes outside the order Caryophyllales including *Pucinellia tenuiflora* (Zhang *et al.*, 2017) and *Ziziphus roteundifolia* (Gupta *et al.*, 2002). As such, we can assume this is a generalized trait present among halophytes, and not specific to betacyanic halophytes.

4.5.2 Alternative mechanisms for salt tolerance

Salt stress is a complex trait involving a range of intertwining mechanisms to reduce cytosolic Na^+ concentrations. When able to, plants control the distribution of toxic Na^+ to organs, tissues and cells where it will cause less damage (Bojorquez-Quintal *et al.*, 2014). A significant group of halophytes, the recretophytes, have evolved specialized epidermal structures called salt glands to store and exlcude salt (Dassanayake, 2017). At present these specialized structures are reported to occur in four divisions of flowering plants; Caryophyllales, Asterids, Rosids, and Poaceae. Salt glands and salt bladders differ in the mechanisms through which salt is secreted in to the environment. Salt glands directly secrete salts to the surface of leaf. Comparatively, salt badders colllect salt in the vacuole of a specialized bladder cell, everntually these bladders cell rupture and release stored salt. Within the Caryophyllales, epidermal bladder cells have been reported in *Mesembryanthemum australe,* a betacyanic ice plant in the family Aizoaceae. Additionally, several species in the family Amaranthaceae are also documented to have salt bladders; *Atriplex lentiformis, Bienertia sinuspersici* and *Chenopodium quinoa.* In Limonium, a model example of recretohalophytes, toxic ions are not only sequestered in vacuoles but also secreted to the outside through salt glands; this process should also contribute to their salt tolerance. Currently *Disphyma australe* are not reported to possesses salt glands and further investigations will be required to confirm if they are present and the contribution of these structues to over all salt tolerance.

Table 4.2 Na+ distribution patterns recorded for betacyanin producing plants from available literature.

Species	Family	Salinity level (NaCl)	Structures tested	NaCl treatment	Methods	Na^+ accumulation highest	Reference
Disphyma australe	Aizoaceae	0 mM and 200 mM	Roots Stems Leaves	5 weeks	Atomic Absorption Spectrometry (Thermo Scientific iCE 3500 AAS)	Leaves	(Jain, 2016)
Limonium sp.	Plumbaginaceae	0, 200, 400, 800 mM NaCl	Roots Leaves	1 month	PFP7 flame photomoter (Jenway Inc., Burlington, VT, USA)	Leaves	(Al Hassan *et al.*, 2017)
Mesembraynthemum crystallinum	Aizoaceae	0, 400 mM NaCl	Roots Leaves	3, 8 and 24 h	Expression of vacuolar H^+ - ATPase genes	Leaves	(Low *et al.*, 1996)
Sesuvium portulacastrum L.	Aizoaceae	0, 100, 200, 300, 400, 500 mM NaCl	Leaves stems	30 d	Flame atoic absorption spectrophotometer	Leaves	(Muchate *et al.*, 2016a)
Portulaca oleracea L.	Portulacaceae	0, 50, 100, 150 mM NaCl	Roots Stems Leaves	Progressively increased over 60 d	Flame spectrophotometer	Stems Leaves	(Sdouga *et al.*, 2019)

A reduced capacity to tolerate high salinity in the substrate is further evidenced by the tissue waster content (TWC%) of each morph (Table. 4.1). TWC% can be assessed as an indicator stress when exposed to adverse conditions. TWC% was unaffected by an increased NaCl upto 400 mM NaCl. At 800 mM NaCl TWC% was significantly reduced in both the green and red morphs. Similarily, two seperate studies on *Sesuvium portulacastrum,* a facultatic halophyte also in the family Aizoaceae, reported that when subjected to increasing concentrations of NaCl in the soil subtrate *S. portulacastrum* exhbited depressed TWC% at concentrations >400 mM NaCl (Lokhande *et al.,* 2013; Muchate *et al.,* 2016). Comparatively, the TWC in selected *Limonium* species watered with 800 mM NaCl was calculated to be ca. 80%. Like *S. Portulacastrum* and *D. australe* these plants experienced a marked reduction in TWC% at concentrations >400 mM NaCl in all species studied (Al Hassan *et al.*, 2017).

Although TWC% was reduced at 800 mM NaCl, the final values were all above 90%. Compared to the TWC of ca. 80 % calculated for *Limmonium* species (Al Hassan *et al.*, 2017) and 73-83% reported for *S. Portulacastrum* (Lokhande *et al.*, 2013). This inidicates a strong resistance to leaf dehydration, and subsequently a strong capacity to tolerate high concentrations of NaCl in the substrate compared with other halophyte species. Madawala *et al* (2014) studied the relative growth of *D. australe* along side three other ice plant taxa. This study confirmed the previous assertion, reporting that *D. australe* demonstrated a greater capacity to tolerate high concentrations in NaCl in the growth medium.

4.5.3 K^+/Na^+ homeostasis

It is commonly accepted that salt stress causes K^+ defficieny in shoots (Shabala *et al.*, 2010). In saline environments Na^+ influx is mediated by pathways that typically function in K^+ transport. Discrimination between Na^+ and K^+ is difficult as the ionic radii of Na^+ and K^+ in their hydrated forms is similar (Nguyen *et al.*, 2015). In plants K^+ is integral for growth, tolerance to biotic and abiotic stress. (Ahmad & Maathuis, 2014) Subsequently, a stable K content at increased salinities is an indicator of salinity tolerance. K content in roots increased in response to salt stress in the two *D. australe*

morphs compared to the controls at 200 mM NaCl. This pattern of K accumulation in dicotyledonous halophyte was observed in four *Limonium* taxa Researchers found that salt stress increased Na content of tolerant cultivators to a lesser degree than salt sensitive taxon. But, a more stable K content was found in in the salinity tolerant varieties (Al Hassan *et al., 2017*).

When NaCl was absent from the treatment (0 mM NaCl), the green-leafed morphs had a high K^+/Na^+. Several studies have reported that halophytes grown in the absence of NaCl accumulate high concentrations of K^+, to substitute for Na^+. While the presence K^+ in tissues is posited to be an important feature of salt tolerant plants (Isayenkov & Maathuis, 2019). It cannot substitute Na^+ in all halophytes, and will not stimulate growth. This was reflected in *S.maritima* in which excess K^+ in the growth medium (340 mM KCl) produced only 28% of the dry mass of plants growing in the equivalent concentration on NaCl. Similar findings were reported by (Yao *et al.*, 2010), the authors stipulated that long term KCl exposure significantly inhibited growth in *Chenopodium album.*

5 Discussion

5.1 Introduction

In this thesis I present three studies that compare and contrast the response of green- and red-leafed morphs of *D. australe* to increasing salinity. Green and red morphs differ only in the production of betacyanin in the vegetative tissues of the red morph when exposed to salinity and high light. It is due to this feature that *D. australe* is a suitable model species to study the role of betacyanin pigmentation in salinity tolerance mechanisms. The discovery that the distribution of green and red morphs correlates with substrate salinity in natural populations on Wellington's south coast (Jain, 2016) ignited my interest researching how betacyanin pigmentation functions in salinity tolerance within this species. Studies in this area postulate that epidermal betacyanins function in photoprotection where they serve to ameliorate excitation pressure on chloroplasts, thereby reducing the propensity for photo inhibition and photo oxidation when compromised by salinity (Jain *et al.*, 2015). Other areas determining plant development in high saline environments have not been thoroughly examined with respect to betacyanin.

The main results contained within my thesis collectively support the following two conclusions:

1. ***Distribution is determined by overall competitive ability***

In this study I considered the possibility that differences in salinity tolerance might explain the distribution of *Disphyma australe* along a salinity gradient. The distinct formation of vegetative zones is a common feature in coastal plant communities, where the distribution of plants can be attributed to both abiotic factors, such as light, temperature and salinity, and abiotic factors such as competition. As salinity is the dominant factor determing growth in these environments, it is assumed that plant distribution is explained by the physiologcial tolerance limits of plants at different stages of development, and not necessarily by biotic interactions such as competition. Previous studies have suggested that zonation along salinity gradients is driven by a plant's overall competitive ability, rather than its capacity to tolerate salinity alone (Crains *et al.,* 2004;

Madawala *et al.,* 2014). Chapter 2 evaluated the capacity of red and green morphs to accumulate below ground biomass in the presence of increased substrate salinity. Where, irrespective of the light screening capabilities of betacyanin, the red morphs were able to maintain growth as salinity increased. Comparatively, the green morphs experienced an inhibited capacity to develop root biomass as NaCl increased.

Furthermore, the results indicate that the early stages of development, germination, early root growth, are the developmental phases most salt sensitive to salinity in the both green and red morphs of *D. australe,* as it has been established for most plants, glycophytes and halophytes alike (Al Hassan *et al.,* 2017). This is evidenced by the relative tissue water content (TWC%) of the green and red morphs during early growth, compared to the plants with a well established root systems studied in chapter 4. TWC% in the early growth phase of seedling establishment and root growth (Table 2.1) was consisntly lower than the TWC values recorded for mature plants with a well developed root system treated with higher concentrations of NaCl (200, 400, 800 mM) documented in Table 4.1. Additionally, Both morphs of *Disphyma australe* experienced inhibited germination under increasing levels of NaCl. In non-saline conditions the red morphs exhibited a consistently lower mean germination (MGT) than their green counter-parts at all treatment levels. This behvaiour towards accelerated growth under low saline conditions affords competitive advantage for establishment. As the rapid onset of germination confers advantages for early root development and growth under optimal conditions.

2. There is an apparent trade-off between high salt tolerance and growth rate at low salinities

The results of this study further support evidence that betalain and specifically betacyanin affords a greater capacity to tolerate high salinity in the root zone. Although this could come at a trade-off to exploit optimal (low saline conditions) as shown in chapter 2 and chapter 4. In chapter 2 I evaluated the capacity of red, green and green morphs with a light screening red filter to develop roots in increasing salinities in which light stress was applied. I aimed to separate the photo protective function of epidermal betacyanins from the possible physiological traits they may afford such as effective ROS scavenging. While the results of this study supported previous findings that betacyanin alleviates the adverse effects of photo inhibition, such as ROS mediated leaf senescence under high salinity.

They revealed a potential trade-off between high salt tolerance and reduced growth rate in low saline conditions. As only the green morph exhibited stimulated root growth when NaCl was absent from the growth medium. *Amaranthus cruentus L.,* a betacyanin producing dicot, was evaluated for the effects of water stress on leaves with and without betacyanin accumulation under high light. The researchers described evidence for photoinhibition when subjected to water stress, such as: reduced relative water content and decreased Chl content. This effect was reported to be greater in leaves where betacyanin was present. The authors postulated that the reduced Chl content reported in water stressed-betacyanic leaves may facilitate the degree of light attenuation by increasing the relative abundance of betacyanin to Chl (Nakashima *et al.*, 2011). Implying that the betacyanin producing plants may experience a trade-off in which they accumulate betacyanin in the epidermis at the cost of Chl abundance. As sustained Chl content is fundamental for photosynthesis and subsequent carbon fixation for growth (Bose *et al.*, 2017). This may offer an explaination for the reduced growth rate observed in the red morph when NaCl was absent from the growth medium (Fig. 2.3). As in these studies betacyanin synthesis appeared to be preferentially induced by high light over high salinity (Fig. 2.8).

The light screening effect of betacyanin was further evident in the green morph with the red filter applied. As this group also expereinced an inhibited capacity develop roots in the absence of NaCl, as shown by the final DW of the root system that was significantly different from the green morph without the filter, but did not differ from the red morph (Fig. 2.3). However, the explaination given above in which Chl abundance is reduced through the accumulation of epidermal betacyanin does not explain this observation, and another explaination is needed. It has been speculated that betacyanin pigmentation may hinder photosynthetic light acquisition, as green light is the primariy wavelength absorbed and utlised for carbon fixation (Nakashima *et al.*, 2011).

In chapter 4 I assessed the capability of green and red *D. australe* morphs to maintain K^+/Na^+ homeostasis under increasing NaCl concentrations. In fitting with the hypothesis, the red morphs displayed a greater ability to maintain homeostasis under increased NaCl in the substrate. However, the ratio in K^+ to Na^+ was greatest for the green morphs when

NaCl was absent from the growth medium. These findings, in concert with the results from chapter 2, suggest that while the red morphs perform better at increased NaCl, this trait may come as a trade-off for tolerance to conditions in which NaCl is absent entirely. For distribution purposes this indicates a differential ability to tolerate not only increased salinity, but also decreased salinity.

In coastal habitats plants are subject to temporal and spatial variation in both airborne and substrate salinity (Sucre & Suárez, 2011; Madawala *et al.*, 2014), and reduced availability of fresh water (Sucre & Suárez, 2011). The capacity of plants to overcome these conditions may determine their establishment and distribution along a salinity gradient (Madawala *et al.*, 2014). Where high salinity will increase the competitive ability of salt tolerant plants relative to less tolerant plants. If it is well established that halophytes grow rapidly in saline conditions (Flowers & Colmer, 2008). Why then, do they not frequently occur in low-saline environments? It is generally assumed that in environments devoid of salinity they are outcompeted by less tolerant counter-parts. This is often documneted as the stimulated growth of halophytes when NaCl is present at low concentrations. As has been shown in *Sesuvium portulacastrum,* a betacyanic halphyte in the Aizoaceae family. When treated with incresaing concentrations of NaCl (0, 200, 400, 600 800 mM), all clone lines of *S. portulacastrum* exhbited stimulated growth at 200 mM NaCl as inidicated by shoot length at TWC%. (Lokhande *et al.*, 2013). This implies that the maximum relative growth rate of salt tolerant halophytes at low salinity would be less than that their less tolerant counterparts (Rozema & Schat, 2013).

Reduced growth rate is an established strategy for plants growing in supra-optimal conditions. Especially in xerophytes, such as *Agave* spp. survive drought with very low growth rates (Flowers & Colmer, 2008; Davis & Long, 2015). In these conditions osmotic adjustment is achieved by solutes synthesized by the plant (Sucre & Suárez, 2011). If so then the low vacuolar content of Na^+ and Cl^- may cause reduced turgor with reduced growth as a consequence. This may inturn be restored at increased salinity allowing for increased vacuolar concentrations of inorganic Na^+ and Cl^- (Rozema & Schat, 2013) Flowers & Colmer (2008) postulated why growth declines at high salinities, they determined that although unclear, there are several possibilities. Such as: reduced C

fixation and altered photosynthesis and respiration rates due to shifting biomass allocation between leaf, stem and root. Another possibility is that growth may decline as a result of reduced turgor, which is dependent on high concentrations of ions in the apoplast. Further possibilities relate to osmotic adjustment, such as an inability to effeciently accumualte and/or distribute nutrients or synthesize sufficient organic solutes or the engrgetic demands of ion compartmentation. Additionally, energy is required to synthesis ions which are not readily available in the environment (Sucre & Suárez, 2011). These possibilities may also translate to the reduced growth rate, and subsequent lower frequencies of salt-tolerant halophytes in low saline conditions.

5.2 *Conclusion*

Salt adversely affects plant growth and development through the disruption of physiological processes during key development phases such as, germination, vegetative growth and reproduction (Byrt *et al.*, 2018). As such, understanding how attributes, such as pigmentation enhance salt adaptation mechanisms is crucial to improving agricultural productivity. Within the next 25 years, it is projected that salinity will affect 30% of the world's agricultural land (Borsai et al., 2017). Recent work has emphasized the potential for improved crop vigour through the identification of traits and physiological mechanisms (Shabala, 2013; Deinlein *et al.*, 2014; Wu *et al.*, 2015; Himabindu *et al.*, 2016; Munns *et al.*, 2016; Jaime-Pérez *et al.*, 2017). Alternatively, other studies have sought to evaluate how the employment of alternative crops could contribute to food production in the future (Foley *et al.*, 2011; Panta *et al.*, 2014). Arguing that the use of halophytes and other non-traditional crops may be a viable alternative for commercial agriculture. Both of these options would ease the pressure on the requirement of good quality land and fresh water for food production. By allowing for agricultural production in marginal habitats, using low-quality and/or brackish water for irrigation (Munns & Gilliham, 2015; Borsai *et al.*, 2017).

However, the genetic improvement of crops is widely considered as the most promising mechanism to improve crop stress tolerance (Borsai et al., 2017). Therefore, understanding how traits and trade-offs among them, repsond to environmental change and their impact for stress tolerance is paramount. In this study I have sought to

demonstrate how betacyanin accumulation may improve salinity tolerance in *Disphyma australe*, a dicotyledonous halophyte native to New Zealand. As stated earlier betacyanin is one of two classes of betalains, a unique pigmentation found only within the core Caryophyllales. Betacyanin accumulation has previously been demostrated to enhance salinity tolerance in *Suaeda salsa* (Wang *et al.*, 2007), *Amaranthus cruentus L.* (Nakashima *et al.*, 2011) and in *Disphyma australe* (Jain *et al.*, 2015; Jain, 2016). These studies have similarly reported that betacyanin accumulation serves a photoprotective function, by screening saturated light, thereby shielding chloroplasts from excess excitation pressure, and facilitating physiological mechanisms associated with salinity tolerance, such as ROS scavenging.

Disphyma australe is novel, as it exhibits colour dimorphism of green- and red-leafed morphs among natural populations. Upon closer inspection these two morphs are distributed along a salinity gradient, where the frequency of red-leafed morphs positively correlates with increased substrate salinity (Jain *et al.*, 2015; Jain, 2016). Through the process of this study I aimed to understand how the previously stated mechanisms may function to determine the distribution of green- and red-leafed *Disphyma australe* morphs along a salinity gradient. Consequently, I designed this study to focus on three key areas of plant development in saline environments, which had not previously been reported for *Disphyma australe*: germination, sustained root development and growth, and ion content under increased salinities. Taken together the reults of this study support previous findings, attribute imporved salinity tolerance to betacyanin accumulation in vegetative tissues (Jain *et al.*, 2015; Jain, 2016). Additionally, they have highlighted how salinity stress during early developmental phases may contribute to distribution along a salinity gradient. Noting, that epidermal betacyanin facilitates the maintenance of root growth and K^+/Na^+ homeostasis under increased salinities. However, this enhanced tolerance to saline conditions may inhibit growth in low-saline environments, where green-leafed morphs may succeed.This trade-off may contribute to the distribution of morphs, indicating that the high frequency of green morphs where salinity is low could be a consequence of their competitive ability to exploit these conditions. Further the results of study, have supported previous findings which report that germination and early seedling establishment are the most sensitive phases to salt stress (Al Hassan *et al.*, 2017).

Therefore, the ability to over come salinity stress during the stages may contribute to the distribution of *Disphyma australe* along a salinity gradient.

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