Competition in *Arabidopsis thaliana* (L.) Heynh., behaviour of *Mimosa pudica* L. and a new method to characterize roots demonstrated with *Helianthus annuus* L.

by

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Abstract

In this dissertation, I address 5 problems in the discipline of plant ecology: two problems in plant competition, two problems in plant behaviour and one problem in the phenotyping of plant roots.

First, we directly test Darwin's competition-relatedness hypothesis with a pairwise competition experiment using 14 accessions of *Arabidopsis thaliana* (L.) Heynh. We do not find support for Darwin's contention that more closely related individuals compete more fiercely, or its underlying assumptions. In fact, under higher resource conditions, we show that kin compete less fiercely than strangers, and interestingly this pattern goes away with lower resource conditions. Within the context of our modern understanding of the genetics of traits, these results challenge a key assumption in ecology.

Second, the transitivity of a competitive hierarchical network is examined using data from the same experiment with *A. thaliana*. We show that intransitivity is a structural component of the competitive networks here because there were no true dominants, patterns of dominance changed with nutrient levels, and there were competitive reversals. We determined the relationship between the frequency of reversals, competitive equivalence and the length of reversals, each with respect to accession diversity. We find that all of these intransitive characters increased with accession diversity. This result has implications for coexistence based on current theoretical models of intransitivity.

Third, we quantify the cost of a plant defense in an experiment with *Mimosa pudica* L. using a unique leaf touching device. *Mimosa pudica* is a plant that defensively closes its leaves when triggered by touch. There is a trade-off to this behaviour as there are increased energetic costs and photosynthetic opportunity costs with greater frequency of leaf closing. With the leaf touching machine, we found that touch itself stimulated growth. However, comparing daytime touching that stimulated leaf closure to nighttime touching that did not stimulate leaf closure, we find that there were costs in reproductive allocation attributable to the frequency of leaf closure. These results contribute to a growing documentation of the cost component of plant defensive strategies.

Fourth, we fill some of the gaps in our knowledge in the behavior of *M. pudica* by measuring the effects of competition, nutrients and rhizobia on leaf re-opening behaviour over a 9 month period. We found that each of the treatments had an effect on plant size, but competition alone had an effect on inflorescence production. In the presence of a competitor, and as plant size increased, leaflets of *M. pudica* took longer to re-open upon stimulated closure. This adds to a catalogue of complex behavioural responses in *M. pudica* that has garnered the attention a broad spectrum of behaviourists.

Lastly, we present a new method using digital image correlation (DIC) to characterize plant root traits, traits that are generally difficult to observe. This method is novel because we use the measurement and analysis of soil to infer the movement and position of roots. We demonstrate the effectiveness of this method in

characterizing the static and dynamic traits of *Helianthus annuus* L. roots. This application of DIC promises to be an effective, time-efficient, cost-efficient and likely scaleable approach to studying roots.

Dedication

To my mom, Kyongja Cho, my dad, Sinh Vinh and Grace Lee.

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Chapter 1 – Introduction

The bread-and-butter of plant ecologists is to study how various biotic (e.g. competitors, mutualists, herbivores) and abiotic (e.g. nutrients, water, light, temperature) factors directly and interactively affect plants at the individual, population or community level. While there is nothing wrong with this approach, common experience shows that a good empirical study may account for around 50% of the variation. A survey of this phenomenon over a broad swath of ecological and evolutionary studies showed that 80% of studies explained less than 20% of variation (Moller and Jennions 2002). Why is our explanatory power so low? Two obvious reasons include context dependence and noise. In ecological studies, particularly field studies, each site and year are different and significant variation comes from the inability to fully account for how effects and interactions change due to this variation. Similarly, noise from one-off factors (e.g. hail storm) that are neither replicable nor predictable affect most studies in some way. Nevertheless, as we learn more about the details of ecological processes it seems likely that there are significant factors that are often not being accounted for.

In this dissertation, I examine five non-conventional questions in plant ecology. In Chapters 2 and 3, I look at two broad organizing concepts in plant competition: Darwin's competition-relatedness hypothesis and transitive competitive hierarchies. In Chapters 3 and 4, I present two studies on *Mimosa pudica*, first looking at the costs associated with its leaf closing defense, and second looking at how the plant's leaf re-opening behaviour is affected by competition, nutrients and rhizobia. In Chapter 5, I introduce a new method for tackling the age old problem of characterizing plant root traits. Each chapter was a collaboration with people in addition to my supervisors. My collaborators were: Chapter 2: Dr. Anne Weisbach

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In *The Origin of Species*, Darwin asserts that closely related individuals would compete more severely than less closely related individuals (Darwin 1968). This hypothesis has been called the competition-relatedness hypothesis and it has long been accepted as ecological doctrine (Cahill et al. 2008). It has been presumed to be true partly because its assumptions seem so reasonable: more closely related individuals are more similar in traits, and greater similarity in trait results in greater competitive struggle. However, there have been remarkably few empirical tests of this hypothesis or its assumptions (Cahill et al. 2008, Violle et al. 2011, Kunstler et al. 2012, Narwani et al. 2013). In Chapter 2, I present an experiment that was designed to test this hypothesis directly and its underlying assumptions using a full pairwise competition experiment with 14 accessions of *Arabidopsis*.

Another organizing concept in the study of competition is that of competitive hierarchies (Keddy and Shipley 1989). They were originally advocated as a pragmatic means by which knowledge about competitive abilities could be scaled from species to communities (Keddy 1990). This ability to scale was contingent on the transitivity of competitive hierarchies (Silvertown and Dale 1991) and there was a strong belief that transitive hierarchies were the norm (Keddy and Shipley 1989, Grace et al. 1993, Shipley 1993). However, more recently, interest has been growing about intransitive hierarchies because of theoretical work that has shown that intransitivity could promote coexistence in communities (Frean and Abraham 2001, Laird and Schamp 2006). But, neither transitivity or intransitivity has been studied extensively with empirical data because few experimentally parameterized competitive hierarchical networks of even modest size (>10 nodes) have been published (e.g. Goldsmith

1978). In Chapter 3, I look at a 13 accession *A. thaliana* competitive hierarchical network and analyze in detail some of its intransitive properties.

Mimosa pudica, the sensitive plant, is well known for its characteristic rapid leaf closing behaviour (Burkholder and Pratt 1936). This behaviour is thought to have a defensive benefit for the plant (Eisner 1981). Plant defense in general is considered to be costly because of the allocational trade-off with other important processes such as growth and reproduction, but the specific cost of a defensive strategy is often difficult to parse from these other processes (Baldwin et al. 1990, Agrawal 2000). In *M. pudica*, the cost of the rapid leaf closing behaviour comes at the energetic cost of opening and closing leaves, as well as the opportunity cost of reduced photosynthesis (Hoddinott 1977). In Chapter 4, I present a study that quantifies the individual level cost associated with the increased use of the rapid leaf closing defense

Mimosa pudica is becoming one the model organisms in plant behavioural ecology. The leaf re-opening behaviour has been the focus of most recent studies and many complex behaviours have been reported including evidence for elementary forms of learning such as habituation (Gagliano et al. 2014). However, baseline data about how the leaf re-opening behaviour changes with ecological factors that affect plants is lacking. In Chapter 5, I look at how *M. pudica* leaf re-opening behaviour changes with competition, nutrients and rhizobia using a full factorial experiment.

Roots are about half the biomass of an average plant, but they are certainly the harder half to study. It has been said that there have been greater efforts in developing ways to study roots than the actual study of roots (Harper et al. 1991). Roots are critical for a plant's resource acquisition, stability, storage and interactions with other organism (Kroon and Visser 2003), and any way to easily determine root traits is immensely valuable to all varieties of plant biologists (Downie et al. 2015,

Kuijken et al. 2015). In chapter 6, I present a novel approach to this problem where the static and dynamic traits of roots are inferred from the analysis of soil movement using an application of digital image correlation (DIC) analysis (Chu et al. 1985, Pan et al. 2009). Photographic images of soil are analyzed with DIC to determine where and how much the soil moves, thereby revealing the placement of roots and their activity.

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Chapter 2 - Testing Darwin's competition-relatedness hypothesis

Introduction

In the third chapter of *The Origin of Species*, "Struggle for Existence", Charles Darwin observes that the Malthusian doctrine applies to the entire natural world. Given the potential of populations to grow exponentially and the constraint of finite resources: "more individuals are produced than can possibly survive, [and] there must in every case be a struggle for existence…" (p. 117)(Darwin 1968). This is a collective struggle that suppresses the natural rates of increase of all organisms. According to Darwin, the strength of this struggle is not arbitrary, but instead "... the struggle almost invariably will be most severe between the individuals of the same species, for they frequent the same districts, require the same food, and are exposed to the same dangers. In the case of varieties of the same species, the struggle will generally be almost equally severe…" (p.126)(Darwin 1968). Darwin generalizes his assertion by stating that the struggle between species of the same genus is more severe than the struggle between those of different genera, and extends this to other "allied forms" or broader taxonomic groups (p. 127)(Darwin 1968). This relationship of increasing competitive struggle with increasing relatedness has been called the competition-relatedness hypothesis (Cahill et al. 2008, Mayfield and Levine 2010, Violle et al. 2011) and can be considered a generalization of Darwin`s Naturalization Hypothesis (Darwin 1968, Duncan and Williams 2002).

The competition-relatedness hypothesis has been foundational in the development of key ecological and evolutionary ideas including: the competitive exclusion principle (Gauze 1934, Hardin 1960); limiting similarity (MacArthur and

Levins 1967); niche conservatism (Peterson et al. 1999, Wiens and Graham 2005); and coexistence in communities (Chesson 2000a, 2000b). Despite the importance of this hypothesis, empirical tests have been rare. Thus far, there has been both support for (e.g. Violle et al. 2011) and against (e.g. Cahill et al. 2008, Burns and Strauss 2011, Kunstler et al. 2012, Narwani et al. 2013) the hypothesis.

The hypothesis, as Darwin articulates, is built upon two fundamental assumptions (Figure 2.1) (Darwin 1968). The first assumption is that more closely related individuals are more similar in trait (Figure 2.1, arrow B). The second assumption is that individuals that are more similar in trait will compete more fiercely (Figure 2.1, arrow C). If both assumptions are true, increasing relatedness will result in greater competitive struggle (Figure 2.1, arrow A). Greater competitive struggle results in the suppression of the natural rate of increase of organisms (Figure 2.1, arrow D). The purpose of this experiment was to test explicitly Darwin's competition relatedness hypothesis, as well as its two underlying assumptions.

In contrast to the competition-relatedness hypothesis, kin-based theories have hypothesized the possibility of reduced antagonism among the most closely related individuals (Hamilton 1964, West et al. 2002). This is also a competition-relatedness hypothesis, but in which the relationship between the two is inverted. We will refer to this alternative as the "Hamilton" version of the competition-relatedness hypothesis. Kin-based theory applies to very closely related individuals that are indeed considered kin, but this Hamilton version is a valid alternative to Darwin's competition-relatedness hypothesis amongst these close relatives, even in the plant kingdom (Tonsor 1989, Kelly 1996, Donohue 2003, Dudley and File 2007, Biedrzycki and Bais 2010). The Hamilton alternative hypothesis is based on two requisites: individuals are able to recognize kin and that kin recognition modifies an individual's response to competitors. In this experiment, we test the Hamiltonian alternative, but do not test the requisites.

Methods

To directly test the competition-relatedness hypothesis (Fig. 2.1, arrow A) we required: a measure of competition and a measure of relatedness between competitors. To measure competition we conducted a full pairwise design competition experiment at 2 nutrient levels with 14 accessions from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH) stock (Table 2.1). Relatedness among the accessions was estimated as the number of shared alleles using 114 variable loci of the SNP data generated by Nordberg *et al.* (2005).

The ABRC accessions used in the experiment included 13 accessions of *Arabidopsis thaliana* (L.) Heynh. and one accession of *A. shokei* (Table 2.1). Before starting the competition experiment, stock seeds were propagated under standardized growth conditions to control for maternal effects (Postma and Ågren 2015, Videvall et al. 2015). We grew plants in uniform soil (LC1 Sunshine Mix, Sun Gro Horticulture, Agawam, MA), nutrient (Nutricote Type 100, NPK 14:14:14, Chisso-Asahi Fertilizer Co., Toyko) and environmental conditions (24 ^{o}C, 16h:8h day:night light) in a climate controlled growth chamber (Biotron facilities, Department of Biological Sciences, University of Alberta). Plants in this propagation were isolated with a cellophane sleeve around each pot and monitored daily for about 70 days. Seeds were harvested from individual plants when siliques were ready to dehisce. The seeds from this propagation were then used in the subsequent competition experiment.

We potted the pairwise competition plants and no-competition plants following the methods of Cahill *et al.* (Cahill et al. 2005). In total there were 105

pairwise competition combinations each replicated 5 times at both low and high nutrient levels. There were 14 no-competition single plant treatments each replicated 10 times at both low and high nutrient levels. We included two nutrient levels because nutrients can affect plant traits and competitive interactions (e.g. Wang et al. 2010). The appropriate nutrient levels, as well as other pot conditions, were determined in a series of pilot studies (Cahill et al. 2005). High and low nutrient levels were established using Hoagland's solution at 1:1 and 10:1 dilutions, respectively, applied 3 times weekly at 1 mL per application (Epstein 1972). Plants were uniformly bottom watered using a customized Rainbird™ (Rain Bird Corporation, Azula, California) watering system with one watering tube per tray of 32 pots. All pots were arranged in a randomized block design.

Our primary response variable was the reproductive output of individual plants which we were able to directly measure as the number of siliques produced per plant. Siliques were counted when each plant completed fruiting. We estimated the plant traits of size and phenology because prior work with *A. thaliana* identified both plant size (Cahill et al. 2005) and phenology (Brachi et al. 2012) as important competitive traits. They are also generally considered to be among the most important traits in influencing competitive outcomes between plants (Keddy 2001). Size was estimated by the diameter of rosettes measured at 4 weeks after seeding. At this time, all plants were germinated and leafy but had not bolted. Because the plants were all seeded on the same day, size is also an estimate of relative growth rate, another trait often associated with plant competitive outcomes (Keddy 2001). Phenology was estimated by the day of bolting which was determined with daily censuses. We chose bolt day because it is a discrete and significant event in the life cycle of an individual plant.

Analyses

We used competitive struggle as the index of competition between ecotypes i and j:

$$\mathrm{CompetitiveStruggle}(i, j) = 1 - \left(\frac{\mathrm{ReproductiveOutput}(i|j)}{\mathrm{ReproductiveOutput}(i_{solo})} + \frac{\mathrm{ReproductiveOutput}(j|i)}{\mathrm{ReproductiveOutput}(j_{solo})}\right)/2$$

where i|j is ecotype i competing with ecotype j and i_{solo} is one plant of ecotype i growing by itself. This index is analogous to aggressivity (Weigelt and Jolliffe 2003) and captures Darwin's notion that the "struggle for existence" is a "collective struggle" that impacts all organisms at once rather than one individual at a time. We found no significant change in the results when we ran our analyses using common focal-plant specific competition measures such as competitive effect and competitive response (Goldberg and Fleetwood 1987, Wang et al. 2010).

The relationships between competitive struggle, relatedness and plant traits were analysed by linear regression. The overall comparison of competitive struggle amongst intra- inter- ecotypic and interspecific groups were analysed using Kruskal-Wallis tests due to the imbalance in group sizes. All analyses were conducted using SPSS 20 (IBM Corp., Armonk, NY).

Results

Is there trait variation amongst accessions?

There was substantial variation amongst accessions in the number of siliques produced, rosette size and bolt day when grown alone (Figure 2.2). One accession (7) did not produce siliques at low nutrient conditions, but excluding this accession there was a greater than 2 fold difference in silique production between the least and most productive accessions at both low and high nutrients. The mean size of rosettes varied even more with a 7-9 fold range in size differences at both nutrient levels. Mean bolt day ranged from 22 days to 55 days which is similar for both nutrient levels. Overall, high nutrient levels resulted in greater mean silique production for 10 out of 14 accessions, but did not consistently affect rosette size or bolt day.

Does competition matter? (Figure 2.1, Arrow D)

Competition was significant in reducing the reproductive output of plants as almost all accessions produced fewer numbers of siliques with than without competition (Figure 2.3). The sole exception was accession 7 as it did not produce siliques without competition at low nutrients. The reduction in silique production due to competition ranged from a ~15%-60% reduction at low nutrient levels and a ~10-65% reduction at high nutrient levels.

Is competitive struggle explained by the relatedness of individuals? (Figure 2.1, Arrow A)

Relatedness, estimated by the number of shared alleles, did not explain the variation in competitive struggle at either low (n=62 r^2=0.005 p=0.578) or high nutrient levels (n=74 r^2=0.000 p=0.979) (Figure 2.4). This was determined using the observations of the 13 accessions of *A. thaliana* in inter-ecotypic competition. Intra-ecotypic competition amongst *A. thaliana* and competition with *A. shokei* were not distributed evenly across the relatedness axis and therefore analysed separately (Figure 2.7). The value of n is smaller for low nutrients levels because at low nutrients, and in the absence of competition, accession 7 did not produce siliques and as a consequence competitive struggle could not be calculated.

Are more closely related individuals more similar in trait? (Figure 2.1, Arrow B)

Closely related individuals were not more similar in traits the less related individuals (Figure 2.5). Relatedness had a significant relationship with size under low nutrients conditions (n=62 r^2=0.09 p=0.018), but increasingly related individuals were more different in size, not more similar (Figure 2.5, top left). With high nutrient conditions there was no relationship between size and relatedness (n=74 r^2=0.001 p=0.755). Relatedness also did not explain bolting day at low (n=62 r^2=0.012 p=0.403; Fig. 3c) or high (n=74 r^2=0.009 p=0.428) nutrient levels. The mean trait differences amongst accessions was calculated with plants in the absence of competition.

Do individuals more similar in trait compete more fiercely? (Figure 2.1, Arrow C)

In 3 out of 4 cases, there was no relationship between competitive struggle and the similarity in traits (Figure 2.6). There was a significant relationship between competitive struggle and size differences at high nutrient levels (n=74 r^2=0.134

p=0.001), but not at low nutrients levels (n=62 r^2=0.003 p=0.682). At high nutrients, the competitive struggle increased as the mean difference in size among accessions decreased (Figure 2.6, bottom left). There was no relationship between competitive struggle and bolt day at low (n=62 r^2=0.001 p=0.807) or high (n=74 r^2=0.012 p=0.347) nutrient levels.

What is the relationship of competition and relatedness over a broader range of relatedness?

With low nutrient conditions, there was no difference between intra-ecotypic, inter-ecotypic and interspecific competition (KW, p=0.808). However, with high nutrient conditions, competitive struggle was weaker among closely related intra-ecotypic competitors (kin) than among inter-ecotypic competitors (strangers) (KW, p=0.046) and among interspecific competitors (KW, p=0.010). That is to say, more closely related individuals competed less fiercely than less closely closely related individuals. The difference in competitive struggle between inter-ecotypic and interspecific groups at high nutrients levels was not significant (KW, p=0.109).

Discussion

Darwin's competition relatedness hypothesis

These data do not show support for Darwin's competition relatedness hypothesis (Figure 2.4). There are two ways we could have reached this conclusion for unintended reasons that must be addressed. First, if the overall effect of

competition was either consistently small or non-variable, the range of data on the y-axis in Figure 2.4 would be compressed and we would not have been be able to find a non-zero slope. This was not the case as there was a strong and variable effect of competition (Figure 2.3). Second, if there was very little overall trait variation, this would have had the effect of range compression on all the trait difference axes in Figures 2.5 and 2.6 and thereby limited our ability to test both assumptions of the hypothesis. This was not the case as accessions had a 9-fold difference in rosette size and a 2-fold difference in bolt day (Figure 2.2).

Therefore, we focus on the two underlying assumptions to explain why our data does not support the competition-relatedness hypothesis. The first assumption is that more closely related individuals are more similar in trait. Neither differences in rosette size nor differences in bolt day supported this assumption (Figure 2.5). The second assumption is that individuals with more similar traits would compete more fiercely. Size differences at high nutrient levels support this assumption, as greater similarity in size resulted in increased competitive struggle (Figure 2.6). However, size differences at low nutrients, or differences in phenology, did not explain competitive struggle (Figure 2.6). The failure to support the competition-relatedness hypothesis is a consequence of the lack of general support for the assumptions necessary to Darwin's assertion.

Why might these results have implications beyond this one experiment? The main reason is that we benefit from an understanding of the genetics of traits that Darwin did not have. We chose to look at size and phenology because they were specifically recognized to be important in this system (Cahill et al. 2005, Brachi et al. 2012), but also because they are generally important in plant competition (Keddy 2001). Size and phenology, however, are well known quantitative (Venable and M. 1989, Kelly 1993, Kuittinen et al. 1997), epistatic (Doust et al. 2014) and plastic (Schlichting 1986) traits, the kind of traits that tend not to be phylogenetically

conserved. If traits important in competition are not phylogenetically conserved, then there cannot be an *a priori* expectation of how competition should vary among close relatives based on traits (Duckworth et al. 2000, Mayfield and Levine 2010, Grime 2012, Cahill 2013, Bennett et al. 2013). In systems where the traits related to competition are highly conserved, and where similarity enhances competition, it is still expected to find support for Darwin's competition-relatedness hypothesis (e.g. Schluter 1993, Grant and Grant 2006, Violle et al. 2011). It is unknown whether such conditions are common or rare, but we hypothesize that among vascular plants (Cahill et al. 2008) they are likely rare because at least some often important competitive traits are not phylogenetically conserved (Bennett et al. 2013).

A possible reason why plants are more likely to compete based on traits that are not phylogenetically conserved is that plants primarily compete over a small number of resources: water, light and mineral nutrients (Grace 1995). The physical partitioning of the use of these resources is limited by the need for plants, regardless of phylogenetic position, to have a specific and relatively conserved set of morphological and physiological characteristics to take up water and nitrate, or absorb a photon of light. This contrasts the form-function variation found in the beaks of finches (Grant and Grant 2006), the jaws of sticklebacks (Schluter 1993), or the mouths of protists (Violle et al. 2011), where the competitive function of a trait is contingent upon a diversity of available resources. In plants, key competitive traits such as size and phenology cannot be separated from other basic biological functions and therefore the evolutionary history of these traits vis-à-vis competition are muddled by the effects of other selective forces.

Hamilton's alternative hypothesis

Looking across a broader range of relatedness by including intra-ecotypic and interspecific competition data, we find some support for the Hamilton alternative to the competition-relatedness hypothesis. Under high nutrient conditions, competitive struggle was weakest amongst intra-ecotypic kin when compared to both amongst inter-ecotypic strangers and amongst interspecific competitors (Figure 2.7). This result contributes to a steadily growing set of evidence that plants use kin-based information in an ecological context (Tonsor 1989, Kelly 1996, Donohue 2003, Dudley and File 2007, Biedrzycki and Bais 2010, Biedrzycki et al. 2010, Semchenko 2014). Under low nutrient conditions, we did not observe the same pattern as intra-ecotypic kin competed as much amongst inter-ecotypic strangers or amongst interspecific competitors (Figure 2.7). As far as we are aware, this is the first documentation in plants of increased competition among kin with a shift to low resource conditions, something often observed in animals. As resources become more limiting, there can be an increased aggression among kin, such as in the case of increased siblicide among chicks of various siblicidal birds (Mock and Parker 1998). Interestingly, many of the characteristics common to siblicidal birds: resource competition, competitive disparity, weaponry and spatial confinement (Mock et al. 1990) are shared by the plants in this experiment. Overall, at both high and low nutrient levels, we do not find support for Darwin's hypothesis across this broader range of relatednesss.

Combined, these results contribute to a body of empirical work (e.g. Cahill et al. 2008, Kunstler et al. 2012, Narwani et al. 2013) that challenges the universality of the competition-relatedness hypothesis, a deeply held ecological assumption. Advances in our understanding of the genetic basis for traits, inform us not to automatically accept the fundamental assumptions upon which Darwin's hypothesis is constructed. Moving forward, it may be useful to integrate additional theories (e.g. Hamilton 1964) to our current framework for understanding competition and coexistence.

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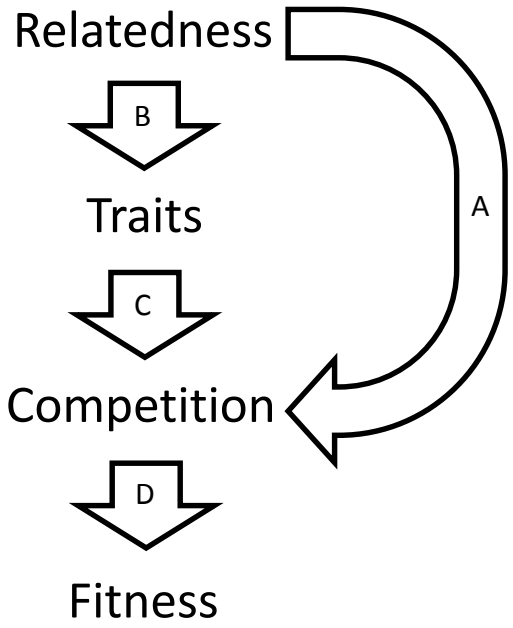
Table 2.1 The ABRC accessions used in experiment. Accession number, the first column, is the number used in this manuscript to identify ABRC accessions.

Accession Number	ABRC Accession Number	Background Line
1	CS1676	*A. shokei*
2	CS28055	*A. thaliana* Bay-0
3	CS28067	*A. thaliana* Berkeley
4	CS28166	*A. thaliana* Col-0
5	CS28167	*A. thaliana* Col-0
6	CS28195	*A. thaliana* Ct-1
7	CS28387	*A. thaliana* Kin-0
8	CS28443	*A. thaliana* Lc-0
9	CS28445	*A. thaliana* Ler-0
10	CS28467	*A. thaliana* Lip-0
11	CS28502	*A. thaliana* Mt-0
12	CS28650	*A. thaliana* Pog-0
13	CS28780	*A. thaliana* Tsu-0
14	CS28782	*A. thaliana* Tsu-1



Arrow	Predicted Relationship	Results
A	Increased competitive struggle with greater relatedness	Fig 2.4
B	Increased trait similarity with greater relatedness	Fig 2.5
C	Increased competitive struggle with greater trait similarity	Fig 2.6
D	Decreased fitness with greater competitive struggle	Fig 2.3

Figure 2.1 A graphical representation of the competition-relatedness hypothesis and its assumptions. Arrow A is the hypothesis, Arrows B & C are the assumptions of the hypothesis, and Arrow D is the consequence of the hypothesis.

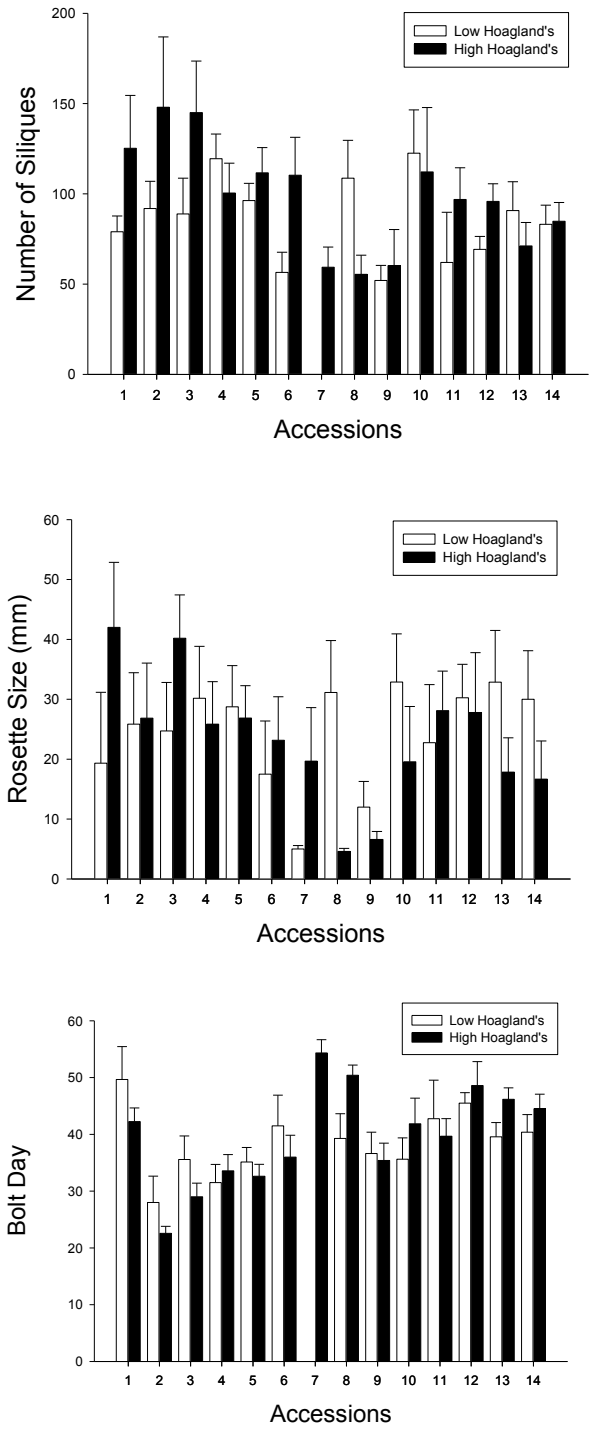


Figure 2.2 Trait variation of 14 ABRC accessions used in this experiment under high and low nutrient conditions in the absence of competition. The accessions are specified in Table 2.1. Error bars give ±1 SE from the means.

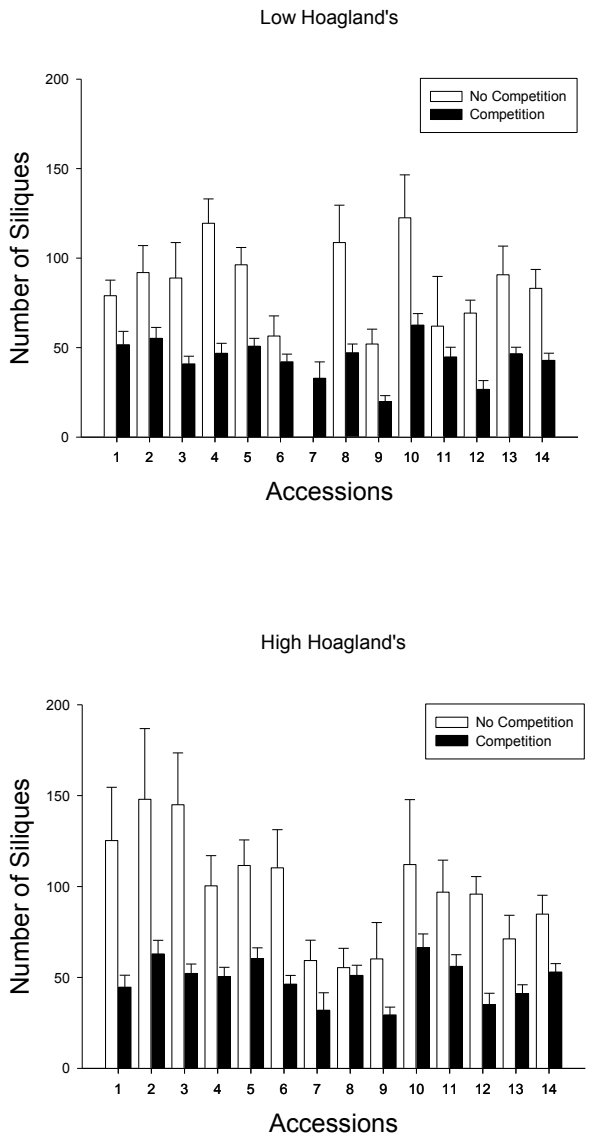


Figure 2.3 The effect of competition on silique production for 14 ABRC accessions used in this experiment under low (top panel) and high (bottom panel) nutrient conditions. The accessions are specified in Table 2.1. Error bars give ±1 SE from the means.

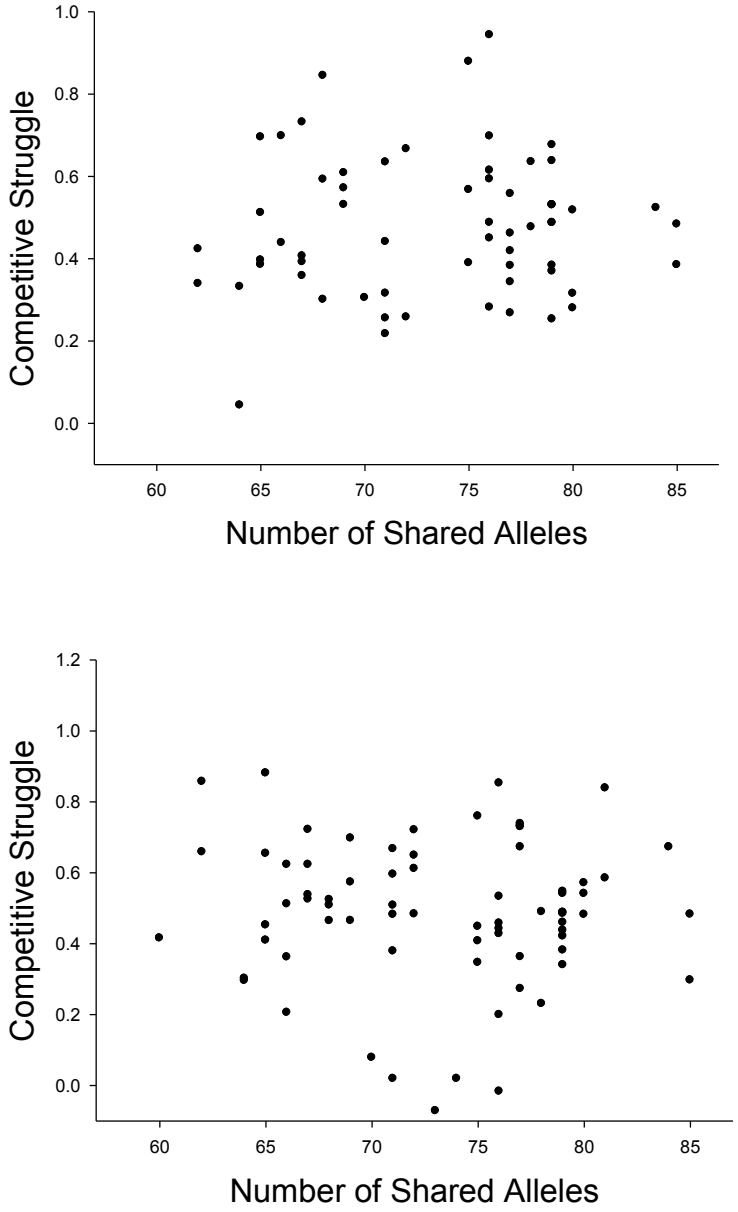


Figure 2.4 The relationship between competition and relatedness under low (top panel) and high (bottom panel) nutrient conditions. The number of shared alleles did not explain the variation in competitive struggle at low (n=62 r^2=0.005 p=0.578) or high nutrient levels (n=74 r^2=0.000 p=0.979).

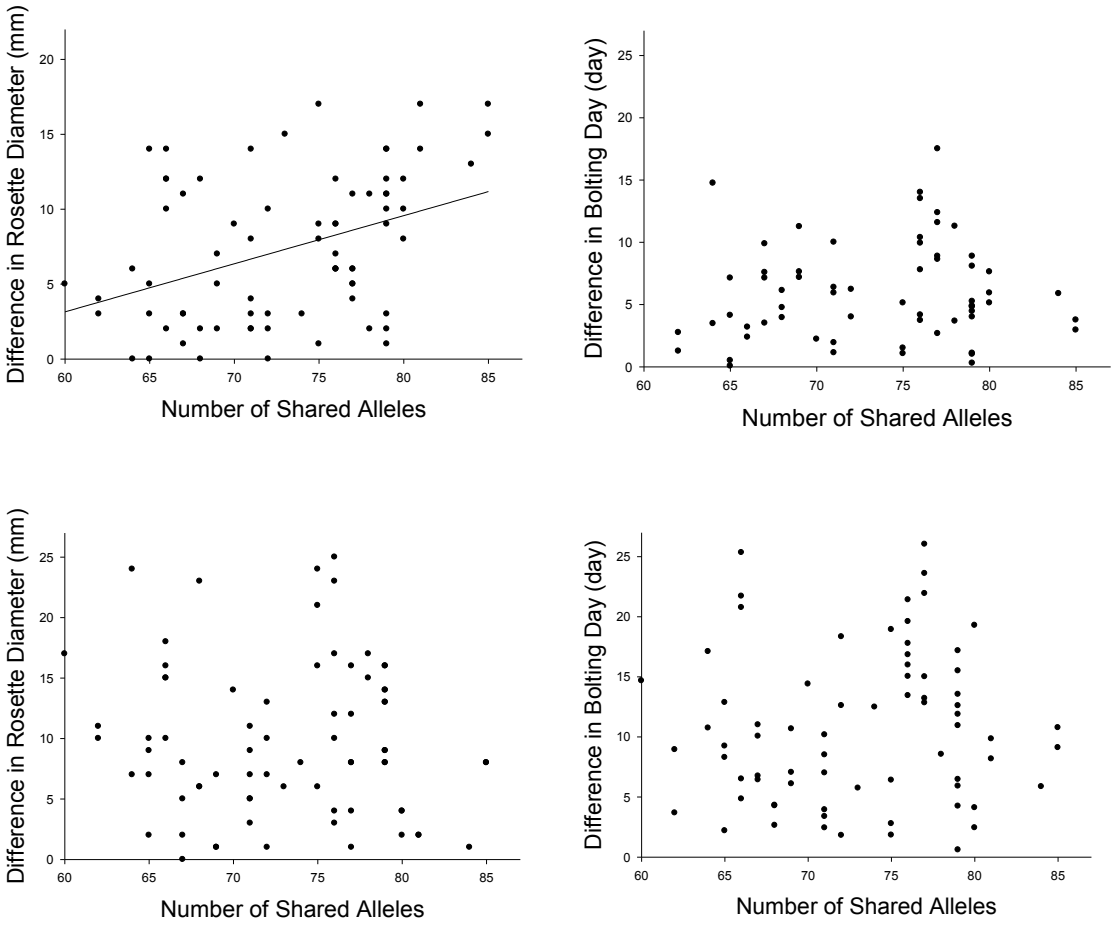


Figure 2.5 The relationship between the differences in plant traits (rosette size and bolt day) and relatedness. The low nutrient conditions are on the top row and high nutrient conditions are on the bottom row. There was a significant relationship between size differences and relatedness with low nutrients (n=62 r^2=0.09 p=0.018), but not with high nutrients (n=74 r^2=0.001 p=0.755). There was no relationship between bolting day and relatedness at low (n=62 r^2=0.012 p=0.403) or high (n=74 r^2=0.009 p=0.428) nutrient levels.

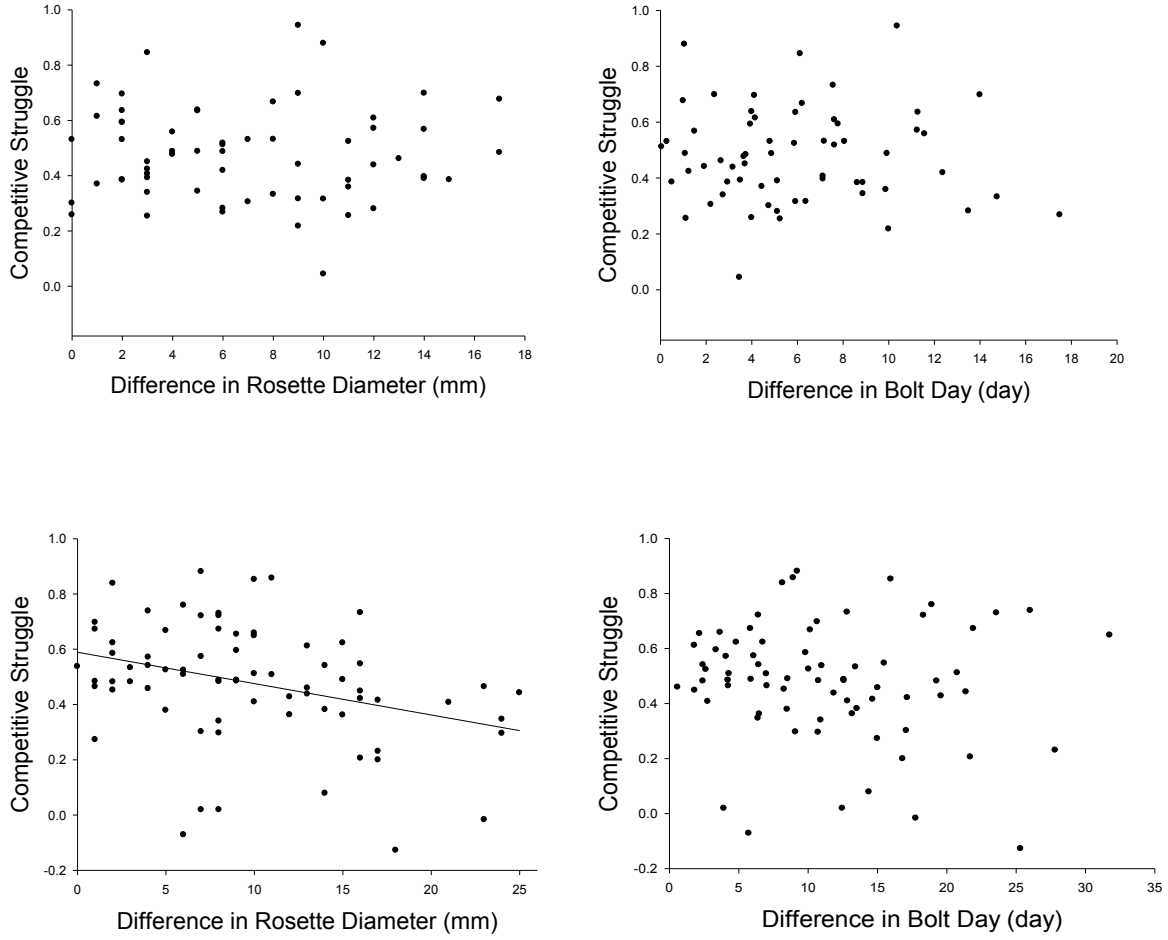


Figure 2.6 The relationship between competitive struggle and the differences in plant traits (rosette size and bolt day). The low nutrient conditions are on the top row and high nutrient conditions are on the bottom row. There was a significant relationship between competitive struggle and size differences with high nutrients (n=74 r^2=0.134 p=0.001), but not with low nutrients (n=62 r^2=0.003 p=0.682). There was no relationship between competitive struggle and bolt day at low (n=62 r^2=0.001 p=0.807) or high (n=74 r^2=0.012 p=0.347) nutrient levels.

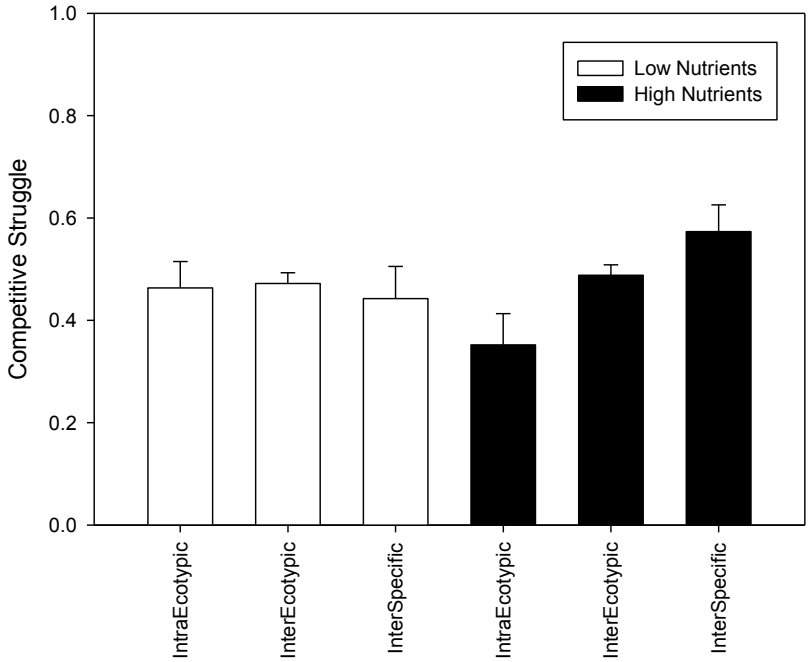


Figure 2.7 Competitive struggle amongst relatedness groups for low and high nutrient conditions. Error bars give ±1 SE from the means. Under low nutrient conditions, there was no difference between the groups (KW, p=.808), but under high nutrient conditions, competitive struggle was weakest in closely related competitors (intra-ecotypic *A. thaliana* or kin) when compared to both competition amongst ecotypes (inter-ecotypic *A. thaliana* or strangers) (KW, p=0.046) or competition with an interspecific competitor (*A. shokei*) (KW, p=0.010). The difference between inter-ecotypic and interspecific groups at high nutrient levels was not significant (KW, p=0.109).

Chapter 3 – Intransitivity, reversals and competitive equivalence in competitive networks

Introduction

A fundamental challenge of studying ecological interactions such as competition has been the difficulty in scaling up from species to communities (Keddy 1990). For n species, the number of possible pairwise interactions is n(n-1)/2. With a community of 25 species, there are 300 possible pairwise interactions to consider even before accounting for other potentially important factors such as abiotic conditions, spatial structure or individual variation.

In the study of competitive interactions, a major effort to bridge the gap between species and communities was the work done on competitive hierarchies (Keddy and Shipley 1989, Goldberg and Landa 1991, Keddy 2001). If competition is important, and if organisms could be ranked consistently according to their competitive ability, then the relative abundance of those species in a community should reflect their competitive ability (Silvertown and Dale 1991). In other words, having determined a competitive hierarchy it would not be necessary to observe all the pairwise interactions. One ecological justification for this argument, shaped by a plant-centric view, was that a hierarchy could be largely based on plant size, as size was considered to be the driving trait in determining competitive outcomes (Gaudet and Keddy 1988).

Hierarchies are any kind of ranking. The work on competitive hierarchies, however, focused on a specific subset called transitive hierarchies (Keddy and

Shipley 1989). Transitive hierarchies are internally consistent hierarchies, such that if $a_1>a_2$ and $a_2>a_3$ then $a_1>a_3$. An everyday example of transitive rankings are the values on playing cards: Ace>King>Queen>Jack>10 ... >2 where rankings are internally consistent (e.g. A>Q>9>2, K>10, etc.) no matter how many cards you start with or look at. The alternative is an intransitive hierarchy, a hierarchy that is not internally consistent. For example, if $b_1>b_2$ and $b_2>b_3$ then the inequality $b_1<b_3$ is not consistent with the first two inequalities. If a competitive hierarchy is intransitive it is not obvious how to directly translate species specific competitive abilities to community relative abundances because of the ranking inconsistencies.

Ecologists recognized the concept of intransitivity in competition relatively early (Lewontin 1968, Gilpin 1975, May and Leonard 1975). Recent theoretical developments have greatly increased the appreciation of its potential ecological importance, albeit not addressing the problem of scaling from species to communities. For example, simulation models have demonstrated that coexistence is promoted by even modest amounts of intransitivity, levels comparable with existing field data (Laird and Schamp 2006). Furthermore, increasing the amount of intransitivity, as well as combining intransitivity with spatial structure, both lead to increased coexistence (Laird and Schamp 2006, 2008). A particularly counterintuitive result is that intransitivity can lead to the least competitive species having the greatest abundance, and being the least likely to go extinct, due to intransitive dynamics where abundance of one species is determined by the invasive ability of another in a spatial model of a 3-species competitive cycle similar to the game of rock-paper-scissors (Frean and Abraham 2001).

Empirical work on competitive hierarchies has been equivocal. Reanalyses of competition studies (Keddy and Shipley 1989, Shipley 1993, Shipley and Keddy 1994) and experiments (Mitchley and Grubb 1986, Miller and Werner 1987, Grace et al. 1993, Keddy et al. 1994) have supported the notion of transitive hierarchies and

their importance. However, hierarchical rankings can change based on environmental context (Suding and Goldberg 2001) and how competition is defined and measured (Goldberg and Landa 1991, Howard and Goldberg 2001). Experiments have also directly demonstrated intransitivity in competitive hierarchies and recognized the implications for coexistence (Russ 1982, Taylor and Aarssen 1990, Dormann 2007). Using a new method to quantify intransitivity from species abundance data (Ulrich et al. 2014), intransitivity was found to be important in the maintenance of species richness in dryland and grassland systems over a large scale (Soliveres et al. 2015). Overall, there has been a growing acceptance that even though competitive hierarchies are not often fully transitive, competitive rankings can still be important predictors of community abundance (Keddy et al. 2000, Fraser and Keddy 2005, but see Engel and Weltzin 2007). At the same time, with the recognition that competitive intransitivities are common, the focus has shifted to their importance for community coexistence (Laird and Schamp 2006).

The purpose of this study was to parameterize empirically a competitive hierarchical network with direct measures of competition and a relatively large number of nodes, and then to analyze this network. To do so, we used a tournament among 13 *Arabidopsis thaliana* (L.) Heynh. accessions at 2 nutrient levels (cf. Aarssen 1989, Taylor and Aarssen 1990). With this data, 1) we characterized the intransitive structures of this hierarchy. We focused on competitive reversals, situations when lower ranked individuals outcompeted higher ranked individuals. 2) We determined how intransitive properties of hierarchies changed with increasing or decreasing diversity. The properties we looked at were reversal frequency, competitive equivalence and reversal length, potentially different aspects of intransitivity as will be discussed later. 3) We interpreted our empirical results in context of current theoretical models of intransitivity and coexistence.

Materials and Methods

To measure the effect of competition amongst 13 accessions of *A. thaliana,* we used a full pairwise competition design of 78 accession pairs at 2 nutrient levels (see Chapter 2). The experiment took place in a temperature (24 ^{o}C) and light (16:8 day:night) controlled growth chamber (Biotron facilities, Department of Biological Sciences, University of Alberta) to mitigate the potentially confounding effects of changing environmental conditions.

The 13 accessions were obtained from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH) (Table 3.1). ABRC stock seeds were propagated using uniform soil (LC1 Sunshine Mix, Sun Gro Horticulture, Agawam, MA), nutrient (6 balls of Nutricote Type 100, NPK 14:14:14, Chisso-Asahi Fertilizer Co., Toyko) and growth conditions to minimize potential maternal effects (Postma and Ågren 2015, Videvall et al. 2015). Each pot was isolated with a cellophane sleeve and monitored daily for about 70 days. Seeds were harvested when siliques were ready to dehisce on individual plants. The seeds from this propagation were used in the subsequent competition experiment.

We setup the pairwise competition plants and no-competition plants following the methods of Cahill *et al.*(2005). The 78 pairwise competition combinations were replicated 5 times at both low and high nutrient levels. The 13 no-competition single plant treatments were replicated 10 times at both low and high nutrient levels. High and low nutrient levels were achieved by using Hoagland's solution at 1:1 and 10:1 dilution, respectively, applied 3 times weekly at 1 mL per application (Epstein 1972). The appropriate nutrient levels were determined in a series of pilot studies prior to Cahill *et al.* (2005). Plants were uniformly bottom watered using a customized Rain Bird™ (Rain Bird Corporation, Azula, California) watering system with one watering

tube per tray of 32 pots. All pots were arranged in a completely randomized block design. As a direct measure of reproductive output, the number of siliques was counted on each plant at the completion of fruiting and averaged for each accession.

Analyses

We used a measure of competitive response (Goldberg and Fleetwood 1987, Weigelt and Jolliffe 2003) to characterize the strength of competition.

$$\text{Competitive response (i.j)} = \frac{\text{reproductive output (i|j)}}{\text{reproductive output } (i_{solo})}$$

where i|j is reproductive output of i when competing with j and i_{solo} is reproductive of one plant of i growing by itself with no competition. This ratio is the fraction of reproductive output of plants in competition compared to plants without competition. From the competition experiment we generated a 13x13 matrix representing the competitive response of each accession to each other accession for the high nutrient treatment. One accession (7) did not produce siliques in the no-competition low nutrient treatment. As a consequence, a 12x12 competitive response matrix was analysed for the low nutrient treatment. We analysed the high nutrient data for the same 12 accessions, but do not present this data because it is a subset of the 13x13 matrix.

For each competition matrix, we first generated every possible combination of n x n submatrices where n is the number of accessions. For the high nutrient treatment, n ranged from 3 to 13 with 13Cn combinations of each n x n submatrix

(Table 3.2). For the low nutrient treatment, n ranged from 3 to 12 with 12Cn combinations of each n x n submatrix (Table 3.3).

For each submatrix, we ranked each accession by using a net in/outdegree method. If the competitive response(i|j) > competitive response(j|i) then i was deemed to be dominant to j because the effect of i on j was greater than the effect of j on i. By convention, we tabulated this result by incrementing the outdegree count of i by 1 and incrementing the indegree count of j was by 1. The (total number of outdegrees) – (total number of indegrees) for each accession determined the rank of that accession in the submatrix where higher net values represented higher positions in the competitive hierarchy. In the case of ties in the net number of in/outdegrees, ranks were averaged between accessions. In the end, a competitive hierarchical ranking was determined for every possible submatrix size and combination.

Based on the competitive ranking of each submatrix, we conducted an exhaustive search for reversals as a measure of intransitivity. Reversals were counted by tabulating the number of times that the ranking of i and j contradicted the pairwise competitive outcome between i and j. In other words, a reversal was detected if i was ranked higher than j, but competitive response(i|j) < competitive response(j|i), or if i was ranked lower than j, but competitive response(i|j) > competitive response(j|i).

With the number of reversals calculated for each submatrix, we determined how the proportion of reversals changed with accession diversity by dividing the number of reversals observed by the total number of possible pairwise competitive interactions, or edges, for each number of accessions (n).

$$\text{Proportion of reversals} = \frac{\text{observed number of reversals}}{\text{number of matrix combinations x number of edge matrix}}$$

Competitive equivalence is defined here as rank equivalence in the hierarchy. To determine how competitive equivalence changed with accession diversity (n), we computed the number of unique ranks observed in each submatrix as a proportion of the number of possible ranks (n). We subtracted this value from 1 to make the index intuitive (i.e. it goes up when competitive equivalence goes up). Greater rank equivalence amongst accessions, therefore results in fewer of the possible ranks being occupied. For example, given 7 accessions, there are 5 unique ranks if the ranking is [1,3,3,3,5,6,7]. Competitive equivalence would be: 1-(5/7) or 0.286.

$$\text{Competitive equivalence} = 1 - \frac{\text{observed number of unique ranks}}{\text{possible number of ranks}}$$

For each reversal, the length of that reversal (rank difference) was also tabulated. For example, the length of a reversal is 5 if a 7th ranked accession "beats" a 2nd ranked accession. The distribution of the length of reversals was calculated for each level of accession diversity (n). To determine whether increased length of reversals could be explained by competitive asymmetries, the relationship between the two was analyzed, where competitive asymmetry was measured as:

$$\text{Competitive asymmetry}(i,j) = \text{competitive response } (i|j) - \text{competitive response } (j|i)$$

All analyses were conducting using R (R Core Team 2015) in the R Studio IDE (R Studio Team 2015). Packages 'abind' (Plate et al. 2015) and 'MASS' (Ripley et al. 2015) were used for their matrix manipulation tools.

Results

The overall hierarchical structure

The hierarchical structure of the high nutrient competitive network (Figure 3.1) and low nutrient competitive network (Figure 3.2) were computed based on the number of outdegrees – indegrees for each accession. In Figures 3.1 and 3.2, the graphical convention being used is that the node at the tail end of the arrow is competitively dominant to the node at the head end of the arrow. The arrows displayed in Figures 3.1 and 3.2 are a subset of all possible arrows, as each node was only drawn with the strongest outgoing and incoming arrow. Comparing Figure 3.1 to Figure 3.2, the hierarchical rankings clearly changed with nutrient level, but the magnitude of rank change depended on the specific accession (e.g. compare ecotype 2 which changed rank a lot between nutrient levels vs. ecotype 11 which did not).

Are there dominants and subordinates?

There are no true dominants or subordinates in either competitive network. As illustrated in Figures 3.1 and 3.2, each node or accession has at least one incoming and one outgoing arrow. The implication is that each accession is dominant or subordinate to at least one other accession and therefore neither network is wholly transitive.

Is the hierarchy transitive?

In addition to the lack of a true dominant or subordinate, we tabulated the number of reversals, or the number of times a lower ranked node was competitively dominant to a higher ranked node, as a measure of intransitivity (Tables 2.2, 2.3). A few examples are represented in Figures 3.1 and 3.2 as outgoing arrows that are pointed upwards from a lower rank node to a higher rank node. In the high nutrient competitive network there were 12 reversals in total and in the low nutrient competitive network there were 11 reversals in total, each of which is by definition an intransitive edge. The cases for an accession diversity of 3 were not reported in Tables 2.2 and 2.3. These are special cases because by the ranking method used here, there are no reversals but only intransitive cycles (like rock-paper-scissors). With high nutrient levels there were 36 cycles in 286 possible 3x3 competition matrices analyzed, and with low nutrient levels there were 31 cycles in 220 possible 3x3 competition matrices.

Does the amount of intransitivity change as a function of diversity?

The number of reversals at each accession diversity for the high and low nutrient conditions are presented in Table 3.2 and Table 3.3, respectively. All things equal, the absolute number of reversals should change with the number of matrices tested and the number of possible edges per matrix. Therefore, the number of reversals as a proportion to the possible number of edges was calculated (Figures 3.3 and 3.4). In both high and low nutrient conditions, the proportions of reversals increased from ~2% to ~15% as the accession diversity increased. As the number of reversals are tabulations of all possible combinations they are exact values for this set of data (i.e. no error bars).

Does competitive equivalence change with diversity?

Competitive equivalence increased with greater accession diversity at both nutrient levels (Figures 3.5 and 3.6). The range of values and the general shape of the increase was similar between both nutrient levels. Specifically, incremental increases in competitive equivalence were greater at lower numbers of accessions than at higher number of accession (i.e. there is a plateau).

Does the length of reversals change with diversity?

The median reversal length does not change with accession diversity for high nutrient treatments (Figure 3.7) but increases with increased accession diversity for low nutrient treatments (Figure 3.8). The distribution of reversal lengths around the median widens with increased diversity at high nutrient levels (Figure 3.7), but not at low nutrient levels (Figure 3.8). With increasing diversity, the 90^{th} percentile of reversal lengths increases 2.5 fold in the high nutrient treatment and around 35% in the low nutrient treatment. Overall, there is a greater proportion of long reversals at high nutrient levels than at low nutrient levels, and the maximum reversal length is greater at high nutrient levels than at low nutrient levels.

Is there a relationship between competitive asymmetry and the length of a reversal?

The peak length of reversals generally occurs between accessions that have a low, but not the lowest, levels of competitive asymmetry between them (Figures 3.9 and 3.10), although the pattern is more pronounced at high nutrient conditions than low nutrient conditions. With high nutrients, maximum reversal lengths of 10 peaks at

competitive asymmetry of 0.2, while at low nutrients maximum reversal lengths of 7 peaks at competitive asymmetry of 0.3.

Discussion

Intransitivity

There are 3 related pieces of evidence that demonstrate that intransitivity is structural to the competitive network analyzed here (Figures 3.1 and 3.2). First, there are no true dominants or subordinates, as no accession is competitively superior or inferior to every other accession. Second, the hierarchical ranking and structure changed with nutrient levels, indicating that the interactions are contingent and not absolute. Third, using reversals as a direct indicator of intransitivity, reversals were common features of the hierarchies regardless of how the 13 accessions were subsampled (Tables 3.2 and 3.3). Therefore, our data does not show a transitive competitive hierarchy. In fact, a literal examination of most classic experimental studies analyzing hierarchies (e.g. Mitchley and Grubb 1986, Miller and Werner 1987, Goldberg and Landa 1991) do not show complete transitivity. Most natural systems are structured and heterogeneous and it is expected that interactions and hierarchies shift based on local environmental conditions (e.g. Suding and Goldberg 2001). It seems likely that true dominance and transitivity would only exist in well-mixed homogenous conditions (e.g. Gauze 1934, but see Shipley 1993).

Nevertheless, it is possible for partially transitive hierarchies to scale to predictions of community abundances (Keddy et al. 2000, Fraser and Keddy 2005). For this to occur, there must exist a relatively stable hierarchy within the range of

environmental conditions experienced by some community, and the partially transitive ranking must map to community abundance. It is actually the latter condition that is more stringent as many factors can impact community structure (Ricklefs and Schluter 1994), and competition may not drive communities even if it is an important component (Silvertown and Dale 1991, Engel and Weltzin 2007).

Relationships with diversity

At both high and low nutrient levels the proportion of reversals increased with increasing accession diversity with ~15% of all possible edges being reversals in the full hierarchy (Figures 3.3 and 3.4). In both cases, the fastest incremental increase in proportion with each incremental increase in diversity was at lower diversity levels. But, at the lower nutrient levels the gradient of incremental increase was steeper at the lower diversity levels (e.g. proportion of ~13% was reached at a diversity of 8 in low nutrient treatments, but at a diversity of 11 in high nutrient treatments). As far as we are aware, this is the first estimation of reversals as a function of diversity.

If a hierarchy is completely transitive, there would be as many ranks as there types of organisms (e.g. genotypes, species). The number of possible ranks observed as a fraction of the number of possible ranks is an indicator of both intransitivity but also of competitive equivalence. The more accessions that share a ranking (i.e. a tie) the greater the competitive equivalence. At both high and low nutrients, there was a steep increase in competitive equivalence at lower diversities, and the value plateaued around 0.35 (Figures 3.5 and 3.6). Based on our ranking algorithm, the theoretical maximum of competitive equivalence is just under 0.5 given a hierarchy of at least 2 levels.

The distribution of reversal lengths was the characteristic most different between high and low nutrient treatments (Figures 3.7 and 3.8). In particular, the range of the upper 25% percent of observations increased dramatically with high nutrients and almost did not increase at low nutrients. That is to say, the maximum length of reversals tracked the increase in diversity at high but not low nutrient levels. Looking at the outliers in Figure 3.8, the longest reversal lengths occurred at diversity of 9/10, not at the maximum of 12.

Implications for coexistence

Each of the graphs with the x-axis of accession diversity (Figures 3.3-3.8) can be examined in the direction of increasing diversity, or decreasing diversity. Theoretical models have established that increasing intransitivity can result in greater coexistence basically because dominance is mitigated by reversals (Laird and Schamp 2006). Here, we show that intransitivity increases with diversity, implying that more diverse communities might be more stable due to intransitivity alone. This would be a unique mechanism to add to the diversity-stability debate (McCann 2000). The converse is also true. Decreasing diversity accelerates the decrease in community stability because of the decreased intransitivity, but also because the incremental decrease in intransitivity accelerates as diversity decreases.

Competitive equivalence is another characteristic of competitive interactions in a community context that is thought to facilitate coexistence (Ferrell 2004). Essentially, competitive ties facilitate coexistence because equivalents cannot competitively exclude each other. Plants may show relatively high degrees of competitive equivalence because they compete over the same basic resources (Grace 1995, Hubbell 2001) and their sedentary behaviour (Pacala 1988). Like the reversals, the proportion of competitive equivalence also increased with increasing diversity

(Figures 3.5 and 3.6). We do not fully understand the relationship between the amount of equivalence and stability, but intuitively, increases in competitive equivalence should result in increasing coexistence. If that is the case, by the same argument as the reversals, decreases in diversity accelerate the reduction of community stability.

The importance of reversal length is the least understood of the hierarchy characteristics we analyzed. It seems possible that longer reversals are in some way more intransitive than shorter reversals because they represent the greatest contraction of rank distances. That is to say, longer reversals reduce the average path length between two nodes more than short reversals. If this is the case, increasing maximum reversal lengths (Figure 3.7) could also contribute to greater stability. However, at lower nutrient levels, we do not see this pattern (Figure 3.8). This clearly demonstrates the sensitivity of competitive hierarchical networks to abiotic conditions, but what does it mean? Given that the proportion of reversals and competitive equivalence were mostly similar at both nutrient levels, the distribution of reversal length to competitive asymmetry (Figures 3.9 and 3.10) shows that at low nutrient levels there was less range in competitive asymmetry, but a higher median value. In other words, at low resource levels the median competitive asymmetry increased which may have contributed to the reduction of maximum reversal lengths by reducing the range of net (out-in) degrees.

Are reversals, competitive equivalences and reversal lengths additive in effect, or are they different facets of the same thing? For example, both intransitivity and equivalence are thought to enhance coexistence independently, but what is their joint effect? The difference in the reversal length-diversity relationship between nutrient treatments, when the patterns of reversal-diversity and equivalence-diversity were similar, suggests that they are probably not all aspects of the same thing. There is an obvious need to further explore the implications and interactions of these intransitive

properties, as they may have direct consequences for our ideas of coexistence. In particular, testing the applicability of these results to a field context would be essential to determining whether intransitivity has implications for conservation practices.

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Table 3.1 The ABRC accessions used in experiment. Accession number, the first column, is the number used in this manuscript to identify ABRC accessions.

Accession Number	ABRC Accession Number	Background Line
1	CS28055	*A. thaliana* Bay-0
2	CS28067	*A. thaliana* Berkeley
3	CS28166	*A. thaliana* Col-0
4	CS28167	*A. thaliana* Col-0
5	CS28195	*A. thaliana* Ct-1
6	CS28387	*A. thaliana* Kin-0
7	CS28443	*A. thaliana* Lc-0
8	CS28445	*A. thaliana* Ler-0
9	CS28467	*A. thaliana* Lip-0
10	CS28502	*A. thaliana* Mt-0
11	CS28650	*A. thaliana* Pog-0
12	CS28780	*A. thaliana* Tsu-0
13	CS28782	*A. thaliana* Tsu-1

Table 3.2 The observed number of reversals as a function of diversity for the high nutrient treatments. The number of matrix combinations and the number of edges per matrix were used to calculate the proportion of reversals in relation to the total number of possible edges in Figure 3.3.

Accession Diversity (n)	Observed Total Sum of Reversals	Number of nxn Matrix Combinations (13Cn)	Number of Edges per Matrix (n)(n-1)/2
4	110	715	6
5	608	1287	10
6	1683	1716	15
7	2911	1716	21
8	3378	1287	28
9	2710	715	36
10	1498	286	45
11	548	78	55
12	120	13	66
13	12	1	78

Table 3.3 The observed number of reversals as a function of diversity for the low nutrient treatments. The number of matrix combinations and the number of edges per matrix were used to calculate the proportion of reversals in relation to the total number of possible edges in Figure 3.4.

Accession Diversity (n)	Observed Total Sum of Reversals	Number of nxn Matrix Combinations (12Cn)	Number of Edges per Matrix (n)(n-1)/2
4	80	495	6
5	463	792	10
6	1221	924	15
7	1900	792	21
8	1876	495	28
9	1184	220	36
10	464	66	45
11	105	12	55
12	11	1	66

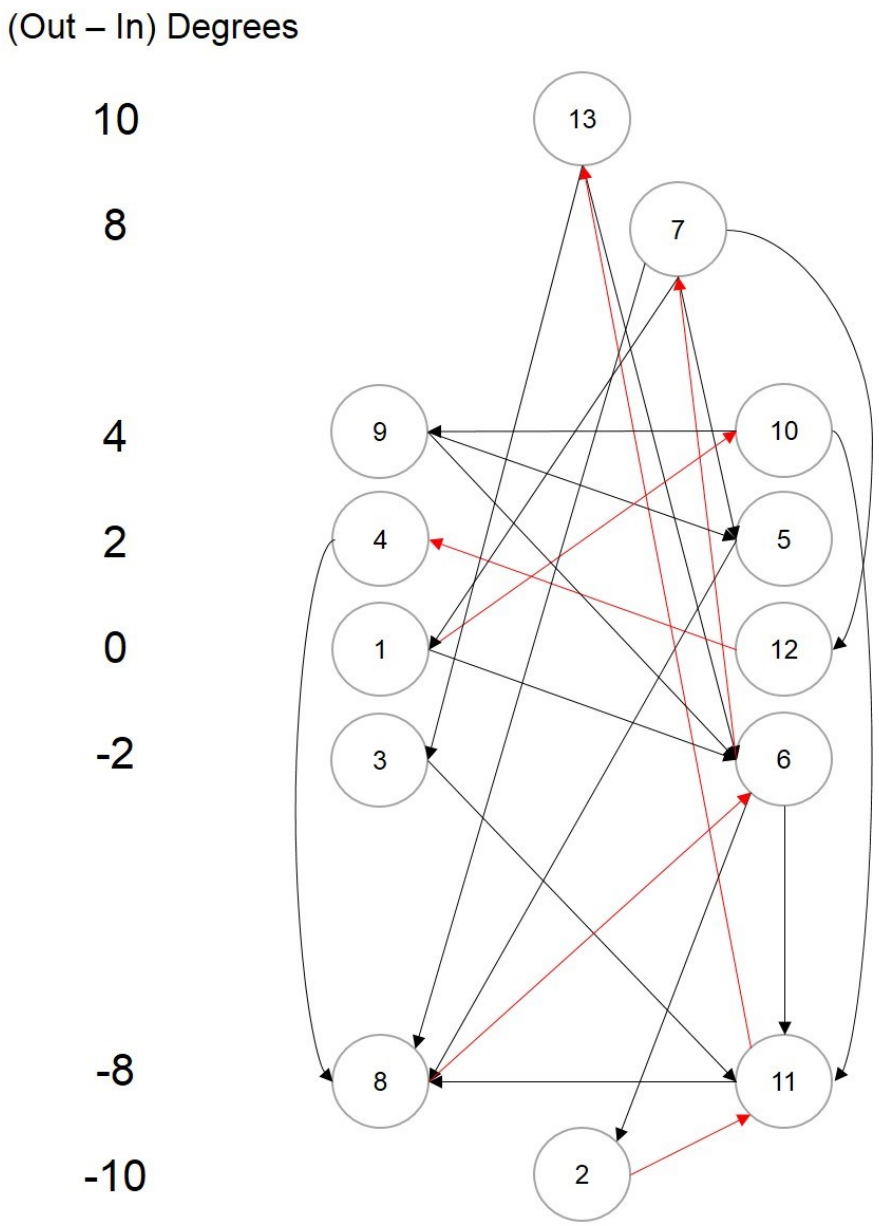

Figure 3.1 Representation of the competitive hierarchical network of 13 accessions of *A. thaliana* grown at high nutrient levels. Accession numbers (Table 3.1) are indicated in the circles (nodes) and each node was drawn only with the indegree and outdegree associated with the largest competitive response. Reversals are highlighted with red arrows. The left column of net (out-in)degrees corresponds to the nodes at the same level in the figure.

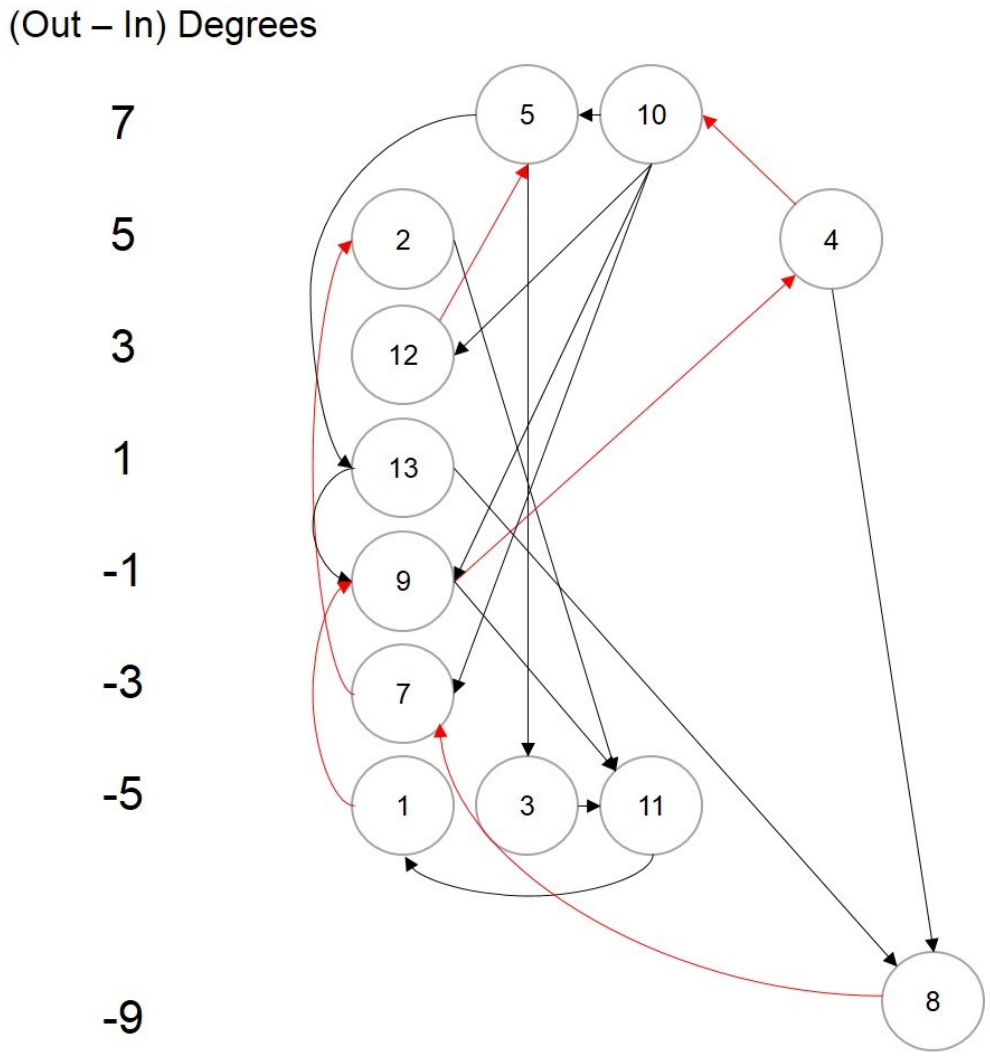

Figure 3.2 Representation of the competitive hierarchical network of 12 accessions of *A. thaliana* grown at low nutrient levels. Accession numbers (Table 3.1) are indicated in the circles (nodes) and each node was drawn only with the indegree and outdegree associated with the largest competitive response. Reversals are highlighted with red arrows. The left column of net (out-in)degrees corresponds to the nodes at the same level in the figure.

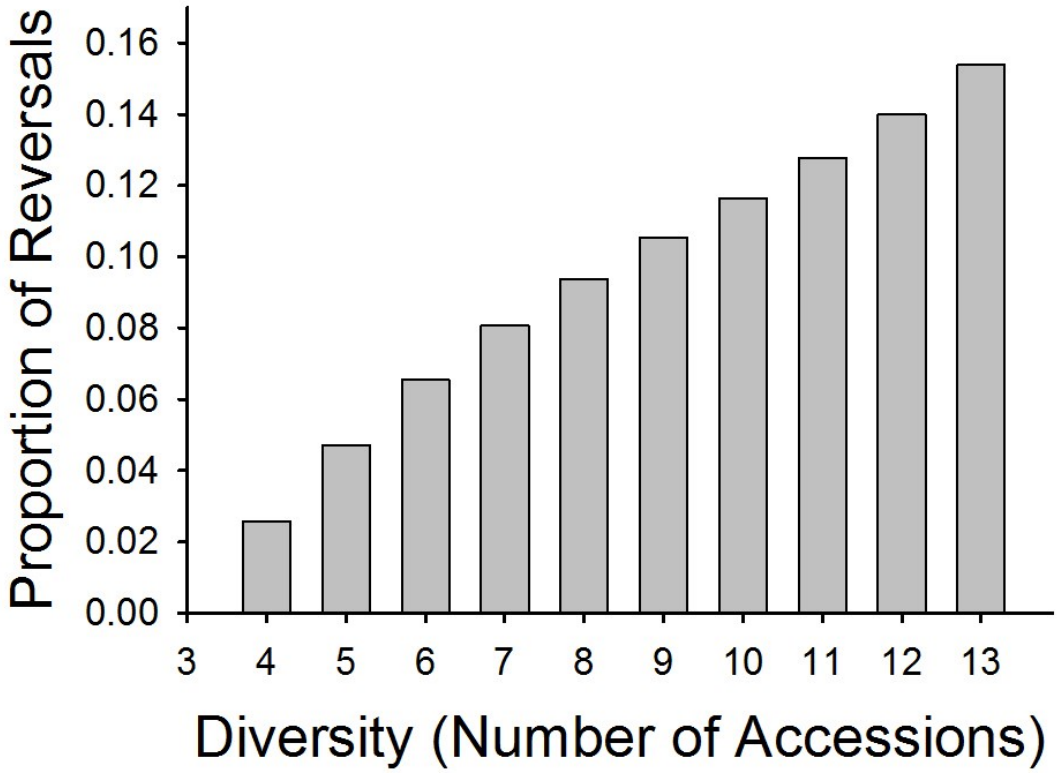

Figure 3.3 The proportion of reversals as a function accession diversity for the high nutrient treatments. The proportion was calculated as the number of observed reversals divided by the possible number of edges. Error bars are not reported because the values are tabulations of all possible combinations.

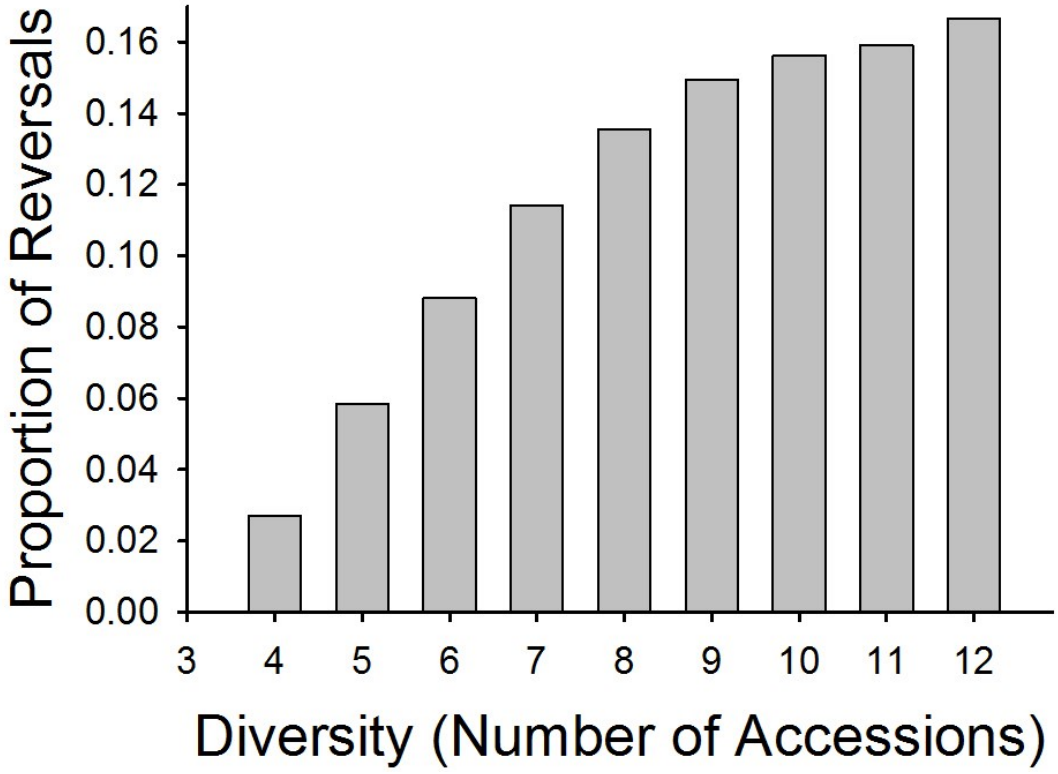

Figure 3.4 The proportion of reversals as a function accession diversity for the low nutrient treatments. The proportion was calculated as the number of observed reversals divided by the possible number of edges. Error bars are not reported because the values are tabulations of all possible combinations.

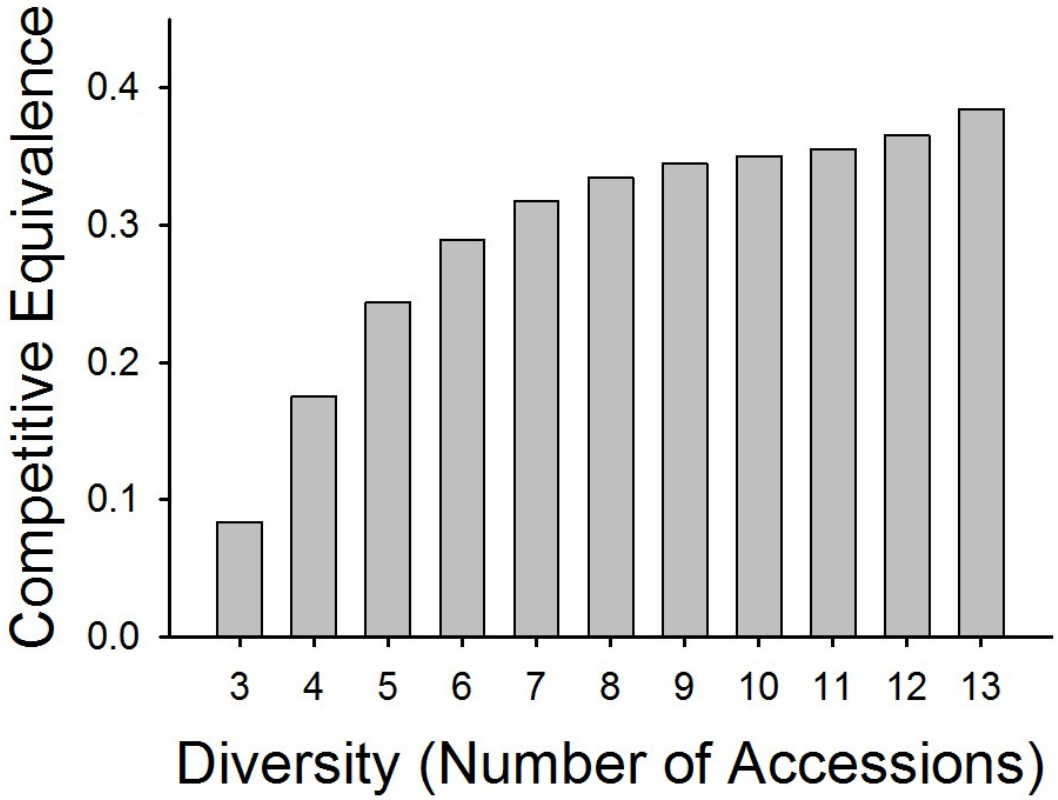

Figure 3.5 The relationship between competitive equivalence and accession diversity for high nutrient treatments. Competitive equivalence is a measure of how many unique competitive ranks were observed as a fraction of the possible number of competitive ranks. Error bars are not reported because the values are tabulations of all possible combinations.

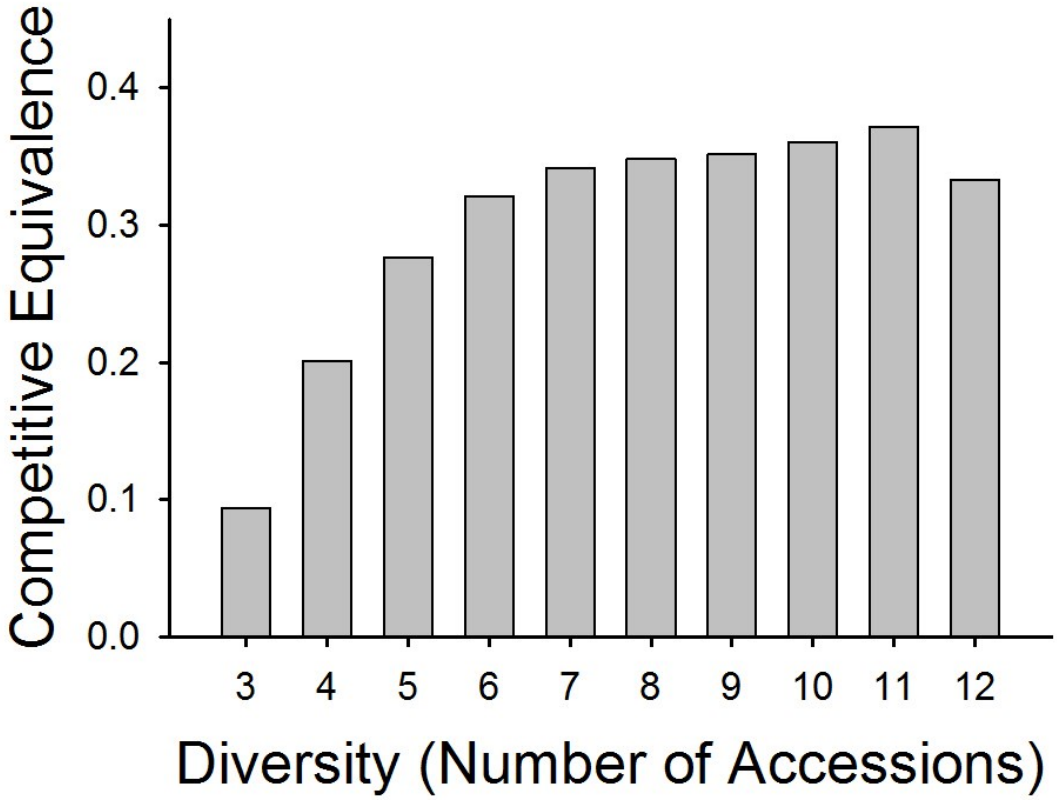

Figure 3.6 The relationship between competitive equivalence and accession diversity for low nutrient treatments. Competitive equivalence is a measure of how many unique competitive ranks were observed as a fraction of the possible number of competitive ranks Error bars are not reported because the values are tabulations of all possible combinations.

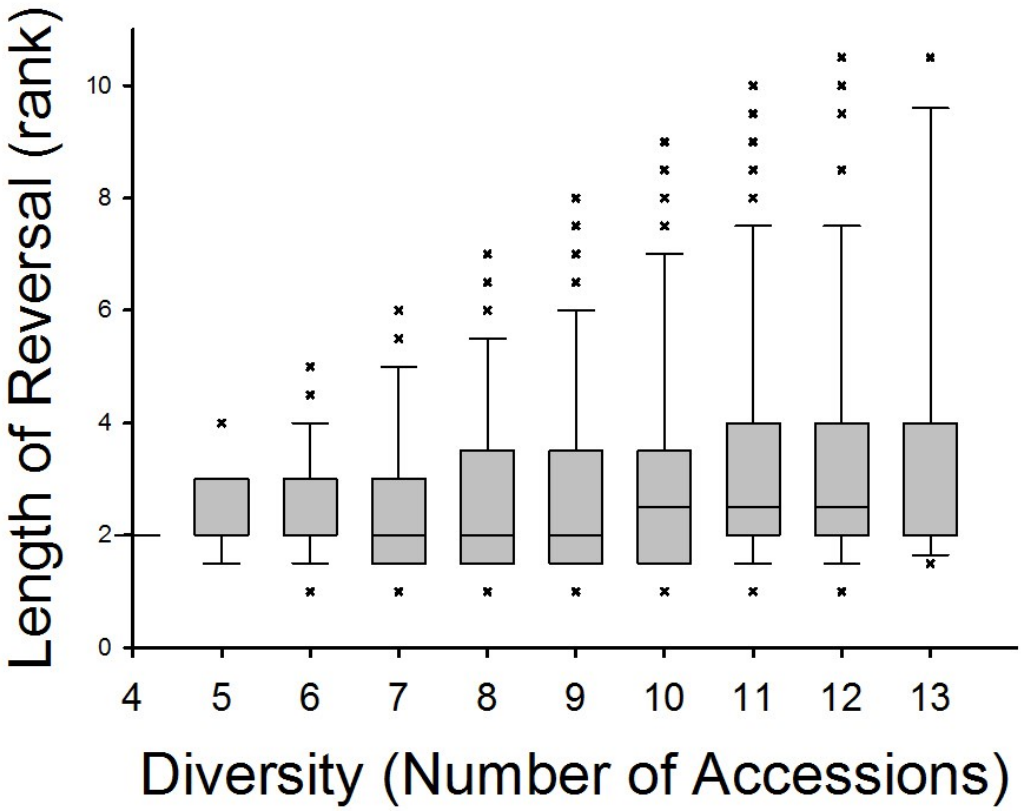

Figure 3.7 The distribution of reversal lengths as a function of accession diversity for the high nutrient treatments. The line inside each grey box indicates the median; the lower and upper edge of the grey box mark the 25th and 75th percentile, respectively; the lower and upper bar mark the 10th and 90th percentile, respectively; and outliers are indicated by an asterisk.

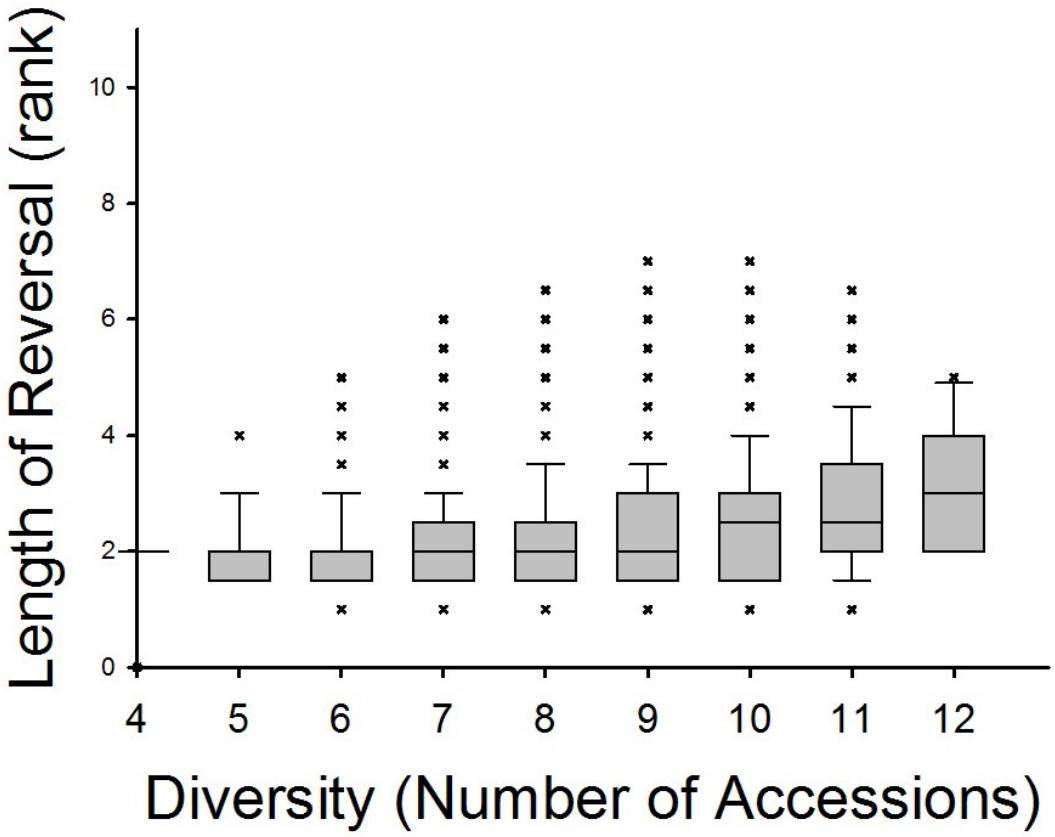

Figure 3.8 The distribution of reversal lengths as a function of accession diversity for the low nutrient treatments. The line inside each grey box indicates the median; the lower and upper edge of the grey box mark the 25th and 75th percentile, respectively; the lower and upper bar mark the 10th and 90th percentile, respectively; and outliers are indicated by an asterisk.

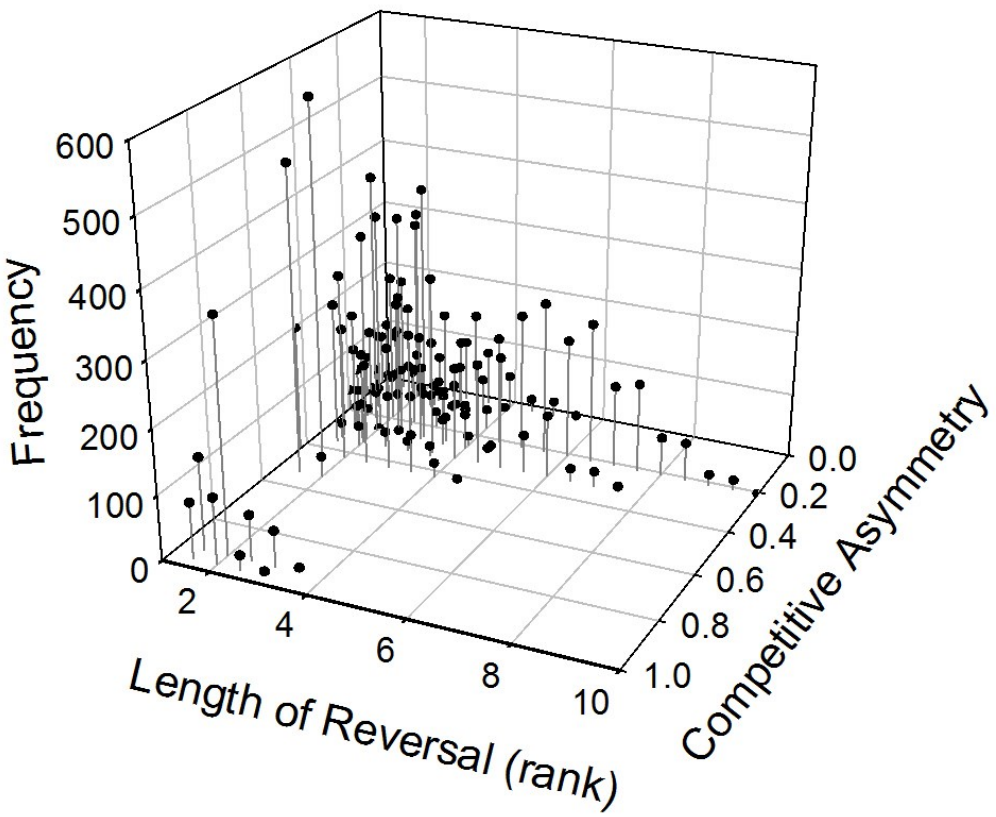

Figure 3.9 The relationship between competitive asymmetry and the length of reversals for high nutrient treatments. The number of observations of are plotted in the z-axis as frequency. The scales on the competitive asymmetry axis were reversed to increase the visibility of points.

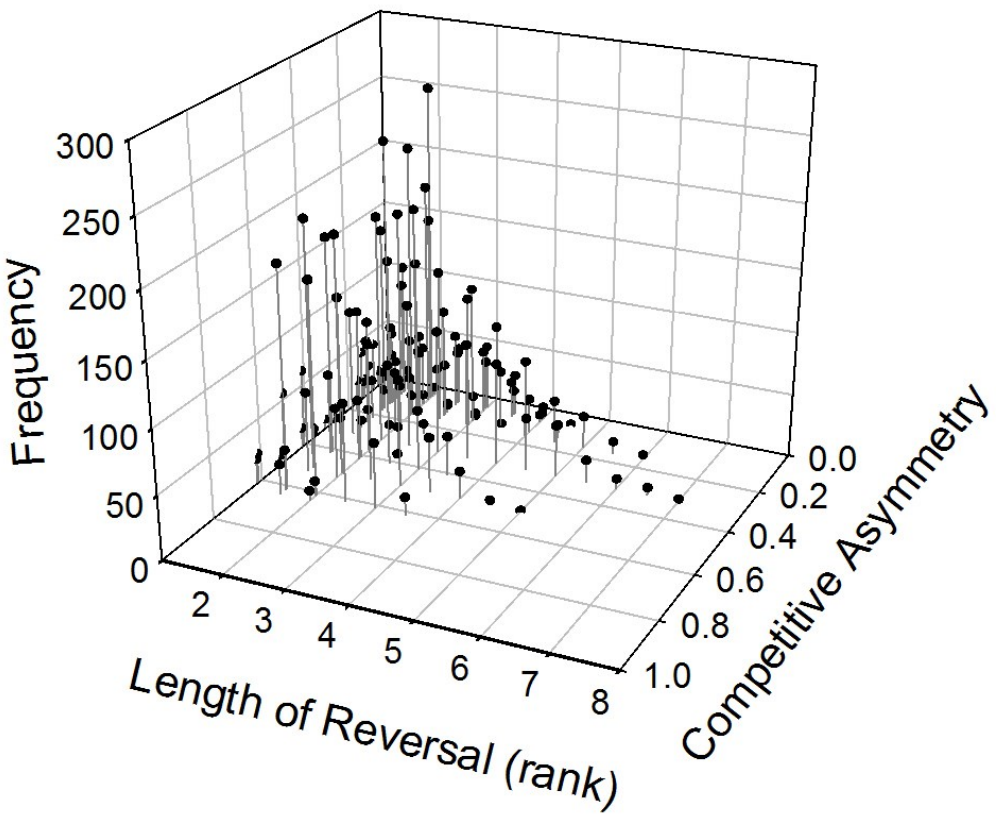

Figure 3.10 The relationship between competitive asymmetry and the length of reversals for low nutrient treatments. The number of observations of are plotted in the z-axis as frequency. The scales on the competitive asymmetry axis were reversed to increase the visibility of points.

Chapter 4 - Photosynthetic opportunity cost and energetic cost of rapid leaf closure defense in *Mimosa pudica* L. (Fabaceae)

Introduction

Without some form of defense, plants are a veritable all-you-can-eat salad bar for herbivores. The increase in plant fitness attributable to plant defense has been demonstrated across a wide range of strategies. These include: physical defenses such as waxy cuticles (Kerstiens 1996), spines (Belovsky et al. 1991) or trichomes (Levin 1973); crypsis (Fadzly and Burns 2010); constitutive and induced chemical defenses (Karban and Baldwin 1997); and interactions with mutualists (Janzen 1966), predators (Dicke and Sabelis 1987) and parasitoids (De Moraes et al. 1998). However, efforts to quantify the costs associated with using a particular defensive strategy are fewer (Coley et al. 1985, Agrawal et al. 1999) and the costs have been harder to detect (Brown 1988, Agren and Schemske 1993, Gianoli and Niemeyer 1997, Agrawal 2000, Karban 2011). Our objective here was to describe the individual level cost of defense in *Mimosa pudica* L. (Fabaceae), a plant that has a rapid leaf closure defense particularly amenable to observation.

Mimosa pudica, commonly called the sensitive plant, is a perennial shrub native to Central Brazil which has now become a pantropical weed (Gehlot et al. 2013). It grows to 15-100 cm in height, has woody stems at the base, bipinnate leaves and flowers occur in globular inflorescences. It is well known for the rapid movement of its leaflets and petioles via the loss of turgor in specialized extensor cells (Braam 2005) triggered by mechanical or electrical stimulation (Volkov et al. 2010) which operates in conjunction with the actin cytoskeleton (Fleurat-Lessard et al. 1993, Kameyama et al. 2000, Kanzawa et al. 2006). A putative function of this rapid movement is the dislodgement of small invertebrate herbivores, while the

potential benefits of having leaves closed include: crypsis to mammalian herbivores and greater exposure of protective spines (Eisner 1981). In terms of cost, there is an energetic cost associated with the movement itself, such as the use of ATP to pump ions required for the function of aquaporin transporters (Fleurat-Lessard et al. 1997). Once the leaves are closed, the main cost is the opportunity cost of reduced photosynthetic activity. Photosynthetic rates decline 40% when leaves of *M. pudica* are closed (Hoddinott 1977). These two costs increase with increased stimulation of movement and longer periods of closure, respectively.

The opening-closing leaf behaviour of *M. pudica* has been shown to take less time with factors such as the amount of previous touching (Applewhite 1972, Gagliano et al. 2014), light availability (Applewhite and Gardner 1971, Jensen et al. 2011), and take longer with factors such as leaf age (Amador-Vargas et al. 2014) and simulated herbivory (Cahill et al. 2013). Based on the findings of these prior studies, we recognized that changes in leaf behaviour had the potential to mediate the realized cost of the leaf closure response on a relatively short time scale, and for this reason we included measures of behavioural response in addition to measures of physical traits.

We specifically addressed the following questions: (i) What is the cost of repeated leaf closure in *M. pudica* as measured by changes in biomass and biomass allocation? (ii) What is the effect of repeated leaf closure on plant traits? (iii) What is the effect of repeated leaf closure on leaf re-opening behaviour *M. pudica*? Given the direct costs of movement (Fleurat-Lessard et al. 1997) and photosynthetic opportunity costs of closure (Hoddinott 1977), our basic expectation was that plants with leaves stimulated to close more often and stay closed longer would be smaller (less biomass) than control plants that did not incur the same costs.

Materials and Methods

In the greenhouse facilities of the Department of Biological Sciences, University Alberta (53.5244 ºN, 113.5244 ºW), 5 *Mimosa pudica* L. (Fabaceae) seeds (Stokes Seeds, Thorold, ON) were planted in each of 60 4-inch pots containing a sieved mix of 15% sand, 15% top soil, 70% Sunshine LC1 soil mix (Sun Gro Horticulture, Agawam, MA) and fertilized with 5 grains of slow release fertilizer (Nutricote Type 100, NPK 14:14:14, Chisso-Asahi Fertilizer Co., Toyko). Germination occurred after approximately 3 weeks in dark conditions and pots were then thinned to one individual per pot as necessary. Plants were given 1 month to grow under ambient greenhouse conditions, during which time they were exposed a light breeze from a fan on a daily basis to prevent spindly growth. 11 plants were then randomly assigned to each treatment. Plant height and width was measured at the start of the treatment period.

To determine the effect of repeatedly inducing rapid leaf closure, we grew plants under one of three treatment conditions: Treatment (1), control, no direct touching of plants. Treatment (2), Day Touch, was used to determine the combined effect of increased leaf-closure and touching on plant response. Day Touch plants were touched continuously for 15 minutes per hour at 9 touches per minute for 8 daylight hours per day. The frequency of touch did not allow full leaflet re-opening during the 15 minutes. Treatment (3), Night Touch, was used to determine the effect of increased touching alone without the potential for photosynthetic opportunity cost and leaf closure costs, as *M. pudica* leaflets are naturally closed at night. Night Touch plants were touched continuously for 15 minutes of touching per hour at 9 touches per minute for 8 night-time hours per day. It was necessary to account for the possible effect of touch as previous studies have demonstrated that touch can increase plant

growth (Jaffe 1973, Cahill et al. 2002) and other plant traits such as tissue rigidity (Braam 2005).

The plants were physically touched by a soft plastic sheet hanging on a custom-made automated belt system with a basic design similar to a recreational ski hill chair-lift (Figure 4.1). Industrial mixer motors driving the belt were speed controlled by a Variac™ and off-the-shelf timers controlled on-off functionality. The bottom of the plastic sheet was cut into vertical strips 3 cm long and 1.5 cm wide so as to increase contact area while reducing entanglement with the plant, particularly with the spines. The intent of the design was to touch all the leaves of a plant and stimulate closure. All plants including the controls had some perturbation from watering (every ~3 days) and rotation of pots (every 3 weeks) within the greenhouse which sometimes stimulated a leaf closure event, but this was much less than the amount from the deliberate touching. Other methods of stimulating leaf closure were attempted including fans, shaking, and manual touching, before choosing this design for effectiveness and reliability.

After 80 treatment days, we measured a suite of individual plant traits such as number of inflorescences, height, widest breadth, number of leaflets and length of longest spines on the longest stem to characterize changes in morphology. Plants were harvested to determine root, shoot and inflorescence biomass. Harvested roots were washed over a fine sieve to remove soil and all biomass was dried for 24 hours in a 60 ^{o}C drying oven prior to weighing.

Plant leaf-opening behaviour of each plant was observed at days 10 and 17 after the start of the treatment period. Following a previously developed protocol (Jensen et al. 2011), we measured the length of time for leaflets to re-open to ¾ maximum breadth post-stimulation. Using calipers, leaflet width was measured at the widest point when fully opened to define maximum breadth, and using a handheld stopwatch, the time for leaflets to re-open to ¾ of this maximum breadth was

measured after stimulation. The level of photosynthetically active radiation (PAR) was determined using an AccuPAR LP80 ceptometer (Decagon, Pullman, WA) just prior to stimulation. We measured PAR levels because the length of time to ¾ re-open increases as light levels increase (Jensen et al. 2011).

The biomass data was analysed as an ANCOVA with biomass as the response variable, treatment as a fixed factor and the initial height and width of the plants as covariates. Linear regression was used to analyze the relationship between various plant traits and plants size. The behavioural data was analysed as a repeated measures ANCOVA with time to ¾ re-open as a response variable and repeated measure, treatment as a fixed factor, PAR as a covariate and observation day as the within-subject factor (Winer 1971). All data was tested for normality before analysis with SPSS version 20 (IBM Corp., Armonk, NY).

Results

The effect of leaf closure and touching on biomass

The total biomass of plants that were touched was 60-75% greater than the untouched control plants regardless of whether the plant was touched during the day or night (ANCOVA: $F_{2,22}$=4.786, p=0.019) (Figure 4.2a). Root (ANCOVA: $F_{2,22}$=3.917, p=0.035) and shoot (ANCOVA: $F_{2,22}$=4.433, p=0.024) biomass differences between touched and untouched plants differed proportionately to the total biomass (Figure 4.2b,c). However, the number of inflorescences (ANCOVA: $F_{2,22}$=6.017, p=0.008) was 65% greater and inflorescence biomass (ANCOVA: $F_{2,22}$=5.266, p=0.014) almost double in Night Touch plants compared to control and Day Touch (Figure 4.3b,c).

The relationship between biomass and plant traits

Overall, plants with greater total biomass produced more inflorescences; plants with twice the total biomass produced approximately twice as many inflorescences (Linear Regression: #Inflorescences = 7.211 $Biomass_{(g)}$ – 1.539, df_{error}=25, r^2=0.596, p<0.001) (Figure 4.3a). Other traits increased linearly with increasing total biomass: Height ($Height_{(cm)}$ = 2.522 $Biomass_{(g)}$ + 7.962, df_{error}=25, r^2=0.337, p=0.01); Width ($Width_{(cm)}$ = 6.798 $Biomass_{(g)}$ + 7.636, df_{error}=25, r^2=0.672, p<0.001); Number of leaflets (#Leaflet = 4.165 $Biomass_{(g)}$ + 4.749, df_{error}=25, r^2=0.685, p<0.001); Longest spine length ($SpineLength_{(cm)}$ = 0.307 $Biomass_{(g)}$ + 3.187, df_{error}=25, r^2=0.157, p=0.04). For example, each 100% increase in biomass resulted in approximately 15% longer longest spine length.

The effect of leaf closure on plant behaviour

The times to ¾ re-opening of leaflets on control plants and Day Touch plants were similar across PAR (400-700 nm) levels of 50 to 250 μmol m^{-2} s^{-1} (repeated measures ANCOVA: df=1,11.9, p=0.968) and grouped accordingly in Figure 4.4. In these two groups, time to ¾ re-open increased with higher PAR levels and on average each 100% increase in PAR resulted in approximately 25% longer ¾ re-open times (linear regression: y = 0.917x + 157.1, df_{error} = 24, r^2 = 0.326, p=0.002). In Night Touch plants, time to ¾ re-open also increased with higher PAR levels and on average each 100% increase in PAR resulted in approximately 25% longer ¾ re-open times (linear regression: y = 0.763x + 101.7, df_{error} = 19, r^2 = 0.329, p=0.007). However, control and Day Touch plants had on average 35% longer ¾ re-open times compared to Night Touch plants for any given PAR level (repeated measures ANCOVA: $F_{2,20.7}$=6.395, p=0.007).

Discussion

The effect of stimulated leaf closure and touching on biomass and plant traits

Contrary to expectations, Day Touch plants that were stimulated to incur greater photosynthetic opportunity cost and leaf movement costs from repeated leaf closure and opening did not have lower total biomass than control plants (Figure 4.2a). The Day Touch plants also did not have lower total biomass than Night Touch plants, and both groups of touched plants had greater total biomass than control plants (Figure 4.2a). The effect of touch on changing root-shoot allocation has been observed in field studies (Cahill et al. 2002), but our data show an increase in total biomass, not just allocation. We do not believe this result to be spurious as physiological work on *Medicago truncatula* (Fabaceae), a confamilial of *M. pudica*, demonstrated an 18% increase in total biomass with 10 seconds of slight touching 3 times a week for 5 weeks (Tretner et al. 2008), a level of touching far less than imparted here. Tretner et al. (2008) found that the slight touching of the shoots resulted in a 4.4 fold increase in MtAOC1 transcript levels in shoot tissue and 2.4 fold increase in root tissue 60 minutes after touching. Allene oxide cyclase (AOC) is a catalyst of the jasmonic acid biosynthesis pathway which can influence growth and development.

Although all touched plants were similar in total biomass, the photosynthetic opportunity cost and leaf movement costs manifested itself in the approximately 50% reduction of reproductive allocation of Day Touch plants compared to Night Touch plants (Figure 4.3 b,c). This result is interesting because it deviates from two overall patterns. The first pattern is that the plant traits that we measured: height, width, number of leaflets, longest spine length and number of inflorescences (Figure 4.3a) were all positively correlated with biomass (see results text). The second pattern is that Day Touch and Night Touch plants were similar in total biomass (Figure 4.2a). Together, this suggests that accounting for the basic effect of touch, the

photosynthetic opportunity cost and energetic cost of increased leaf movement cost incurred by Day Touch plants has reduced the energy plants allocated to reproduction. We do not rule out the possibility that the timing of touch alone may affect allocation, but the functional result is that plants stimulated to close their leaves during the day reduce their reproductive allocation. Fitness is ultimately gauged relative to competitors in a given environment, but reproductive allocation is often correlated with changes in fitness (Bazzaz et al. 2000).

Why are control plants, without the expected opportunity and energetic costs, smaller and producing fewer inflorescences? Smaller plant size and lower total reproductive allocation both are indicative of reduced fitness in a competitive context (Harper 1994, Keddy 2001). One possibility is that biomass, allocation and the plant traits we measured did not capture the full costs associated with the touch response. Slower growth and lower biomass could have resulted from a higher investment in traits we did not measure, such as anti-herbivore chemical defenses, where slower growing individuals may invest more highly (Coley et al. 1985). In a pharmacological context, *M. pudica* was found to produce substantial amounts of flavonoids (Ahmad et al. 2012) so this potential exists. More mechanistically, one explanation that was suggested to us is that the movement of leaflets in *M. pudica* increases the activity of salicylic acid (Dédaldéchamp et al. 2014) which stimulates sugar and amino acid uptake (Bourbouloux et al. 1998). In conjunction with the touch induced changes in photoassimilate uptake (Fromm and Eschrich 1988), extended periods of repeated touching could lead to short term metabolic shifts resulting in longer term carbon accumulation.

The effect of stimulated leaf closure on plant behaviour

In all treatments, increases in PAR were positively correlated to increases in time to ¾ re-open. This relationship has been interpreted as higher light availability affording greater herbivore avoidance behaviour (Jensen et al. 2011). The slope of the

relationship between PAR and time to ¾ re-open is similar across all treatments suggesting that the basic behavioural response of plants to various light levels does not change. However, the leaflets on control and Day Touch plants close for ~35% longer than Night Touch plants for a given PAR level (Figure 4.4). Comparing Day Touch and Night Touch plants to account for touch, plants with shorter ¾ re-open times, and therefore lower behaviourally mediated photosynthetic opportunity cost, correlate with plants with greater reproductive biomass production. We do not rule out the alternative explanation that plants with more inflorescences simply re-open their leaflets more rapidly, although intuitively it seems less likely.

These results add to the growing documentation of: (1) the cost component of plant defensive strategies; (2) the potentially important role of touch as a signal for plant growth and development in an ecological context; (3) the value of understanding plant behavioural traits in interpreting plant responses to their environment.

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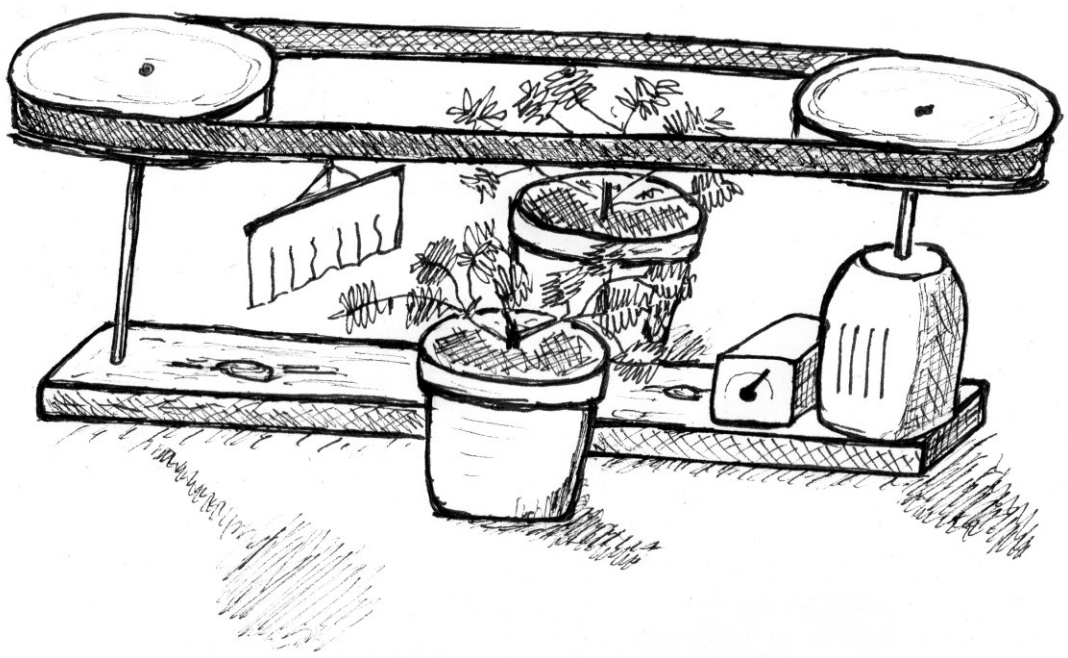
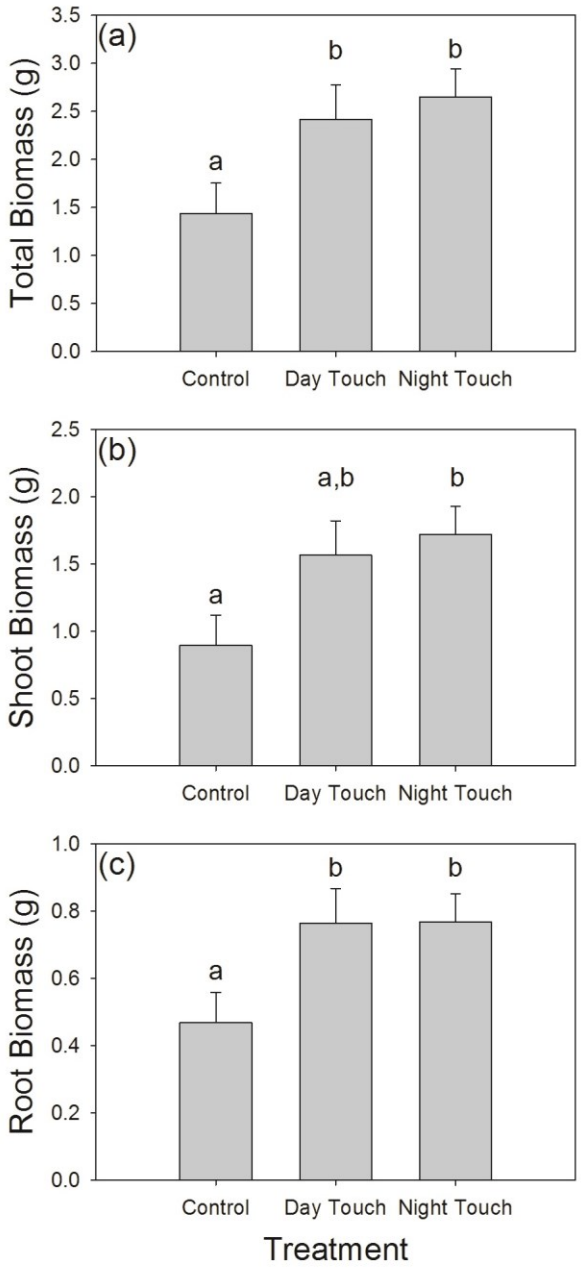
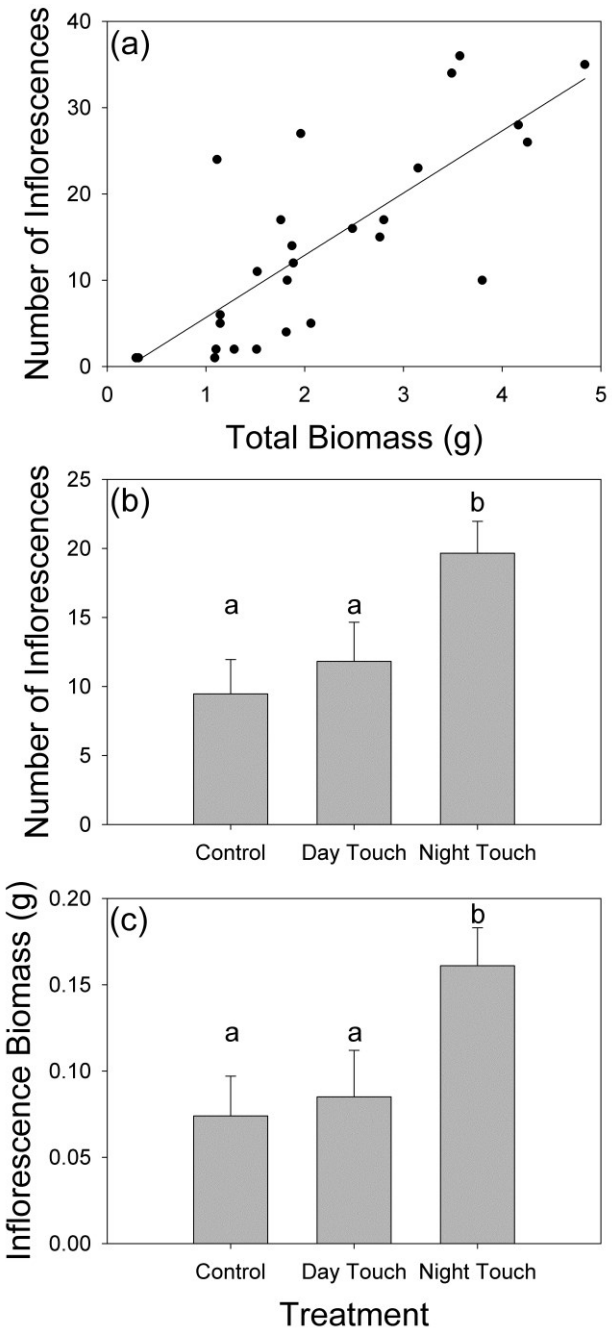


Figure 4.1 Set-up of the automated *M. pudica* touch stimulation device. An industrial motor that is speed controlled by a Variac™ drives a belt running between 2 pulleys. A thin plastic sheet attached to the belt touches the plants. A commercial timer controls the on-off functionality.



Figures 4.2 a, b, c Mean biomass response of *M. pudica* plants exposed to one of three treatments: (a) Control (no touching); (2) Day Touch (15min/hour at 9 touches/min for 8 hours during the day); (3) Night Touch (15min/hour at 9 touches/min for 8 hours during the night). **Figure 4.2a** Total biomass is aboveground biomass + belowground biomass + floral biomass (± 1 SE). **Figure 4.2b** Mean aboveground biomass (± 1 SE). **Figure 4.2c** Mean belowground biomass (± 1 SE). Differences in means ($p<0.05$) are indicated by letters above bars.



Figures 4.3 a, b, c Reproductive allocation of *M. pudica* plants exposed to one of three treatments: (1) Control (no touching); (2) Day Touch (15min/hour at 9 touches/min for 8 hours during the day); (3) Night Touch (15min/hour at 9 touches/min for 8 hours during the night). **Figure 4.3a** Overall relationship (all plants) between total biomass and number of inflorescences ($y = 7.211x - 1.539$, $r^2=0.596$, $p<0.001$). **Figure 4.3b** Mean inflorescence counts (± 1 SE). **Figure 4.3c** Mean floral biomass (± 1 SE). Differences in means ($p<0.05$) are indicated by letters above bars.

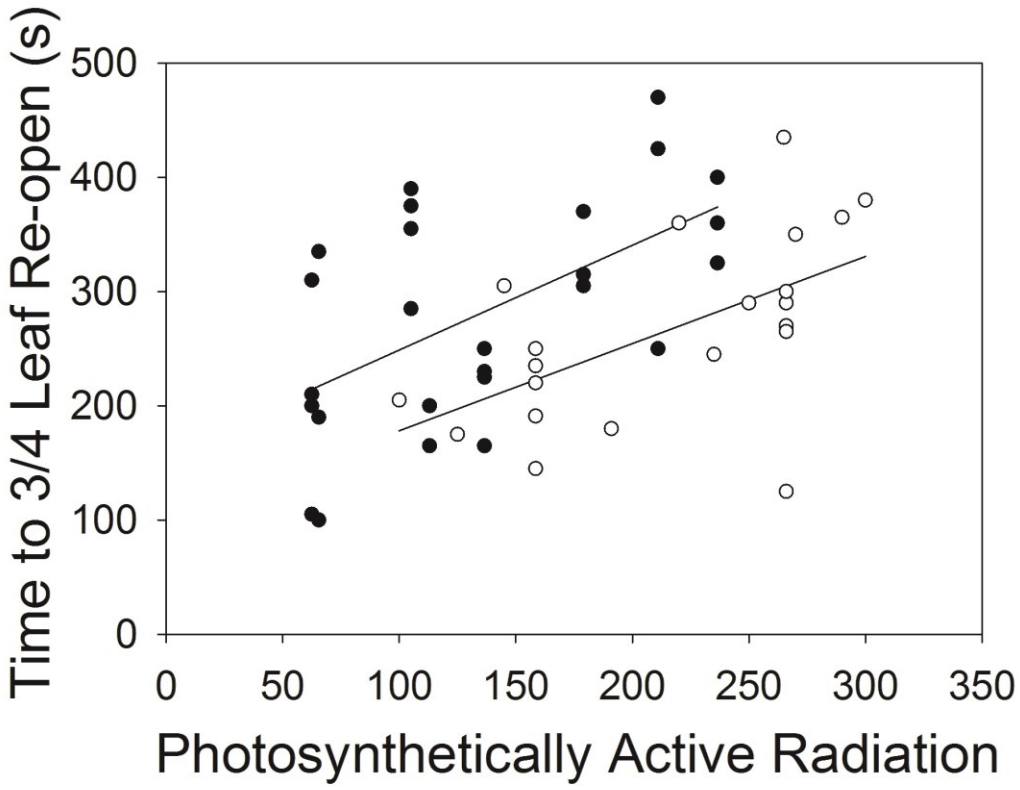


Figure 4.4 Length of time (seconds) required for *M. pudica* leaflets to reopen to 75% of their original breadth after touch stimulation as a function of photosynthetically active radiation (μmol m^{-2} s^{-1}). Closed circles: control and Day Touch ($y = 0.917x + 157.1$, $r^2=0.326$, $p = 0.002$). Open circles: Night Touch ($y = 0.763x + 101.7$, $r^2=0.329$, $p=0.007$).

Chapter 5 – Effect of competition, nutrient, rhizobia and size on leaf opening behaviour of *Mimosa pudica* L.

Introduction

A mainstay in a plant behaviourist's repertoire of seminar humour is to show a photograph of a plant and say: "Here is a video of a plant … in action!" In writing, it has been a different story, as plant behaviourists have traditionally emphasized the parallels between plants and animals (e.g. Darwin and Darwin 1881). There is now a substantial corpus of research that has moved past mere parallels to demonstrating the complexity of plant responses to events or changes in the environment (Silvertown and Gordon 1989, Novoplansky 2002, Karban 2008, Cahill 2015). Particularly well documented are applications of behavioural theory to understanding the machinations of root foraging (Hutchings and de Kroon 1994, de Kroon and Mommer 2006, McNickle and Cahill 2009, Cahill et al. 2010, Cahill and McNickle 2011). But, the downside to doing at least some of this work has been that it is at times genuinely arduous and technically challenging to quantify things such as root traits. A pragmatic plant behaviourist may indeed hope to study a plant that does something interesting and important, but in a way that is convenient to observe.

Mimosa pudica L. (Fabaceae) is becoming one of the model organisms for plant behaviour largely due to its charismatic, but also biologically important, rapid leaf closing behaviour (Abramson and Chicas-Mosier 2016). Leaves close and petioles drop when shocked by mechanical, chemical, thermal or electrical stimuli (Burkholder and Pratt 1936). It is thought that this behaviour is defensive in nature, either to shake off small invertebrates, or to become cryptic and expose sharp spines to mammalian herbivores (Eisner 1981). The trade-off is that photosynthetic rates

decline 40% when leaves are closed due to the reduced surface area for collecting light (Hoddinott 1977). The mechanism of rapid leaf closure in *M. pudica* has been extensively studied at the cellular level. Triggered by stimulus, and mediated by aquaporin transporters and the actin cytoskeleton, turgor loss in specialized extensor cells causes leaf closure and petiole drop (Fleurat-Lessard et al. 1993, 1997, Kameyama et al. 2000, Braam 2005, Kanzawa et al. 2006).

The specific behavioural trait most studied in *M. pudica* has been the length of time required by a plant to re-open its leaves after a stimulus. Leaf closure and opening is important because it is at the intersection of both the plant's defense and light foraging. The trait is easily elicited and observed, and demonstrates a remarkably complex set of consistent responses. For example, light availability (Fondeville et al. 1966, 1967, Applewhite and Gardner 1971, Applewhite 1972, Jensen et al. 2011, Gagliano et al. 2014), previous touching and leaf age (Amador-Vargas et al. 2014), simulated herbivory (Cahill et al. 2013) and past resource availability (Gagliano et al. 2014), all have been shown to both increase and decrease leaf re-opening times. However, how this behaviour changes with some basic ecological factors has not been characterized making the comparison of these results difficult.

The purpose of this experiment was to determine how competition, nutrient levels and rhizobial inoculation affects the leaf re-opening behaviour of *M. pudica*. Each of these three factors are often important in the growth and fitness of plants (Harper 1994), and have the potential to affect leaf re-opening behaviour based on nutrient state dependence (Nonacs 2001). We hypothesize that plants with lower nutrient availability may require less photosynthesis to maximize plant growth and therefore have slower leaf re-open times. We included a rhizobia treatment as we had previously observed nodule formation, and *M. pudica* in wild populations are known to form N_2-fixing rhizobial associations (Klonowska et al. 2012, Bontemps et al.

2015). The social component of competition could also affect re-opening behaviour through by physical contact, shading or chemicals.

Materials and Methods

To test the effect of nutrient level, competition and rhizobia inoculant on *Mimosa pudica* L. (Fabaceae) we conducted 2x2x2 full-factorial design experiment with high and low nutrient levels, with and without competition, and with and without inoculation of soil with rhizobia. There were 21 replicates of each treatment, set up as a randomized block design. The experiment was conducted over a period of 9 months in the greenhouses at the University of Alberta, Department of Biological Sciences (53.5244 ^{o}N, 113.5244 ^{o}W).

Plants were grown in 5 inch Azalea pots that were sterilized with bleach (3%) then rinsed 3 times prior to use. The soil used was approximately 1L of sieved 1:1 sand:top soil mix that was autoclaved 3 times (65 minute sterilization cycle, 1 day between each cycle) prior to use. The seeds (Stokes Seeds, Thorold, ON) were scarified for 15 minutes in 98% sulfuric acid (H_2SO_4), rinsed 7 times in autoclaved MilliQ water (Millipore Corp, Billerica, MA), then sterilized for 15 minutes in 3% Clorox bleach, and rinsed 7 times in autoclaved MilliQ water (Klonowska et al. 2012). Tremendous care was used in the use and disposal of the sulfuric acid including wearing safety attire and using a fume hood, especially in mixing the acid with water (most of the acid was poured off into a disposal container before the seeds were added to the rinsing water). Seeds were then soaked in autoclaved MilliQ water for 2 hours prior to seeding.

Seeds were divided into 2 groups: those to be tossed with an active, non-*Mimosa* specific blend of rhizobial (*Rhizobium leguminosarum* biovar viceae, *R.*

leguminosarum biovar *phaseoli*, *Bradyrhizobium* sp.) inoculant (McKenzie Seeds, Garden Inoculant, A.E. McKenzie Co, Brandon, Manitoba) and those to be tossed with the same inoculant that was autoclaved. The latter was to control for the non-biological components of the inoculant. Equal numbers of pots were seeded with 6 inoculated or non-inoculated seeds establishing the rhizobia treatment prior to the nutrients or competition treatments. Plants were regularly misted and covered with a clear plastic sheet to maintain humid conditions. First germination occurred at 4 days and at 14 days there was sufficient germination (~40% overall) to establish the remaining treatments. Pots were thinned to one plant per pot, except the competition treatment pots that were thinned to two individuals per pot. Three times a week, low nutrient plants were given 3 mL of 10% Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma-Aldrich, St. Louis, MO) and high nutrient plants were given 3 mL of 100% Hoagland's solution. On June 23, 2015, random individuals from each of the 8 treatment combinations were then placed into one of 21 blocks for total of 168 plants. Plants were watered daily as the soil mix drained well. Supplemental greenhouse lighting of 8 hours per day (11:30 am – 7:30 p.m.) started on Nov. 14, 2015 to compensate for shorter winter day lengths.

The effect of the competition, nutrient and rhizobia treatments on plant size was estimated by measuring the height and maximum width of the plants on 9 monthly observation days, immediately after the behavioural assay on each plant. The number of inflorescences produced by each plant was the summation of daily inflorescence production over the entire experimental period. Daily inflorescence production was determined by a daily census. It was necessary to census at this frequency because inflorescences last one day.

The effect of the competition, nutrient and rhizobia treatments on the leaflet re-opening behaviour was determined using monthly behavioural assays that totaled 9 observations days starting on July 20th, 2015. Adapting a previously used protocol

(Jensen et al. 2011, Cahill et al. 2013), leaflets were stimulated by touch to close and the time required for them to re-open to ¾ of maximum breadth (¾ re-open) was measured. The primary change in protocol was to observe one block of 8 plants simultaneously to mitigate the variation in light levels among plants within blocks since the leaf re-opening behaviour changes with light intensity (Jensen et al. 2011). Basically, plants of each treatment combination within a block were raced against each other.

To have relatively consistent levels of soil moisture during testing, plants were watered approximately 3 hours before observations started. The observation period was selected to span across peak daily light intensity (~noon-2 p.m.). The level of photosynthetically active radiation (PAR) (400-700 nm wavelength) was measured using an AccuPAR LP80 ceptometer (Decagon, Pullman, WA) every 2 minutes during a trial and averaged for analysis. To start a trial, a randomly selected block was chosen and light levels were measured just above the plants. Using fresh nitrile laboratory gloves, all the leaflets of the plants in that block were then stimulated to close and the time to ¾ re-open of the focal leaf (see below) of each plant was measured using a stopwatch (Jensen et al. 2011). This method required 2 observers working together to implement. The observers were blind to the specific treatment combination, but it was not possible to blind the competition treatments.

After the initial observation period in July, plants had multiple potential focal leaves. For this reason, in the August trial, we compared the time to ¾ re-open of the two newest fully developed leaves on about 1/3 of the plants. Based on the consistency and qualitative similarity of ¾ re-open times of the second newest leaf to other leaves, we chose this leaf to be the focal leaf for all subsequent trials. The coloration of the newest leaf was often a lighter green than the other leaves, even though leaflets were able to open and close.

We used a mixed model to analyze the effect of the treatments on plant size with final plant size (March max width x height) as the response variable, competition, nutrient and rhizobia treatments as fixed effects and block as a random effect. Similarly, the effect of the treatments on inflorescence production was analyzed with total number of inflorescence produced per plant as the response variable.

To examine the effect of the treatments on leaf re-opening behaviour, we modelled the response variable of time to ¾ re-open as a function of: the fixed effects of competition, nutrient and rhizobia; the random effects of block and observation date; and the covariates of light and size. Marginal means were estimated with size (max width x height) = 79.7 cm^2 and PAR (light) = 198.4 $\mu mol\ m^{-2}\ s^{-1}$. To help interpret the effect of plant size, PAR, and observation date on time to ¾ re-open, we looked at the individual effect of each of these variables on time to ¾ re-open with linear regression and ANOVA. All analyses were conducted using SPSS 20 (IBM Corp., Armonk, NY).

Results

The absence of competition, higher nutrients and inoculation with rhizobia resulted in larger *M. pudica* plants after 9 months (Table 5.1). Without competition, mean size was 43% larger; with higher nutrient levels, mean size was 20% larger; and with rhizobia inoculation, mean size was 24% larger (Figure 5.1). Block was also a significant factor with plants on one side of the greenhouse being larger than at the other end of the greenhouse. This was likely due to air circulation patterns that dried the soil in pots faster in some areas.

Competition reduced the number of inflorescences produced by plants over 9 months, while nutrient level and inoculation with rhizobia did not change inflorescence production (Table 5.2). Competition reduced the mean number of inflorescences produced by 46% (Figure 5.2). Higher nutrients increased mean inflorescence production by 17% but this percentage was not significant.

Leaves closest to the shoot apex opened slower than leaves second closest to the shoot apex (Wilcoxon Signed Ranks, $n=65$, $p<0.001$). Although physically fully formed, some of the newer leaves had a lighter green colour relative to the other leaves on a plant.

Competition, size, observation date and block affected the leaf re-opening behaviour of the *M. pudica* plants (Table 5.3). Competition increased the mean time to ¾ re-open of leaflets by 8%, estimated as a marginal mean (size = 79.7 cm^2 and PAR (light) = 198.4 $\mu mol\ m^{-2}\ s^{-1}$) (Figure 5.4). Larger plants tended to open leaves more slowly ($y=.769x+230$, $r^2=.206$, $p<0.001$) (Figure 5.5). There was a trend for slower leaf ¾ re-opening with increased light intensity ($y=.242x+252.1$, $r^2=.015$, $p<0.001$) (Figure 5.6), although not significant in the overall model. There was appreciable variation in average time to ¾ re-open depending on the observation date (Group 1: Sept., Oct., Nov., Dec.; Group 2: July, Jan., Mar.; Group 3: Aug; Group 4: Feb) (Figure 5.7).

Discussion

Effects on traits

As expected, competition decreased plant size and higher nutrient levels and rhizobia inoculation increased plants size (Table 5.1, Figure 5.1) (Harper 1994). The effect of competition was on average the greatest as plants grown with competition were the smallest group, and plants grown without competition were the largest group. The effect of these three treatments on the average production of inflorescences followed the same pattern but only the effect of competition was significant (Table 5.2, Figure 5.2). The soil was a nutrient poor mix of sand and top soil so it is somewhat surprising that the nutrient effect on inflorescence production was not more pronounced after 9 months.

Effects on behaviour

Competition resulted in *M. pudica* plants taking longer to re-open their leaves after stimulated closure (Table 5.3, Figure 5.4). Three possible mechanisms that could explain this observation include: First, if the effect of competition on re-opening behaviour was strictly mediated by nutrient levels, it would be expected that the relative behaviour of no-competition plants:competition plants would be similar to the relative behaviour of high nutrient plants:low nutrient plants, but this was not the case (Figure 5.4). Second, previous work has shown that *M. pudica* plants can be habituated to touch (Gagliano et al. 2014). It is likely that competition plants had more physical contact with neighbouring plants than plants grown alone even in the relatively calm conditions of a greenhouse. Thus, it is a reasonable possibility that the longer re-open times with competition were a product of habituation. Third, the change in re-opening behaviour could have been triggered by changes in the Red:Far Red ratio. Reflection of light by plants increases the far red wavelengths of light, and the ratio of these wavelengths to red wavelengths is known to be an important signal of the presence of plant competitors (Ballaré et al. 1990, Ballaré 1999). Work by Fondeville (Fondeville et al. 1966, 1967) and Applewhite (Applewhite and Gardner 1971, Applewhite 1972), using $CuSO_4$ light filtering methods similar to Ballaré

(Ballaré et al. 1990) demonstrated a strong sensitivity of *M. pudica* to light in the far red (720 nm) range. More importantly, it was shown that this sensitivity to 720 nm wavelengths had a direct effect on leaf re-opening behaviour (Fondeville et al. 1966, 1967). Although the critical ratio of R:FR was never tested, this certainly suggests that changes in re-opening behaviour could possibly be a response to a modification of the R:FR ratio due to competing neighbours. Longer re-open times in response to competition has the potential adaptive value of increasing photosynthesis and therefore

Neither nutrient levels nor rhizobia directly affected time to ¾ re-open (Table 5.3, Figure 5.4), even though they had an effect on size (Table 5.1, Figures 5.1). Size had an effect on re-open times (Table 5.3, Figure 5.5), so it is possible that nutrients and rhizobia (and competition, for that matter) had an indirect effect on behaviour mediated by size. Nevertheless, the lack of direct effect suggests that the nutrient state, assuming that the primary effect of rhizobia is on nutrient state (Stewart 1967, van Rhijn and Vanderleyden 1995), is decoupled from the energetic state which has been shown to affect behaviour (Applewhite and Gardner 1971, Jensen et al. 2011).

Larger plants took longer to re-open their leaves (Table 5.3, Figure 5.5) implying that smaller individuals exposed themselves to greater potential risk quicker after a leaf closing stimulus. Over the size range observed, an average doubling in size increased the time to re-open by ~30%. Increased risk taking behaviour is often observed in animals trying to minimize the time spent at vulnerable size classes (e.g. Biro et al. 2005). Similarly, plants often face tremendous pressure to grow because the competition for light is asymmetrical and taller individuals are able to capture a disproportionate amount of light (Weiner 1990). *Mimosa pudica* plants are able to exhibit rapid leaf closing behaviour from their first pair of leaves, when plants are 1-2 cm tall. Plants at this early stage may take greater risks in light foraging to mitigate the potential for being outcompeted for light. Smaller plants are also naturally more

cryptic, especially to mammalian herbivores. So, it is also possible that smaller *M. pudica* individuals are able to quickly re-open their leaves without significant functional increases in risk.

The effect of light was confounded with the observation date. This is the likely reason we did not observe an overall relationship between light intensity and time to ¾ re-open reported in previous studies (Jensen et al. 2011, chapter 4). Ideally, observation dates would have had the same mean and variance of light values. Over 9 months, seasonal variation in light availability resulted in 3-fold differences in average light levels over observation days. Looking only at the relationship of light intensity to re-open times (Figure 5.6), there is a marginal trend with a positive slope that is in agreement with past observations. In hindsight, we appreciate the obvious seasonality in greenhouses, but it should be noted that most controlled growth chamber facilities (including ours) are unable to replicate the light intensity of the sun, and this was the primary reason we chose to conduct our experiment in the greenhouse.

Competition and size can be added to a growing list of factors that contribute to determining the leaf re-opening behaviour in *M. pudica*. While this strengthens an appreciation for the complexity of plant behaviour, it also suggests the need to formalize and standardize protocols for plant behavioural observations to facilitate the direct comparison of empirical studies. For example, based on what is known, standardizing plant size, choice of focal leaf (Amador-Vargas et al. 2014), pre-observation conditions (Gagliano et al. 2014) and accounting for light intensity (Jensen et al. 2011) and light quality (Applewhite and Gardner 1971) would probably be a reasonable first step for behavioural studies with *M. pudica*.

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Table 5.1 The effect of competition, nutrients, rhizobia and block on *M. pudica* size (height x max width) after 9 months.

Factor	df	F	p
Competition	1	18.991	0.000
Nutrients	1	4.435	0.048
Rhizobia	1	5.771	0.026
Block	20	6.592	0.017
Competition*Nutrients	1	0.002	0.965
Competition*Rhizobia	1	0.008	0.930
Competition*Block	20	1.297	0.444
Nutrients*Rhizobia	1	0.292	0.595
Nutrients*Block	20	1.826	0.320
Rhizobia*Block	20	0.868	0.625

Table 5.2 The effect of competition, nutrients, rhizobia and block on number on inflorescences produced by *M. pudica* over the 9 months of the experiment.

Factor	df	F	p
Competition	1	32.450	0.000
Nutrients	1	3.589	0.080
Rhizobia	1	0.259	0.619
Block	19	2.042	0.287
Competition*Nutrients	1	0.666	0.435
Competition*Rhizobia	1	0.194	0.669
Competition*Block	12	0.415	0.935
Nutrients*Rhizobia	1	0.190	0.671
Nutrients*Block	13	1.104	0.431
Rhizobia*Block	13	0.959	0.522

Table 5.3 The effect of competition, nutrients, rhizobia and block the time to ¾ re-open of leaflets of *M. pudica* after stimulated closure.

Factor	df	F	p
Competition	1	11.169	0.001
Nutrients	1	2.078	0.150
Rhizobia	1	0.323	0.570
Block	20	2.616	0.000
PAR (light)	1	1.635	0.201
Size	1	125.210	0.000
Date	7	90.015	0.000
Competition*Nutrients	1	0.959	0.328
Competition*Rhizobia	1	2.946	0.086
Nutrients*Rhizobia	1	0.117	0.732

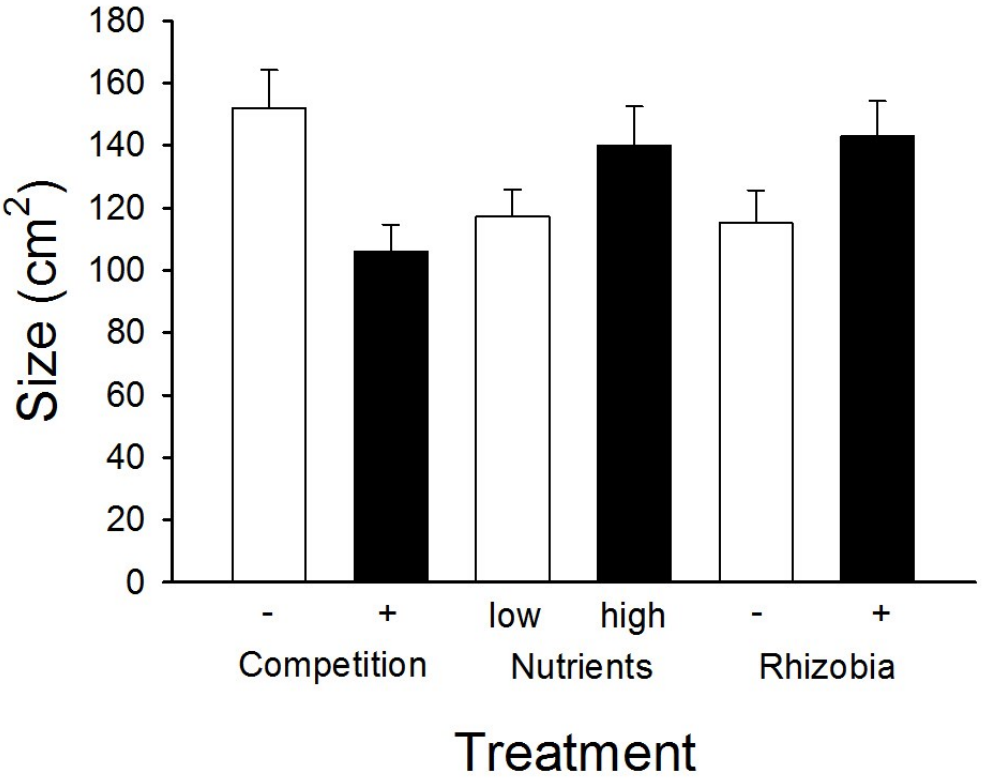

Figure 5.1 The effect of competition, nutrients and rhizobia on size (height x max width) of *M. pudica* plants after 9 months. Error bars give ±1 SE from the means. Each pair of bars is significant different. Results of analyses are found in Table 5.1.

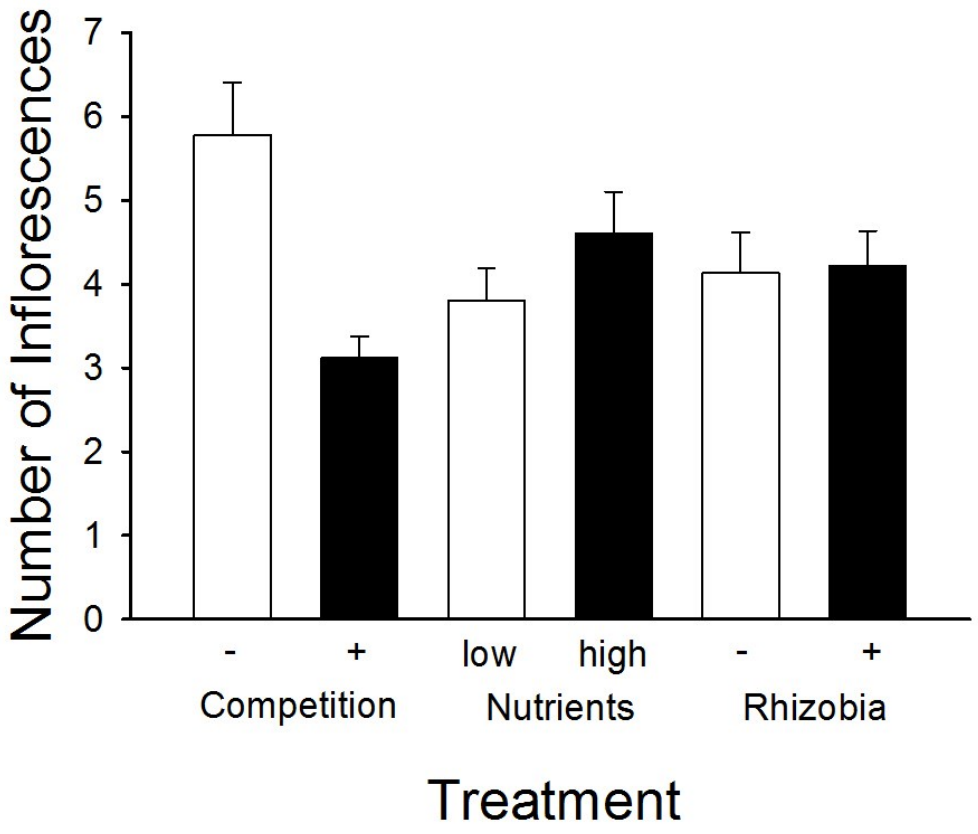

Figure 5.2 The effect of competition, nutrients and rhizobia on number on inflorescences produced by *M. pudica* over the 9 months of the experiment. Error bars give ±1 SE from the means. Only the competition treatment bars are significantly different. Results of analyses are found in Table 5.2.

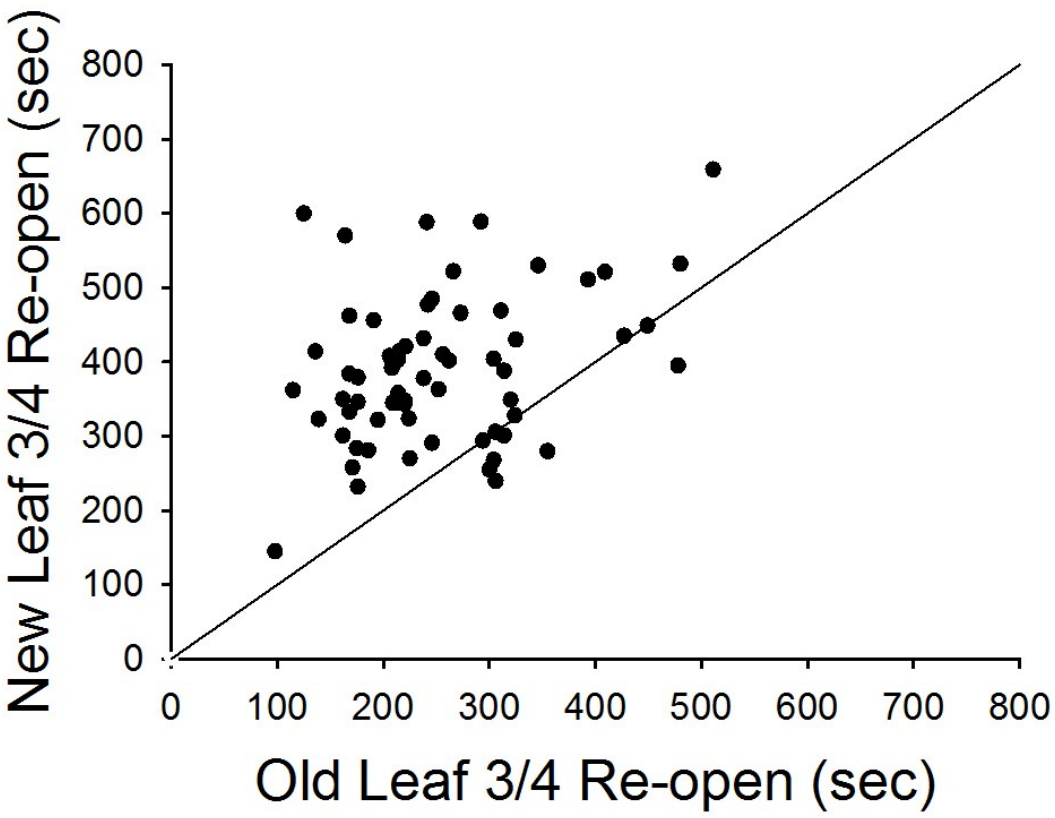

Figure 5.3 Paired comparison of time to ¾ re-open of leaflets of *M. pudica* after stimulated closure. New leaf = fully formed leaf nearest the shoot apex. Old leaf = fully formed leaf second closest to the shoot apex. Line is a 1:1 reference line.

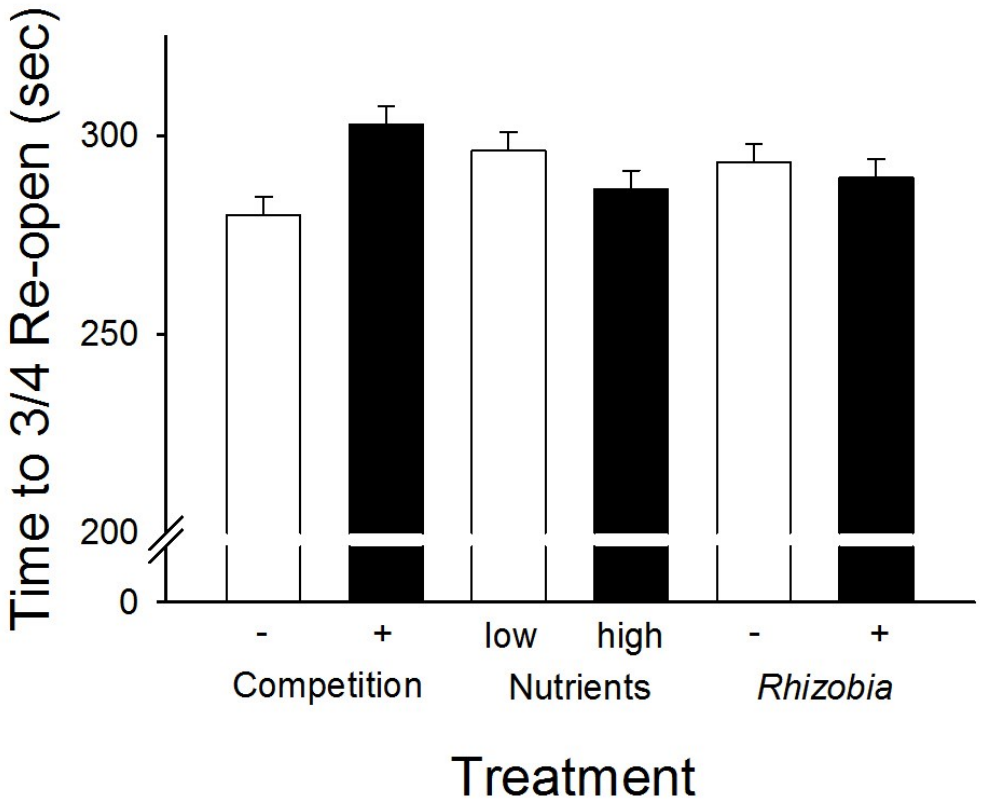

Figure 5.4 The effect of competition, nutrients and rhizobia time to ¾ re-open of leaflets of *M. pudica* after stimulated closure. Error bars give ±1 SE from the means. Only the competition treatment bars are significantly different. Results of analyses are found in Table 5.3. Marginal means were estimated with size (max width x height) = 79.7 cm^2 and PAR (light) = 198.4 μmol m^{-2} s^{-1}.

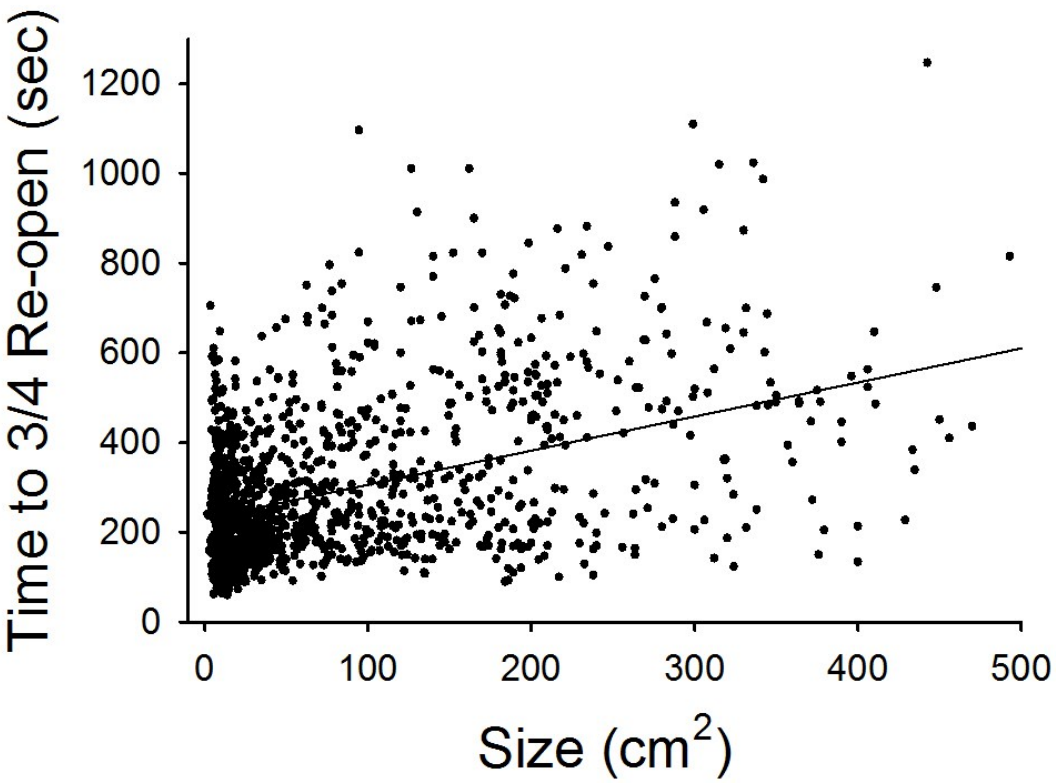

Figure 5.5 Average length of time to ¾ re-open of leaflets of *M. pudica* after stimulated closure as a function of plant size (height x max width). Regression line: $y=.769x+230$, $r^2=.206$, $p<0.001$.

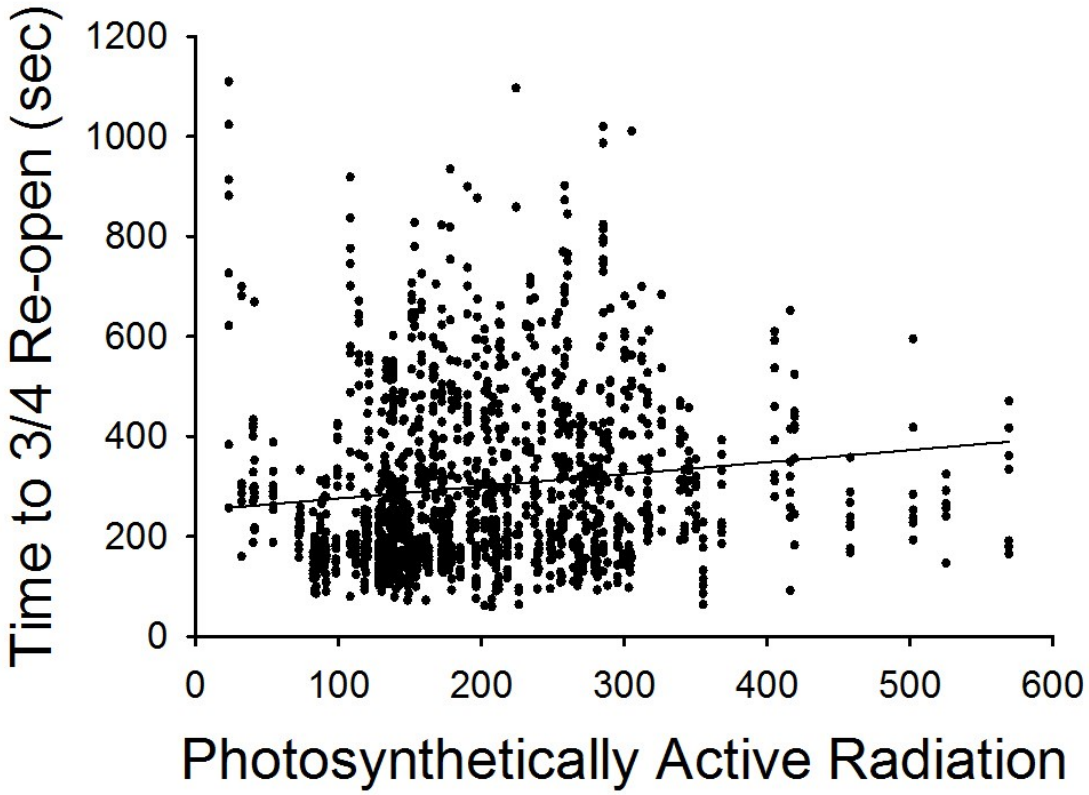

Figure 5.6 Average length of time to ¾ re-open of leaflets of *M. pudica* after stimulated closure as a function of photosynthetically active radiation (μmol m^{-2} s^{-1}). Regression line: y=.242x+252.1, r^2=.015, p<0.001.

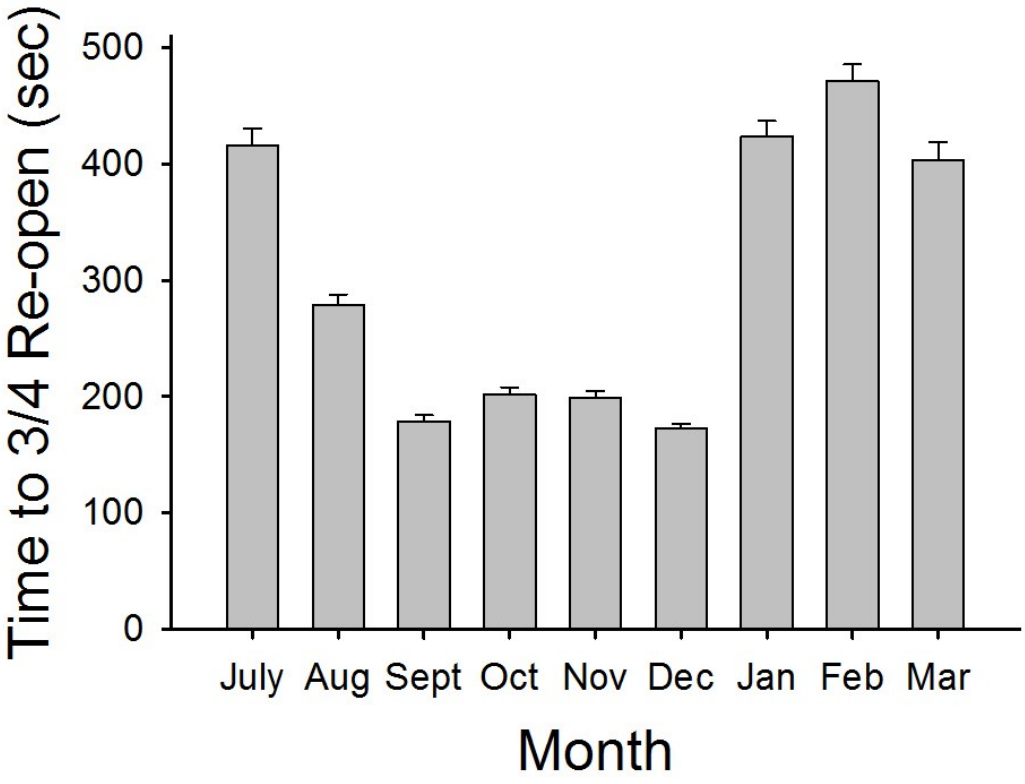

Figure 5.7 Average length of time to ¾ re-open of leaflets of *M. pudica* after stimulated closure over the 9 months of observations. Error bars give ±1 SD from the means. Group 1: Sept., Oct., Nov., Dec.; Group 2: July, Jan., Mar.; Group 3: Aug; Group 4: Feb.

Chapter 6 - A novel method for the characterization of static and dynamic root traits using digital image correlation

Introduction

The characterization of static and dynamic belowground plant traits is challenging because direct observation is obstructed by the very soil required for natural plant growth (Harper et al. 1991). Here, we present a method to study these traits by using the measurement and analysis of the displacement of the soil itself to infer the movement and position of roots. We believe that this method is both additive and complimentary to current techniques and has the potential of being scaleable to high-throughput applications.

There has been a tremendous effort to study the belowground parts of a plant despite the obvious obstacles (Downie et al. 2015). This is because essential plant functions are associated with these parts, such as the acquisition of water and nutrients, as well as functions such as structural stability and energy storage (Kroon and Visser 2003). Further, the physical contact points for a broad spectrum of ecological interactions involving plants are situated belowground. For example, mycorrhizal interaction with fungi (Malloch et al. 1980), rhizobial interactions with bacteria (Hirsch 1992) and allelopathic interactions with other plants (Bais et al. 2006). Understanding how plants respond belowground is therefore vital for understanding the basic and applied biology of plants.

There have been 5 general approaches employed to studying plant roots (Atkinson 1991, Mancuso 2012, Downie et al. 2015, Kuijken et al. 2015): (1) Destructive methods involving the separation of entire root systems from soil, either by in-situ excavation (e.g. Mackie-Dawson and Atkinson 1991) or by ex-situ root washing (e.g. Bucksch et al. 2014) (2) Destructive methods involving the sampling of

a small fraction of roots, such as by coring (e.g. Wasson et al. 2016) (3) Use of a soil substitute for growth, typically transparent, that allows for in-situ root observation. Examples include transparent soil (Downie et al. 2012) and agar or agar-like systems (Clark et al. 2011). (4) Non-invasive imaging of root systems visible from a transparent pot surface. The imaging can be done by: a camera (e.g. Belter and Cahill 2015); a minirhizotron (e.g. McNickle and Cahill 2009, Karst et al. 2012); or a scanner system (e.g. Adu et al. 2014). (5) Non-invasive imaging that is capable of penetrating the soil surface such as MRI (e.g. Metzner et al. 2014) and CT scanning (e.g. Lontoc-Roy et al. 2005, Flavel et al. 2012). It should be noted that a rapid development in computational and analytical techniques aimed at maximizing the extraction of root information has paralleled the development of these experimental methods (e.g. Cai et al. 2015, Hatzig et al. 2015, Kalogiros et al. 2016).

The method we introduce here is an approach that is fundamentally different because the target of the imaging is the soil medium itself, rather than the roots. Soil can be imaged precisely with relatively low cost and effort, and as a non-destructive process it is possible to take images of soil repeatedly over the course of an experiment. We extract information about root position and movement by analyzing the small movements of the soil in these images (e.g. Figure 6.4, top right) using digital image correlation (DIC). In the relatively closed system of a plant pot, the only thing moving the soil are the roots.

Digital image correlation is an image analysis technique that has been used in a wide range of applications such as in remote sensing (Anuta 1970), electronics (e.g. optical laser mouse (Gordon et al. 2002)), the study of mechanics (Chu et al. 1985) and orthodontics (Melenka et al. 2013). For a DIC analysis, it is necessary to generate a sequence of at least 2 images to analyze. The images are of some region of interest, usually with one image taken before a deformation event and the other after. In the example on Figure 6.1, the two photos were taken before and after a pencil was run through the middle of a Petri dish of soil. A critical characteristic of this soil is the

contrast of the white stone particles to the black top soil. Each of the white stone particles is a distinct feature in the soil because of this contrast. The two images, before and after, are then compared to see how features changed or moved between the images. The algorithm that implements this comparison maximizes the correlation of feature position between images (Pan et al. 2009). With the maximum correlation, the most parsimonious set of movements that would transform the first image to the second image can be determined. This set of movements is a vector field, the displacement vector field. The displacement vector field itself can be visualized on top of an image of the soil to show how features moved from one image to the next (Figure 6.1, right). The direction and length of vectors indicate the direction and magnitude of the displacement (L). From the vector field, the longitudinal (ε_x), transverse (ε_y) and shear (ε_{xy}) components of strain, $\varepsilon = \Delta L$ /L, can be determined by dividing the distance features moved (ΔL) by a chosen reference scale or displacement (L). Strain itself is a dimensionless measure of deformation or relative displacement. The advantage of using strain is that the resolution of the analysis can be scaled by L, whereas displacement is an absolute measure. The spatial distribution of strain over the region of interest (i.e. the original photographs) can be plotted, making a strain map. We are looking at these strain maps to infer static and dynamic root traits.

Our objective was to develop an experimental and analytical protocol for root characterization using DIC analysis. Specifically, we wanted to: 1) determine the experimental conditions under which images necessary for DIC analysis could be produced; 2) apply DIC analysis to soil images to generate strain maps; 3) validate the characterization of roots determined by the new method

Methods

There are 3 stages in our method: 1) setting up the plant experiment, 2) image capture, and 3) image analysis.

Setting up the plant experiment

The key design constraint in implementation was the combination of shoots needing light, roots needing dark, and the need to maintain visual access to the soil for imaging. Our basic approach was to grow plants in a transparent pot inside a blackout chamber such that the shoots emerged from the top of the chamber, while the roots and soil were inside the chamber and accessible for photography (Figure 6.2). All our work took place in a climate controlled growth chamber (Department of Biological Sciences, University of Alberta) set at 24ºC and a 16h:8h day:night cycle.

The blackout chamber was constructed out of an extruded aluminum frame for general stability, but also for the strength necessary to support the weight of 10 watered pots. Blacking out was achieved using multiple layers of cardboard, aluminum foil, plywood and light blocking tape, all covered with a laser blocking fabric (Figure 6.2). The front face of the chamber was only covered with the fabric for ease of access. The length of the chamber was chosen to maximize the use of the growth chamber space. The width and height, however, had to accommodate the distance and angle required between the pots and camera.

We made 8.5 inch x 11 inch x ¼ inch pots, or "window boxes", out of pieces of ¼ inch thick clear acrylic sheets held together with stainless steel nuts and bolts (Figure 6.2, top left, Figure 6.5, top left). This thickness of acrylic was required so that the pot walls would not deform due to the pressure from root growth. Seam surfaces were lined with Teflon tape to seal the pot. Sets of 5 pots were bolted to an acrylic sheet which was then mounted into the blackout chamber at a ~35-40^{o} angle

(Figure 6.2, top left). 2 sets of 5 pots could be mounted in the blackout chamber at one time. This angle was previously used to facilitate root visualization as gravitropism enhances root visibility on the imaging surface (Belter and Cahill 2015). We did not test whether this angle was necessary for the DIC method as we wanted to be able to compare our method with previous methods.

We tested our method using sunflower (*Helianthus annuus* L.), tomato (*Solanum lycopersicum* L.) and velvet leaf (*Abutilon theophrasti* Medik), but we present the results with sunflower here. We used a series of pilot studies to determine the required pot size for plants to grow to the desired developmental stage while maintaining plant health and normalcy of growth form. In developing this method, we also tested different soil blends to determine the optimal black top soil:crushed white stone ratio for the purposes DIC image analysis. In the end, we chose a 70:30 black top soil:crushed white stone mix (Figure 6.4, left). This mix is very similar to the standard soil mix we regularly use for plant experiments, other than with the substitution of the white crushed stone for sand. We chose to use the white crushed stone because it offered superior contrast. Prior to seeding, we placed 3 balls on slow release fertilizer (Nutricote Type 100, NPK 14:14:14, Chisso-Asahi Fertilizer Co., Toyko) on one side of each pot (6 cm deep, 6 cm from the edge of the pot) to see determine if asymmetry of root proliferation could be detected by this method. Each day, plants were carefully given 15 mL of water with plastic pipettes to minimize the potential of soil turnover with watering (Figure 6.2, bottom left). To determine the effect of watering alone on soil movement, we also imaged control pots that were watered but did not have plants. We found that watering had no direct effect on soil movement other than in the upper 1 cm of the soil.

Image capture

The purpose of the image capture stage is to take repeated images of a region of interest over the experimental time period, with the least possible photography

related movement, distortion or lighting variation, between images. Ideally the lens surface is parallel to and centered on the region of interest so that there is minimal edge-to-edge distortion. Regardless, spherical aberration requires correction at the image processing stage using a calibration target of dots 4 mm in diameter and 10 mm apart (Figure 6.3, bottom left). The calibration target is affixed to the region of interest (i.e. taped to the pot) and a photograph is taken of the target before each image capture of the soil and roots. Each pot had a horizontal and vertical strip of 4 dots (4 mm in diameter, 10 mm apart) placed on the edge (Figure 6.3, top right) to landmark the pot position in image processing.

A camera mount was built out of MDF board, empty pipette tip boxes and retort stand clamps to guide the positioning of the camera (Figure 6.3, top row). Pipette tip boxes were screwed into the frame of the blackout chamber and used as a physical template to position the camera mount (Figure 6.3, bottom right). Positional accuracy was ±2 mm, which was adequate, but not ideal because of the increased image processing effort downstream. The mount and positioning system placed the camera at an angle of ~35-40^{o} (to match the angle of the pots) and laterally centered on each pot, while the focal point of the lens was around 45 cm away from the soil surface.

Daily photographs were taken for 21 days per pot with an off-the-shelf DSLR (24 MP Nikon D5200, 50mm f/1.8F AF-S NIKKOR FX, Nikon Canada, Mississauga, ON) illuminated with a Polaroid macro LED ring flash for Nikon (5500K, Polaroid Corporation, Minnetonka, MN). The shutter was triggered with a remote control (Nikon ML-L3) and uncompressed NEF format images were transferred to a Windows laptop using digiCamControl (www.digicamcontrol.com).

DIC analysis

The series of images captured in the previous stage are first processed in preparation for analysis then analyzed using DIC software. The protocols here are an adaptation of the methods described previously (Melenka and Carey 2015).

As required by the DIC software, NEF format images were batch converted into greyscale TIFF images using Adobe Photoshop CC (Adobe, San Jose, CA). Using a commercial DIC software package (DaVis 8.2.0, LaVision GmBh, Gottingen, Germany), distortions and displacements in the image series were corrected using the calibration targets associated with each image, as well as the reference markers on each pot. The objective was to make the series of soil images from one plant into a temporal stack of the exact same region of interest. A filter was then used to enhance the contrast between the black soil and the particles of white crushed stone. Image correlation analysis was performed using a multi-pass approach, first at a 128x128 pixel correlation, then a 64x64 pixel correlation with 25% overlap pixel correlation. By this process, discrimination of movement of about 0.001 mm is possible. Displacement vector fields generated in DaVis 8.2.0 (e.g. Figure 6.4 right) were then analyzed using a MATLAB based DIC post-processing toolbox (MATLAB 2015b, MathWorks, Natick, MA) to compute strain.

Longitudinal strain was computed as:

$$\varepsilon_x = \frac{\Delta L_x}{L}$$

And, maximum normal strain was calculated as:

$$\varepsilon_1 = \frac{\varepsilon_x + \varepsilon_y}{2} + \sqrt{\left(\frac{\varepsilon_x - \varepsilon_y}{2}\right)^2 + \left(\varepsilon_{xy}\right)^2}$$

where, L is displacement, and strain was computed in the longitudinal (ε_x), transverse (ε_y) and shear (ε_{xy}) directions.

Strain values were spatially positioned to generate strain maps (Figures 6.5-6.9). Strain was computed either referenced to the first image in a series (Figures 6.6 and 6.7) or referenced to the start of a period of interest. In Figure 6.9, analysis was referenced to the first day of 3 successive weeks to produce cumulative strain maps corresponding to those weeks. From the strain maps, we can directly determine root system architecture metrics such as maximum root depth, maximum root width, or depth of maximum root width (see Belter and Cahill 2015 for more metrics). Alternatively, a localized region of interest can be compared. In Figure 6.8, we analyzed two 20 mm x 60 mm regions corresponding to the area in each pot that did or did not have nutrients. In those regions, average maximum strain values were computed over the course of 21 days to determine differences in localized root activity. DIC analysis has outstanding analytical flexibility because of the ability to reference analysis to any temporal start point or to localize analysis to any spatial region.

Results

The roots of each plant were processed by full excavation and scanning for comparison with the DIC analysis. The figures presented here are the analysis of the images for the plant in Figure 6.4. Figure 6.4 shows a comparison of the complete sunflower plant and pot, a photograph of the soil, the scan of the excavated root and a

strain map generated by DIC analysis using maximum normal strain. The detection of the visible roots by DIC is evident from the comparison. Additionally, DIC analysis was able to detect roots under the soil surface, as highlighted by the comparison of the regions outlined by the red boxes in Figure 6.4. A length of root is clearly visualized on the strain map that is not visible in the soil photo, but observed in the excavation.

From a DIC analysis of strain in the horizontal or longitudinal (ε_x) direction on we are able to detect root growth over the observation period (Figure 6.5). Direct measurement of root growth in the vertical or horizontal direction can be made from this series of strain maps. Unique to using DIC analysis, however, we are able to observe the movement and strain of the soil itself as highlighted by the red and blue regions in Figure 6.5. The red regions represent tensile strain (pulling out), while the blue regions represent regions of compressive strain (pushing in). There is a correlation between regions of dense tensile strain (red) with compressive strain (blue) indicating a tendency for roots to push towards the central vertical axis of the plant (i.e. a line drawn straight down from the plant shoot) as they grow.

The analysis of the same images using maximum normal strain reveals greater detail, but more noise due to the increased sensitivity of this measure (Figure 6.6). We are not able to easily interpret the movement of the soil because we are analyzing the 3 dimensions of strain, but using maximum normal strain permits a greater sensitivity to movement overall. From both Figures 6.5 and 6.6 we can easily resolve one day's growth and therefore root growth can be directly measured from the series of strain maps. We did not take photographs at a time interval shorter than one day so we could not determine the minimum time interval over which root growth can be resolved.

From the DIC analysis using maximum normal strain, we subsequently analyzed the activity in localized region of the pots (Figure 6.7), one containing

nutrients (red box) and the other without (blue box). A lower resolution was used to filter out some noise. A time series of the average maximum normal strain in each region over 21 days illustrates the difference in root activity in each region. The region with nutrients had average strain levels around 25% higher than the no nutrient region after one week due to increased root proliferation in that region, as confirmed by excavation.

The analysis of strain can be referenced to different time intervals. For example, to determine differences in root growth in weeks 1, 2 and 3, we analyzed the corresponding one week blocks of images. In Figure 6.8, cumulative strain in those one week blocks of images were visualized to highlight the region of growth each week. The region of active growth moves deeper and wider each week.

Discussion

We have shown here that the characterization of both the static and dynamic traits of roots is possible with the application of DIC analysis to a plant experimental system. We have demonstrated the ability to measure basic root system architecture traits important for plant fitness (Lynch 1995, Orman-Ligeza et al. 2014) by directly examining or analyzing strain maps (Figures 6.6-6.9)

Pros and cons

The challenge in developing methods to study roots has long been appreciated (Harper et al. 1991). The importance of doing so has spurred a plethora of new methods (Mancuso 2012, Downie et al. 2015) but each method, including the one presented here, is not completely ideal. What is ideal? A realistic way to answer this question is to compare root methods to what is possible with shoot methods; both to give inspiration for what might be possible, but also to recognize what is not. With

shoots, access to the shoot is generally not limited, therefore we are able to apply methods ranging from counting leaves to fluorescent microscopy. However, the choice of method is strictly based on purpose, and so it is not reasonable to compare the low cost of leaf counting to the cost of a microscope if one needs to look at cells. Similarly, the range of root methods from root washing to CT scans are not meant to be compared because the intent is different. We contend that the largest gap in root methods is on the end of the spectrum most similar to counting leaves or measuring stem height - the low cost, high volume work necessary for collecting ecological, agricultural and general phenotype information. The method presented here may help address this gap.

The main drawback of the DIC method as developed here is the current requirement to have a pot that is relatively 2-dimensional to concentrate root activity near a surface to be imaged. Nevertheless, for many applications such as phenotyping, this is probably not a severe constraint especially if the experimental conditions are optimized for the plant of interest. If it is essential to use wider pots, one possible solution is to combine images from a mini-rhizotron with the DIC analysis of those images (Mackie-Dawson and Atkinson 1991).

There are 7 advantages of the DIC method:

1) Consistent, good quality results. The fundamental difference distinguishing the DIC method from other methods is that all analyses are based on images of the soil, specifically images of particles in the soil. Set up correctly, the soil in a pot will always have to move when there is a root. This alleviates the need to contend with interpreting roots that appear and disappear across an image surface in the analysis phase allowing for greater consistency and quality of results.

2) It is time efficient once an implementation is optimized. The photography, even done manually, is not time consuming. However, the major efficiency is in the analysis phase. As long as a given region of interest has been

consistently imaged, a DIC analysis can be reliably batch processed. This avoids the need to do manual root tracing, or run root finding algorithms. Once again, choosing the soil to be the target of analysis allows for this automation.

3) It is fully complimentary to other imaging methods and excavation. The same images used for DIC can be root traced or otherwise analyzed (e.g. Cai et al. 2015, Hatzig et al. 2015) in necessary, and the pots can be excavated at an end point for biomass information.

4) It is additive to other imaging methods. New data about the movement and response of the soil itself is generated by the DIC analysis (Figure 6.6). This is potentially important for understanding the mechanics of soil, as well as the biomechanics of roots.

5) It is relatively low cost. Currently, the most expensive component is the proprietary DIC software (DaVis 8.2.0), but there are low cost open source and MATLAB options (e.g. Ncorr, www.ncorr.com). The DSLR and lens are the next most expensive items but a lower resolution camera can be used with no problems, as long as a lower resolution analysis is acceptable.

6) The analysis is adaptable. DIC analysis is flexible to many different kinds of questions because the analysis can be referenced to any temporal start point, or localized to any spatial region, within the constraints of the images available.

7) Promises to be scaleable. There are several reasons why this application of DIC is scaleable for root phenomics: a) it is relatively easy to design a system to consistently image soil; b) the technology already exists to accurately and efficiently capture large numbers of images for plant phenomics (Furbank and Tester 2011, Brown et al. 2014); c) images of soil can be automatically analyzed using well-

established DIC algorithms and software packages (Melenka et al. 2013, Melenka and Carey 2015).

Future directions

By simultaneously tracking the image by image movement of thousands of little stone particles, we are able to infer the movement of plant roots within the closed system of a pot using DIC analysis. The implementation and extension of this basic concept can be imagined in many ways. Certainly, high throughput phenomic applications are logical and important extensions of this novel method, albeit potentially expensive.

However, the most interesting applications may be relatively rudimentary low cost implementations targeted at answering unique questions about roots that were not easy to answer before. For example, many questions about the dynamic response of roots to experimental treatments have been time consuming and often frustrating to answer in the past. The effectiveness, flexibility, low cost and time efficiency of the DIC root analysis method will hopefully facilitate creativity in the study of roots.

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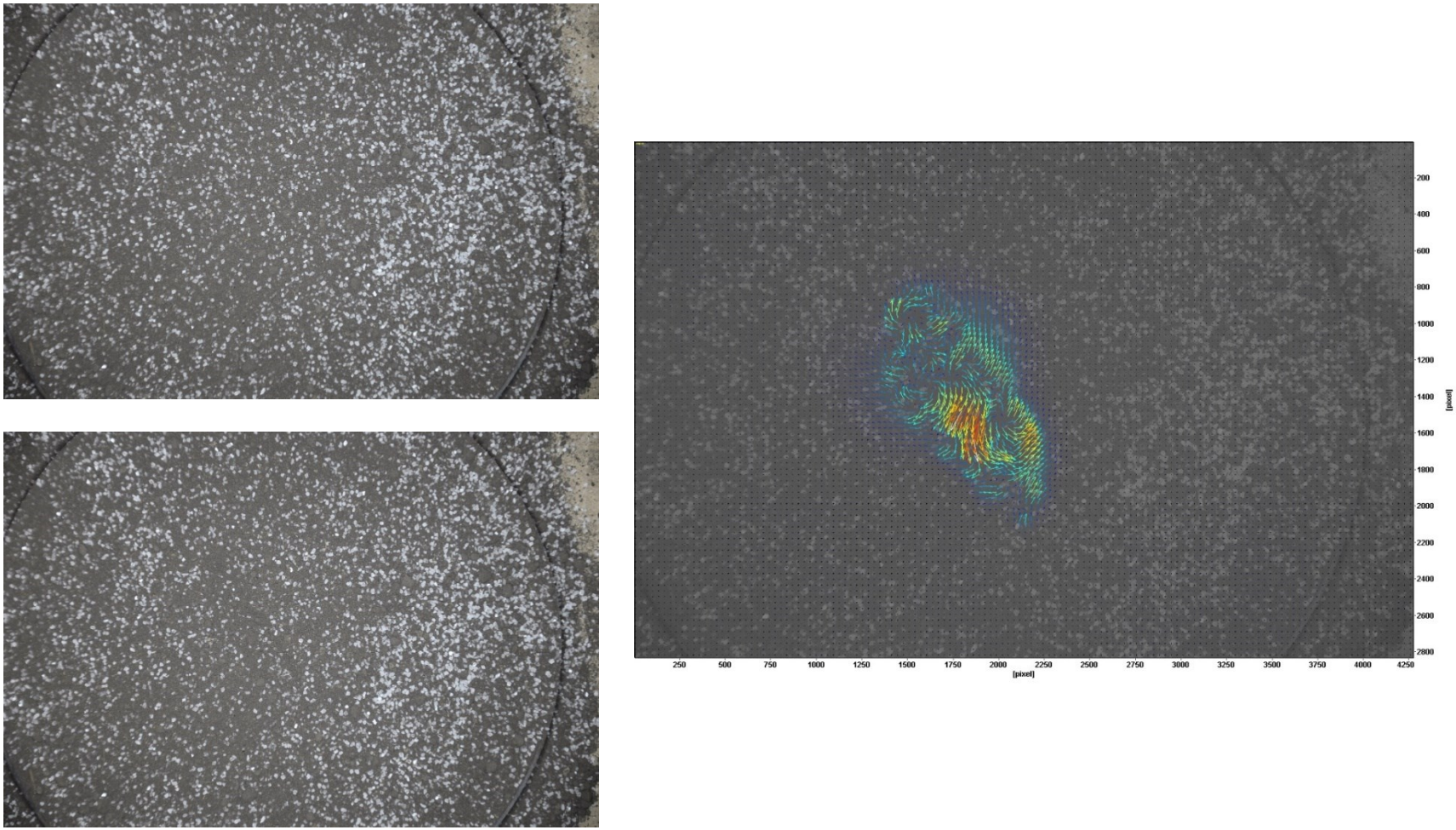
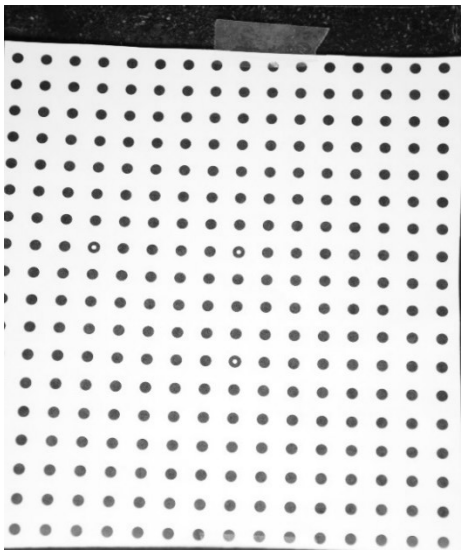


Figure 6.1 Top left: Photo of Petri dish filled with black top soil and white crushed stone before deformation. **Bottom left:** Same Petri dish after a pencil has been run through the middle. **Right:** Visualization of the displacement vectors characterizing the movement of the soil superimposed upon the image of the Petri dish (image by Garrett Melenka).

Figure 6.2 Photographs of the experimental set up. **Top left:** Attachment of pots inside blackout chamber at an angle of ~37°. Chamber was constructed out of extruded aluminum for stability and blacked out with cardboard, plywood, aluminum foil, light blocking tape and laser blocking cloth. **Top right:** Top of same pots visible from outside the blackout chamber. **Bottom left:** Watering of plants (visible are tomato and sunflower plants). **Bottom right:** The blackout chamber.



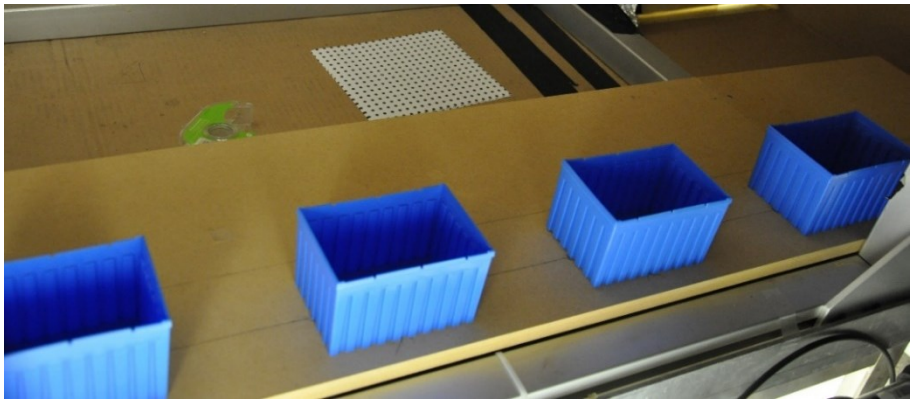


Figure 6.3 Photographs of the camera setup **Top left:** Camera rig and flash mount. To prevent glare, flash was mounted at an angle off center to the lens. Camera was angled up to be flush with the inclined pots. **Top right:** Camera rig and pots. Photographs were taken in the dark with the laser blocking material down and a remote control. **Bottom left:** Calibration target (dots 4 mm diameter, 10 mm apart) **Bottom right:** Pipette tip boxes screwed into the frame used to consistently position rig for each pot.

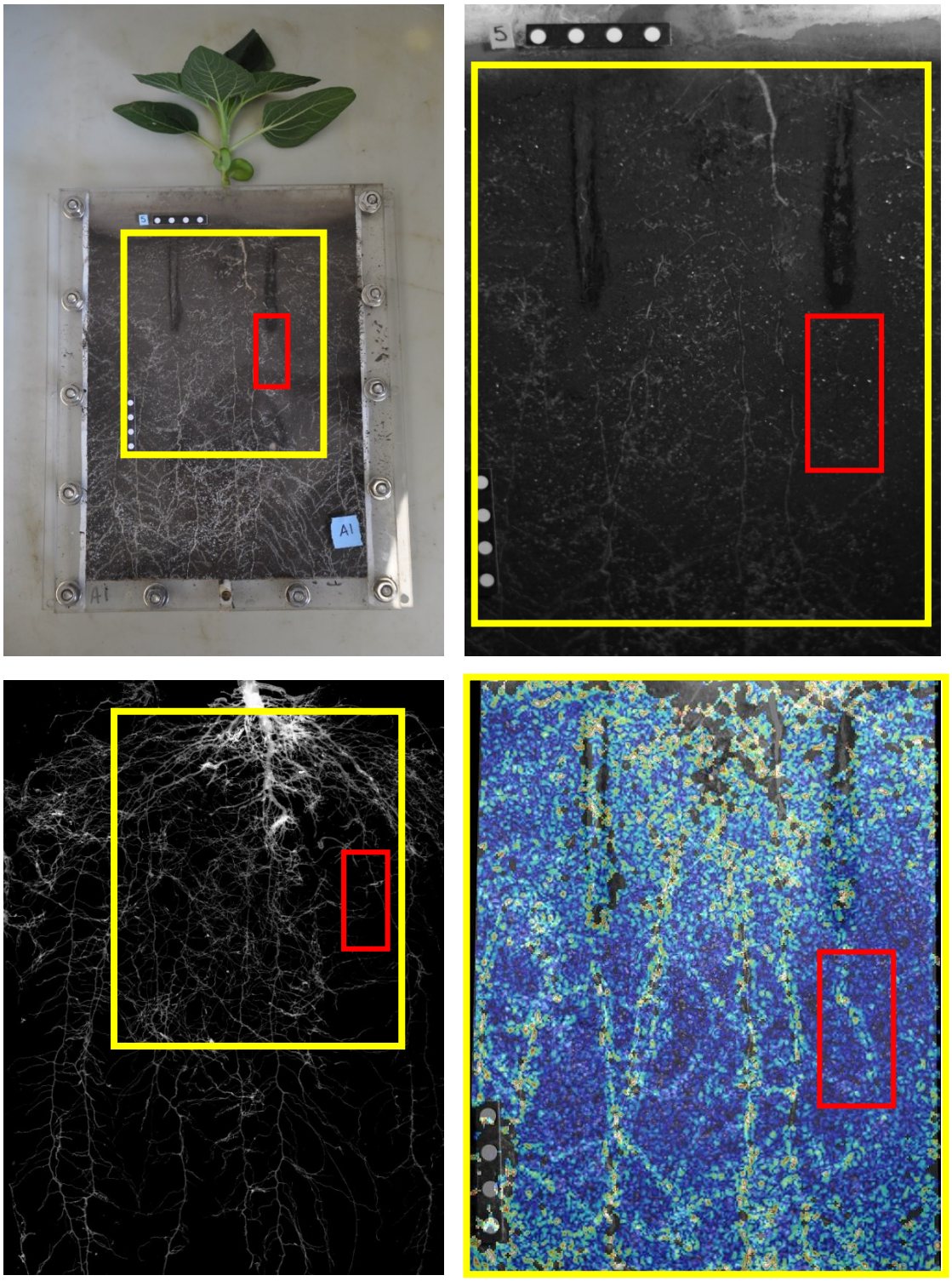


Figure 6.4 Comparison of images of roots from one sunflower (*H. annuus*) plant. The regions outlined by the yellow and red boxes approximate the same region in each image. **Top left:** Whole plant and window box. **Top right:** Photograph of soil and roots. DIC was performed on a series of such images. **Bottom left:** Scan of an excavated root. The roots shifted position in excavation. **Bottom right:** Strain map generated by a DIC analysis (image by Garrett Melenka).

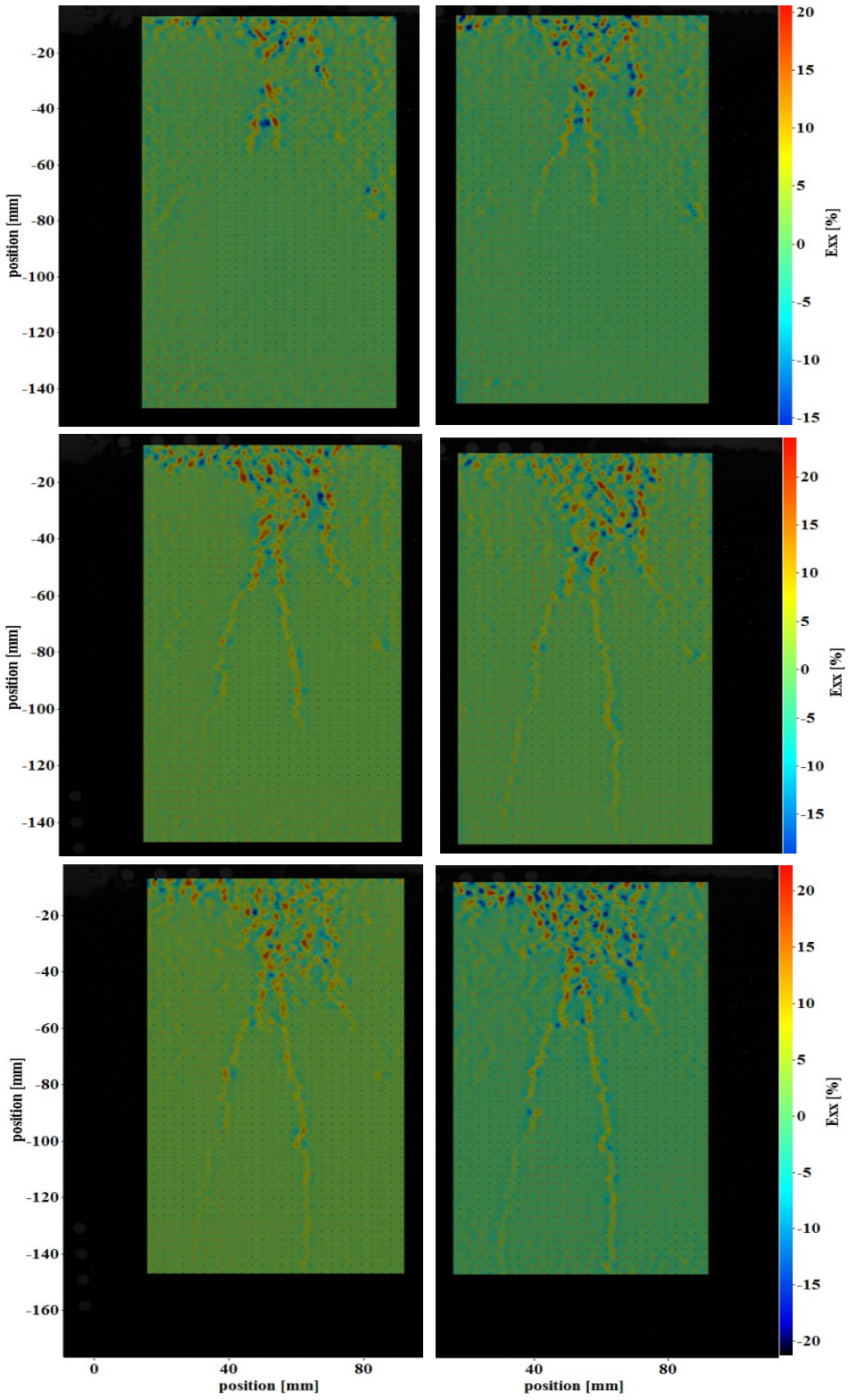


Figure 6.5 Strain maps generated by a DIC analysis showing root growth of a *H. annuus* over 6 days. **Top to bottom, left to right:** Days: 1,2,3,4,5,6. Longitudinal strain was used. Red regions indicate tensile strain and blue regions indicate compression strain in the horizontal direction. Images by Garrett Melenka.

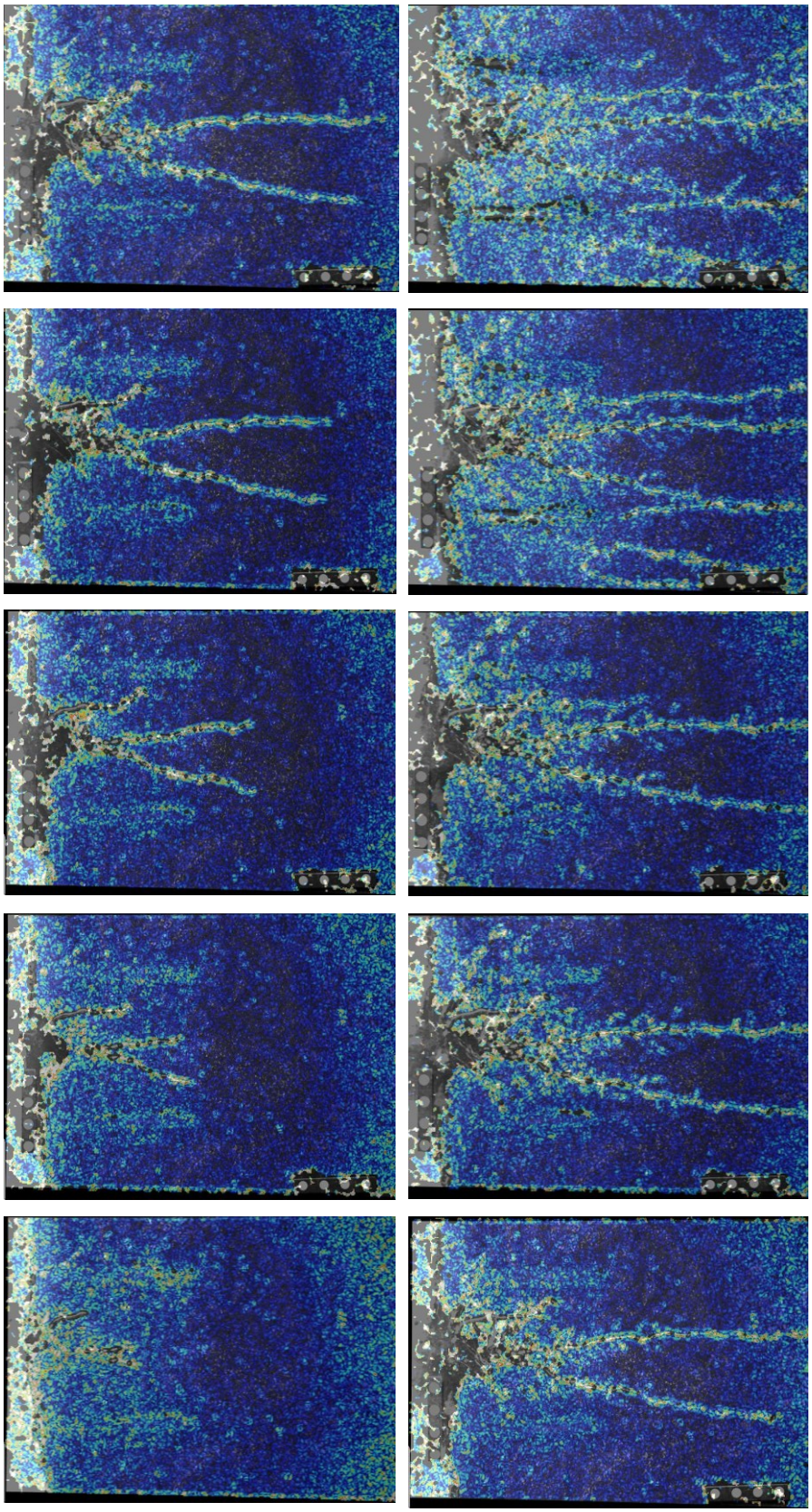


Figure 6.6 Strain maps generated by a DIC analysis showing root growth of a *H. annuus* plant over 3 weeks. **Top row, left to right:** strain maps from days: 1,2,3,4,5 **Bottom row, left to right:** strain maps from days: 6,8,10,13,16. Maximum normal strain was used. Images by Garrett Melenka.

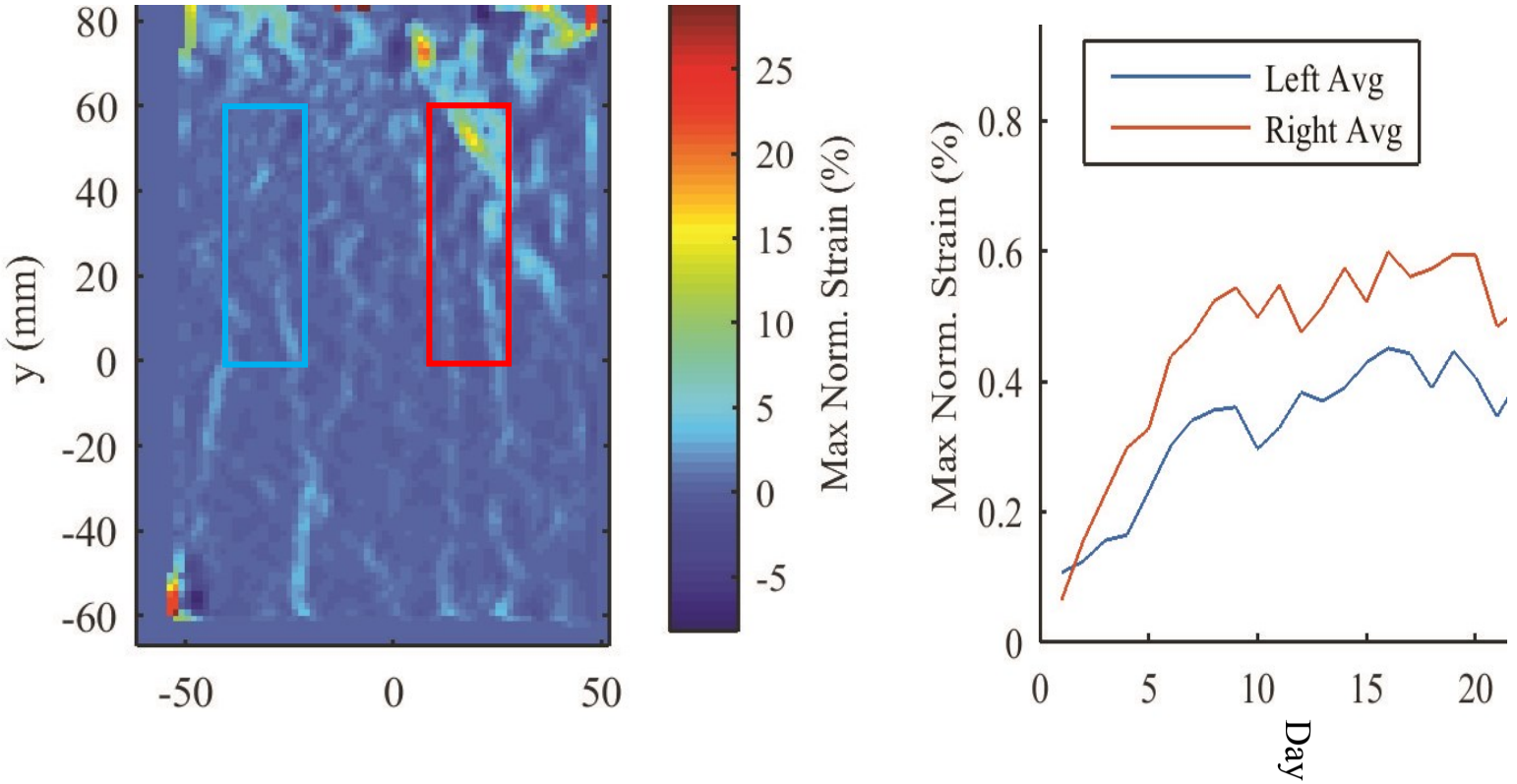


Figure 6.7 Root growth (*H. annuus*) in localized regions of a pot analyzed in strain maps generated by a DIC analysis. Comparison of average maximum normal strain in the left region (blue box, no nutrients) with the right region (red box, nutrients) over 21 days show higher strain on the nutrient side. Images by Garrett Melenka.

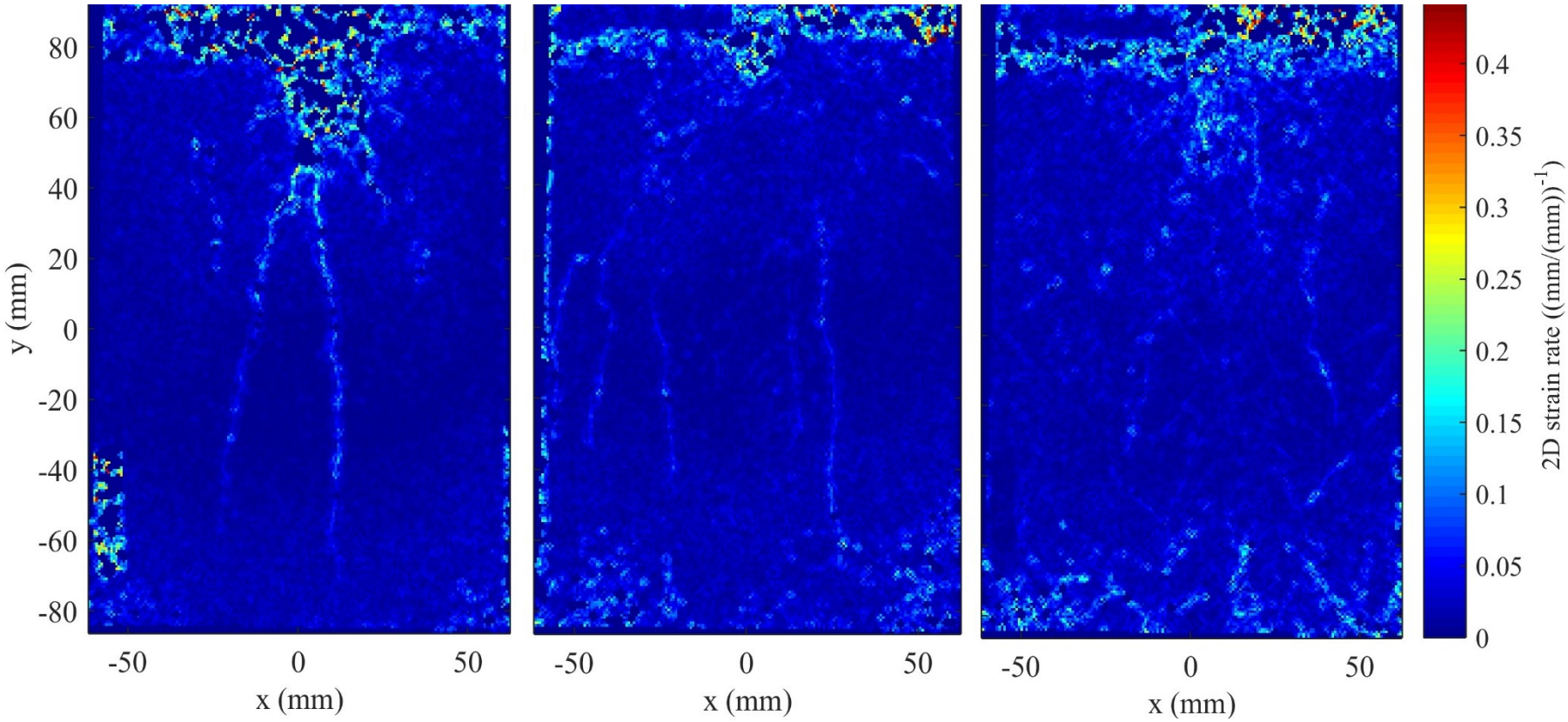


Figure 6.8 Strain maps generated by DIC analysis showing root growth of a *H. annuus* plant over three successive 1 week periods. **Left:** days 1-7 **Center:** days 8-14 **Right:** days 15-21. Images by Garrett Melenka.

Chapter 7- Conclusions

In this chapter, I will speculate on some of the implications of the work presented in the previous chapters, as well as provide some forward guidance for how the work could be extended.

In Chapter 2, I presented the results of an experiment specifically designed to test Darwin's competition-relatedness hypothesis (Cahill et al. 2008, Mayfield and Levine 2010, Violle et al. 2011), a hypothesis that is at the foundation of many ecological theories (e.g. Hardin 1960, MacArthur and Levins 1967, Chesson 2000). This experiment did not support the hypothesis that closely related individuals compete more fiercely, or its assumptions. Instead, there was evidence that at high nutrient levels intra-ecotypic kin compete less with each other than amongst inter-ecotypic strangers. However, at low resource levels, the antagonism amongst kin increased such that there was no difference in competition amongst kin or strangers. Further tests of Darwin's hypothesis to determine the conditions under which it holds are necessary. However, our results suggest that there are gaps in our current theories of competition and coexistence that need to be addressed. One direction that could be pursued would be to better integrate existing theories, such as behavioural theories of kin relationships, with competition theory in order to explain these gaps.

In Chapter 3, I analyzed an empirically determined competitive hierarchical network and described the discovery of some surprising structures and patterns. In particular, the frequency of competitive reversals and competitive equivalence both increased with diversity. Based on current theoretical models, this has direct implications for coexistence (Laird and Schamp 2006). The length of competitive reversals also changed with diversity, but the nutrient levels determined the nature of this change. One specific implication for plant ecologists is that the commonly used

technique of phytometers needs to be reevaluated because hierarchical rankings are sensitive to choice of phytometer. It is an open question whether combinations of phytometers can be chosen to be generate representative competitive rankings. On a broader scale, the conservation implications of intransitivity may be significant. The results here suggest that diversity, genetic diversity in this case, increases intransitivity and potential stability of a competitive network. Conversely, the degradation of diversity in the network accelerates the loss of stability in the system. Critically, intransitivity seems to be intrinsic to competitive networks and therefore more fundamental than other mechanisms of stability and coexistence that are extrinsic to the system. However, the role of intransitivity in natural ecosystems needs to be studied directly. Future theoretical work includes modelling the direct effect of competitive reversals, competitive equivalence and reversal length on community coexistence, as well as analyzing existing data sets for these intransitive traits.

In Chapter 4, I showed that there is a reproductive allocation cost to the leaf closing behaviour in *M. pudica.* All plants that were touched grew bigger, but the ones that were touched during the day produced fewer inflorescences than those that were touched at night. This finding adds to a relatively short list of studies that have quantified the cost of a plant defensive strategy (e.g. Coley 1986, Baldwin 1998, Agrawal et al. 1999). An unexpected finding was that touch itself promoted the growth of plants. One implication is that plant behaviour has a role in mitigating costs of defensive function. This differs from the bulk of plant behaviour studies that focus on the optimization of benefits (e.g. nutrient acquisition). In the future, it would be interesting to examine how defensive strategies, chemical, physical and behavioural are intertwined. The *Mimosa* system would be ideal to pursue this approach some pilot studies we have conducted seem to indicate that there are substantial chemical defenses, in addition to the obvious spines and leaf-closing behavior.

In Chapter 5, I showed that both competition and plant size increased the time it takes for *M. pudica* plants to re-open their leaves after stimulated closure. This result adds to the growing list of factors to which *M. pudica* responds behaviourally. Moving forward, as experiments with *M. pudica* test subtler questions of behaviour, such as whether there is rudimentary learning in plants (Gagliano et al. 2014), the need to standardize observation conditions is apparent because of the sensitivity of the plant to different factors. From the plant behavioural perspective, there are two implications. First, plant size, often a factor important to other plant functions (e.g. reproduction) and interactions (e.g. competition) is also important in behaviour. Second, there is a social context for plant behaviour as neighbours affect how plants re-open. One logical extension of the current work is to try to determine the mechanism for how competition affects leaf re-opening. In particular, cues that maybe signaling competitors could be tested by altering R:RF ratios and separating roots/shoots.

In Chapter 6, I present a new method for the characterization of roots based on the application of DIC analysis (Chu et al. 1985, Pan et al. 2009, Melenka and Carey 2015). In this method, the movement of particles in soil were analyzed to infer the position and movement of *H. annuus* roots. Both the position, as well as the daily proliferation of roots were visible in strain maps. Localized areas were also analyzed within a single pot to demonstrate the analytical flexibility of this method. The focus of this method on the analysis of soil movement is inherently more reliable than methods that concentrate on roots because the soil moves in response to roots near the surface whether they are visible or not. The development of this method has continued since the work presented here was completed. The system has been shifted to a dedicated camera system, dramatically reducing the noise introduced by variation between pictures. Further efforts to reduce cost, simplify the software analysis pipeline and scaling up the method are all necessary to increase the usefulness of our method. However, the method is sufficiently developed that we are now using the method to run experiments.

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