Parental Effects in *Senecio vulgaris*

A thesis submitted to the University of London for the degree of Doctor of Philosophy

By

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I hereby declare that this thesis and the work presented in it, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy and entitled 'Parental effects in *Senecio vulgaris*', represents my own work and has not been previously submitted to this or any other institution for any degree, diploma or other qualification. Where I have consulted the work of others, this is always clearly stated

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Abstract

Parental effects are traits that are passed between generations of organisms to help the next generation survive. They have been shown to be present in plants but little is understood about their ecological value.

Only a few ecological studies account for parental effects in their experimental design and there is much debate about whether parental effects are fully controlled for before beginning an experiment. Furthermore, there are few studies into the influence of mycorrhizal colonisation and insect herbivory on parental effects. These factors have been shown to affect plants within generations, so it was hypothesised that these influences could be manifest between generations. This is the first study to test the effects of mycorrhizas and herbivory on parental effects over multiple generations.

The first part of this thesis explores how long parental effects were visible for over multiple generations of *Senecio vulgaris* and whether the addition of mycorrhizas and aphid herbivory altered any of the effects observed. Parental effects were observed over multiple generations instead of the one-generation that was suggested by previous literature. The presence of aphids and mycorrhizas altered the growth of offspring, for example herbivory in the parental generation delayed growth in the offspring generation, while mycorrhizas in the previous generation increased the growth rate of the offspring. The alteration was dependent upon the trait being measured.

The second part of this thesis explores the mechanisms for passing on the parental effects. One potential mechanism explored was the vertical transmission of endophytic fungi but none of the parental effects could be attributed to the endophytic community present within the plants. The other was the epigenetic change to DNA methylation. Methylation of DNA seems to alter the parental effects on a plant's growth rate, but many other life history parameters were unchanged.

The results highlighted that parental effects persist across multiple generations of *Senecio vulgaris*, so many ecological studies are not accounting for these traits. This could have consequences not only for the growth of plants in natural communities, but also for the conduct of many controlled experiments.

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Chapter One

General Introduction

1.1 Transgenerational effects

Transgenerational effects are traits passed between generations of plants to help the next generation's survival. The effects can include adaptation of plant defences to pests or pathogens (Agrawal, 2002; Rasmann *et al.*, 2012; Vivas *et al.,* 2013) or increased seedling survival (Elwell *et al.*, 2011). Transgenerational effects can be called epigenetic effects based upon of the mechanisms to pass effects between generations or parental effects based on where the effects originate from.
Epigenetics is defined as the 'stably heritable phenotype resulting from changes in the chromosomes without alterations to the deoxyribonucleic acid (DNA) sequence' (Berger *et al.*, 2009). Several mechanisms are thought to cause these chromosomal changes, such as methylation of DNA or structural changes to histones (Heard & Martienssen, 2014).
Parental effects – maternal or paternal depending upon the contribution made by the parent – are beyond the equal chromosomal contribution expected. Within generation effects can mask transgenerational effects, which makes studying the effects difficult. Parental effects are generally weaker, so less commonly observed than within generation effects (Groot *et al.*, 2016).
Latzel (2015) compared how ecological studies controlled for parental effects with many studies not controlling for the effects at all. If parental effects were controlled for, then one generation was grown prior to the start of the experiments. However, Latzel deemed this insufficient and suggested two generations of plants need to be grown prior to the start of the experiments to control for parental effects. His findings were based on growing only two generations of plants to check that parental plants were controlled for, so there may still be residual parental effects visible in the third generation of plants.

1.1.1 Epigenetics and methylation of DNA

Epigenetic changes are capable of having long-term stability through alteration of DNA structure. One of these structural changes is the addition or removal of methyl groups on cytosine residuals (Herman & Sultan, 2016). The addition or removal of

methyl groups can change transcription on specific loci, with the transcriptional changes influencing many factors of plant development.

Methylation of DNA may increase a plant's tolerance to some environmental conditions when the offspring experiences the same conditions as the parental plants. Zebularine (demethylating agent) was used on *Polygonum persicaria* (lady's thumb) to explore what influence DNA methylation had on drought tolerance between generations (Herman & Sultan, 2016). Eliminating methyl groups (demethylation) removed the epigenetic effects of drought tolerance but did not significantly change the phenotypic expression in control plants. Many studies have addressed environmental parental effects being controlled by epigenetics (Seffer *et al.*, 2013; Baulcombe & Dean, 2014; Meyer, 2015), however epigenetic effects on adaptation between generations is still debated in the literature (Akst, 2017). It has been suggested that vertical transmission of symbionts could be a potential mechanism for parental effects to be transferred between generations (Gundel *et al.*, 2017).

1.1.2 Parental effects and the environment

Abiotic and biotic environmental factors present in the parental generation can influence the phenotype of the progeny (Elwell *et al.*, 2011; Vivas *et al.*, 2013; Latzel, 2015). Parental effects are known to affect progeny germination time, flowering and seed weight in *Arabidopsis thaliana* (Elwell *et al.*, 2011). However, it was not stated what environmental conditions caused these effects to occur. So, it could be that the general environmental conditions can trigger these effects or a specific environmental condition, such as extreme temperature ranges (Vivas *et al*., 2013).

Higher provisioning of resources from parental plants to progeny leads to a higher chance of seedling survival and better defence against pathogens (Vivas *et al.*, 2013). This higher provisioning is able to occur due to parental plants being grown in favourable environmental conditions. *Pinus pinaster* (maritime pine) seedlings from parental plants grown in 'good' (mild temperature, adequate moisture and well drained, deep soil) environmental conditions were able to better reduce a

fungal infection than seedlings from parental plants grown in 'poor' conditions (extreme temperature ranges, high wind exposure and thin, waterlogged soil) (Vivas *et al.*, 2013).

1.1.3 Parental effects and insect herbivores

Plants attacked by insect herbivores in the parental generation are known to pass on traits to the progeny generation to better help the next generation of seedlings survive insect herbivore attacks (Agrawal *et al.,* 1999; Agrawal, 2002; Rasmann *et al.*, 2012).

The defensive phenotype is partly determined by the maternal environment and this phenotype can alter seedling growth (Agrawal, 2002). For example, seedling biomass of *Raphanus raphanistrum* (wild radish) was increased in plants whose parents had experienced insect herbivory (*Pieris rapae*) (small white butterfly). At the same time, larval weight of *P. rapae* was reduced when the larvae fed upon the progeny seedlings whose parents had been attacked (Agarwal, 2002).

Parental effects can affect multiple phenotypes at the same time, so it was interesting that parental effects caused by insect herbivory seemed to override any other effects being passed on (Alba *et al.*, 2016). Without insect attack, *Verbascum thapsus* (great mullein) seeds had higher percentage germination when produced by plants in a warmer climate than seeds produced by plants grown in a colder climate. With insect herbivory, the difference in percentage germination between the climates disappeared (Alba *et al.*, 2016).

Changes in defence phenotype could be caused by rapidly inducing defences (Agrawal *et al.*, 1999). Epigenetic changes and defence signalling through either jasmonic or salicylic acid pathways in *Arabidopsis thaliana* caused the increase in defence priming (Rasmann *et al.*, 2012), which could cause the progeny generation to be better defended than plants in the parental generation. The majority of studies into parental effects and insect herbivory use chewing insects to induce plant defences, with few looking into the effects of other insect feeding types, such as sucking or mining.

1.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are from the phylum Mucoromycota (Spatafora *et al*., 2016) and form symbiotic relationships with roughly 80% of vascular plants (Schüßler *et al*., 2001). AMF associate predominantly with annual and perennial grasses and herbs. The symbiosis is normally highly mutualistic, with AMF contributing phosphorus, nitrogen and other nutrients in return for the carbon produced by the plant (Wright, 2005).

Fungal spore germination happens under similar conditions needed for plant germination. The rate of fungal growth is increased through intensification of hyphal branching when the hyphae encounters root exudates (Akiyama *et al.*, 2005). When the hyphae encounter the root or root hair, they form an appressorium on the root epidermis and hyphal penetration occurs using pressure. Specialised hyphal tissue form arbuscles where nutrient exchange takes place (Smith & Read, 2008). Another structure found when arbuscular mycorrhizas are present is the vesicles are believed to be small round storage organelles (Olsson, 1999).

1.2.1 Arbuscular mycorrhizal fungi and the environment

AMF cause a variety of changes to the plant including altering resistance to environmental stressors (Hu *et al.*, 2017; Miransari, 2017; Saxena *et al.*, 2017; Tuheteru & Wu, 2017). Drought stressed *Lycium barbarum* (matrimony vine) was found to be more tolerant to the stress when colonised by AMF, this was through increased transpiration rate and stomata closure (Hu *et al.*, 2017). Under moderate water stress, AMF colonised plants outperformed control plants through increased transpiration rate, while under severe water stress AMF colonised plants maintained normal photochemical processes but had increased sugar levels compared to non-AMF plants (Hu *et al.*, 2017). In waterlogged conditions, AMF plants have promoted growth and biomass through improved nutritional status compared to non-AMF plants (Tuheteru & Wu, 2017). AMF associated plants respond better to salt stress in a pot trail due to hyphae extending the reach of the

roots beyond the salt stressed area; increase root surface area; maintain osmotic balance between sodium and potassium; and facilitate nutrition absorption (Saxena *et al.*, 2017). Heavy metal stress can affect a lot of plants, but AMF plants are better able to tolerate heavy metals using increased expression of stress genes and allocating heavy metals to different tissues in the plants (Miransari, 2017). If AMF colonisation was able to alter a plant's tolerance to certain environmental conditions, then mycorrhizal colonisation may be able to influence the next generation of plants through adaptation of the parental plants.

1.2.2 Arbuscular mycorrhizal fungi and insect herbivory

AMF have been observed to change the plant defences against insect pests (Gange *et al.*, 1999; Koricheva *et al.*, 2009). In contrast to chewing insects, sucking insects often respond positively to mycorrhizal colonisation of their host. For example, *Myzus persicae* (peach-potato aphid) weight and fecundity were increased when feeding on AMF colonised plants (Gange *et al.*, 1999). The presence of AMF could change the plant physiology to increase aphid feeding, such as enlarging the phloem. An enlarged phloem would be easier for the aphids to find with the stylet mouthpieces (Simon *et al.*, 2017). AMF root colonisation decreases as the plant ages. Wheat plants showed a decrease in root colonisation when colonisation was measured in plants between 7 and 48 days post inoculation (Simon *et al.*, 2017). However, this only occurred in certain cultivars of wheat so it is not universal to all plants. A decrease in root colonisation could mean that mycorrhizal effects vary through the plant's lifetime.

Generalist feeders are negatively affected by AMF colonisation while specialist feeders respond more positively to colonisation (Koricheva *et al.*, 2009). Specialist feeders have adapted to specific host plants so the insect should have also adapted to their host plant being colonised by AMF. The increase in plant defences could be why generalist feeders are negatively affected. AMF colonisation has the ability to increase the priming of plant defences through the jasmonic acid pathways giving a quicker defensive response to pests (Jung *et al.*, 2012).

1.2.3 Arbuscular mycorrhizal fungi and parental effects

AMF are only transmitted between members of the same generation and not between parent and offspring (horizontal transmission) (Genkai-Kato & Yamamku, 1999). However, there has been research showing that AMF colonisation in the previous generation of plants can affect the progeny generation (West, 1995; Koide & Lu, 1995; Heppell *et al.*, 1998).

Total percentage carbon, nitrogen and phosphorus in *Senecio vulgaris* (common groundsel) seeds decreased with AMF colonisation particularly in the progeny generation (West, 1995). The presence of AMF and the exchange of nutrients should mean that more resources can be placed into the seeds, due to the nutrient exchange between fungi and plant. However, there is some debate over whether seed chemistry is affected by external sources (Fenner, 1986). A decrease in percentage germination of *Senecio vulgaris* seemed to be associated with the decrease in nutrient content (Fenner, 1986). This led to the conclusion that these extra nutrients help the seed to germinate.

AMF alteration of seed nutrition could affect other growth parameters. Offspring of AMF associated *Abutilon theophrasti* (velvetleaf) were larger than offspring from non-AMF associated plants (Heppell *et al.*, 1998). The size difference was magnified as the experiment progressed. The AMF offspring were also found to have a greater chance of survival and a larger production of seeds than the non-AMF associated offspring. This led to the conclusion that AMF association in the previous generation leads to an increased competitive ability in the offspring (Heppell *et al*. 1998).

Quality versus quantity of seeds is a reproductive dilemma for the plant. When seed quality and quantity were increased, *Avena fatua* (common wild oat) offspring grew rapidly with a superior ability to absorb nutrients (Koide & Lu, 1992; Koide & Lu, 1995). The experiments lasted 75 days so these effects could be short-term, ensuring the seedlings initial survival until they are properly established. Increased phosphorus uptake due to AMF colonisation caused phosphorus levels in the seeds to increase (Koide & Lu, 1992). *Campanula rotundifolia* (harebell), germinated from seeds with increased phosphorus, had larger leaf area than plants from seeds with

no increase in phosphorus (Nuortila *et al.*, 2004). Phosphorus levels in maternal plants decreased when their seed phosphorus levels increased, suggesting that parental plants sacrificed their own health to ensure the next generation's survival. Seed quantity increased in *Lycopersion esculentum* (tomato) when seed phosphorus levels increased (Poulton *et al.,* 2001A; Poulton *et al.*, 2001B; Poulton *et al.*, 2002). Increasing seed quantity could increase the chances of the next generation's survival, although the percentage germination was not checked the germination percentage may not have changed.

1.3 Endophytes

An endophyte is a symbiotic fungus or bacterium that lives within the plant tissue for some of its lifecycle, while causing no visible sign of disease (Carroll, 1988; Clay, 1988; Isaac, 1992; Wilson, 1993).

1.3.1 Endophytes and anti-herbivory effects

Various species of endophytes are known to cause anti-herbivory effects. While entomopathogenic endophytes are known to attack and seriously disable or kill insects, there are non-entomopathogenic endophytes that also have anti-herbivory effects (Gange *et al.*, 2012; Hartley *et al.*, 2015).

Endophytes present within *Cirsium arvense* (creeping thistle) cause chemical changes in the leaf, which are similar to chemical changes that occur when the leaf is under attack from insect pests (Hartley *et al.*, 2015). This can cause a significant reduction of foliar feeding insects on *C. arvense* when these endophytes are present (Gange *et al.*, 2012). The generalist-feeding insect (*Mamestra brassicae*) (cabbage moth) was more affected by the chemical changes than the specialist feeder (*Cassida rubiginosa*) (thistle tortoise beetle). This was also true with mycorrhizal fungi and insect feeding type (Koricheva *et al.*, 2009).

On the other hand, interactions between endophytes within the plants can cause positive effects on the insect herbivores (Gange *et al.*, 2007). A mixture of

endophytes present in *Leucanthum vulgare* (ox-eye daisy) caused an increase in leaf mines. The specificity of the endophyte is thought to reduce insect attack while high volumes of endophytes present within the same plant could counteract the anti-herbivory effect of specific endophytes (Gange *et al.*, 2007). The likelihood of a plant having only one endophyte present is low, so interactions between multiple endophytes present in the same plant have to be considered (Hodgson *et al.*, 2014). In six species of forbs including *S. vulgaris*, there were over ten species of endophytes present in each forb species (Hodgson *et al*., 2014), so this could stop anti-herbivory effects from being seen.

1.3.2 Endophytes and vertical transmission

Vertically transmitted (parent to offspring transmission from the parent directly to the embryo) organisms are common in plants with huge potential to influence phenotypes and fitness of progeny (Gundel *et al.*, 2017). Vertical transmission of endophytic fungi from the parental plants to progeny is thought to occur through the seed of both grasses (Gundel *et al.*, 2012; Gilbert *et al.*, 2015; Wiewióra *et al.*, 2015) and forbs (Hodgson *et al.*, 2014; Quesada-Moraga *et al.*, 2014). The plant that formed the subject of all experiments in this thesis, *S. vulgaris* has vertical transmission of endophytes with multiple species of endophytes entering the seed through the parental pollen tube (Hodgson *et al.*, 2014). Endophytic fungi can be found in the plant at any life stage, but fungal growth was more likely to be lost when the seed germinated. Endophytes being lost at any life stage, suggests that vertical transmission is imperfect (Afkhami & Rudgers, 2008; Hodgson *et al.*, 2014).

1.3.3 Endophytes and mycorrhizal colonisation

Both endophytes and AMF take carbon from the host plants so when both are present within the same plant they may compete for the same resources (Chen *et al.*, 2007). Most research into the interaction of AMF and endophytes has been performed on grass species.

The presence of mycorrhizas in two *Lolium perenne* (perennial ryegrass) cultivars (high sugar and control) reduced concentrations of endophytes and alkaloids in the leaf blades, but the reduction depended upon the phosphorus concentration in the plants (Liu *et al.*, 2011). In the control ryegrass cultivar, foliar endophyte inoculation caused a reduction in AMF percentage root colonisation but this did not occur in the high sugar ryegrass cultivar (Liu *et al.*, 2011). The results of this study suggested that the outcome of competition between endophytes and mycorrhizas depends upon the carbohydrate content of the host plant (Liu *et al.*, 2011).

Poa bonariensis was found to have a larger number of endophytes colonising it than AMF within the roots (Novas *et al.*, 2009). The AMF had a positive response to endophyte colonisation in *Poa* with fungal features, i.e. hyphae and arbuscules, being more abundant in plants with endophytes present (Novas *et al*., 2009). The study went on to explore whether the percentage of plant infected with endophytes had an impact on the endophyte and mycorrhizal interaction. Percentage root colonisation by AMF was larger in plants that were 100% infected by endophytes. However, the percentage of arbuscules was most increased in the plants infected only 50% by endophytes compared to both 100% endophyte infected plants and the endophyte free plants (Novas *et al.*, 2009). So the interaction between two types of fungi is very dependent on external factors, and the amount of fungal colonisation in the plant.

1.4 Common Groundsel, *Senecio vulgaris*

1.4.1 Biology and lifecycle

Common Groundsel, *Senecio vulgaris* L. (1753), is a herbaceous annual from the Asteraceae family. It is native to the UK and is most commonly found in open and rough ground growing upon almost any soil type.

S. vulgaris is a well-known common garden weed. It seems to react strongly to environmental stressors with visible changes in vegetative growth, such as a variable flower and leaf shape. The leaf shape depends upon how much soil disturbance the plant experiences during growth (Bosbach *et al.*, 1982; Grime *et al.,*

1988). While the plant has the ability to flower throughout the year, its reproductive ability is also affected by environmental stress with flowering times becoming irregular when the plant is under stress (Harper & Ogdon, 1970). Stressful environmental conditions have the ability to alter the length of the plants' lifecycle with it being possible under 'ideal' environmental conditions for the lifecycle to be completed in five to six weeks (Salisbury, 1962).
Common Groundsel has high selfing ability where inbreeding causes populations to stabilise with less variability in undisturbed habitats (Grime *et al.*, 1988). This high selfing ability ensures that a suitable genotype is kept within the population. Each plant is capable of producing on average between 1,000 and 1,200 seeds within its lifecycle. The seeds are lightweight with a pappus for wind dispersal. These features of the life cycle, plasticity and reproductive biology meant that *S. vulgaris* was chosen as the model plant system for all experiments reported in this thesis.

1.4.2 Chemical defences

Inducible defences in the form of pyrrolizidine alkaloids are present within *S. vulgaris* (Hartmann et al., 1989). Pyrrolizidine alkaloids are naturally occurring alkaloids found in over six thousand species of plants with more than 660 alkaloids identified (Fu *et al.*, 2010). Pyrrolizidine alkaloids are known for the characteristic pyrrolizidine (tertiary alkaloid, see Figure 1.1). In *S. vulgaris* the plant synthesise N-oxides and store the alkaloids as N-oxides (Hartmann *et al.*, 1989). The N-oxides are chemical intermediates and play a role in the multistep synthesis of pyrrolizidine alkaloids (Hartmann *et al.*, 1989).

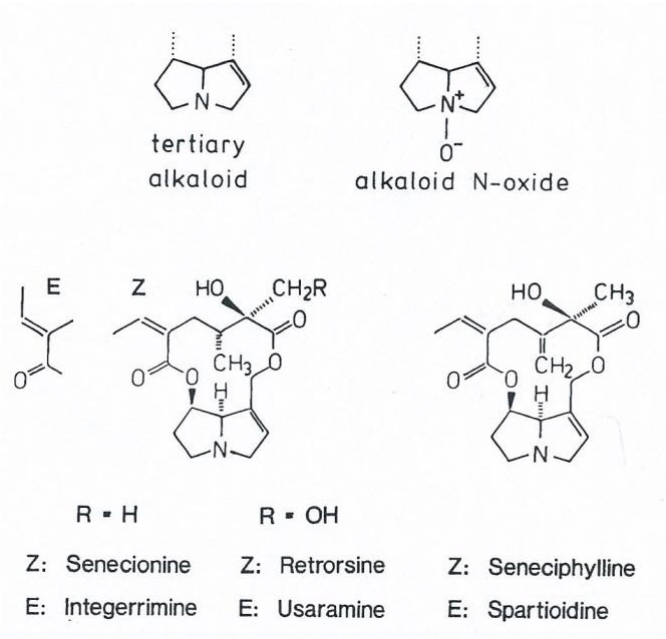

Figure 1.1. Structure of pyrrolizidine alkaloids found in *Senecio vulgaris* (Hartmann *et al.,* 1989).

They are well known chemical defences that a plant can use to defend against insect herbivory. Over half of the pyrrolizidine alkaloids cause hepatotoxicity (Schoental & Kelly, 1959), with some of these alkaloids being present within the insect herbivore's body even after they have stopped feeding on the plant. The n-oxides of the alkaloids present in Groundsel are synthesised in the roots and transported to specific sites via tissue transport. The alkaloids can be found in all areas of an individual plant with levels varying between tissue types (Hartmann et al., 1989). Chemical defences are expensive to produce and maintain so the plant may only defend specific tissues leading to the variation (Theis & Lerdau, 2003). Sending chemical defences to areas that are more likely to be targeted by pests or tissues is important for the plant's survival.

The effects of fungal symbionts and chemical defences in *S. vulgaris* has not been explored, however there is some research in *Jacobaea vulgaris* (formerly known as *Senecio jacobaea*) (ragwort), which is a close relative of *S. vulgaris*. Chemical defences were increased in root tissue of AMF colonised plants when compared to non-AMF colonised plants (Hill *et al*., 2018). However, the increase in four pyrrolizidine alkaloids was in the roots of AMF colonised plants and not the vegetative tissue.

An endophyte present in *J. vulgaris* lowered pyrrolizidine alkaloids and changed their composition (Nuringtyas, 2013). Changing the composition of alkaloids could increase the variation of pyrrolizidine alkaloids present, leading to consequences in plant defences against generalist and specialist pests and pathogens.

1.5 Peach-Potato Aphid, *Myzus persicae*

1.5.1 Biology and lifecycle

The peach-potato aphid, *Myzus persicae* (Sulzer, 1776), is a sap feeding insect that uses its stylet mouthparts to pierce between cells to the host plants' phloem. This polyphagous insect has a wide host range including *S. vulgaris* (CABI, 2015) and was used as the herbivore in all experiments within this thesis. It is normally green in colour and on average between 1.2mm to 2.1mm.

The lifecycle of *M. persicae* is very dependent upon the climate but it can be completed within 10 to 12 days. Four to thirteen yellowish eggs, which are roughly 0.6mm long and 0.3mm wide, are laid on *Prunus* spp. Once hatched the nymphs quickly turn yellow in colour and resemble the parthenogenic, nymph producing adults. Asexual reproduction length and the adult reproductive age vary greatly (Horsfall, 1924; MacGillivray & Anderson, 1957). On average females give birth asexually to nymphs when they are six to seventeen days old and will continue to reproduce for roughly 14 days. Asexual reproductive behaviour is favoured in most climates around the world (CABI, 2015).

When aphid densities increase, apterous aphids are produced and start to disperse to almost any nearby plant (Van Emden *et al.*, 1969). The cycle will then begin again with eggs being laid on the new host plants.

1.5.2 Myzus persicae and plant chemical defences

M. persicae are known to have induced defensive responses in *Arabidopsis thaliana* with aphids present on infested leaves reducing progeny production (De Vos &

Jander, 2009). Aphid progeny feeding on plant tissue that was not previously infested did not exhibit a reduction in progeny production. In *Nicotiana tabacum* (cultivated tobacco) and *Arabidopsis thaliana*, certain aphid salivary proteins were released into the phloem while feeding. It is believed that these salivary proteins trigger the plant's defensive response (Elzinga *et al*., 2014).

Molyneux *et al.* (1990) found pyrrolizidine alkaloids and their n-oxides from *S. vulgaris* were present in the honeydew of *M. persicae.* Honeydew is a sticky substance excreted by aphids because they feed upon the sugary fluid within the plant's phloem. The presence of alkaloids in *M. persicae* honeydew suggests that they do cause chemical defences to be induced in *S. vulgaris* when feeding from the phloem.

1.6 Aims and objectives

The overall aims of this thesis were divided into two main themes: to investigate 1) which environmental conditions cause parental effects and 2) establish the transmission mechanism(s) of any effects between generations.

The first theme investigated whether the addition of symbiotic mycorrhizas or insect herbivores causes parental effects to be passed between generations of plants. This was explored by testing plant and insect development through multiple generations. Specifically the objectives were to determine:

- If parental effects are passed between generations of *Senecio vulgaris* and if so, for how many generations
- If the presence of insect herbivores causes changes in plant defences over multiple generations
- If the presence of insect herbivores affects plant development over multiple generations
- If the symbiotic relationship between plant and mycorrhizal fungi affects plant development over multiple generations

The second theme explored the possible mechanisms for these effects to be transferred between generations. Specifically, the objectives were to determine:

- If vertical transmission of endophytes causes transgenerational effects in the progeny generations
- If methylation of DNA causes environmental parental effects to be passed between generations

Chapter Two

Methods and Materials

2.1 Introduction

Methods and materials were kept the same throughout all the experiments and these methods are included here. The main techniques used were: culturing *Senecio vulgaris*, culturing *Myzus persicae*, setting up different treatment comparisons, visualisation of mycorrhizas and analysis of the endophytic community.

2.2 Culturing *Senecio vulgaris*

Senecio vulgaris seeds were collected from wild plants growing around the Salisbury Plain area in autumn 2014. These seeds were planted into identical individual pots containing 165g of John Innes (Norwich, Norfolk) Grade 3 compost. The grade 3 compost is designed for established plants and shrubs. It contains loam, peat, sand, and ground limestone, hoof and horn meal, superphosphate and potassium sulphate. The plants were grown in controlled conditions (20^{o}C and 16 hours daylight). The pots were placed randomly on workbenches and were moved once a week to try and reduce environmental variation. The wild seeds were grown with no additions, i.e. mycorrhizal inoculum and insect herbivores, until they produced seeds. The seeds were collected for use in the main experiment and the plants were destroyed.

For the experiments, four seeds, from plants grown in control conditions for one generation, were planted in a pot containing 165g of John Innes Grade 3 compost. The plants were all grown in the same controlled conditions (20^{o}C and 16 hours daylight) until they had produced seed. The pots were placed randomly onto workbenches and moved once a week to reduce environmental variation. Once the plants had developed true leaves, they were weeded out so only one plant remained per pot. There were four treatments per generation with twenty plants per treatment. The control plants, referred to as C (Figure 2.1), had 5mls autoclaved Rootgrow (PlantWorks Ltd., Sittingbourne, Kent) mycorrhizal inoculum added 2cm below the topsoil at the time of sowing. The inoculum was autoclaved so the fungal spores were killed and only the clay particles remained. The mycorrhizal treatment,

referred to as AM (Figure 2.1), had 5mls of Rootgrow inoculum added 2cm below the topsoil of each pot. Plants that were to be treated with aphids, referred to as H for herbivory (Figure 2.1), had 5mls autoclaved Rootgrow inoculum added 2cm below the topsoil of each pot before the seeds were sown. Once plants in this treatment had developed buds, aphids were placed onto the plants for fecundity measurements (Section 2.3; Section 6.2.1). The final treatment group was the combined treatment, referred to as HAM (Figure 2.1), with 5mls of Rootgrow mycorrhizal inoculum added 2cm below the topsoil of each pot. Once these plants had developed buds, the aphids were added (Section 2.3; Section 6.2.1). At the end of each generation, seeds were collected from each plant and stored in paper envelopes. These seeds were used to plant the next generation.

2.3 Culturing *Myzus persicae*

Myzus persicae were obtained from Rothamsted Research, Harpenden. They were kept in a large insect cage in controlled conditions (20^{o}C and 16 hours daylight). The insects were reared upon Chinese cabbage (*Brassica rapa*). The Chinese cabbage was grown from seeds (Premier Seeds Direct, Salisbury) planted in John Innes Grade 3 compost. *M. persicae* were cultured continuously throughout the experiments, with pots of Chinese cabbage being replaced as it died.

2.4 Setting up different treatment comparisons

To observe whether a change to the treatments was a cause of transgenerational effects, a separate group of treatments were set up for each generation after the first generation. The seeds from the non-control treatments were taken from each treatment group and grown under control conditions (Section 2.2), while some seeds were also grown under the same treatment conditions as the previous generation. For example (Figure 2.1), seeds from generation one H plants were taken and some were grown in control conditions (2B) while other seeds from generation one H treated plants were used to grow the generation two H plants, 2B was compared to generation 2 H to see if there was any difference when the seeds

were not subjected to the same treatments. Other environmental conditions were still kept the same (Section 2.2).

2.5 Visualisation of arbuscular mycorrhizal fungi

The roots were collected from each plant and stored in 70% ethanol until they were ready to be analysed. For visualization, roots were washed in tap water to remove any visible signs of soil. Roots were placed into labelled square mesh tissue cassettes (Thermos Fisher Scientific, Waltham, USA). They were cleared in 10% potassium hydroxide (KOH) in a water bath at 75^{o}C for 10 minutes. KOH was discarded and the roots were thoroughly washed with tap water. A modified ink staining method (Vierheilig *et al*., 1998) was used to visualise the AMF. Parker (Newhaven, UK) washable Quink was mixed with 1% hydrochloric acid (HCl) and distilled water in the ratio 84.4:15:0.6. Cleaned root samples were added to the stain in a heated water bath at 75^{o}C for 15 minutes. The stain was discarded and the roots samples were made into slides. The slides had distilled water added to prevent the roots from drying out and the coverslip was sealed with clear nail varnish.

Percentage root length colonised was obtained with the cross-hair eyepiece method (McGonigle *et al.*, 1990). Roots were spread evenly across the slide and observed at x200 magnification. Each root piece at the centre of the eyepiece (cross-hair) was observed for presence and absence of fungal features (hyphae, vesicles and arbuscles) and recorded. Approximately 100 views were counted for each root sample.

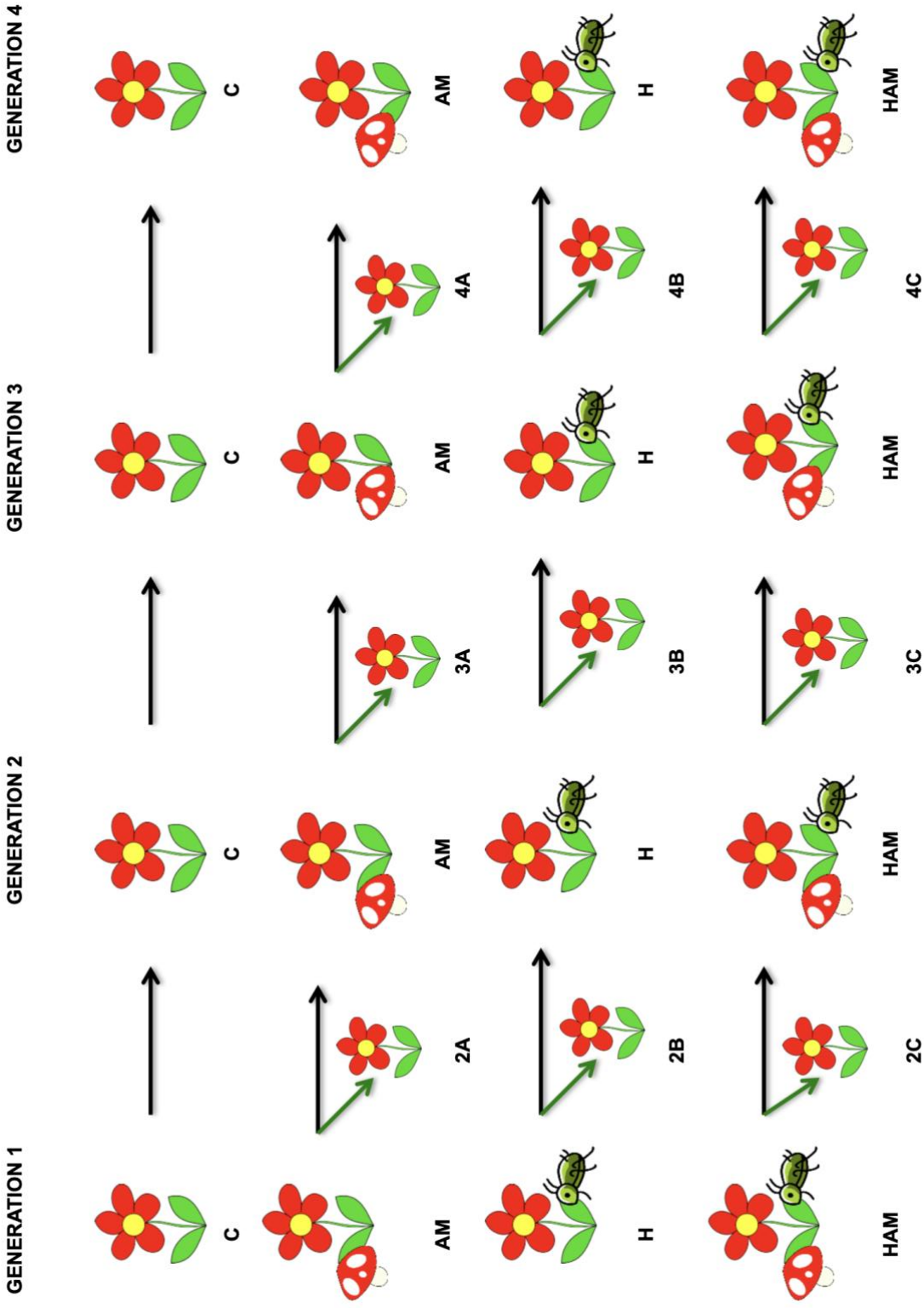

Figure 2.1. Diagram showing how the treatments were set up for each experiment. The mushroom represents mycorrhizal treatment and the aphid represents aphid infestation.

2.6 Analysis of the endophyte community

Three leaves were taken at random from five plants in each treatment group. These leaves were subjected to fragment plating to ensure only endophytes are identified. The fragment plating method was adapted from Schulz *et al*. (1993). Leaves were dissected into two small fragments and subjected to surface sterilisation and plating of the fragments onto a nutrient rich agar (potato dextrose, PDA). Surface sterilisation was achieved by immersing the leaf fragment into 100% ethanol for 30 seconds; sterile water for 30 seconds; bleach solution 1:3 for 2 minutes; 100% ethanol for 30 seconds and sterile water x4 for 30 seconds each. Each leaf segment was transferred using sterile forceps onto PDA containing 80mg L^{-1} streptomyocin sulphate and 60mg L^{-1} penicillin G added to inhibit bacterial growth. Leaf presses were also performed, to check that the sterilisation process had removed all epiphytic fungi. Plates were incubated at room temperature and endophytes were subcultured onto potato carrot agar (PCA). The fungal isolates were removed from the PDA plates soon after they appeared to avoid contamination by other endophytes. The PCA plates were used to induce sporulation, which aids identification.

Dr B.C. Sutton (ex-CABI) identified the isolated fungi through visualisation of the fungal spores and hyphae. The fungi that could not be identified through visualisation were sent for molecular analysis. CABI (Centre for Agriculture and Biosciences International) ran molecular analysis following their in-house methods. All samples were checked for purity. Molecular assays were carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Once DNA was extracted, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA in vitro. The quality of the PCR product was assessed using gel electrophoresis. PCR purification step was carried out to remove unutilised dNTPs, primers, polymerase and other PCR mixture compounds. This obtained a highly purified DNA template used for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using

BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilised fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeExTM 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionised formamide Hi-DiTM (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing was undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following the sequencing, identifications were undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database from the European Bioinformatics Institute (EBI).

Chapter Three

Parental effects on *Senecio vulgaris* development time

3.1 Introduction

This experiment investigated the effects of the environment on *Senecio vulgaris* development time over multiple generations. This addressed the first theme of this project; which environmental conditions cause parental effects to occur. It specifically explored the objectives; does the presence of insect herbivores cause changes in plant development over multiple generations and does the symbiotic relationship between plant and mycorrhizas cause changes in plant development over multiple generations.

Parental effects on early growth have been explored (Elwell *et al.*, 2011; Moriuchi *et al*., 2016; Walter *et al*., 2016) but no studies have explored the influences of specific environmental factors, such as insect herbivory and/or mycorrhizal colonisation on plant development time, in any species of plant including *S. vulgaris* (Roach & Wulff, 1987).

There is research into seed nutrition of *S. vulgaris* and early growth (Fenner, 1986). Fenner (1986) found that external nutrients had more effect upon *S. vulgaris* growth than any parental effects, so there may not be any parental effects controlling changes in development time. But other research found that maternal *S. vulgaris* grown in poor soil conditions produced seeds that took longer to germinate (Aarssen & Burton, 1990). The seedlings that were grown from seeds produced by parental plants in poor soil conditions, survived longer than other seedlings (Aarssen & Burton, 1990). It was believed that the seedlings were able to live longer by 'waiting' in the seedling stage and pausing development. Seedlings could pause development to gain enough nutrients before continuing development (Aarssen & Burton, 1990). Aarssen and Burton's (1990) study suggests that there is a connection between parental effects and *S. vulgaris* lifecycle development time.

Early seedling growth appears to be dependent upon materials stored within the seeds (Roach & Wulff, 1987) with both the plant's genetics and environmental effects influencing seedling growth (Latter, 1971). Changes in germination time have been previously explored through changes in seed dormancy (Gray & Thomas, 1982; Garbutt & Witcombe, 1986). Roach and Wulff's (1987) review of previous literature suggested that seed coat thickness was linked to changes in seed

dormancy. A thinner seed coat reduced seed dormancy therefore decreasing time to germination. The parental plant could have the ability to influence the seed coat thickness.

Parental effects on leaf development are debated and not heavily researched. Time to leaf emergence has been briefly explored, wherein maternal environment has been seen to influence leaf emergence (Latter, 1971). Maternal environment effects on leaf emergence differed between species, even closely related species (Edwards, 1970). Edwards (1970) found that leaf emergence in different *Lolium* spp. was linked to both the seedling's genetics and the maternal environment, so can be unpredictable.

Flowering time has been the main focus of experiments into parental effects upon development time changes. There is much debate in the literature about whether flowering time was under the plant's own genetic control or under parental influence. Some species were found to have flowering time influenced by both the plants own genetics and the parental environment (Hayward, 1967). In some species, flowering time was found to be only influenced by the plant's own genetics (Lawrence, 1964; Thomas, 1967; Kotecha, 1979) or only influenced by the parental environment (Hayward & Breese, 1968; Hayward & Nsowah, 1969; Edwards & Emara, 1970; Jinks *et al.*, 1972; Singh & Murty, 1980).

There has not been much focus in the literature upon time taken to reach other development stages, so it could be that other life stages are affected by changes in development time caused by parental effects.

The alteration of a plant's development time could have significance in other ecological and agricultural experiments. In ecological studies, rapid cycling plants are useful to run fast/multiple experiments in a small space of time. In agriculture, rapid cycling of plants could allow for quicker crop production time, e.g. rapid cycling in spring wheat accelerating crop development time (Boontung, 2017).

Parental effects have been seen to affect plant development time in other species (Elwell *et al.*, 2011; Moriuchi *et al*., 2016; Walter *et al*., 2016) so it was hypothesised that parental effects would alter *S. vulgaris* development time. In addition, if this hypothesis was upheld, the addition of aphids and/or mycorrhizal fungi may cause

further effects on *S. vulgaris.* Overall it was hypothesised that the environment of the previous generation affects the development time of the progeny generation.

3.2 Methods

3.2.1 Main experiment development measurements

The main experiment was to explore whether the previous generation affects the development time of the progeny generation. It involved growing four generations of *S. vulgaris* under the same environmental conditions (Section 2.2). Each generation had a total of 80 plants grown. There were four separate treatment groups with 20 plants in each group per generation. The four treatment groups were C, AM, H and HAM (Section 2.2; Figure 2.1). In each generation, each plant in each treatment group was checked for when the plant reached set lifecycle stages. Development stages were set as: germination – first visible signs of growth; true leaf – first sign of a true leaf; budding – first sign of a bud; flowering – first flower; seeding – first seed production. The dates these development stages were reached were recorded. Days taken to reach these lifecycle stages were calculated from the date of sowing.

3.2.1.1 Statistical analysis of main experiment

Analysis of development time of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
Differences in time taken to reach certain development stages over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence and generation as the main effects.

3.2.2 Comparing changes to treatments and the impact on development time

Changes to treatments and the impact on development time were set up following the protocol in Section 2.4 and Figure 2.1. Each generation from generation one had seeds from treated plants (AM, H and HAM) grown under control conditions (Section 2.4; Figure 2.1). There were ten plants grown per treatment and three treatment groups used per generation. In each generation, each plant in each treatment group was checked for when the plant reach set lifecycle stages. Development stages were set as: germination – first visible signs of growth; true leaf – first sign of a true leaf; budding – first sign of a bud; flowering – first flower; seeding – first seed production. The dates these development stages were reached were recorded. Days taken to reach these lifecycle stages were calculated from the date of sowing.

Differences between the plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment, i.e. generation two C was compared to 2A, 2B & 2C (Figure 2.1).

The results were also compared to the next generation plants grown under treated conditions, i.e. generation two AM plants were compared to 2A.

3.2.2.1 Statistical analysis of comparing changes to treatments

Analysis of development time of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.

Differences between the plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment. A one-way, repeated measures ANOVA was performed, employing parental treatment as the main effect.

Differences in time taken to reach certain development stages over treatment groups compared to controls were tested using a one-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence as the main effects for the control versus AM experiment and H experiments. Differences in

time taken to reach certain development stages over HAM treatments versus control plants were tested using a two-way, repeated measures ANOVA, employing aphid absence/presence and AMF absence/presence as the main effects.

3.3 Results

The results explore changes in the development time (time to germinate, develop true leaves, buds, flowers and seeds) of control (C), mycorrhizal only (AM), aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over multiple generations.

3.3.1 Main generation results

Germination time differed between generations ($F_{3,282}$ = 15.984, p<0.001). The main point was germination time increased between generations one and three, but decreased again in generation four. Overall, plants from the first generation germinated quicker than plants from any other generation. Plants in the third generation took the longest time to germinate. The effects of generation and aphid presence had a significant interaction ($F_{3,282}$ = 6.358, p<0.001). Seeds from aphid attacked (H and HAM) parents were faster to germinate in the second and fourth generations than seeds from non-attacked parents (C and AM plants). Aphid attacked (H and HAM) plants were slower to germinate than the non-attacked (C and AM) seeds in the first and third generations (Figure 3.1).

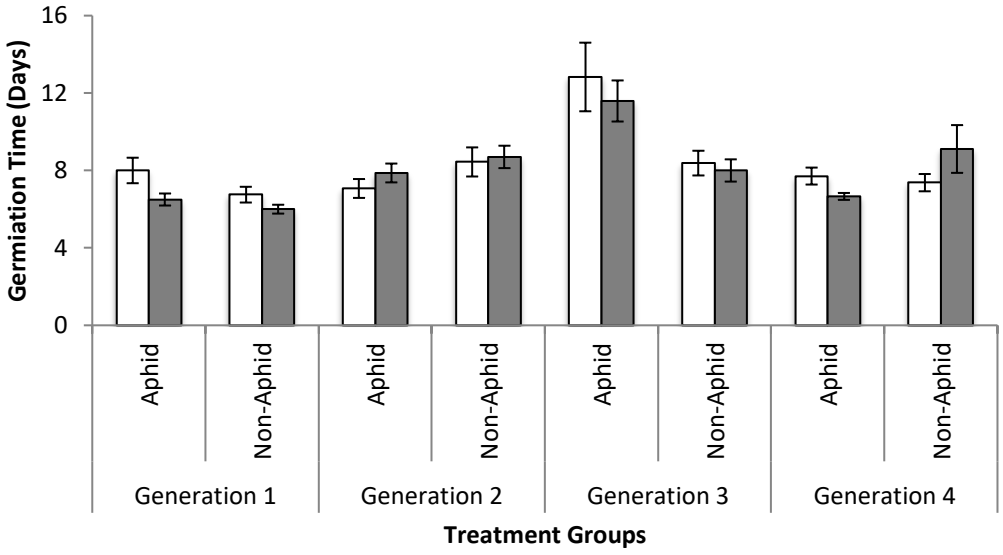

Figure 3.1 Mean time taken for a seed to germinate from day of sowing (germination time) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

The key finding was that plants in generation four were quicker to produce true leaves than plants in any other generation ($F_{3,292}$ = 14.901, p<0.001). Over all generations, plants from mycorrhizal (AM and HAM) parents produced true leaves slower than non-mycorrhizal (C and H) plants, ($F_{1,292}$ = 7.04, p<0.01). Meanwhile, plants attacked by aphids (H and HAM) produced true leaves slower than those not attacked (C and AM) ($F_{1,292}$ = 8.481, p<0.01). Aphid attacked plants (H and HAM) in the second and third generations produced true leaves slower than non-attacked (C and AM) plants, with this being the opposite in the first and fourth generations, leading to a significant interaction term ($F_{3,292}$ = 29.546, p<0.001) (Figure 3.2).

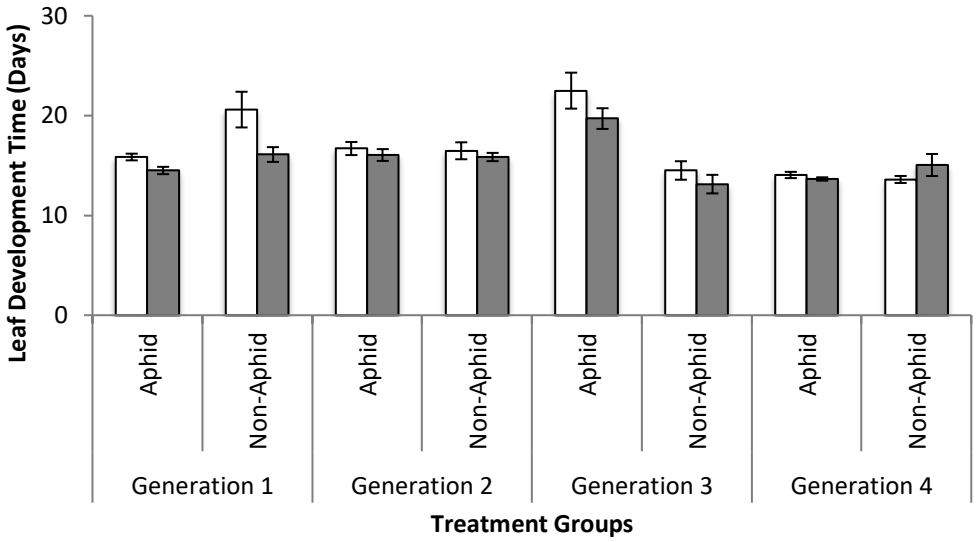

Figure 3.2 Mean time taken for plants to develop true leaves from date of sowing (true leaf development time) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

The key finding of these results was the presence of aphids decreased the time taken from planting for the plant to produce buds ($F_{1,296}$ = 13.891, p<0.001). Bud development time was significantly influenced by the interaction of generation and aphid treatments ($F_{3,296}$ = 75.923, p<0.001). In generations one and two, aphid attacked plants (H and HAM) took less time to produce buds than non-attacked plants (C and AM). The opposite was true in generations three and four with aphid attacked plants (H and HAM) producing buds slower than non-attacked plants (C and AM) (Figure 3.3).

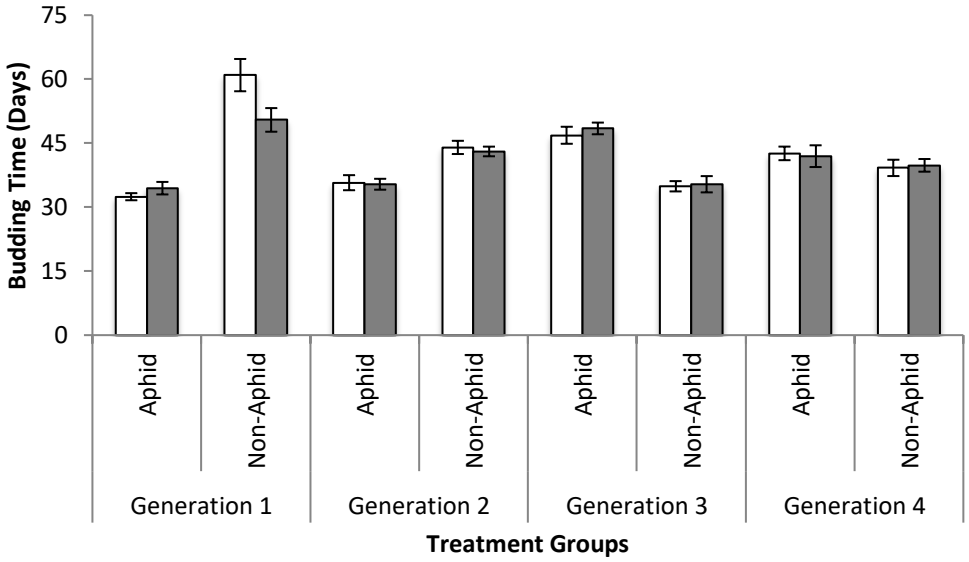

Figure 3.3 Mean time taken for bud development from date of sowing (budding time) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

A key finding was that plants in generation two took a shorter time to produce flowers from date of sowing than other generations ($F_{3,289}$ = 6.138, p<0.001). Over all generations, the presence of aphids decreased the time taken to flower ($F_{1,289}$ = 7.063, p<0.01). In generation one and two, aphid attacked plants (H and HAM) took a shorter time to flower than the non-attacked plants (C and AM). However, non-attacked plants (C and AM) in the third and fourth generations took a shorter time to flower than aphid attacked plants (H and HAM), leading to a significant interaction term ($F_{3,289}$ = 58.338, p<0.001) (Figure 3.4).

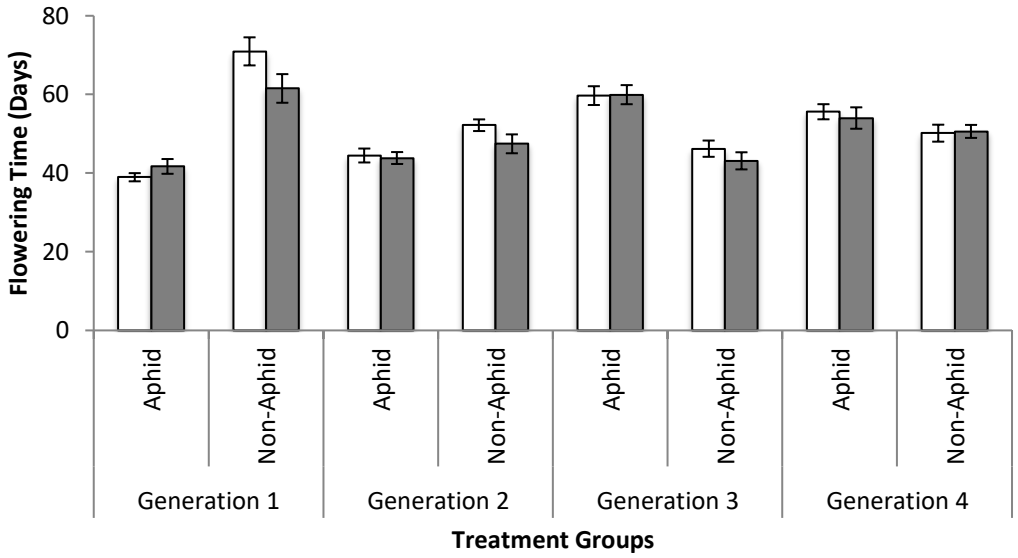

Figure 3.4 Mean time to develop flowers from date of sowing (flowering time) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

The key finding was that plants in generation one produced seeds in a shorter time than plants in other generations ($F_{3,261}$ = 7.690, p<0.001). Over all generations, aphid attacked plants (H and HAM) produced seeds in a quicker time than the non-attacked plants (C and AM) ($F_{1,261}$ = 9.379, p<0.01). Over all generations, the presence of mycorrhizas increased the seeding time when compared to non-mycorrhizal plants ($F_{1,261}$ = 4.066, p<0.01). There was a significant interaction

between generations and aphid treatments in time taken for plants to seed ($F_{3, 261}$ = 58.325, p<0.001). In generation one, the presence of aphids slowed the seeding time, especially in HAM. Plants from the AM were slower than plants from any other treatment group in that generation. In generation two, the presence of aphids also sped up seeding time. Plants in C had a slower seeding time than those in AM. The H group had a faster seeding time than any other treatment group within generation two. In generation three, the presence of aphids slowed seeding time. The AM plants were slower than C. In generation four, aphid presence on the plants also slowed seeding compared to plants with no aphids present. C had a slower seeding time than AM but HAM plants had the slowest seeding time of all within generation four. Generation one HAM plants had the quickest seeding time, while AM plants in generation one had the slowest seeding time. All of this led to a significant interaction in the analysis ($F_{3,261}$ = 4.013, p<0.01) (Figure 3.5).

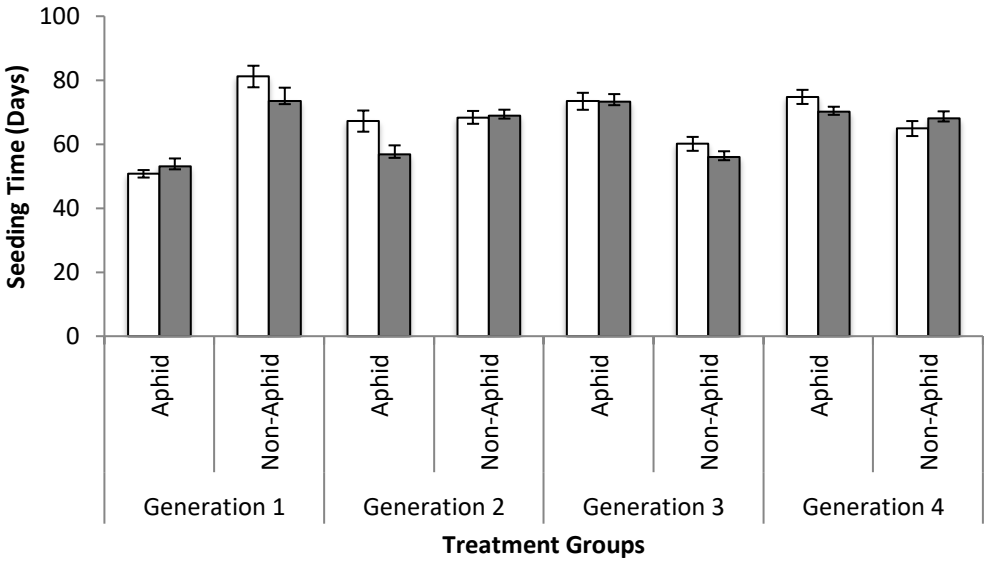

Figure 3.5 Mean time for plants to set seed from date of sowing (seeding time) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

3.3.2 Changing treatments and the impact on development time

Development time of generation two C plants was compared to 2A, 2B and 2C plants (Figure 2.1). The same occurred with generation three C plant development time being compared to development time of 3A, 3B and 3C plants and generation four C plant development time being compared to 4A, 4B and 4C plant development time (Figure 2.1).

Generation two control (C) plants were significantly different to 2A, 2B and 2C plants for leaf development, budding, flowering and seeding development time. However, germination time did not differ significantly between generation two control (C) plants and 2A, 2B and 2C plants. Interestingly, the only significant difference in development time between generation three control (C) plants and 3A, 3B and 3C was in the time taken to germinate. Development time between generation four control (C) plants and 4A, 4B and 4C plants differed significantly from germination through to the time take to set seed (Table 3.1).

	GENERATION TWO			**GENERATION THREE**			**GENERATION FOUR**		
	F	**P**	**Summary**	**F**	**P**	**Summary**	**F**	**P**	**Summary**
Germination	1.425	>0.05		**6.81**	**<0.05**	**3A, 3B & 3C increased time**	**4.2**	**<0.05**	**4A, 4B & 4C decreased time**
Lead development	**4.435**	**<0.05**	**2A, 2B & 2C decreased time**	0.02	>0.05		**8.27**	**<0.01**	**4A, 4B & 4C decreased time**
Budding time	**41.78**	**<0.001**	**2A, 2B & 2C decreased time**	0.31	>0.05		**10.9**	**<0.01**	**4A, 4B & 4C decreased time**
Flowering time	**5.658**	**<0.05**	**2A, 2B & 2C decreased time**	0.52	>0.05		**9.06**	**<0.01**	**4A, 4B & 4C decreased time**
Seeding time	**12.7**	**<0.001**	**2A, 2B & 2C decreased time**	0.02	>0.05		**9.83**	**<0.01**	**4A, 4B & 4C decreased time**

Table 3.1 A, B and C treatments from each generation compared with the generations control treatment to determine whether the effects being seen are from within generation or transgenerational effects. Significant differences indicated by bold text.

Mycorrhizal plants in generation two from mycorrhizal parents took longer to produce buds from sowing than their controls (treatment 2A) ($F_{1,23}$=11.49, p<0.01). This difference in time taken to achieve life stages was seen from the budding with the generation two AM being slower to produce flowers ($F_{1, 25}$= 12.06, p<0.01) and seeds ($F_{1,21}$= 6.644, p<0.05) than 2A plants (Figure 3.6).

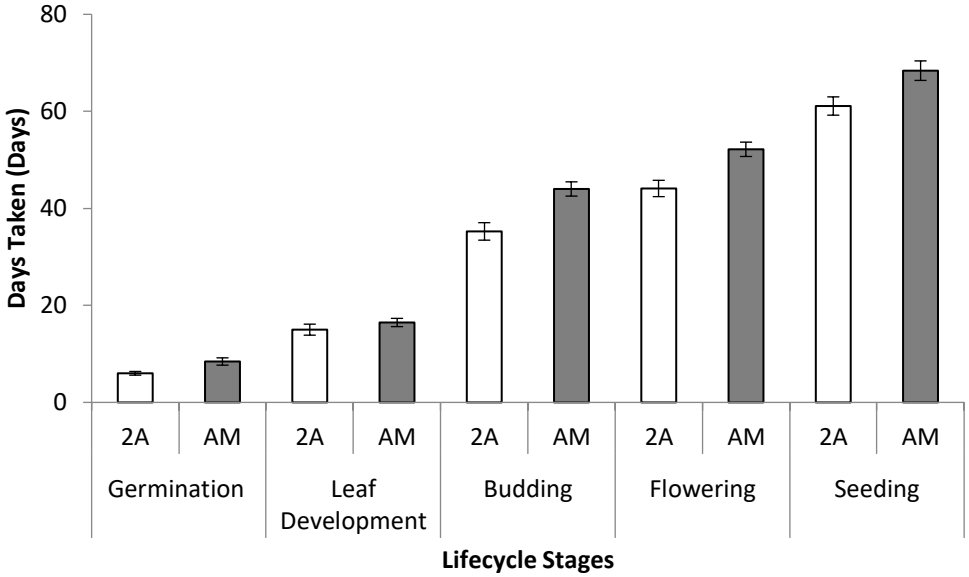

Figure 3.6 Mean time for 2A and generation two AM plants to reach each life stage. White represents 2A and grey represents generation two AM. Bars represent means +/- SE.

Aphid plants in generation two from aphid treated parents took less time to produce leaves from sowing than their controls (treatment 2B) ($F_{1, 28}$ = 13.66, p<0.001) (Figure 3.7).

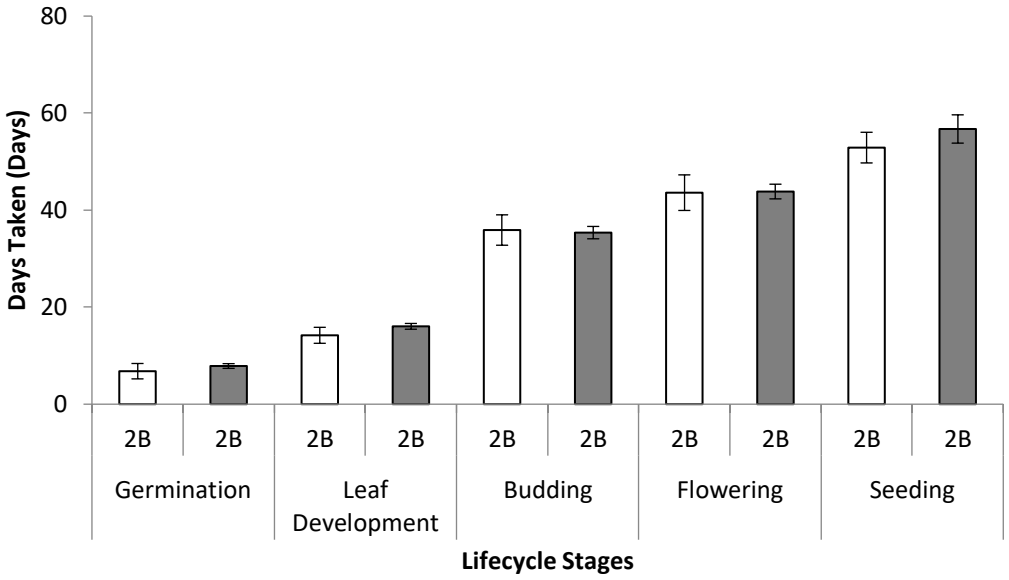

Figure 3.7 Mean time for plants from 2B and plants from generation two H to reach each life stage. White represents 2B and grey represents generation two H. Bars represent means +/- SE.

Combined mycorrhizal and aphid plants (HAM) in generation 2 from combined parents took longer to germinate ($F_{1, 27}$ = 14.3, P<0.01), develop true leaves ($F_{1, 28}$= 45.31, p<0.001), and set seed ($F_{1, 26}$ = 5.264, p<0.05) from sowing than their controls (treatment 2C) (Figure 3.8).

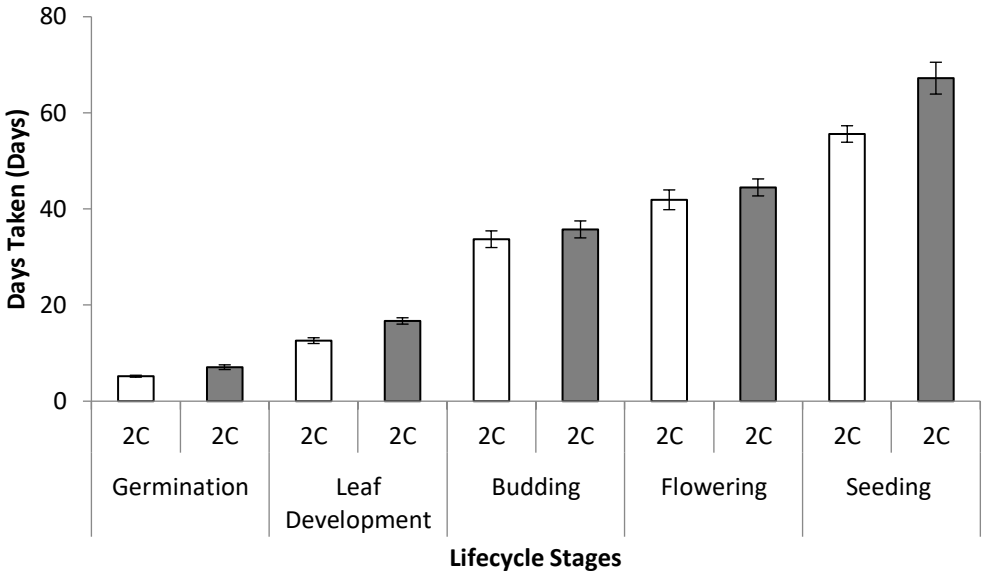

Figure 3.8 Mean time for plants from 2C and plants from generation two HAM to reach each life stage. White represents 2C and grey represents generation two HAM. Bars represent means +/- SE.

Plants from generation three AM and 3A showed no significant difference in time taken to reach each life stages (data in Appendix I).

Aphid attacked plants in generation three from aphid attacked parents took longer to develop true leaves from sowing than their controls (treatment 3B) ($F_{1, 28}$=5.943, p<0.05). This difference never recovered as the plants developed, meaning that bud development ($F_{1, 28}$ = 42.6, p<0.001), flower development ($F_{1, 26}$ = 27.56, p<0.001) and setting seed ($F_{1, 23}$ = 23.94, p<0.001) took significantly longer in plants from generation three H than plants from 3B (Figure 3.9).

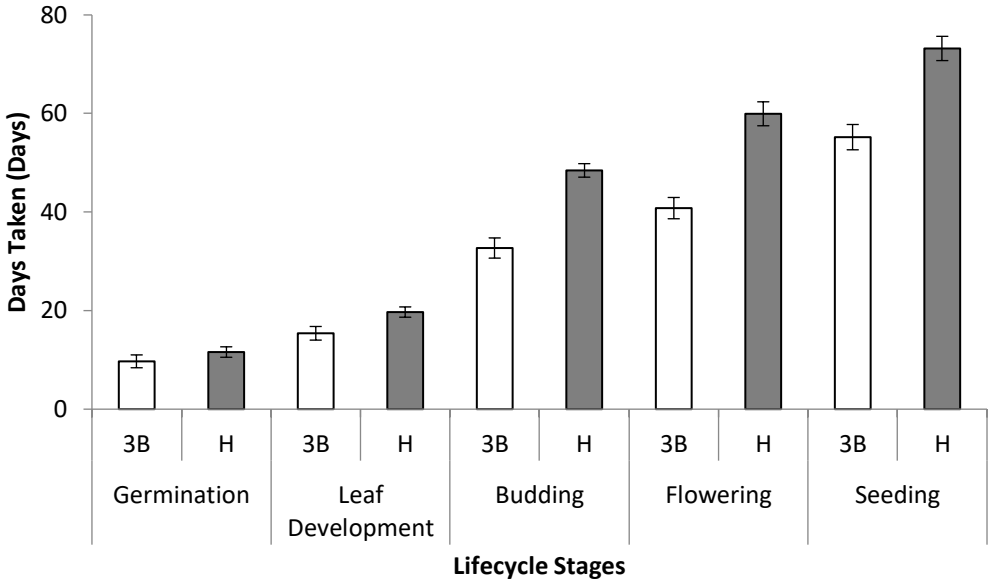

Figure 3.9 Mean time for plants from 3B and plants from generation three H to reach each life stage. White represents 3B and grey represents generation three H. Bars represent means +/- SE.

Combined mycorrhizal and aphid (HAM) plants in generation three from HAM parents took longer to develop true leaves from sowing than their controls

(treatment 3C) ($F_{1, 26}$=512.07, p<0.001). This difference did not change as the time taken for plants to develop buds ($F_{1, 28}$ = 44.07, p<0.001), produce flowers ($F_{1, 28}$= 19.11, p<0.001 and set seed ($F_{1, 25}$ = 14.83, p<0.001) took significantly longer in plants from generation three HAM than the plants from 3C (Figure 3.10).

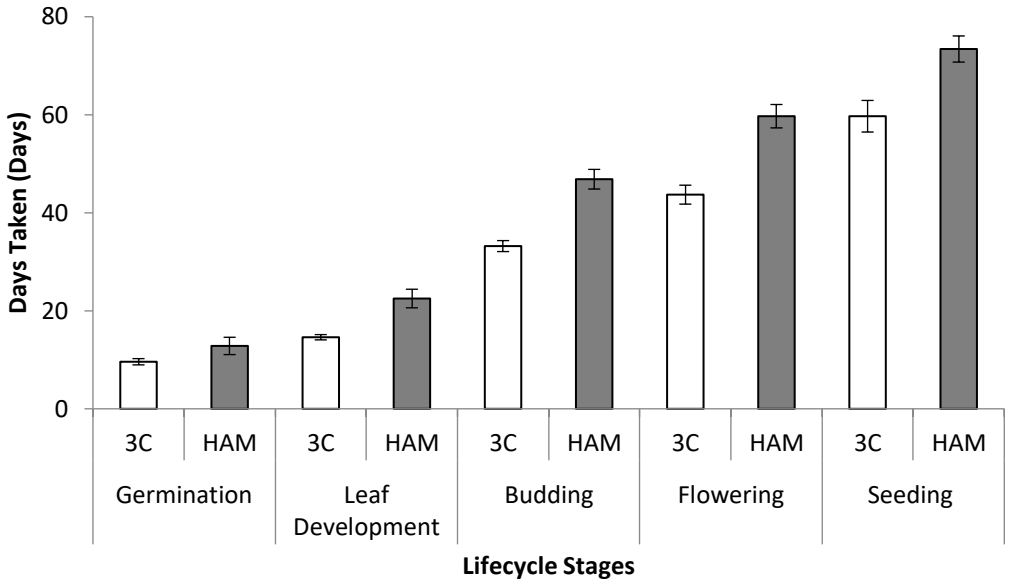

Figure 3.10 Mean time for plants from 3C and plants from generation three HAM to reach each life stage. White represents 3C and grey represents generation three HAM. Bars represent means +/- SE.

Mycorrhizal plants in generation four from mycorrhizal parents did not differ in their development times until the end of their lifecycle when compared to their controls (treatment 4A). Seed development was significantly quicker in 4A than the generation four AM ($F_{1, 27}$ = 5.793, p<0.05) (Figure 3.11).

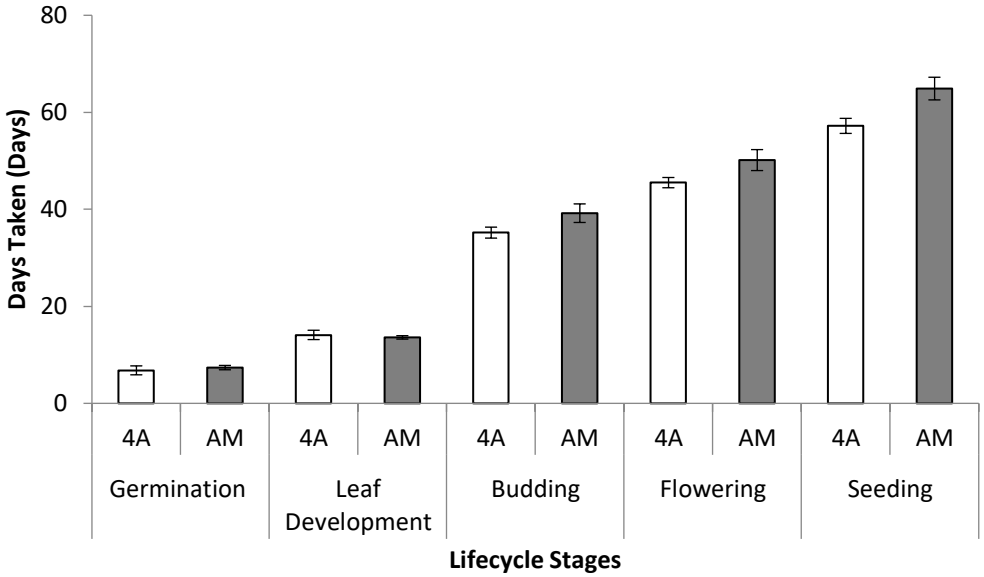

Figure 3.11 Mean time for plants from 4A and plants from generation four AM to reach each life stage. White represents 4A and grey represents generation four AM. Bars represent means +/- SE.

Aphid treated plants in generation four from aphid treated parents took longer to develop from sowing than their controls (4B). From germination the plants from generation four H took longer to reach each life stage than the plants from 4B ($F_{1, 26}$= 10.16, $p<0.01$). This continued through true leaf development ($F_{1, 28}$ = 37.88,

p<0.001), bud development ($F_{1, 28}$ = 27.06, p<0.001), flower development ($F_{1, 28}$ = 24.6, p<0.001) and setting of seeds ($F_{1, 25}$ = 42.36, p<0.001) (Figure 3.12).

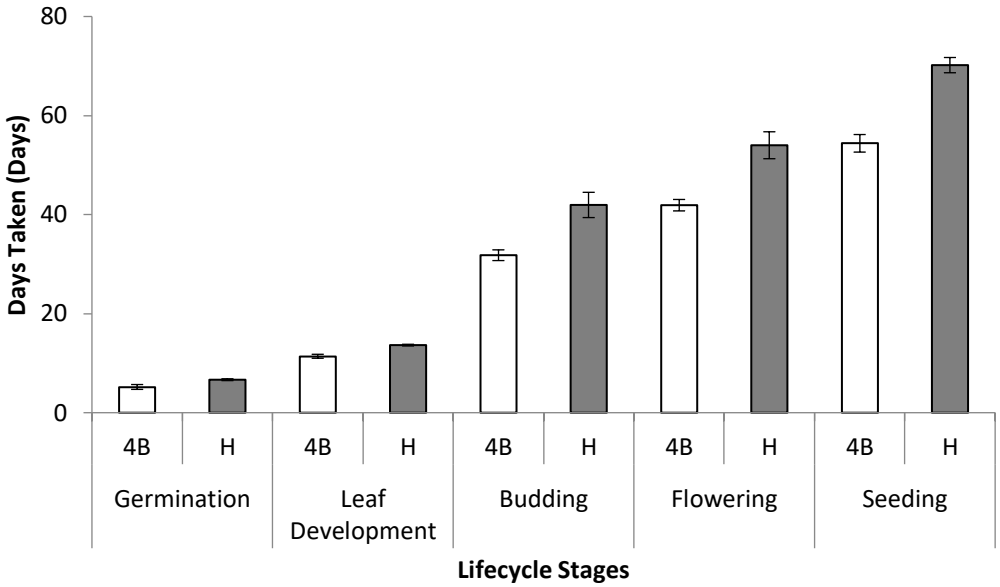

Figure 3.12 Mean time for the plants from 4B and plants from generation four H to reach each life stage. White represents 4B and grey represents generation four H. Bars represent means +/- SE.

Combined mycorrhizal and aphid (HAM) plants in generation four from HAM parents took longer from bud development than their controls (treatment 4C). The seeds from 4C were significantly quicker in bud development ($F_{1, 28}$ = 28.82,

p<0.001), flower development ($F_{1, 28}$= 23.89, p<0.001) and seed production ($F_{1, 27}$= 30.86, p<0.001) than the plants from generation four HAM (Figure 3.13).

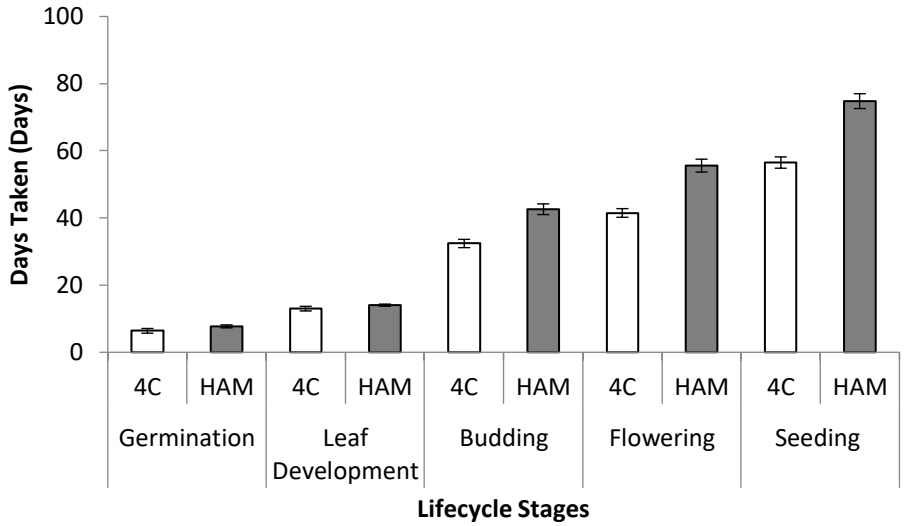

Figure 3.13 Mean time for plants from 4C and from generation three HAM to reach each life stage. White represents 4C and grey represents generation four HAM. Bars represent means +/- SE.

3.4 Discussion

3.4.1 Germination time

Interestingly, analysis of germination time of the main experiment showed a significant difference between the sequential generations of *S. vulgaris.* Germination time increased between generations one and three, but decreased again in generation four. The change in time to germinate may be due to epigenetic changes in genes regulating seed dormancy through deacetylation and methylation of DNA (Nonogaki, 2014). These epigenetic changes can remove or put into place the inhibiting factors that prevent or slow germination (Nonogaki, 2014). The environment was kept the same for each generation, so the plant could be passing on information for a better survival in the same environment (Latzel *et al*., 2010). H and HAM plants also increased germination time in the second and fourth generations when compared to C and AM plants. This was reversed in the first and

third generations. The first generation plants had not come from plants that were affected by insect herbivory, nor were they experiencing herbivory at the time of germination. The third generation plants may not have had the parental effects for defence passed on with the parental effects being lost after the second generation. An increase in the second and fourth generations could be due to plants in the previous generations using parental effects to increase plant defences and to not speed up germination. There did not seem to be any cumulative effects with the results fluctuating between generations. It has been shown that seedlings from parental plants under stress are able to delay development (Aarssen & Burton, 1990), so the parental generation being stressed by insect herbivores may cause a delay in the development of seedlings. Germination time is linked to seed dormancy and it could be that the parental plants are able to increase seed dormancy so the seeds do not germinate in the same environment immediately (Bernareggi *et al.*, 2016). Third generation seeds may have had a slower germination time as the parental (second generation) plants had lost the parental effects, or the plants had experienced two generations of the same treatments, which could cumulate to cause an effect. A parental effect could be that there are more resources to place into the seed meaning more nutrients for the seedling to develop (Hanley, 2004). Insect herbivory has been linked to a decrease in seed size, with less resources being placed into the seed (Hanley, 1998). So, parental plants that are less affected by insect herbivory are likely to be better equipped to increase resource allocation to the seeds (Hanley, 1998), therefore these seedlings may germinate faster.

3.4.1.1 Changing treatments and the impact on germination time

Germination time in plants grown in 3A, 3B, 3C, 4A, 4B and 4C was significantly different to generation two control (treatment C) and generation three control (treatment C) respectively. This suggests that there were parental effects being passed between treated parental plants to the offspring from generation two and three parents. There were no significant effects being passed from the first to second generation. These results suggest that either parental effects do not always

occur between generations and are imperfect or that the effects are visible after multiple generations.

Plants from generation three HAM were slower to germinate than the 3C plants. Growing with the same treatment (aphid and mycorrhizal presence) as the parent could hinder the offspring's development times. Plants from generation four H were slower to germinate than 4B. Although the treatments were kept the same in the parental generations over multiple generations, there did not seem to be any cumulative effects. Germination time differed between seeds grown in the same conditions as the parental plants and seed grown in different conditions to the parental plants, suggesting that the environment is not playing a role in the changes to development time. Instead of the same environmental conditions causing the changes in germination time, it could be due added treatments expended the energy of the parental plant to cause germination time to differ. The results imply that there was a transgenerational interaction occurring between generations and aphid presence.

3.4.2 True leaf development time

Leaf development in generation four was significantly quicker than all the other generations. There seems to be no cumulative effects, as development time did not reduce gradually over all generations but fluctuated. It could be that there are only short-term parental effects that ensure quick germination but disappear before the seedlings develop true leaves or nutrient availability to the seedling masks the effects (Hanley, 2004). The seedling depends upon nutrients stored mostly in the cotyledon. Therefore, the nutrient reserves may be the reason that the development time is changed and transgenerational effects differing could be altering the seed nutrient reserves (Koide & Lu, 1995). The treatment groups did play a role in changes to true leaf development. Over all the generations, AM and HAM plants that were always treated with mycorrhizas produced true leaves slower than C and H plants. The percentage root colonisation of mycorrhizas was low (Section 7.3.1) so this could affect what was being seen in the growth of *S. vulgaris*. However, low colonisation can still benefit plants (Gange & Ayres, 1999) and it may

be that *S. vulgaris* is a plant that requires only low colonisation levels to benefit from the symbiosis. Colonisation percentage could also be influenced by the species of mycorrhizas. A mixture of five species was used for these experiments so one species or a mixture may have colonised the roots, causing different effects even in percentage colonisation (Robinson-Boyer *et al.*, 2014). From previous research, it would have been predicted that mycorrhizal colonised plants would have a faster development time. Association with mycorrhizas means that the plant is able to gain extra nutrients, particularly phosphorus. The mycorrhizal colonisation in the previous generation may increase the nutrients passed onto through the seed (Koide & Lu, 1992; Koide & Lu, 1995). However, it has been shown that mycorrhizal colonisation can cause a depression in growth of the host plant (Jin *et al*., 2017), so this depression in growth may have been the cause of the delay. Mycorrhizal colonisation may also become parasitic (Purin & Rillig, 2008), so at the beginning of plant development the mycorrhizas may be taking more nutrients from the plant than the plant is gaining from the mycorrhizas and so delaying development. Third generation H and HAM plants were the slowest to produce true leaves, and this may be a random occurrence, as this was not seen in any other generation.

3.4.2.1 Changing treatments and the impact on true leaf development time

Time taken to develop true leaves in plants from 2A, 2B, 2C, 4A, 4B and 4C was significantly different to generation two control and generation four control plants respectively. This suggests that there are parental effects being passed from treated parental plants to the next generation and that the effects being seen are not just within-generation effects. The parental effects appear between the first generation parents to the second generation progeny and from the third generation parents and the fourth generation progeny but not between second generation parents and third generation progeny. This is highly suggestive that transgenerational effects for leaf development are temporary over one generation and not seen over multiple generations. However, if ecological studies are growing two generations to combat parental effects (Latzel, 2015), the parental effects may reappear, as seen in these results.

Plants from generation two HAM were slower to develop true leaves than the plants from 2C. This could be a knock-on effect from the slow germination. It could also be that seed size is playing a large role in the development of these plants. Seed size has been explored in Section 5.3.1.3. The seed is used as a nutrient reserve, which a seedling depends upon to develop (Hanley, 2004). A larger seed suggests a larger nutrient reserve that could be used to increase development time of seedlings. Seed size has also been linked to the epigenetic change of DNA methylation (Varga & Soulsbury, 2017). In that study it was found that methylation of DNA by the parental plant caused an increase in the seed size. Meanwhile, insect herbivory on the parent plant can reduce seed size (Hanley, 1998). In the third generation, H and HAM took longer to develop true leaves than 3B and 3C plants. There was no difference in germination time, suggesting that development was delayed between germination and true leaf development. This delay could be the plant expending more energy on plant defences than growth. Plant chemical defences are energy expensive (Neilson *et al.*, 2013), so the plant may not be able to focus upon growth while making these chemicals. The same occurred in generation four H treated plants compared to 4B plants. This showed a general pattern with results repeating over multiple generations. The presence of aphids and mycorrhizas may delay true leaf development with symbiosis of the mycorrhizas taking carbon from the plant and the aphids taking sugars from the plant's phloem.

3.4.3 Bud development time

Bud development was affected by the presence of aphids with generation three H and HAM plants being the slowest to produce buds. The budding time fluctuated between generations, so it does not appear to be a cumulative effect of aphid herbivory delays budding by taking nutrient from the plant. The nutrient fluctuations caused by the insect herbivory could delay plant development, which could reduce the energy the plant has to develop buds (Myers & Sarfraz, 2017), thus causing a development delay.

3.4.3.1 Changing treatments and the impact on bud development time

Time taken to develop buds in plants from 2A, 2B, 2C, 4A, 4B and 4C was significantly different to generation two control and generation four control plants (treatment C) respectively, which was also observed with true leaf development. This further suggests that if ecological experiments are trying to control for transgenerational effects for development time then one generation is needed and not two as suggested in Latzel (2015).

When comparing seeds that were subjected to control and treated conditions, generation two AM, generation three H, generation three HAM, generation four H and generation four HAM plants took longer to produce buds than 2A, 3B, 3C, 4B and 4C respectively. In some cases this may be a knock-on effect from the plants in the treatment group having slower development time in general before bud development (generation three H, generation three HAM and generation four H plants) than the plants in their controls (3B, 3C, 4B). But generation four HAM plants were not affected by the slow development so something must have specifically delayed budding. It could be the interaction of mycorrhizal colonisation and insect herbivory reducing the resources that the plant has for growth, especially reproduction. However in generation two H treated plants had quicker budding time than the 2B plants. It could be that the plants had more energy to put into reproduction and growth, or that there are parental effects passed from the first generation those speeds up development. The generation two H plants would have experienced herbivory for both generations, so it could be that the effect of herbivory accumulates to produce a quicker bud development time.

3.4.4 Flower development time

Flowering is important for reproductive success, so time taken to flower is important to the plants as the shorter the time the longer the plants have to

reproduce before dying. Time taken to flower decreased between generation one and two. It was assumed that plants would speed up time throughout all generations until flowering to increase reproduction time but this did not occur. However, flowering has been explored with epigenetic changes to chromatin (Sun *et al.*, 2014). A delay in flowering time was found to be an epi-mutant of the flowering wageningen (*FWA*) gene in *Arabidopsis thaliana* (Soppe *et al*., 2000), which led to prevention or delay of proper flowering. This suggests that the *S. vulgaris* plants after generation two may have experienced epigenetic changes especially to the *FWA* gene, as the plants flowering was delayed. Insect herbivory seems to have a large effect on time taken to flower on the *S. vulgaris* grown in the experiments. Insect herbivory has been shown to consistently cause a decrease in time taken to flower (Jordan *et al.*, 2015), but in these experiments the presence of aphids caused an increase in time taken to flower. However, the decreases were dependent upon generation. The later generations three and four had increased flowering time in H and HAM plants compared to C and AM plants in the same generations. This could be where parental effects helping to alter the effects of aphid herbivory on flowering time. The parental effect seems to be only present between two generations and disappear.

3.4.4.1 Changing treatments and the impact on flower development time

Time taken to develop flowers in plants from 2A, 2B, 2C, 4A, 4B and 4C was significantly different to generation two control and generation four control plants respectively. This pattern was also observed in true leaf development and bud development.

Plants from generation two AM, generation three H, generation three HAM, generation four H and generation four HAM were slower to produce flowers than their control counterparts (2A, 3B, 3C, 4B and 4C). This showed a general pattern, with the results being repeated over different generations and treatments. It seems that mycorrhizal presence and/or aphid presence can alter flowering time. Mycorrhizal colonisation has been shown to change flowering time with certain species of mycorrhizal fungi used caused the change in flowering time (Liu *et al.*,

2017). Specific species of mycorrhizas would cause a delay in flowering time, while other species would speed up the time taken to flower (Liu *et al.*, 2017). The experiments in this thesis used a mixture of mycorrhizal species and the roots were not analysed to find out which specific species was colonising the plant, so it could be that the difference in flowering time between mycorrhizal plants is due to the species of mycorrhizas colonising the roots. It could also be that these delays are knock-on effects from previous slow development.

3.4.5 Seed development time

There were significant interactions between generation, aphid presence and mycorrhizal presence in the time to produce seed. Speeding up the time to set seed means that the plant can create the next generation faster. Over all generations AM and HAM plants had a slower seed development time. This was not expected as mycorrhizal presence allows the plant to have greater access to nutrients and growth was shown increased in the mycorrhizal plants compared to the non-mycorrhizal plants (Wright, 2005). Mycorrhizal presence has been shown to increase a plants time flowering, which would cause a delay in time to set seed (Young *et al*., 2015). However, the percentage root colonisation of mycorrhizas was low (Section 7.3.1) so this could affect what was being seen in the growth of *S. vulgaris*. A mixture of five species was used for this experiment so one species may be colonising the roots or a mixture causing different effects even in percentage colonisation (Robinson-Boyer *et al.*, 2014) and so on the effects upon plant development time. Aphids feeding on the phloem have been shown to consistently delay flowering time (Jordan *et al.*, 2015), so it may be that there is a knock on effect with delays in time taken to seed. Aphids could have caused a delay in the setting of seeds with the plants having reduced nutrition so taking longer to develop. The changes over generations may be the parental effects taking place to alter the development time. Time to flower is key for reproduction, so the plants may be elongating flowering time, so waiting to produce more flowers before setting seed. There did not seem to be any cumulative effects over multiple

generations even though seed development time was delayed after generation one.

3.4.5.1 Changing treatments and the impact on seed development time

Time taken to develop seeds in plants from 2A, 2B, 2C, 4A, 4B and 4C was significantly different to generation two control and generation four control plants respectively. This was also observed in true leaf, bud and flower development time. This does suggest that development time parental effects should be controlled with the growth of one generation prior to the start of ecological studies (Latzel, 2015). Plants from generation two AM, generation three H, generation three HAM, generation four H and generation four HAM were all slower at producing seeds than their control counterparts (2A, 3B, 3C, 4B and 4C). This showed a general pattern with the results being repeated between generations and treatment groups. The treatment groups had slower development time than their control counterparts, so maybe the treatments of aphids and mycorrhizas are delaying the development time more than any other potential effect. Between generation three and four H and 3B and 4B, time to set seed was decreased, so maybe there is a cumulative effect from multiple generations of aphids that is speeding up seed development. Generation three and four HAM and 3C and 4C were different with the 4C speeding up time to develop seeds but generation four HAM slowing time to develop. The herbivory and mycorrhizal treatments within the generation could be interacting with the parental effects to cause some of the effects seen. However, generation two HAM treated plants were quicker at producing seeds than the plants grown from 2C. The effects seen on seed development time in generation two HAM plants may have been caused by within-generation effects caused by the specific species of mycorrhiza colonising the plant. A study by Liu *et al.* (2017) found that specific species of mycorrhiza could cause development time to speed up. This study used a mixture of five mycorrhizas, but not necessarily all of the mycorrhizas would have colonised the same plant at once.

3.5 Conclusion

The hypothesis of parental effects in *S. vulgaris* changing development time was upheld, with both aphids and mycorrhizas producing different times for the next generation to reach certain stages of a plant's lifecycle. Epigenetic effects have already been linked to changes in germination time and flower development time in previous experiments (Nonogaki, 2014; Sun *et al.*, 2014), but other development stages showed significant transgenerational effects including time to develop true leaves and to set seed that have not been linked to specific epigenetic changes. The secondary hypothesis of mycorrhizal and aphid presence causing changes in *S. vulgaris* development time was also upheld, especially with the interaction in development time for setting seed. Overall, the generation of the plant and aphid presence affected development time of the plants more than mycorrhizal colonisation. Mycorrhizal colonisation of the plant roots was very low which could be why mycorrhizal colonisation did not affect development time as much as aphid presence and the generation. Transgenerational effects have been seen specifically in germination and flowering in previous literature but these experiments show that development of true leaves and timing of setting seeds should be explored further.

Chapter Four

Parental effects on mature plant size of *Senecio vulgaris*

4.1 Introduction

This experiment investigated whether final plant size measurements are affected between generations by the presence of insect herbivores and/or mycorrhizal fungi. The final plant size measurements in this thesis were defined as plant height, dry biomass, leaf number, flower number and mean flower size. It addressed the first theme of this project; which environmental conditions cause parental effects to occur. It specifically explored three of the objectives; are parental effects being passed between generations of *Senecio vulgaris*; does the presence of insect herbivores affect plant development over multiple generations and does the relationship between plant and mycorrhizas affect plant development over multiple generations.

Transgenerational effects on final plant size traits have been explored in *S. vulgaris* on final leaf number (West, 1995); in *Achnatherum inebrians* on plant height, dry biomass and leaf number (Herman *et al.*, 2012); and *Calluna vulgaris* (common heather) for plant height (Walter *et al.*, 2016). Interestingly there are some final plant size parameters that have not been explored in the context of transgenerational effects. There is no literature that explores effects on flower size and little information on flower number.

There are limited studies into life history events and transgenerational effects in *S. vulgaris.* West (1995) found that phosphorus levels, rust infection and mycorrhizal colonisation in the previous generation affected the leaf number of *S. vulgaris*. There was a significant increase in leaf number between the two generations. The effects of rust infection on leaf production were less obvious in mycorrhizal plants grown in medium phosphorus than non-mycorrhizal plants grown in the same P conditions (West, 1995). The findings suggest that leaf number can be affected by multiple environmental conditions in the previous generation.

Changes to dry biomass have been linked to transgenerational effects in previous literature. Over multiple generations the offspring of drought stressed *Polygonum persicaria* (lady's thumb) had a larger dry biomass than the offspring of non-stressed plants (Herman *et al.*, 2012). In that study, the effects of drought stress were cumulative over two generations. Dry biomass was increased over multiple generations of control and salt stressed *Arabidopsis thaliana* (Groot *et al.*, 2016). This experiment again suggests that transgenerational effects on life history events

can occur over multiple generations, though the effects did not seem to be cumulative (Groot *et al.*, 2016).

Transgenerational effects can influence multiple final plant size parameters in the same plant with different outcomes, e.g. height increasing but leaf number decreasing in the grass *Achnatherum inebrians* (Xia *et al*., 2018). This suggests as many plant final size parameters as possible should be monitored in plants to discover the true extent of parental effects in plants such as *S. vulgaris*.

Plant biomass of *A. inebrians* was affected by endophyte presence over multiple generations (Xia *et al.*, 2018). The endophyte, *Epichloë gansuensis*, (which is transmitted vertically via the seeds) caused higher biomass in drought stressed plants over multiple generations, probably due to transgenerational effects and *E. gansuensis* causing an increase in the offspring's height but decreased plant leaf number (Xia *et al*., 2018). Vertical transmission has also been shown to occur in *S. vulgaris* (Hodgson *et al.*, 2014), so it could be that endophytes are a mechanism for transgenerational effects upon certain life history traits.

Studies into transgenerational effects on plant height have found that it was not significantly altered between generations of barley (*Hordeum vulgare*) (Walters & Paterson, 2012) or in *C. vulgaris* (Walter *et al.*, 2016). This suggests that there would be no transgenerational effects on *S. vulgaris* plant height. However, *C. vulgaris* is a dwarf shrub species and barley is a crop plant, so the type of plant may have an effect on which final plant size parameters are influenced and how they are affected.

Leaf number was decreased in *Arabidopsis thaliana* from heat stressed parents, but the leaves were larger in size (Migicovsky *et al.*, 2014), so there may be transgenerational effects altering leaf number in *A. thaliana*. A decrease in leaf number of *Brassica rapa* was also seen when the plant was attacked by herbivores with the decrease lasting for multiple generations (Kellenberger *et al.*, 2018). In the same study, it was found that leaf number was not affected by insect herbivores either within a generation or between generations (Kellenberger *et al.*, 2018). This suggests that in *S. vulgaris* the transgenerational effects of herbivory in the previous generation could last over multiple generations. Any changes to leaf

number may not be caused by the aphid infestation within the generation of plant being observed.

Mycorrhizal colonisation was found to increase plant height, flower size and flower number in *Leucanthemum vulgare* (oxeye daisy) (Gange *et al.*, 2005) and in *Chrysanthemum morifolium* (florist's daisy) root colonisation by AMF increased plant height and flower size (Sohn *et al.*, 2003). Meanwhile, *Abutilon theophrasti* (velvetleaf) showed increased leaf number in mycorrhizal colonised plants compared to non-colonised plants, but the leaf area was more affected by AMF colonisation (Lu & Koide, 1994). Flower number, height and dry biomass of *Crossandra infundibuliformis* (firecracker flower) were increased when the plant was colonised with AMF (Vaingankar & Rodrigues, 2014). However in *Cucumis sativus* (cucumber) mycorrhizal colonisation had no effect on plant biomass, flower size or flower number (Barber *et al.*, 2013). There seems to be multiple studies in many species that suggest that mycorrhizal colonisation can affect the final plant size and there are links to mycorrhizal colonisation in the parental generation having effects on the offspring of *S. vulgaris* (West, 1995). This does suggest that there would be a parental effect caused by mycorrhizal colonisation on the final plant size of *S. vulgaris*.

Parental effects have been seen to affect plant life history events in other species (Holeski *et al.*, 2013; Migicovsky *et al*., 2014; Walter *et al.*, 2016) so it was hypothesised that parental effects would alter *S. vulgaris* height, dry biomass, leaf number, flower number and flower size. In addition, if this hypothesis were upheld, the addition of aphids and/or mycorrhizal fungi may cause further effects on *S. vulgaris.* Overall it was hypothesised that the environment of the previous generation affects the final parameters of the progeny generation.

4.2 Methods

4.2.1 Main experiment final plant size measurements

The main experiment was to explore whether conditions experienced by the previous generation affects the progeny generation's mature plant size. It involved growing four generations of *S. vulgaris* under the same environmental conditions (Section 2.2). Each generation had a total of 80 plants grown. There were four separate treatment groups with 20 plants in each group per generation. The four treatment groups were C, AM, H and HAM (Section 2.2; Figure 2.1). In each generation, each plant was measured to obtain results.

4.2.1.1 Flower number

This involved counting each flower head that produced seeds and counting the flowers that had not produced seeds when the plant was harvested at the end of each generation. Those numbers were combined to obtain the total flower number.

4.2.1.2 Leaf number

The numbers of true leaves on each plant were counted at the time of harvest.

4.2.1.3 Flower size

Five random flowers per plant were chosen to measure when the plant was still growing. Digital callipers were used to measure the flowers from the tip to the bottom. The measurements were averaged to gain the mean flower size per plant.

4.2.1.4 Height

When the plants were harvested, each individual plant was measured using digital callipers to give an accurate height. The plants were measured from the tip of the highest flower to the start of the plant roots.

4.2.1.5 Dry biomass

Once the plants had been harvested and other measurements taken, the plants were dried at 60^{o}C in a drying over for 48 hours. The plants were placed into hole punched envelopes for this drying process so all vegetation could be weighed. Once the plants were dried, they were taken out of the envelopes and weighed to obtain the dry biomass.

4.2.1.6 Statistical analysis of main experiment

Analysis of mature plant size parameters of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation. Differences in the measured parameters over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence and generation as the main effects.

4.2.2 Comparing changes to treatments and the impact on mature plant size

Changes to treatments and the impact on development time were set up following the protocol explained in Section 2.4 and Figure 2.1. Each generation from generation one had seeds from treated plants (AM, H and HAM) grown under control conditions (Section 2.4; Figure 2.1). There were ten plants grown per treatment and three treatment groups used per generation. In each generation, each plant had all the parameters defined in Section 4.2.1 measured in the same way.

Differences between the plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment, i.e. generation two control was compared to 2A, 2B & 2C.
The results were also compared to the next generation plants grown under treated conditions, i.e. generation two AM plants were compared to 2A.

4.2.2.1 Statistical analysis of comparing changes to treatments

Analysis of development time of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
Differences in the measured mature plant size parameters over treatment groups compared to controls were tested using a one-way, repeated measures ANOVA, employing aphid absence/presence or AMF absence/presence as the main effects for the control versus AM experiment and H experiment. Differences in the measured life history events over HAM treatments versus control plants were tested using a two-way, repeated measures ANOVA, employing aphid absence/presence and AMF absence/presence as the main effects.

4.3 Results

The results explore changes in the final plant size (flower number, leaf number, flower size, height and dry biomass) of control (C), mycorrhizal only (AM), aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over multiple generations.

4.3.1 Main experiment on mature plant size

4.3.1.1 Flower number

Overall, the main finding was plants in the second generation had fewer flowers than those in other generations ($F_{3,290}$ = 15.095, p<0.001). In generations one and two, aphid attacked plants (H and HAM) had more flowers than non-attacked plants (C and AM) but in generations three and four the reverse occurred leading to significant interaction in the analysis ($F_{3,290}$ = 10.998, p<0.001) (Figure 4.1).

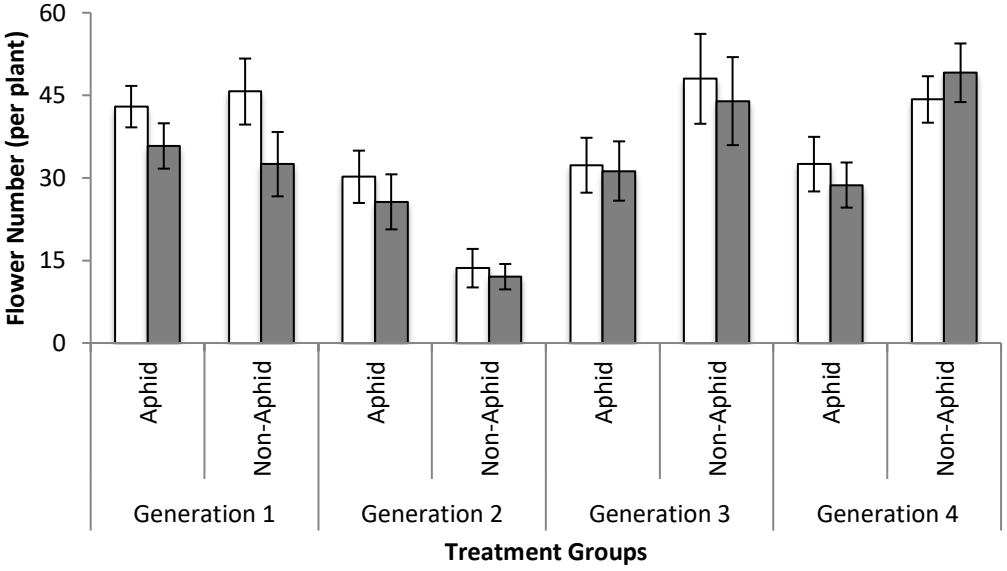

Figure 4.1 Mean total number of flowers produced per plant per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

4.3.1.2 Leaf Number

The key finding was the leaf number of plants in generations one and two was lower than the leaf number in generations three and four ($F_{3,299}$ = 32.261, p<0.001). Leaf number showed a significant interaction of mycorrhizal addition and generation ($F_{3,299}$ = 2.861, p<0.05). The mycorrhizal plants (AM and HAM) in

generations one and two had an increased leaf number compared to the non-mycorrhizal plants (C and H), with the opposite effect occurring in generations three and four. The presence of aphids on the plants in generation one caused a decrease in the number of leaves per plant compared to the non-attacked plants (C and AM). The opposite effect was observed on plants in generations three and four, leading to a significant interaction ($F_{3,299}$ = 7.527, p<0.001) (Figure 4.2).

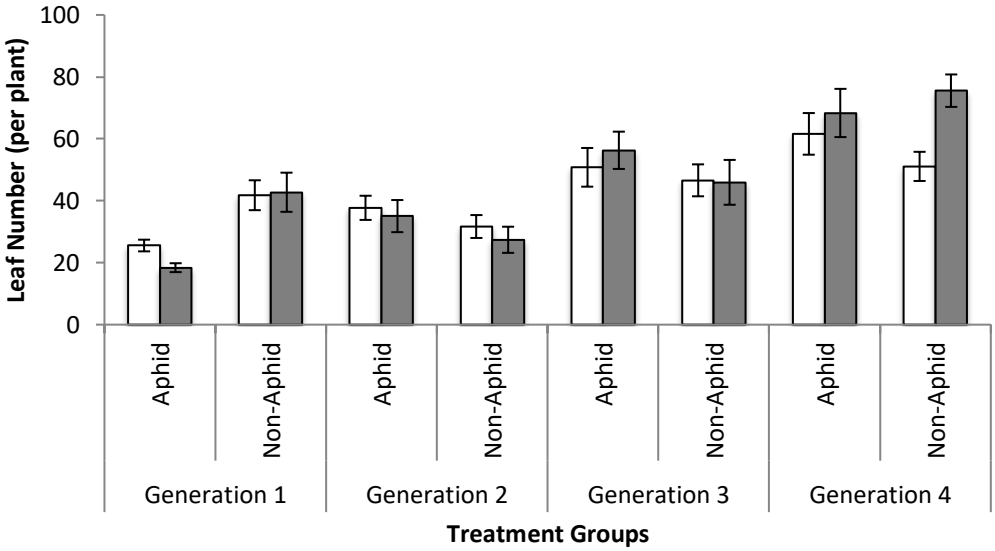

Figure 4.2 Mean number of leaves per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

4.3.1.3 Flower Size

The key finding was plants in generation one produced the smallest flowers compared to the other generations ($F_{3,287}$ = 41.215, p<0.001). Over all generations, mycorrhizal plants had larger flowers than plants that were not colonised by mycorrhizas ($F_{3,287}$ = 5.030, p<0.05). Mycorrhizal plants (AM and HAM) in generations one, two and four had larger flowers than non-mycorrhizal plants (C and H) in those generations. The same happened with aphid attacked plants (H and HAM) in generations one and two, leading to a significant interaction ($F_{3,287}$ =

10.492, p<0.001). Flower size was significantly affected by the interaction of generation, mycorrhizal and aphid treatments ($F_{3,287}$ = 4.001, p<0.01). Generation one control (C) plants had the smallest flowers while generation four AM colonised plants had the largest flowers (Figure 4.3).

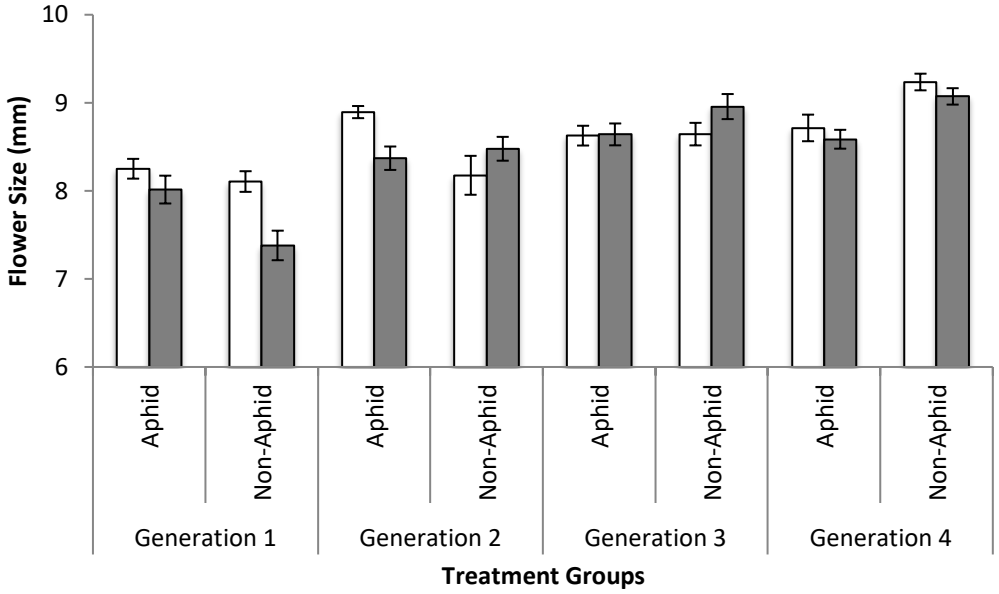

Figure 4.3 Mean flower size per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

4.3.1.4 Height

The key conclusion was that plants in generation two were shorter than those in any other generation ($F_{3,298}$ = 23.171, p<0.001). In generation two, aphid attacked plants (H and HAM) were taller than non-attacked plants (C and AM). In all other generations the aphid attacked plants were shorter than the non-attacked plants, leading to a significant interaction ($F_{3,298}$ = 3.353, p<0.05) (Figure 4.4).

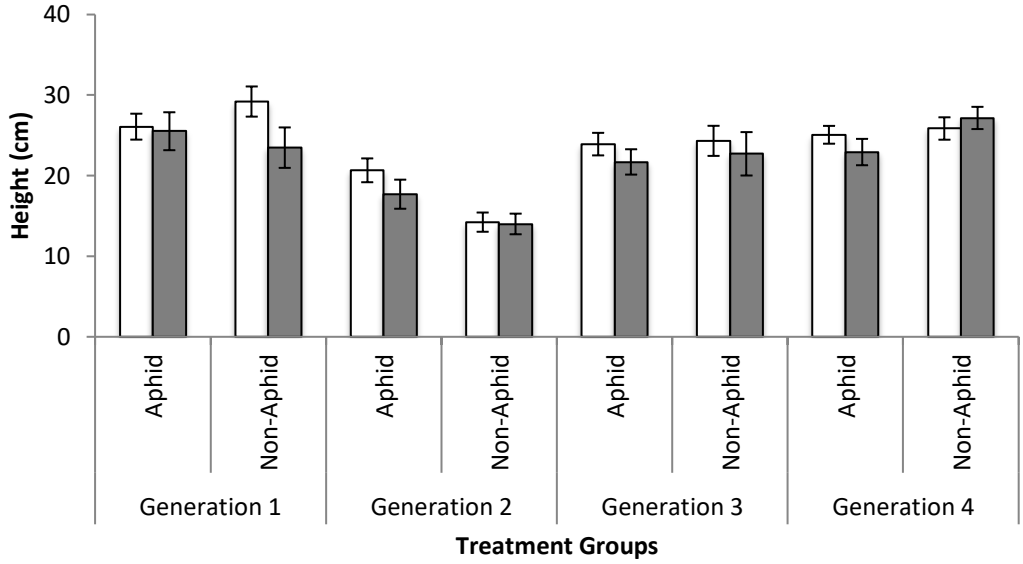

Figure 4.4 Mean height per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

4.3.1.5 Dry biomass

The main point was that plants in generations one and four had greater biomass than plants in generations two and three ($F_{3,298}$ = 15.737, p<0.001). Over all generations, mycorrhizal plants (AM and HAM) had higher biomass than non-mycorrhizal plants (C and H) ($F_{1,298}$ = 4.376, p<0.05). Over all generations, herbivore attacked plants generally had a biomass larger than those without aphids ($F_{1,298}$ = 4.852, p<0.05). Dry biomass was significantly affected by the interaction of aphid

treatments and generations ($F_{3,298}$ = 11.67, p<0.001). In generations one and two, H and HAM plants had increased biomass when compared to C and AM plants. However, in generations three and four, the opposite occurred where H and HAM plants had a reduced the biomass compared to C and AM plants (Figure 4.5).

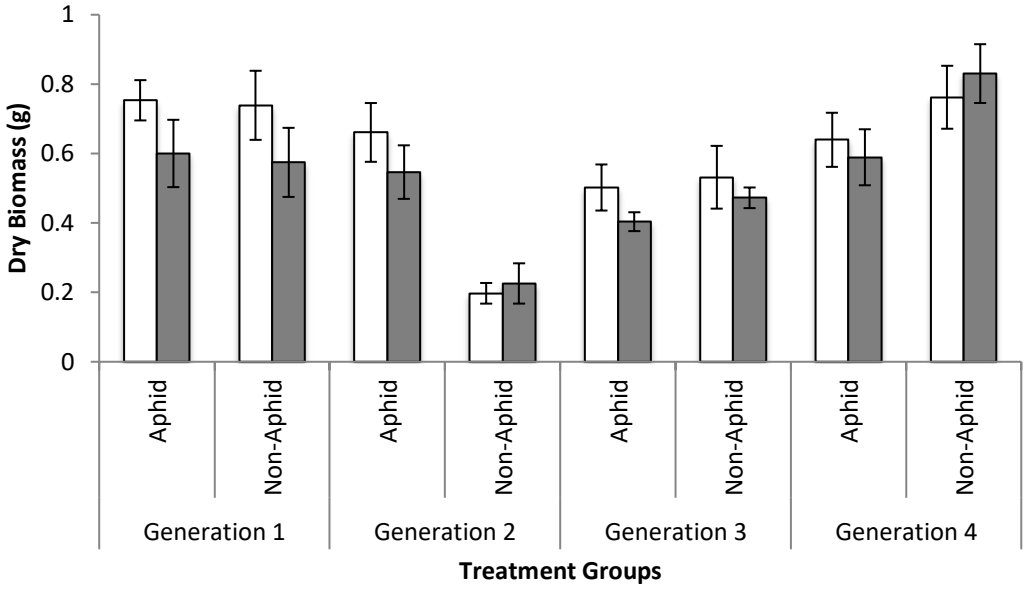

Figure 4.5 Mean dry biomass per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

4.3.2 Changes to treatments and the impact on mature plant size

Final plant size of generation two control (treatment C) plants was compared to 2A, 2B and 2C plants. The same occurred with generation three control final plant size being compared to final plant size of 3A, 3B and 3C plants and generation four control final plant size being compared to 4A, 4B and 4C final plant size. Generation two control plants were significantly different to 2A, 2B and 2C plants for all final plant size parameters. Interestingly, only flower number, height and dry biomass was significantly different between generation three control plants and 3A,

3B and 3C. The only final plant size parameter to differ between generation four control and 4A, 4B and 4C plants was the leaf number (Table 4.1).

	GENERATION TWO			GENERATION THREE			GENERATION FOUR		
	F	**P**	**Summary**	**F**	**P**	**Summary**	**F**	**P**	**Summary**
Flower Number	**58.97**	**<0.001**	**2A, 2B & 2C increased**	0.376	>0.05		0.121	>0.05	
Leaf Number	**9.15**	**<0.01**	**2A, 2B & 2C increased**	0.611	>0.05		**25.75**	**<0.001**	**4A, 4B & 4C decreased**
Flower Size	**5.987**	**<0.05**	**2A, 2B & 2C increased**	0.329	>0.05		0.747	>0.05	
Height	**41.14**	**<0.001**	**2A, 2B & 2C increased**	**4.427**	**<0.05**	**3A, 3B & 3C increased**	0.027	>0.05	
Dry Biomass	**24.09**	**<0.001**	**2A, 2B & 2C increased**	**10.41**	**<0.01**	**3A, 3B & 3C increased**	0	>0.05	

Table 4.1 A, B and C treatments from each generation compared with the generations control treatment to determine whether the effects being seen are from within generation or transgenerational effects. Significant differences indicated by bold text.

For clarity, due to the number of possible comparisons, only the significant results have been included in this section and in the figures (additional figures in Appendix II).

4.3.2.1 Flower Number

Mycorrhizal plants in generation two from mycorrhizal parents produced significantly less flowers than their controls (treatment 2A) ($F_{1,26}$ = 31.48, p<0.001) The same occurred with significantly more flowers produced by the plants from 2C than plants from generation two HAM ($F_{1,27}$ = 5.414, p<0.05). Furthermore, there were significantly more total flowers ($F_{1, 26}$ = 12.68, p<0.001) per plant from 3B than plants from generation three H and the same occurred in the fourth generation with significantly more flowers ($F_{1, 28}$ = 21.66, p<0.001) per plant from 4B than plants from generation four H (Figure 4.6).

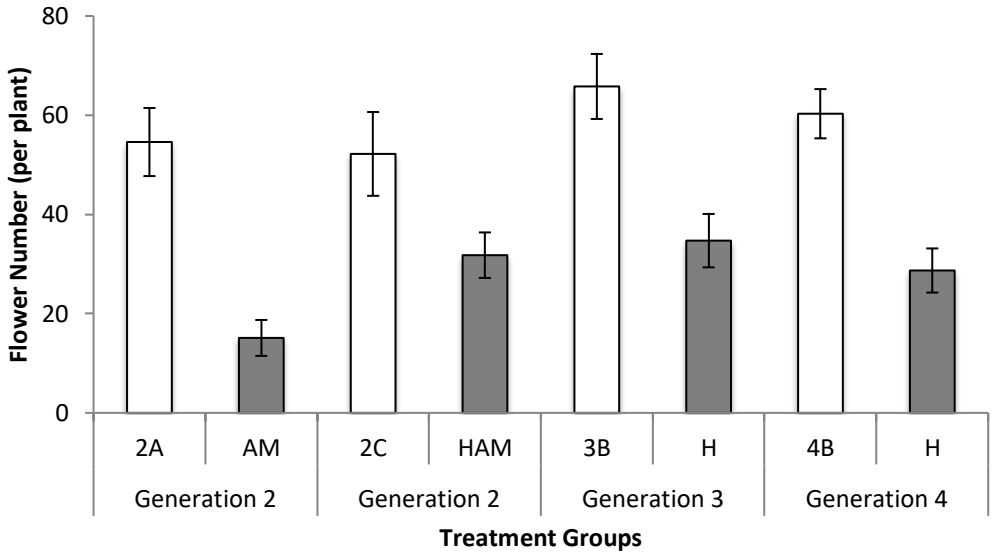

Figure 4.6 Mean number of flowers for plants grown from 2A, 2C, 3B, 4B and generation two AM, HAM, generation three H and generation four H. White represents 2A, 2C, 3B or 4B and grey is for the AM, HAM or H treatments. Bars represent means +/- SE.

4.3.2.2 Leaf Number

Generation four HAM plants from HAM parents produced significantly more leaves per plant than their controls (treatment 4C) ($F_{1,28}$ = 18.08, p<0.001) (Figure 4.7).

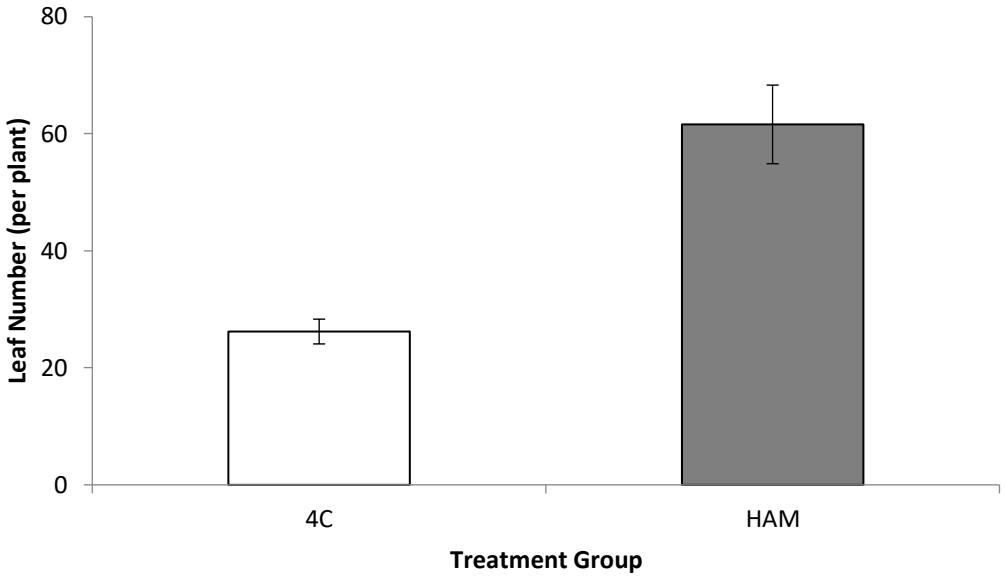

Figure 4.7 Mean leaf number for plants grown from 4C and generation four HAM. White represents 4C and grey represents generation four HAM. Bars represent means +/- SE.

4.3.2.3 Flower Size

There were significantly smaller flowers produced by the plants from generation two mycorrhizal plants from mycorrhizal parents than their control (treatment 2A) ($F_{1,26}$ = 6.356, p<0.05) (Figure 4.8).

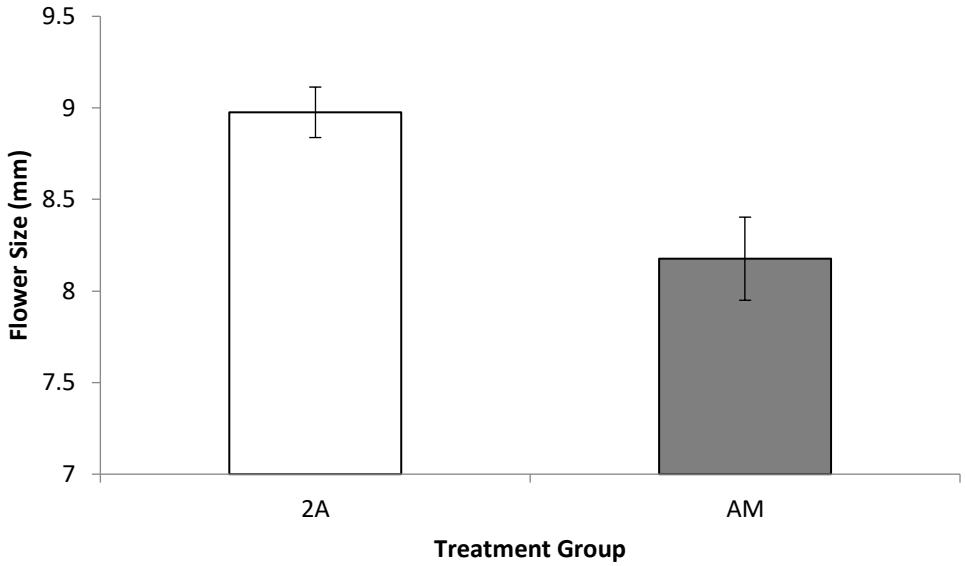

Figure 4.8 Mean flower size for plants from 2A and generation two AM. White represents 2A and grey represents generation two AM. Bars represent means +/- SE.

Generation two H plants from H parents produced significantly smaller flowers than their controls (treatment 2B) ($F_{1,26}$ = 6.665, p<0.05). This also occurred between generation three H plants when compared to their control (treatment 3B) ($F_{1,27}$ = 11.86, p<0.005). Generation four H plants also followed the same pattern with their control (treatment 4B) producing larger flowers ($F_{1,28}$ = 9.899, p<0.005) (Figure 4.9).

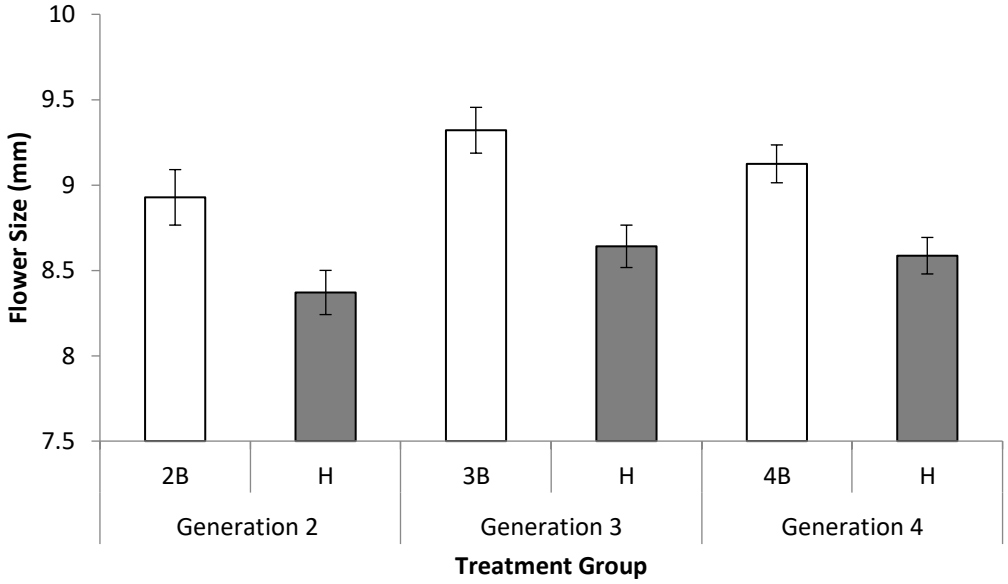

Figure 4.9 Mean flower size (mm) for plants from 2B, 3B and 4B and generations two, three and four H. White represents 2B, 3B and 4B and grey represents generations two, three and four H. Bars represent means +/- SE.

4.3.2.4 Height

Generation two AM plants from mycorrhizal parents were significantly shorter than their controls (treatment 2A) ($F_{1,27}$=31.48, p<0.001). The same occurred with generation two H plants from aphid infested parents were significantly shorter than their controls (treatment 2B) ($F_{1,27}$=7.95, p<0.01). The pattern continued with generation two HAM plants being significantly shorter than their controls (treatment 2C) ($F_{1,26}$=4.877, p<0.01). Interestingly, in generation three and four H plants from aphid infested parents the height continued to be significantly shorter than their controls (treatments 3B and treatment 4B respectively) ($F_{1,28}$ = 25.12, p<0.001) ($F_{1,28}$ = 6.765, p<0.05) (Figure 4.10).

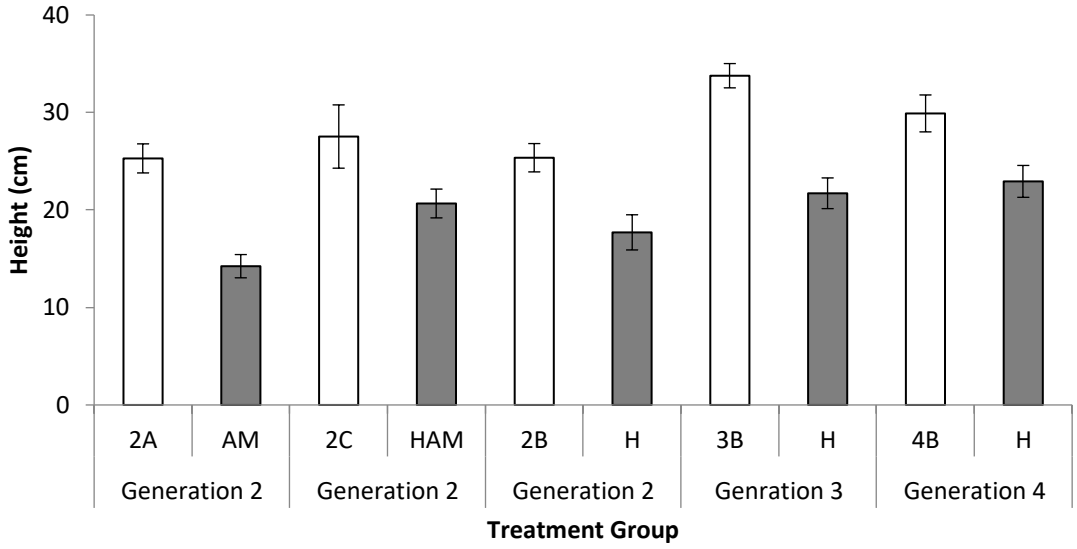

Figure 4.10 Mean height (cm) for plants from 2A, 2C, 2B, 3B and 4B and plants from generation two AM, H and HAM, generation three H and generation four H. White represents 2A, 2B, 2C, 3B and 4B and grey represents generation two AM, H and HAM, generation three H and generation four H. Bars represent means +/- SE.

4.3.2.5 Dry biomass

Mycorrhizal plants from mycorrhizal parents had a significantly lower dry biomass than their controls (treatment 2A) ($F_{1,27}$ = 19.59, p<0.001). The same pattern occurred with the control (treatment 3B) having a significantly larger dry biomass

than generation three H plants ($F_{1,28}$ =59.57, p<0.001). Aphid infestation also caused a lower biomass in generation four H plants than their control (treatment 4B) ($F_{1,28}$ =7.705 p<0.001). In generation three the HAM plants had a significantly lower dry biomass than 3C controls ($F_{1,28}$ =5.632, p<0.05) (Figure 4.11).

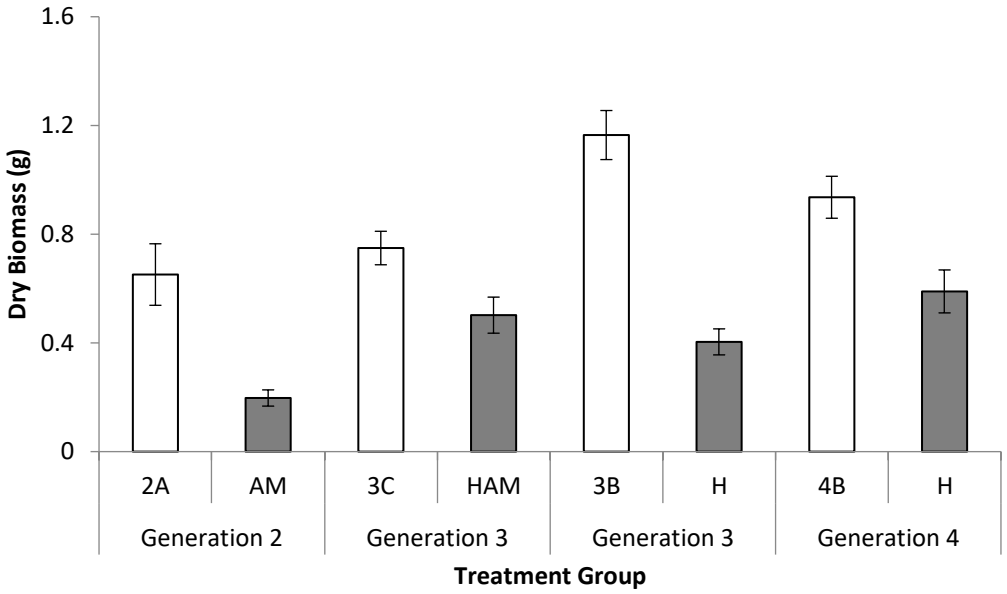

Figure 4.11 Mean dry biomass for plants grown from 2A, 3C, 3B and 4B and from generation two AM, generation three HAM, generation three H and generation four H. White represents 2A, 3C, 3B and 4B and grey represents generation two AM, generation three HAM, generation three H and generation four H. Bars represent means +/- SE.

4.4 Discussion

The results strongly suggest that parental effects can manifest themselves in mature plants in subsequent generations and that differences seen in plant development (Chapter 3) follow on to affect mature plant size.

4.4.1 Flower Number

The number of flowers was influenced by the presence of aphids across the multiple generations. Previous literature had debated whether there were parental effects acting upon flower number, with one experiment finding no significant effects from insect herbivory or transgenerational effects (Kellenberger *et al.*, 2018). These results show that there is an effect from insect herbivory that is passed between generations in *S. vulgaris*. The flower number changing could be due to an epi-mutation that is linked to DNA methylation (Soppe *et al.*, 2000). A mutation in the *FWA* gene can lead to a failure in flower development. If the flowers are not able to develop properly, it could lead to the inability to set seed. The increase in flower number between generations could be linked to an increase in flowering time. Time taken to flower was seen to reduce between generations, especially generations one to two (Section 3.3.1; Figure 3.4). This could cause an increase in the flower number, as there is an increase in the length of time the plant can produce flowers (Lu & Koide, 1994).

Plants from H and HAM in the first and second generation had a shorter time to flowering than C and AM plants. This could be why there is an increase in the number of flowers produced by generation one and two H and HAM plants. The change in generation three and four also links to aphids decreasing flowering time in generations three and four (Section 3.3.1; Figure 3.4). However, in previous literature, the presence of *Diuraphis* (*Holcaphis*) *holci* (grass feeding aphids) reduced the flowers produced and even stopped *Holcus mollis* from flowering if the aphids were present in high numbers (Crawley, 1989), so this may explain the decrease in flowers on aphid present plants in other generations of *S. vulgaris*.

Damage to bud production when the aphids were placed onto the plant could lead to a reduction in flowering or even stop flowering in grasses (Crawley, 1989). This could be true in these plants, as aphids were placed onto the plant during bud development and the duration of bud development was decreased in plants with aphids present (H and HAM) in generations one and two (Section 3.3.1; Figure 3.3). This was reflected in plants with aphids present in generation one and two producing more flowers, so maybe the aphids had less time to damage the buds as the time spent developing buds was decreased in the first two generation. This

switched in generation three and four with bud development time increasing in H and HAM plants (Section 3.3.1; Figure 3.3) and there were less flowers produced on plants with aphids present (H and HAM).

When exploring other types of insect herbivory, moths were found to cause a decrease in flower number in the next generation (Sletvold & Grindeland, 2008). It was believed that this was due to diminished returns from placing a lot of energy into flower production. To reduce the energy that is being lost when the flowers were destroyed, the number of flowers produced was reduced (Sletvold & Grindeland, 2008).

Mycorrhizal colonisation has been shown to increase flower number (Vaingankar & Rodrigues, 2014), but was not seen in the *S. vulgaris* grown. Mycorrhizal colonisation was not seen to really alter development time either, so the changes in flower number could mainly be linked to alterations to *S. vulgaris* development time.

4.4.1.1 Changes to treatments and the effect on flower number

Flower number in plants grown in 2A, 2B and 2C was significantly different to generation two control (treatment C). This suggests that there were parental effects being passed between by the parental plants treated with aphids and/or mycorrhizas to the offspring between generations one and two. These results suggest that parental effects are mainly visible over one generation but disappear, supporting previous work (Latzel, 2015). From this only one generation could be grown before ecological experiments start and the two generations suggested in Latzel (2015) are not necessary. Intriguingly, this is different to parental effects on development time seen in Table 3.1 where parental effects were seen over multiple generations or seen to disappear and reappear over multiple generations. Mycorrhizal and aphid colonisation within the generations seem to mask the parental effects on flower number, especially as flower number was measured at the end of the lifecycle of *S. vulgaris.* It could be that the increase in flower number in 2A, 2C, 3B, and 4B is the visible parental effects being passed on and these are clearer when no other treatment causes in-generation effects. Between 3B and 4B

and generation three H and generation four H, there was a decrease in the flowers produced. It could be the cumulative effect of aphids being present on the parental plants for multiple generations causing the flower numbers to decrease. Between generations two and three H plants the presence of aphids went from speeding up development time to decreasing development time (Section 3.3.1). This may be why there is a decrease in the flowers produced as aphids start to slow development time therefore slowing the time the plant has to produce flowers.

4.4.2 Leaf Number

Leaf number was seen to increase over the generations of *S. vulgaris* grown. Development time to reach the final stage (seed production) was faster in generation one than subsequent generations (Section 3.3.1; Figure 3.5). It could be that slowing down overall development time caused the increase in leaf number. A slower development time could give the plant time to grow more leaves. In this experiment, mycorrhizal colonisation was shown to increase the leaf number for the first two generations but then decreased in subsequent generations. AMF has been shown to increase leaf number in *Zingiber officinale* (ginger) possibly by increasing phosphorus uptake by the plant (Dos Santos *et al.*, 2010). However, over multiple generations leaf number increased more in non-mycorrhizal colonised *Abutilon theophrasti* than the mycorrhizal *A. theophrasti* (Lu & Koide, 1994). This could be occurring in *S. vulgaris* where there was an increase over multiple generations, but the influence of mycorrhizal colonisation is hidden by the general parental effects or in-generation effects. It could also be due to low colonisation of *S. vulgaris* in these experiments (Section 7.3.1). Aphids attacking *S. vulgaris* caused a decrease in leaf number in the first generation grown, however this was reversed in generations three and four. Stressed plants have been shown to have fewer leaves, but those leaves were larger in area (Migicovsky *et al*., 2014). This was in heat stressed plants, but insect herbivory is known to cause stress in plants so this could be true in *S. vulgaris* attacked by aphids. This was reversed after generation one in the experiments, which was not expected. Previous literature suggested that leaf number would be significantly decreased by herbivory and that the effects of

this are visible over multiple generations (Kellenberger *et al*., 2018). It could be that parental effects are causing this to not be seen and instead helping the next generations to increase leaf production.

4.4.2.1 Changes to treatments and the effect on leaf number

Leaf number in plants from 2A, 2B and 2C was significantly different to generation two control plants (treatment C). This suggests that there are parental effects being passed from treated parental plants to the next generation. The effects being seen are not just in-generation effects for at least generation two results. The parental effects appear between the first generation parents to the second generation progeny but not between other generation. This is highly suggestive that transgenerational effects for leaf number are temporary over one generation and not seen over multiple generations. For removing the parental effects on leaf number, these results suggest that only one generation needs to be grown and not multiple generations, as suggested by Latzel (2015).

4.4.3 Flower size

Flower size was influenced by the interaction of generations, mycorrhizal colonisation and aphid presence. There is no research into the parental effects or epigenetic effects on flower size. There is little research into the effects of mycorrhizal colonisation and/or the presence of insect herbivores effects on flower size. Mycorrhizal colonisation has been shown to increase flower size of *Chrysanthemum morifolium* (florist's daisy) (Sohn *et al.*, 2003) and *Leucanthemum vulgare* (ox-eye daisy) (Gange *et al.*, 2005). The increase in *C. morifolium* size was caused by AMF but the size of the effect was dependent upon colonisation timing (Sohn *et al*., 2003). But any form of mycorrhizal colonisation independent of timing did cause an increase in *C. morifolium* size (Sohn *et al*., 2003). The presence of aphids caused flower size to increase in generations one and two. It could be that *S. vulgaris* had longer to flower so it could spend longer producing larger flowers. This

is evident with plants from generation one and two producing flowers quicker when aphids were present (Section 3.3.1; Figure 3.4).

4.4.3.1 Changes to treatments and the effect on flower size

Flower size from 2A, 2B and 2C was significantly different to generation two control plants (treatment C). This suggests that there are parental effects being passed from treated parental plants to the next generation and that the effects being seen in generation two are not just in-generation effects. Transgenerational effects for flower size appear to be temporary over one generation and not seen over multiple generations. It may be that ecological experiments controlling for parental effects with only one generation will control for parental effects on flower size and multiple generations are not required.

Plants from 2A, 2B, 3B and 4B all produced larger flowers than the generation two AM, generation two H, generation three H and generation four H respectively. This suggests that the treatments in each generation are causing the flower size to decrease compared to the controls. Flower size did increase between the generations of H plants, so it could be that there is a cumulative effect of constant aphid herbivory on the parent over multiple generations that increases the flower size. The B control producing larger flowers than H plants suggests that in generation the aphids are causing a decrease in flower size. But without aphid herbivory the parental effects are evident.

4.4.4 Height

Height decreased between generation one and two but rose again between generations two and three. Plants in generation one reached time to seed quicker than any other generation, which gave the other generations longer to develop (Section 3.3.1; Figure 3.5). Longer development could have influenced height as the plant would have longer before the final development stage to grow (Section 3.3.1; Figure 3.5). The presence of aphids affected the plants height over multiple generations of *S. vulgaris*. While generation 2 plants were the shortest, the aphid

attacked plants (H and HAM) within that generation were taller than the non-attacked plants (C and AM). This did not occur in other research into the effect of aphids upon plant height (Bing *et al.*, 1991; Bayram & Tonga, 2017; Stanton *et al.*, 2017). Corn leaf aphids feeding upon maize seedlings caused the mature plant height to decrease (Bing *et al*., 1991). This suggests that the timing of insect herbivory does not matter and that aphid attack at any stage of plant growth can reduce plant height. The addition of methyl jasmonate, which is used in plant chemical defences, led to the reduction of plant height and aphid number on wheat (Bayram & Tonga, 2017). The production of plant chemical defences is energy expensive and so is aphids' taking resources from the plant. So the plant may not have enough resources to grow taller. A few experiments have found that transgenerational effects did not alter stature (Walters & Paterson, 2012; Walter *et al*., 2016). However, one study linked the presence of a specific endophyte (*Epichloë gansuensis*) and transgenerational effects to increases in plant height (Xia *et al.*, 2018). It could be that there is a similar endophyte present within *S. vulgaris* that affects height over multiple generations, especially as endophytes have been shown to have vertical transmission in this species (Hodgson *et al.*, 2014) (Section 7.3.3). It was surprising that there were no mycorrhizal effects and may be a result of low levels of colonisation in the roots (Section 7.3.1).

4.4.4.1 Changes to treatments and the effect on plant height

Height in plants grown in 2A, 2B, 2C, 3A, 3B and 3C was significantly different to generation two control and generation three control plants. This suggests that there were parental effects being passed between by the treated parental plants to the offspring between generations one and two and two and three. It was thought that parental effects are mainly visible over one generation and then disappear (Latzel, 2015). However, there were still some parental effects passing between generations two and three but the effects in this study did not continue further. It could be that the effects slowly disappear over multiple generations, so the suggestion by Latzel (2015) to grow plants for two generations before starting the experiment would ensure that parental effects on height were removed. This

differed to other parameters measured in this chapter, so it appears that to effectively control for parental effects, the specific parameters must be considered before starting experiments.

The 2A, 2C, 2B, 3B and 4B were taller than generation two AM, generation two HAM, generation two H, generation three H and generation four H, especially in all generations of H treatments. It suggests that the parental effects of aphid herbivory can cause a reduction in plant height in the progeny generation. In the H treatments, height seemed to generally increase, so there may be a cumulative effect of aphid herbivory through the generations.

4.4.5 Dry biomass

Dry biomass was affected by aphid presence over the multiple generations with generation one and two H and HAM plants having a lower dry biomass and this reversing in subsequent generations. H and HAM plants were shown to decrease development time for the first two generations and to generally decrease the time taken to seed (Section 3.3.1; Figure 3.5). The decrease in development time could mean that there is less time for the plants to increase their biomass. The changes in leaf number were not seen to cause the changes seen in dry biomass. However, the decrease in flower number in generation two may explain the decrease in dry biomass in generation two. The presence of AMF generally caused an increase in dry biomass with AMF presence found to increase plant biomass through an increase in phosphorus uptake (Hu *et al.*, 2014). Maternal effects were found to cause different effects in different species of trees (Holeski *et al.*, 2013), which may also occur in forb species. Herbivory in the maternal generation of *Populus* spp. caused an increase in plant biomass in the next generation. There was a trade off between maintenance of offspring growth and offspring photochemical defences. In the *Populus* spp. it was found that the 'decision' to increase growth, at the expense of defence was taken (Holeski *et al.*, 2013). It could be that *S. vulgaris* is putting more resources into growth and decreasing the amount of resources put into chemical defence. Over three generations of *Polygonum persicaria* (lady's thumb), the offspring of stressed individuals had a larger biomass than the offspring

of non-stressed plants (Herman *et al.*, 2012). The effects were found to be cumulative over two generations. This could be the case with *S. vulgaris*, as the increase in dry biomass on H and HAM plants occurred in the third and fourth generations. These plants had been experiencing aphid herbivory over multiple generations so the effects could be cumulative. The presence of aphids has been shown to decrease the biomass in one generation of plants (Babikova *et al.*, 2014), but there was no interaction between aphid attack and AMF colonisation.

4.4.5.1 Changes to treatments and the effect on dry biomass

Dry biomass in plants in 2A, 2B, 2C, 3A, 3B and 3C was significantly different to generation two control and generation three control. This follows the same pattern as plant height, suggesting that to control for parental changes in dry biomass multiple generations of the plant must be grown.
Plants in 2A, 2B, 2C, 3A, 3B and 3C had considerably higher dry biomasses than their controls. The treatments of mycorrhizal colonisation and aphid herbivory could be reducing the dry biomass when interacting with parental effects.

4.5 Conclusion

The hypothesis of parental effects in *S. vulgaris* changing final plant size parameters was upheld, with both aphids and mycorrhizas producing different final parameter results. The number of generations the parental effects occurred over differed to those seen in chapter three (Table 3.1). In chapter three the effects disappeared but reappeared over multiple generations. However for final plant size parameters the parental effects disappeared after one or two generations but only reappeared for final leaf number. Some of these results may be linked to other alterations found in development time (Section 3.3.1). Shortening of flowering time could change the results of the final parameters especially flower number. The secondary hypothesis of mycorrhizal and aphid presence causing changes in *S. vulgaris* final parameters was also upheld, especially with the interaction in final parameters for flower size. Overall, mainly generation and aphid presence affected final

parameters of the plants, which could be due to mycorrhizal colonisation fluctuating between plants. Parental effects of final parameters are occurring in *S. vulgaris* and the generations that these last for depends upon the parameter being measured. This plays an important role in the setup of ecological experiments. The amount of generations grown before the experiment starts could depend upon the parameter that is being measured.

Chapter Five

Parental effects of *Senecio vulgaris* upon the seeds

5.1 Introduction

This study investigated the effects of the environment on *Senecio vulgaris* seeds over multiple generations, which addressed the first theme of this project; which environmental conditions cause parental effects to occur. It specifically explored the objectives; does the presence of insect herbivores cause changes in plant development over multiple generations and does the symbiotic relationship between plant and mycorrhizas cause changes in plant development over multiple generations. The variables measured in this experiment were seed carbon, nitrogen and phosphorus, seed weight and percentage germination.

Seeds are known for their role in the passing of genetic information to the next generation. The mechanisms for passing on parental effects are thought to be through the seed, either through epigenetic changes to the DNA structure (Michalak *et al.*, 2013) or vertical transmission of endophytes (Hodgson *et al.*, 2014).

To ensure survival of the next generation, the maternal plants could provision the seeds to give them a better chance of survival. The provisioning of these seeds may be linked back to the environment of the maternal plants. The colonisation of the roots by mycorrhizal fungi could increase the provisioning of the seeds. Maternal phosphorus supply and AMF colonisation were seen to affect the nitrogen and phosphorus supply of *Abutilon theophrasti* (velvetleaf) (Lewis & Koide, 1990). It is believed that AMF infection modified the way that seed phosphorus was allocated, with higher levels of phytate being placed into the seeds from AMF colonised plants than seeds from plants grown in soil with additional phosphorus added (Koide & Lu, 1992). Nitrogen and phosphorus is translocated to the seedlings via the seeds after germination (Milberg & Lamont, 1997), so the higher levels of phosphorus and nitrogen in the seed could increase the seedlings survival.

The studies reviewed by Maschinski & Whitham (1989) showed that insect herbivores can in certain cases benefit the plant by increasing seed production (Hendrix, 1984) and in others it can cost the plant by reducing reproduction potential (Rockwood, 1973; Inouye, 1982; Louda, 1984; Whitham & Mopper, 1985). The costs of insect herbivory were mainly seen in crop systems compared to natural

systems. Whether insect herbivory was costly or beneficial to the seed production of the plant depended upon the timing of the insect herbivores and the nutrients available to the plants (Maschinski & Whitham, 1989).

Variation in seed mass has been linked to increasing progeny's ability to cope with unpredictable but recurrent instabilities in their environment (Capinera, 1979). *S. vulgaris* is subjected to the same environment over multiple generations in these experiments, so it could mean little variation in the seed size. Alternatively, variation may exist as a form of 'insurance', to ensure that the progeny can cope with anything unpredictable that might occur. In *Pastinaca sativa* (wild parsnip), seed mass variation on the same plant caused differences in seedling growth and seed germination (Hendrix, 1984). This study found that smaller seed size was linked with a lower growth rate but a quicker germination. In *S. vulgaris* the size of the seed may correlate or cause alterations in the plant growth rate and the germination time of the seeds. In annuals, it is believed the addition of mycorrhizal fungi does not alter the seed size, as the reproduction strategy is to maintain seed quality through consistency (Fenner, 1986; Peat & Fitter, 1993). Insect herbivores as a stressor have repeatedly been shown to decrease seed size (Hanley, 1998). For example, Cinnabar moth (*Tyria jacobaeae*) caterpillar herbivory upon *Jacobaea vulgaris* (ragwort) cause the seeds produced from regrowth to be lighter than seeds produced from unattacked areas of the plant (Crawley & Nachapong, 1985). The lighter seeds produced less competitive seedlings than the heavier seeds.

Changes in germination have important consequences with a plant's quality and development being altered. *Sinapis arvensis* (charlock mustard) had reduced seed mass due to flooding and the lighter seeds had a reduced germination rate (Luzuriaga *et al.*, 2006). Any changes in the plant's growth rate in this chapter could be due to seed mass altering seed germination. *S. vulgaris* seeds can be infertile, dead or dormant (West, 1995). So measuring the percentage germination can be a good way to understand the quality of the seeds being produced, especially over multiple generations.

Parental effects are transmitted through the seed. The mechanisms thought to cause this are either epigenetic changes to the DNA (Berger *et al.*, 2009) or through vertical transmission of endophytes (Gundel *et al.*, 2017). It was hypothesised that

parental effects would alter the chemistry and/or size of seeds produced by *S. vulgaris*. In addition, if this hypothesis were upheld, the addition of aphids and/or mycorrhizal fungi may cause further effects on the seeds of *S. vulgaris.* Overall, it was hypothesised that the environment of the previous generation affects the development of the seeds.

5.2 Methods

5.2.1 Main experiment measurements

The main experiment was to explore whether the previous generation affects the progeny's generations seed development. It involved growing four generations of *S. vulgaris* under the same environmental conditions (Section 2.2). Each generation had a total of 80 plants grown. There were four separate treatment groups with 20 plants in each group per generation. The four treatment groups were C, AM, H and HAM (Section 2.2; Figure 2.1). In each generation, the seeds from each plant in each treatment group were analysed for:

5.2.1.1 Seed carbon and nitrogen

To measure the percentage of carbon and nitrogen present in a mg of seed material, seeds were oven dried at 60^{o}C for 48 hours and ground to a fine powder. Roughly 10mg of ground seeds were weighed out into tin capsules (CE instruments, Wigan, UK) and sealed. Replicate number was lower than 20 per treatment group for seed chemistry, as a certain weight had to be achieved for each test to be run. The plants did not produce enough seeds individually to meet the weight requirements to run the tests so the samples were pooled. Nitrogen and carbon content were calculated by combustion-gas chromatography using NC soil analyser flash EA 1112 series with a CHNS configuration. The sample was introduced by an autosampler connected to a quartz reactor in a furnace at a temperature of 900^{o}C. The sample was burnt and the CO_2 and NO_2 from oxidation were transported in a carrier gas (helium), separated by the gas chromatography column and measured

by the thermal conductivity detector. The individual gases were separated and eluted as N_2 and CO_2. There were quality controls (Sulphanilamide STD) (CE instruments, Wigan, UK) with known nitrogen and carbon concentrations added to the autosampler throughout the sample run. The nitrogen and carbon concentrations of the quality controls were checked against the standards to ensure the results were not drifting through the sample run.

5.2.1.2 Seed phosphorus

To measure the percentage of phosphorus present in a mg of seed material, seeds were oven dried at 60^{o}C for 48 hours and ground to a fine powder. To analyse the seed phosphorus content the method was adapted from West (1995). Replicate number was lower than 20 per treatment group for seed chemistry, as a certain weight had to be achieved for the test to be run. The plants did not produce enough seeds individually to meet the weight requirements to run the tests so the samples were pooled. Ground seeds needed to be run through a digestion procedure. 0.2g of ground seeds were weighed into a digestion vessel with 6mls of concentrated nitric acid. In the Mars Xpress (CEM technologies, Buckingham, UK) microwave, the temperature was ramped to 140^{o}C over 10 minutes (power = 1200W) and held for 20 minutes. The digestion vessels were left to cool for about one hour until they were roughly 55^{o} C. Once the digestion was complete, the vessels were opened carefully in the fume cupboard. Once opened, distilled water and the vessel contents were filtered into a 50ml volumetric flask.
The samples were run through the Skalar segmented flow analyser comprised of SA 1050 random access autosampler, chemistry unit SA 4000, SA853 SFA interface with a digital photometer head and Flowaccess software package. The ammonium hepta molybdate and potassium antimony (III) oxide tartrate react in an acidic medium with diluted solutions of phosphate to form an antimony-phospho-molybdate complex. This complex is reduced by L (+) ascorbic acid to an intensely blue-coloured complex which is measured spectrometrically at 880nm. The temperature of the reaction was kept constant. A standard was used throughout the sample run, so that any drift was corrected for. The standard was made by

dissolving 4.3937g of potassium orthophosphate (KH_2PO_4) in 800mls deionised water. The standard was diluted down from 1000ppm to 8, 6, 4, 2, 1 and 0 to be used throughout the sample run.

5.2.1.3 Seed weight

At least five mature seeds from each replicate plant were weighed and the mean was calculated from the weight.

5.2.1.4 Percentage germination

The method was modified from West (1995). The experiment was set up with ten 85mm petri dishes per plant with five seeds in each petri dish. These petri dishes were each filled with 0.5cm of damp sand. The five seeds were placed at random into the sand. The petri dishes were sealed and placed in a dark cupboard to prevent any light reaching them. The experiment was checked daily for the first five days and then left until day thirteen, where it was checked for the final time. The percentage of seeds that had germinated was recorded at each check.

5.2.1.5 Statistical analysis of the main experiment

Analysis of experiments on seeds from *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
If the sample sizes were unbalanced, two different forms of coding were used to ensure the data were interpreted correctly. It was suggested that weighted means were used to perform the multi-way ANOVA using the anova() function in the car package in R (Quick, 2010). The order of the independent variables was checked by running the ANOVA multiple times to ensure hierarchical order was achieved (Quick, 2010). Even though this is a type I analysis, by running it multiple times it becomes a type II analysis. The second form of analysis used unweighted means but the same technique. The results were compared to ensure that all precautions were

taken with the data. Fox (2018), stated that using type III analysis was to be avoided when using the car package and anova() function in R.
Differences in seed chemistry, weight and germination over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence and generation as the main effects.

5.2.2 Comparing changes to treatments and the impact on seed traits

Changes to treatments and the impact on seed parameters were set up following the methods outlined in Section 2.4 and Figure 2.1. Each generation from generation one had seeds from treated plants (AM, H and HAM) grown under controlled conditions (Section 2.4; Figure 2.1). There were ten plants grown per treatment and three treatment groups used per generation. In each generation all the parameters described in Section 5.2.1 were measured in the same way.
Differences between the plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment, i.e. generation two control was compared to 2A, 2B & 2C.
The results were also compared to the next generation plants grown under treated conditions, i.e. generation two AM plants were compared to 2A.

5.2.2.1 Statistical analysis of comparing changes to treatments

Analysis of seed chemistry, weight and germination of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
If the sample sizes were unbalanced, two different forms of coding were used to ensure the data were interpreted correctly. It was suggested that weighted means were used to perform the multi-way ANOVA using the anova() function in the car package in R (Quick, 2010). The order of the independent variables was checked by running the ANOVA multiple times to ensure hierarchical order was achieved (Quick, 2010). Even though this is a type I analysis, by running it multiple times it

becomes a type II analysis. The second form of analysis used unweighted means but the same technique. The results were compared to ensure that all precautions were taken with the data. Fox (2018), stated that using type III analysis was to be avoided when using the car package and anova() function in R.

Differences between the plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment, i.e. 2A, 2B and 2C was compared to generation two C. A one-way, repeated measures ANOVA was performed, employing parental treatment as the main effect.

Differences in seed nitrogen, carbon, phosphorus, seed weight and germination over treatment groups compared to controls were tested using a one-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence as the main effects for the control versus AM experiment and H experiment. Differences in seed nitrogen, carbon, phosphorus, seed weight and germination over HAM treatments versus control plants were tested using a two-way, repeated measures ANOVA, employing aphid absence/presence and AMF absence/presence as the main effects for the control versus HAM plants.

5.3 Results

The results explore changes in seed parameters (seed carbon, seed nitroge, seed phosphorus, seed weight and percentage germination) of seeds produced by control (C), mycorrhizal only (AM), aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over multiple generations.

5.3.1 Main generation experiments

5.3.1.1 Seed carbon and nitrogen

Carbon percentage differed between the generations ($F_{3,40} = 18.083$, $p<0.001$). The key conclusion was generation two had the lowest percentage of carbon present per mg of seed material when compared with seed material produced by plants in

the other generations (Figure 5.1). The presence of aphids in generations one, two and four significantly increased the seed carbon ($F_{3,40} = 4.087$, $p<0.05$). The opposite occurred in generation three, where the presence of aphids on the parental plant significantly reduced the seed carbon concentrations. There was a significant interaction between all three variables ($F_{3,40} = 2.959$, $p<0.0437$). In generations one, two and three, AM plants produced seeds with higher concentrations of seed carbon compared to the C plants. When aphids were present on the plants, the opposite occurred.

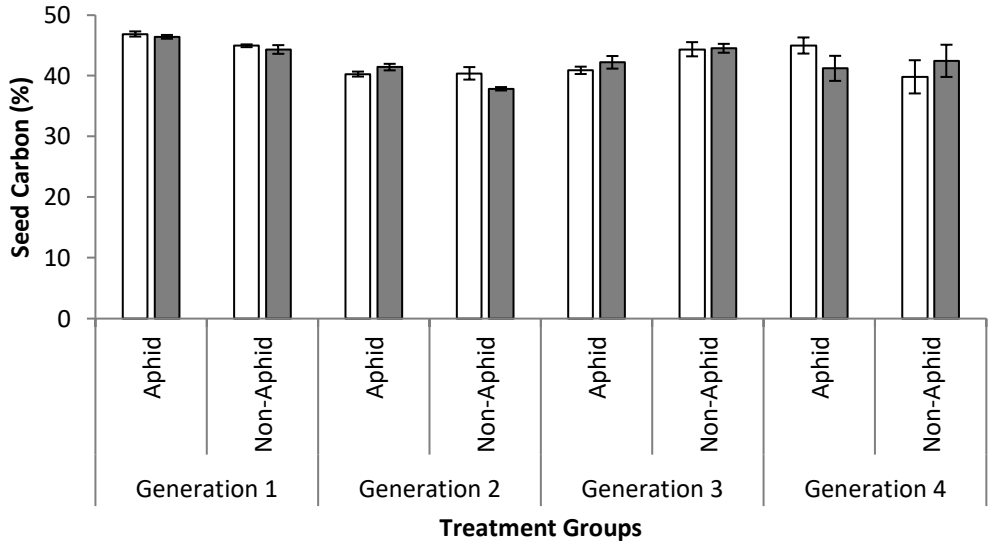

Figure 5.1 Mean percentage of carbon per mg of seed material (seed carbon) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

A key finding was seed material produced by plants in generations three and four had a much higher percentage of nitrogen present when compared to the other generations, especially generation two seed material ($F_{3,40} = 6.485$, $p<0.05$). Plants from H and HAM treatment groups in generations one and four had much higher percentages of nitrogen present in the seeds than plants C and AM treatment groups in those generations. The opposite is true in the second and third generations, leading to a significant interaction ($F_{3,40} = 10.323$, $p<0.001$). There was

a significant interaction between all three variables ($F_{3,40} = 6.935$, $p<0.001$). AM plants in generations one, two and three produced seeds with higher concentrations of seed nitrogen than C plants. The opposite occurred between H and HAM plants (Figure 5.2).

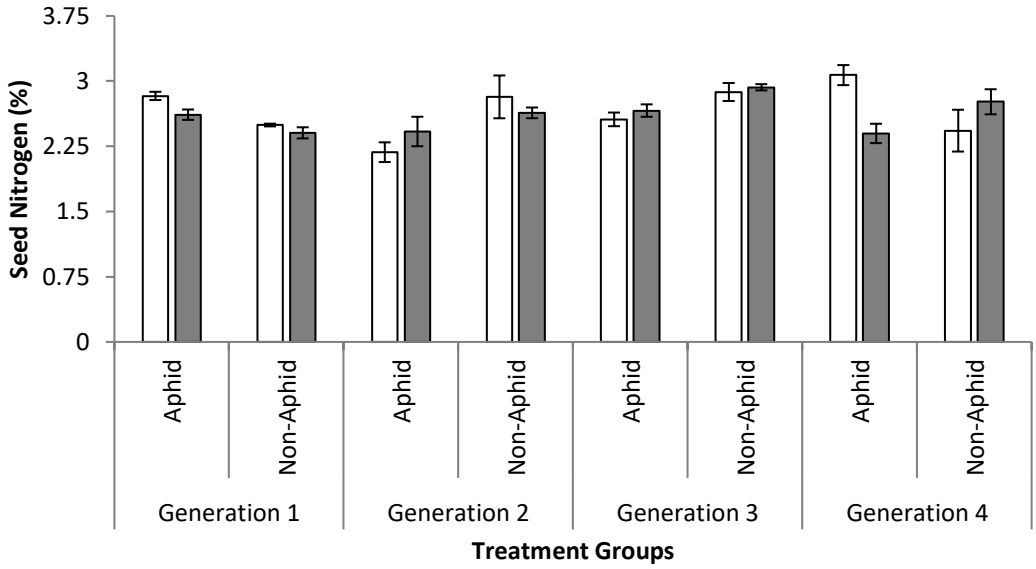

Figure 5.2 Mean percentage of nitrogen per mg of seed material (seed nitrogen) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

5.3.1.2 Seed phosphorus

Due to 0.2g of dried seed material being needed for each replicate, the non-aphid treated generation two plants only had one replicate. This meant that an ANOVA could not be run. The results suggest that plants in generation two and three had considerably higher percentages of phosphorus present within the seed material when compared to the first and fourth generation plants. Over all the generations, mycorrhizal plants seem to have higher percentages of phosphorus in the seeds than non-mycorrhizal plants. Seeds produced by plants without aphids seemed to

have a higher percentage of phosphorus present per mg of seed material than those with herbivores over all the generations (Figure 5.3).

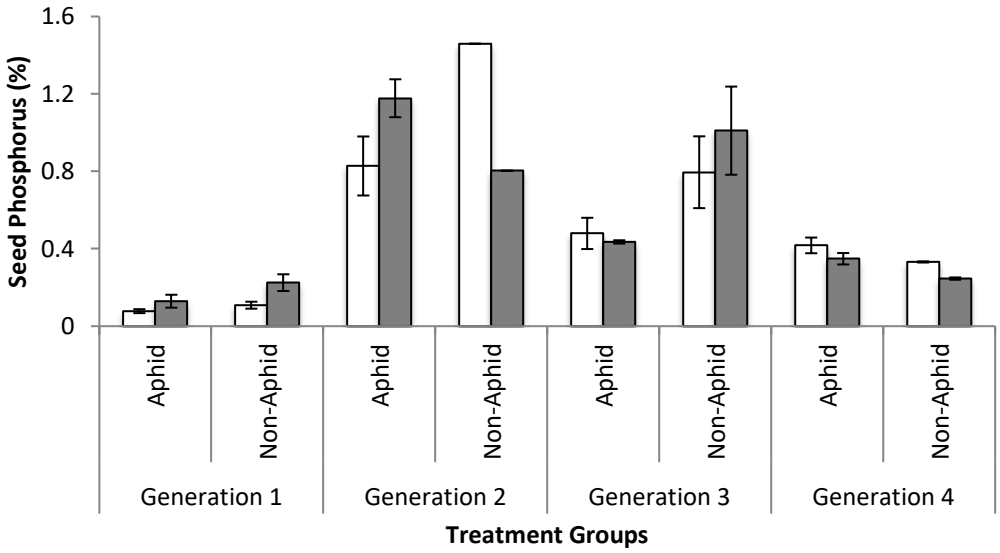

Figure 5.3 Mean percentage of phosphorus per mg of seed material (seed phosphorus) per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

5.3.1.3 Seed weight

The main point of this result was generation two plants produced the lightest seeds compared to the other generations, ($F_{3,266}$ = 24.144, p<0.001). The presence of aphids caused a change in seed weight between the generations, which led to a significant interaction ($F_{3,266}$ = 3.491, p<0.05). H and HAM plants in generation two had increased seed weight compared to C and AM plants. The seed weight of H and HAM plants in generations three and four was decreased when compared to C and AM plants in those generations (Figure 5.4).

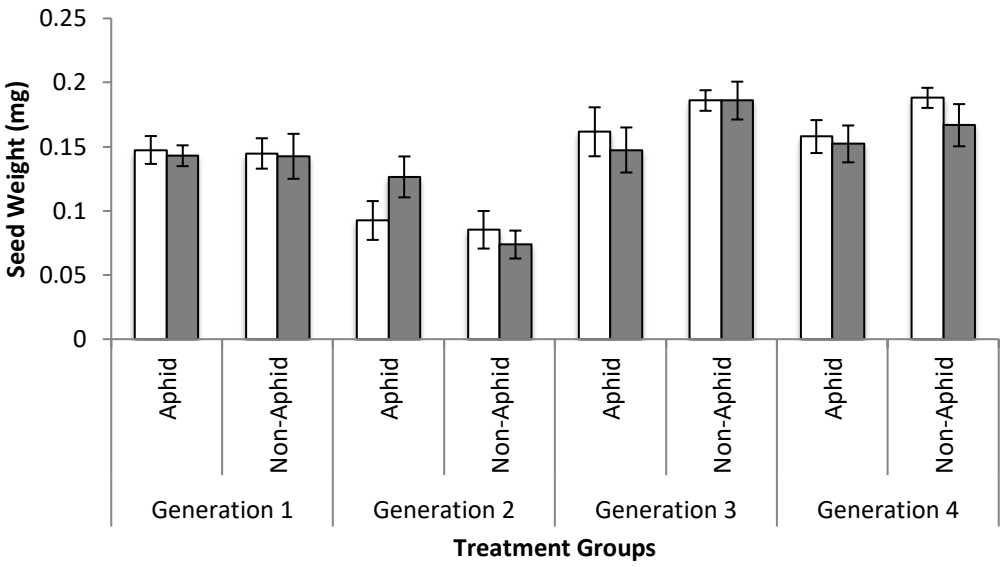

Figure 5.4 Mean seed weight per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

5.3.1.4 Percentage germination

The key conclusion from this result was plants in generation two had fewer seeds germinate within 13 days than any other generation ($F_{3,127}$ = 14.72, p<0.001). Over all generations seeds from mycorrhizal plants showed higher germination levels

than those from uncolonised parents ($F_{1,127}$ = 6.902, p<0.01). In generations one, three and four, mycorrhizal plants (AM and HAM) plants had higher percentage germination than C and H plants, but in generation two the reverse was true, leading to a significant interaction ($F_{3,127}$ = 4.828, p<0.01). Aphid attacked plants in generation one and two produced seeds with a higher percentage germination than non-attacked plants ($F_{3,127}$ = 6.586, p<0.001). However, in generations three and four the opposite happened where non-attacked plants (C and AM) had the higher percentage germination (Figure 5.5).

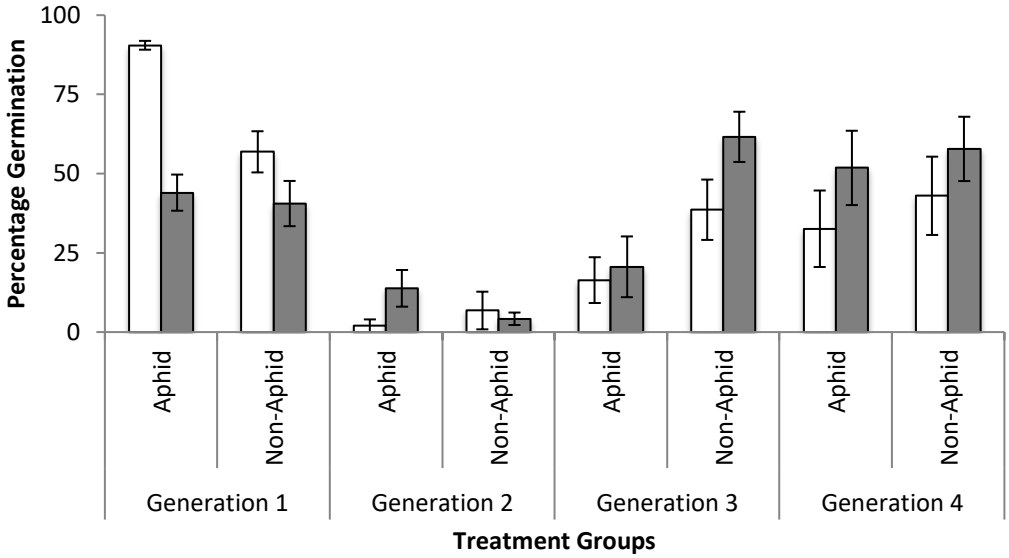

Figure 5.5 Mean percentage germination per treatment group in each generation of *S. vulgaris* grown. White represents addition of mycorrhizas (AM and HAM treatments) and grey represents absence of the fungi (C and H treatments). Bars represent means +/- SE.

5.3.2 Changing treatments and the impact on the seeds

Each parameter measured for generation two control plants was compared to 2A, 2B and 2C plants. The same occurred with generation three control plant seed parameters being compared to development time of 3A, 3B and 3C plants and generation four control seed parameters being compared to 4A, 4B and 4C.

Seed nitrogen, seed weight and percentage germination of seeds produced by generation two control plants were significantly different to the seed nitrogen, seed weight and percentage germination of seeds produced by 2A, 2B and 2C plants (Table 5.1).

	Generation Two			Generation Three			Generation Four		
	F	**P**	**Summary**	**F**	**P**	**Summary**	**F**	**P**	**Summary**
Seed Carbon	**7.62**	**<0.05**	**2A, 2B & 2C increased**	2.963	>0.05		1.187	>0.05	
Seed Nitrogen	0.724	>0.05		0.929	>0.05		0.142	>0.05	
Seed Weight	**26.97**	**<0.001**	**2A, 2B & 2C increased**	0.035	>0.05		0.787	>0.05	
Percentage Germination	**25.9**	**<0.001**	**2A, 2B & 2C increased**	0.163	>0.05		0.061	>0.05	

Table 5.1 A, B and C treatments from each generation compared with the generations control treatment to determine whether the effects being seen are from within generation or transgenerational effects. Significant differences indicated by bold text.

For clarity, due to the number of possible comparisons, only the significant results are included in this section and in the figures (additional figures in Appendix III).

5.3.2.1 Seed carbon and nitrogen

Seeds from 3B had higher seed carbon than generation three H seeds ($F_{1,5}$ = 13.57, $p<0.05$) (Figure 5.6).

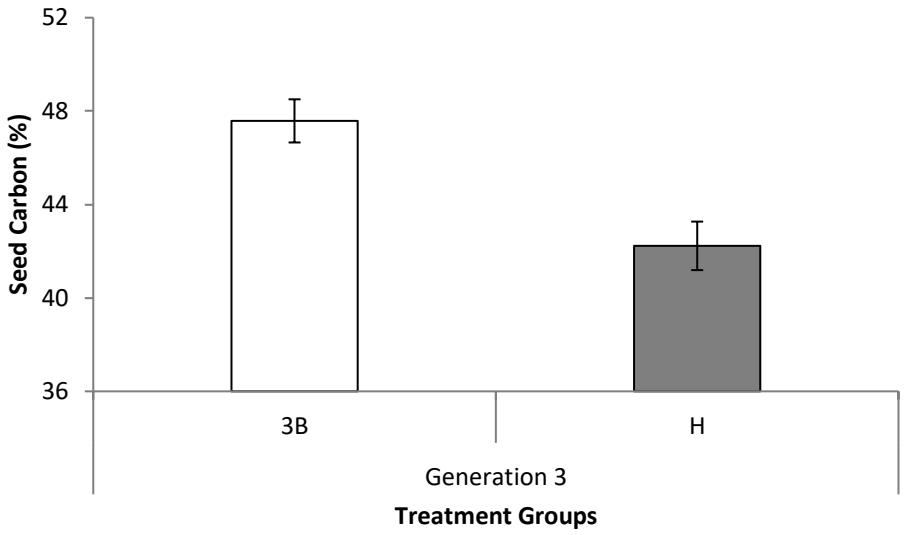

Figure 5.6 Mean seed carbon concentrations for seeds produced by plants grown from 3B and generation three H. White is 3B and grey is for the H treatment. Bars represent means +/- SE.

Seeds from 2C had a higher seed nitrogen than generation 2 HAM seeds ($F_{1,5}$ = 15.27, $p<0.05$). Seeds from 4B had higher seed nitrogen than generation four H seeds ($F_{1,4}$ = 30.18, $p<0.01$) (Figure 5.7).

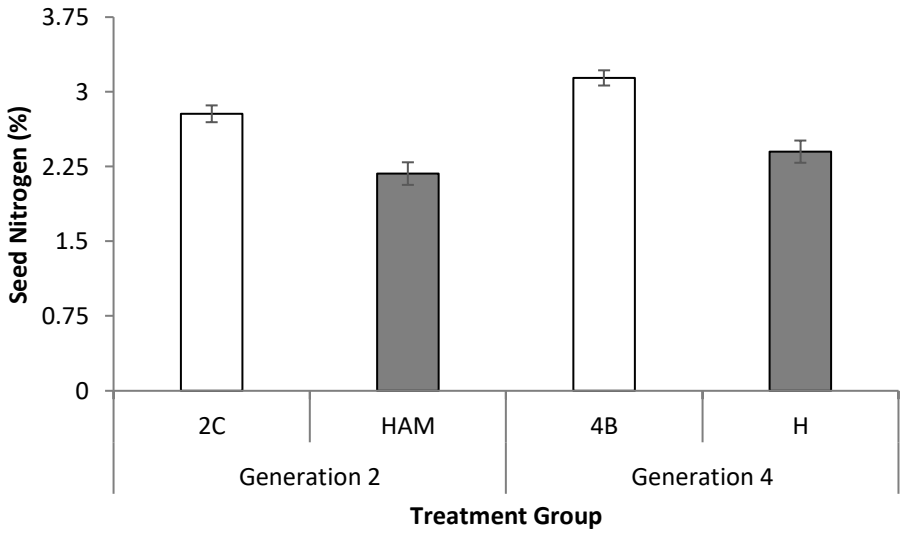

Figure 5.7 Mean seed nitrogen concentrations for seeds produced by plants grown from 2C or 4B and generation two HAM or generation 4 H. White is 2C or 4B and grey is for the HAM or H treatments. Bars represent means +/- SE.

5.3.2.2 Seed phosphorus

Seeds from 2B had lower seed phosphorus than generation two H seeds ($F_{1,4}$ = 36.91, p<0.01). The same occurred with seeds from 2C and generation two HAM seeds ($F_{1,5}$ = 16.33, p<0.01). Seeds from generation three H had higher percentage phosphorus than the 3B seeds ($F_{1,2}$ = 548.9, p<0.01) (Figure 5.8).

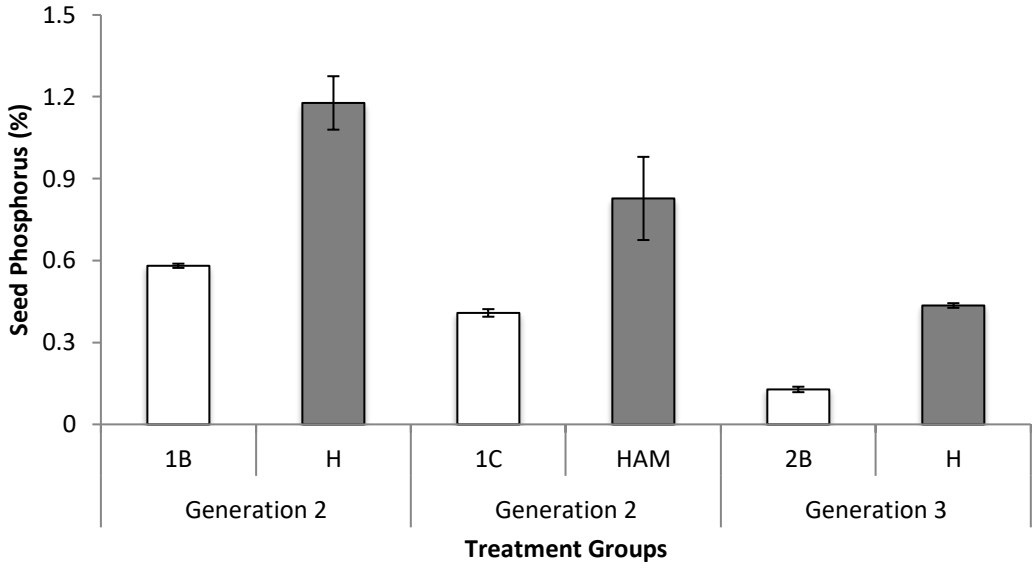

Figure 5.8 Mean seed phosphorus concentrations for seeds produced by plants grown from 2B, 2C and 3B and generation two H, HAM and generation three H. White is 2B, 2C or 3B and grey is for the H or HAM treatments. Bars represent means +/- SE.

5.3.2.3 Seed weight

In generation two AM, H and HAM plants the weight of their seeds was less than those produced from plants in the 2A ($F_{1,24}$ = 5.601, p<0.05), 2B ($F_{1,22}$ = 4.655, p<0.05) and 2C treatments, ($F_{1,26}$ = 9.127, p<0.01). The same occurred with generation three H seeds being lighter than seeds from 3B plants ($F_{1,26}$ = 6.688, p<0.05) (Figure 5.9).

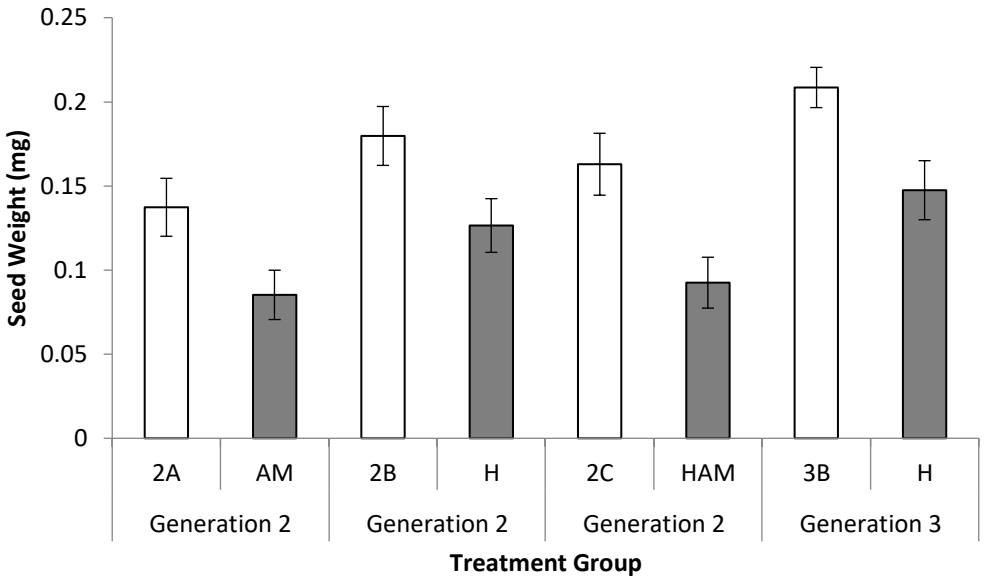

Figure 5.9 Mean seed weight (mg) for seeds produced by plants grown from 2A, 2B, 2C, 3B and 3B and generation two AM, H, HAM and generation three H. White is 2A, 2B, 2C and 3B and grey is for the AM, H or HAM treatments. Bars represent means +/- SE.

5.3.2.4 Percentage germination

Seeds from generation two H, generation three HAM and generation four HAM had a lower percentage germination than seeds from 2B ($F_{1,6} = 8.134$, $p<0.05$), 3C ($F_{1,6} = 7.925$, $p<0.05$) and 4C ($F_{1,9} = 5.474$, $p<0.01$) (Figure 5.10).

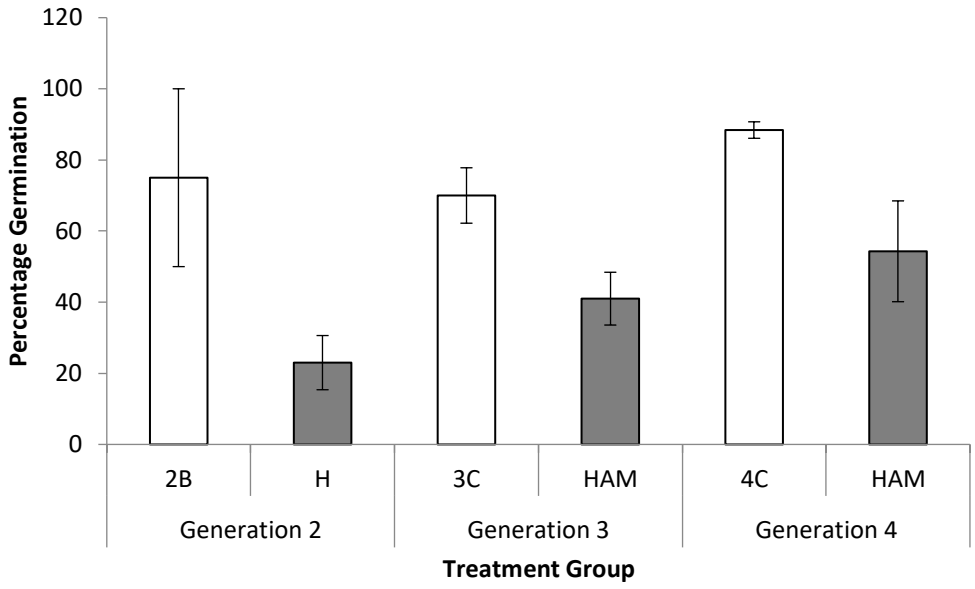

Figure 5.10 Percentage germination for seeds produced by plants grown from 2B, 3C and 4C and generation two H, HAM and generation three HAM. White is 2B, 3C and 4C and grey is for the H or HAM treatments. Bars represent means +/- SE.

5.4 Discussion

Parental effects and the length of time they are visible for in seeds of *S. vulgaris* depend upon the parameter being measured. Previous literature into transgenerational effects on seed parameters suggest that there is some maternal control through epigenetic changes, especially regulation through DNA methylation allowing maternal control of seed development (Berger & Chaudhury, 2009; Zhang *et al.*, 2010). However, the maternal control of seed development is very complex and involves both epigenetic and genetic controls (Chaudhury & Berger, 2001), so may not always be visible in these experiments.

5.4.1 Seed carbon

Seed carbon percentage was significantly lower in generation two than the other generations but the addition of mycorrhizas and/or aphids did not have an effect on seed carbon. The effects did not appear to be cumulative. *S. vulgaris* is an annual plant and the strategy is to keep mineral nutrient content of seeds consistent

(Fenner, 1986). In *S. vulgaris* the nutrient content was kept the same even when there was a change in the external supply (Fenner, 1986). The treatments may be changing the supply of nutrients but the plant itself is keeping the nutrient content similar. However, this does not explain the variation between generations. But these changes could be linked to other alterations causing a reduction in seed carbon.

5.4.1.1 Changing treatments and the impact on the seed carbon

Seed carbon concentrations produced by generation two control plants was significantly different to that of seeds produced by 2A, 2B and 2C. This suggests that there is a transgenerational effect between the first and second generation. This agrees with the literature that transgenerational effects are short-term and disappear after one generation (Latzel, 2015). It also suggests that only one generation is needed to control for parental effects on seed carbon and not two before starting ecological experiments (Latzel, 2015). Seeds from generation three H plants contained a lower concentration of seed carbon compared to treatment 3B. The controls and treated groups did not differ in any other treatment or generation.

5.4.2 Seed nitrogen

Seed nitrogen was increased in generations three and four compared to other generations and none of the effects appear to be cumulative. Nitrogen is translocated from the seed to the plant after germination in *Eucalyptus* (Milberg & Lamont, 1997). The study found that plants rely on nutrients from the seed in early growth more than nutrients from the soil. This could be true in *S. vulgaris* where the seedling relied upon seed nutrients for early growth. *S. vulgaris* was quicker to develop true leaves in generation four plants (Section 3.3.1). This quicker development time in seedlings could be due to higher nitrogen levels in generation four. Increased nitrogen levels could also be linked to the increase in dry biomass in

generation four compared to generations one and two (Section 4.3.1.5) but it does not explain the increase in generation one dry biomass.
Nutrient loading is known to be through the phloem and regulated by the source tissue (Zhang *et al.*, 2007). *Myzus persicae* is a sap-sucking aphid that feeds from the phloem. Aphids could reduce the nutrients being loaded into the seeds. It could explain the decrease in seed nitrogen in generation two. However there were aphids present on each generation but there was no increase in nitrogen in seeds from generation one.

5.4.2.1 Changing treatments and the impact on seed nitrogen

There was no difference between the generation control (treatment C) and their controls (A, B and C) for seed nitrogen. This suggests that the effects on nitrogen levels by herbivory are an affect caused within the generation and not a parental effect from the previous generation. Seed nitrogen differed twice between plants grown in control conditions (2C and 4B) compared to their treated counterparts (generation 2 HAM and generation 4 H). This was not seen repeatedly, suggesting that this was not linked to transgenerational changes and instead caused by in generation effects.

5.4.3 Seed phosphorus

Due to lack of replicates in generation two this could not be analysed statistically. The potential differences in nitrogen, carbon and phosphorus results could be linked to no nutrients following the same pathway to the seed for nutrient loading (Sale & Campbell, 1980). Phosphorus content seemed to follow the same increase in second-generation seeds in *S. vulgaris* (West, 1995). Maternal phosphorus supply and AMF increased the phosphorus supply to the seeds of *Abutilon theophrasti* (velvetleaf) (Lewis & Koide, 1990). This also appeared to occur in these experiments even with a lack of mycorrhizal colonisation (Section 7.3.1). AMF colonisation has been seen to modify the way seed phosphorus levels were allocated in *A. theophrasti* (Koide & Lu, 1992). In *A. theophrasti* the increased phosphorus levels

were not seen to increase seedling performance (Lewis & Koide, 1990). Interestingly there was a decrease in dry biomass in generations two and three (Section 4.3.1.5), which was coincidental with an increase in phosphorus levels. *Arabidopsis thaliana* with lower phosphate levels induced jasmonic pathways and this increases plant chemical defences (Khan *et al.*, 2016). However, there appeared to be no effect of increased phosphorus levels on *S. vulgaris* chemical defences. The fecundity of the aphids feeding upon the aphids feeding upon *S. vulgaris* was not altered by the increase phosphorus levels in generation two and three (Section 6.3.1).

5.4.3.1 Changing treatments and the impact on seed phosphorus

Due to lack of replicates, parental effects on seed phosphorus and how long they last for could not be analysed.

Treatments in generation two H and HAM and generation three H plants had higher seed phosphorus levels than their controls (treatments 2B, 2C and 3B respectively). These results suggest that the treatments of aphids and/or mycorrhizas in each generation are causing an increase in seed phosphorus levels in addition to the parental effects. The seed phosphorus did seem to decrease from generation 2 H to generation 3 H and their respective controls, so there may be cumulative decrease over multiple generations.

5.4.4 Seed weight

Seeds produced by plants from generation two were considerably lighter than seeds from any other generation. The effects did not appear to be cumulative. Variation in seed size can result in progeny that can effectively cope with unpredictable but recurrent instabilities in their environment (Capinera, 1979). The variation in *S. vulgaris* seed weight could help the plant to cope with variations of insect attack and also whether there is any mycorrhizal colonisation or not. Mycorrhizal colonisation was low and did vary in these experiments (Section 7.3.1), therefore making it unpredictable. Increased maternal nutrients in *Campanula*

americana (American bellflower) caused an increase in seed mass (Galloway, 2001). Nutrient content of *S. vulgaris* in these experiments was not measured but seed nitrogen and carbon levels did follow the same pattern as seed weight, suggesting that there was increase maternal nutrition. Mycorrhizal colonisation did not affect seed weight, which agrees with previous literature on *S. vulgaris* and seed weight (Peat & Fitter, 1993; West, 1995). Aphid presence was seen to increase the seed weight in generation two but decrease the weight in generations three and four. Timing and nutrient availability to the herbivore can affect the cost or benefit of the herbivore (Maschinski & Whitham, 1989), while the timing was kept the same for each generation, the aphid feeding intensity may have differed. The effects of aphids on seed weight had no apparent relationship to an increase in the seed nutrient levels in *S. vulgaris*. The aphids were placed onto the plant at the same time each generation, so that does not account for why the seed weight varied over multiple generations and with aphids present.

5.4.4.1 Changing treatments and the impact on seed weight

The average weight for seeds produced by generation two control plants was significantly different to that of seeds produced by 2A, 2B and 2C. This suggests that there is a transgenerational effect between the first and second generation. This agrees with the literature that transgenerational effects are short-term and disappear after one generation (Latzel, 2015). It also suggests that only one generation is needed to control for parental effects on seed weight and not two before starting ecological experiments (Latzel, 2015).

Plants in generation two AM, H, HAM and generation three H had decreased seed weights compared to their controls (treatments 2A, 2B, 2C and 3B). There seemed to be no cumulative effect between 2B and 3B. The differences in generation two may be linked to transgenerational effects from the previous generation. However, the difference between generation three H and 3B suggest that insect herbivory in the third generation caused a decrease in seed weight.

5.4.5 Percentage germination

Percentage germination was lowest in seeds produced by generation two plants and no effects seemed cumulative. Interestingly low percentage germination correlated with slower germination rate, as seeds from generation two parents were the slowest to germinate (Section 3.3.1; Figure 3.1). Seed mass has been used to explain variation in percentage germination in *Sinapis arvensis* (velvetleaf) (Luzuriaga *et al.*, 2006). Seeds from *S. vulgaris* in generation two of the experiments reported were the lightest so that may explain a decrease in percentage germination. Changes in germination are known to affect plant development and quality of *S. arvensis* (Luzuriaga *et al.*, 2006). In the *S. vulgaris* grown there were no significant effects on the development or quality of seedlings in generation three (Section 4.3.1). However, interestingly plants in generation two had a reduced number of flowers compared to other generations, so maybe these flowers also produced poorer quality seeds (Section 4.3.1.1). The seed produced with low percentage germination were very different to the seeds that germinated. The failed seeds were much lighter in weight, creamy white in colour (Plate 5.1) and very thin compared to the seeds that germinated (Plate 5.2). The seeds were between 0.1 and 1mm in size for both 'failed' and 'healthy' seeds. This suggested that they were dead and therefore unable to germinate.

Plate 5.1 'Failed' seeds produced by *S. vulgaris.* Pappus present.

Plate 5.2 'Healthy' seeds produced by *S. vulgaris.* Pappus absent.

Previous work suggests that the colonisation of the roots by AMF caused no effects on percentage germination (West, 1995). However, in these experiments there was a significant effect on percentage germination by mycorrhizal colonisation. Plants colonised by mycorrhizas in generation one, three and four produced seeds with higher percentage germination than seeds produced by plants from H and C treatment groups. It may be that the number of dead seeds was increased in non-mycorrhizal plants. So they were not able to germinate which affected percentage germination. It could also be that the seed coat is altered by the mycorrhizal colonization so the seed dormancy mechanisms are altered.

5.4.5.1 Changing treatments and the impact on percentage germination

The percentage germination for seeds produced by generation two control plants was significantly different from that of seeds produced by 2A, 2B and 2C. This suggests that there was a transgenerational effect between the first two generations, which agrees with the literature that transgenerational effects are short-term and disappear after one generation (Latzel, 2015). It also suggests that only one generation of plants is needed to control for parental effects on percentage germination and not two before starting ecological experiments (Latzel, 2015).

Generation two H, generation three HAM and generation four HAM all had lower percentage germination than their controls (treatment 2B, 3C and 4C respectively).

It could be that there was a parental effect from maternal plants attacked by aphids, as there was a significant parental effect between generation one and two. A higher percentage of seeds germinating may mean a higher chance of the offspring surviving. There was no cumulative effect between generation three and four HAM plants. The lack of significance to the overall control treatments for these generations suggests that it is the treatments within the generation that was causing these differences and not parental effects.

5.5 Conclusion

The hypothesis of parental effects in *S. vulgaris* changing seed parameters of chemistry, weight and percentage germination was upheld for some of the parameters, with both aphids and mycorrhizas producing different results. Seed carbon and nitrogen did not appear to be affected by parental effects (Table 5.1). The number of generations the effects occurred over differed to those seen in chapters three (Table 3.1) and four (Table 4.1). In chapter three the effects disappeared but reappeared over multiple generations. However in chapter four the parental effects disappeared after one or two generations but only reappeared for final leaf number. For seed parameters the parental effect either appeared after multiple generations or disappeared after only one generation. Some of these results may be linked to other alterations found in development time (Section 3.3.1). Shortening of true leaf development could be caused by changes in the seed nutrient levels, or changes to dry biomass may have been caused by changes to seed nutrient levels (Section 3.3.1; Section 4.3.1.5). Alterations to the seed weight were also seen to correlate with changes to the percentage germination of the seeds within this chapter. The secondary hypothesis of mycorrhizal and aphid presence causing changes in *S. vulgaris* final parameters was also upheld, especially with the interaction in final parameters for flower size. Overall the presence of aphids and/or mycorrhizas over multiple generations were seen to alter the results for seed parameters. Parental effects on seed quality do occur in *S. vulgaris* but the generations that these last for, depended upon the parameter being measured. This has important implications for the set-up of ecological experiments. The

number of generations grown before the experiment starts could depend upon the parameter that is being measured, which was also shown in Table 4.1.

Chapter Six

Senecio vulgaris* parental effects upon *Myzus persicae

6.1 Introduction

This study investigated the effects of *Senecio vulgaris* on *Myzus persicae* over multiple generations, which addressed the first theme of this project; which environmental conditions cause parental effects to occur. It specifically explored the objective; does the presence of insect herbivores cause changes in plant defences over multiple generations. *M. persicae* growth rate, teneral adult weight, population increase and chemical defences in *S. vulgaris* were measured.

S. vulgaris synthesises pyrrolizidine alkaloids (Hartmann *et al*., 1989). The nitrogen based plant defences are produced in the roots of the plants and transported to specific plant tissues that need defending (Hartmann *et al.*, 1989). Defence against insect herbivores is important to ensure the plant's survival and its ability to reproduce.

Myzus persicae, a generalist sap-sucking herbivore (Berry *et al.*, 1998), induces plant chemical defences through the salivary proteins that come into contact with the plant when the aphid is feeding (Elzinga *et al.*, 2014). Plant chemical defences in *Arabidopsis thaliana* caused the aphid herbivores to have decreased progeny production (Elzinga *et al.*, 2014). *M. persicae* are known to induce pyrrolizidine alkaloids in *S. vulgaris* (Molyneux *et al.*, 1990), so potentially the chemical defences would reduce fecundity in the aphids.

Mycorrhizal colonisation has been shown to increase the alkaloids present in *Jacobaea vulgaris* roots, but not the alkaloids present in the above ground vegetation (Hill *et al.*, 2018). However, AMF colonisation has been linked to improving a plant's tolerance to the insect herbivore. Multiple species of milkweed (*Asclepias*) had increased tolerance to insect herbivores when phosphorus was increased by AMF colonisation (Tao *et al.,* 2016). Tao *et al.* (2016), found that increased nitrogen from AMF colonisation increased the production of chemical defences, however if growth rate was increased then chemical defence production decreased. This suggests that there is a trade-off between plant growth and defences. AMF colonisation can cause differing effects on insect performance (Vannette & Hunter, 2009). In a review on AMF colonisation, crop plants and insect performance it was found that the increase in nutrients from AMF inoculation can

increase plant defences, but it can also increase insect performance (Vannette & Hunter, 2009). When mycorrhizas colonise the plant, they modify plant defences through priming the jasmonic acid pathway (Pozo *et al.*, 2010; Jung *et al.*, 2012). The priming of jasmonic acid pathways could lead to quicker production of chemical defences when the plant is under attack. The presence of mycorrhizas may increase *S. vulgaris* defences and/or tolerance of insect herbivores, which could lead to insect herbivores having a reduced fecundity.

Plant defences have been shown to pass between generations of plants (Agrawal, 2002; Holeski *et al.*, 2012; Latzel *et al.*, 2012). Larval weight of *Pieris rapae* (small cabbage white butterfly) was reduced when insects were feeding upon progeny from an attacked parental *Raphanus raphanistrum* (wild radish) (Agarwal, 2002). It was believed that rapidly induced defences were the cause of the reduced larval weight (Agrawal, 2002). The priming of defences due to the previous generation being attacked by insect herbivores could occur in the experiments reported here, especially as the plants were subjected to insect herbivory over multiple generations. In *Arabidopsis thaliana* changes in heritable variation in plant defences were linked to epigenetic changes through DNA methylation (Latzel *et al.* 2012). Parental effects have been seen to affect plant defences (Agrawal, 2002; Holeski *et al.*, 2012; Latzel *et al.*, 2012) so it was hypothesised that parental effects would alter *S. vulgaris* chemical defences. If this hypothesis were upheld, the alteration in plant chemical defences would decrease the fecundity of aphids feeding upon *S. vulgaris.* In addition, if these hypotheses were upheld, the addition of mycorrhizal fungal spores to *S. vulgaris* may cause further effects on *M. persicae* fecundity. Overall it was hypothesised that the environment of the previous generation affects the chemical defences of the progeny generation.

6.2 Methods

This experiment involved growing four generations of *S. vulgaris* under the same environmental conditions (Section 2.2). There were two treatment groups grown per generation, with forty plants in total and twenty in each treatment group. The two treatment groups were H and HAM (Figure 2.1). The addition of mycorrhizas

over multiple generations was monitored to see if the symbiosis had any benefit on the plant and or the insect herbivore. From the first generation of HAM plants, all the seeds used came from mycorrhizal colonised parents.

6.2.1 Setting up aphid fecundity experiments

The experiments were started once the plants had begun bud production because if the plants were any younger the aphids could kill them. The method was adapted from Leather & Dixon, (1984). Three adult *M. persicae* from the culture (Section 2.3) were placed onto a specific leaf of each plant. The aphids were unable to move from the leaf due to 'Oecotak' barrier glue (Oecos Ltd, Kimpton, UK) being placed around the petiole of each leaf. The aphids were left on the leaves until they had produced nymphs, which could take up to 3 days. Once nymphs were produced, the adults were removed from the plant. The nymphs were weighed and placed back onto the specific leaf. The nymphs were left until they reached the teneral adult life stage. Teneral adults were removed from the leaf, weighed and placed back onto the same leaf to begin reproduction. New nymphs were counted daily and removed from the plant, to reduce overcrowding. The plants were checked daily for new nymphs until the adult stopped reproducing or died.

6.2.1.1 Teneral weight

Each aphid used in the aphid fecundity experiments were weighed once they reached the teneral adult life stage.

6.2.1.2 Mean relative growth rate

Aphid weight differences between the life stages of nymph and teneral adult were recorded to calculate the mean relative growth rate. The mean relative growth rate (MRGR) was calculated by the equation given by Leather & Dixon (1984):

$$MRGR = \left(\frac{\ln(teneral\ adult\ weight) - \ln(birth\ weight)}{Days\ taken\ to\ reach\ teneral\ adult\ weight}\right)$$

Equation 6.1 Equation to calculate the mean relative growth rate of aphids (Leather & Dixon, 1984).

6.2.1.3 Intrinsic rate of increase

To calculate the intrinsic rate of population increase (r_m), the time taken to reach adulthood and produce their first nymph was recorded, after which the number of nymphs produced per day was recorded

$$r_m = 0.74(\frac{\ln(FD)}{D})$$

Equation 6.2 The r_m is calculated using the time taken from birth to produce the first nymph (D) and the number of nymphs produced over a period equivalent to time D (FD) starting at the production of the first nymph. A constant obtained from the mean pre-reproductive times for numerous aphid species (Wyatt & White, 1977) is used in the equation.

6.2.1.4 Statistical analysis

Analysis of aphid fecundity measurements was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
Differences in the aphid fecundity measurements over treatment groups in each generation were tested using a two-way, repeated measures ANOVA, employing AMF absence/presence and generation as the main effects.

6.2.2 Comparing the effect of changing treatments

Development time (Section 6.2.1.1), mean relative growth (Section 6.2.1.2) and intrinsic rate of increase (Section 6.2.1.3) were measured on aphids feeding upon non-mycorrhizal plants grown from seeds produced by HAM plants. This was attempted to see if the mycorrhizal colonisation was having any effect on potential parental effects being passed between generations.

6.2.3 Measuring Senecio vulgaris chemical defences

Pyrrolizidine alkaloid analysis was attempted on *S. vulgaris* after it was harvested. Harvested plant material was oven dried at 60^{o}C for 24 hours as it reduced the variability in the material.

Dried plant material was ground to a powder. Samples (1g) were weighed into 15ml round-bottomed glass tubes and extracted with 0.05M sulphuric acid (5ml) plus an internal standard in an ultrasonic bath for 15min. The internal standards used were the alkaloids monocrotaline and nicotine, and therefore of similar chemical structure as the secondary chemical defences present within *S. vulgaris*.

The sample was centrifuged at ~4000g for 10minutes. The supernatant was decanted into another round-bottomed glass tube. The pellet was re-extracted with another 5ml of sulphuric acid but no more standard, centrifuged and added to the supernatant already extracted. 500mg zinc dust was added to the extract and agitated for three hours at room temperature. After three hours, the sample was centrifuged, and filtered if necessary. The cation exchange solid phase extraction column was prepared using a vacuum chamber, with 6ml methanol followed by 6ml 0.05M H_2SO_4 run through the extraction columns. The extract was loaded, washed with 3ml water followed by 3ml methanol and eluted with 6ml ammoniated methanol into a glass test tube. Ammoniated methanol was prepared by connecting a septum-capped bottle of 50ml 0.880 ammonia to a bottle containing 50 ml methanol via syringes and silicone tubing and a gas line being used to bubble ammonia vapour into the methanol, with another needle. The gas was bubbled

through for roughly half hour. The ammoniated methanol was dried down under nitrogen. The residue was taken up by 1ml ethyl acetate into a 2ml GC vial.
The samples were analysed by gas chromatography-mass spectrometry (GC-MS), which identified the alkaloids and quantified the chemicals present. One microliter of sample was injected into the GC-MS through the inlet. The inlet has a constant flow with a solvent delay for 5 minutes. The solvent delay was needed as the samples were taken up in ethyl acetate. The sample vaporised in the oven for 2 minutes at 50^{o}C, it was passed through the GC column at 200^{o}C for 15 minutes, and the gas was passed through again for 4 minutes at 300^{o}C and finally passed through the column for 8.3 minutes at 325^{o}C. After the GC phase, the sample was run through the mass spectrometer to scan for low mass parameters of 33 and high mass parameters of 350.

6.3 Results

The results explore changes in aphid development (teneral weight, MRGR and r_m) when feeding on aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over multiple generations.

6.3.1 Aphid fecundity experiments

6.3.1.1 Teneral weight

Teneral weight in generations one and four was significantly higher than that in generations two and three ($F_{3,102}$= 7.692, p<0.01). Mycorrhizal colonisation had no effect on teneral weight of aphids raised upon *S. vulgaris* (Figure 6.1).

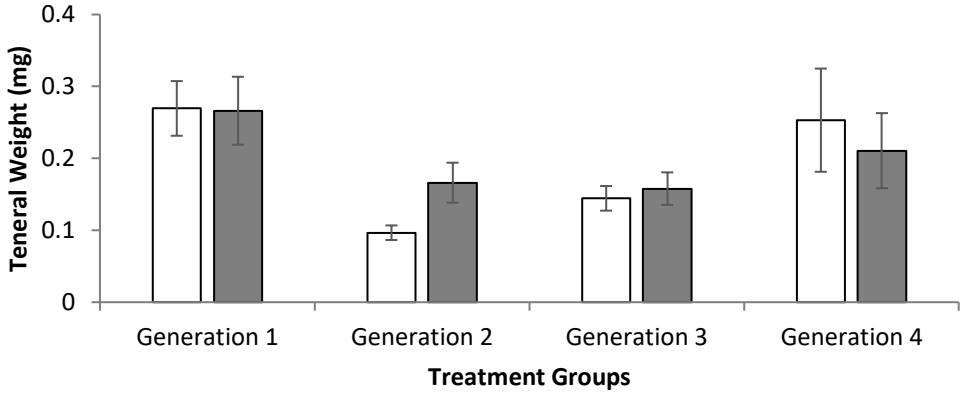

Figure 6.1 Teneral weight for *M. persicae* raised on control and mycorrhizal plants in each generation of *S. vulgaris*. White represents mycorrhizal plants and grey represents control (uncolonised) plants. Bars represent means +/- SE.

6.3.1.2 Mean relative growth rate

Mycorrhizal colonisation had no effect on MRGR of aphids raised upon *S. vulgaris*. Insect herbivory upon *S. vulgaris* that had experienced aphid herbivory over multiple generations had no effect on MRGR of the aphids (Figure 6.2).

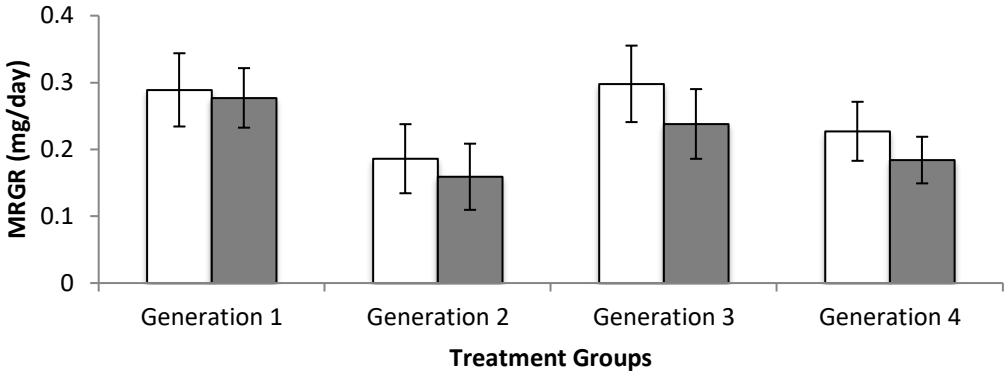

Figure 6.2 Mean relative growth rate (MRGR) for *M. persicae* raised on control and mycorrhizal plants in each generation of *S. vulgaris*. White represents mycorrhizal plants and grey represents control (uncolonised) plants. Bars represent means +/- SE.

6.3.1.3 Intrinsic rate of growth

Mycorrhizal colonisation had no effect on intrinsic rate of population increase (r_m) of aphids raised upon *S. vulgaris*. Insect herbivory upon *S. vulgaris* that had experienced aphid herbivory over multiple generations had no effect on r_m of the aphids (Figure 6.3).

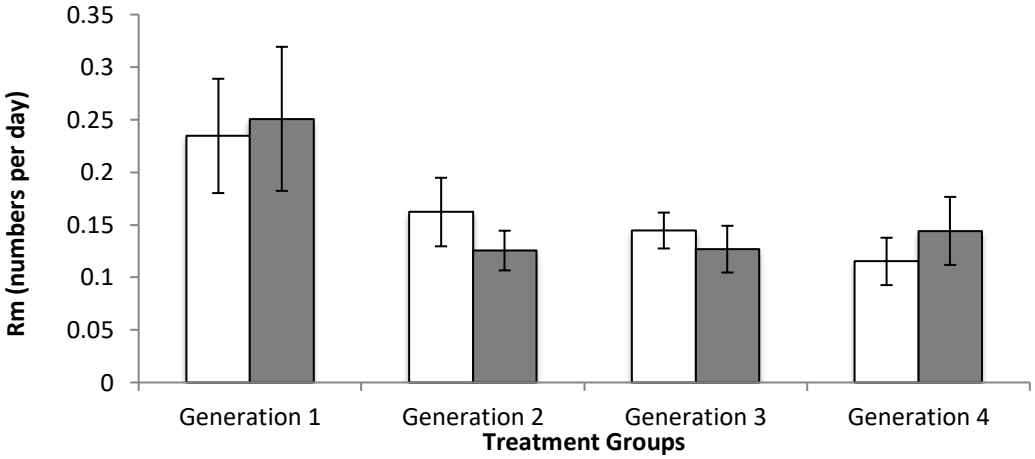

Figure 6.3 Mean intrinsic rate of population increase (r_m) for *M. persicae* raised on control and mycorrhizal plants in each generation of *S. vulgaris*. White represents mycorrhizal plants and grey represents control (uncolonised) plants. Bars represent means +/- SE.

6.3.2 Changing the treatment and the effect on aphid fecundity

No results were obtained, as wasps parasitized the aphids so the fecundity experiments were stopped. No results were obtained for any generation as multiple generations of this treatment group were grown at the same time. The results that were collected are not shown, as they would not be an accurate representation.

6.3.3 Measuring plant chemical defences

Regrettably this could not be performed. Firstly, the dried samples from generation one were accidentally disposed of by another person. Secondly, the methods used seemed to have multiple flaws that took too long to work around or were never totally resolved. The original internal standard was monocrotaline as it was very similar to the pyrrolizidine alkaloids present within *S. vulgaris*. This standard did not show any peaks on the GC-MS results, which could never be explained even when the amount of monocrotaline was increased. A pure solution of monocrotaline was run through the GC-MS in ethyl acetate and this still failed to produce any peaks. Thin layer chromatography (TLC) was used to see if there was a problem with the monocrotaline. TLC separates non-volatile compounds (Lewis & Moody, 1989) using a solvent with different substances in the analyte moving up at different rates so that separation was achieved (Vogel *et al.*, 1989). TLC showed that there was monocrotaline present in the samples so it was not being lost before going through the GC-MS. The GC-MS was checked with the internal coil replaced, a new pump was added and the sample was injected into the GC-MS without the use of the autosampler. Changes to the GC-MS still did not result in the peak for monocrotaline being seen. It was then decided to try a new internal standard of pure nicotine. Even after all these alterations, the samples still did not consistently show the internal standard. It appeared at random quantities or not at all. Standards of the pyrrolizidine alkaloids known to appear in *S. vulgaris* were run through the GC-MS and even these did not consistently produce peaks. TLC was used to explore where the pyrrolizidine alkaloids in the samples were being lost. It was found that they were not being removed from the cation exchange column.

The method was altered to increase the amount of ammoniated methanol used to elute the sample. Even after all these fixes there were still flaws. The pyrrolizidine alkaloids did not always consistently appear, in a run of twenty samples roughly one would show peaks. Due to inconsistent results and no accurate internal standard, it was decided to not continue this analysis.

6.4 Discussion

6.4.1 Aphid fecundity

Teneral weight of *M. persicae* was significantly lower when feeding on plants from generations two and three. The dry biomass of *S. vulgaris* varied between generations with plants from generations one and four having a higher dry biomass than plants from generations two and three (Section 4.3.1.5). *M. persicae* was shown to have decreased teneral weight when shoot dry biomass was increased (Gange *et al.*, 1999). There is some evidence that seed nutrient levels and nutrient levels in the parental plant's leaves are similar (Shibles & Sundberg, 1998). Seed nitrogen concentrations only varied between generation four and the other generations (Section 5.3.1.1), which suggests that the change in teneral weight is not linked to changes in nitrogen levels. Seed carbon levels were higher in generations one and four than generations two and three (Section 5.3.1.1). High carbon levels can reduce insect performance (Awmack & Leather, 2002). *Aulacorthum solani* (foxglove aphid) responded to changes in the C:N ratio but the response was dependent upon the host plant species (Awmack *et al.*, 1997). In *Vicia faba* (broad bean) the increased carbon caused a reduction in nymph production but in *Tanacetum vulgare* (tansy) the increased carbon levels caused a decrease in development time. In *Polyommatus icarus* (common blue butterfly) feeding upon *Lotus corniculatus* (bird's-foot trefoil), nitrogen levels remained the same but carbohydrate levels were increased and the development time of *P. icarus* was decreased (Goverde *et al.*, 1999). Carbon based sterols are known to affect insect performance. Green apple aphid (*Aphis pomi*) densities were increased when the aphids fed apple foliage that had been treated with a sterol inhibiting fungicide

(Biggs & Hagley, 1988). So, the changes in aphid teneral weight could be due to the changes in plant carbon levels and therefore changes in aphid food quality.

M. persicae MRGR and r_m were not significantly affected by feeding upon multiple generations of *S. vulgaris* that had experienced insect herbivory. *S. vulgaris* has a trade-off between production of chemical defences and reproduction and the effect that 'wins' the trade-off is very unpredictable (Frischknecht *et al.*, 2001). The unpredictable nature of this trade-off could be why no effects were seen.

M. persicae has been shown to have increased fecundity and intrinsic rate of population increase when feeding on fertilised plants compared with non-fertilized plants (Jansson & Smilowitz, 1986; Stafford *et al*., 2012). The aphid is known to demonstrate a significant preference for plants with higher nutrient content (Van Emden & Bashford, 1971). There is a small amount of evidence that leaf nitrogen levels are related to seed nitrogen levels in soybeans (Shibles & Sundberg, 1998), but it is unknown if this occurs in *S. vulgaris*. Mycorrhizal colonisation is well known to increase the nutrient content of plants including nitrogen, so this potentially could have altered aphid population increase between non-mycorrhizal and mycorrhizal plants. However, there was no significant difference between control and mycorrhizal plant seed nitrogen and carbon levels (Section 5.3.1.1) and so this may be the most likely reason why no difference was found in aphid performance on mycorrhizal and non-mycorrhizal plants.

It is known that food supply to the aphids is a major cause for changes in aphid development and fecundity. High amino acid (nitrogen) content caused *Drepanosiphum platanoidis* (sycamore aphid) to be increased in size and have a higher fecundity when feeding upon *Acer pseudoplantus* (sycamore) (Dixon, 1970). When the amino acid production ceased, the reproduction of *D. platanoidis* stopped too (Dixon, 1970). The same was seen with seasonal amino acid changes in wheat and *Rhopalosiphum padi* (bird cherry-oak aphid) development (Weibull, 1987). However, it is thought that plant nutrient levels did not fluctuate too greatly between generations of *S. vulgaris* in this experiment. Decreases in plant quality have been linked to the production of winged morphs in aphids (Dixon & Glen, 1971) and in *M. persicae* (Harrewijn, 1976). No winged aphids developed during

these experiments, which suggests that plant food quality did not decrease too much during these experiments.

M. persicae is known to develop faster and have a greater fecundity with fluctuating temperatures (Davis *et al.*, 2006). The lack of changes to fecundity may be due to the fact that the environment in the CT room was the same between generations. The optimal temperature for *M. persicae* to develop was found to be 26.7^{o}C (Davis *et al.*, 2006), which is much higher than the CT room temperature. This may be one of the reasons why the development of *M. persicae* differs from the literature (Horsfall, 1924; MacGillivray and Anderson, 1957).

Aphid fecundity on mycorrhizal plants was not significantly different to that on control plants, even within generations. This was not expected, as previous literature has found that phloem feeding insects benefitted from mycorrhizal colonisation (Koricheva *et al.*, 2009). This was not expected as *M. persicae* is a generalist feeder and was found in to be positively affected by mycorrhizal colonisation when feeding on *Plantago lancelota* (ribwort plantain) (Gange *et al.*, 1999). Mycorrhizal colonisation was low in the *S. vulgaris* used in these experiments (Section 7.3.1), so it could be that the low colonisation meant the plant did not receive any benefit from the symbiosis. This could have led to no difference in aphid reproduction feeding upon H and HAM plants. Mycorrhizal colonisation has been shown to physically enlarge the vascular bundle size in wheat and this was thought to be a reason for altered aphid fecundity and development (Simon *et al.*, 2017). Low colonisation could have stopped the mycorrhizas from physically altering the plant. The interaction between plants, aphid and mycorrhizas is very complex (Guerrieri & Digilio, 2008). Tomato plants colonised by *Glomus mossae* were able to dramatically reduce *Macrosiphum euphorbiae* (potato aphid) reproduction, due to increased plant defences (Guerrieri *et al.*, 2004). However, it was concluded that the responses of aphid reproduction seem dependent upon the species of plants, mycorrhizas and aphids (Guerrieri & Digilio, 2008). Often inoculation of a single species of arbuscular mycorrhizas seem to affect the insects feeding upon the colonised plants while multiple species of AMF seem to not affect the insect feeding on the plant (Gadhave *et al.*, 2016). It could be that the

combination of five different species of arbuscular mycorrhizas, *S. vulgaris* and *M. persicae* do not have any observable effects on plant defence responses.
Plant responses to aphids have been found to be slower than that of chewing insects (Guerrieri & Digilio, 2008), due to the precise and selective nature of their feeding (Schoonhoven *et al.*, 2007). Their salivary proteins interacting with calcium to 'hide' the wound from the plant (Will *et al.*, 2007). It could be that the plant response to aphid feeding was too slow to be observed before the plants died. Also, there are some cases where large numbers of aphids were able to feed on a plant without any observable symptoms (Guerrieri & Digilio, 2008), which happened to tomato plants with *M. euphorbiae* (Battaglia *et al.*, 2013).

6.4.2 Plant chemical defences

It was believed that the parental effects for defence priming would override other parental effects being passed between the generations. This was found to be true with chewing insects and increased germination in *Verbascum thapsus* (great mullein) (Alba *et al.*, 2016). Other parental effects were seen in other experiments in this thesis (Table 3.1; Table 4.1; Table 5.1). This suggests that if there were any parental influences on plant chemistry, they did not override the other parental effects.
A meta-analysis of transgenerational effects of defences in both animals and plants found that there is only weak evidence for anticipatory parental effects of defences being passed between generations (Uller *et al.*, 2013). If anticipatory parental effects occurred, then the effect caused in the progeny generation was very subtle (Uller *et al.*, 2013). It could be that there were no parental effects being passed between generations or the effects being passed were too subtle to pick up with the small amount of data collected.
In a review by Holeski *et al.* (2012) it was shown that several species of plant can pass defence priming to the next generation. *Raphanus raphanistrum* (wild radish) showed resistance to specialist feeding caterpillars (Agrawal, 2002); *Arabidopsis thaliana* was shown to reduce *Pieris rapae* (small cabbage white butterfly) herbivore performance when feeding on progeny from attacked plants (Rasmann *et*

al., 2012). However, transgenerational effects for plant defences have not been seen in *S. vulgaris*. In this experiment, there was no significant difference in aphid fecundity even after the plants had experienced multiple generations of insect herbivory. So, there may be no parental effects from *S. vulgaris* to decrease *M. persicae* performance.

6.5 Conclusion

The hypothesis that parental effects would alter *S. vulgaris* chemical defences was unable to be tested, as the pyrrolizidine alkaloid levels could not be analysed. The secondary hypothesis of parental effects in *S. vulgaris* altering aphid fecundity was rejected, with no significant difference in fecundity measurements over multiple generations of *S. vulgaris*. The hypothesis of mycorrhizal colonisation in the host plant affecting changes in aphid fecundity was also rejected, as there was no significant difference between control and mycorrhizal plants within or over multiple generations. Overall, there was no effect of parental effects or mycorrhizal colonisation on the fecundity of the aphids feeding upon *S. vulgaris*.

Chapter Seven

Parental effects and fungal presence in *Senecio vulgaris*

7.1 Introduction

These experiments investigated the effects of insect herbivores and time upon mycorrhizal fungal colonisation and community of endophytes present in each generation of *Senecio vulgaris*, which addressed the second theme of this project; transmission mechanisms of parental effects. It specifically explored the objective; vertical transmission of endophytes causing parental effects to occur in the progeny generations.

Fungal communities within plants are well known to benefit the host plant. A meta-analysis showed mycorrhizas within host plants influenced the insect herbivore performance but it was dependent upon the type of feeding, the diet of the insect and the specific fungus involved (Koricheva *et al.*, 2009). A meta-analysis into the effects of the endophyte *Neotyphodium coenophialum* found the host grass vegetation became toxic to insect herbivores when the endophyte was present (Saikkonen *et al.*, 2010).

There are fewer studies into the effects of insect herbivory upon mycorrhizal colonisation than studies into the effects of mycorrhizal colonisation on insect herbivores (Gehring & Whitham, 2002). Very few of those studies looked into the effects of both insect herbivores and mycorrhizas upon each other within the same study system (Gehring & Whitham, 2002).

In the majority of cases, above ground herbivores reduced mycorrhizal colonisation and altered the fungal community composition within the roots (Gehring & Whitham, 2002). However, it was found that the reaction of mycorrhizas to insect herbivores depends on the species of mycorrhizas (Gehring & Whitham, 2002). A meta-analysis did find that the presence of insect herbivores reduced the percentage of mycorrhizal colonisation in the roots by 3%, which was not deemed biologically relevant (Barto & Rillig, 2010). However, when looking at different species of plants it was found that forbs did not show any significant change in root colonisation when attacked by insects (Barto & Rillig, 2010). While it is still debated about why insect herbivory can cause the mycorrhizal root colonisation to decrease, there is no evidence that it is due to a reduction in carbon available to the roots and the mycorrhizal fungi, as might be expected (Barto & Rillig, 2010).

The effects of the timing of post inoculation root harvest and the levels of mycorrhizal colonisation in the roots has not been studied in *S. vulgaris*. In certain varieties of wheat it was shown that mycorrhizal colonisation decreased as the date of harvest was further from the date of mycorrhizal inoculation (Simon *et al.*, 2017). The plants in the experiments reported in this thesis were all harvested at roughly 90 days post inoculation. So an experiment was set up to see whether this would affect the mycorrhizal colonisation levels seen within the roots.

Endophytes are groups of fungi that live inside plant tissue and cause no visible signs of disease (Rodriguez *et al*., 2009). However much of the literature suggests that these endophytes can alter the plant's performance against insect herbivores. Non-entomopathogenic endophytes have also been found to have anti-herbivory effects (Gange *et al.*, 2012; Hartley *et al.*, 2015).

Endophytes present within *Cirsium arvense* (creeping thistle) cause chemical changes in the leaf, which are similar to chemical changes that occur when the leaf is under attack from insect pests (Hartley *et al.*, 2015). This can cause a significant reduction of foliar feeding insects on *C. arvense* when these endophytes are present (Gange *et al.*, 2012). *S. vulgaris* has been shown to contain many different species of endophytes within the leaves (Hodgson *et al.*, 2014), which could include fungi that have anti-herbivory effects.

In herbaceous species, there is evidence that fungi from ubiquitous taxa e.g. *Cladosporium*, can increase resistance of the host plant to insect herbivores (McGee, 2002; Jaber & Vidal, 2010; Gange *et al*., 2012). In legumes it was shown that the enhanced plant defences were through changes in alkaloid production (Oldrup *et al.*, 2010; Ralphs *et al*., 2011; Cook *et al.*, 2013). The plant-endophyte mutualism can benefit herbaceous plants by offering protection against pathogens through induced plant resistance and anti-herbivory effects against insects (Currie *et al.*, 2014).

The majority of endophytes are believed to enter the plant through horizontal transmission, from the environment as air-borne spores landing directly on to the plant (Rodriguez *et al*., 2009). However, vertical transmission of endophytes through the seed has been shown to occur in a range of herbaceous plants, including *S. vulgaris* (Hodgson *et al.*, 2014). The vertical transmission of certain

species of endophytes could be the mechanism for some parental effects being passed between generations (Gundel *et al.*, 2017). *Undifilum oxytropis*, an endophyte, is transmitted vertically through the seed and can increase the host plant's (*Astragalus* and *Oxytropis* spp.) (locoweeds) resistance to insect herbivores through changes to alkaloid production (Oldrup *et al.*, 2010; Ralphs *et al.*, 2011). Vertical transmission of an unidentified fungus in *Ipomoea carnea* (pink morning glory) was also shown to cause similar alterations to the alkaloid production (Cook *et al.*, 2013). This may be a mechanism for parental effects for plant defences to be passed through multiple generations of plants.

The effect of insect herbivory on mycorrhizal colonisation is much debated in the literature (Barto & Rillig, 2010), so it was hypothesised that there would be no significant difference in mycorrhizal root colonisation between the non-attacked and attacked plants in this study. It was predicted that mycorrhizal colonisation in *S. vulgaris* would be affected by the time taken to harvest post mycorrhizal inoculation, as it might reasonably be expected that the fungus would grow through the root system as it extends. Vertical transmission of endophytes is thought to be a mechanism for the transmission of parental effects to the progeny generation (Gundel *et al.*, 2017), so it was hypothesised that there would be a significant difference in the endophyte community between generations and treatments to correspond with significant differences in the parental effects seen in previous chapters. Overall it was hypothesised that fungal communities within the plant would have a positive effect on the parental effects seen in the next generation. The positive effects could be a reduction in insect herbivory on the plant or increased plant nutrition or by causing some of the parental effects to be transferred from parent to progeny plant.

7.2 Methods

7.2.1 Mycorrhizal colonisation experiments

7.2.1.1 Setting up mycorrhizal colonisation experiments

The experiment involved growing four generations of *S. vulgaris* under the same environmental conditions (Section 2.2). Each generation had a total of 80 plants grown for this experiment. There were four separate treatment groups with 20 plants in each group per generation. The two mycorrhizal treatment groups were AM and HAM (Section 2.2; Figure 2.1), where there was an addition of a commercial inoculum to the soil (Section 2.2). There were two non-mycorrhizal treatments that were used in the experiments as controls (treatments C and H).

7.2.1.2 Measuring mycorrhizal colonisation

All plants in the AM and HAM treatments in each generation were checked for mycorrhizal colonisation following the method laid out in Section 2.5. A random sample of plants in the non-mycorrhizal treatments (C and H) for each generation was also checked for mycorrhizal colonisation following the visualisation of arbuscular mycorrhizal fungi protocol (Section 2.5).

7.2.1.2.1 Statistical analysis

Analysis of mycorrhizal colonisation of *S. vulgaris* roots was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
If the sample sizes were unbalanced, two different forms of coding were used to ensure the data were interpreted correctly. It was suggested that weighted means were used to perform the multi-way ANOVA using the anova() function in the car package in R (Quick, 2010). The order of the independent variables was checked by running the ANOVA multiple times to ensure hierarchical order was achieved

(Quick, 2010). Even though this is a type I analysis, by running it multiple times it becomes a type II analysis. The second form of analysis used unweighted means but the same technique. The results were compared to ensure that all precautions were taken with the data. Fox (2018), stated that using type III analysis was to be avoided when using the car package and anova() function in R.
Differences in total colonisation, hyphal, arbuscular and vesicle colonisation over treatment groups in each generation were tested using a two-way, repeated measures ANOVA, employing aphid absence/presence and generation as the main effects.

7.2.1.3 Measuring the change in colonisation percentage over age of the plant when harvested

Five replicates of *S. vulgaris* were grown under controlled environmental conditions with Rootgrow inoculum added to the soil at the time of sowing (Section 2.2) for seven different time points between inoculation and harvest. The time points between inoculation and harvest were 30, 40, 50, 60, 70, 80 and 90 days. Once harvested, the roots were stored in ethanol and followed the protocol set out in Section 2.5 to visualise the mycorrhizal fungi.

7.2.1.3.1 Statistical analysis

Analysis of mycorrhizal colonisation of *S. vulgaris* roots was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.
If the sample sizes were unbalanced, two different forms of coding were used to ensure the data were interpreted correctly. It was suggested that weighted means were used to perform the multi-way ANOVA using the anova() function in the car package in R (Quick, 2010). The order of the independent variables was checked by running the ANOVA multiple times to ensure hierarchical order was achieved (Quick, 2010). Even though this is a type I analysis, by running it multiple times it becomes a type II analysis. The second form of analysis used unweighted means but

the same technique. The results were compared to ensure that all precautions were taken with the data. Fox (2018), stated that using type III analysis was to be avoided when using the car package and anova() function in R.
Differences in total colonisation, hyphal, arbuscular and vesicle colonisation over treatment groups in each generation were tested using a one-way, repeated measures ANOVA, employing time between inoculation and harvest as the main effect.

7.2.2 Endophyte experiments

7.2.2.1 Setting up endophyte community experiments

Four generations of *S. vulgaris* were grown under the same environmental conditions (Section 2.2). Each generation had a total of 80 plants grown. There were four separate treatment groups with 20 plants in each group per generation. The four treatment groups were C, AM, H and HAM (Section 2.2; Figure 2.1). In each treatment in each generation, a random sample of plants was used to analyse the endophyte community using the method laid out in Section 2.6. To check which species of fungi were present within the environment at the time of the experiments, there were two PDA plates left open in the environment for 24 and 48 hours respectively when the plants in each generation were grown. These plates were left to grow and the fungal growth placed onto PCA plates using the method in Section 2.6. Species of fungi that were isolated from the plants and from the environment were identified using either method explained in Section 2.6.

7.2.2.2 Statistical analysis

Isolation frequency of each fungus in each plant was calculated by dividing the total number of isolates of that endophyte species by the total number of isolates of all species isolated.
Species abundance and species richness, using plants as replicates, were analysed in R Studio 3.3.3. Normality tests were performed on whole data sets and data

were transformed if necessary using lambda calculated by Box-Cox transformation. Differences in species richness and species abundance over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence and generation as the main effects.
Principal community analysis was performed in CAP4 (Seaby & Henderson, 2014). Non-MDS analysis and ANOSIM analysis was used to analyse the community composition of endophytes found in the plants from each generation and treatment group.

7.2.2.3 Comparing changes to treatments and the impact on the endophyte community

Changes to treatments and the impact on the endophyte community were set up using methods in Section 2.4 (Figure 2.1). Each generation from generation two onwards had seeds from treated plants (AM, H and HAM) grown under control conditions (Section 2.4; Figure 2.1). There were ten plants grown per treatment and three treatment groups used per generation. In each generation, four plants in each treatment group had endophytes isolated using the protocol in Section 2.6. To check which species of fungi were present within the environment at the time of the experiments, there were two PDA plates left open in the environment for 24 and 48 hours respectively when the plants in each generation were grown. These plates were left to grow and the fungal growth placed onto PCA plates using the method in Section 2.6. Species of fungi that were isolated from the plants and from the environment were identified using either method explained in Section 2.6.

7.2.2.4 Statistical analysis for comparing changes to treatments and the impact on the endophyte community

Isolation frequency of each fungus in each plant was calculated by dividing the total number of isolates of that endophyte species by the total number of isolates of all species isolated.

Analysis of the endophyte community of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation. Differences between the species abundance/species richness of endophytes in plants grown from treated parents were compared to a true control in each generation where the parent was not subjected to any treatment. A one-way, repeated measures ANOVA was performed, employing parental treatment as the main effect.

Differences in species richness and abundance over treatment groups compared to controls were tested using a one-way, repeated measures ANOVA, employing aphid absence/presence or AMF absence/presence as the main effects for the control versus AM experiment and H experiment. Differences in species richness and abundance over HAM treatments versus control plants were tested using a two-way, repeated measures ANOVA, employing aphid absence/presence and AMF absence/presence as the main effects for the control versus HAM plants.

7.3 Results

The results explore the effects of the fungal communities within the plants (mycorrhizal colonisation and endophyte colonisation) within control (C), mycorrhizal only (AM), aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over multiple generations.

7.3.1 Mycorrhizal colonisation experiments

The key conclusion was there was no significant difference in total root colonisation by arbuscular mycorrhizal fungi between any of the treatments or generations. No mycorrhizal colonisation was seen in the control plant roots. The percentage root colonisation in plants that received inoculum was very low with there being only a maximum of 3% of the root colonised. There seems to be a significant difference in total colonisation of the roots between attacked plants and non-attacked plants in generation three (Figure 7.1), however only two plants out of twenty were found to have any mycorrhizal colonisation in this generation. The low number of replicates and the low amount of total root colonisation means the results should be treated with caution.

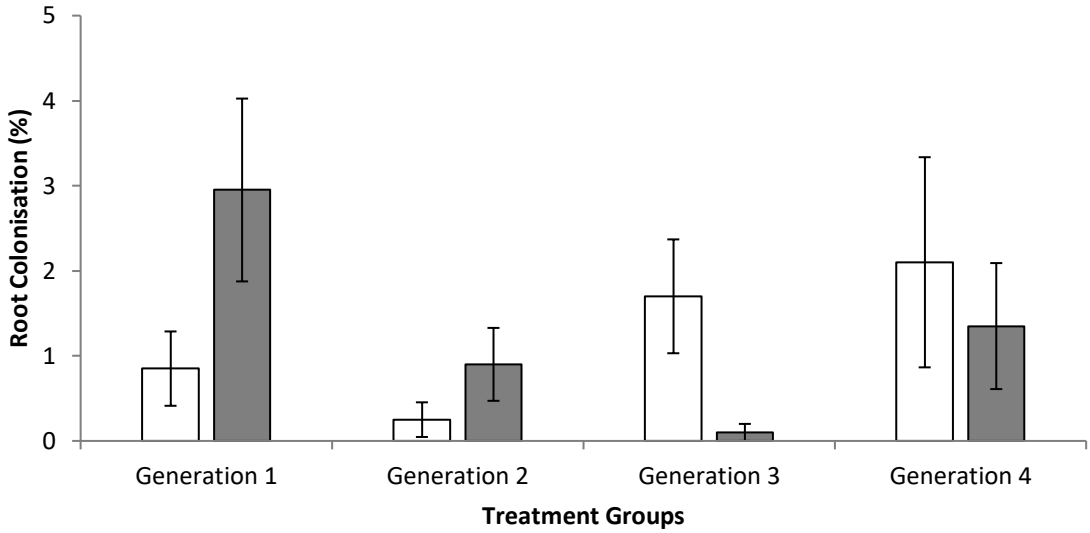

Figure 7.1 Mean total percentage of root colonised by arbuscluar mycorrhizal fungi per treatment group in each generation of *S. vulgaris* grown. White represents mycorrhizal only plants (AM treatments) and grey represents addition of aphid herbivores (HAM treatments). Bars represent means +/- SE.

The key point was there was no significant difference in hyphal colonisation between any of the treatments or generations. There seemed to be a significant difference in total colonisation of the roots between attacked plants and non-attacked plants in generations one and three (Figure 7.2), however the levels of colonisation were again incredibly low and so mean the results should be treated with caution.

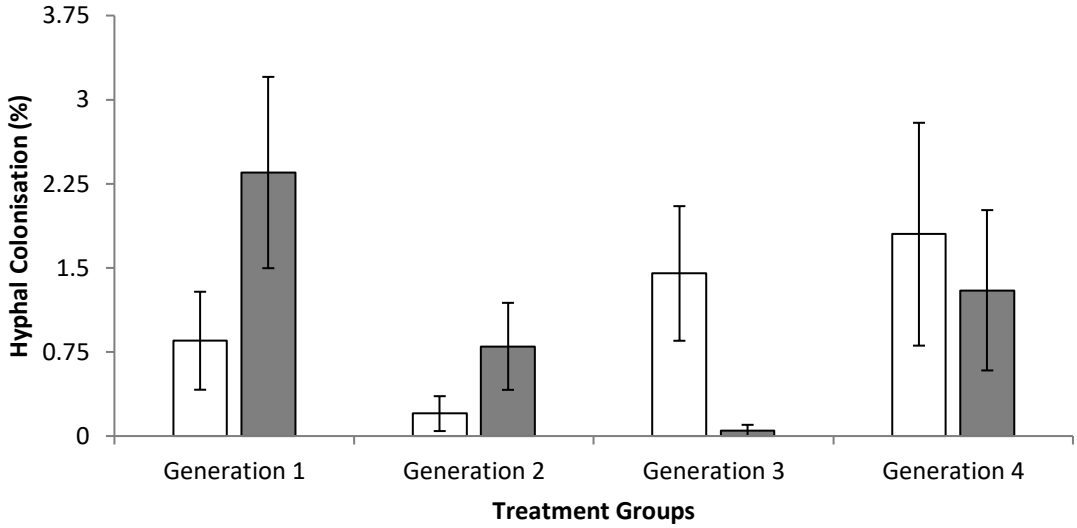

Figure 7.2 Mean percentage of roots colonised by hyphae per treatment group in each generation of *S. vulgaris* grown. White represents mycorrhizal only plants (AM treatments) and grey represents addition of aphid herbivores (HAM treatments). Bars represent means +/- SE.

There was no significant difference in vesicle colonisation between any of the treatments or generations. However, there were only eight plant roots with vesicles visible, which greatly skewed the results (Figure 7.3).

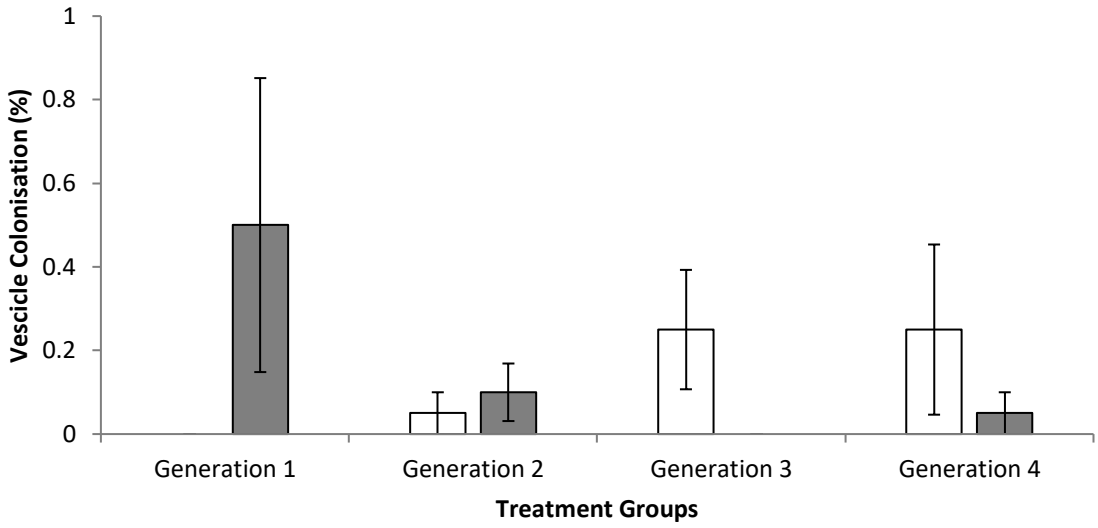

Figure 7.3 Mean percentage of root colonised by vesicles per treatment group in each generation of *S. vulgaris* grown. White represents mycorrhizal only plants (AM treatments) and grey represents addition of aphid herbivores (HAM treatments). Bars represent means +/- SE.

There were so few arbuscles recorded that no statistical analysis was attempted (data shown in Appendix IV).

7.3.2 Mycorrhizal colonisation changes over time

The main finding was there were no changes made to the methods for inoculating the plants or for visualising the mycorrhizal fungi. Total root colonisation was increased in this experiment compared to the root colonisation observed in Section 7.3.1. There was no arbuscular mycorrhizal fungi found in the roots of the control plants. Total root colonisation by AMF did not significantly alter over time as plants grew (Figure 7.4).

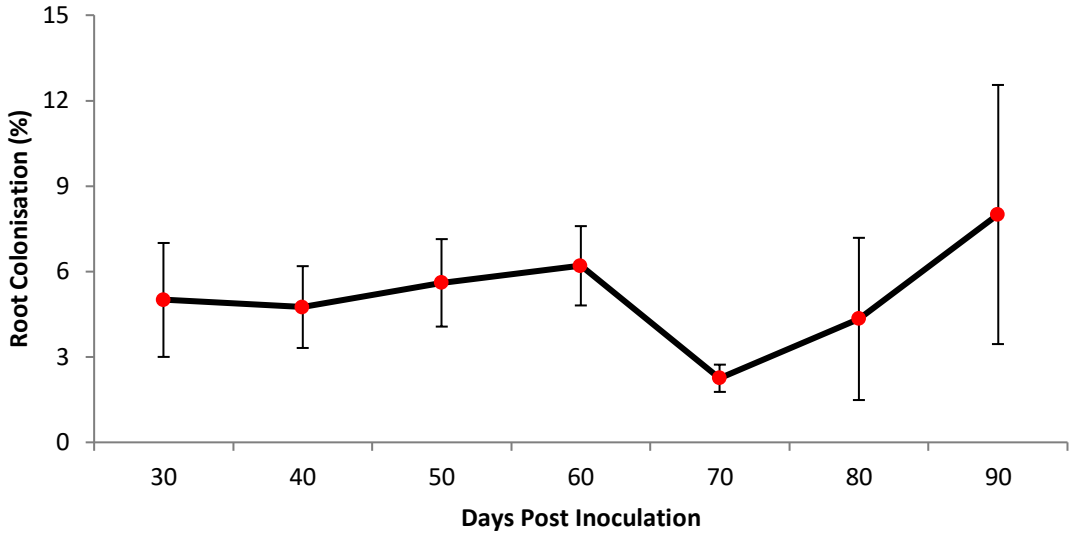

Figure 7.4 Mean total root colonisation by arbuscular mycorrhizal fungi over the lifetime of *Senecio vulgaris*. Bars represent means +/- SE.

7.3.3 Endophytes

7.3.3.1 Isolation frequency

A total of 20 different endophyte species was found in the *S. vulgaris* plants used through the experiments in this thesis. A key finding in this experiment was there was no one species of endophyte was found in every generation or treatment group. *Acremonium psammosporum* was found in the most treatment groups and isolated from all treatments in generation three and four. *Chaetomium* spp. and

Simpicillium lamellicola were both isolated from all treatments in generation four (Table 7.1).

7.3.3.2 Species richness and abundance

There was no significant difference in species richness between the different generations and treatment groups. The same occurred with species abundance. For clarity, there are no figures shown in this section due to lack of significant results.

	Generation One				Generation Two				Generation Three				Generation Four			
	C	AM	H	HAM	C	AM	H	HAM	C	AM	H	HAM	C	AM	H	HAM
Acremonium bacterocephalum	**8.25**	**12.5**	0	0	0	0	0	0	0	0	0	**6.25**	0	0	0	0
Acremonium fusidioides	0	0	0	0	**8.3**	0	0	0	0	0	0	0	0	0	0	0
Acremonium psammosporum	**8.25**	0	0	0	0	0	0	0	**6.25**	**41.7**	**25**	**14.6**	**29.125**	**29.125**	**12.5**	**20.75**
Acremonium recifei	0	0	0	0	0	0	**55.5**	0	0	0	0	0	0	0	0	**6.3**
Acremonium spp.	0	0	0	0	0	0	**27.8**	0	**8.3**	0	0	0	0	0	0	**2**
Acremonium strictum	0	**25**	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bionectria ochroleuca	0	0	0	0	0	0	0	**4.125**	0	0	0	0	0	0	**9**	**4.5**
Chaetomium cochliodes	**41.75**	0	0	0	**33.3**	0	0	0	0	0	0	**6.25**	0	0	0	0
Chaetomium globosum	0	0	0	0	**8.3**	0	0	0	0	0	0	0	0	0	0	0
Chaetomium spp.	0	0	0	0	0	0	0	0	0	0	0	0	**16.6**	**10.4**	**4.9**	**6.5**
Cladosporium cladosporioides	0	0	0	0	**8.3**	**6.6**	0	0	**18.75**	0	0	0	0	**25**	0	**2**
Cladosporium spp.	0	0	0	0	0	0	0	0	**4.125**	0	0	0	0	0	**9**	**4.5**
Cladosporium sphaerospermum	**8.25**	0	0	0	0	**30**	0	0	0	0	0	**17**	0	0	**12.9**	**29**
Clonostachys rosea	0	0	0	0	**8.3**	0	0	0	0	0	0	0	0	0	0	0
Penicillium olsonii	0	0	0	0	0	**10**	0	0	0	0	0	0	0	0	0	0
Penicillium section *Chrysogena*	0	0	0	0	**8.3**	**6.6**	0	0	0	0	0	0	0	0	0	0
Rhyparobius cookei	0	0	0	0	**8.3**	**6.6**	0	0	0	0	0	0	0	0	0	0
Simplicillium lamellicola	**8.25**	**12.5**	0	0	0	0	0	0	0	0	0	**6.25**	**16.6**	**10.4**	**4.9**	**6.5**
Thielavia heterothallica	0	0	0	0	0	**6.6**	0	**16.7**	0	0	0	0	0	0	0	0
Trichoderma spp.	0	0	0	0	**8.3**	0	0	0	0	0	0	0	0	0	0	0

Table 7.1 Mean isolation frequency (%) of endophytes found in each treatment in each generation of *Senecio vulgaris.*

7.3.3.3 Environmental endophytes

The key conclusion of these results was the majority of species that were isolated from the leaves of the experimental plants (Table 7.1) were also found in the environment when the plants were growing. No species of endophyte was found consistently in the environment for every generation of *S. vulgaris* grown. There were less endophytes found in the environment in generations two, three and four (Table 7.2), than species of endophytes found within the plants (Table 7.1).

	Generation One	**Generation Two**	**Generation Three**	**Generation Four**
Acremonium psammosporum			PRESENT	
Acremonium recifei		PRESENT		
Bionectria ochroleuca	PRESENT		PRESENT	PRESENT
Cladosporium cladosporioides	PRESENT		PRESENT	PRESENT
Cladosporium spp.	PRESENT		PRESENT	PRESENT
Cladosporium sphaerospermum				PRESENT
Clonostachys rosea	PRESENT		PRESENT	PRESENT
Penicillium olsonii		PRESENT		
Penicillium spp.	PRESENT		PRESENT	PRESENT
Thielavia heterothallica				PRESENT
Trichoderma spp.	PRESENT			

Table 7.2 Species of endophytes found present in the environment when each generation of *Senecio vulgaris* were grown.

7.3.4 Changing treatment and the impact of the endophyte community

7.3.4.1 Species richness and abundance

The main finding was there were more species of endophytes per plant (1.5 species) isolated from plants in generation three control plants (treatment C) than from plants in treatments 3A, 3B and 3C (0.67 species per plant) ($F_{1,16}$=5.442, $p<0.05$). There was no significant difference in species abundance and/or species richness between the generation three and four controls than the 4A, 4B and 4C treatments. Another key finding was plants from 3B had a higher number of species of endophytes present (1 species per plant) than plants from generation three H (0 species per plant) ($F_{1,8}$=9.6, $p<0.05$).

7.3.4.2 Isolation Frequency

No one species of endophyte was isolated from every treatment group. *Acremonium psammosporum*, *Acremonium strictum, Cladosporium cladosporioides* and *Cladosporium sphaerospermum* were all found in two different treatment groups but these were not the same two (Table 7.3).

	3A	3B	3C	4A	4B	4C
Acremonium psammosporum	0	**62.5**	0	0	0	**25**
Acremonium strictum	0	0	0	**87.5**	**75**	0
Bionectria ochroleuca	0	0	0	**4**	0	0
Cladosporium cladosporioides	**50**	0	**25**	0	0	0
Cladosporium spp.	0	0	0	**4**	0	0
Cladosporium sphaerospermum	0	**12.5**	**25**	0	0	0
Clonostachys rosea	0	0	0	**4**	0	0

Table 7.3 Mean isolation frequency (%) of endophytes found in treatments 3A, 3B, 3C, 4A, 4B and 4C of *Senecio vulgaris.* Treatments 3A, 3B, and 3C are grown from treated plant seed produced by plants in generation two (AM, H and HAM respectively). Treatments 4A, 4B, and 4C are grown from treated plant seed produced by plants in generation three (AM, H and HAM respectively).

7.3.4.3 Environmental endophytes

All treatments were grown in the CT room at the same time, so the same species of endophytes were isolated from each generation's environment PDA plates. The majority of the endophytes isolated from within the plants' leaves (Table 7.3) were also found in the environment. Not all the treatments had the same endophytes and none of the treatments had all the endophytes found in the environment isolated from the leaves (Table 7.4).

	3A, 3B & 3C	4A, 4B & 4C
Acremonium psammosporum	PRESENT	PRESENT
Acremonium strictum	PRESENT	PRESENT
Cladosporium spp.	PRESENT	PRESENT
Clonostachys rosea	PRESENT	PRESENT
Penicillium spp.	PRESENT	PRESENT
Trichoderma spp.	PRESENT	PRESENT

Table 7.4. Species of endophytes found present in the environment when treatments 3A, 3B, 3C, 4A, 4B and 4C of *Senecio vulgaris* were grown.

7.3.5 Community analysis

The ANOSIM statistical analysis did show significant differences between the communities of endophytes ($p<0.01$), however when looking at the non-metric MDS output, it seems that the three outliers are skewing the results. These outliers are from generation one AM (g1m10) and generation two C (g3Con6 and g3Con7). So it is most likely that the analysis of the endophyte community found that the community within the different treatments and between generations and treatments were similar (Figure 7.5).

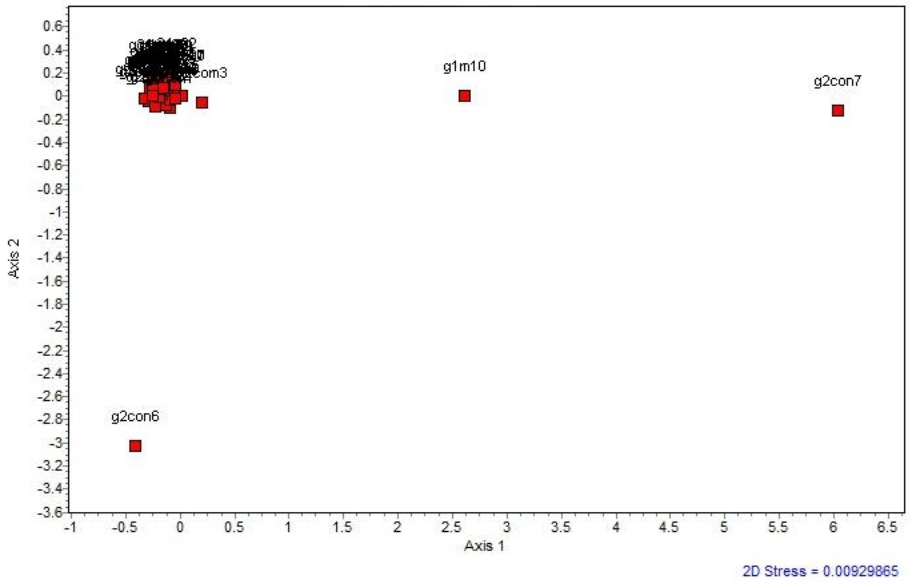

Figure 7.5 Non-metric MDS analysis of the endophyte communities isolated from *Senecio vulgaris* grown for the experiments in this thesis.

7.4 Discussion

7.4.1 Mycorrhizal colonisation

S. vulgaris has been shown form symbiotic relationships with mycorrhizas (Harley & Harley, 1987), however it could be that species within the commercial inoculum used do not colonise the roots of *S. vulgaris*. There was low colonisation of *S. vulgaris* roots by mycorrhizas in previous transgenerational experiments, 29.9% root colonisation in the parental generation and 14.5% in the progeny generation (West, 1995). However this is still very high compared to the percentage germination in these experiments. The amount of mycorrhizal inoculum used was based upon Rootgrow's recommendations for the pot sized used in these experiments (Section 2.2), but this may have been too low for *S. vulgaris*. This has been shown in *Datura stramonium* (jimsonweed) where increasing the inoculum concentration caused an increase in the mycorrhizal colonisation percentage (Garrido *et al.*, 2010). Mycorrhizal colonisation was shown to cause benefits to certain plant and seed parameters (Section 3.3.1; Section 4.3.1.3; Section 4.3.1.5; Section 5.3.1.2; Section 5.3.1.4), so it could be that even low colonisation by

mycorrhizas in this species can still be beneficial to the plants (Gange & Ayres, 1999). A mixture of five mycorrhizas were used in the mycorrhizal inoculum, which could have led to low root colonisation. Generally one species of mycorrhiza dominates the root colonisation and there can be competition from other species especially if the species are inoculated at the same time (Jansa *et al.*, 2008). The competition from multiple species trying to colonise the root at the same time can make interpreting the results difficult (Jansa *et al.*, 2008).

These experiments did explore the effects of insects on mycorrhizal colonisation and mycorrhizal colonisation on insect herbivores (Chapter 6), which does not always happen in the literature. In the meta-analysis by Barto & Rillig (2010), it was shown that mycorrhizal colonisation in forbs was not affected by insect herbivory. In this experiment, insect herbivory had no effect upon the percentage of root colonised, which agrees with the findings of Barto & Rillig, (2010). The results from both suggest that in *S. vulgaris* each factor has no effect upon the other, which could be due to percentage colonisation achieved being too low to pick up any of the effects.

The results in this experiment show that there are both endophytes and mycorrhizas present within the same plant. In the grass, *Lolium mulitiflorum,* plants were infected with a specific grass endophyte, *Neotyphodium occultans,* had decreased mycorrhizal colonisation (Omacini *et al.*, 2006). An endophyte found in these experiments may have the same effect on mycorrhizal colonisation in forbs, so the low colonisation in these experiments may be due to symbiosis from two different fungi. The opposite was found to be true in *Cirsium arvense* (creeping thistle), where endophyte colonisation was reduced by the presence of mycorrhiza fungi (Eschen *et al.*, 2010).

7.4.1.1 Mycorrhizal colonisation changes over time

The mycorrhizal colonisation percentage was extremely low in the plants used in experiments within this thesis. It was initially believed to be due to the late harvest of the plants (>90 days post inoculation) as mycorrhizal colonisation decreased over time in other plant species (Simon *et al.*, 2017). This is thought to be due to the

fungus proliferating more slowly as the roots develop, which creates a dilution effect (Simon *et al.*, 2017). However, in *S. vulgaris* the colonisation did not appear to change over the life of the plant. This is highly suggestive that the low colonisation seen above was not due to the timing of harvest post inoculation. The roots in plants used in the time experiment had a significantly higher colonisation than roots in the main experiment. There was no change in the environment or the experiment set up. One cause may be the change in Rootgrow batch between the experiments. The spore counts were checked and did not differ significantly, however other biological symbionts present (e.g. bacteria) within the batch may have caused the changes seen.

7.4.2 Endophytes

7.4.2.1 Endophytic community from the main generation plants

The majority of species of endophytes found within the plants were also found within the environment in the CT room. This suggests that the majority of fungi were being horizontally transmitted from the environment to the plants. *S. vulgaris* was found to vertically transmit endophytes between parental and progeny generation (Hodgson *et al.*, 2014). *Cladosporium cladosporioides* and *C. sphaerosperum* were both found to be vertically transmitted in *S. vulgaris* (Hodgson *et al.*, 2014). Both of these species of endophytes were found in plants used for the experiments in this thesis. *Acremonium psammosporum* was found in plants from all treatment groups in generations three and four, but only found in the environment in generation three. *A. psammosporum* appearing in all the treatments in generation four but not on the environmental PDA plates suggests that the fungus was passed from plants in generation three through the seed to plants in generation four. *Simplicillium lamellicola* was found in generations one, three and four but not in the environment. This may be due to some form of vertical transmission especially between generations three and four. Transmission of endophytes from the parental plant through the seed to the offspring has been seen to occur in *S. vulgaris* (Hodgson *et al.*, 2014). Transmitting endophytes is

known to be an imperfect process with many being lost at each plant life stage (Afkhami & Rudgers, 2008), which may explain why the endophyte was lost between generation one and two. However, it could be that *S. lamellicola* was present in the environment but not at the time when the environmental plates were placed in the CT room. It is believed that plants can 'choose' which endophytes to pass onto the next generation with the parental plant controlling which endophytes are absent and present (Gundel *et al*., 2017), which could explain why some species of endophytes are present over multiple generations, while others are only present for one generation.

7.4.2.2 Changing treatments and the impact of the endophyte community

Again, the findings suggest that the majority of endophytes are being horizontally transmitted from the environment to the plants in this experiment. Generation three control plants (treatment C) had a higher number of species present than plants from treatments 3A, 3B and 3C (plants grown from seed produced by generation two AM, H and HAM treated plants) (Figure 2.1). This is probably due to the plants growing at different times with different environmental endophytes present. Endophytes are able to transfer horizontally to the plants through airborne spores. If there are more fungal spores present in the environment then there may be more horizontal transmission occurring, as the more spores present, the more likely the plant is to become infected. This could cause a difference in species richness between generations as each generation had a different number of endophyte species isolated from the environmental PDA plates. However, there was no difference in the number of species found in the environment when either plants from 3B or plants from generation three H were growing. This suggests that the plants in generation three H may have accepted less of the environmental endophytes through 'choice' (Gundel *et al.*, 2017).

The vertical transmission of endophytes may cause symbiont-mediated transgenerational effects that could be a reason for plant adaptations to changing environments (Gundel *et al.*, 2017). There was a suggestion that *Acremonium psammosporum* was passed between generations, as it was not always present in

the environment, and this fungus could trigger some transgenerational effects to occur. Unfortunately there is no literature on *A. psammosporum* and its interactions with plants so whether it has any role in transgenerational effects is unknown. Its close relation *Acremonium strictum* has been studied closely. The fungus has been isolated in the seeds of different crop plants, however it is thought that the fungus infects the seeds through the soil and not through vertical transmission (Leslie, 2008). *A. strictum* has been shown to cause a high mortality in seeds (Zida *et al.*, 2008), so the presence of this endophyte being passed vertically through the seed could cause a low percentage germination (Section 5.3.1.4). The fungus is also a mycoparasite, which does stop the growth of the potato pathogen *Helminthosporium solani* and reduces mycelial growth (Rivera-Varas *et al.*, 2007). The mycoparasitic properties may also extend to mycorrhizal fungi within the roots and cause a reduction in root colonisation. In *Maclura cochinchinensis* (cockspur thorn), *A. strictum* is an endophytic fungus that can be isolated from the leaves. The endophyte is known to provide and moderate an anti-herbivory response (Zhou *et al.*, 2014). *Cladosporium cladosporioides* was not found in the environment when plants in treatments 3A and 3C were grown, however the fungus was also present in generation two AM plants. *C. cladosporioides* is known to be vertically transmitted in *S. vulgaris* (Hodgson *et al.*, 2014), so it could easily have been vertically transmitted from generation two AM to treatment 3A (Figure 2.1). *C. cladosporioides* is a very common fungus that colonises plant material and soil (Deshmukh & Rai, 2005; Flannigan *et al.*, 2011). It is known to produce anti-fungal metabolites (Wang *et al.*, 2013). These anti-fungal metabolites could cause a reduction in the number of endophytes isolated from the plants with *C. cladosporioides* present and it could also cause a reduction in mycorrhizal colonisation.

7.4.2.3 Community analysis

The community analysis of endophytes isolated from plants in these experiments seemed insignificant suggesting that there is no difference in the communities between each treatment and generation. The communities of endophytes between

each treatment group and generation do not seem to be the reason for some of the consistent parental effects seen as they were not similar between generations or treatments. However, the presence of specific endophytes within specific plants may still cause parental effects between those plants and their progeny.

7.5 Conclusion

The hypothesis that insect herbivory would have no effect on the percentage of roots colonised by mycorrhizas was upheld. There was no significant difference in percentage colonisation by any mycorrhizal feature between the aphid attacked plant roots and plant roots from non-attacked plants. The hypothesis that there would be significant difference in the endophyte community between generations and treatments was also not upheld. The ordination analysis did not find a difference in the endophyte communities in different treatments and different generations. This further suggests that the parental effects seen in previous chapters (Table 3.1; Table 4.1; Table 5.1) are not due to the presence of fungal endophytes.

Chapter Eight

DNA methylation and parental effects in *Senecio vulgaris*

8.1 Introduction

This experiment investigated whether methylation of DNA affects the growth of the progeny generation, addressing the second theme of this project; searching for possible mechanisms that cause environmental parental effects. It specifically explored the objective of whether DNA methylation may cause parental effects to be passed between the generations.

An epigenetic change to the DNA structure is the addition or removal of methyl groups on cytosine residuals (Herman & Sultan, 2016), which has been shown to pass stably between one generation of plants to the next (Akst, 2017). This addition or removal of methyl groups can change transcription on specific loci, with the transcriptional changes potentially altering environmental parental effects (Herman & Sultan, 2016). The research into DNA methylation started from McClintock's research, where it was believed transposable elements present near genes can change the expression of those genes from one generation to the next but in a reversible way (McClintock, 1950). Plants generally leave their epigenome intact, whereas animals wipe the majority of methyl markers when reproduction is taking place (Akst, 2017). By leaving the epigenome intact, plants are able to inherit alleles that are altered by methyl groups which can change phenotypes, such as drought tolerance (Herman & Sultan, 2016).

To explore the influence of DNA methylation on drought tolerance in the progeny generation, zebularine (a demethylating agent) was used on *Polygonum persicaria* (lady's thumb) (Herman & Sultan, 2016). Demethylation was found to remove the epigenetic effects of drought tolerance, but did not significantly change the phenotypic expression in non-drought stressed plants.

Epialleles, alleles altered by epigenetic changes, can regain the 'wild-type' methylation after several generations (Johannes *et al.*, 2009). This was shown in *Arabidopsis thaliana* and the adaptations to salt stress (Wibowo *et al.*, 2016). Five generations of *A. thaliana* were grown in different degrees of salt stress with epigenetic changes being seen within the first generation, however the phenotypic changes were not observed until late second/third generation (higher germination and plant survival rates) (Wibowo *et al.*, 2016). The effects were not magnified over

multiple generations, which suggested that there is a limit to epigenetic changes and when the plants were subjected to 'normal' salt conditions the methylation reverted back to the wild-type (Wibowo *et al.*, 2016). Phenotypic expression occurs a while after the epigenetic change has happened and that these epigenetic changes are quick to revert back to wild-type.

There is much debate in the literature over whether epigenetic changes to the methyl groups are due to environmental adaptation, especially as epigenetic changes are quick to snap back to the wild-type (Akst, 2017). However, Zhang *et al.*, (2010) believed that epialleles can be inherited, which if visible for selection could cause adaptation and evolution over time.

The type of reproduction the plant undergoes is believed to cause differences in the epigenetic occurrences observed. In *Taraxacum officinalis* (dandelion), DNA methylation was seen to modify root/shoot biomass, phosphorus content, leaf morphology and stress tolerance in the offspring of stressed parents (Verhoeven & vanGrup, 2012). Chemical suppression of DNA methyltransferase blocked any of these effects being seen, which suggests that the transgenerational effects were observed due to DNA methylation. Dandelions have the ability to self-fertilise (selfing) and this was thought to be a factor as to why the epigenetic effects were observed so quickly (Verhoeven & vanGrup, 2012). *Senecio vulgaris* is known to self-fertilise (Grime *et al.*, 1988). So if any epigenetic effects are occurring they may be seen as quickly, as they did in the closely-related *Taraxacum*.

Many studies have linked epigenetic changes to phenotypic changes. Decreased DNA methylation was shown to reduce plant size, decreased fertility and altered flowering time in *A. thaliana* (Finnegan *et al.*, 1996). Flowering time has been shown to be affected by DNA methylation in *A. thaliana* (Soppe *et al.*, 2000; Baulcombe & Dean, 2014). Flowering is repressed by the *FWA* gene, which represses the genes required to switch the meristem to a floral fate (Soppe *et al.*, 2000; Baulcombe & Dean, 2014). Seed yield has also been shown to be increased using DNA methylation in *Brassica napus* (rapeseed) (Hauben *et al.* 2009).

The effect of environmental stresses on DNA methylation is debated in the literature (Akst, 2017), however it was shown to occur in the selfing dandelion (Verhoeven & vanGrup, 2012). Therefore, it was hypothesised that there would be

some transgenerational effects that could be altered due to the use of a chemical that stops DNA methylation. Further, if there are any transgenerational effects that are altered by the use of a demethylation drug, the most likely to be altered would be related to reproduction, i.e. elongating flowering time (Soppe *et al.,* 2000) and seed production (Hauben *et al.*, 2009).

8.2 Methods

8.2.1 Set up of the experiment

To explore whether DNA methylation was causing any of the differences in parameters between generations, an experimental method was adapted from Herman & Sultan (2016). The parental generation was grown from seeds collected for generation one of the main experiment. For the parental generation, four seeds, from plants grown in control conditions, were planted in a pot containing 165g of John Innes Grade 3 compost. The plants were all grown in the same controlled conditions (20^{o}C and 16 hours daylight) until they produced seeds. The pots were placed randomly onto workbenches and moved once a week to reduce environmental variation. Once the plants had developed true leaves, they were weeded out so only one plant remained per pot. There were four treatments per generation with twenty plants per treatment. The control plants, referred to as C (Figure 8.1), had 5mls autoclaved Rootgrow (PlantWorks Ltd., Sittingbourne, Kent) mycorrhizal inoculum added 2cm below the topsoil. The inoculum was autoclaved so the fungal spores were killed and only the clay particles remained. The mycorrhizal treatment, referred to as AM (Figure 8.1), had 5mls of Rootgrow inoculum added 2cm below the topsoil of each pot. Plants that were to be treated with aphids, referred to as H for herbivory (Figure 8.1), had 5mls autoclaved Rootgrow inoculum added 2cm below the topsoil of each pot before the seeds were sown. Once plants in this treatment had developed buds, aphids were placed onto the plants for fecundity treatments (Section 2.3; Figure 8.1). The final treatment group was the combined treatment, referred to as HAM (Figure 8.1), with 5mls of Rootgrow mycorrhizal inoculum added 3Cm below the topsoil of each

pot. Once these plants had developed buds, the aphids were added (Section 2.3; Section 8.1). At the end of each generation, seeds were collected from each plant and stored in paper envelopes. These seeds were used to plant the next generation. The progeny generation was grown in the same conditions as the parental generation. The same treatment groups were used; however each treatment was split into two with half being treated with zebularine (demethylating agent) and half not (Figure 8.1). To treat with zebularine, the method was adapted from Herman & Sultan (2016), as transplanting the seedlings from agar to pots resulted in few seedlings taking. The adaptation was started two days after the seeds were sown using the demethylating drug zebularine (Sigma-Aldrich, Gillingham, UK). Over six days, 45μM of zebularine was watered into each zebularine treated pot. This concentration has been previously shown to not disrupt plant development (Herman & Sultan, 2016).

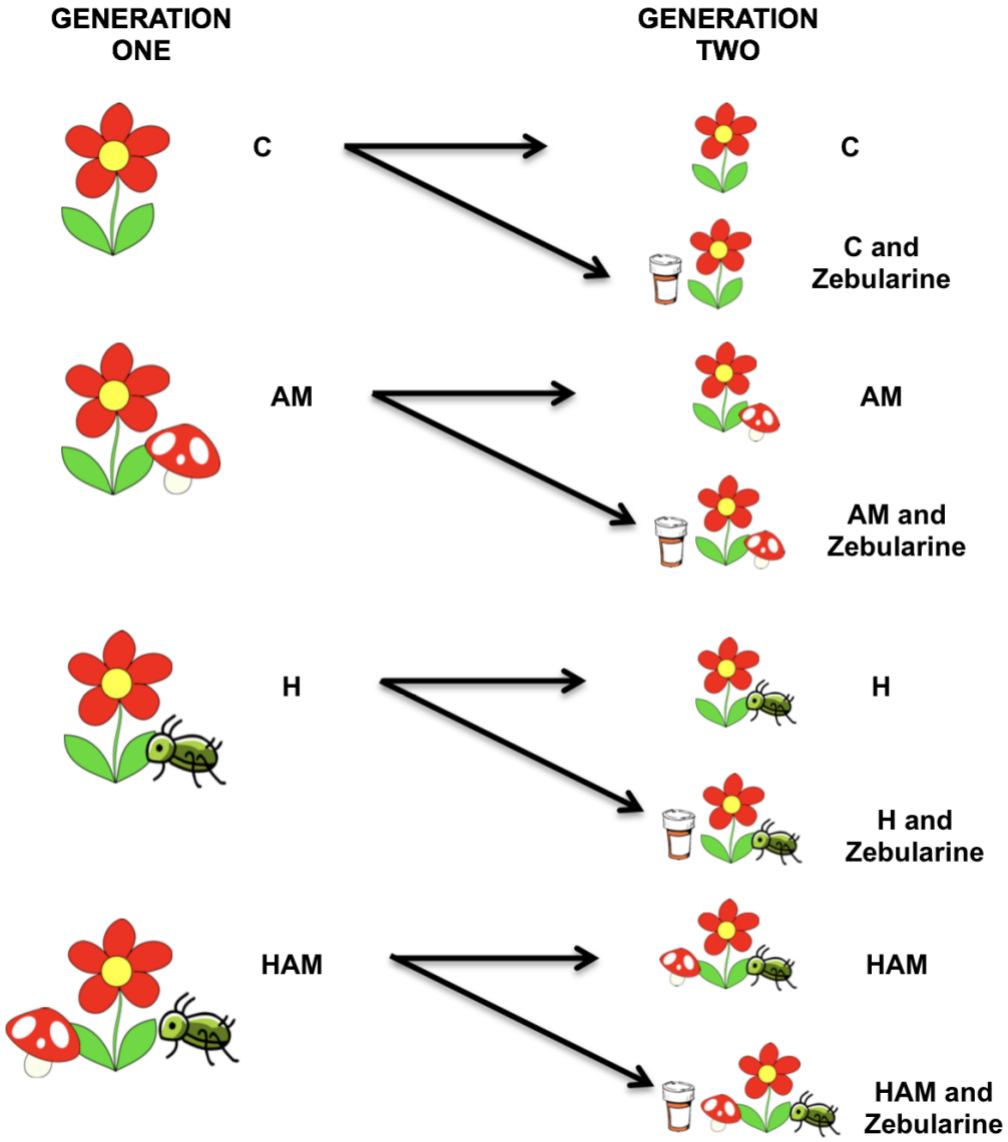

Figure 8.1 Diagram showing how the treatments were set up for the demethylation experiment. The mushroom represents mycorrhizal treatment, the aphid represents aphid infestation and the drug bottle represents zebularine treatment.

8.2.2 Measurement parameters

8.2.2.1 Lifecycle parameters

Data for germination, true leaf development, bud development, flower development and seed development times were collected as described in Section 3.2.1.

8.2.2.2 Mature plant size parameters

Data were collected for flower number (Section 4.2.1.1), leaf number (Section 4.2.1.2), flower size (Section 4.2.1.3), height (Section 4.2.1.4) and dry biomass (Section 4.2.1.5).

8.2.2.3 Seed parameters

Data were collected for seed carbon and seed nitrogen (Section 5.2.1.1), seed phosphorus (Section 5.2.1.2), seed weight (Section 5.2.1.3) and percentage germination (Section 5.2.1.4). Replicate number was low for seed chemistry methods (seed carbon, nitrogen and phosphorus), as a certain weight had to be achieved for each test to be run. The plants did not produce enough seeds individually to meet the weight requirements to run the tests so the samples were pooled.

8.2.2.4 Aphid parameters

Data were collected for teneral weight (Section 6.2.1.1), mean relative growth rate (MRGR) (Section 6.2.1.2) and intrinsic rate of population increase (r_m) (Section 6.2.1.3).

8.2.2.5 Mycorrhizal fungi parameters

Mycorrhizal colonisation was visualised and measured using the method described in Section 2.5.

8.2.3 Statistical analysis

Analysis of these parameters of *S. vulgaris* was performed in R Studio 3.3.3. Normality tests were performed on whole data sets and data were transformed if necessary using lambda calculated by Box-Cox transformation.

If the sample sizes were unbalanced, two different forms of coding were used to ensure the data were interpreted correctly. It was suggested that weighted means were used to perform the multi-way ANOVA using the anova() function in the car package in R (Quick, 2010). The order of the independent variables was checked by running the ANOVA multiple times to ensure hierarchical order was achieved (Quick, 2010). Even though this is a type I analysis, by running it multiple times it becomes a type II analysis. The second form of analysis used unweighted means but the same technique. The results were compared to ensure that all precautions were taken with the data. Fox (2018), stated that using type III analysis was to be avoided when using the car package and anova() function in R.

8.2.3.1 Lifecycle, mature plant size and seed parameters

Differences in these parameters over treatment groups in each generation were tested using a four-way, repeated measures ANOVA, employing aphid absence/presence, AMF absence/presence, zebularine absence/presence and generation as the main effects.

8.2.3.2 Aphid parameters

Differences in the aphid fecundity measurements over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing AMF absence/presence, zebularine absence/presence and generation as the main effects.

8.2.3.3 Mycorrhizal parameters

Differences in total colonisation, hyphal, arbuscular and vesicle colonisation over treatment groups in each generation were tested using a three-way, repeated measures ANOVA, employing aphid absence/presence, zebularine absence/presence and generation as the main effects.

8.3 Results

The results explore whether DNA demethylation alters any of the parameters explored in previous chapters of this thesis using control (C), mycorrhizal only (AM), aphid only (H) and combined mycorrhizal and aphid (HAM) treated plants over two generations.

For clarity, due to the number of possible comparisons, only the significant results have been included in this section and in the figures (additional figures shown in Appendix V).

8.3.1 Lifecycle parameters

Plants in all treatments in generation one took longer to germinate than plants in all treatments in generation two ($F_{1,101}$=1598.072, p<0.001).The key finding was in generation two, plants treated with zebularine took longer to germinate than plants not treated with zebularine ($F_{1,101}$=5.310, p<0.05) (Figure 8.2).

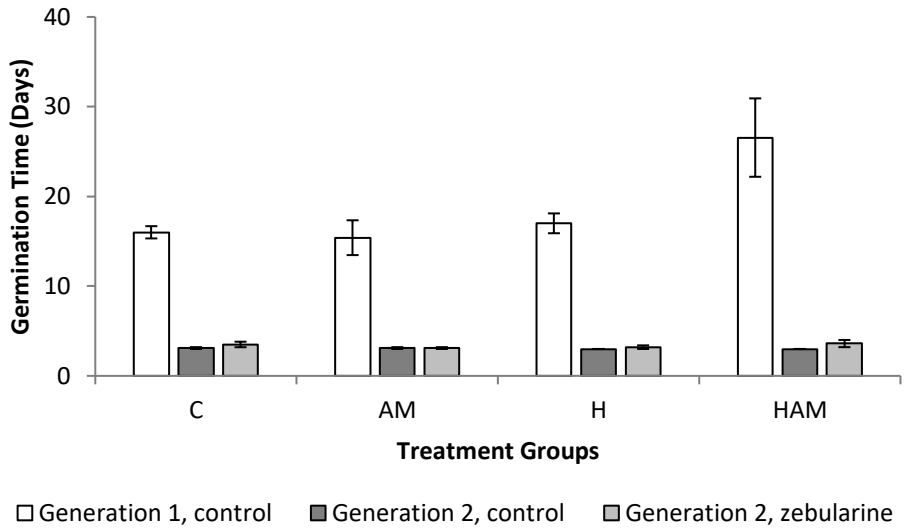

Figure 8.2 Mean time taken to germinate from date of sowing per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

Generation one plants in all treatment groups took longer on average to develop true leaves than generation two plants ($F_{1,105}$=941.981, p<0.001). The standard errors were very small for generation two plants, as there was very little difference between the days taken to develop true leaves (Figure 8.3).

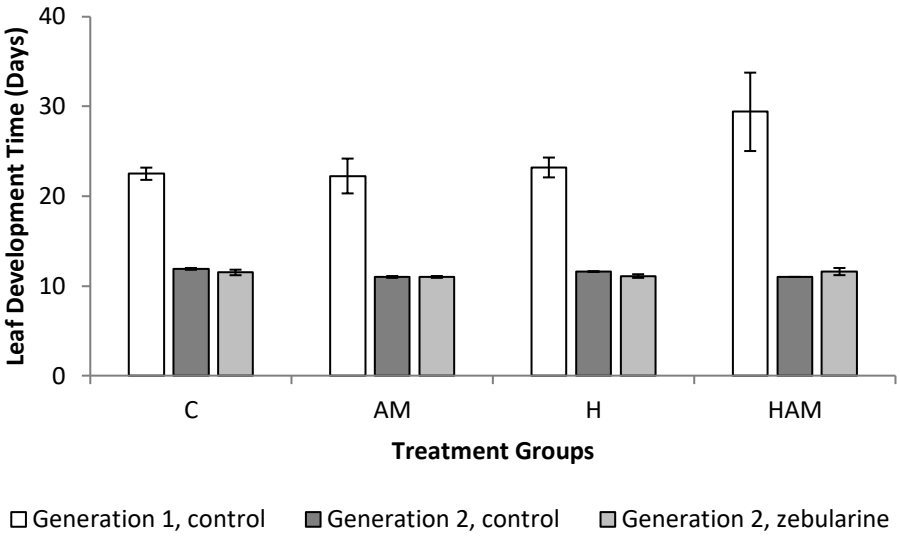

Figure 8.3 Mean time taken to develop true leaves from date of sowing per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

Across treatments, generation one plants took the longest to develop buds from the date of sowing ($F_{1,106}$=117.756, p<0.001). The main point was in generation two, zebularine treated plants took longer to develop buds than non- treated plants ($F_{1,106}$=12.100, p<0.001). Mycorrhizal plants (treatments AM and HAM) in generation one took significantly longer to produce buds than mycorrhizal plants in generation two ($F_{1,106}$=6.422, p<0.05) (Figure 8.4).

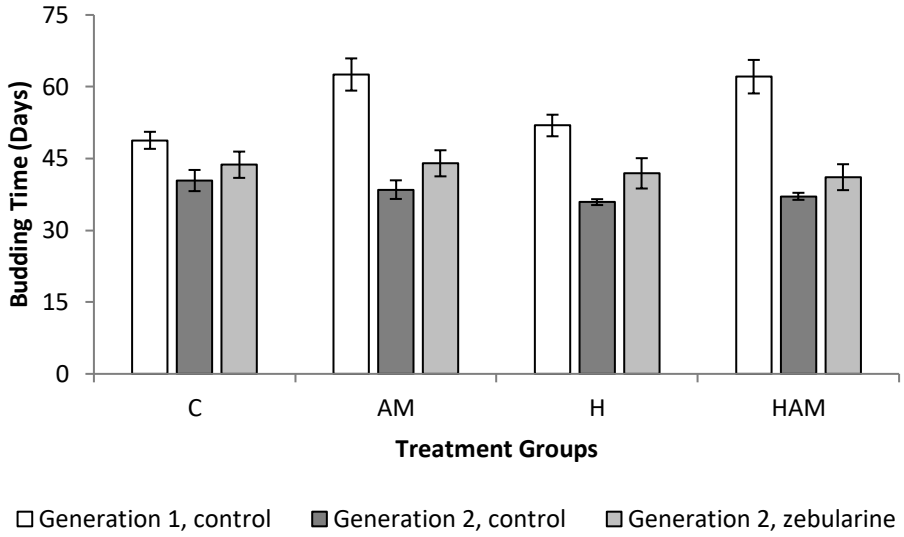

Figure 8.4 Mean time taken to develop buds from date of sowing per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

Plants in generation one took longer to produce flowers than plants in generation two ($F_{1,105}$=56.780, p<0.001). The main conclusion from this parameter was generation two, zebularine treated plants took longer to produce flowers than plants not treated ($F_{1,105}$=12.733, p<0.001). Plants colonised with mycorrhizas in generation one took significantly longer to produce flowers than mycorrhizal plants in generation two (treatments AM and HAM) ($F_{1,105}$=7.926, p<0.01) (Figure 8.5).

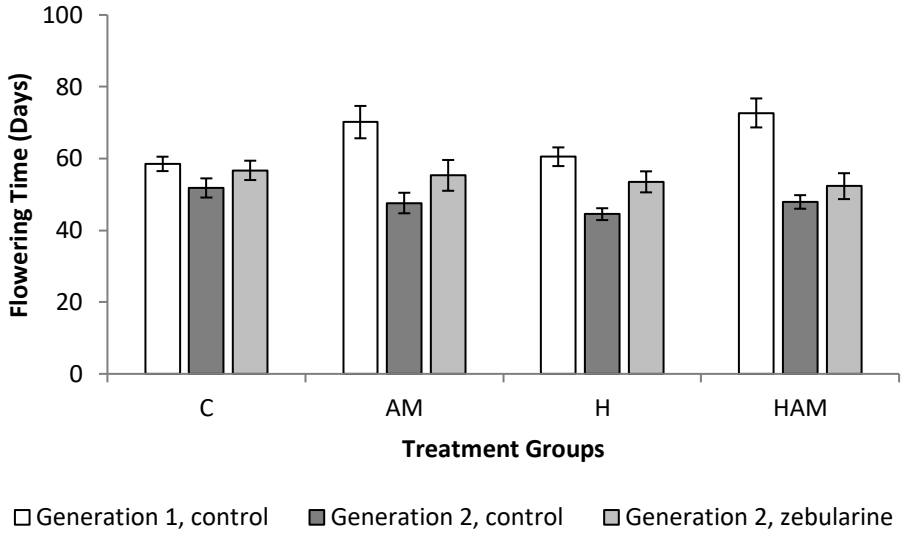

Figure 8.5 Mean time taken to develop flowers from date of sowing per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

Plants in generation one took significantly longer to set seeds than plants in generation two ($F_{1,82}$=14.256, p<0.001). Mycorrhizal treated plants (treatment groups AM and HAM) in generation one took longer to set seeds than plants colonised by mycorrhizas in generation two ($F_{1,82}$=15.126, p<0.001) (Figure 8.6).

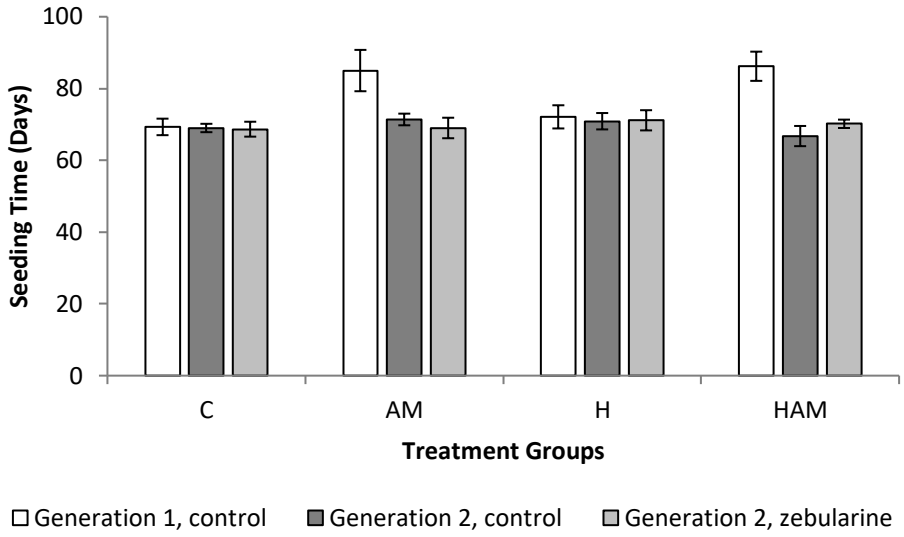

Figure 8.6 Mean time taken to set seeds from date of sowing per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.2 Mature plant size parameters

8.3.2.1 Flower Number

Plants in generation one produced more flowers than those in generation two ($F_{1,105}$=13.410, p<0.001). In the mycorrhizal only treatment, plants in generation one produced more flowers than plants in generation two ($F_{1,105}$ = 5.706, p<0.05). Meanwhile, aphid attacked plants produced more flowers in generation one than in generation two ($F_{1,105}$ = 7.020, p<0.01) (Figure 8.7).

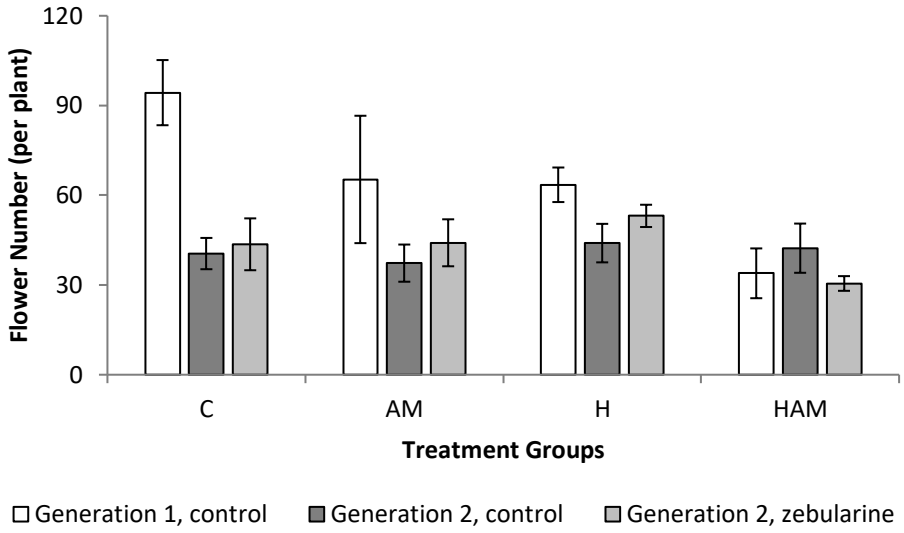

Figure 8.7 Mean number of flowers produced per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.2.2 Leaf number

There was a significant interaction between generation, mycorrhizal colonisation and aphid attack ($F_{1, 107} = 7.793$, $p<0.01$). Leaf number increased in plants in generation two AM and H treatments compared to the previous generation, whereas it decreased between generation one and two C treatments. The combined treatment of mycorrhizas and aphids (treatment HAM) did not see a change in leaf number between generation one and two (Figure 8.8).

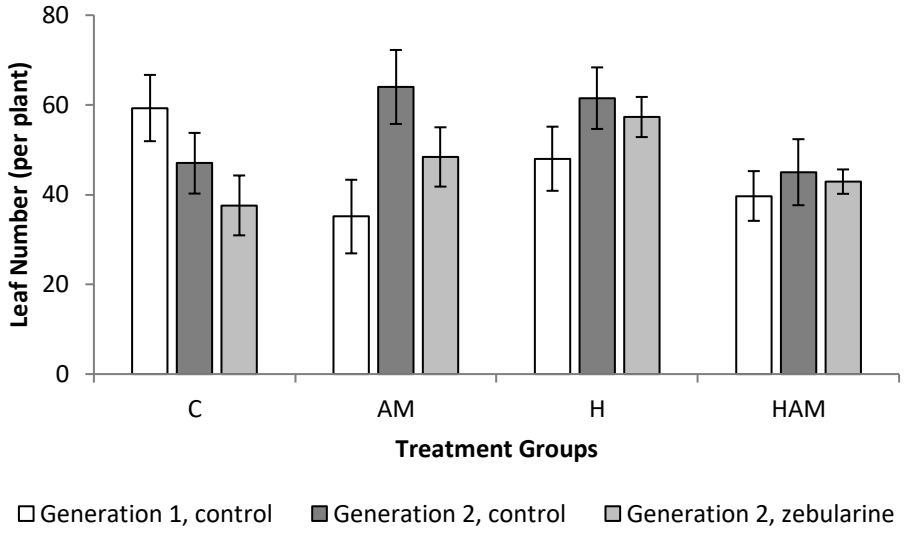

Figure 8.8 Mean number of flowers produced per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.2.3 Height

Overall mycorrhizal plants (treatments AM and HAM) were shorter than non-mycorrhizal plants (treatment C and H) ($F_{1, 107}$ = 9.791, p<0.01). Generation one C plants were taller than generation two C plants, whereas generation one HAM plants were shorter than generation two HAM plants ($F_{1, 107}$=12.801, p<0.001) (Figure 8.9).

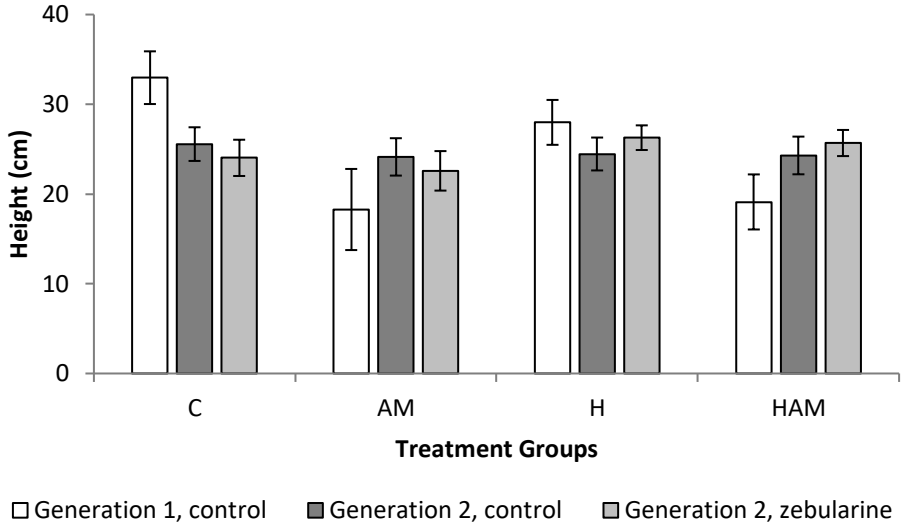

Figure 8.9 Mean height per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.2.4 Dry biomass

Mycorrhizal plants (treatments AM and HAM) were lighter overall than non-mycorrhizal plants (treatments C and H) ($F_{1, 106} = 8.346$, $p<0.01$), which is a contrast to the other findings in Section 8.3.2. Generation one C plants were heavier than plants in generation two C, whereas generation one HAM plants were lighter than plants in generation two HAM plants ($F_{1, 106} = 10.334$, $p<0.01$) (Figure 8.10).

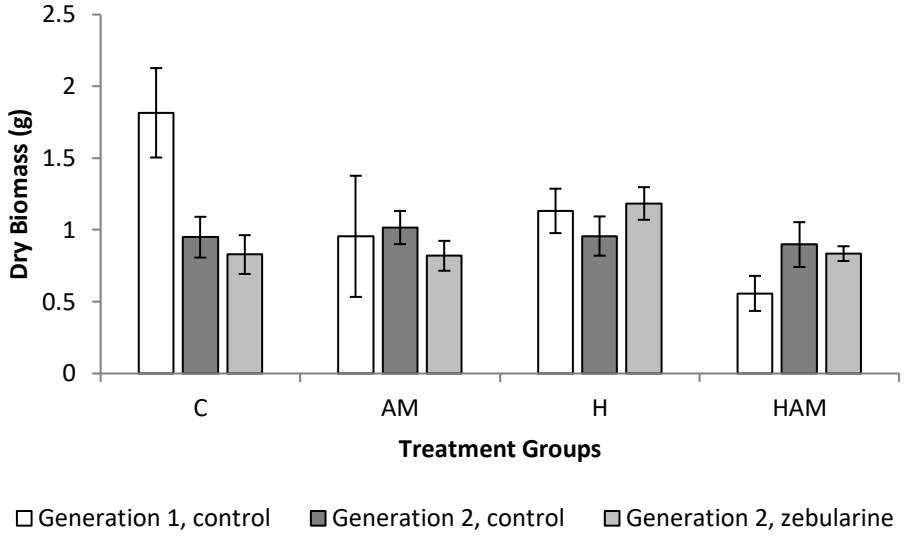

Figure 8.10 Mean dry biomass per plant per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.3 Seed parameters

8.3.3.1 Seed weight

Overall plants in generation one produced heavier seeds than plants in generation two ($F_{1, 91}$ = 9.450, p<0.01). There was a significant interaction between mycorrhizas and aphids ($F_{1, 91}$ = 4.106, p<0.05) with the combined treated plants (treatment HAM) produced heavier seeds overall, than mycorrhizal only (treatment AM) plants (Figure 8.11).

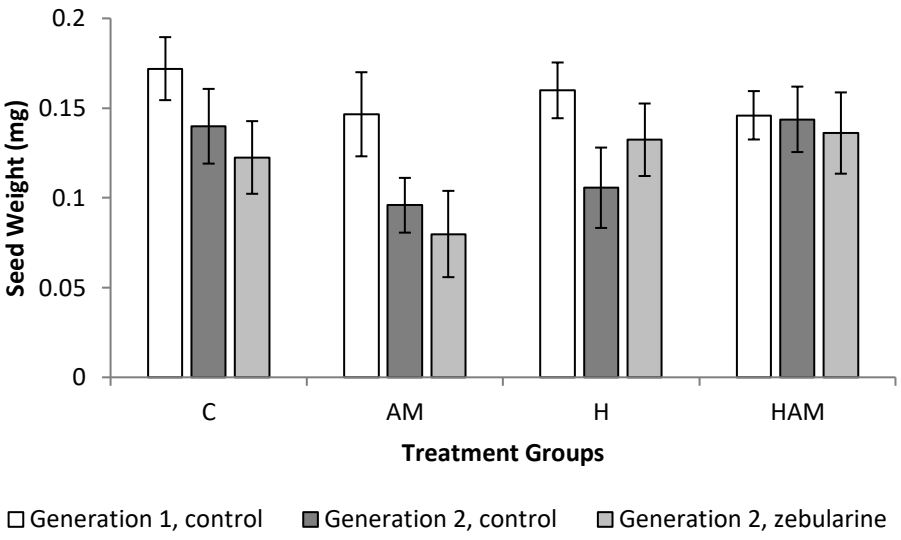

Figure 8.11 Mean seed weight per treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.3.2 Percentage germination

Overall plants in generation one produced seed which had a higher percentage germination than seeds from plants in generation two ($F_{1, 26} = 56.971$, $p<0.001$). Meanwhile, plants that were colonised by mycorrhizas (treatments AM and HAM) produced seeds with a lower percentage germination than plants that were not colonised by mycorrhizas (treatments C and H) ($F_{1, 26} = 5.414$, $p<0.05$) (Figure 8.12).

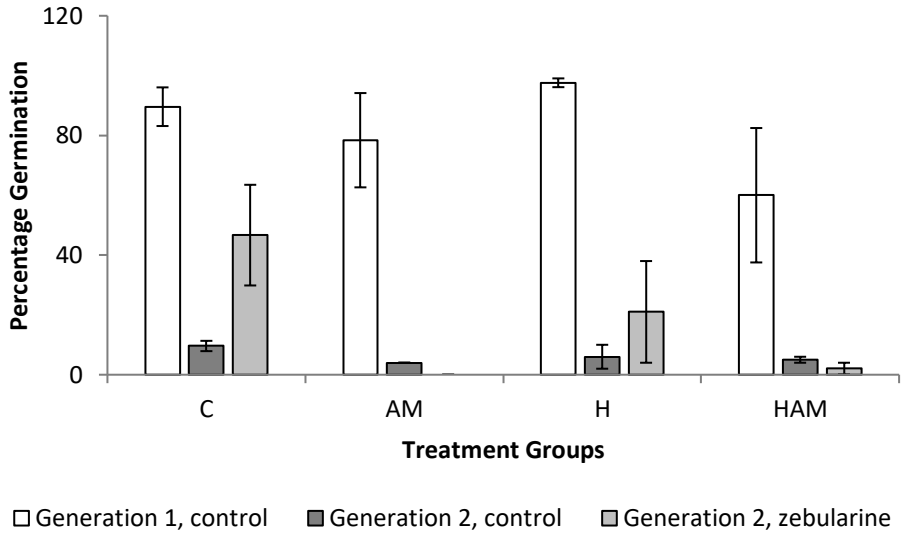

Figure 8.12 Mean percentage germination of seeds produced in each treatment group over two generations of *Senecio vulgaris* grown. Bars represent means +/- SE.

8.3.4 Aphid parameters

8.3.4.1 Teneral Weight

Due to aphid death before they reached the teneral adult stage, there was only one aphid replicate for generation two zebularine treatment. This means the ANOVA could not be run. However, Figure 8.13 seems to show in generation one there was no difference in teneral weight between aphids feeding on mycorrhizal and non-mycorrhizal plants, whereas in generation two there was a difference between aphids feeding upon mycorrhizal and non-mycorrhizal plants in both treatment groups. The aphids feeding upon mycorrhizal plants were heavier than the aphids feeding on non-mycorrhizal plants in the same treatment group (generation 2, control). The opposite occurred between the teneral weights of aphids feeding upon mycorrhizal and non-mycorrhizal plants in generation two, zebularine (Figure 8.13).

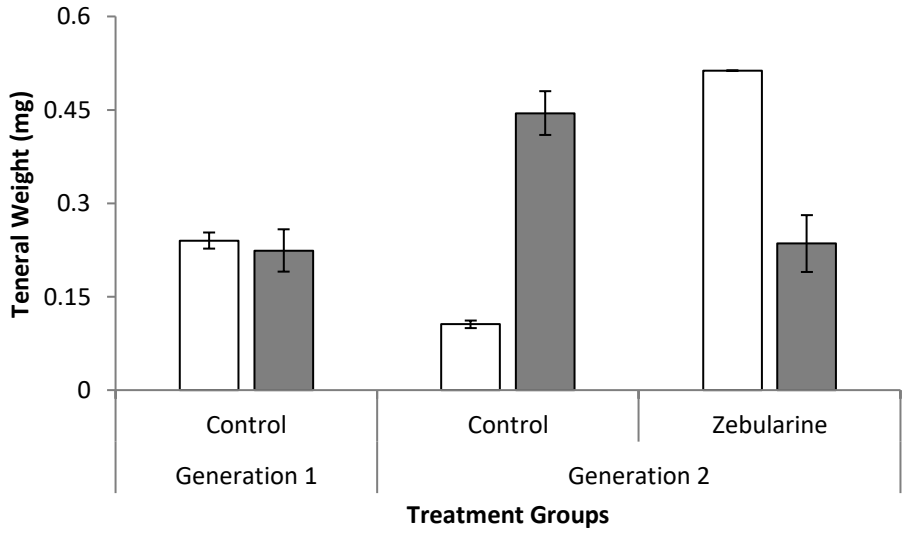

Figure 8.13 Mean teneral weight of aphids feeding upon plants in each treatment group over two generations of *Senecio vulgaris* grown. White represents control (uncolonised) plants and grey represents mycorrhizal plants. Bars represent means +/- SE.

8.3.4.2 Mean relative growth rate

Due to aphid death, there was only one aphid replicate for generation two zebularine treatment. This means the ANOVA cannot be run, however looking it the results it seems that aphids feeding upon plants in generation one had a larger mean relative growth rate (MRGR) than aphids feeding upon plants in generation two. Aphids feeding upon mycorrhizal plants in generation two had a higher MRGR than aphids feeding upon the non-mycorrhizal plants in the same treatment group (generation two, control). The opposite occurred in the generation two, zebularine group where aphids feeding on mycorrhizal plants had a lower MRGR than the aphids feeding upon the non-colonised plants (Figure 8.14).

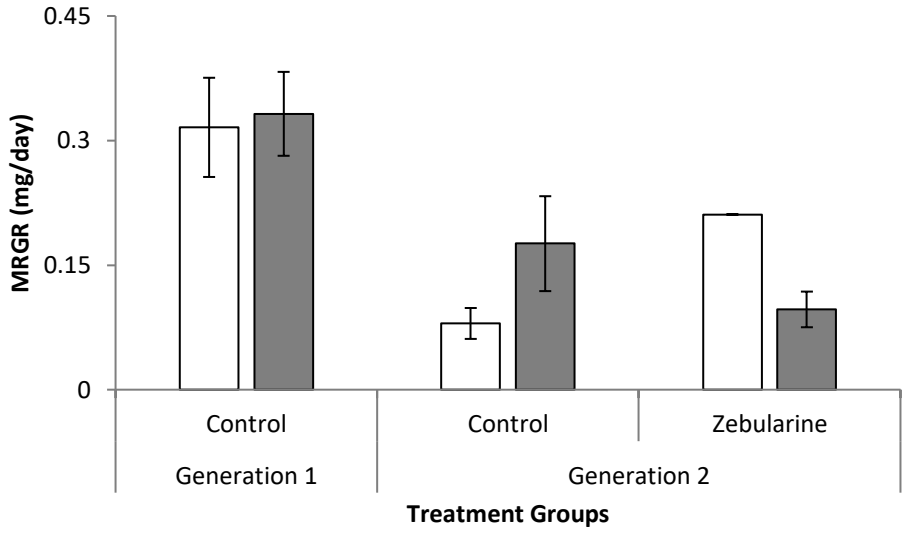

Figure 8.14 Mean relative growth rate of aphids feeding upon plants in each treatment group over two generations of *Senecio vulgaris* grown. White represents control (uncolonised) plants and grey represents mycorrhizal plants. Bars represent means +/- SE.

8.3.5 Mycorrhizal parameters

8.3.5.1 Total colonisation

Plants in generation one had a higher percentage of the root colonised by mycorrhizal fungi than plants in generation two ($F_{1, 25}$ = 119.212, p<0.001) (Figure 8.15). The levels of colonisation seen in generation one were some of the highest observed in this thesis.

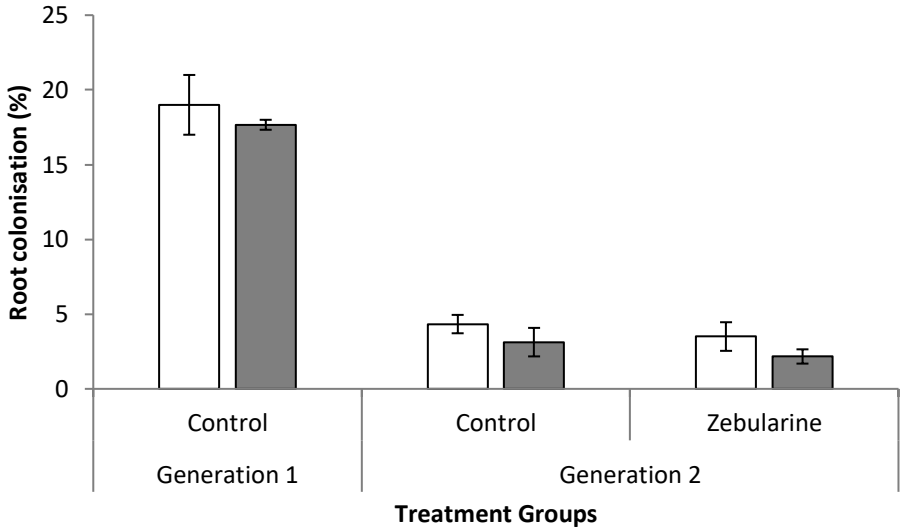

Figure 8.15 Mean root colonisation in plants in each treatment group over two generations of *Senecio vulgaris* grown. White represents non-aphid attacked plants and grey represents aphid attacked plants. Bars represent means +/- SE.

8.4 Discussion

The mycorrhizal and aphid treatments were not believed to have been affected by the zebularine treatment. The zebularine treatment was a temporary treatment that was meant to last 6 days. The aphids were placed onto the plant long after the treatment had ended and the plants had likely experienced DNA methylation within the generation. The dosage of zebularine used was shown (Herman & Sultan, 2016)

to not disrupt plant development, so it was assumed that this would not affect fungal spores present within the soil.

8.4.1 Lifecycle parameters

There was no significant difference between treatment groups, whereas in previous experiments aphid attacked plants (treatments H and HAM) did germinate quicker than non-attacked (Section 3.3.1). Overall, plants treated with zebularine in generation two took longer to germinate than generation two non-zebularine treated plants. There was no difference seen between non-zebularine and zebularine treated plants and the treatment groups (C, AM, H and HAM). This suggests that the difference in the results between the two experiments is not due to DNA methylation. An active demethylation pathway is known to be initiated during seed development in *Arabidopsis thaliana* (Kawakatsu *et al.*, 2017) and *Brassica rapa* (Lu *et al.*, 2006). In both species there were cycles of hypo and hypermethylation just before germination with Lu *et al.* (2006) suggesting that this is a necessary step towards transcriptional activation in gene expression which can contribute to developmental gene regulation. In common pear (*Pyrus communis*), it was found that DNA methylation increased the time it took for the seeds to germinate (Michalak *et al.*, 2013). However, this was not seen in these experiments. It could be that there is a parental effect in place that sped up the time to germination but removing the methylation meant that the germination time was returned to 'normal'. Germination time was longer in generation one that generation two, which is the opposite of what occurred in previous experiments (Section 3.3.1). The seeds used to grow generation one were dry stored for over two years, which could be why germination was slower as dormancy had to be broken. *Senecio vulgaris* seeds have been shown to not survive long with many seeds dying even in undisturbed soil (Roberts & Feast, 1973), so it is understandable that germination took longer.

The development of true leaves took longer in generation one than generation two, but there was no significant difference between these two generations seen in previous experiments (Section 3.3.1). This could be a knock-on effect from the slow

germination experienced. Such an effect from germination may have caused generation one plants to take longer to produce buds than plants from generation two. Mycorrhizal colonisation delayed development of buds compared to non-mycorrhizal plants, which was also observed in previous experiments (Section 3.3.1). This is interesting as the plants in this experiment experienced a much higher level of root colonisation than the other experimental plants (Section 7.3.1). The symbiotic relationship between the plant and AMF can also become parasitic especially at the beginning of the plant's lifecycle (Schmidt *et al.*, 2011). The seedlings may have delayed development due to the AMF using the plant's carbon. Plants treated with zebularine took longer to develop buds than non-treated plants in generation two. *Chrysanthemum* cultivars 'He Hua Xian Zu' and 'Qiu Shui Chang Liu' with non-altered methylation experienced a drop in DNA methylation when buds were produced compared to plants that had methylation altered by short day treatments (Li *et al.*, 2016). The same drop was seen in *Azalea* cultivars 'Johanna' and 'Blaauws Pink' and this was linked to DNA methylation and deacetylation acting together to differentiate the apical meristem to floral development (Meijón *et al.*, 2010). It could be that the purposely demethylated plants (generation 2, zebularine) had slower bud production due to the low levels of DNA methylation. DNA methylation could have been influencing bud production time. This idea is backed up further with Richards' (1997) finding that hypomethylation (very low levels of methylation) can cause a delay in flowering. However, it could also be a consequence caused by zebularine treated plants taking longer to reach other life stages.

The time taken to produce flowers was also affected by mycorrhizal colonisation. Mycorrhizal plants in generation one took longer to produce flowers than mycorrhizal plants in generation two. Mycorrhizal colonisation was not observed to affect flowering time in the previous experiment (Section 3.3.1), however in this experiment mycorrhizal colonisation was much higher (Section 7.3.1). Some species of mycorrhizas can cause a delay in flowering time, while other species can speed up the time taken to flower (Liu *et al.*, 2017). There was a mixture of mycorrhizal species used in these experiments and the roots were not analysed to find out which specific species was colonising the plant. So it could be that the difference in

flowering time between generations of mycorrhizal plants was due to the particular species of mycorrhizas colonising the roots. Plants treated with zebularine took longer to develop flowers than non-treated plants in generation two. This is the opposite of what was expected, as an epi-mutant (*FWA* gene) has been shown to stop or delay flowering (Soppe *et al.*, 2000). Demethylation using zebularine should have removed this epi-mutant if it was present, therefore speeding up flowering. It could be that the demethylation treatment was only effective in the short term and methyl groups had been added to alleles before flowering began.

Zebularine treatment did not affect the time taken for the plant to set seed. This was to be expected as DNA methylation was not found to play an important role in regulating significant genes involved in seed development in *Arabidopsis* (Lin *et al.*, 2017). Generation one plants took longer to develop seeds, which may be due to slow development throughout the lifecycle. The plants in generation one of previous experiments were the quickest to produce seeds (Section 3.3.1), however they were the fastest to germinate so the difference in germination time may be a reason for this. Mycorrhizal plants in generation one took longer to produce seeds than mycorrhizal plant in generation two. This could be due to mycorrhizal plants in generation one taking longer to flower and so taking longer to set seed than mycorrhizal plants in generation two. However, in previous experiments AMF colonised plants in generation one were faster to produce seeds than generation two plants (Section 3.3.1). In the previous experiment, generation one was faster overall, so that may be why the AMF colonised plants were faster in the previous experiment. It could also be linked to the lower mycorrhizal colonisation in the previous experiments (Section 7.3.1).

8.4.2 Mature plant parameters

8.4.2.1 Flower number

Plants in generation one produced a higher number of flowers produced than plants in generation two, which also occurred in previous experiments (Section 4.3.1.1). Plants in generation one took longer to develop, so had longer to produce

flowers than plants in generation two. Aphid attacked plants in generation one produced more flowers than aphid attacked plants in generation two. However in the previous experiments (Section 4.3.1.1) both generations one and two had increased number of flowers when the plants were attacked by aphids. Foxglove pug moths (*Eupithecia pulchellata*) have been found to cause a decrease in flower number in the next generation of *Digitalis purpurea* (lady's glove) (Sletvold & Grindeland, 2008). The decrease was thought to be due to diminished returns from placing a lot of energy into flower production. The number of flowers produced was reduced to reduce the energy that was lost when the flowers were destroyed (Sletvold & Grindeland, 2008). Mycorrhizal colonised plants in generation one produced fewer flowers than mycorrhizal colonised plants in generation two, which was not seen in the previous experiments (Section 4.3.1.1). Mycorrhizal colonisation was much lower in the previous experiments (Section 7.3.1), which may be why the difference was not observed. The results in this experiment agree with much of the literature looking into mycorrhizal colonisation, with plants having an increased flower number when colonised by mycorrhizas (Gange *et al.*, 2005; Vaingankar & Rodrigues, 2014). However, none looked into the effects of mycorrhizas on flower number over multiple generations. It could be that there is a cumulative effect of mycorrhizal colonisation on flower number.

There was no effect from demethylating the plant on flower number. There is no literature specifically on this topic, however DNA methylation is meant to cause an epi-mutant in flower production (Soppe *et al.*, 2000). If there was an epi-mutant present that delayed or stopped flowering, it would be expected that the flower number could decrease due to lack of time to flower or flower production being stopped. This was not observed, suggesting that this epi-mutant was not present in the plants used in this experiment and is less likely to be an explanation for the results in Section 4.3.1.1.

8.4.2.2 Leaf number

Leaf number increased in plants in generation two AM and H treatments compared to the previous generation, whereas leaf number decreased between generation one and two C treatments. Previous experiments found that leaf number in generation one aphid attacked plants was lower than aphid attacked plants in generation two (Section 4.3.1.2), which agrees with the results from this experiment. The combined treatment of mycorrhizas and aphids (treatment HAM) did not see a change in leaf number between generation one and two. Mycorrhizal colonisation was previously seen to increase leaf number between generation one and two in previous experiments in this thesis (Section 4.3.1.2).
DNA demethylation had no effect on leaf number. There is no literature on DNA methylation affecting leaf number and DNA methylation affecting the genes used to develop leaves. This all suggests that any changes in leaf number were not due to the epigenetic effect of DNA methylation.

8.4.2.3 Height

Plants in generation one controls were taller than the same plants in generation two, which was also seen in other experiments in this thesis (Section 4.3.1.4). However, in this experiment there was no effect from aphid attack, whereas in the previous experiments aphid attacked plants in generation two were taller. Mycorrhizal plants were shorter than non-mycorrhizal plants, which was not expected. Previous experiments in this thesis did not find any effect from mycorrhizal colonisation, but this may be due to the low colonisation levels (Section 7.3.1). There were no studies into the effects of AMF colonisation on plant height over multiple generations, however the majority of studies found within a generation the plant height was increased when AMF was present (Sohn *et al.*, 2003; Gange *et al.*, 2005; Vaingankar & Rodrigues, 2014). There was reduced mycorrhizal colonisation in generation two, so this may have caused the effects of mycorrhizal colonisation on plant height to be lessened.

There was no effect of demethylation by zebularine on the plant height in this experiment. This was not expected as previous literature found DNA methylation in *Arabidopsis* altered plant height (Zhang *et al.*, 2013). However, this was seen in drought stressed plants, so the parameter being altered could be down the type of stress the plant is under and whether it would be beneficial to alter the plant height.

8.4.2.4 Dry biomass

In previous experiments, generation one plants had a higher dry biomass than plants in other generations (Section 4.3.1.5), but this was not seen in these experiments. Mycorrhizal colonisation caused an increase in dry biomass between the generations of the plants. This was also seen in other experiments in this thesis (Section 4.3.1.5). Interestingly, dry biomass is a function of the other parameters measured such as height and leaf number. Mycorrhizal colonisation resulted in the plant height and flower number decreasing between generations, however leaf number of mycorrhizal plants did increase between generations. The increase in leaves on mycorrhizal plants between generations seems to be the cause of the increase in dry biomass.

Aphid presence was shown to increase dry biomass in generations one and two of previous experiments (Section 4.3.1.5). In this experiment, aphid attack had no effect upon the dry biomass.

The application of zebularine had no effect on the dry biomass of the plants. This was not expected as some of the literature found biomass was affected by DNA methylation in dandelions (Verhoeven & vanGrup, 2012). However, the results of this study disagreed with other findings in epigenetics studies, mainly that phenotypic changes are slow to be observed (Akst, 2017). These results suggest that the changes in biomass of *S. vulgaris* observed in Section 4.3.1.5 and Section 8.3.2.4 were not due to DNA methylation.

8.4.3 Seed parameters

8.4.3.1 Seed weight

Generation one plants produced heavier seeds than those in generation two. This was also observed in previous experiments in this thesis (Section 5.3.1.3). However, compared to Section 5.3.1.3, plants that were colonised by mycorrhizas (treatment AM) produced lighter seeds. This may be a result of the increased mycorrhizal colonisation in this experiment compared to previous experiments (Section 7.3.1). Seeds from aphid attacked plants in generation two were heavier than seeds from attacked plants in generation one in the previous experiment (Section 5.3.1.3). Zebularine application had no effect on seed weight, suggesting that any changes in seed weight was not due to DNA methylation. There is no literature into the effects of DNA methylation and seed weight.

8.4.3.2 Percentage germination

Plants in the first generation produced seeds which had a higher percentage germination than seeds from plants in generation two. In previous experiments in this thesis (Section 5.3.1.4), seeds from generation two also had the lowest percentage germination. The lower percentage germination by seeds from plants in generation two could be due to the lower seed mass that was observed. These two parameters have already been linked with lower seed mass causing lower percentage germination (Luzuriaga *et al*., 2006). It did seem that seeds from generation two plants were more likely to be 'failed' (Plate 5.1) than 'healthy' (Plate 5.2). Plants that were colonised by mycorrhizas (treatments AM and HAM) produced seeds with a lower percentage germination than plants that were not colonised by mycorrhizas (treatments C and H). This is the opposite to what was observed in previous experiments (Section 5.3.1.4). This could be a result of mycorrhizal colonisation being higher in these experiments than previous experiments (Section 7.3.1). Different levels of mycorrhizal colonisation have been shown to have different levels of benefits (Gange & Ayres, 1999), so it could be that

the level of colonisation in this experiment were too high to cause benefits to seed germination. There was a drop in percentage germination of mycorrhizal offspring in the second generation of *S. vulgaris* (West, 1995). In the previous experiment, aphid attacked plants produced seeds with a higher percentage germination than non-attacked plants (Section 5.3.1.4). This was not observed in this experiment. The application of zebularine had no effect upon the percentage germination of seeds. This is interesting, as DNA methylation was shown to be heavily involved in the germination process (Lu *et al.*, 2006; Michalak *et al.*, 2013; Kawakatsu *et al.*, 2017). It could be that thirteen days is enough time for the slow germination due to lack of DNA methylation to not be noticed, or the plant has methylated alleles within the generation so the seed has DNA methylation. Methylation has shown to quickly revert back to the wild-type (Wibowo *et al.*, 2016), so it is not unexpected that within a generation the methyl groups reappear.

8.4.4 Aphid parameters

In the previous experiment, teneral weight was reduced in aphids feeding on generation two plants when compared to aphids feeding upon generation one plants (Section 6.3.1.1). This did not appear to occur in these experiments, however the results were not analysed statistically due to lack of aphids in generation two, zebularine treatment group. However, from looking at the results both teneral weight and mean relative growth rate of aphids feeding upon mycorrhizal plants were increased compared to the aphids feed on non-colonised plants in generation two, control. The opposite occurred with mycorrhizal colonisation causing aphid performance to be decreased compared to aphids feeding upon the non-mycorrhizal plants in generation two, zebularine. The effect of aphids feeding upon zebularine treated, mycorrhizal colonised plants should be explored further, as the results look very interesting. Mycorrhizal colonisation was seen to have no effect on aphid parameters in the previous experiment (Section 6.3.1). Mycorrhizal colonisation was much lower in the previous experiment (Section 7.3.1), so that could be why there was no effect in those experiments. There is currently no literature of DNA methylation and symbiotic fungi interacting to affect insect

herbivore performance. It could be that the removal of DNA methylation means that the effects of AMF on insect herbivores or the plants defences are more visible. Previous literature did show that DNA methylation in *Arabidopsis* can change the plants ability to respond to the pathways involved in defence hormones (jasmonic and salicylic) (Latzel *et al.*, 2012). These changes in the defence pathways could be why there was a decrease in MRGR between generation one and two.

8.4.5 Mycorrhizal parameters

Total root colonisation was higher than colonisation levels seen in Section 7.3.1. The environmental conditions were kept the same between both experiments, however a different batch of Rootgrow was used, so that may be why there was a different colonisation percentage. Plants in generation one had a higher colonisation rate than plants in generation two, which was also seen in West (1995).

The application of zebularine had no effect on the colonisation percentage, which was expected as there is no evidence that DNA methylation affects symbiotic relationships.

8.5 Conclusion

The hypothesis that some of the effects seen between generations of *S. vulgaris* would be altered by DNA demethylation was upheld. Demethylation of the DNA did affect the plant's development time, especially germination and bud development. It also seems to have affected aphid parameters, which was most interesting. There has been no literature into the interaction of DNA methylation and symbiotic fungi, especially affecting insect herbivores, so this is worth exploring further with more replicates. Interestingly, the hypothesis that DNA demethylation would affect seed production was not upheld, however the effect on flowering time was upheld. This suggests that some of the parental effects seen in previous chapters can be explained by DNA methylation, especially changes to development time.

Chapter Nine

General Discussion

9.1 Summary of outcomes

The aim of this PhD was to firstly find out if the presence of insect herbivores and/or mycorrhizal fungi causes parental effects to occur in *Senecio vulgaris* and secondly to try and find the transmission mechanisms for any of the effects seen between generations. A summary of the original objectives with the consequent outcomes can be found in Tables 9.1 and 9.2.

Objectives	Outcomes
Are parental effects passed between generations of *Senecio vulgaris* and if so, how many generations?	Yes, certain parameters measured in Chapters 3, 4 and 5 were passed between the generations. The length the parental effects lasted depended upon the parameter being measured.
Does the presence of insect herbivores cause changes in plant development over multiple generations?	Yes, all parameters measured in Chapters 3 and 4 were affected by insect herbivores over multiple generations. In Chapter 5, seed nitrogen, seed carbon, seed weight and percentage germination were affected by insect herbivores over multiple generations.
Does the presence of insect herbivores cause changes in plant defences over multiple generations?	Unfortunately the method for measuring plant defences was never perfected, so this could not be measured. The population growth parameters of the aphids were not significantly different between generations suggesting the defences did not change.
Does the symbiotic relationship between plant and mycorrhizal fungi affect plant development over multiple generations?	Chapter 4, leaf number and flower size were affected by mycorrhizas over multiple generations. Chapter 5, percentage germination was affected by the relationship over multiple generations.

Table 9.1 Summary of objectives and outcomes of the first aim in this thesis.

Objectives	Outcomes
Does vertical transmission of endophytes cause parental effects in the progeny generations?	No. Individual plants may be affected by the vertical transmission of certain endophytes, however there was no community of endophytes being passed between the treatment group generations.
Does methylation of DNA cause environmental parental effects in the progeny generations?	Yes, Chapter 8 the lifecycle parameters were seen to be affected by the treatment of zebularine. The time taken for germination, budding and flowering to take place was seen to increase when DNA was demethylated.

Table 9.2 Summary of objectives and outcomes of the second aim in this thesis.

9.2 Environmental triggers of parental effects

9.2.1 Parental effects and length of visibility

The main aim of this thesis was to determine if parental effects did occur in *Senecio vulgaris* and how long they are visible for. Latzel (2015), had stated that ecological studies are not properly correcting for parental effects. Before his study, if an ecological study was correcting for parental effects then only one generation of the plant was grown before the study was started. However, Latzel (2015) stated that at least two generations of the plants needed to be grown to correct for parental effects as they can remain visible over multiple generations.

The results from this thesis both agree and disagree with his findings. The common parameters of plant growth that are measured in these experiments were found to show visible parental effects over a varying number of generations (Table 9.3). The results suggest that to control for parental effects, the number of generations grown before an ecological study depends upon the parameters that are going to be measured in that particular study.

Parental effects could be reducing the stress experienced by the plants, for example by increasing seed nutrition the seedling experiences less stress associated with nutrition during early development. The reduction of stress the progeny experiences could be to such an extent that parental effects are triggered to a lesser extent in formation of the subsequent generation. This has not been explored in any of the previous literature, however it is assumed that parental effects are triggered by stress to cause adaptation of plant defences to pests or pathogens (Agrawal, 2002; Rasmann *et al.*, 2012; Vivas *et al.,* 2013) or increased seedling survival (Elwell *et al.*, 2011), therefore potentially reducing stress experienced by the progeny. If the progeny is experiencing less stress when the plants set seed, then it suggests that the parental effects will be triggered to a lesser degree than the previous generation parental effects. However offspring from those plants may be less prepared to deal with stress, so retrigger parental effects. This may be why parental effects seem to only last a couple of generations before disappearing (Latzel, 2015) or why the results show that parental effects disappear and reappear.

	Generation 1 to 2	Generation 2 to 3	Generation 3 to 4
Chapter Three			
Germination time	No	**Yes**	**Yes**
True leaves development time	**Yes**	No	**Yes**
Budding time	**Yes**	No	**Yes**
Flowering time	**Yes**	No	**Yes**
Seeding time	**Yes**	No	**Yes**
Chapter Four			
Flower number	**Yes**	No	No
Leaf number	**Yes**	No	**Yes**
Flower size	**Yes**	No	No
Height	**Yes**	**Yes**	No
Dry biomass	**Yes**	**Yes**	No
Chapter Five			
Seed carbon	No	No	No
Seed nitrogen	No	No	No
Seed weight	**Yes**	No	No
Percentage germination	**Yes**	No	No

Table 9.3 Summary of when and how long parental effects were observed for each parameter measured in this thesis.

9.2.2 Parental effects and insect herbivory

There have been few studies into the effect of insect herbivores on a plants development over multiple generation. Previous studies have looked at the effect of insect herbivory on plant development between only two generations. This study

has shown that the presence of insect herbivores can cause parental effects to be passed between generations of *S. vulgaris*. Aphids had a major effect on the plants' development time and final size parameters, both within generations and between generations (Table 9.4). However, the presence of insect herbivores had less of an effect upon seed parameters, especially seed chemistry. The effect on seed weight when insect herbivores are present on the plant could have a major knock-on to the growth of the next generation plants. The effect of insect herbivores on a large number of parental effects suggests that correction for parental effects is needed before starting ecological experiments. Especially, if the seeds being used are from parental plants that could have experienced insect herbivory.

Unfortunately, the effect of insect herbivores presence on plant chemical defences over multiple generations could not be measured. There did not seem to be any significant changes to aphid population growth when feeding upon different generations, which suggests that the plants did not use parental effects to increase plant defences between generations. However, there were changes in the plants development and final plant size parameters between generations so the plant could be focusing on tolerance of the insects through growth rather than increasing chemical defences.

9.2.3 Parental effects and mycorrhizal fungus

Colonisation by mycorrhizas has been shown to not transfer between generations (Genkai-Kato & Yamamku, 1999), however previous research had shown that mycorrhizal effects can be seen between a parental and progeny generation (West, 1995). This thesis was exploring the effects of mycorrhizal colonisation over multiple generations. There were only four parameters seen to be affected by mycorrhizal colonisation over multiple generations (Table 9.5). Mycorrhizal colonisation was very low in these experiments, so there could be more parental effects caused by mycorrhizal fungi if the plant experienced a higher level of colonisation.

The time taken to develop seeds and flower size were both affected by the presence of both mycorrhizal fungi and insect herbivores. This interaction shows

that parental effects can be affected by multiple environmental factors. This makes it hard to predict what parental effects are going to be seen when multiple conditions vary while the plants are growing. The effects differing between parameters shows that the parameters being measured must be considered before controlling for parental effects in ecological studies.

Parameter	Parental effect?	Effect seen
Chapter Three		
Germination time	**Yes**	Aphid attacked plants faster to germinate in second and fourth generations but slower in first and third generations.
True leaves time	**Yes**	Second and third generation aphid attacked plants slower to produce true leaves but faster in the first and fourth generations.
Budding time	**Yes**	Generation three and four attacked plants slower at developing buds than attacked plants in the first and second generations.
Flowering time	**Yes**	Generation one and two attacked plants produced flowers in a shorter time but the opposite was true in generations three and four.
Seeding time	**Yes**	Generation one aphid attacked plants were significantly faster at developing seeds than aphid attacked plants in other generations
Chapter Four		
Flower number	**Yes**	Plants in generations three and four produced more flowers when attacked by aphids. The opposite was true in generations one and two.
Leaf number	**Yes**	Generation three and four attacked plants produced more leaves than generation one plants.
Flower size	**Yes**	Generation three attacked plants produced smaller flowers than the attacked plants in other generations.
Height	**Yes**	Generation two attacked plants were taller than non-attacked plants. The opposite was seen in the other generations
Dry biomass	**Yes**	Generations one and two attacked plants had a higher dry biomass. Generations three and four had lower dry biomass.
Chapter Five		
Seed carbon	No	Generation three attacked plants produced seeds with a lower seed carbon content
Seed nitrogen	**Yes**	Generation one and four plants produced seeds with a high seed nitrogen content.
Seed phosphorus	No	N/A
Seed weight	**Yes**	Generation two attacked plants had a higher seed weight. Generations three and four had a lower seed weight.
Percentage germination	**Yes**	Generations one and two plants produced seeds with a higher percentage germination when attacked by aphids. The opposite was true in generations three and four.

Table 9.4 Parameters that were affected by the presence of insect herbivores over multiple generations and the effects that the insect herbivores had.

Parameter	Parental effect?	Effect seen
Chapter Three		
Germination time	No	N/A
True leaves time	No	N/A
Budding time	No	N/A
Flowering time	No	N/A
Seeding time	**Yes**	There was a three-way interaction with mycorrhizas and aphids over multiple generations. AM plants in generation one were the slowest to produce seeds. However, in generations two, three and four the AM or HAM plants were not the slowest treatment groups to produce seeds.
Chapter Four		
Flower number	No	N/A
Leaf number	**Yes**	Generations one and two plants produced plants with more leaves when the plants were colonised by mycorrhizas. The opposite occurred in generations three and four.
Flower size	**Yes**	Mycorrhizal plants in generations 1, 2 and 4 had larger flowers. This did not occur in generation two.
Height	No	N/A
Dry biomass	No	N/A
Chapter Five		
Seed carbon	No	There was a three-way interaction with mycorrhizas and aphids over multiple generations. generations one, two and three, AM plants produced seeds with higher concentrations of seed carbon compared to the C plants. When aphids were present on the plants, the opposite occurred.
Seed nitrogen	No	There was a three-way interaction with mycorrhizas and aphids over multiple generations. AM plants in generations one, two and three produced seeds with higher concentrations of seed nitrogen than C plants. The opposite occurred between H and HAM plants.
Seed phosphorus	No	N/A
Seed weight	No	N/A
Percentage germination	**Yes**	In generations one, three and four, mycorrhizal plants (AM and HAM) plants had higher percentage germination but the reverse happened in generation two.

Table 9.5. Parameters that were affected by the presence of mycorrhizas over multiple generations and the effects that the mycorrhizal colonisation had.

9.3 Mechanisms for transmission of parental effects

9.3.1 Vertical transmission of endophytes

Multiple species of endophytes are known to transfer vertically between parent and progeny in forb species, including *S. vulgaris* (Hodgson *et al.*, 2014). Certain species of fungal endophytes have been shown to produce anti-herbivory effects (Gange *et al.*, 2012; Hartley *et al.*, 2015). It was originally hypothesised that communities of endophytes would be dissimilar between each treatment group within a generation, but the endophytic community within a certain treatment group would be similar between the generations therefore causing some of the parental effects seen in this thesis. However this was found to not occur, with the results showing that the endophyte community was generally similar in every plant used in this thesis and there was no significant differences between treatment groups. From these results it is believed that while vertical transmission of endophytes in individual plants can cause some parental effects, this was not seen on a larger scale with multiple plants in the same treatment groups. However, these results were obtained by growing plants in a controlled environment, where the atmosphere seemed generally constant in the species of endophyte present. The endophytic community obtained from plants grown in field conditions should be considerably more varied.

9.3.2 DNA methylation and parental effects

Epigenetics and the effects on plant development has been previously researched especially DNA methylation (Herman & Sultan, 2016). However, there is much debate in the literature over whether DNA methylation creates a true long-term phenotypic change due to how quickly the epigenetic changes revert back to the 'wild-type' (Akst, 2017).

In these results, only plant development time appeared to be affected by the application of a DNA demethylation drug (zebularine). Germination time, bud and flower development time were all increased when the plant DNA was

demethylated. This suggests that DNA methylation plays a role in speeding up *S. vulgaris* development and is the mechanism for the quickening of plant development time between generations. However, DNA methylation was not seen to affect any other parameter measured in this study.

There are still many parameters that were influenced by parental effects in these studies, where a mechanism for these changes has not been found. These parental effects could be caused by other epigenetic changes, for example histone modification or chromatin modification.

9.4 Recommendations

The results in this thesis suggest that ecological studies should be correcting for parental effects before the study is started, unless the growing conditions of the parental plants for multiple generations are known before the seeds were collected. None of the parameters measured showed the same pattern for the visibility of parental effects. The parameters being measured in the studies should be known before the study is started, so the number of generations grown corrects for the specific parameters being measured.

Plant breeding is very important, both for agriculture and commercial use. Some of the parameters observed to be affected by parental effects in this study could be beneficial to plant breeders. Speeding up development time was shown to be a parental effect in this study and if replicated it is a desirable trait for plant breeding. The faster the plants develop, the quicker they can be harvested and sold. Other parameters in this study that were affected by parental effects could also be beneficial when breeding plants, such as increased flower number or size for commercial growers.

In agriculture, the knowledge of which parental effects are passed through the seed could be beneficial especially if the growing conditions for the next are similar to the parental plant. It could increase the yield of the crops or speed up the development time, as these parameters have been shown to be affected by parental effects in other plants.

9.5 Future work

DNA methylation is thought to take many generations before phenotypic changes are visible (Akst, 2017), but some changes were observed between parent and progeny generation in this thesis. The DNA methylation experiment could be continued for a few more generations to observe whether more phenotypic changes are visible or the 'wild-type' of DNA methylation is reverted back to.

This thesis used *Senecio vulgaris*, which has the ability to self-fertilise. Selfing plants may have different parameters altered by parental effects compared to species of plants that cross-pollinates. Cross-pollinating plants have been shown to pass parental effects between generations (Trueman & Turnbull, 1994), so it is not thought that the parental effects would disappear. However, there may be differences in which parental effects are visible and how long they are visible for. This experiment could be replicated in an agricultural crop species, to see if the same parental effects are seen over multiple generations. Certain parental effects, such as the increase in dry biomass could be very beneficial in agriculture. However, these experiments should be run in the field, as this would replicate the actual conditions the plants would experience, so the parental effects may differ both in the parameters affected and longevity of the effects.

It would be interesting to carry out the same experiment but in the field. The experiments were all carried out in a controlled environment, however in a field experiment the environmental conditions can be highly variable. This should greatly affect the visibility of parental effects and may give different result dependent upon the environment experienced especially over multiple generations. There have been studies into parental effects in the field (Alba *et al.*, 2016). However the parental effects observed were greatly affected by environmental conditions, such as temperature and so easily lost. In the field, the longevity of parental effects may not be the same as in controlled conditions. An interesting way to observe parental effects in the field is to two very different communities of the same species of plant, to compare the effect of different environments on the visibility of parental effects (Alba *et al.,* 2016).

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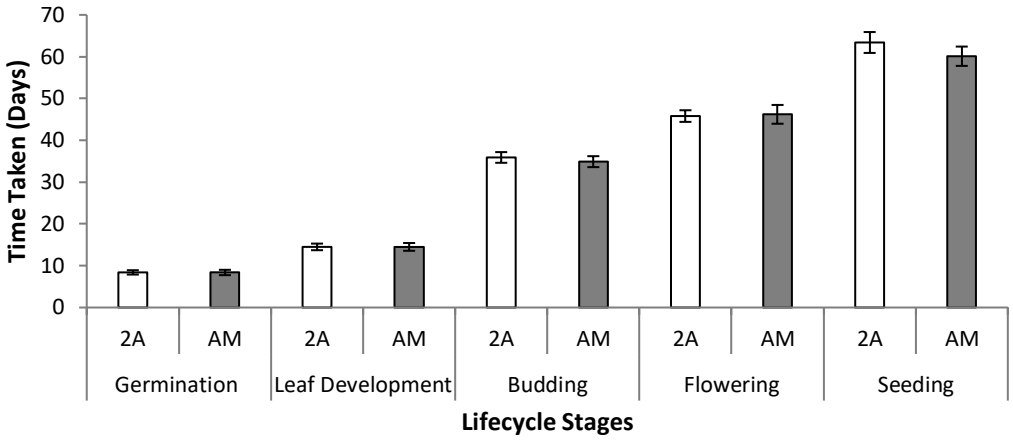
Appendix I

A.1 Changing treatments and the impact on development time

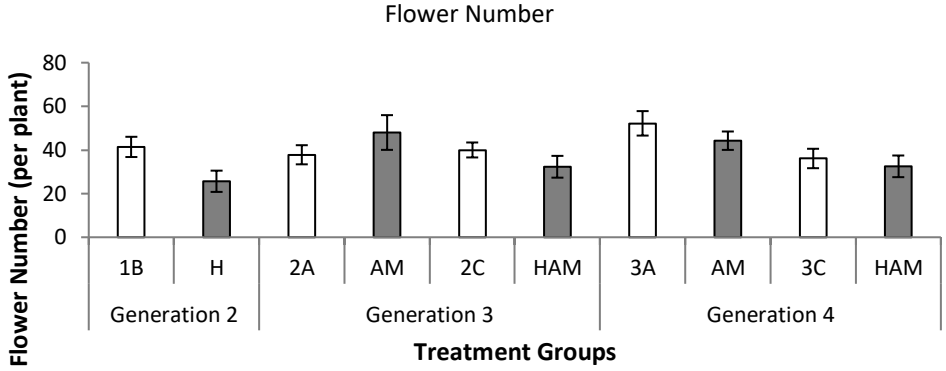
Plants development time in the generation three AM treatment group was compared to control treated plants (2A), as the seeds were from the same parental plants. There were no significant correlations between the variables, as seen with standard error bars (Chapter 3). Treatments 2A now refers to treatments 3A within the thesis.

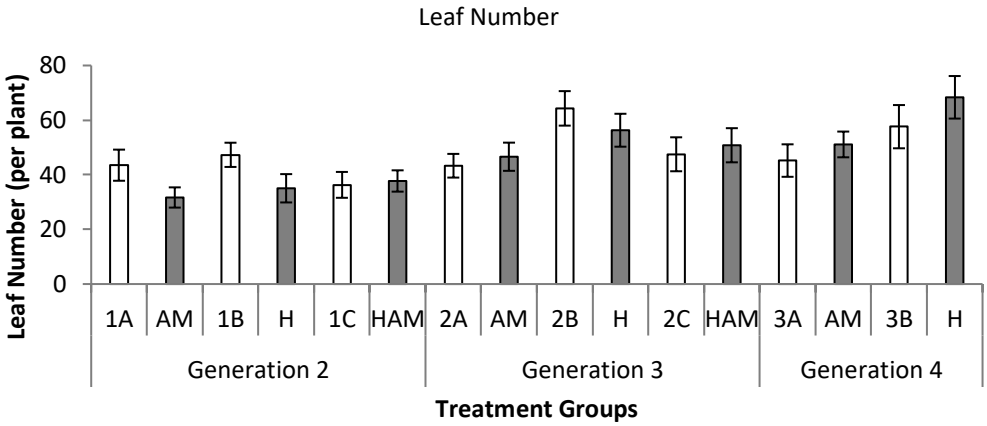


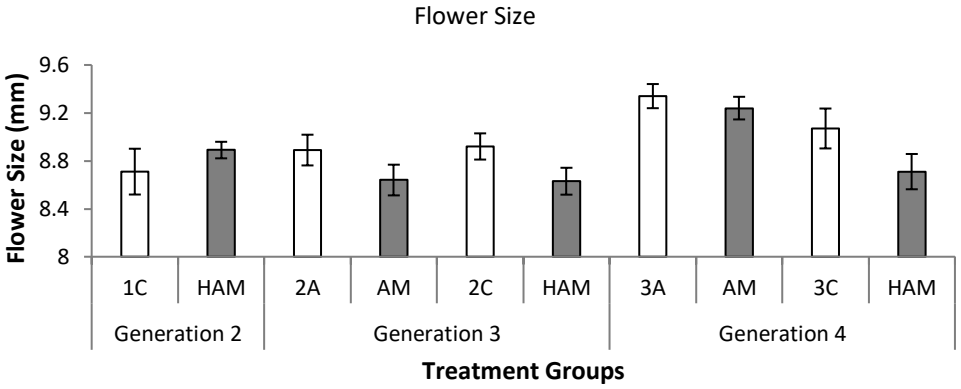
Appendix II

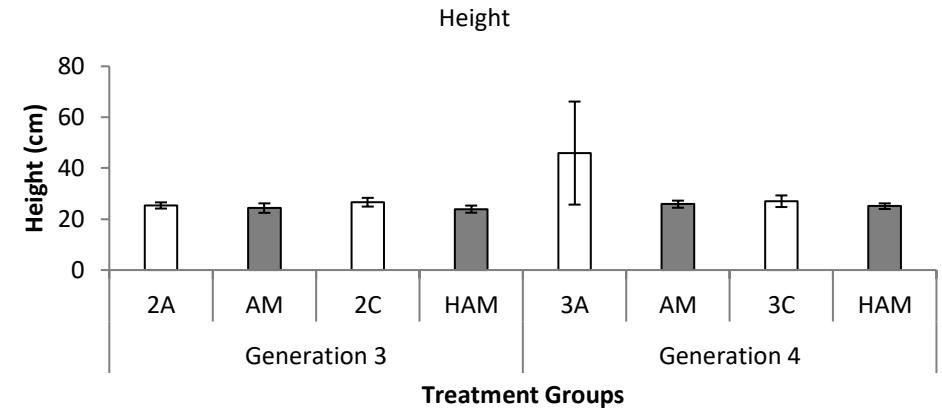
A.2 Changing treatments and mature plant size parameters

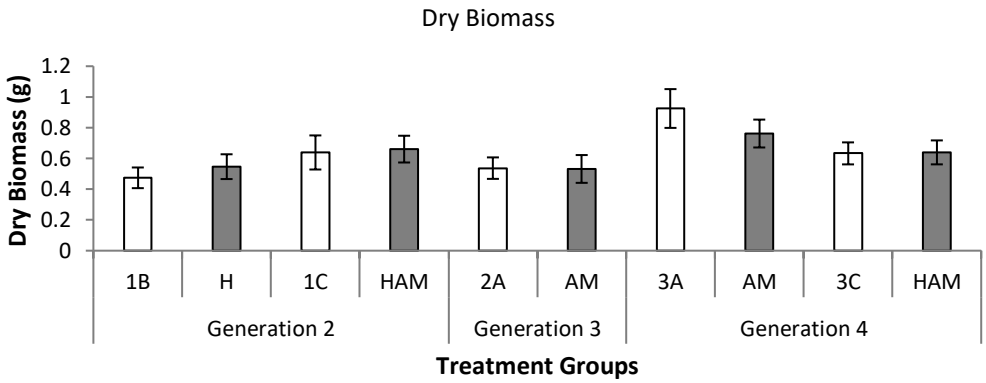
Plants mature plant size was measured for different parameters by comparing plants from the same parental treatment group that was subjected to either control conditions (white) or the same treated conditions (grey) as the parent. There were no significant correlations between the variables, as seen with standard error bars (Chapter 4). The treatment numbers have been altered, so treatment 1A has changed to 2A, 1B to 2B, 1C to 2C, 2A to 3A, 2B to 3B, 2C to 3C, 3A to 4A, 3B to 4B and 3C to 4C.






Flower Size
Flower Size (mm)
9.6
9.2
8.8
8.4
8
1C
HAM
2A
AM
2C
HAM
3A
AM
3C
HAM
Generation 2
Generation 3
Generation 4
Treatment Groups

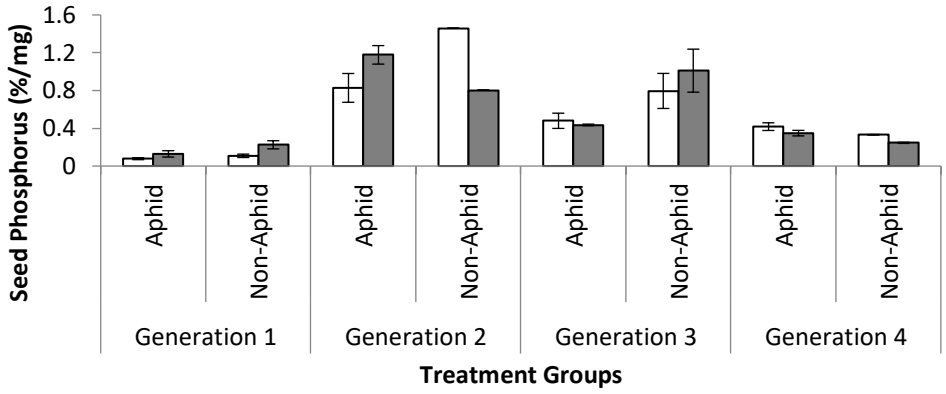

Height
Height (cm)
80
60
40
20
0
2A
AM
2C
HAM
3A
AM
3C
HAM
Generation 3
Generation 4
Treatment Groups


Dry Biomass
Dry Biomass (g)
1.2
1
0.8
0.6
0.4
0.2
0
1B
H
1C
HAM
2A
AM
3A
AM
3C
HAM
Generation 2
Generation 3
Generation 4
Treatment Groups

Appendix III

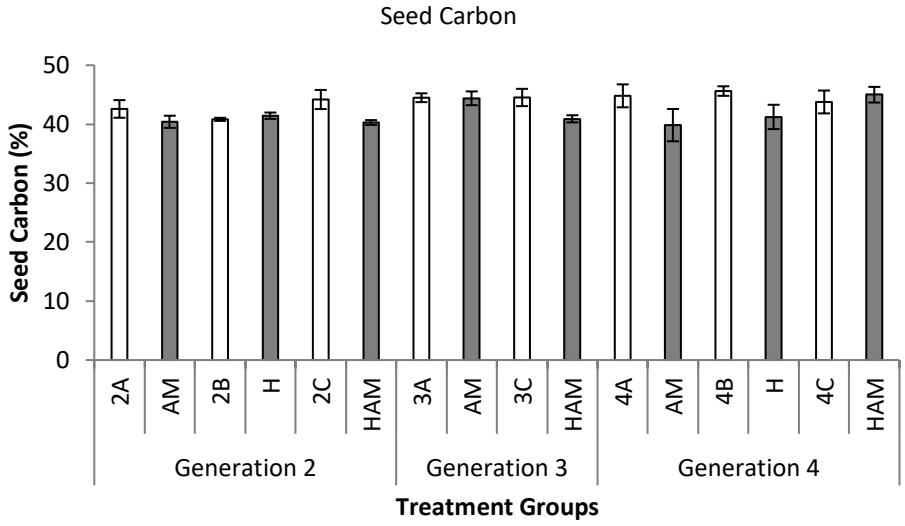
A.3.1 Seed phosphorus

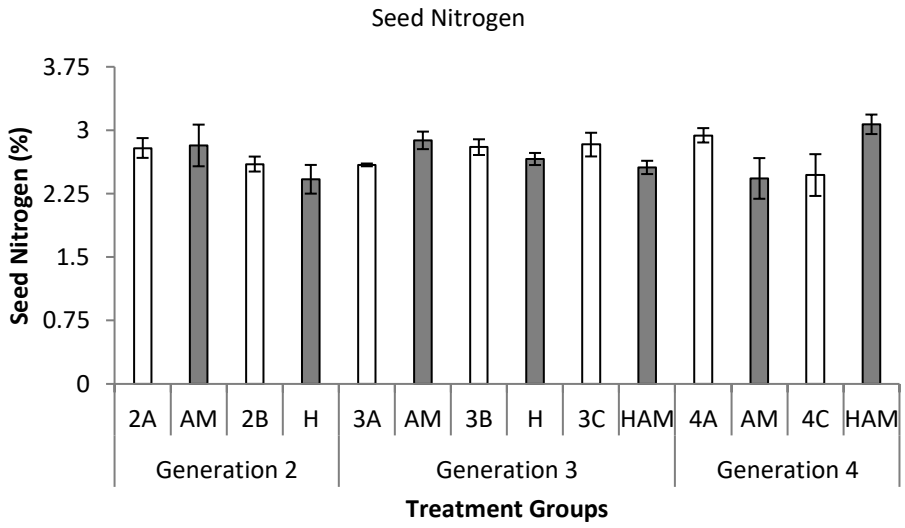
Seed phosphorus measurements were excluded due to lack of replicates (Chapter 5).

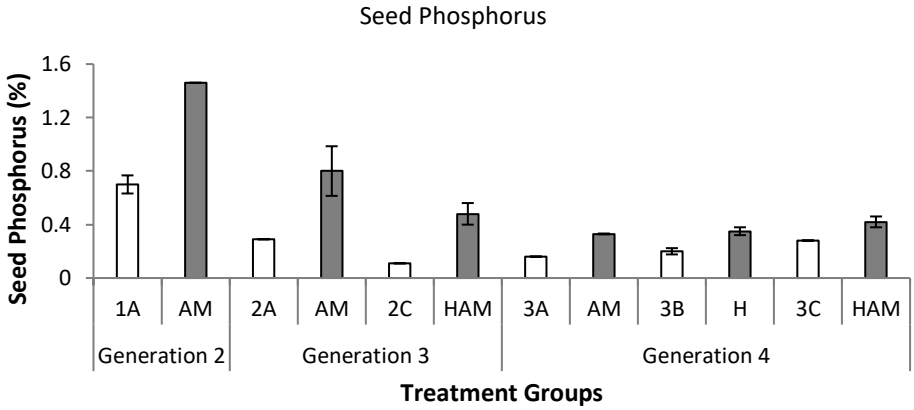


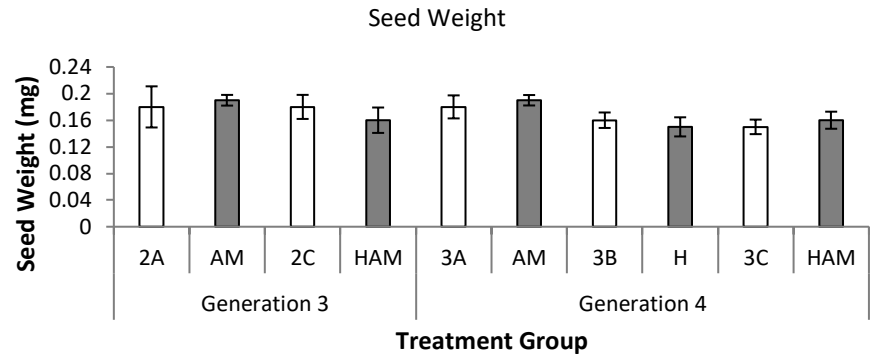
A.3.2 Changing treatments and the effects on seed measurements

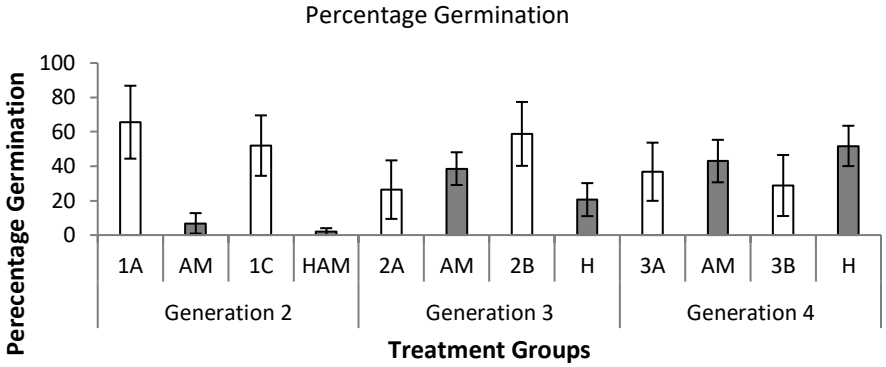
Plants seed chemistry, weight and percentage germination was measured for different parameters by comparing plants from the same parental treatment group that was subjected to either control conditions (white) or the same treated conditions (grey) as the parent. There were no significant correlations or the there was a lack of replicates between the treatments, as seen with standard error bars (Chapter 5). The treatment numbers have been altered, so treatment 1A has changed to 2A, 1B to 2B, 1C to 2C, 2A to 3A, 2B to 3B, 2C to 3C, 3A to 4A, 3B to 4B and 3C to 4C.


Seed Carbon
Seed Carbon (%)
50
40
30
20
10
0
2A
AM
2B
H
2C
HAM
3A
AM
3C
HAM
4A
AM
4B
H
4C
HAM
Generation 2
Generation 3
Generation 4
Treatment Groups


Seed Nitrogen
Seed Nitrogen (%)
3.75
3
2.25
1.5
0.75
0
2A
AM
2B
H
3A
AM
3B
H
3C
HAM
4A
AM
4C
HAM
Generation 2
Generation 3
Generation 4
Treatment Groups


Seed Phosphorus
Seed Phosphorus (%)
1.6
1.2
0.8
0.4
0
1A
AM
2A
AM
2C
HAM
3A
AM
3B
H
3C
HAM
Generation 2
Generation 3
Generation 4
Treatment Groups

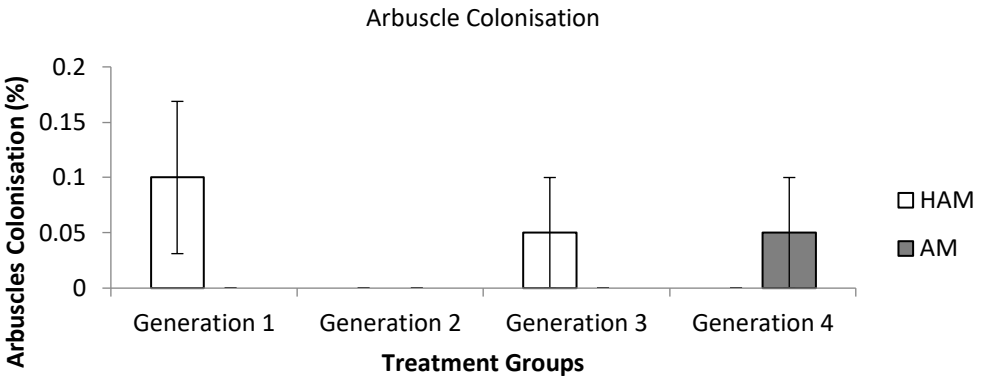

Seed Weight
Seed Weight (mg)
0.24
0.2
0.16
0.12
0.08
0.04
0
2A
AM
2C
HAM
3A
AM
3B
H
3C
HAM
Generation 3
Generation 4
Treatment Group


Percentage Germination
Perecentage Germination
100
80
60
40
20
0
1A
AM
1C
HAM
2A
AM
2B
H
3A
AM
3B
H
Generation 2
Generation 3
Generation 4
Treatment Groups

Appendix IV

A.4 Mycorrhizal colonisation experiment

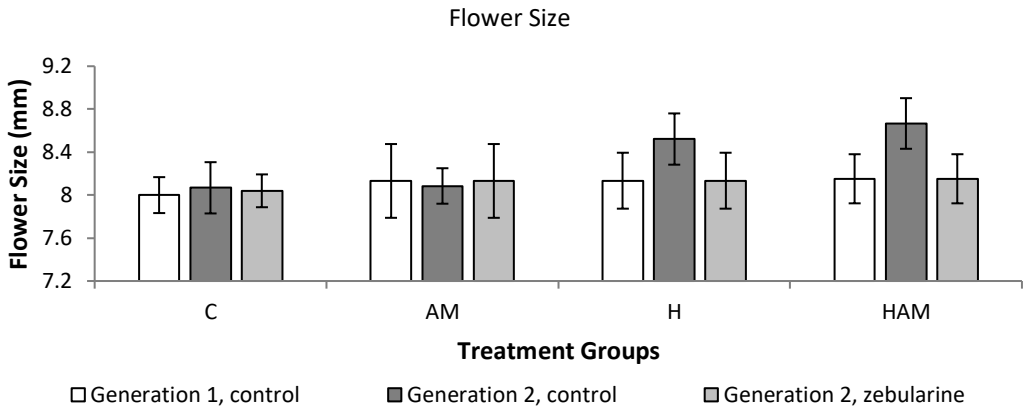
Arbsucle colonisation was not shown due to lack of replicates (Chapter 7).

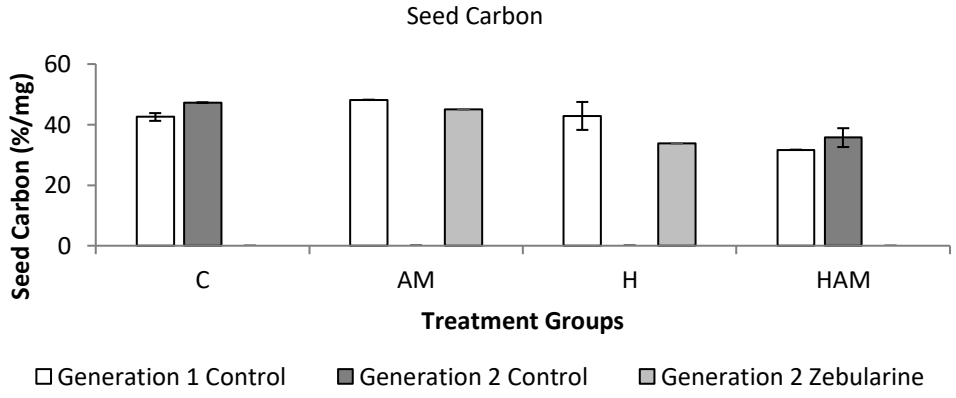


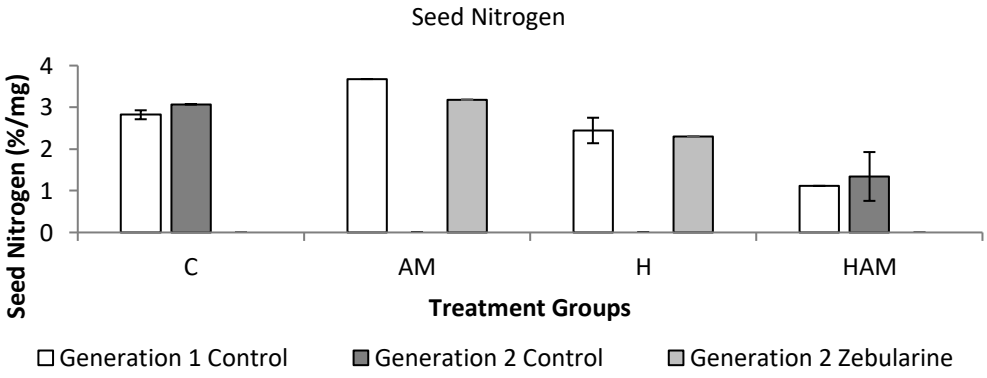
Appendix V

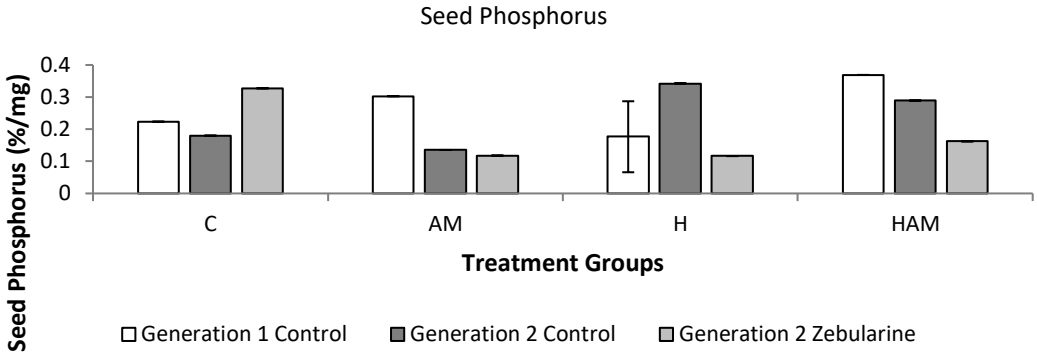
A.5 DNA methylation experiment

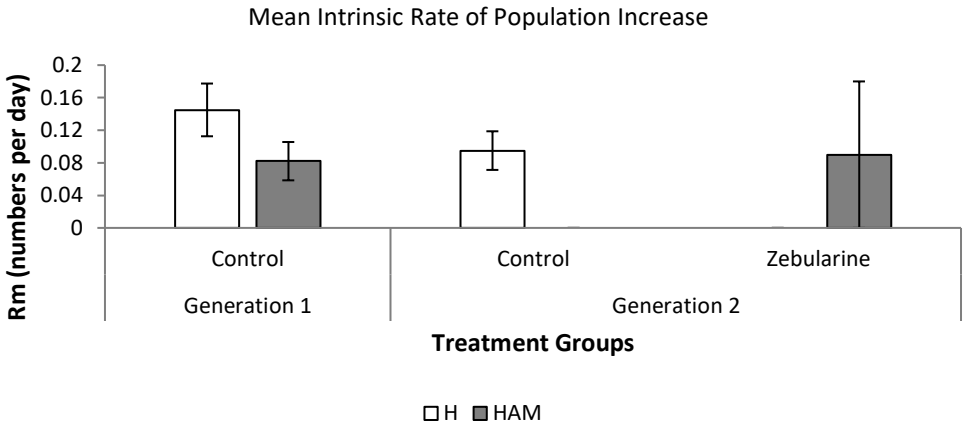
Parameters with non-significant or not enough replicates are shown below (Chapter 8).

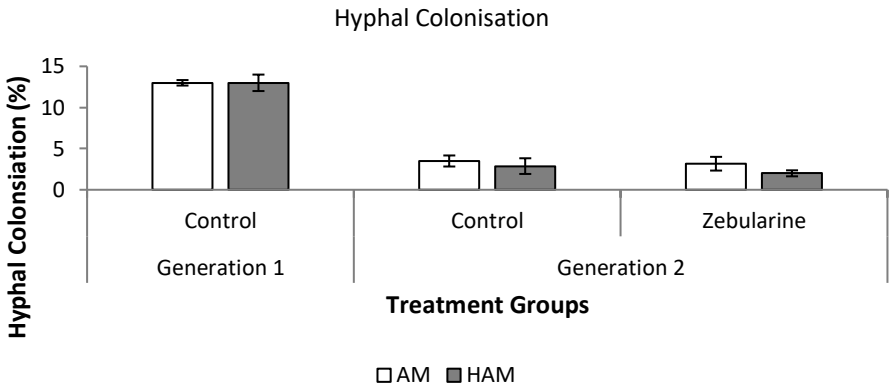


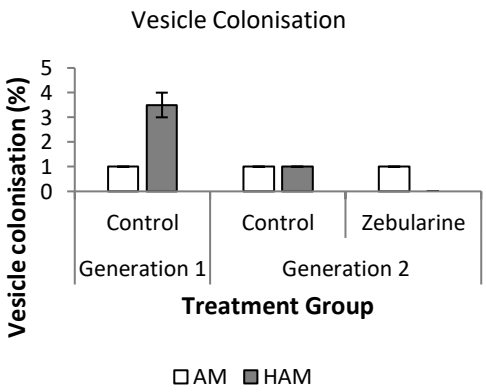


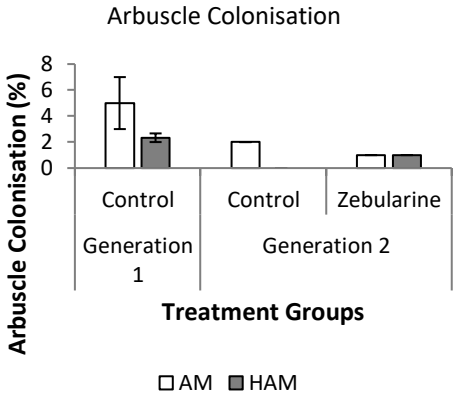



Seed Phosphorus
Seed Phosphorus (%/mg)
0.4
0.3
0.2
0.1
0
C
AM
H
HAM
Treatment Groups
Generation 1 Control
Generation 2 Control
Generation 2 Zebularine


Mean Intrinsic Rate of Population Increase
Rm (numbers per day)
0.2
0.16
0.12
0.08
0.04
0
Control
Control
Zebularine
Generation 1
Generation 2
Treatment Groups
H
HAM


Hyphal Colonisation
Hyphal Colonsiation (%)
15
10
5
0
Control
Control
Zebularine
Generation 1
Generation 2
Treatment Groups
AM
HAM


Vesicle Colonisation
Vesicle colonisation (%)
5
4
3
2
1
0
Control
Control
Zebularine
Generation 1
Generation 2
Treatment Group
AM
HAM


Arbuscle Colonisation
Arbuscle Colonisation (%)
8
6
4
2
0
Control
Control
Zebularine
Generation 1
Generation 2
Treatment Groups
AM
HAM