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Genetic basis of winter oilseed rape resistance to the cabbage stem flea beetle (PhD)

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This is the final report of a 72-month project (21120064) that started in October 2017: Genetics of the interaction between rapeseed and the cabbage stem flea beetle (*Psylloides chrysocephala*). The work was funded by a contract for £70,500 from AHDB Cereals & Oilseeds. The total project value was £116,452.

Contents

Abstract

The cabbage stem flea beetle (CSFB; *Psylliodes chrysocephala*) is a major pest of Brassicaceae, in particular, winter oilseed rape (WOSR; *Brassica napus*). After the 2013 EU-wide moratorium on the use of neonicotinoids, CSFB numbers dramatically rose in the United Kingdom (UK), with a near complete ban of the use of neonicotinoids across the EU declared in April 2018. In 2015, resistance to pyrethroids, the remaining chemical control method, was confirmed in Germany, Denmark, and the UK. As a result, farmers have been left with no single viable control for CSFB and are moving away from growing WOSR, with a decrease of 50% in national area between 2013 and 2023. WOSR is one of the most profitable crops in the UK and a reduction in the area grown is detrimental to the economy and UK farming.

A large gap in knowledge of the genetics underlying phenotypic responses to CSFB is preventing advancements in development of new resistant/tolerant varieties of WOSR. Previous research attempts have been made to reveal resistance and tolerance traits to flea beetles in general, however few have focused specifically on CSFB adults. Many experiments have been focused on large, field-trial-scale observations and, as such, are impacted by environmental interactions and insect availability.

Within this paper, we present controlled environment, choice, adult CSFB feeding trials on a diverse population of *Brassica napus*, comprising spring, semi-winter and WOSR, kale and swede types.

We identified variation in adult CSFB feeding across the panel. Lines showing extreme variation, and therefore increased or decreased adult CSFB feeding, and the resulting F_1, have been confirmed within non-choice and field environments, suggesting palatability is genetic and robust across environments.

Using genome-wide association and gene expression association approaches, we have identified loci associated with adult CSFB palatability. Utilising the relationship to the model plant *Arabidopsis*, we have tested potential candidate genes controlling palatability.

Our results support the development of CSFB-Brassica interactions and identify the potential for breeding for reduced palatability as a component of integrated pest management.

1. Introduction

Oilseed rape (*Brassica napus*; OSR) is an agricultural crop species that is part of the Brassicaceae family and results from a recent hybridisation event between *Brassica rapa* (turnips and mustards) and *Brassica oleracea* (vegetable brassicas such as broccoli, cabbage and cauliflower) (Chalhoub et al., 2014). OSR is a highly important crop being the second biggest contributor to vegetable oil globally (European Commission, 2018).

In the United Kingdom (UK), OSR was the 5th most produced crop in 2022 (in terms of hectares, DEFRA). The area of oilseed rape in the UK has been increasing since the crops commercialisation in the 1970s, but has declined rapidly, going from 741,920 ha in 2012 to 365,721 ha in 2022 (DEFRA). This is largely due to changes in policy leading to stricter regulations on pesticide usage, including the withdrawal of neonicotinoids and increasing threats from invertebrate pests (Scott and Bilsborrow, 2019).

1.1. Invertebrate herbivores of Brassicas

OSR faces many invertebrate pests. For Europe, the most significant pests are the brassica pod midge (*Dasineura brassicae*), rape stem weevil (*Ceutorhynchus napi*), cabbage stem weevil (*Ceutorhynchus picitarsis*), pollen beetle (*Brassicogethes aeneus*) and the cabbage stem flea beetle (*Psylliodes chrysocephala*) (Zheng et al., 2020).

The brassica pod midge (BPM) feeds predominantly on the pods of *B. napus*. Larvae feed inside the pod and can cause significant yield losses through splitting of pods resulting in loss of seed (Meakin and Roberts, 1991). The BPM is an understudied pest of oilseed rape leaving large gaps in the understanding of its biology, thus there are limited effective control measures in place to tackle it (Hausmann, 2021). The rape stem weevil (RSW) is also a major pest for oilseed rape. Larvae feed within the main stems of plants and can cause significant distortions, leading to stunted growth and lodging of the crop (Juran et al., 2011). The cabbage stem weevil (CSW) causes similar issues as with RSW (Juran et al., 2011), but with larvae starting by feeding in the petioles before moving into the main stems of the crop (Alford, 2003).The pollen beetle has received more research attention continues to become an increasing problem for oilseed rape agriculture as pesticide resistance develops (Zimmer et al., 2014). Adult beetles feed on young, unopened buds which causes the plant to drop them, leaving podless stems (Williams and Free, 1978). Furthermore, if plants make it through this stage of herbivory, beetles will continue to feed on open flowers (Williams and Free, 1978), which can result in severe yield losses. The invertebrate pest species which has been highlighted as the most problematic and threatening to oilseed rape production across Northern Europe is the cabbage stem flea beetle (CSFB) (Zheng et al., 2020).

1.2. The cabbage stem flea beetle (CSFB)

The cabbage stem flea beetle (CSFB), a member of the Chrysomelidae, is a small black, iridescent beetle about 4mm long with enlarged femurs, allowing them to jump (Figure 1.1). CSFB feed on many Brassicaceae species native throughout Northern Europe but are most problematic for winter oilseed rape (WOSR) (Zheng et al., 2020). They have an annual life cycle (Figure 1.2) which aligns to the WOSR cropping cycle, although multiple generations per year are possible in captive populations (personal observations). This indicates that their life cycle is phenotypically plastic to environmental conditions.

Figure 1.1. Photographs of the adult cabbage stem flea beetle, with the right image demonstrating the enlarged femurs on the back pair of legs used for jumping.

Adult CSFBs migrate during the autumn (in temperatures 16°C and above) into newly sown oilseed rape fields (Figure 1.2A) and can travel 3-4km during this migration (Ebbe-Nyman, 1952, as cited by (Williams, 2010)). What initiates the start of this migration still is not fully understood, but Tixeront et al., (2023) determined increasing temperatures and decreasing air humidity correlate with the numbers of beetles caught during migration events. It is also unknown how CSFB detect suitable host crop fields during migration. However, Bartlet et al., (1999) identified sensilla on CSFB antennae that likely have an olfactory role, indicating the possibility of detecting volatiles of food sources in the air.

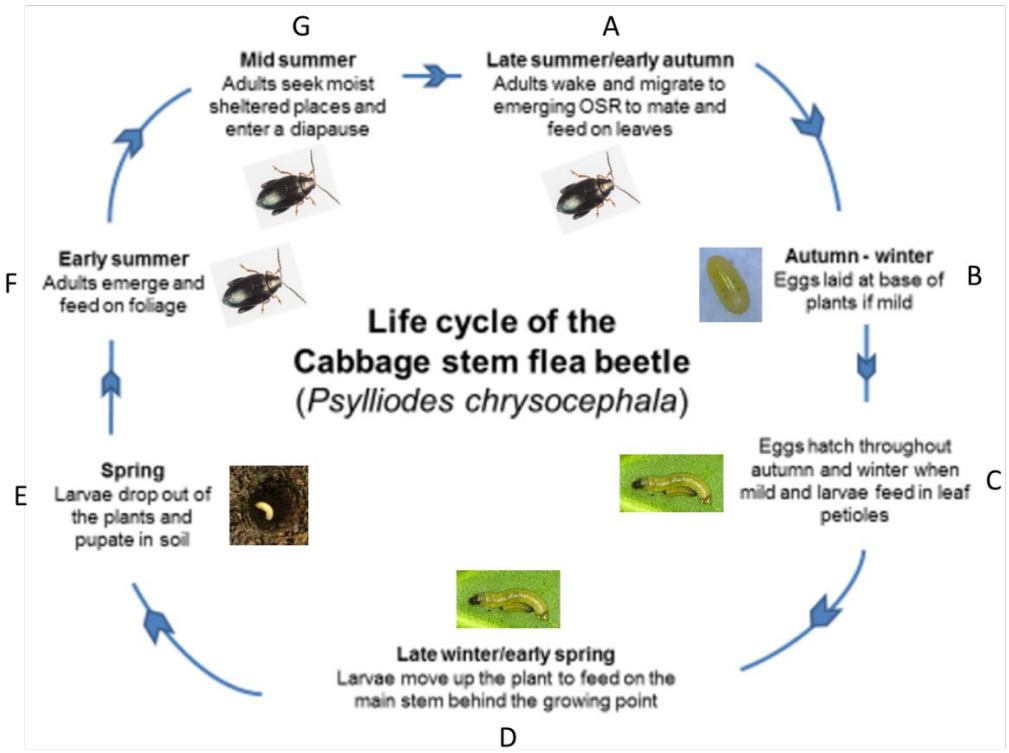

Figure 1.2. The annual life cycle of CSFB in the UK (adapted from Nicholls, 2016) with letters denoting the key life stages throughout the year.

Upon arrival in the crop beetles feed on seedling cotyledons, creating a distinctive ‘’shot holing’’ feeding pattern (Figure 1.3). At this stage the young WOSR crop is particularly vulnerable to high levels of CSFB herbivory and can be completely decimated if the shoot apical meristem (growing point) is consumed. Furthermore, if coupled with other non-favourable factors, such as warm and dry weather conditions preventing rapid germination and growth, the crop can be lost before establishment. During this period of feeding, flight muscles are degraded, female ovaries mature, and mating occurs (Ebbe-Nyman, 1952, as cited by (Williams, 2010)).

Figure 1.3. Examples of CSFB shot holing damage on cotyledons (left) and true leaves (right).

During autumn, female CSFB lay eggs on the soil at the base of plants (Figure 1.2B). If conditions remain mild enough oviposition can continue into the winter months (Sáringer, 1984 as cited by Højland et al., 2015; Mathiasen et al., 2015b). Eggs are oval and cream coloured, about 0.5mm long (Figure 1.4a). Time taken to hatching is temperature dependent, ranging from about 37 days at 10°C to 70 days or more at less than 6°C (Alford, 1979; Mathiasen et al., 2015b).

Upon hatching throughout autumn and winter, larvae (Figure 1.4b) burrow into petioles and stems of nearby host plants and feed throughout the winter into spring (Figure 1.2C and D) (Alford, 1979). Larvae are susceptible to cold conditions, especially if exposed to temperatures under -5°C for a number of continuous days in a row (Mathiasen et al., 2015a; Emery et al., 2023). However, the UK rarely experiences prolonged periods of cold under -5°C, particularly in the South-East where CSFB are most prominent. Damage throughout this winter larval period is equally as detrimental as adult herbivory and can lead to complete plant collapse (Figure 1.5).

Figure 1.4a: CSFB egg, 1.4b: first instar and third instar CSFB larvae.

Figure 1.5. Examples of stem damage where the plant has completely collapsed (left) and the inside of a stem that has been mostly consumed (right).

Larvae go through three instars (Figure 1.4b for an example of a first and third instar larvae) before tunnelling out of the plant and burying themselves in the first few centimetres of soil for pupation (Figure 1.2E and Figure 1.6) (Alford, 2003). Depending on environmental conditions in the field,

pupation takes about eight to 12 weeks (Sáringer, 1984, as cited by Højland et al., 2015). Adult CSFBs begin to emerge in May where they remain in the crop and feed on the leaves (Figure 1.2F) (Williams & Carden, 1961, as cited by Ortega-Ramos et al., 2022b). In mid-late summer, during the warmest months, beetles go through a period of aestivation, sheltering under lumps of soil, rocks or in plant matter on the ground (Figure 1.2G) (Sivčev et al., 2016). When conditions begin to cool in autumn beetles become active again, migrating to newly sown crops and starting the life cycle once more.

Figure 1.6. CSFB pupae in soil.

Examining the CSFB life cycle, it is evident that the pest presents a two-level problem for WOSR cropping. Adults attack young, vulnerable seedlings, and if plants manage to growth through this stage, they are then infested with larvae feeding within the petioles and stems. This two-fold damage from CSFB makes this pest particularly important to control but since changes in policy surrounding pesticide usage there has been little viable protection for the agricultural community.

1.3. Chemical control of CSFB

Until 2013, the primary control for CSFB on oilseed rape was the use of seed treatments, including systemic neonicotinoid pesticides, that work by attacking the insect nerve system, resulting in paralysis (Simon-Delso et al., 2015). However, an EU-wide moratorium in December 2013 was imposed for neonicotinoid use on flowering crops. In 2014, some areas of the UK (including Suffolk, Bedfordshire and Cambridgeshire) were so badly affected by CSFB that the ban was temporarily suspended in 2015. However, despite the struggles for the oilseed rape growing community, the ban on neonicotinoids was made permanent and extended to other non-flowering crops, such as sugar beet, across the EU (European Commission, 2018).

The withdrawal of neonicotinoids was supported by evidence of negative effects on wild bee populations, specifically reducing the growth and reproduction of colonies (Rundlöf et al., 2015). Neonicotinoid pesticides influence also extends to birds, with research demonstrating that birds

consuming neonicotinoids either directly through treated seed or indirectly via insects show reduced migration ability and significant weight loss (Eng et al., 2017; Hallmann et al., 2014). These pesticides are even more problematic due to high levels of persistence in soils and leaching into water systems, thus spreading to other environments (Goulson, 2013).

As an alternative remaining chemical control growers are advised to use pyrethroid insecticides in the form of a foliar spray. Pyrethroids have been a commonly used insecticide since their introduction in the 1970s and are found in household insecticides. They are non-systemic and short-lived, requiring contact with the pest to be effective. Pyrethroids act on the insect nerve system by binding to and disrupting voltage-gated sodium channels but other targets, including voltage-gated calcium and chloride channels, have been implicated as secondary sites of action for a subset of pyrethroids (Soderland, 2012). Multiple amino acid substitutions at a pyrethroid binding site have been associated with insecticide resistance in house flies (Soderland, 2012). This target-site mutation, known as knockdown-resistance (kdr or super-kdr) has been found in CSFB populations in Germany (Zimmer et al., 2014) and is correlated with resistance levels to pyrethroids (Højland et al., 2015). Højland et al. (2015) further confirm presence of kdr in UK CSFB populations. Additional metabolic resistance, based on a detoxification mechanism via overexpression of a cytochrome P450, has also been observed. Willis et al. (2020) also confirmed kdr in the UK, demonstrating pyrethroid resistance levels remain high in the South-East but have additionally spread further North and West. Højland and Kristensen (2018) investigated pyrethroid resistance in 15 field populations of CSFB in Denmark, demonstrating that populations in southern Denmark have also been shown to carry kdr, and some of these CSFB populations showed reduced susceptibility to pyrethroids. Although there was a correlation between pyrethroid susceptibility and kdr in CSFB in Denmark, it was not statistically significant. Højland and Kristensen (2018) therefore suggest that a metabolic resistance, such as the one discovered in UK populations, may also be playing a role.

Stara and Kocourek (2019) support the notion of an unidentified metabolic resistance also being present among some CSFB populations. Using glass vial experiments to expose beetles to pesticides, they reported high susceptibility of CSFB populations from two localities of Czech Republic to six different pyrethroids, indicating they do not possess kdr. However, in the same experiment, these two CSFB populations do appear to be resistant to the neonicotinoid thiacloprid. Therefore, it is important to consider the local population of CSFB before countries implement the use of difference pesticides to prevent the evolution of resistance.

Overall, a combination of neonicotinoid withdrawal and spreading pyrethroid resistance has left oilseed rape growers with no viable control for CSFB (Zhang et al., 2017). Scott and Bilsborrow (2019) address the impacts of neonicotinoid withdrawal in England on oilseed rape production with survey data on WOSR production area, damage from CSFB and management techniques used to

control pest damage. Notably, they report large county and yearly variation in crop losses in the 2014/2015 and 2015/2016 growing seasons, making implemented pest management approaches challenging. Additionally, Scott and Bilsborrow (2019) report significant costs of controlling CSFB in the 2014/2015 and 2015/2016 growing seasons, £25.2 million and £23.3 million, respectively (cost of chemicals and applications, crop loss and re-drilling of lost areas). (Dewar, 2017) also exemplify changes in UK oilseed rape cropping, reporting a sharp drop in production levels between 2015 to 2016. The drastic decline in UK OSR cultivation is evident when examining data from DEFRA, which demonstrates a drop from 741,920 ha in 2012 to 365,721 ha in 2022. This has led to a deficit in UK oilseed rape production, meaning imports are now necessary and often from countries which still have access to neonicotinoid pesticides (Ortega-Ramos et al., 2022a).

Therefore, it is clear that the cost of controlling CSFB along with the perceived (and real) lack of control for CSFB and substantial temporal and spatial variation in CSFB incidence is severely impacting the viability of growing OSR in the UK. Alternative controls are desperately needed as reliance on environmentally damaging chemicals are no longer a sustainable option. There is encouragement to take up Implemented Pest Management (IPM) strategies to combat CSFB, and the research community is focusing on discovering effective control methods.

1.4. Integrated pest management (IPM) strategies for CSFB control

Integrated pest management (IPM) is a sustainable, science based, decision making process that combines biological, cultural, physical and chemical tools to identify, manage and reduce risks from pests. The focus is on managing pest levels rather than eradication, keeping crop damage below an economic impact (Dara, 2019). Often represented as a triangle, at its base IPM relies on cultural controls, such as the enhancement of natural enemies or choice of resistant or tolerant cultivars; the use of forecasting systems and threshold values for decision support and finally responsive crop control, first using biological or biophysical control. Chemical control is utilised as a last resort. The research community is looking to identify alternative control methods to support IPM approaches to OSR cultivation.

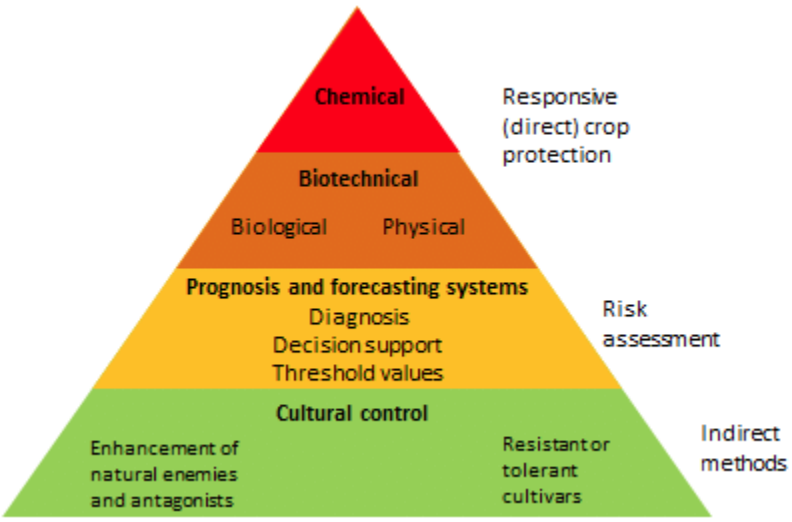

Figure 1.7. The IPM 'triangle' showing progression from Indirect to responsive crop protection (Slunge et al., 2015).

1.4.1. Cropping strategies

Companion cropping, or intercropping, where OSR is grown along with another crop species that acts as a ''trap'' with CSFB preferring to feed on the companion over the focal crop. Recent work by Seimandi-Corda et al. (2023) demonstrated the effectiveness of growing cereals (wheat and oat) alongside OSR in four field trials in the UK and Germany. They observed a significant reduction in adult CSFB to oilseed rape when grown with these companion crops. Additionally, they observed this effect with legumes and mustard, but this was only found in one field trial. There were no consistent effects of companion cropping on larval load.

Other research has had success in identifying an effect of companion cropping on larval loads. Barari et al. (2005a) reported that a UK OSR field trail sown with a trap of crop turnip rape had significantly fewer CSFB larvae than OSR alone. Furthermore, they demonstrated that the turnip rape had significantly higher loads of CSFB larvae compared with OSR when cropped together. More recent work also demonstrates the benefit of companion cropping with faba bean and grass pea for the reduction of larval CSFB (Breitenmoser et al., 2022). Results are promising for companion cropping as part of an IPM strategy, but more research is needed on optimal species and how to best implement companion cropping into agricultural practices.

Other IPM research has focused on a variety of other farming practices, such as adding straw mulch cover to the crop. This has been demonstrated to result in significantly less adult CSFB feeding damage in oilseed rape crops but again, does not appear to impact larval load (Seimandi-

Corda et al., 2023). Using reduced or minimal tillage has also been demonstrated to reduce adult CSFB herbivory, particularly if the previous crops stubble is left, in addition to reducing larval infestation (Ulber & Schierbaum-Schickler, 2003, as cited by Ortega-Ramos et al., 2022b). Grazing or mowing off has also been demonstrated to effectively reduce larval load, but timing is crucial as if performed after crop extension there is a negative impact on final yield of the crop (White et al., 2020).

Date of drilling has also been addressed, with earlier sowing dates (from late July to early- mid August) being particularly beneficial for OSR crop establishment before adult CSFB migration (Alves et al., 2015; Personal communications, Sacha White and Steve Ellis). However, earlier sowing dates have also been associated with higher larval loads late in the oilseed rape growing season, potentially due to adults having a longer window for egg oviposition (Conrad et al., 2021). Generally, one of the most important factors for sowing is the presence of adequate amounts of soil moisture, to aid establishment and growth to withstand CSFB herbivory (Alves et al., 2015; Personal observations).

Location of cropping has also been subject to investigation, and it is generally regarded in the growing community that crops should not be sown nearby to an area that was previously used to grow OSR, due to there being a potential reservoir of CSFB. Recent research has also indicated that growing next to woodlands can lead to an increase in adult CSFB migration into crops, as numbers of aestivating beetles was significantly higher in these borders compared with others, such as flower strips (Pigot et al., 2023).

Therefore, whilst there is encouraging evidence for manipulation of farming practices to control levels of adult and larval CSFB infestations, further research is required to better understand the mechanisms behind these benefits. Furthermore, more research is needed to refine the timing and combinations of such practises to provide the greatest levels of protection.

1.4.2. Biological controls

Another area of IPM research has explored the impact of use of biological controls on CSFB. Entomopathogenic fungi (EPF) have received limited research attention for combating CSFB in OSR. However, some early research from Butt et al. (1992, as cited by Hoarau et al., 2022) found one strain of *Metarhizium anisopliae* delivered 100% mortality to adult CSFB in laboratory assays after 14 days of exposure. Unfortunately, subsequent tests revealed that after repeated exposure to *M. anisopliae* effectiveness dropped significantly and thus would not be suitable to develop as a biocontrol. Two further M. *anisopliae* strains delivered 88% and 73% mortality to CSFB, however, these have not been tested in field conditions (Butt et al., 1994, as cited by Hoarau et al., 2022). Currently, there are no publications on the effectiveness of EPF in field conditions for CSFB, thus

more work is required to identify suitable strains that maintain effectiveness temporally and in agricultural settings.

There has been slightly more research focused on entomopathogenic nematodes (EPN) with some recent promising discoveries. Price et al., 2023 identified four species of EPN which caused a 70% or greater mortality in adult CSFB under laboratory conditions, the most successful being *Steinernema carpocapsae* providing 80% mortality after six days. Further tests on the addition of adjuvants, designed to protect EPN in field environments, determined how they affected their survival. Some adjuvants appeared to compromise survival of EPNs, thus further research is required to select optimal combinations to protect EPNs and test their efficacy under field conditions.

Other recent research has highlighted the effectiveness of using EPNs for CSFB larval control. Godina et al., (2023) showed spraying plants with three different strains of EPN resulted in significantly lower levels of live CSFB larvae compared with non-sprayed control OSR plants. Treatment of plants with *Steinernema feltiae* lead to 82% mortality of CSFB larvae and reaffirm the ability of *S. carpocapsae* to infect adult CSFB. Testing the effectiveness of EPN in four fields showed that the cold activated EPN strain, *Heterorhabditis bacteriophora* resulted in the highest reductions of CSFB larvae at 45%. However, results from the field were highly variable and thus again highlights the need for further research on the efficacy of EPNs in agricultural systems.

Natural predators and parasitoids of CSFB have also received some, but limited, research attention as a mode of IPM. Having the ability to jump, adult CSFB face few threats from predators and parasitoids. However, Jordan et al., 2020 demonstrated the parasitoid wasp, *Microctonus brassicae*, infests UK CSFB populations. The parasitoid slowly stalks an active adult CSFB before ovipositing an egg into the body through a gap in the beetle's elytra. In captivity, an average parasitism rate of 44% was observed. M*icroctonus brassicae* could be reared for inundative (released) biocontrol however, this is time consuming and costly. Optimisation of this process and improved understanding of field efficacy and natural populations of this parasitoid, combined with conservation and support is required to fully exploit such natural enemies.

Other research has focused more on CSFB larval parasitoids as these are more common. Barari et al. (2005b) identified *Tersilochus microgaster* for the first time in the UK as an endoparasite of CSFB larvae in the field, but only found low levels of parasitism. Ulber et al. (2010) later discovered 11% levels of parasitism for *T. microgaster* in a UK field trial, which was an encouraging sign that this parasitoid may be spreading naturally. However, research also indicates that certain farming practices, such as tillage, are harmful to beneficials (Nilsson, 2010). Furthermore, there are consistent negative effects of pesticide use on these natural populations of beneficials (Geiger et

al., 2010). Farming practices will need to be carefully balanced to be able to fully monopolise on the benefits of parasitoids and predators as modes of biocontrol.

Finally, biocontrol in the form of RNA interference (RNAi) are now being considered. Recent work has demonstrated feeding adult CSFB oilseed rape leaf discs coated with double stranded RNA targeting the gene *sec23* (a gene involved in endoplasmic reticulum-golgi transport), resulted in 76% mortality in pre-aestivation beetles (Cedden et al., 2023). This dropped to 56% when fed to post-aestivated beetles. Therefore, whilst this is promising research it highlights the importance of timing for potential RNAi foliar sprays.

Overall, as demonstrated above, a combination of these agricultural and biocontrol practices may work as part of an IPM system but require more research to make them practically viable for growers to implement. Additionally, these practices may not be enough on their own to control CSFB in a post-neonicotinoid system.

1.5. Phenotyping for resistance/tolerance traits in brassicas

Phenotyping of resistance or tolerance traits in plants is the first step in breeding or developing more robust OSR varieties. Variation in plant traits have been indicated to be involved in brassica defence against herbivory.

1.5.1. Physical resistance traits

Leaf epicuticular waxes provide protection, either by providing physical or chemical barriers to feeding. Bodnaryk (1992a,b) investigated feeding behaviour of *Phyllotreta cruciferae*, the crucifer flea beetle (a major pest which feeds on canola and other brassicas), on high leaf wax species (>1000mg kg-1), including *Brassica oleracea* and *Brassica napus*, and low leaf wax species (<240mg kg-1), including *Brassica rapa*, *Brassica juncea* and *Sinapis alba*. They demonstrate varieties with waxy leaves receive significantly lower levels of flea beetle feeding compared with low leaf wax species. Notably, the most fed upon species was *S. alba* but this demonstrated high tolerance to flea beetle feeding. Furthermore, all species with high leaf wax had edge feeding only, and all those with low leaf wax showed random feeding patterns across the leaf. This aligns with personal observations where mature *B. napus* leaves receive much less damage from CSFB adults, with damage confined to the edges, compared with less waxy leaves such as *B. rapa*. *Phyllotreta cruciferae* feeding was also recorded on *B. napus* mutants, with reduced leaf wax levels. Feeding on these mutants was 1.6-2.2 times higher and occurred in a random pattern compared to wildtype where >96% of damage was found on the edge of leaf (Bodnaryk, 1992a). Finally, manually removing wax from leaves of *B. oleracea* and *B. napus* by gentle rubbing with cotton buds also increased *P. cruciferae* herbivory, with initially beetles only feeding at the edges of leaves until wax was removed.

Research by (Lambdon et al., 1998) addressed the influence of leaf waxes on CSFB herbivory in field trials, demonstrating a correlation between increased leaf waxes and reduced adult beetle feeding. Little research has related plant waxes to larval feeding or colonisation, but Nielsen (1977) reports that larvae of *Phyllotreta nemorum*, the turnip flea beetle, struggle to enter leaf petioles with high levels of wax (as cited by Lambdon et al., 1998).

Therefore, leaf epicuticular wax has been identified as a resistance trait against adult flea beetle herbivory and potentially larvae but there has been little recent research into this trait. Leaf waxiness traits have been attributed to the CC genome originating from *B. oleracea* (CC), which is also present in *B. napus* (AACC) (Bodnaryk, 1992a). It is possible that leaf waxes may be a suitable target for creation of transgenic material or conventional breeding programmes. Based on leaf epicuticular wax research winter OSR has high leaf wax so should be relatively more resistant to flea beetle feeding compared with other Brassicaceae. However, reports from growers and research since the neonicotinoid ban in 2013 demonstrate that flea beetle damage has drastically increased. Therefore, it's unlikely leaf waxiness traits alone are enough to protect from flea beetle herbivory.

Trichomes help prevent insect herbivory by acting as a physical barrier between the insect and plant (Gavoski et al., 2000). Soroka et al. (2011) use a transgenic line, named ''Hairy1'', to indicate trichomes act as anti-herbivory traits to the flea beetle *P. cruciferae*. They created Hairy1 by inserting genes from *Arabidopsis thaliana* into Westar, a spring *B. napus* variety. In the laboratory adult beetles fed significantly more on cotyledons and 2nd true leaves of Westar compared to Hairy1. Field results support this result, with Hairy1 receiving equal or less feeding damage compared to its parental lines and plants grown from insecticide treated seeds, indicating trichomes may be as effective at deterring adult *P. cruciferae* feeding as pesticides.

Other research reports *Brassica villosa*, a species endemic to Sicily with high trichome density, being resistant to damage from *Phyllotreta striolata*, the striped flea beetle (Palaniswamy and Bodnaryk, 1994, as cited by Gavloski et al., 2000). However, trichomes are not present on seedling *B. villosa* and thus failed to protect them from *P. striolata* adult feeding at their most vulnerable developmental stage (Gavoski et al., 2000). Trichomes on pods of *S. alba* have been shown to receive insignificant amounts of damage from *P. cruciferae* whilst growing next to plants which have fewer hairs and show pod damage (Lamb, 1980). Furthermore, removal of pod hairs significantly increases feeding damage from flea beetles. However, another further study on *Barbarea vulgaris* resistance to *Phyllotreta nemorum* did not find trichome presence to be associated with flea beetle resistance (Kuzina et al., 2011).

Overall, research into trichomes indicates that they can reduce feeding in some species of flea beetle and that it is possible to create transgenic lines with higher levels of trichomes. However,

there is no published research addressing trichomes as resistance trait to the CSFB or flea beetle larvae.

Seed size has also been indicated to influence incidence of flea beetle herbivory. Plants grown from small seed for both *S. alba* and *B. napus* suffered a higher proportion of mortality compared to those grown from large seed. In a study examining *P. cruciferae* herbivory, 45% of *S. alba* and 100% *B. napus* seedlings were killed when they when grown from small seeds, compared to only 9% and 28% killed respectively when grown from large seeds (Bodnaryk and Lamb, 1991). As adult CSFBs attack plants as they emerge from the soil, quick establishment is essential to outgrow damage from pests. Larger seeds have more resources, allowing plants to grow away more quickly and tolerate more damage. Selectively breeding plants for large seed is desired by growers and it may help protect crops from flea beetle herbivory when they are most vulnerable.

1.5.2. Chemical resistance traits

Glucosinolates are secondary metabolites that typically act as plant defence compounds to deter herbivores from feeding on plant material and are characteristic to Brassicaceae. Upon plant tissue damage they are hydrolzed by the enzyme myrosinase into bioactive compounds including isothiocyanates, thiocyanates, nitriles, goitrin and epithionitrile (Ishida et al., 2014). Isothiocyanates have been shown to be toxic to both generalist and specialist insect herbivores (Wittstock, 2003). Despite this, in laboratory experiments, Bartlet and Williams (1991) indicate that glucosinolates act as a feeding stimulant rather than deterrent for adult CSFBs, showing that they only feed on plants with glucosinolates. Furthermore, adding glucosinolates to agar stimulated flea beetle feeding and increasing glucosinolate content increased the amount of feeding (Bartlet et al., 1994). Giamoustaris and Mithen (1995) support this with field experiments of 28 lines of *B. napus* with altered glucosinolate levels. Lines with increased glucosinolate content received more damage from adult CSFB. However, research indicates that total glucosinolate level alone does not control flea beetle herbivory.

Glucosinolates can be divided into three groups; aliphatic, indole, and aromatic based on the structure of their amino acid precursors. Considerable phenotypic variation for glucosinolate level and types exist with *B. napus* (Liu et al., 2020, Kittipol et al., 2019). Both indolic and aliphatic glucosinolates frequently appear in the literature in relation to flea beetle feeding. Glucobrassicin, an indolic glucosinolate, positively influenced CSFB feeding on *B. napus* (Bartlet et al., 1994) but negatively influenced *Phyllotreta* spp. feeding on *S. alba* (Bohinc et al., 2013). Many of the glucosinolates identified to increase flea beetle feeding by Bohinc et al. (2013) are aliphatic glucosinolates. Giamoustaris and Mithen (1995) identified *B. napus* lines with reduced levels of butenyl glucosinolates were less susceptible to CSFB feeding. This is supported by reduced herbivory from *Pyllotreta* spp. recorded by Soroka & Grenkow (2013) of canola quality *S. alba*,

which has reduced levels of butenyl glucosinolates compared to standard *S. alba*. Additionally, Bohinc et al. (2013) identify 3-butenyl glucosinolates to stimulate feeding of *Pyllotreta* spp. on *B. napus*. However, other research focusing on the CSFB did not show any significant effect of aliphatic glucosinolates on feeding damage (Bartlet et al., 1996). Therefore, further research is required to better understand how glucosinolate profiles influence flea beetle herbivory.

More recent research has focused on improving understanding as to why and how flea beetles feed on plants with glucosinolates. Glucosinolates themselves are not toxic, however upon herbivory plants hydrolyse these using the myrosinase enzyme to create toxic isothiocyanates (Brown and Hampton, 2011). However, these defence compounds do not act as a deterrent or protectant for specialist insects that feed solely on the *Brassicaceae*. *Phyllotreta striolata* has been demonstrated to selectively accumulate glucosinolates and hydrolyses them to isothiocyanates with their own myrosinase system (Beran et al., 2014). Furthermore, they demonstrate that the major substrates for the insect myrosinase enzyme are aliphatic glucosinolates, rather than indolic or aromatic glucosinolates. Taking this, with previous research, it appears that flea beetles may be attracted to brassica varieties which have higher levels of aliphatic glucosinolates as they can utilise them for themselves. This may explain why flea beetles seem to have higher feeding rates on brassicas with higher overall glucosinolate content, even though this is considered a plant defence compound against herbivory.

Unlike *P. striolata*, adult CSFB do not demonstrate myrosinase activity and only around 26% of glucosinolates are sequestered or desulfidised, indicating that isothiocyantes are taken up from the plant when feeding (Beran et al., 2018). Instead, Beran et al. (2018) demonstrated that adult CSFB detoxify isothiocyanates by conjugating them to glutathione, which has previously been recorded in other invertebrates (Jeschke et al., 2016). However, what happens to 40% of ingested glucosinolates still remains unknown, indicating that conjugation of isothiocyanates to glutathione may not be the only method of detoxification. Beran et al. (2018) further demonstrate that CSFB adults, pupae and larvae all have similar glucosinolate profiles, but eggs have profiles more similar to that of the host plant. This suggests that adults are transferring glucosinolates to eggs, indicating that CSFB sequestration of glucosinolates has an ecological purpose. Finally, as *Phyllotreta* and *Psylliodes* have different methods to overcome glucosinolate defences, it is likely that they evolved separately and therefore should be studied at a species-specific level.

Ahn et al. (2019) demonstrated glucosinolate sulfatases (GSS) activity in adult CSFB occurs mainly in the gut membrane and has strongest activity towards sinalbin, a relatively uncommon benzenic glucosinolate which is found in *S. alba* (Agerbirk et al., 2008). Such strong activity towards sinalbin is surprising given that CSFB have a wide source of Brassica food plants and that *S. alba* does not appear to be a preferred food source (personal field observations). GSS activity converts sinalbin into desuflo-sinalbin, allowing 80% to be safely excreted, indicating that this

specific glucosinolate is not sequestered by CSFB (Ahn et al., 2019), unlike others previously reported by Beran et al. (2018). It is therefore important to consider that different food plants may require different detoxification mechanisms.

Other recent research has attempted to address performance of CSFB larvae on different plants and the role of glucosinolate profile of plants (Doering and Ulber, 2020). Larval weight and number recovered from plants did not differ between the four, oilseed rape (OSR) varieties tested. Significantly fewer larvae were recovered from *S. alba* and larvae in *S. alba* appeared to have slower development compared with OSR varieties. Interestingly, Doering and Ulber (2020) also report no correlation between larval weight and glucosinolate content of plants but do see a positive correlation between larval weight and certain glucosinolates, specifically progoitrin (aliphatic) and 4-hydroxyglucobrassicin (indolic). This collection of research considering glucosinolates highlights the need to consider individual species, life stages within species and target food source to advance our understanding in pest mechanisms for overcoming plant defences.

1.6. Utilising resistance traits via plant breeding

Gavloski et al. (2000) also conducted laboratory experiments to identify 11 cultivars of *S. alba* seedlings as consistently resistant to *P. cruciferae* feeding, in contrast to *B. napus*, *B. rap*a and *B. juncea* cultivars which showed no consistent resistance. *Sinapis pubescens* was also tested and shown to be susceptible to flea beetle feeding, indicating that resistance/tolerance is very species specific. Having identified *S. alba* as showing resistant properties they created 308 hybrids of *S. alba* crossed with *B. napus*. Out of these, 34 show some resistance to flea beetle damage; they were damaged significantly less than the control in at least one out of four replicates. However, only one hybrid showed consistent resistance across all four replicates. Despite only one resistant hybrid being identified, it demonstrates that resistant traits can be bred into *B. napus* from relatives, such as *S. alba*, via conventional breeding methods. This indicates that traits such as increased trichomes, leaf wax and seed size could also be bred into *B. napus* to create plants with multiple lines of defence. Gavloski et al. (2000) demonstrate that the amount of *S. alba* DNA in the hybrids does not correlate with resistance to flea beetle feeding. The genes or mechanisms by which this resistance is conferred has not been identified.

1.7. Identifying the genetic control of herbivory in the Brassicacae

Identifying phenotypic traits which confer resistance or tolerance, such as pubescence or glucosinolate profiles, is beneficial however, to support breeding and easily select for resistance, identifying the genes, genetic variation and mechanisms controlling resistance is key. Genetic mapping aims to identify the regions of the genome in which these genes causing the differences

in plant phenotypic responses are located. Through this, linked genetic markers can be identified for marker assisted selection which allow rapid selection of the desirable genotype at the seedling stage.

There are different methods used to discover variation of genes associated with specific traits. These include genome wide association studies, which utilise large panels of diverse lines, or quantitative trait loci (QTL) mapping in a cross between two parents which segregate for the trait of interest.

Little research has focused on identifying genomic regions or candidate genes for resistance to flea beetles in general and currently none focus on the CSFB. However, some research has attempted to uncover underlying genetic variation of *Barbarea vulgaris* resistance to *Phyllotreta nemorum*. Kuzina et al. (2011) created a F_2 segregating hybrid population from two parental lines of *B. vulgaris* – Glabrous type (G-type) which is hairless and resistant to *P. nemorum* larvae, and Pubescent type (P-type) which is hairy and susceptible to *P. nemorum* larvae. Following mapping of this population they identified two QTLs for flea beetle resistance and both resistance alleles were inherited from the G-type parent. Interestingly, these resistance QTLs colocalised with QTLs for saponins, a group of detergent like chemicals which are naturally found in plants and have a role in plant defence. Therefore, it's likely that these saponins play a role in G-type *B. vulgaris* resistance to flea beetle larvae. This research demonstrates that it's possible to identify areas of the genome associated with resistance and traits which may be linked to resistance. Kuzina et al. (2011) observed synteny between linkage groups conferring resistance to flea beetles in *B. vulgaris* and areas of the *Arabidopsis thaliana* genome, giving the potential for exact genes to be identified which underlie the resistance and become more comparable to other plant species.

1.8. Aims and objectives

In summary:

Following the loss of chemical controls IMP approaches are required to help manage insect pests. Genetic resistance represents one potential component of IPM and can be due to either physical or chemical traits. Several phenotypic characteristics have been associated with flea beetle feeding in the *Brassicacae* however the genetic basis of traits controlling herbivory and the related mechanisms have not been identified. There is no published research on underlying genetic basis of resistance to CSFB in Brassicas.

Overall, the aims of this project were to identify biological and genetic traits which confer resistance to cabbage stem flea beetle within *Brassica napus*, to aid development of more targeted pest management approach. More specifically, the objectives are as follows;

1. Phenotype diverse *B. napus* germplasm for differences in adult and CSFB palatability by;

    a. Developing a protocol for reliably recording levels of adult herbivory,

    b. Identifying feeding variation within germplasm,

    c. Confirming if herbivory differences are maintained in the field.

2. Identify genetic variation underlying tolerance or resistance to CSFB by;

    a. Mapping genetic variation linked to tolerance/resistance phenotypes identified in laboratory assays,

    b. Selecting candidate gene(s) for further investigation.

3. Improve understanding of CSFB feeding behaviour and life cycle.

2. Identification of *Brassica napus* genotypes with phenotypic variation in adult CSFB herbivory

2.1. Introduction

Although there has been research into phenotypic traits associated with adult CSFB herbivory, to date, there is limited exploration into the underlying genetics conferring these traits. Genome wide association studies (GWAS) utilise panels of diverse lines to identify statistical association of genetic variation with the trait of interest. Such studies have been applied successfully in crops such as maize, rice, barley, wheat and brassica to identify the control of traits including flowering time, yield components and glucosinolate content (Zheng et al., 2021; Greenwood et al., 2024; Tsai et al., 2020, Han et al., 2022; Miller et al., 2019; Kittipol et al., 2019).

GWAS requires both the genotyping and phenotyping of a large population of diverse individuals. Genomic technologies now allow rapid, cost-effective identification of genetic variation. Phenotyping technique should be high-throughput, reproducible, quantifiable, and non-invasive (Bazakos et al., 2017). However, within the field phenotyping insect resistance is subject to positional, regional, and annual variability in pest availability, linked to seasonal life cycle and subject to environmental effects. Laboratory screening is reliant on in-house rearing or

maintenance of collected field insects, the development of robust screening methodologies, but removes environmental effects and controls for pest pressure.

Here, we present results of quantitative data on adult CSFB feeding damage on *B. napus*. Using a novel feeding choice assay, we screened a genetically diverse panel of *B. napus* varieties for variation in CSFB herbivory. Preliminary findings of lines showing extremes in variation were confirmed in refined laboratory assays. Finally, we demonstrated differences in CSFB herbivory were retained in a field environment, supporting results from our laboratory assays.

2.2. Methods

2.2.1. Insect collection and husbandry

Beetles were collected from the field by sweep netting or gathering decaying leaf matter in the summer and autumn. Additionally, whole OSR plants were collected in winter and spring and potted up and contained in a bread bag to allow adults to emerge in the spring/summer. Alternatively, these plants were contained in plastic bags and allowed to decay, resulting in larval evacuation. These larvae could then be collected and applied to intact plants. Field collected beetles were kept separately from laboratory cultures until one generation had passed. These offspring were then incorporated into the laboratory population.

Within the laboratory population, adults were collected from whole plants contained within bread bags and placed in plastic boxes with ventilation holes lined with damp blue roll. They were provided with fresh Chinese cabbage leaves weekly. Here beetles would lay eggs down the sides of the blue roll, allowing them to be collected and applied to the base of intact, bagged oilseed rape or Chinese cabbage plants. Larvae fed within these plants and adults were collected upon emergence and moved to boxes for egg laying/collection.

All beetles used in experiments were laboratory reared for at least one generation and maintained in a controlled environment room in the John Innes Centre insectary (22°C:22°C and 16h day length). Additionally, as CSFBs have a four-to-six-week period of aestivation two weeks after eclosion (Alford, 2003 and personal observations), beetles were screened for feeding activity five to seven days prior to inclusion in a feeding assay. To screen, beetles were starved for 24 hours before introducing single beetles to a Chinese cabbage leaf disc on agar and allowing them to feed for 48 hours. If the beetle had consumed more than 5% of the leaf disc area it was deemed a ‘’feeder’’ and used in subsequent assays.

2.2.2. Plant material - *Brassica napus* Diversity Fixed Foundation Set (DFFS)

The *Brassica napus* Diversity Fixed Foundation Set (DFFS) consists of 101 lines originating from a genetically diverse set of material produced at the University of Warwick within the Oilseed RapE Genetic Improvement Network (OREGIN), (Teakle, G., University of Warwick, https://www.brassica.info/resource/plants/diversity_sets.php). The DFFS comprises a range *of B. napus* genotypes; winter oilseed rape (WOSR), spring oilseed rape (SOSR), Kale, Fodder, Exotic, Synthetic and Swede types, giving the set its rich genetic diversity (see Appendix 1 for details of specific varieties and crop types). From these 101 *B. napus* lines, 96 were chosen for our baseline six-way choice experiment for exploring adult CSFB feeding preferences.

To generate seedlings for use in CSFB feeding choice assays, seeds were germinated in petri dishes with damp blue roll before being pricked out into small pots and grown for a further six days (23°C:20°C, 16h day length). After these 6 days, at the expanded cotyledon stage, prior to emergence of true leaves, the seedlings were ready to use in CSFB feeding assays.

2.2.3 Six-way choice assays

To screen the 96 lines of the diversity set with replicates in an efficient manner multiple choice tests were performed.

2.2.3.1 Design and process of running six-way choice assays

A number of adaptations were made to the petri dish assay (initially designed by Anna Jordan, John Innes Centre insectary) before developing the final choice-chamber set up displayed in Figure 1.8. Assays ran for 48 hours, the same duration as other researchers have previously selected when running laboratory feeding experiments with flea beetles (Bartlet et al., 1996; Soroka et al., 2011). To aid with seedlings wilting throughout the duration of the assays, assays were covered with cling film to help retain moisture in the soil. Additionally, halfway through the assay (at 24 hours) plants were watered with 1.5ml water using a syringe and needle directly into the soil to prevent them from desiccating. Furthermore, the inclusion of agar in the base of the petri dish kept moisture levels higher in the chamber. The agar also prevented beetles from squeezing through gaps to escape as the lid could slot into the agar creating a tight seal.

To set up the six-way choice assays, plant pots containing seedling from the DFFS were slotted into groves in the base of the petri dish and standard water agar as demonstrated in Figure 1.8b. Choice chambers consisted of two plants of six different *B. napus* genotypes from the DFFS, with replicates opposite each other (Figure 1.8a), making 12 plants in total per assay. *Brassica napus* variety Matador appeared in every chamber as a control line and additionally replaced any missing lines. Each *B. napus* genotype appeared in three separate chambers according to an alpha design,

to account for interactions between accessions within chambers. Six beetles were introduced to the chamber after being starved for 24 hours, giving a 2:1 ratio of plants per beetle. Chambers ran for 48 hours in a CER at 22°C:22°C and 16h day length.

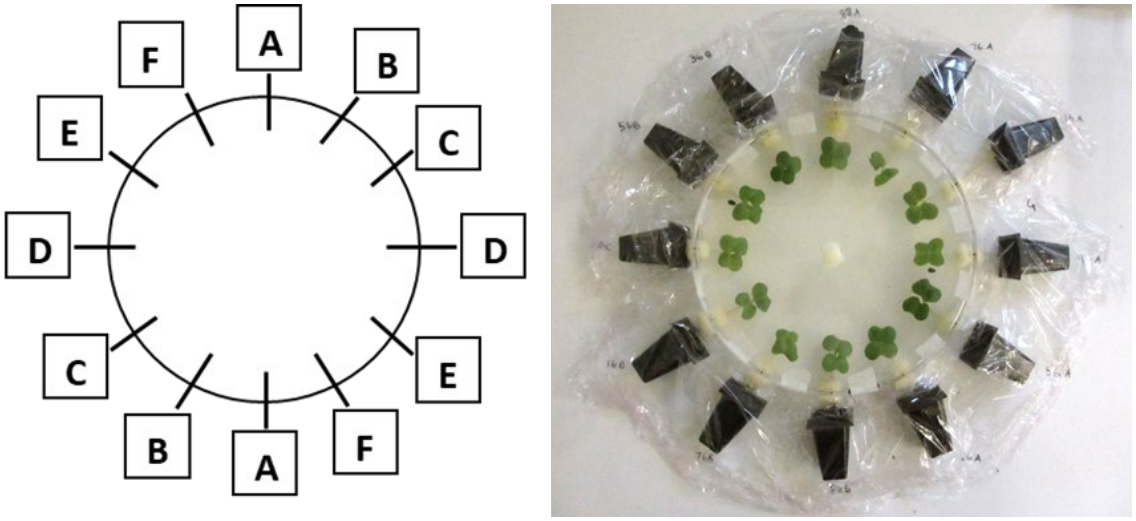

Figure 2.1 Six-way choice chambers for screening CSFB herbivory showing a) the layout with the letters representing two replicate seedlings of each gentoype opposite each other within an assay and b) the final choice chamber assay design with seedlings slotted into agar. The lid is secured with micropore tape and hole in centre, to add the beetles, is sealed with a foam bung. Clingfilm covers the plant pots to help keep soil damp.

After 48 hours of feeding, beetles were removed, and plants scored for CSFB feeding damage. Cotyledons were removed from seedlings and laid out on white paper to aid with scoring. Damage to cotyledons was visually estimated to the nearest 5%. If damage was present but less than 5% a score of 1% was given. This was to enable recording of the fact some damage had occurred, even if minimal, as this could provide important information about the beetles feeding behaviours.

2.2.3.2 Statistical analysis of six-way choice assays

The experiment ran over the course of three blocks, organised by an alpha design. All 96 *B. napus* genotypes from the DFFS were screened for CSFB herbivory, each appearing once per three blocks. The alpha matrix was designed to ensure that the same lines would not appear together in subsequent assays. In total 60 assays were run, giving three replicated per *B. napus* genotype, except for the control line Matador which appeared in all 60 assays. *Brassica napus* genotypes were randomly assigned to numbers, making the experiment blind.

Percentage data was LOGIT+ transformed for analysis using the formula in Equation 1. Data was analysed using Genstat software (VSN International, 2015).

$$ln\frac{x+1.25}{101.25-x}$$

Equation 1. Logit+ transformation equation

A two-way ANOVA was run, to assess whether total damage for control variety Matador varied significantly between blocks, assays and whether an interaction was observed. To examine differences in herbivory levels between genotypes, damage means were analysed with a linear mixed model fitted via the restricted maximum likelihood method (REML), with the fixed effect of Line (*B. napus* variety) as the main effect of interest and Block (as per the alpha design), Block.Date (accounting for variation between dates within a block) and Block.Date.Assay (accounting for variation between assays (individual choice-chambers) within dates within a block), as random effects. The model is summarised in Equation 2. The same model was used to assess differences in damage between *B. napus* crop types, where instead of ''Line'', ''Crop type'' is used. Due to restrictions of this REML variance components analysis, we do not generate F statistics or p values for random effects.

$$\mathrm{lmer}(\mathrm{formula} = \mathrm{Trait} \sim 1 + \mathrm{Line} + (1|\mathrm{Block} + \mathrm{Block.Date} + \mathrm{Block.Date.Assay})$$

Equation 2. Linear mixed model used to analyse six-way choice assays of DFFS *B.napus* varieties. Fixed effects = *B. napus* line or crop type. Random effects = Block (three blocks from an alpha design), Block.Date (for variation between dates within blocks) and Block.Date.Assay (for variation between assays within dates within blocks). Trait = total percentage damage.

Adjusted mean percentage scores were back-transformed using the EXPIT+ function (Equation 3) to visualise data.

$$x = \frac{(101.25e^{y} - 1.25)}{(1 + e^{y})}$$

Equation 3. EXPIT+ back-transformation equation

2.2.4 Two-way and non-choice assays of genotypes showing extreme variation in CSFB herbivory

After conducting the baseline experiment assessing adult CSFB feeding differences between 96 *B. napus* genotypes from the DFFS using six-way choice chamber assays, we selected two to investigate further. Altasweet (S) was selected for demonstrating higher levels while Apex-93_5 X Ginyou_3 (R) for demonstrating lower levels of CSFB feeding damage.

2.2.4.1 Experimental design of two-way and non-choice assays

Six-way choice assays may not allow beetles to distinguish differences between *B. napus* genotypes, maybe due to the effects of mixed volatile signals within the chambers. Therefore, we conducted further experiments using a similar assay set up but with either a two-way choice or no choice assay.

Two assay setups were piloted for two-way choice assays, either alternating seedlings of each variety or a chamber split into 50:50 of each variety, with one in the left half of the petri dish and the other in the right. From this we were able to discern that a half and half setup gave clear distinctions in CSFB herbivory and thus selected this method. Figure 2.2 demonstrates this setup. In no choice assays, CSFB were offered 12 seedlings of the same *B. napus* variety, either R or S. Plants and beetles were prepared in the same way as in six-way choice assays, other than plants being grown in the same CER (22°C:22°C and 16h daylength) where the assays were run. Assays were also conducted and scored in the same way as previously described.

2.2.4.2 Statistical analysis of two-way and non-choice assays

To investigate differences in herbivory levels between S and R, damage means were compared with a two-way ANOVA. Percentage data was LOGIT+ transformed for analysis. Two-way choice and no-choice assays were treated as separate experiments for analysis. Rstudio software was used for analysis (http://www.rstudio.com/).

2.2.5 Three-way and non-choice assays of genotypes showing extreme variation in CSFB herbivory and their F_1 offspring

It is not known whether the genetic basis of reduced CSFB feeding is recessive or dominant therefore assays for S and R genotypes were re-run alongside individuals from their F_1 cross cotyledon percentage damage using image analysis as developed by Thursfield (2022).

2.2.5.1 Development of F_1 plant material between S and R lines

A F_1 cross of S and R genotypes was generated by growing these parental *B. napus* genotypes to maturity in a glasshouse. They were then monitored for flowering and first blooms removed. Five to 10 unopened buds reaching maturity were emasculated before, using a fine paint brush, pollen was taken from the anthers of one parent and applied to the just exposed stigma of the other. Pollinated flowers were contained in a perforated bag and secured with a paper tie to ensure pollinating insects could not access the buds/flowers. These were left to mature and when ready cut from the plant and threshed to obtain the seed.

A subset of these seeds, plus S and R parental genotypes were grown in a glasshouse to obtain leaf material for DNA extraction using the Edwards DNA extraction protocol (Edwards et al., 1991). PCR and electrophoresis of BBSRC SSRC microsatellites (Lowe et al., 2003) segregating between the parents showed the F_1 crosses had been successful.

2.2.5.2 Sexing of beetle material for F_1 screens

Adult CSFBs were obtained from captive populations and screened for feeding activity as previously described. However, differences in herbivory amounts between male and female beetles (Thursfield, 2022) directed us to control the sex ratio of beetles entering assays. Beetles were sexed by examining their tarsal segments under a microscope. Females were differentiated from males by having a triangular shaped tarsal segment and males broader, heart shaped tarsal segments. Six beetles were used in a 1:1 ratio of males and females.

2.2.5.3 Experimental design of three-way and non-choice assays

Assays were conducted in the same petri dish setup as described previously in section 2.2.3.1. No-choice assays consisting of either one seedling of S, R or the F_1 cross of these parental genotypes. Three-way choice assays of these lines were arranged as demonstrated in Figure 2.9. Assays were arranged in a replicated, randomised design.

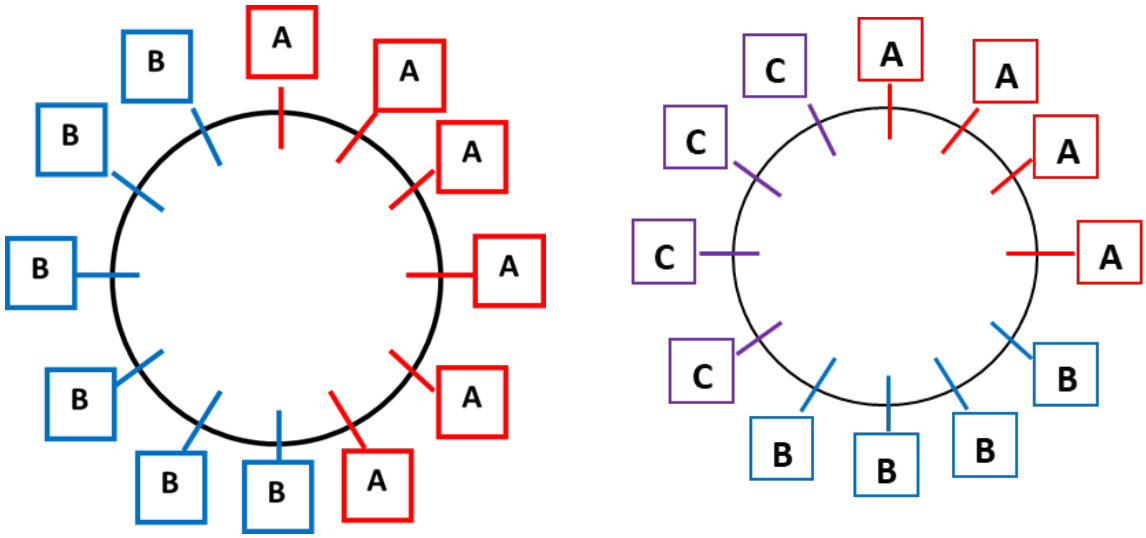

Figure 2.2. The layout of *B. napus* genotypes in a) two-way and b) three-way choice chambers, with the letters representing replicate seedlings of each genotype in the assay.

Total percentage damage to cotyledons was scored again by visual estimates to the nearest 5%. However, we additionally used ImageJ software to obtain more accurate, computerised scores for percentage damage to cotyledons. The process involved placing cotyledons removed from seedlings onto a PVC A4 sized plastic board and scanning them. These images were then thresholded and interpolated in ImageJ, before running a pipeline script (Lucy Thursfield, 2022) to extract percentage loss of cotyledon area (i.e. percentage damage scores).

2.2.5.4 Statistical analysis of three way and non-choice assays

To compare means for total percentage damage to cotyledons for S, R and the F_1 cross of these genotypes, data was analysed with a two-way ANOVA. As data was a percentage score it was LOGIT+ transformed. Three-way choice and no-choice assays were treated as separate experiments for analysis. Additionally, visually estimated scores and computerised scores were analysed separately. These were then correlated to see how visual estimates related to scores from image analysis via ImageJ. Analyses were conducted in Rstudio (http://www.rstudio.com/).

2.2.6 Confirmation of phenotypic variation under field trial conditions

Conducting controlled laboratory experiments enabled us to identify variation in palatability across different *B. napus* genotypes. However, we next wanted to better understand differences in damage in a more natural environment and thus conducted two field trials.

2.2.6.1 Plant material, field treatments & layout, 2019

In total *eight B. napus* genotypes were selected for inclusion in these field trials. Based upon data collected from laboratory adult CSFB feeding assays, we selected six extreme *B. napus* lines from the DFFS, our R and S genotypes plus one further line showing reduced feeding DFFS1, two showing higher feeding, DFFS2 and 3, and a line having an intermediate to high level of damage in laboratory, DFFS4. In addition to these *B. napus* lines from the DFFS, we included two commercial varieties, C1 and C2.

To increase our chances of successful data collection, in 2019 we ran field trials at two locations: one at The Morley Agricultural Foundation, in Morley St. Botolph (about three miles away from Wymondham, Norfolk) and one at the John Innes Centre field station, in Bawburgh (about two and a half miles away from the main John Innes Centre (JIC) site). Both were drilled in a replicated, randomised complete block design (Figure 2.10), with same seed densities used (roughly 864 seeds per plot). Both trials were ploughed the day prior to drilling and harrowed the morning of drilling. After drilling (drill depth one to two centimetres) the trials were rolled, and no insecticides used throughout the duration of the trials. Fertilisers and herbicides were used to aid the establishment of *B. napus* seedlings.

Drilling date varied between the two trials, the Morley trial was drilled on 23/08/2019 and Bawburgh on 29/08/2019. Unfortunately, the weather following drilling at Morley was very warm and dry, resulting in very poor establishment. Given the lack of establishment and damage from bird activity, the Morley trial was not suitable for any data collection and thus abandoned shortly after drilling. Establishment of the Bawburgh field trial was more successful, likely due to a better soil moisture content and slightly cooler temperatures. Therefore, the Bawburgh site became the only focus for successful data collection of CSFB herbivory. Feeding pressure from adult CSFB was too great for the plots to fully establish, therefore the trial was ended on 01/11/2019 and not taken to yield.

2.2.6.2 Phenotyping of CSFB herbivory and field establishment, 2019

To understand how different *B. napus* lines established in the field, we used a drone image to count number of plants per plot. Drone images were taken on 03/10/2019, 35 days after drilling. Establishment was uncharacteristically poor for the commercial *B. napus* lines C1 and C2, likely due to the poor-quality seed bulked at JIC, therefore these were removed from analyses.

To assess whether there were differences in percentage damage to seedlings in the field, 20 plants were sampled from border runs for each experimental plot 20 days after drilling and brought into the laboratory in petri dishes lined with damp blue roll to keep them from desiccating. Cotyledons were removed and laid out on white plastic making CSFB shot holes clearer, then

visually scored for percentage damage to the nearest 5%. If a seedling had no cotyledons, this was removed from the analysis.

2.2.6.3 Statistical analysis of field data, 2019

Means for establishment (number of seedings per plot) and damage (percentage eaten for 20 seedlings per plot) were both analysed, separately, using a two-way ANOVA. Percentage damage data was LOGIT+ transformed for analysis. These data were then correlated to see if there was a relationship between establishment and damage. Field herbivory scores were further correlated with scores from six-way choice assays to better understand how laboratory derived damage scores related to in field damage scores. Analyses were conducted using Rstudio software (http://www.rstudio.com/).

2.2.6.4 Field trials of 2020 to 2021, plant materials, treatment and layout

After losing the 2019 field trial early due to high levels of pest damage, we endeavoured to run a second round of field trials at Bawburgh field station in 2020 to 2021. These trials consisted of two parts; an untreated trial as performed in 2019, and a pyrethroid treated trial, to help ensure *B. napus* plants would establish given high levels of adult CSFB herbivory. Both trials were treated the same other than pesticide treatment, with 864 seeds per plot drilled. They were ploughed the day prior to drilling, harrowed the morning of drilling and rolled once drilled. Standard fertilisers and herbicides regimes were applied in both insecticide treated and non-treated trials.

Nine *B. napus* genotypes were selected for the 2020 field trials. The same six DFFS genotypes within the 2019 field trial were repeated. In addition, three commercial *B. napus* varieties were selected: C1 and C2 (as with the previous year) and C3. C3 was used to replace plots where seed numbers were limited.

2.2.6.5 Phenotyping of CSFB herbivory and field establishment, 2020

Drone images were used to obtain seedling counts per plot, 25 days after drilling, to better understand how well different *B. napus* lines established in pesticide treated and non-treated trials. Establishment was better than in the 2019 field trial and thus all genotypes could be examined for CSFB damage.

CSFB herbivory was scored in-field by visually estimating percentage damage to ten seedlings cotyledons per plot, 31 days after drilling. A random area within the plot was selected and then a continuous run of plants scored. If a seedling had no cotyledons present it was not included in the analyses.

2.2.6.6 Statistical analysis of field data, 2020

Pesticide treated and non-treated plots were considered as separate trials and thus analysed separately. For both, establishment was analysed by running a two-way ANOVA to compare mean seedling counts per genotype. For the non-pesticide treated trial, seedling count scores from 2020 were correlated with 2019 scores to compare establishment between years.

Mean percentage damage scores for genotypes were analysed by running a two-way ANOVA, investigating pesticide treated and non-treated separately. As with other percentage damage data, it was LOGIT+ transformed for analysis. The non-pesticide treated damage data was correlated with laboratory and field 2019 scores to examine the consistency of herbivory to specific genotype.

For the pesticide treated field trial, it was not appropriate to compare damage scores to those obtained from the laboratory assays or the 2019 field trial as they had not received insecticide treatments. However, we could compare them to the non-pesticide treated 2020 field trial as they were grown side by side and wanted to better understand how insecticide treatment may have influenced CSFB herbivory. For both treated and non-treated trials, CSFB herbivory scores were correlated with establishment to understand if there was a relationship between them. All analyses were conducted in RStudio (http://www.rstudio.com/).

2.3 Results

2.3.1 Variation in CSFB herbivory on *B. napus* seedlings from the DFFS can be observed in six-way choice chambers

The 96 *B. napus* lines from the DFFS, comprises diverse brassica material previously shown to carry variation for multiple traits including glucosinolate content (Harper et al., 2012, Lu et al. 2014, Kittipol et al., 1019) and disease resistance (Jacott et al., 2024). We therefore hypothesised that variation in CSFB herbivory would be present between genotypes. Six-way choice assays were conducted using *B. napus* seedlings according to an alpha design with Matador, as a control line, represented in each chamber. ANOVA of the LOGIT+ transformed CSFB percentage feeding damage (visually estimating cotyledon herbivory levels to the nearest 5%), revealed that there was no significant variation between assays (P=0.5813) and there was no interaction between blocks and assays (P=0.0524), therefore these terms were removed from the model. There was significant variation in total damage score for Matador between blocks (P=0.00157), therefore the data displayed from this experiment is adjusted for between block variation. Data was back-transformed with EXPIT+ for visualisation.

Adjusted mean total herbivory varied between the accessions, ranging from 0.89% to 9.03% for accessions Jaune et Collet Vert and York, respectively (Figure 2.3). Analysis using a linear mixed

model fitted via the restricted maximum likelihood method (REML) (fixed effect Line; random effects Block, Block.Date, Block.Date.Assay), identified no statistically significant difference was present between *B. napus* genotypes (*P*=0.145). All random factors were retained in the model as they explained a large amount of variation observed in total percentage damage.

The diversity set comprises several different crop types including winter, semi-winter and spring OSR, swede, kale, forage and leaf types which will have been under varying selection pressures during crop improvement and may influence palatability. REML analysis with Crop Type as a main fixed effect identified no significant difference between types for total percentage herbivory damage (*P*=0.664). Again, a large component of variation was attributable to random factors and therefore they were retained in the model.

Due to the number of lines and complexity of this experiment requiring a low number of replicates to make phenotyping feasible, we could not conclude whether significant differences were present between *B. napus* varieties for total percentage CSFB feeding damage. However, the distribution suggests that variation in herbivory damage may exist and warrants further investigation. Observation of crop type distribution suggests palatability does not vary between *B. napus* crop types. Lines showing greater and reduced levels of CSFB damage were selected for further, replicated study.

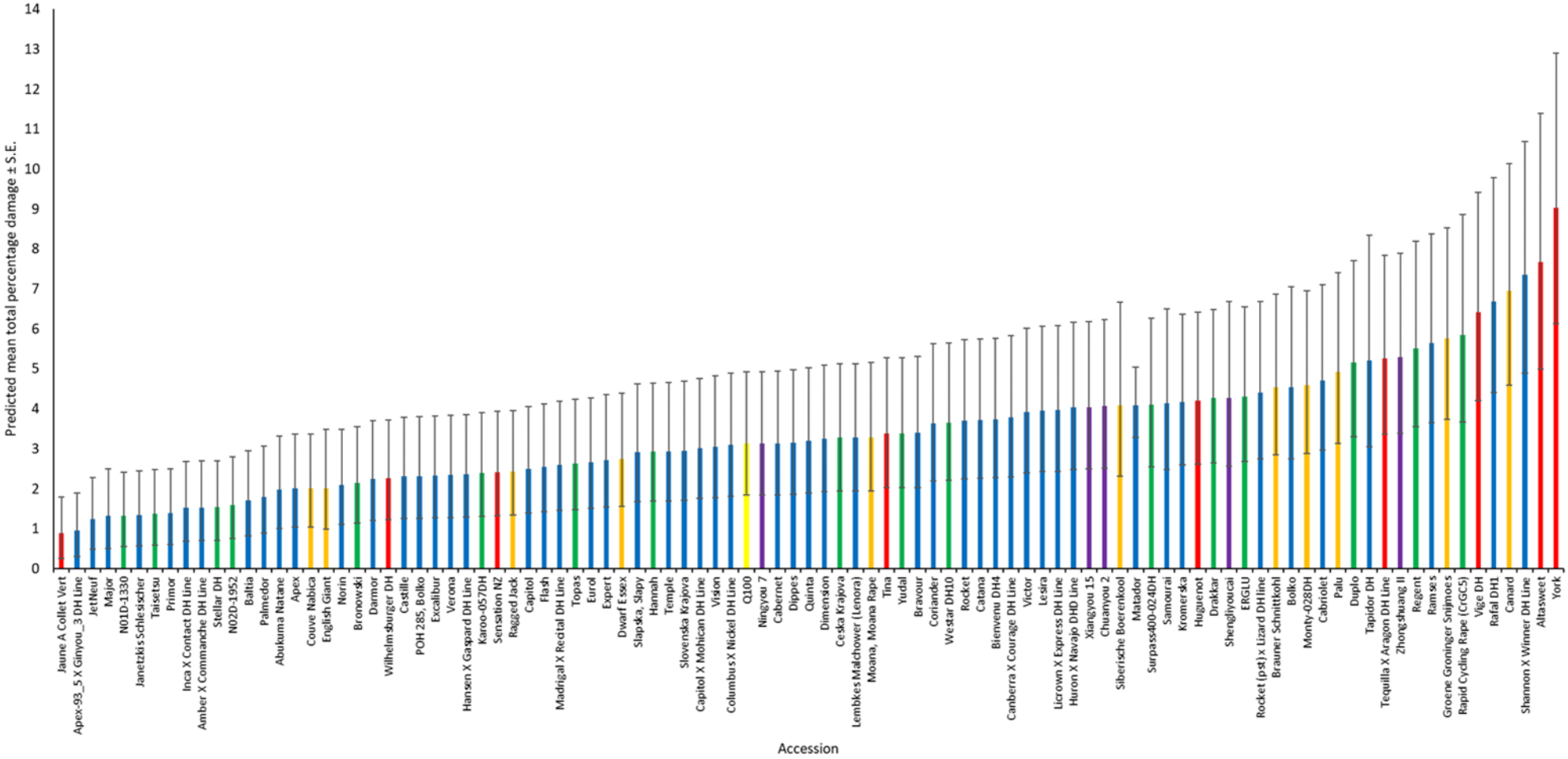

Figure 2.3. Variation in the adjusted mean total percentage damage to cotyledons from adult CSFB herbivory for 96 accessions from the DFFS (± standard error). Colour equates to crop type groupings (Blue = WOSR, Green = SOSR, Red = Swede, Orange = Kale/Forage/Leaf, Yellow = Synthetic, Purple = Semiwinter OSR). See Appendix 1 for specific crop type for each accession. n = 3 except for Matador, where n = 60.

2.3.2 Lines showing extremes in herbivory levels within the DFFS screen show significant differences in feeding within two-way herbivory assays

Focusing on two *B. napus* genotypes exhibiting extreme variation in of adult CSFB herbivory in six-way choice assays, we hypothesised that the line showing high palatability (S) would receive significantly higher levels of total percentage damage compared with the line showing low palatability (R).

To confirm CSFB feeding differences between genotypes, two-way choice chambers were run consisting of six seedlings of each genotype, presenting beetles with a choice between these two accessions only, rather than six. As in the six-way choice chambers described in the previous section, seedlings were visually estimated for percentage total damage. Percentage data was LOGIT+ transformed for analysis. Two-way ANOVA of *B. napus* Line and Block (date of assay), with an interaction term between the two to check that individual *B. napus* lines were not behaving differently to each other on different dates, identified S received significantly more cotyledon damage than R (P=0.00023), with percentage damage scores of 11.80% and 4.73%, respectively (Figure 2.4). A significant difference was observed between blocks (P=0.00707), suggesting the feeding levels varied between assay dates. However, no interaction between *B. napus* line and block was present, allowing this term to be removed from the model, therefore demonstrating that despite differences between weeks feeding differences were always maintained.

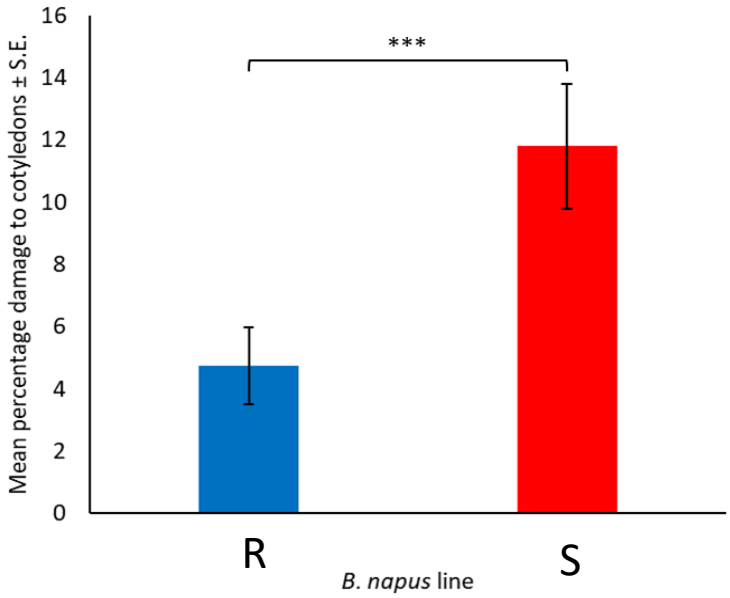

Figure 2.4. Variation in mean total percentage damage (± standard error) to cotyledons between two *B. napus* lines, R and S, in two-way choice assays (***P< 0.001) (n = 5).

2.3.3 Differences in feeding for extreme lines is retained in non-choice herbivory assays

From observing such clear differences in two-way choice chambers, we next wanted to investigate herbivory differences between S and R when beetles were presented with only one food option. Here we hypothesised that feeding differences would be maintained but, despite being only offered one food source, the lack of potential cues stimulating feeding from the S line would make differences more apparent.

To test these hypotheses, non-choice chambers were run with only either S or R seedlings present. The experiment was scored for total cotyledon percentage damage and analysed in the same way as described for two-way choice assays in the previous section.

A two-way ANOVA revealed that S received significantly higher levels of damage ($P< 0.00001$), scoring 19.77%, compared with R at 2.37% (Figure 2.5). Significant differences between Block (assay date, $P=0.00106$) identified feeding differences were present between weeks. There was no significant interaction between *B. napus* line and block ($P=0.59138$), thus the term was removed from the model, demonstrating again that despite differences between weeks, feeding differences were always maintained. The difference between these two genotypes was stronger in non-choice assays than previously observed in six-way and two-way choice experiments (Figure 2.3 and Figure 2.4, respectively).

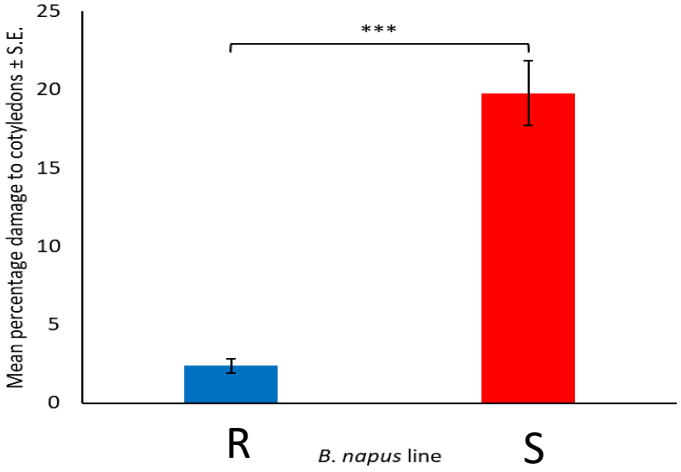

Figure 2.5. Variation in mean total percentage damage (± standard error) to cotyledons between two *B. napus* lines, R and S, in non-choice assays (***$P< 0.001$) (n = 5).

2.3.4 Herbivory assays for an F_1 of a S cross R identifies susceptibility is a recessive trait in three-way choice chambers

To determine the genetic nature of CSFB palatability, F_1 crosses were assessed for levels of feeding damage compared with parental lines. In addition to manual scoring, images of cotyledons were put through image analysis, using an ImageJ pipeline (Thursfield, 2022), with the aim of obtaining a more accurate, computerised score of damage.

Analysis of manual scoring via ANOVA of LOGIT+ transformed data, including a blocking factor of assay date and interaction term between *B. napus* line and block, identified a statistically significant effect of *B. napus* genotype on mean percentage damage to cotyledons (P=0.0436). Block and the interaction term were found to be non-significant thus excluded from the model. Tukey's HSD Test for multiple comparisons revealed that the mean percentage damage score for the F_1 (2.25%) was statistically lower than that of S (6.88%), however, there was no statistically significant difference between the mean damage score of S and R (4.44%) or R and the F_1 crossed line. Data derived from image analysis using ImageJ identified reduced levels of damage when compared to manual scoring of 1.07% for the F_1, 2.02% for R and 3.35% for S (Figure 2.6b), suggesting scoring by eye overestimates the true level of feeding damage. Pearson's correlation coefficient analysis identified significant, positive correlations (R r (14) = 0.965, S r (14) = 0.923 and F_1 r (14) = 0.800; P<0.01) were between the visual estimated scores and computer-generated data. Analysis by ANOVA identified statistically significant differences between lines (P=0.0709) and that feeding assay varied significantly between weeks (P=0.03553). Differences between lines were retained between weeks (interaction n.s, P=0.14465) and so the interaction term was not included in the final model. Tukey's HSD multiple comparisons test confirmed the variation between lines determined by the manual scoring.

Altogether, these results indicate that herbivory damage in the F_1 line resembles the levels seen in the resistant parent, suggesting that resistance to feeding is dominant.

2.3.5 Non-choice assays clarify the recessive nature of increased palatability to CSFB

Alongside the three-way choice assays, reported in the previous section, genotypes R, S and their F_1 cross were assessed for adult CSFB in non-choice herbivory assays using both manual and computational scoring pipelines. Focusing on visually estimated data first, analysis by ANOVA identified a statistically significant effect for *B. napus* line for mean percentage damage to cotyledons in non-choice assays (P< 0.00001) (Figure 2.7a). Block and the interaction term between line and block were observed to be non-significant so removed from the model.

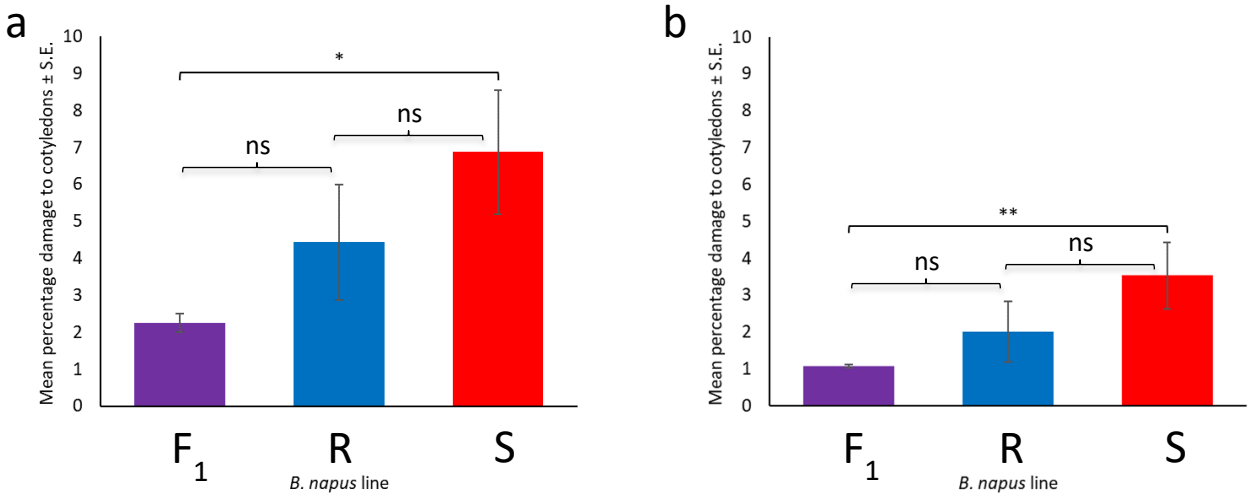

Figure 2.6. Variation in the mean percentage damage to cotyledons (± standard error) from three-way choice chambers for three *B. napus* lines, R, S and F1 cross, with these as parental lines. Data displayed is derived from a) by-eye estimated scores to the nearest 5% (* $p < 0.05$) and b) Image analysis derived scores utilising the software ImageJ (** $p < 0.01$) (n = 4).

Multiple comparison testing via Tukey's HSD revealed that, unlike the damage observed in three-way choice chambers, the mean percentage damage score for S (9.98%), differed significantly ($P< 0.00001$) from that of both R (1.98%) and their F_1 cross (1.67%). This again demonstrated that differences were easier to identify in non-choice than in choice herbivory assays. As previously observed with three-way choice assays, the mean percentage damage scores of the F_1 cross and R did not differ statistically significantly ($P=0.86696$). Analysis of data obtained from ImageJ again identified reduced levels of herbivory when compared with manual scoring damage, with the mean percentage damage score of S at 5.52% being statistically greater ($P< 0.00001$) from that of R (0.95%) and the F_1 cross (0.79%). Consistent with previous results, there was no statistically significant difference between the mean percentage damage scores of R and the F_1 cross. Pearson's correlation coefficient analysis identified significant, positive correlations (R $r(46) = 0.874$, S $r(46) = 0.904$, and F_1 $r(46) = 0.762$) between the visual estimated scores and computer-generated data. The visible trend in reduced feeding for the F_1 line compared to the R parent was not apparent in the non-choice assays.

To conclude, manual scoring results in overestimation of herbivory damage compared to measurement using the ImageJ pipeline. It is recommended therefore, that this approach is utilised for future experiments. As previously observed, non-choice assays provide a greater level of discrimination between choice assays, potentially due to olfactory feeding cues. Adult CSFB

herbivory levels within the F_1 line are similar to the levels seen in the resistant parent, further confirming that resistance to feeding is dominant.

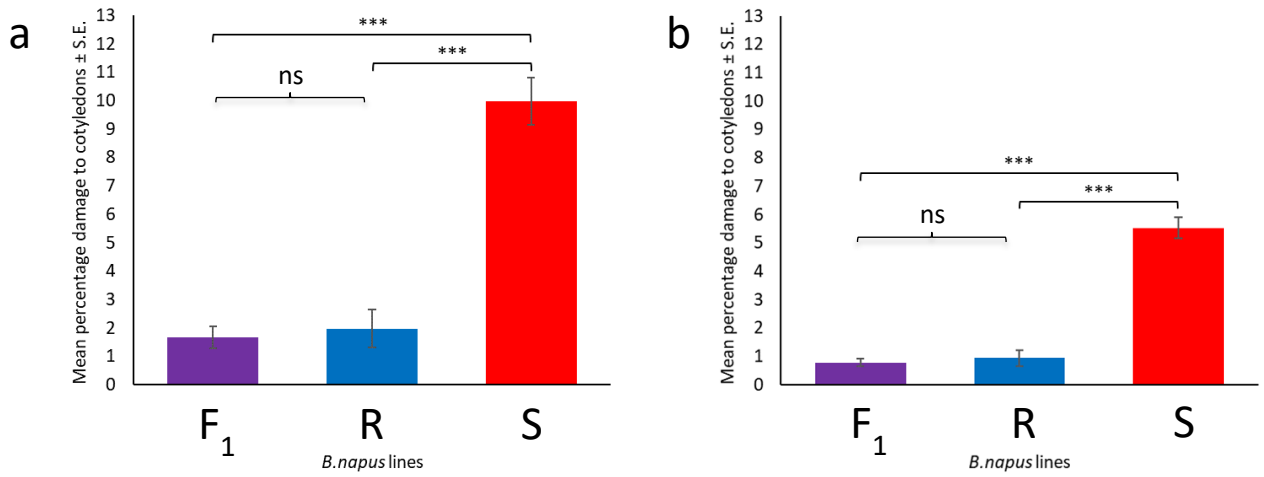

Figure 2.7. Variation in the mean percentage damage to cotyledons (± standard error) from non-choice chambers for three *B. napus* lines, R, S and F1 cross, with these as parental lines. Data displayed is derived from a) by-eye estimated scores to the nearest 5% and b) image analysis derived scores utilising the software ImageJ (*** P<0.001) (n = 4).

2.3.6 Significant differences are observed between *B. napus* genotypes for establishment and feeding damage in 2019

After observing significant differences in feeding damage from adult CSFB in a laboratory setting, we aimed to determine whether these differences were maintained within a field environment and if this variation influenced levels of establishment. Using six *B. napus* lines from the DFFS that showed varying amounts of feeding damage in the laboratory six-way choice assays in a complete randomised balanced block design, we assessed establishment and feeding damage. Due to poor seed quality, the two commercial lines, C1 and C2, were removed from analyses.

Overall, establishment was poor and therefore seedling counts for the field trial were low. Mean seedling counts per plot, obtained from drone images, ranged from 11.6 for S to 50.2 for R (Figure 2.8). Two-way ANOVA revealed a significant difference between both lines and blocks, P< 0.00001 and P=0.0294, respectively. Tukey's HSD identified significant differences between *B. napus* lines (P<0.05), with the two lines classified as having reduced palatability in controlled trials showing improved establishment.

To quantify adult CSFB damage in the field, we sampled 20 seedlings per plot and visually scored percentage damage to cotyledons in the laboratory. Adjusted mean feeding damage ranged from

17.21% to 26.22%, for DFFS1 and DFFS2, respectively (Figure 2.9). A two-way ANOVA demonstrated a strong significant effect of both *B. napus* line and block (*P*<0.00001).

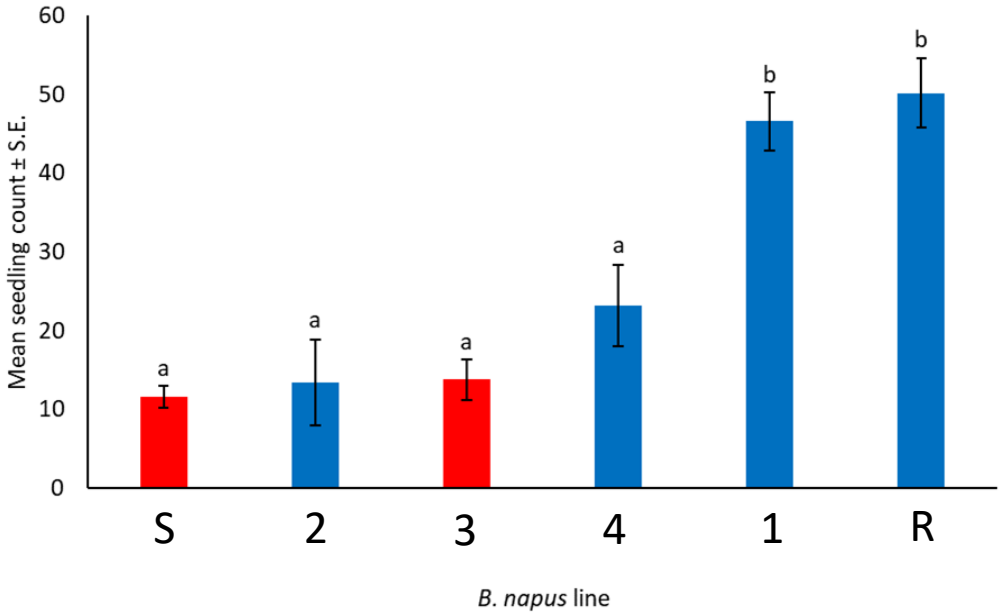

Figure 2.8. Variation in the mean seedling count for six *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 5. Letters denote statistically significant differences between lines determined by Tukey's HSD (*P*<0.05).

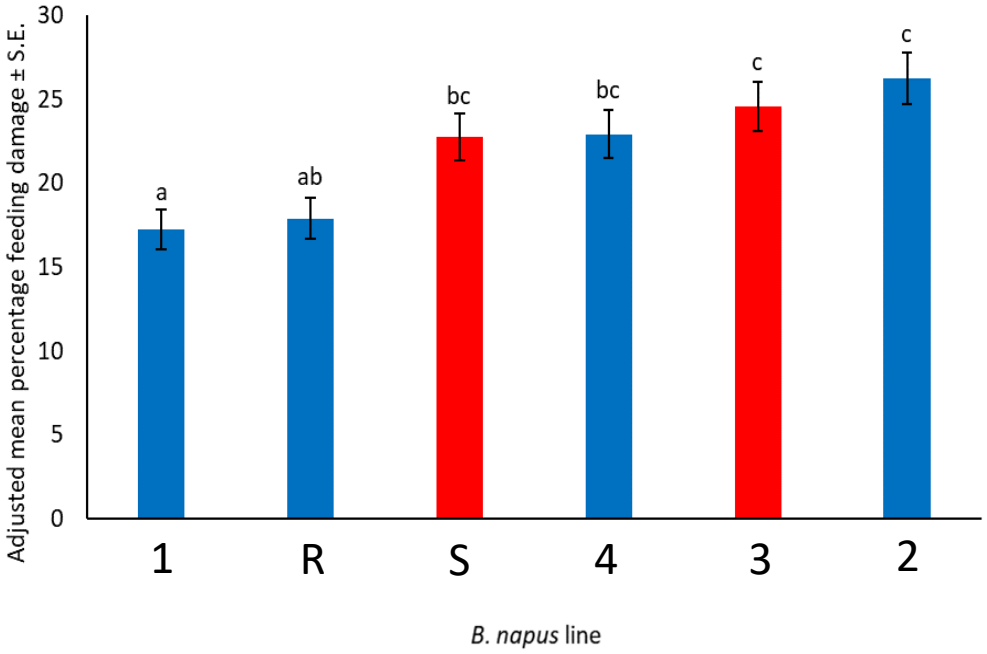

Figure 2.9. Adjusted mean percentage feeding damage for six *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 5. Letters denote statistically significant differences between lines determined by Tukey's HSD (*P*<0.05).

Pearson's correlation coefficient between field damage and laboratory derived scores (from six-way choice chamber assays), revealed a statistically significant positive correlation (r (4) = 0.894, *P*<0.02), indicating that *B. napus* genotypes which received higher levels of damage in the laboratory also received higher damage in the field (Figure 2.10). Higher levels of damage occurred in the field trial compared with laboratory assays. Seedling counts were negatively correlated with damage levels (r (28) = -0.0586, *P*<0.001), indicating plots with higher damage levels showed poorer levels of establishment (Fig 2.10).

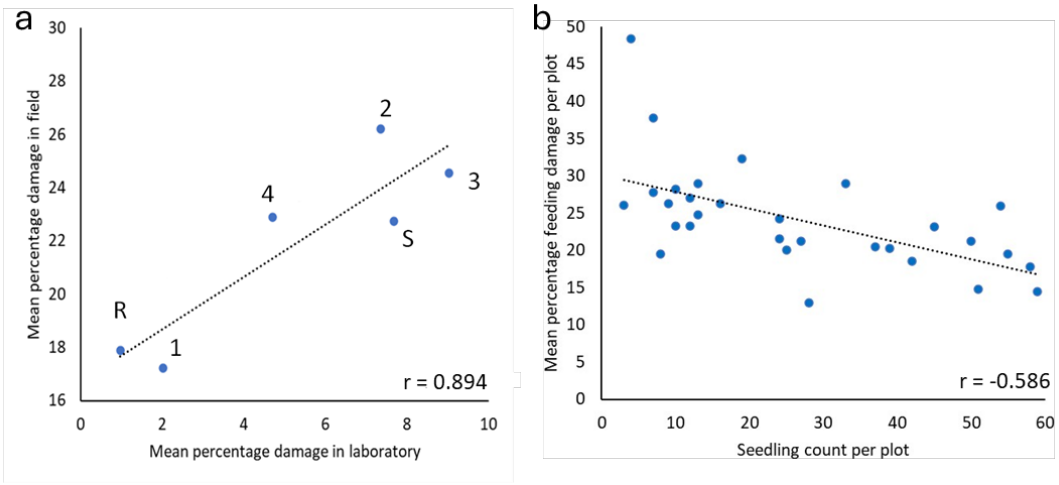

Figure 2.10. Correlation between a) percentage damage from six-way choice laboratory assays and field scores and b) seedling count per plot and percentage feeding damage in 2019.

2.3.7 Significant differences are observed between *B. napus* genotypes for establishment and feeding damage in 2020

After concluding the 2019 field trial shortly after establishment (due to persistent high levels of pest damage), the 2020 repeat trial included an additional pyrethroid treated trail to allow the assessment of larval CSFB damage. We selected the same eight *B. napus* genotypes as the previous year and commercial varieties, C1 and C2. Due to limited seed for some *B. napus* genotypes, an additional commercial line, C3, was included to replace any missing plots of the other eight varieties.

For the untreated trial mean seedling counts scored from drone images ranged from 39.7 to 99.8 per plot, for C1 and C3 respectively (Figure 2.11). Analysing data with a two-way ANOVA of line and block revealed a statistically significant effect (*P*<0.00001) of *B. napus* line on seedling count. Tukey's HSD multiple comparisons test revealed significant differences between *B. napus* lines

(*P*<0.05), with the susceptible line, S, showing significantly poorer establishment (*P*=0.00014) than resistant line R.

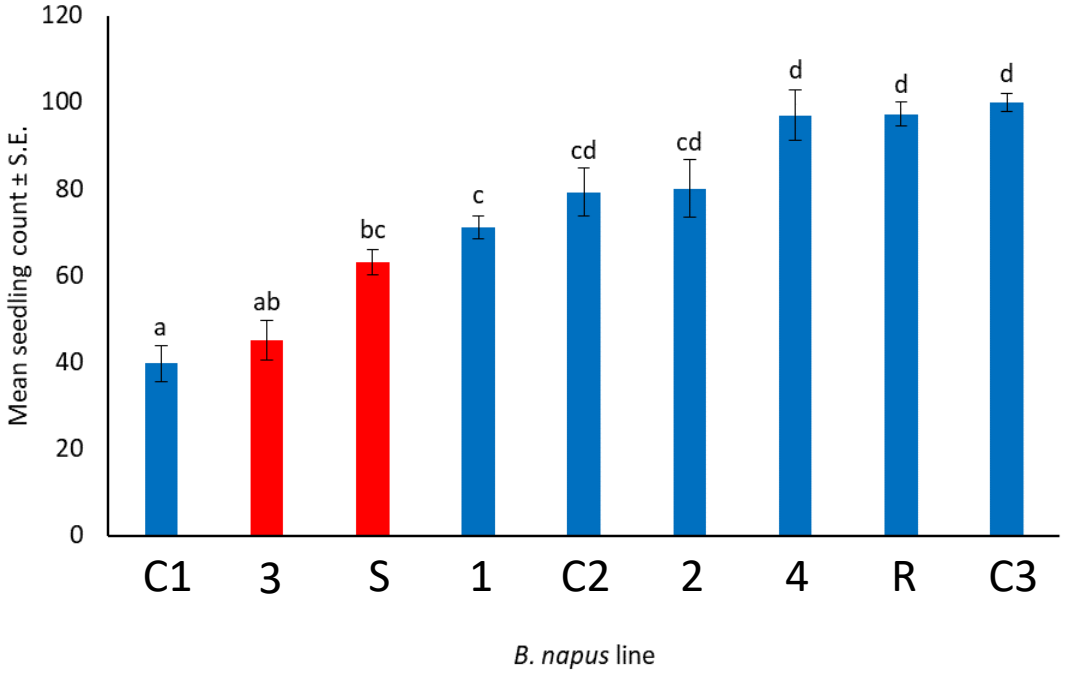

Figure 2.11. Variation in the mean seedling count for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for lines 2, 3 and C1; n = 4 for C2. n = 5 for S, R, 1 and 4; n = 6 for C3. Letters denote statistically significant differences between lines (*P*<0.05).

Damage scores ranged from 10.93% for line 2 to 18.79% for C1 (Figure 2.12). A two-way ANOVA of Line and Block demonstrated a strong, significant effect of *B. napus* line (*P*<0.00001) on percentage feeding damage. Tukey's HSD multiple comparison test revealed statistically significant differences between *B. napus* lines for damage (*P*<0.05) as shown in Figure 2.12). Significant differences were present between R and S lines (*P*<0.00017).

Pearson's correlation coefficient between field damage and laboratory derived scores revealed a non-significant positive correlation between field damage and laboratory damage scores (r = (4) 0.479, *P*=0.337) (Figure 2.16). Again, as in 2019, damage in the field was greater than that observed in the laboratory (Figure 2.16), though lower levels of damage were present compared to 2020 and trials were not- significantly correlated (r = (4) -0.167, *P*= 0.752). Lines showing variable behaviour between years. Seedling counts were negatively correlated with damage levels (r (37) = -0.722, *P*<0.001), again demonstrating plots with higher damage levels showed poorer levels of establishment.

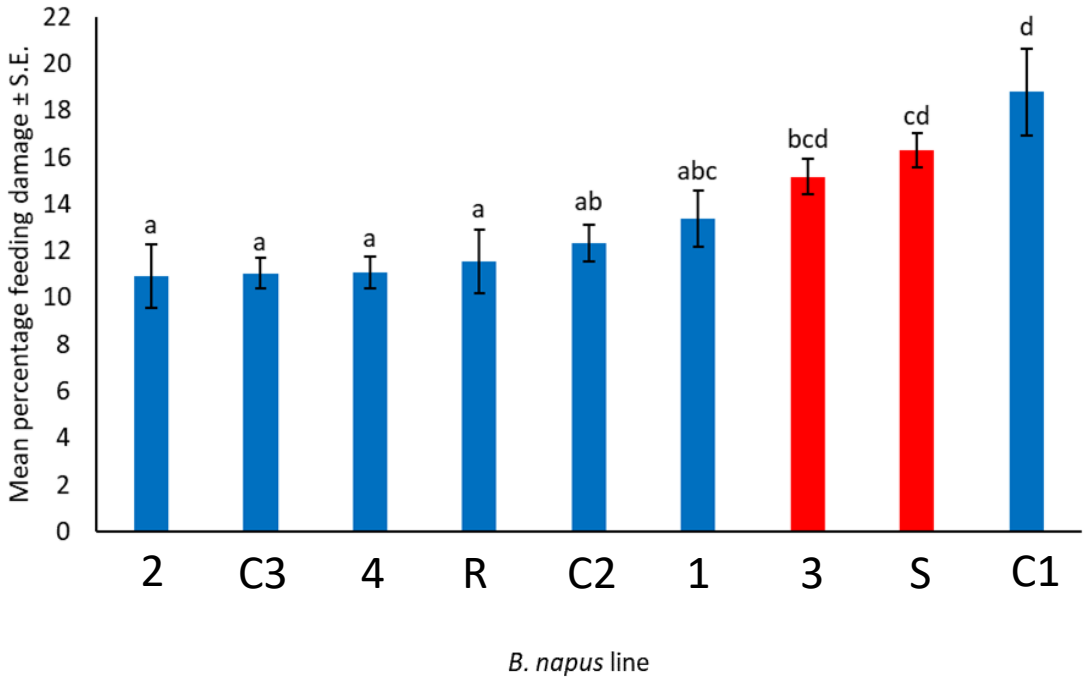

Figure 2.12. Variation in the mean percentage feeding damage for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for lines 2, 3 and C1; n = 4 for C2. n = 5 for S, R, 1 and 4; n = 6 for C3. Letters denote statistically significant differences between lines (P<0.05).

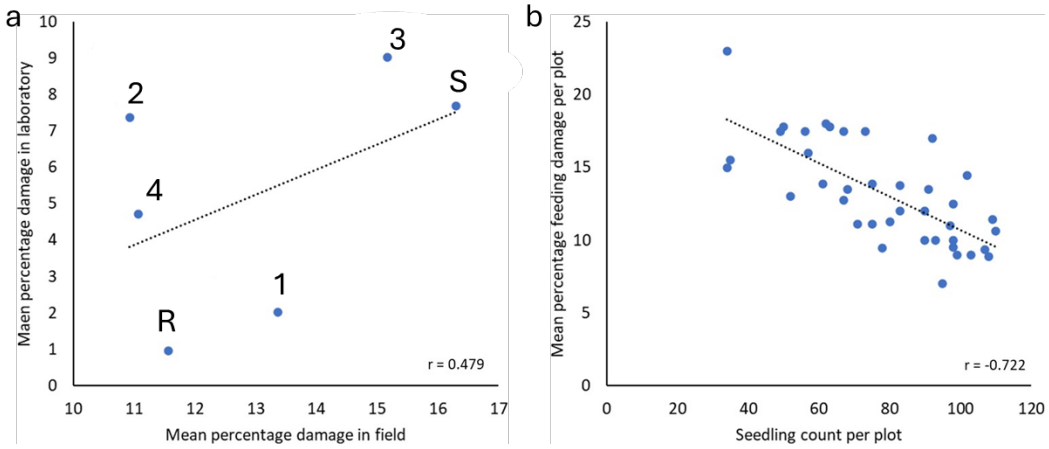

Figure 2.13. Correlation between a) percentage damage from six-way choice laboratory assays and field scores and b) seedling count per plot and percentage feeding damage in 2020.

2.3.8 Significant differences are observed between *B. napus* genotypes for establishment and feeding damage in 2020 pesticide treated trial

Seedling counts from the 2020 pesticide treated field trial indicated a range of establishment, from 39.67 to 99.83 seedlings, for C1 and C3, respectively (Figure 2.14). A higher seedling count was observed for *B. napus* lines in the pesticide treated field trial compared to the non-treated trial. Pearson's correlation revealed a statistically significant positive correlation between the two trials (r = (7) 0.973, *P*=0.00001), indicating that establishment was similar for *B. napus* lines between treated and non-treated plots. Two-way ANOVA demonstrated a statistically significant difference between *B. napus* lines and experimental Blocks, *P*<0.00001 and *P*=0.00554 respectively. Significant differences between *B. napus* lines (*P*<0.05) as determined Tukey's multiple comparisons test are given in Figure 2.14.

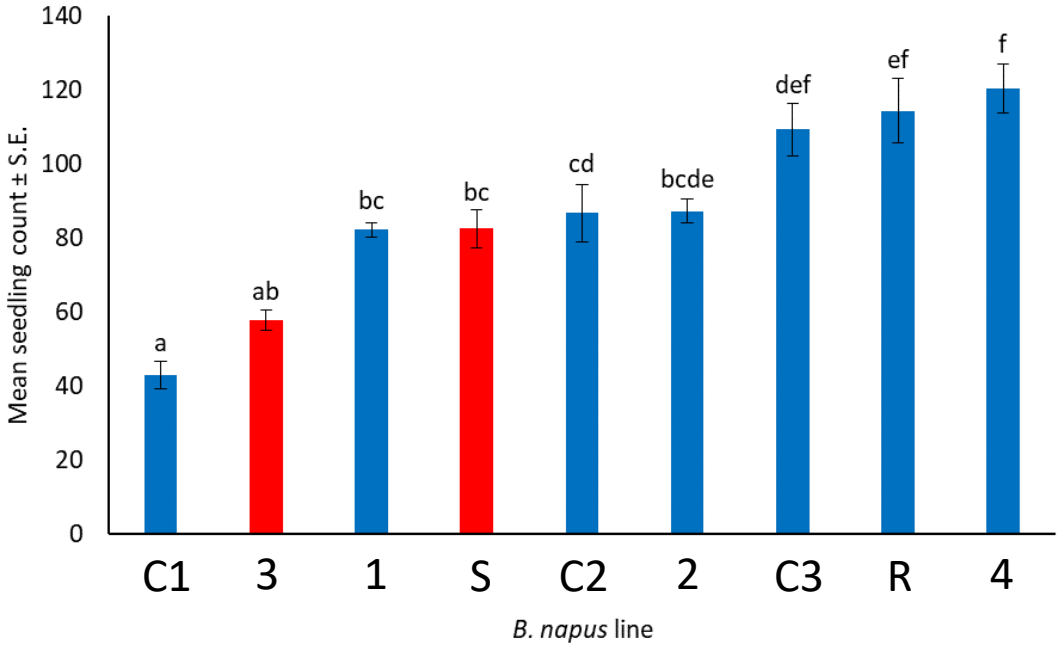

Figure 2.14. Variation in the mean seedling count for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 2 and C1; n = 4 for 3; n = 5 for S, R, C2, C3, 1 and 4. Letters denote statistically significant differences between lines (*P*<0.05)

Variation in feeding damage was reduced compared to the untreated trial, ranging from 9.59% for R to 14.26% for S (Figure 2.15). Two-way ANOVA revealed a statistically significant effect of *B. napus* Line and Block on percentage damage (P<0.0057, P<0.00098). Tukeys HSD multiple comparison test identified a statistically significant difference between, R and S (P< 0.008). Pearson's correlation demonstrated a non-significant association between pesticide treated and non-treated damage scores (r (7) = 0.410, P= 0.273) (Figure 2.16). Treated damage scores were not correlated with laboratory scores as they were deemed non-comparable due to pesticide usage. Correlation with seedling count per plot identified no association between seedling count per plot and seedling damage in the sprayed trial (r = (38) -0.082, P= 0.614).

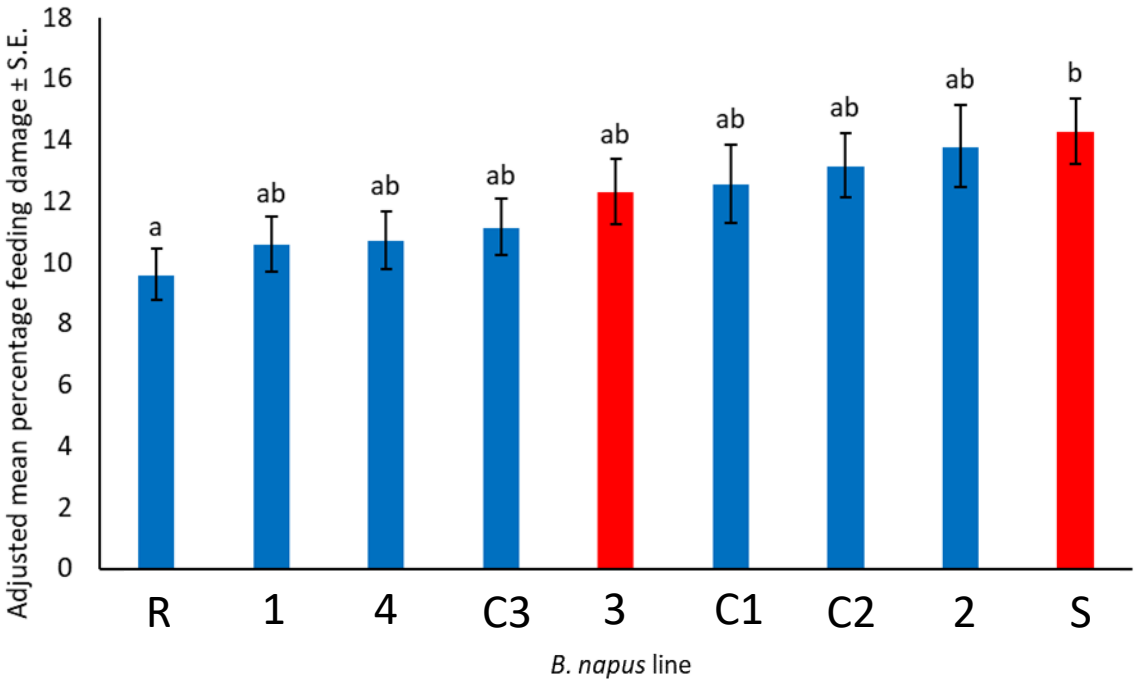

Figure 2.15. Variation in the adjusted mean percentage damage to cotyledons for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for 2 and C1; n = 4 for 3; n = 5 for S, R, 1, 4, C2 and C3. Letters denote statistically significant differences between lines (P<0.05).

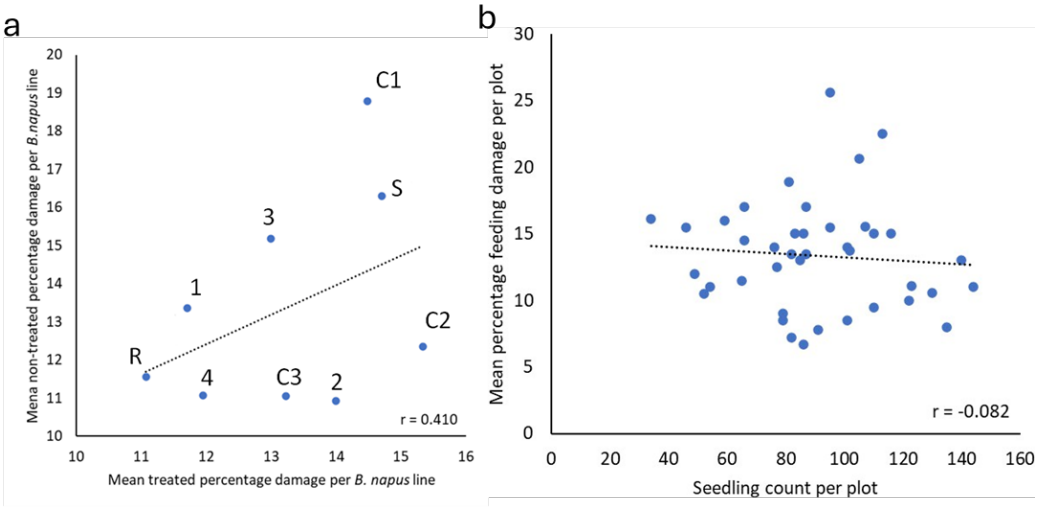

Figure 2.16. Pearsons correlation between a) mean non-pesticide treated and pesticide treated CSFB percentage damage and b) seedling count per plot and feeding damage.

2.4 Discussion

Novel six-way choice assays examining adult CSFB of intact *B. napus* seedlings allowed us to identify variation, albeit non-significant, between varieties under laboratory conditions. This could potentially have been because there were no actual differences in adult feeding preferences however, low replication levels (n = 3) and the large error with multiple choice of food source in a relatively limited spatial range, poor air flow and lack of control for beetle age and sex, will reduce the likelihood of discrimination between varieties. Differentiation between food sources by CSFB in such an environment may be problematic if olfaction is involved in host selection (Henderson et al., 2004), indeed Bartlet et al. (1999) identified that sensilla are likely involved in olfactory sensing on CSFB.

No other research has quantified the CSFB herbivory differences between a large number of *B. napus* lines from a genetically diverse panel under laboratory conditions. Most studies have focused on smaller numbers of *Brassica* varieties, samples of leaf tissue rather than complete plants or other pests of cruciferous crops. However, Barlet and Mithen (1996) do assess differences in CSFB on whole plants between eight *B. napus* varieties. They found a significant difference between lines for number of CSFB shot holes in leaves under laboratory conditions. However, they only discuss these differences further in the context of glucosinolate content and do not find a relationship between CSFB herbivory and glucosinolate content.

Other research has looked at more diverse plant material but not with CSFB. Gavoski et al. (2000) tested a variety of Brassicaceae species, including *B. napus* varieties for resistance against *Phyllotreta cruciferae*, a closely related flea beetle to the CSFB. Using multiple choice assays and

a zero to 10 scoring system for damage to cotyledons, they did not find any repeatable resistance in *B. napus* varieties to *P. cruciferae* herbivory. This is consistent with other studies where they have also failed to find significant differences in damage to *B. napus* against *P. cruciferae* (e.g. Palaniswamy et al., 1997). This is perhaps because there are no differences between these *B. napus* varieties, but also may be that a multiple-choice experimental system is not suitable for revealing differences in flea beetle herbivory. This again may indicate that beetles find it difficult to differentiate between food sources in a relatively air-tight environment, particularly as detection of volatiles is involved in their choice whether to feed them or not (Henderson et al., 2004).

From the preliminary six-way choice assays we were able to select two *B. napus* varieties from the DFFS to further characterise CSFB herbivory. We selected S as a genotype with high, and R with low, levels of CSFB herbivory, indicating a more susceptible and more resistant/tolerance variety, respectively.

Refined assays of either a two-way choice between *B. napus* varieties or no choice, i.e. a single genotype demonstrated significant strong difference between S and R, with S receiving much higher levels of total feeding damage, thus confirming our preliminary finding from six-way choice assays. Interestingly, when beetles were given no choice of food source the difference in feeding levels between the two *B. napus* lines became even more exacerbated, i.e. S received higher and R lower damage in comparison to two-way choices. This further indicates that decisions to feed may be initiated by volatile detection, which can get confused in assays containing multiple *B. napus* varieties.

Whilst there is no previous research into CSFB herbivory on *B. napus* varieties in two-way or non-choice assays of whole plants Soroka et al. (2011) investigated herbivory between two *B. napus* varieties, Westar and a transgenic line with enhanced trichome density. Hairier plants received significantly less damage from flea beetles, indicating that trichomes offer some resistance against herbivory. Investigating the CSFB herbivory response to different plant architectures would also be beneficial, although *P. cruciferae* are smaller than CSFB and likely more impacted by trichomes.

In earlier research, Bodnarky and Lamb (1991) compared *S. alba* variety Ochre with *B. napus* variety Westar in two-way and non-choice assays for *P. cruciferae* herbivory, finding that *B. napus* was fed upon about twice as much as *S. alba*. They also demonstrated a similar result in non-choice assays, but the differences between *B. napus* and *S. alba* we more extreme. This is similar to what we observed when running non-choice assays compared to two-way choice assays. Taken together, although running assays with multiple varieties provides a higher throughput method for screening plant material, this indicates CSFB research into palatability of different *B. napus* varieties may benefit from more non-choice experiments as opposed to multiple choice assays.

Assays performed on S, R and their F_1 hybrid identified that herbivory on the F_1 line resembled the

resistant parent, suggesting within this cross increased susceptibility is a recessive trait. Additionally, the F_1 seedlings were similar in appearance to the R parent (personal observations). If time had permitted, further exploration of the herbivory phenotype and distribution in the F_2 generation would help identify the inheritance and if the trait is multigenic.

In this same experiment, we additionally aimed to test a new cotyledon damage scoring method using the software ImageJ and compare this to human estimated damage scores. Percentage damage scores derived from ImageJ were consistently about half the value of visually estimated scores. However, the scores from ImageJ followed the same pattern as those from visual estimates and a strong, positive correlation was observed between the scoring methodologies, demonstrating both as valid techniques for capturing differences in adult CSFB cotyledon herbivory. ImageJ provides more precise damage values, but visual estimates are still an appropriate way to distinguish differences in *B. napus* palatability. The underlying software has previously been used to assess herbivory damage of other important crop pest species successfully, such as snails (Stawarczyk and Stawarczyk, 2015) and thrips (Visschers et al., 2018). The ImageJ pipeline for quantifying flea beetle damage to crop species as developed by Thursfield (2022) and is now in use to score CSFB damage in collaborating research and breeding organisations.

Field trials in 2019 and 2020 assessing CSFB damage levels for a subset of *B. napus* genotypes from the DFFS and commercial lines confirmed previous laboratory results for adult CSFB herbivory damage. The 2019 trial suffered high levels of pest damage immediately after drilling. Coupled with dry, hot conditions, establishment was generally poor, and the trial prematurely ended despite having two field sites. Establishment was much more successful in the 2020 field trials, with less pest pressure and better weather conditions. Two field trials were drilled at the Bawburgh field site, one treated with pyrethroid pesticides to try and ensure the crop would make it through the overwinter larval CSFB season.

Whilst we observed a significant effect of *B. napus* line on CSFB damage amount, we did not observe a significant difference between S and R. This is potentially attributable to the scoring methodology used, where 20 seedlings were sampled from border runs and cotyledons scored in the laboratory for percentage damage (visual estimates to the nearest 5%). This may have introduced bias, as to select 20 seedlings meant they had to be present, and thus does not give a full representation of how empty or damaged a plot may have been. Seedlings where just small bits of plant material remained (i.e. mostly completely eaten) were likely not selected for scoring back in the laboratory. In 2020, the damage received by S was significantly higher than that of R. Within this trial we scored percentage damage to cotyledons in the field for a continuous run of 10 seedlings, rather than sampling and scoring in the laboratory. Although it was less accurate to score in the field, this methodology may have been more appropriate as seedlings with high levels of damage would not have been missed.

When inspecting drone images, there was a clear difference in plant coverage between plots and *B. napus* varieties. Swede type *B. napus* genotypes had a significantly lower seedling count compared to the R genotype and line1 (that also received low levels of damage in six-way choice laboratory. Establishment and herbivory scores were significantly negatively correlated, indicating that *B. napus* genotypes with lower plant counts also received higher levels of damage. Furthermore, damage in the field in 2019 correlated strongly with damage scores obtained in the laboratory suggesting controlled trials are a good proxy for field adult CSFB resilience. No correlation for establishment or damage data was observed between the 2020 and 2019 field trials. This is perhaps unsurprising given that damage levels were so high in 2019 and demonstrates how variable establishment and CSFB damage can be year on year.

In pyrethroid treated plots, we also observed a significant effect of *B. napus* genotype on the amount of CSFB damage, despite reduced levels of damage and differences being more subtle. It may be that pesticide treatment offered some protection to more susceptible *B. napus* genotypes, and thus lessened differences between them and the more tolerant varieties. Overall, damage scores from pesticide treated plots did not correlate statistically significantly with the non-treated trial. Nonetheless, S was observed to be the most highly damaged *B. napus* variety compared with R, which received the lowest levels of cotyledon herbivory.

Establishment scores differed significantly between *B. napus* genotypes in treated plots but did not correlate with damage scores. However, they did correlate strongly with non-treated establishment scores. Marginally higher establishment scores were achieved for the treated trial compared to the non-treated trial. Taking higher establishment scores with lower damage scores for treated plots, there was an indication that pesticide treatment was providing some protection for these seedlings, albeit minor. Later in the growing season the differences between pesticide treated and non-treated plots became more apparent with differing plant sizes.

From personal scoring of a field trial on behalf of OREGIN (Oilseed RapE Genetic Improvement Network) I observed some variation, albeit limited, in CSFB damage to a panel of 28 *B. napus* genotypes. Incidentally, S was included in this panel and received the third highest amount of damage out of the 28 *B. napus* genotypes (unpublished data). The results presented in this project appear to represent the first time a more susceptible *B. napus* variety S and a more resistant/tolerant variety R, identified in laboratory assays, also maintained these CSFB damage differences in a field environment.

Overall, our results demonstrate that non-choice assays are most suitable for picking out significant differences between *B. napus* genotypes for CSFB feeding traits. However, the choice experiments did successfully provide data for using in genetic mapping via Associative Transcriptomics (AT, see Chapter 3) and allow selection of more and less palatable varieties for further investigation. Both

visual estimates and scores derived from ImageJ analysis are useful techniques for determining these differences. Finally, I demonstrated that line S remains more susceptible, and R more resistant/tolerant, to adult CSFB herbivory damage in the field environment. These methodologies and data offer a basis for determining the genetic control of herbivory in *Brassica* by adult CSFB.

3. Determining the genetic control of adult CSFB herbivory

3.1 Introduction

Previous research has largely focused on identifying phenotypic differences determining insect herbivory levels, with the aim of breeding in potential resistance/tolerance traits. Whilst this has potential for the improvement of crop varieties, modern genetics-based approaches allow the identification of associated regions and genes potentially conferring these observable differences in herbivory.

Genome wide association (GWA) mapping, or linkage disequilibrium mapping, relies on the statistical association of genetic variation in the form of genetic markers at a locus with a phenotypic trait of interest. If genetic variation is in linkage equilibrium the co-occurrence of the trait with a genetic variant will be random. However, if variants are in linkage disequilibrium they will segregate with the trait of interest. For example, in Figure 3.1 across a panel of sequenced individuals, the yellow variant will always be associated with tall plants, whilst the black variant at that locus is associated with dwarf plants. The closer the proximity between variants along a chromosome, the lower the likelihood of recombination between them, therefore, all variants in close proximity to a gene controlling a trait of interest will have an increased statistical association with that trait. By scanning the genome for these statistical associations, we can identify regions harbouring candidates for our trait of interest.

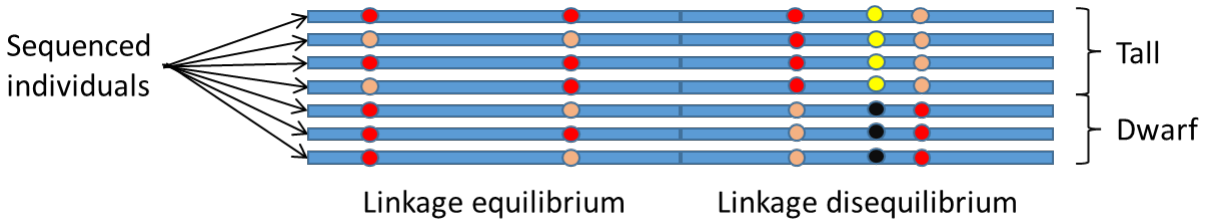

Figure 3.1 Linkage disequilibrium shows genetic variation (red/pink) linked to the causal gene for tall (yellow) or dwarf (black) phenotype.

GWA studies have several requirements. Firstly, this method requires a population of genetically fixed lines encompassing a wide range of genetic diversity. This is to ensure a large number of genetic variants are available for mapping and, because individuals are distantly related or unrelated, it ensures a greater number of potential recombination's are present along a chromosome to allow more fine scale dissection of associated regions. Markers must be arranged in genetic order along the chromosome to support the identification of statistically associated regions. The advent of next generation sequencing approaches, and accompanying computational analyses, have revolutionised the ability to assay large numbers of genetic variants, in a cost-effective manner across mapping populations (Trick et al., 2009, Bancroft et al., 2011), greatly enhancing the efficacy of GWA approaches. Secondly, the population structure and relatedness of the individuals must be determined. Historically, GWA methods were designed for populations of unrelated individuals however, especially within crop species, this is unlikely. Genetic relatedness can prevent the correct identification of causal variants and can result in false positive, or spurious associations. Therefore, it is necessary to adjust for this using a measure of population structure, the Q matrix, and for some statistical approaches, a further measure of relatedness, the K matrix. The last requirement is robust phenotyping data. The quality of these data will determine how accurately the statistical association with the trait of interest can be calculated.

Xuan et al. (2020) investigated the effects of leaf trichomes on herbivory from *Plutella xylostella* larvae, a prolific pest of oilseed rape. Using simple phenotypic association, they demonstrated that hairy leaves were less attractive to larvae than smooth leaves. However, utilising a GWA approach to associate trichome phenotype data with genetic variability within 290 *Brassica napus* genotypes, Xuan et al. identified two regions of the *B. napus* genome linked to hairiness and implicated several genes influencing trichome production. Selection for these genes could be used to assist in breeding programmes for trichome density and resistance to *P. xylostella.*

Associative transcriptomics (AT) is a GWA approach. Variants are identified from expressed genes (mRNA-Seq), reducing the sequence complexity from that represented by the entire genome (Harper et al., 2012). In addition, the mRNA-Seq data can be used to quantify transcript abundance resulting in gene expression markers (GEMs). This allows the detection of associations between gene expression level and trait phenotype. This is a powerful approach within polyploids, where multiple gene copies, or homologues, may results in gene copy number variation via gene loss, duplication and homeologous exchange; or expression variation via homologue silencing and unequal expression of duplicated genes (Adams et al., 2003; Pires et al., 2004). AT has been extensively used in *Brassica* genetics for multiple traits including yield, quality traits and disease resistance (Miller et al., 2019; Kittipol et al., 2019; Fell et al., 2023).

Using an AT approach on 195 *B. napus* genotypes Fell et al. (2023) identified gene loci significantly associated with in light leaf spot (LLS) disease occurrence, including one demonstrating enhanced resistance to LLS. Furthermore, they identified eight gene expression markers, seven of which demonstrated a positive correlation between resistance and gene expression levels, and one where expression resulted in increased susceptibility. This approach offers great potential to aid future crop improvement.

Here we present an AT approach for Cabbage Stem Flea Beetle (CSFB) herbivory traits to screen a diverse panel of *B. napus* varieties for genomic variation and gene expression variation, and ultimately identify potential candidate genes conferring these phenotypic differences for further exploration.

3.2 Methods

3.2.1 Associative transcriptomics for the identification of loci associated with adult CSFB herbivory

Phenotypic scores for CSFB herbivory to cotyledons collected from six-way choice assays using 96 *B. napus* varieties from the DFFS (see section 2.2 Methods) was used to investigate underlying genetic variation linked to CSFB herbivory trait using Associative Transcriptomics (AT).

Analysis was performed using the AT pipeline developed by Harper et al. (2012) which was previously demonstrated for use in mapping traits in *B. napus*. Genotype and expression level data for 95 *B. napus* lines (one line was dropped due to insufficient quality of sequence data) were from published datasets (Trick et al., 2009; Harper et al., 2012).The SNP file for analysis was previously generated from mRNA-seq (Illumina, single end library, 80bp reads) of the first true leaf collected from 21-day old plants grown at 18°C/15°C day/night with a 16hr photoperiod. Plants were sampled at the midpoint of the photoperiod. The Q-matrix was previously generated using STRUCTURE 2.3.3 for Bayesian population analysis. Population structure divided the DFFS panel into two, comprising WOSR and other. GWAS was performed using TASSEL v4 after removing Single Nucleotide Polymorphism (SNP) markers with an allele frequency of less than 0.05. Association was performed using both a generalised linear model (GLM), which uses a least-squares fixed effect model and accounts for population structure using the Q-matrix, and mixed linear model (MLM), which includes fixed and random effects, so also includes kinship, the genetic resemblance between individuals. QQ plots were used to determine the most suitable model for the herbivory data. False discovery rate (FDR) was calculated using the Shiny implementation of the q-value R package as detailed in Storey et al. (2020). Allelic effect and linkage disequilibrium (LD) were investigated for the most significantly associated SNPs. LD was examined by calculating

the mean pairwise r^2 for all markers on the chromosome of the focal SNP. LD was considered if r^2 was greater than 0.15.

Gene expression marker (GEM) associations were determined for CSFB herbivory using linear regression with transcript abundance in Reads Per Kilobase per Million mapped reads (RPKM) as per Harper et al. (2012). Markers with expression less than 0.5RPKM were removed before analysis.

3.2.2 Confirmation of candidate genes via Arabidopsis feeding assays

To investigate the effect of candidate genes CSFB feeding assays were developed and used for phenotyping feeding on *Arabidopsis* mutants.

3.2.2.1 Plant material

IQ67-domain 2 (IQD2; AT5G03040) and ABB8 (IQD1; AT3G09710) loss-of-function mutants (in Columbia (Col-0) and Wassilewskija (Ws-0) wild-type backgrounds, respectively) were obtained from Katharina Bürstenbinder (Leibniz-Institut für Pflanzenbiochmie) and are detailed in Zang et al. (2021) and Levy et al. (2005), respectively. Seeds were germinated and grown in a controlled environment room, under 22°C constant temperature and 16h daylength, for 14 days before being pricked out into custom ‘’pots’’ modified from 50ml Corning centrifuge tube lids (Figure 3.2). These lids were drilled with holes and covered in mesh to allow water uptake from below. Seedlings were grown on for a further week before use in feeding assays.

Figure 3.2. A: photograph demonstrating modifications made to 50ml Corning centrifuge tube lids, with holes drilled in the bottom and covered in mesh to allow contact with water and B: photographic example of *Arabidopsis* seedlings in modified corning tube ‘’pots’’, on damp blue roll to allow watering from below.

3.2.2.2 Insect material

CSFB were used from a laboratory stock population maintained in the John Innes Centre (JIC) insectary and screened for feeding activity six days prior to inclusion in assays (as previously described in Methods 2.2). Four beetles per assay were used, in a sex ratio of 2:2 males and females, after being starved for 24 hours.

3.2.2.3 Arabidopsis CSFB herbivory assays

A petri dish assay was designed alongside Anna Jordan in the JIC insectary as displayed in Figure 3.3. Arabidopsis plants were slotted into holes in an agar base to maintain moisture levels. Prior to inclusion in assays plants were imaged to have an undamaged document of each seedling. Non-choice (i.e. just a single *Arabidopsis* line) assays were run for 48 hours before beetle removal and damage scoring. Imaging was performed upon removal, maintaining the same orientation that they went into the assay, to give a document of overall plant damage. The intention was to use these before and after photographs to obtain changes in greenness and thus damage scores utilising ImageJ software. However, during the 48 hours seedlings grew substantially, meaning that greenness scores often increased despite being subjected to CSFB herbivory.

Instead, approximate percentage damage scores were obtained for six leaves per plant after removal and laying out on a white PVC board (Figure 3.4). Leaves were removing starting at a 12:00 o'clock position moving clockwise so that individual leaves were identifiable in photographs. Additionally, this ensured that the same portion of leaves were scored for all plants removing any selection bias whilst scoring. Images were also taken of cut leaves for potential future image analysis.

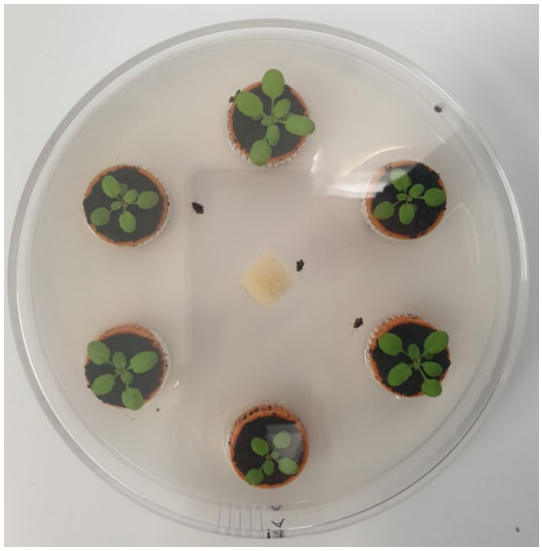

Figure 3.3. Non-choice *Arabidopsis* feeding assays, of whole plants in soil pots inserted into water agar.

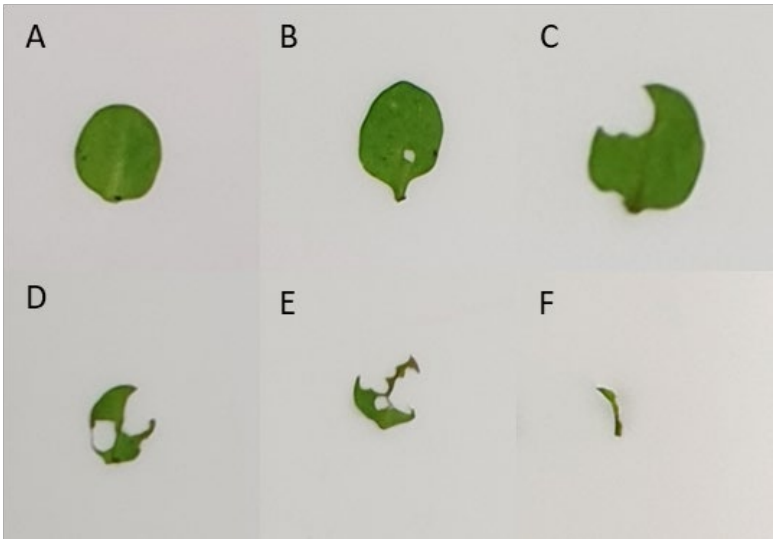

Figure 3.4. Examples of levels of feeding on *Arabidopsis* leaves. A: 0% (no feeding damage occurred). B: 1% (marginal feeding levels occurred but not 0%). C: 25%. D: 50%. E: 75%. F: 100% (or near to no leaf material left).

3.2.2.4 Statistical analysis

Mean feeding damage levels were analysed for the four *Arabidopsis* lines with a two-way ANOVA of *Arabidopsis* line and date of assay and an interaction term between the two. As with previous percentage damage presented in this thesis data was LOGIT+ transformed for analysis. Analysis was conducted using Genstat21.

3.3 Results

3.3.1 Genome wide association analysis identifies two clear loci and homoeologous regions associated with CSFB herbivory

Phenotypic CSFB feeding from six-way choice assays for 95 *B. napus* lines (see Results 2.3.1) were put through an AT pipeline (as per Harper et al. 2012 protocol) to identify genomic regions or gene expression linked to herbivory. Removal of SNP markers with an allele frequency of less than 0.05 resulted in ~255,000 markers for GWAS. Analysis using TASSEL identified a mixed linear model (MLM) as the optimal fit for CSFB herbivory.

No significant markers were identified associated with CSFB herbivory at the FDR of 0.05. However, 16 markers were associated with herbivory at an arbitrary cut off $P<10^{-4}$ (Table 1). Due to the polyploid nature of the *B. napus* genome there is a high degree of similarity between the homeologous A and C genomes. Where allelic polymorphisms are present within homoeologous sequences, upon sequence alignment, these generate an ambiguity code when compared to the reference base. These markers are termed as ‘hemi-SNPs’ i.e. cultivar S may have a C allele,

while cultivar R will have both a C and T allele, so be called as a Y base code. These are effective, dominant markers but cannot be anchored to a specific homoelogue and so are present on both genomes. Therefore, gene models for associated markers in Table 1, are anchored to both the A and C genome. The causal variation may only be present at one of these homeologous loci.

SNP QTL peaks are observed where markers are in linkage disequilibrium (LD) with the causal variant. The statistical association with the trait decreases as the distance from the causal variant increases. Peaks of marker association ($P<10^{-4}$) were observed on homeologous regions of chromosomes A02/C02 and A03/C03 (Figure 3.5), with the majority of 16 associated markers identified being present within these regions. These regions were selected for further investigation.

The most strongly associated marker, JCVI_1507:55, was located at the top of the peak on A03, within a gene model of the orthologues of *Arabidopsis* UBIQUITIN-CONJUGATING ENZYME 30 (UBC30; AT5G56150). Allelic variation at this marker identified *B. napus* genotypes carrying the A allele (n=5) received higher levels of herbivory (Figure 3.6). Furthermore, four out of five of these genotypes carrying this allele were swede morphotypes, suggesting susceptibility within this crop group. The majority of genotypes carried the R ambiguity allele for A and G (n=71). This indicates that genotypes with a G allele at either the A03 or C03 locus receive reduced CSFB feeding.

JCVI_10519:503, the most highly associated SNP for CSFB damage on chromosome A02/C02, is within the *Brassica* orthologue of PSEUDO-RESPONSE REGULATOR 7 (PRR7; AT5G02810). Two further markers within this gene were also associated with CSFB herbivory. The majority of *B. napus* genotypes carried the C allele (n=78) at this marker, which was associated with lower total CSFB herbivory than those with the T allele (n=8).

Table 1. SNPs associated with CSFB total feeding damage phenotypic scores above $P<10^{-4}$.

Brassica SNP marker	A Chromosome	C Chromosome	-Log10*P* value	*P* value	Arabidopsis orthologue	Gene annotation
JCVI_1507:55	A03	C06	5.182	6.57E-06	AT5G56150	Ubiquitin-conjugating enzyme 30
JCVI_4396:57	A03	C03	4.431	3.71E-05	AT5G55850	RPM1-interacting protein 4 (RIN4) family protein
JCVI_22654:430	A06	C07	4.300	5.01E-05	AT5G47970	Aldolase-type TIM barrel family protein
JCVI_18361:964	A03	C03	4.247	5.66E-05	AT5G57850	4-amino-4-deoxychorismate lyase
JCVI_27545:652	A10	C09	4.245	5.68E-05	AT5G14720	Mitogen activated protein kinase kinase kinase kinase 4
JCVI_28000:793	A09	C08	4.240	5.76E-05	AT3G51800	ERBB-3 binding protein 1
JCVI_20195:561	A03	C07	4.207	6.21E-05	AT4G30020	PA-domain containing subtilase family protein
JCVI_6849:531	A09	C08	4.188	6.49E-05	AT3G50790	Esterase/lipase/thioesterase family protein
JCVI_22673:421	A01	C01	4.159	6.93E-05	AT4G37000	Accelerated cell death 2 (ACD2)
EV195955:561	A10	C02	4.070	8.51E-05	AT5G03940	Chloroplast signal recognition particle 54 kDa subunit
JCVI_10519:503	A02	C02	4.063	8.64E-05	AT5G02810	Pseudo-response regulator 7
JCVI_19582:609	A06	C03	4.053	8.85E-05	AT5G65110	Acyl-CoA oxidase 2
JCVI_26321:511	A04	C04	4.053	8.86E-05	AT2G21270	Ubiquitin fusion degradation 1
JCVI_10519:375	Anng	C02	4.052	8.87E-05	AT5G02810	Pseudo-response regulator 7
JCVI_35451:678	A08	C08	4.051	8.89E-05	AT1G14830	DYNAMIN-like 1C
JCVI_10519:335	Anng	C02	4.002	9.95E-05	AT5G02810	Pseudo-response regulator 7

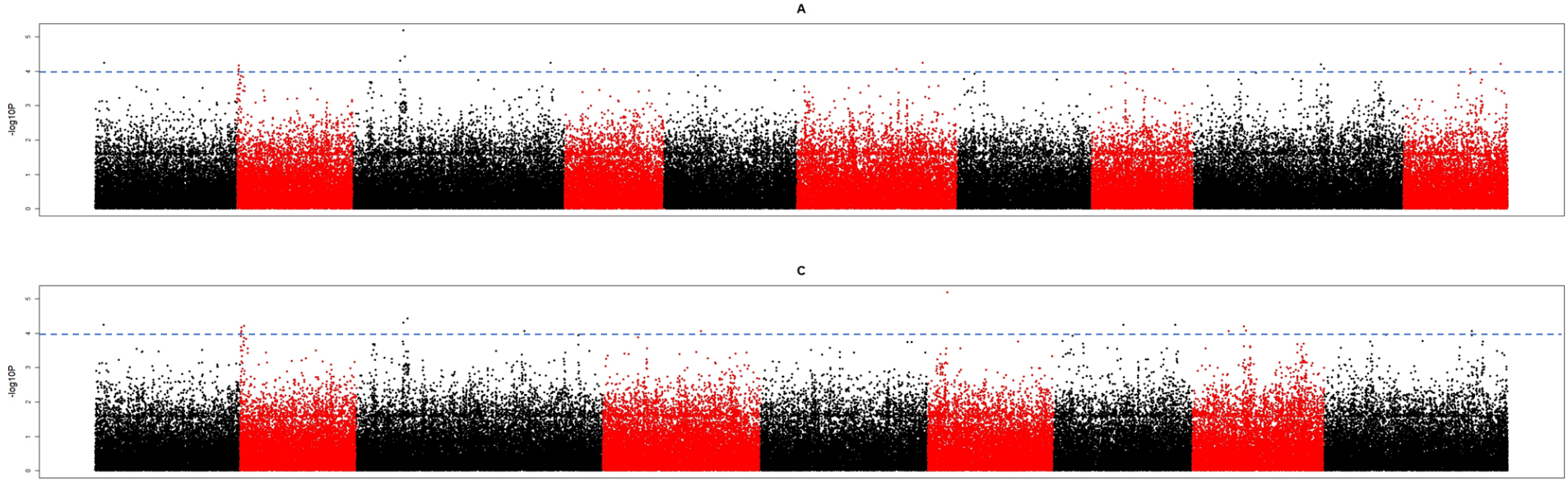

Figure 3.5. Manhattan plot demonstrating SNPs and their association with total CSFB feeding damage. The blue dashed line indicates an arbitrary cut off point of $P<10^{-4}$.

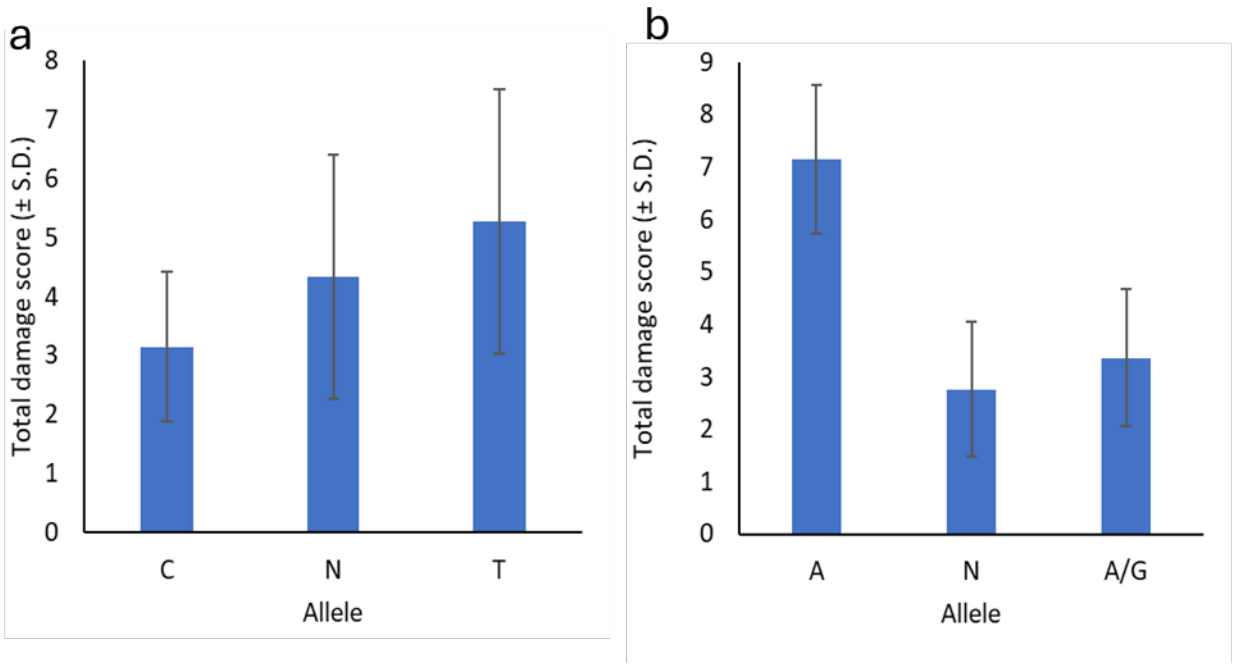

Figure 3.6. CSFB total herbivory and their allele calls for left: JCVI_10519:503 and right: JCVI_1507:55. Note that allele call of ‘’N’’ denotes no allele call.

Whilst statistically associated markers provide valuable information on genetic variation linked to CSFB herbivory, they are not necessarily causing the observed variation and may be linked to the causal genes. LD determines the region to which association with a causal variant extends and can vary across the chromosome and chromosomal positions. Therefore, to determine the extent of LD, and the size of the region where a causal variant may be found, LD analysis was performed for JCVI_1507:55 and JCVI_10519:503 LD analysis against all other markers on one of the homeologous chromosome pairs.

Analysis for JCVI_10519:503, on A02, demonstrated LD with r^2 over 0.15 over a 2.51Mb region covering 902 genes (Figure 3.16A). This region included a number of possible candidate genes including, CV544662, 23Kbp upstream of JCVI_10519:503, encoding an orthologue of *Arabidopsis IQ-DOMAIN 2* (At5g03040). For marker JCVI_1507:55, the most highly associated SNP for herbivory on chromosome A03, no LD was observed therefore a candidate gene region could not be determined.

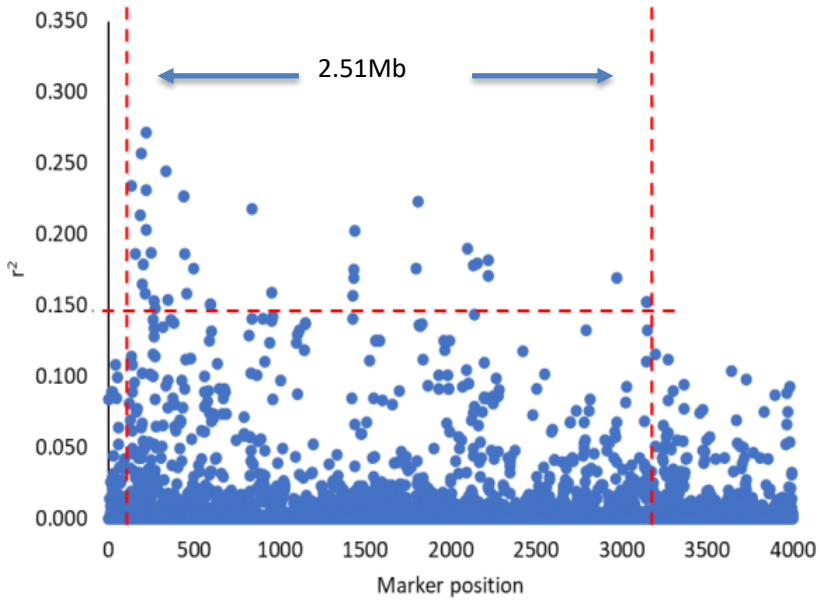

Figure 3.7. Linkage disequilibrium on chromosome A02 for marker JCVI_10519:503 for total CSFB feeding damage demonstrating a LD span of 2.51Mb. The horizontal red dashed line indicates a r^2 cutoff of 0.15 for LD and the vertical red dashed lines indicate the boundaries of LD.

3.3.2 Differences in gene expression are found to be associated with CSFB herbivory

Unlike SNP associations gene expression markers show a direct link between the level of gene expression and the trait of interest. Gene expression analysis via marker regression identified 17 significantly associated GEMs (FDR<0.05, *P*<0.00001) (Table 2). All 17 were positively correlated with CSFB feeding damage, indicating higher gene expression was linked to increased CSFB herbivory.

A peak in gene expression association was present on chromosome A03, with three of the significantly associated GEMs (FDR<0.05, *P*<0.00001) identified being present within this peak and corresponding with the genomic region of the SNP association. The marker at the peak on A03 A_JCVI_17072, a gene encoding LORELEI LIKE PROTEIN (LLP).

Table 2. GEMs associated with CSFB total feeding damage (FDR<0.05).

Gene model	Chromosome	-Log10*P* value	*P* value	Arabidopsis orthologue	Gene annotation
A_JCVI_16258	A09	6.414	3.85761E-07	AT2G16485	NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION, NERD
A_EX137858	A05	6.122	7.5585E-07	AT2G30490	CINNAMATE 4-HYDROXYLASE
C_JCVI_2920	C08	6.053	8.84243E-07	AT1G06460	ALPHA-CRYSTALLIN DOMAIN 31.2,
A_JCVI_27501	A09	5.896	1.2701E-06	AT1G13120	EMBRYO DEFECTIVE 1745
C_EV009430	C06	5.604	2.48732E-06	NH	
A_JCVI_2920	A08	5.561	2.74761E-06	AT1G06460	ALPHA-CRYSTALLIN DOMAIN 31.2,
A_JCVI_23190	A02	5.552	2.80803E-06	AT5G01530	LIGHT HARVESTING COMPLEX PHOTOSYSTEM II
A_EV009430	A01	5.535	2.91614E-06	NH	
A_DY009335	A07	5.460	3.47054E-06	NH	
A_JCVI_17072	A03	5.288	5.15053E-06	AT5G56170	LLG1, LORELEI-LIKE-GPI-ANCHORED PROTEIN 1
A_EV196428	A04	5.248	5.64846E-06	NH	
C_DY009791	C02	5.226	5.9418E-06	NH	
A_JCVI_26505	A09	5.219	6.04641E-06	AT5G48385	FRIGIDA-like protein
C_DY009335	C05	5.162	6.88791E-06	NH	
A_JCVI_36078	A03	5.117	7.64713E-06	AT5G55500	BETA-1,2-XYLOSYLTRANSFERASE
A_JCVI_41243	A03	5.053	8.85939E-06	AT4G33180	alpha/beta-Hydrolases superfamily protein
C_ES266017	Cnn	5.048	8.94753E-06	AT1G53290	GALT9

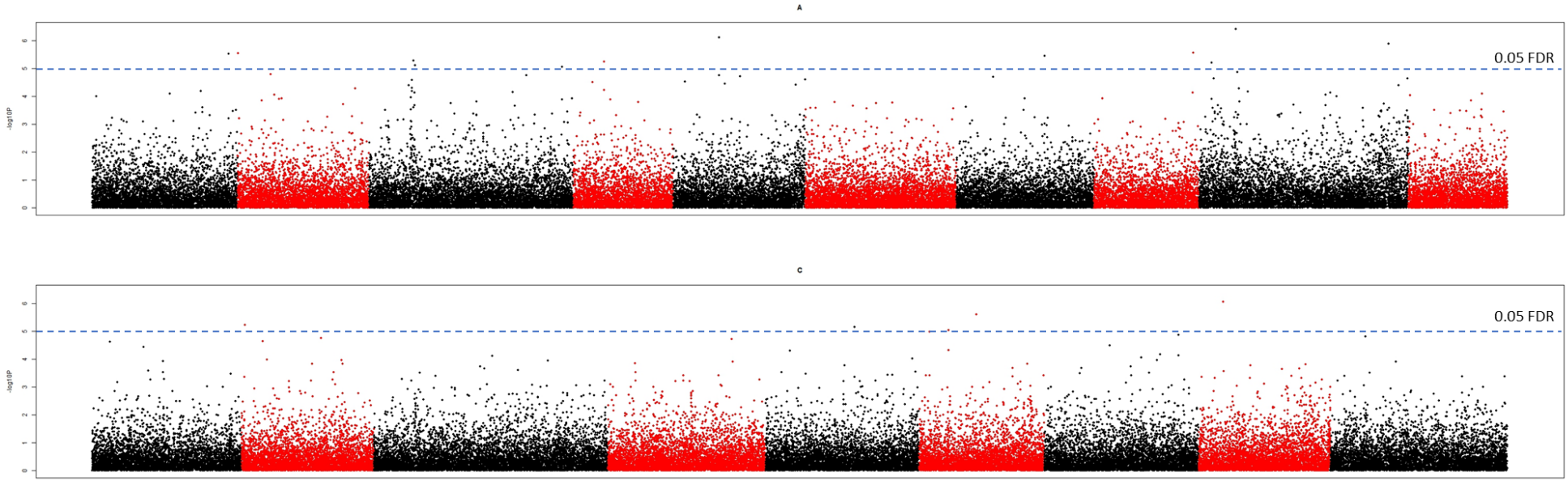

Figure 3.8. Manhattan plot demonstrating GEMs and their association with total CSFB feeding damage. The blue dashed line indicates the 0.05 FDR.

3.3.2 Assays on *Arabidopsis* mutants confirm candidate genes associated with CSFB herbivory in *B. napus*

IQ-DOMAIN 2 (*IQD2*), 20Kbp upstream of JCVI_10519, the most significant associated SNP marker on chromosome A02, was chosen from the 902 genes in LD for further study. Furthermore, *IQD1* (a closely related gene to *IQD2*), has previously been implicated as a plant defence gene and overexpression linked to reduction in insect herbivory (Levy et al., 2005; Barda & Levy, 2022). Therefore, *IQD2* and *IQD1* knockout mutants were selected for testing in CSFB herbivory assays. Non-choice assays were conducted using *IQD2-2*, a knock-out mutant for *IQD2* in a wild-type (WT) background of Columbia (Col-0), *ABB8*, a loss-of-function of *IQD1* mutant in a WT Wassilewskija (Ws-0) background and their WT controls.

Two-way ANOVA identified statistically significant differences in the LOGIT+ transformed CSFB herbivory levels (P= 0.024) were observed between *Arabidopsis* lines (Figure 3.17). Fisher's multiple comparisons testing demonstrated mutant lines *IQD2-2* and *ABB8* received significantly higher levels of damage compared with both wild-type controls Ws-0 and Col-0 (P<0.05). The two mutant lines and two control lines did not significantly differ from each other.

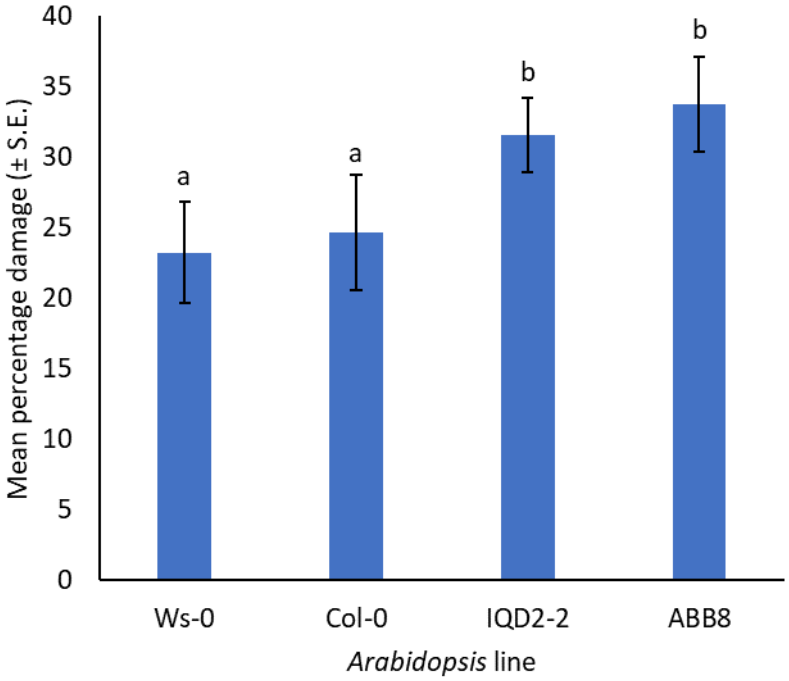

Figure 3.9. Mean percentage CSFB feeding damage for four *Arabidopsis* lines, two controls (Ws-0 and Col-0,) and two mutants (IQD2-2 and ABB8) (± Standard Error, n = 4, P<0.05).

3.4 Discussion

Associative transcriptomics has been widely used in *Brassica* to look at multiple traits including yield, quality and glucosinolate content (Miler et al., 2019; Kittipol et al., 2019). Bankes-Jones (2016) also used this methodology to look at herbivory from the grey field slug (*Derocerus reticulatum*) showing significant associations with glucosinolate level. Here we demonstrate that AT can be successfully used to identify loci associated with total CSFB herbivory.

GWAS did not identify any markers significant at the 0.05 FDR. This was anticipated as accurate due to the potentially quantitative nature of pest herbivory resistance and phenotyping is notoriously difficult, with variation in beetle behaviour affecting phenotype scoring quality. Despite this, SNP QTL peaks of marker association ($P<10^{-4}$) for herbivory damage were observed on homeologous regions of chromosomes A02/C02 and A03/C03, with the majority associated markers being located within these regions, thus providing strong evidence for loci influencing CSFB feeding. Three SNPs within JCVI_10519:503, on chromosome A02/C02, associated with CSFB herbivory were within the *Brassica* orthologue of PSEUDO-RESPONSE REGULATOR 7 (PRR7; AT5G02810). PPR7, is involved in circadian rhythm and though the gene itself does not have any specific role in preventing herbivory, many metabolites that accumulate within plants have cyclical rhythms. Jasmonate, a major plant defence hormone, accumulates under circadian control (Goodspeed et al., 2012). Goodspeed (2013) showed glucosinolate accumulation in *B. oleracea* varied rhythmically across the day, with total glucosinolates accumulating at dawn, being elevated during the day and declining in the evening. Furthermore, when plant circadian rhythms, and therefore metabolite cycling, were out of phase (timing) with the rhythm of their insect herbivore *Trichoplusia ni* (cabbage looper), greater damage and enhanced larval growth was observed, illustrating the role of the clock in protection from plant herbivory (Goodspeed et al., 2013).

Statistically associated markers identified by GWA may not be responsible for the observed phenotype and may be linked to the causal genetic variation. LD for the loci on A02/C02 covered 902 genes. Within this region several other candidate gene were present, including *CDL1* (At5g02800), a gene that regulates brassinosteroid signalling and plant growth but has also been implicated to be involved in immune responses to pathogens (Rao et al., 2018). Calmodulin binding gene, IQD2 (At5g03040) was located 20Kbp upstream of JCVI_10519. There are 33 members of the IQD family that have been identified in *Arabidopsis* and the protein family play a role in calcium signalling (Bürstenbinder et al., 2017). The closest *Arabidopsis* homologue to IQD2, IQD1 (At3g09710), is a positive regulator of glucosinolate accumulation and plant defence responses, with overexpression reducing plant herbivory by *Myzus persicae*, the peach potato

aphid and increasing resistance to the necrotrophic fungus *Botrytis cinerea* (Levy et al., 2005; Barda and Levy, 2022). Our CSFB feeding assays for *IQD1* and *IQD2* mutants show statistically increased herbivory when compared to WT suggesting these genes also play a role in resistance to CSFB. However, the current assay set up demonstrated here is not optimal and therefore further assays should be performed to confirm these preliminary observations.

The most highly associated SNP for herbivory on chromosome A03, JCVI_1507:55 within the orthologues of *Arabidopsis* UBIQUITIN-CONJUGATING ENZYME 30 (UBC30; AT5G56150) was not identified to be within LD with other markers on the chromosome therefore the extent of the candidate gene region could not be determined. However, a peak in gene expression association was also present on chromosome A03, with three of the significantly associated markers (FDR<0.05, *P*<0.00001) identified being present within this peak. Gene expression is unlikely to influence the expression of adjacent genes, therefore the association of the expression of multiple genes in a region is not expected. This can occur however, if there is a region of presence/absence variation or structural rearrangement present at that genomic location within multiple genotypes. Gene expression and presence absence variation in this area should be considered in the future to determine the size of any genetic rearrangements present and therefore number of potential candidates. Sequencing of resistant and susceptible lines will help clarify the genomic architecture in this region.

In total, 17 GEMs were correlated with CSFB herbivory (FDR<0.05). Unlike SNP association, unless an area of gene deletion is suspected, these markers identify potentially causal genes. The GEM marker at the peak on A03 A_JCVI_17072, is an orthologue of *LORELEI LIKE PROTEIN* (*LLP*). LLP has been shown to associate with the receptor kinase FLAGELLIN SENSING 2 (FLS2) to recognise flagellin, a pathogen-associated molecular pattern (PAMP), and activate downstream immune responses. *llg1* mutants show enhanced susceptibility to powdery mildew (*Golovinomyces cichoracearum*) strain UCSC1, oomycete pathogen (*Hyaloperonospora arabidopsidis*) Noco2 and the bacterial pathogen (*Pseudomonas syringae*) pave *tomato* (*Pto*) DC3000, indicating that LLG1 has an important role in plant immunity (Shen et al., 2017; Chen et al., 2022). Associated marker, A_EX137858 on A05, is within an orthologue of *CINNAMATE-4-HYDROXYLASE (C4H,* At2g30490), a gene involved in phenylpropanoid biosynthesis and development (Kim et al., 2021). Mutants of *C4H* accumulate decreased levels of several different classes of phenylpropanoid end-products and exhibit reduced lignin deposition and altered lignin content. These plants also accumulate cinnamoylmalate, a novel hydroxycinnamic ester (Schmiller et al., 2009). The C05 homeologue of *CH4* was also identified by AT as being associated with disease resistance against *Pyrenopeziza brassicae*, light leaf spot, in *B. napus* (Fell et al., 2023), however, unlike our positive correlation, this was negatively correlated with resistance suggesting protection from increased phenylpropanoid and lignin. Associated gene JCVI_2920, an orthologue of *ALPHA-CRYSTALLIN*

DOMAIN 31.2 (*ACD32.1, At1g06460*) on C08, encodes a small heat shock protein. Again, this gene has a role in circadian rhythm (Chandler & Melzer, 2004) but has been demonstrated to be strongly repressed in Poplar trees when exposed to insect herbivory (Ralph et al., 2008). As gene expression for all of these genes demonstrated a positive correlation with CSFB damage, future work should consider testing knockout mutants in *Arabidopsis* or *Brassica* for levels of herbivory.

4 Discussion

4.1 Laboratory feeding assays can effectively identify differences in feeding by CSFB

Insect studies are extremely challenging. Laboratory experiments require a supply of suitable insects for assay, either collected from the field or reared in house. Field collected beetles will be subject to availability due to lifecycle or yearly variation therefore within this project we developed protocol to maintaining an active population of adult CSFB. Maintenance of large populations is challenging however these capabilities are essential for effective controlled CSFB research.

Few studies have quantified the CSFB herbivory differences between large numbers of *B. napus* lines under laboratory or field conditions. Most focus on smaller numbers of *Brassica* varieties, samples of leaf tissue rather than complete plants or other pests of cruciferous crops. Barlet and Mithen (1996) assessed differences in CSFB on whole plants but only for eight varieties eight *B. napus* varieties. They found a significant difference between lines for number of CSFB shot holes in leaves under laboratory conditions. However, they only discuss these differences further in the context of glucosinolate content and do not find a relationship between CSFB herbivory and glucosinolate content. No attempts have been performed to map the basis of differences in CSFB feeding.

Novel six-way choice assays examining adult CSFB herbivory of intact *B. napus* seedlings allowed us to identify variation between varieties under laboratory conditions. These differences were not statistically significant; however, this is likely due to the low discriminatory power of the experiment, with low replication levels (n = 3) and multiple choice of food source in a relatively limited spatial range. Differentiation between food sources by CSFB in such an environment may be problematic if olfaction is involved in host selection (Henderson et al., 2004).

From the preliminary six-way choice assays we were able to select two *B. napus* genotypes from the extremes of the feeding range observed to further characterise CSFB herbivory, S as a genotype with high and R, a genotype with low, levels of CSFB herbivory, indicating a more susceptible and more resistant/tolerance variety, respectively. In either a two-way choice between *B. napus* varieties or no choice, i.e. a single variety, we recorded a strong difference between S and R genotypes, with S receiving much higher levels of feeding damage. This suggests that large scale screening in choice environments is effective at identifying lines showing variation. Interestingly, the differences in feeding are retained and even more apparent when there is no alternative food source offered, suggesting that this would be effective in field environments where no choice is given.

Taken together, although running assays with multiple varieties provides a higher throughput method for screening plant material, this indicates the no choice assay is the most sensitive for screening for herbivory. Further adjustment will allow uptake of this as a phenotyping methodology for CSFB palatability. Indeed, optimised no choice feeding assays have been adopted in the BR2CSFB BBSRC IPA project: A toolkit for breeding resistance to adult and larval herbivory by the cabbage stem flea beetle. This collaboration between the John Innes Centre, Rothamsted Research, AHDB and eight different breeding companies is following up the observations within this study as well as researching larval resistance. Methodology for adult CSFB feeding assays has been transferred to industry and are now being used to screen commercial materials for the breeding of resistant varieties.

Currently the basis of selection for feeding by CSFB and the influence of metabolites is unknown. The difference if feeding levels observed in multi-choice, two-way choice and no choice environments suggests that decisions to feed may be initiated by volatile detection, which can get confused in assays containing multiple *B. napus* varieties (Hendersen et al., 2004). Indeed, Bartlet et al. (1999) identified sensilla likely involved in olfactory sensing on CSFB. Assays to determine the effect on flea beetle behaviour in chambers may provide a greater understanding of the feeding differences. Additionally, using a Y-tube setup and monitoring beetle movement and choice may provide further information.

To determine the genetic nature of herbivory we sought to further clarify differences between S and R and the F_1 of a cross of these parental lines. In both multiple choice and non-choice experiments, CSFB damage to the F_1 did not differ significantly from R parental line. Additionally, the F_1 seedlings were similar in appearance to their R parent (personal observations). Given the visual and damage level similarities, it is possible that the resistance trait of R is the dominant. If time had permitted, further exploration of the segregation of resistance and susceptibility by within the F_2 generation would provide further information on the genetic control of the trait.

In this same experiment, we additionally aimed to test a new cotyledon damage scoring method using an ImageJ based pipeline (Thursfield, 2022) and compare this to human estimated damage scores. Percentage damage scores derived from ImageJ were consistently about half the value of visually estimated scores. A strong, positive correlation was observed between the scoring methodologies, demonstrating both as valid techniques for capturing differences in adult CSFB cotyledon herbivory. ImageJ provides more precise damage values, but visual estimates are still an appropriate way to distinguish real differences in *B. napus* palatability between varieties. ImageJ software has been successfully used to assess herbivory damage of other important crop pest species such as snails (Stawarczyk and Stawarczyk, 2015) and thrips (Visschers et al., 2018). Therefore, we demonstrate that it may be beneficial for future studies to utilise such computation methods for obtaining damage data for CSFB on *B. napus*.

Overall, from laboratory feeding assays, we demonstrate a series of novel adult CSFB feeding assays, which resulted in successful identification of a more resistant and more susceptible genotypes. High-throughput six-way choice assays enabled the selection of extreme *B. napus* genotypes from a large panel of lines however non-choice assays provide much clearer feeding preferences. Utilisation of computational scoring pipelines provide greater accuracy for scoring CSFB feeding damage.

4.2 CSFB herbivory differences in the laboratory are conserved in the field environment

From field trials in 2019 and 2020 assessing CSFB damage levels for a subset of *B. napus* varieties from the DFFS and commercial lines, we observed support for previous laboratory results on adult CSFB herbivory damage. The 2019 trials suffered high levels of pest damage immediately after drilling. Coupled with dry, hot conditions, establishment was generally poor, and the trials prematurely ended, despite having two field sites. Establishment was much more successful in 2020 field trials, with less pest pressure and better weather conditions. These results highlight the impact of the environment on establishment and the need to ensure correct seedbed conditions for sowing (Ortega-Ramos et al., 2021).

The ability to detect significant differences between lines varied between years. This is potentially attributable to the scoring methodology used. In 2019, 20 seedlings were sampled from border runs and cotyledons scored in the laboratory for percentage damage (visual estimates to the nearest 5%). This may have introduced bias, as to select 20 seedlings meant they had to be present, and thus does not give a full representation of how empty or damaged a plot may have been. Seedlings where just small bits of plant material remained (i.e. mostly completely eaten) were potentially less likely to be selected for scoring back in the laboratory. In 2020, we scored percentage damage to cotyledons in the field for a continuous run of 10 seedlings. Although this may reduce observation time and therefore accuracy and used a smaller sample size, this methodology may have been more appropriate as seedlings with high levels of damage would have been recorded. We did not observe a correlation for damage data between the 2020 and 2019 field trials. This is perhaps unsurprising given differences in scoring methodology and the high levels of damage observed in 2019. These data demonstrate how variable CSFB damage can be year on year. Damage in the field in 2019 correlated strongly with damage scores obtained in the laboratory, providing further support for differences between our R and S genotypes. Utilisation of computational scoring pipeline for efficient and accurate damage estimation would be highly beneficial for future studies. Bereciartua-Pérez et al. (2024) used a deep learning pipeline to quantify flea beetle damage and compared this with manual scoring. Image detection of cotyledons was effective however, the training data only allowed for detection of up to 60% damage so heavily damaged plants could not be scored. Occlusion and effects of waterdrops or shine within images

prevented accurate measurement. As with our comparisons with computational scoring, it was found that manual estimation overestimated the damage present (Bereciartua-Pérez et al., 2024). Advances in field phenotyping would be of great benefit to industry for not only selecting lines for breeding but also to monitor field damage levels and advise thresholds. However, accurate measurement of field damage remains a major challenge for field studies.

When inspecting drone images, there was a clear difference in plant coverage between plots and *B. napus* varieties. Swede type *B. napus* varieties had a significantly lower seedling count compared to WOSR lines. Establishment and herbivory scores were negatively correlated, indicating that *B. napus* varieties with higher levels of damage resulted in lower establishment. This shows a direct effect of the level of CSFB damage on field establishment.

In pyrethroid treated plots, we also observed a significant effect of *B. napus* variety on amount of CSFB damage, with R and S lines behaving as expected, despite these differences being more subtle. Establishment scores were also marginally higher in treated plots. Together, this suggests pesticide treatment was effective, offering some protection, albeit minor, to more susceptible *B. napus* varieties, and thus lessening differences between them and the more resistant varieties. Later in the growing season the differences between pesticide treated and non-treated plots became more apparent with differing plant sizes (personal observations), again suggesting benefit from the pesticide treatment. Effectiveness of pyrethroid sprays is known to vary with location, with White and Cowlrick (2016) showing in Lincolnshire and East Yorkshire 85% and 55% of treatments respectively were deemed to provide more than 50% control compared to 0% of treatments in Bedfordshire and Essex. With the rise in resistance against pyrethroids reliance on spraying is advised as a last resort (AHDB, 2024).

Overall, our results demonstrate that differences observed between R and S line in the laboratory are maintained in a field environment. However, our trials demonstrate how challenging establishment can be when conditions are not optimal at sowing and heavy herbivory from pests is present. Pyrethroids still provided some level of protection to support establishment.

4.3 Associative transcriptomics identifies loci associated with CSFB herbivory

Genome wide association analysis has been used for multiple phenotypic traits for multiple crops over the last 20 years however performing such studies for insect resistance are highly challenging. The power of GWAS is reliant of robust phenotyping methodologies which must be performed across large panels of genotypes which may be difficult with insect availability and variability between insects. Despite these challenges recently studies have been published on maize (Badji et al., 2020), soybean (Hanson et al., 2018; Almeida-Sila and Venancio, 2023) and rice (Zhou et al., 2024).

With the limited discrimination between lines detected using 6-way choice chamber we did not observe any SNP markers significantly associated with CSFB herbivory at the 0.05 FDR. However, 16 SNPs associated with CSFB herbivory were identified at $P<0.0004$, with four clearly associated loci detected at two homeologous locations, A02/C02 and A03/C03. The current AT pipeline does not allow discrimination between the highly conserved constituent genomes on *B. napus*. The sequence alignment must allow for the levels of variation between the genotypes whilst being stringent enough to resolve between genomes. Updated genome references and updated mapping approaches may help resolve associations between genomes.

Despite this, calculation of LD allowed discrimination of the A02/C02 region to 902 potential candidate genes. LD was not apparent for the A03/C03 peak however a gene expression marker association peak was present within this area. Gene expression is usually gene specific within a region, therefore multiple associations suggest genomic structure variation from insertion, deletion or homeologous exchange. In the future, genome sequencing approaches will identify potential structural variation and further define and potential candidates within both regions. All the genes identified within Chapter 3 warrant further investigation as potential candidates conferring the causal phenotypic variation in CSFB feeding.

4.4 Arabidopsis assays identify potential genes influencing herbivory

To determine the role of candidate genes in CSFB feeding we developed phenotyping assays for the model plant *Arabidopsis*. As a member of the Brassicaceae and close relative of our crop *Brassica's* it provides an excellent system for testing gene function, with mutants readily available. Hallet et al. (2005) previously used *Arabidopsis* arenas for screening herbivory by *Phyllotreta cruciferae* however plants were only grown to the first second leaf stage and scored visually. The *Arabidopsis* assay set up demonstrated here is not optimal and therefore further assays should be performed to confirm these preliminary observations. Computational scoring systems are being developed to accurately quantify beetle damage on *Arabidopsis*. We attempted to perform this using ImageJ software to capture changes in greenness scores and thus herbivory however, over the 48 hours of the assay, *Arabidopsis* seedlings grew significantly. Experimental and quantification pipelines need adapting to account for plant growth during assays. This method could be adapted and used in future research to phenotypically quantify CSFB herbivory of other *Arabidopsis* mutants for other candidate genes.

Testing both the candidate gene *IQD2* (At5g03040), located 20Kbp upstream of the peak on A02/C02 and the closest *Arabidopsis* homologue, *IQD1* (At3g09710), we demonstrated mutants in both genes show increased herbivory by CSFB when compared to WT, suggesting these genes play a role in resistance. *IQD1* has previously been shown to be involved in herbivory by *Myzus persicae* and resistance to *Botrytis cinerea* (Levy et al., 2005; Barda and Levy, 2022). Following

confirmation in *Arabidopsis*, the effect of these genes should be assessed in *Brassica* either using mutants, genome editing or GM approaches. This is the first indication that it may be possible to select for genes reducing CSFB palatability offering the potential to move towards breeding for CSFB resistance.

4.5 Conclusion

In conclusion, this project demonstrates a research pipeline that successfully identified variation in CSFB herbivory variation exists between *B. napus* genotypes, both in the laboratory and field environment. Future research should further explore the attributes of the S and R genotypes that make them more and less palatable, respectively, to help develop an understanding of the cues for herbivory on OSR and factors controlling levels of feeding. Using AT, we identified candidate genes linked to phenotypic differences. Further work should be performed to dissect the underlying genetics and mechanisms conferring differences in herbivory, including further follow up of the potential candidate genes which may be of use to breed for insect resistance. Developing a deeper understanding of the interaction between insects and plants offers the potential to understand how we can use plant resistance as part of an integrated pest management approach to crop protection.

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