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2023 to 2024

Project title	Varietal resistance to feeding (herbivory) by the cabbage stem flea beetle (CSFB) in oilseed rape		
Project number	21120219		
Start date	21/11/2021	**End date**	21/11/2025

Project aim and objectives

This project aims to determine the genetic controls and mechanisms underlying variation in feeding by adult cabbage stem flea beetle (CSFB) and larval resistance in *Brassica napus*. Working with breeders we will exploit outputs to help develop crops with resilience against CSFB. There are three main objectives:

Objective 1: Characterising the genes associated with adult CSFB feeding and the mechanisms of plant resistance

- Using genome sequences from lines with contrasting CSFB feeding, we aim to identify the genetic causes of variation.
- Further experiments will identify the mechanism(s) by which these genes affect CSFB feeding and thus support breeding for resistance.

Objective 2: Understanding the basis of variation underlying reduced CSFB palatability and exploitation for breeding

- Using a mapping population, we aim to identify loci associated with reduction in feeding.
- Gene expression analysis will be used to investigate plant-CSFB interactions and identify responses induced by CSFB feeding.
- Metabolite analysis will be employed to establish a link between causal loci, gene expression and compounds that influence beetle feeding.
- The germplasm and data will be used with breeding companies to select for adult CSFB feeding resistance.

Objective 3: Understanding the basis of variation in larval resistance to CSFB in *B. napus* and exploitation for breeding

- Using diverse *B. napus* and breeding material, we aim to identify lines showing the lowest adult emergence and characterise the effect on larval development.
- These data will be used to identify loci associated with this resistance.
- This will be combined with gene expression, metabolite analysis and candidate gene studies to develop our understanding of plant-CSFB larvae interactions.
- Resulting knowledge will be shared with breeding companies to accelerate breeding for resistance in oilseed rape (OSR).

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Key messages emerging from the project

1. Methodologies for rearing CSFB and screening germplasm for CSFB damage have been optimised, providing the ability to screen large germplasm collections for adult and larval CSFB susceptibility. These have been successfully transferred to industry. Variation exists in levels of adult CSFB feeding within current varieties and breeder's material. Breeder selection is improving varietal resistance.
3. Previous observed variation in adult CSFB feeding within a diverse collection has been confirmed both in controlled and field conditions. Two regions controlling this variation have been mapped. Candidate genes are being tested.
4. Gene expression analysis has identified plant responses to beetle feeding differ from those observed for mechanical damage suggesting a distinct response to flea beetle damage. Responses have been identified that are specific to resistant lines and genes have been selected for further study.
5. Detailed metabolite analysis on cotyledonary tissues from diverse germplasm screened shows a wide range of sugars, waxes and secondary metabolites are present. This is being used for gene identification and association with herbivory.
6. Controlled environment larval screens on a diverse population have identified a range of variation for larval survival. Extreme differences were not confirmed in further trials. Further work is ongoing.
7. Analysis of data from field trials suggests variation in larval numbers between lines. Final analyses need performing to determine outcomes.

Summary of results from the reporting year

<u>Enabling technologies: Optimisation of CSFB rearing, screening for adult and larval resistance, and transfer of skills to industry</u>

Methodologies for rearing and screening germplasm for CSFB damage have been transferred to industry partners.

The use of AI is currently under development for cotyledon damage (PhD project collaboration Huazhong Agricultural University (HZAU), China) and automatic scoring of larval instars (JIC Bioinformatics).

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Objective 1: Sequencing of parental lines showing variation in adult CSFB feeding

Variation in adult feeding previously observed between the two lines identified in Obj1 was previously shown to be reproducible in both laboratory settings and in the field.

Oilseed rape shows variation in genome organisation, gene copy number and sequence variation. To allow us to identify causal genetic variation linked to differences in feeding it is therefore useful to have genome sequence data from the exact plant material being used for studies. Our high-quality genome assemblies produced for the two lines showing extreme variation in CSFB palatability identified considerable genetic variation between the two lines. These data have allowed us to determine the genes present within the regions associated with CSFB adult feeding (C02, 742 gene models; A03/C03; 384 gene models). This still represents a considerable number of candidates. Gene expression marker analysis has identified six genes where expression is significantly associated with adult CSFB shot holing damage (FDR<0.05). One of these sits within the A03/C03 region and is implicated to be involved in plant defence signaling in *Arabidopsis*.

- Development of assays within *Arabidopsis*, the model plant, for screening potential candidate genes controlling adult feeding and testing of mutants

Arabidopsis is a small model plant with a simple genome for which many resources exist to study gene function. Analysis of selected mutant lines by associated AHDB-funded PhD student, Jessica Hughes, have shown mutants within candidate gene on A02/C02 and a related family member show increased levels of feeding, suggesting gene function supports resistance. Feeding assays in *Arabidopsis* and image analysis have been further optimised to allow higher throughput screening for further testing of candidates. Testing of candidates is underway.

Objective 2: Benchmarking the adult feeding tolerance trait against current commercial material

Results show variation in levels of adult feeding in controlled trials within material provided by our breeding partners. Results of individual lines have been transferred to specific breeding partners to develop an understanding of where their material sits within the distribution of material tested. This suggests that there is likely to have been selection for resistance for adult feeding in recent breeding history. Adult feeding assays performed in the field on breeder's material has also been recorded for both 2021,2022 and 2023 field seasons. Data analysis is still in progress.

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- Population production for the genetic mapping of adult CSFB feeding

~250 DH lines were produced form a cross between a high and low palatable line. Unfortunately, bulking failed to produce the seed quantities required for trialling. 100 lines were selected for further work and these have been bulked at JIC. Lines have been transcriptome sequenced and SNP genotype and gene expression for gene models has been determined. Genetic mapping is underway.

Lines have been phenotyped for flowering characters, plant morphology and trichome variation in both the polytunnel and an unvernalised glasshouse experiment. These data will be used to discover genetic regions associated with these traits. For flowering there is knowledge on the genes controlling this trait so this will act as a positive control to determine the quality of the mapping.

A replicated field trial was established at JIC and sampled for adult CSFB feeding, however beetle numbers were low in the field this autumn. Seed was supplied for a second trial at Rothamsted Research (RRes) but due to timing and weather conditions trial sowing was not completed.

Further population seed is being threshed for distribution to breeding partners as a population developed from a diverse cross.

- Gene expression/metabolite profiling for determining chemical association with adult feeding

Gene expression experiments were previously performed to determine the effects of beetle feeding on gene expression and induction of plant defences when compared to mechanical damage and undamaged control. This analysis was performed at three timepoints (2, 4 and 8 hours) for both our resistant and susceptible lines. Principle component analysis of the RNA-seq data showed clear clustering by treatment suggesting differences were present. Differential gene expression analysis identified the number of genes showing differences in expression increased across the time-period with around 2500 genes being common to all timepoints in the resistant line.

Clustering of these genes produced some clear classes of gene expression behaviour. Genes could be classed as a) being upregulated/downregulated with mechanical damage and feeding, thus showing a general response to damage; b) being specifically upregulated to damage in the resistant line only; c) showing a specific response to beetle feeding in both lines and d) showing a specific response to beetle in either resistant or susceptible lines. This analysis suggested that some of the responses observed by the plant are specific to beetle feeding and there is variation in response between resistant and susceptible lines.

New analysis of patterns of gene expression of these genes across time have identified 5 genes showing time series responses to beetle feeding. These genes have been selected as interesting candidates and are undergoing further analysis.

Gene ontology (GO) term analysis, which looks at genes according to their function and biological process, identified that these genes were involved in a number of plant responses including a) response to jasmonic acid (JA), an important plant hormone involved in host responses to pathogen infection and herbivore damage, b) indole containing metabolic process (defence compound synthesis) and c) response to chitin, a primary component of cell walls in fungi and the exoskeletons of arthropods such as crustaceans and insects. Analysis has been performed to look at hormones salicylic acid (SA), which is antagonistic to JA, and JA levels to confirm the gene expression results. In control samples, SA levels did not differ between varieties. After 2 hrs of beetle feeding SA was elevated in the susceptible variety but this difference was not maintained at 8 and 24 hours. SA levels appear relatively constant across all treatments with no significant changes observed. JA level was very low in control samples but increased upon beetle feeding across time, showing activation of plant defence response. JA was also higher in the susceptible variety at 2hr, with no significant differences were seen at later timepoints. From our results it does not appear that SA induction reduces JA response thus supressing responses leading to susceptibility. There is no difference between genotypes in response across the time course.

Reactive oxygen species (ROS) are involved in the signal transduction process associated with plant growth and defence. ROS is produced by Brassica on application of a chitin solution. No ROS response has been detected in response to beetle extract or frass.

Cotyledon surface compounds and internal metabolites may influence feeding by CSFB. Protocols have been developed for the quantification of surface compounds, such as waxes, phenolics, sugar alcohols (polyols), and internal compounds, such as sugars, glucosinolates and polyphenols (e.g., phenolic acids, flavonoids). These compounds have previously not been measured on cotyledonary tissue. Quantification of these compounds has been performed on the diversity set previously screened to identify adult beetle feeding. These data will be correlated with beetle feeding data and used for genome wide association analysis to identify loci associated with metabolite production.

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Objective 3: Phenotypic analysis of variation in larval infestation in breeding material

In 2021/22, a field trial of 20 varieties (x 5 replicates) was performed at JIC and RRes research sites. Material was assessed for establishment, feeding damage, fresh weight, dry weight, vigour, flowering, yield, seed size and oil content. Larval evacuation experiments in December and February were used to assess variation in larval numbers between lines at JIC. At RRes, analyses were performed monthly to determine differences in larval number and development between lines. Statistical analysis identified variation in larval numbers exists between lines however large variation existed between sites. Analysis of monthly data identified significant differences between the number of larvae between varieties, but these differences were not consistent across months. However, varieties that often have less larvae than the mean between varieties can be identified.

Replicated field trials established for 2022/23 suffered from poor establishment at the JIC site due to unknown underlying soil conditions. Establishment was successful at RRes and assessed for the same variables as in 21/22. To gain further data, this was repeated for the 23/24 season.

Samples from JIC have been imaged and are currently in use to develop and AI scoring pipeline.

- Phenotypic analysis of variation in larval success in diverse *Brassica* material

Previous observations of differences in adult emergence from diverse *Brassica* lines could not be produced in replicated trials. This was believed to be due to variation in hatch rate of eggs for plant infestation. Improved methodology involving the addition of one day old larvae was developed and material taken through to adult emergence for one replicate of the diversity set. However, large variation in adult emergence was observed within the control lines of *B. napus* and *S. alba*.

A new protocol similar to methodology published by Doring and Ulber (2020) has been developed for a more rapid larval assay system. This protocol has been used for screening the diverse panel of lines previously assessed for adult screening. Results show a range of larval recovery across the diversity set, with some lines showing low larval numbers, as observed in *S. alba.* Images have been collected for determining larval instar and size. Lines showing extreme variation in larval survival were assessed in a highly replicated trial, but results showed no significant difference in numbers between lines. This retrial of material was subjected to drought stress during the experiment and the most extreme lines are being retested.

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- Effect of climate perturbation on field larval infestation

Mathiasen et al. (2015) showed significant effects of temperature on egg laying, egg hatching and embryonic development, with warmer temperatures >4°C being more favourable for egg production, development rate and hatching percentage. Modelling data (Ortega-Ramos et al., 2023) showed a high level of importance of daily temperature between December and February with higher temperatures related to higher larval numbers in the spring. Observations from field environmental perturbation trials by heating trial plots with soil warming cables (O'Neill et al., 2019) identified that plots heated across the October period appeared to suffer from larger amounts of larval damage. To test the hypothesis that warmer field temperatures over the winter period results in greater numbers and more developed CSFB, plots were heated during the December and January period, predicted to be critical for spring larval numbers by Ortega-Ramos et al. (2023) to compare larval numbers.

A 2 x 2 factorial field trail was performed using the following treatments – unheated CSFB excluded, unheated CSFB present, heated CSFB excluded, heated CSFB present. Plots were drilled at the end of August and covered to prevent CSFB infestation. Plots for beetle inclusion were uncovered at the start of October to allow beetles to migrate from the surrounding OSR field plots. Plots were then recovered for heating for 8 weeks from the start of December. Plants were assessed for larval infestation at three timepoints – preheating, during heating and post heating.

Winter warming raised ambient plot temperatures by 2°C throughout the heating period. Assessment of larval numbers showed low infestation, with larvae still being present in the exclusion plots but at a much lower level. There was no significant difference in larval numbers between unheated and heated plots where CSFB were present however, where we had tried to exclude CSFB, heated plots contained more larvae post heating.

Larval size was increased in plots where beetles were excluded suggesting where smaller numbers of larvae were present this resulted in an increased in size.

No effect of the heating could be observed on size in the exclusion plots but where CSFB were included larval size was greater post heating.
Higher larval loads were correlated with reduced yield within the plots with an approximate effect of 100g reduction in yield per plot found per two larvae within plant. We observed a difference of 0.46t/ha between our inclusion and exclusion plots.

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- Machine learning for larval counting and assessing larval stage

During the 21/22 field trials assessment of larval numbers resulted in the counting and staging of ~60,000 larvae, an intensive, skilled and time-consuming process. Images have been taken of a large number of larval samples from our field and controlled trials. Pilot training of an AI pipeline at JIC with labelled images of L1, L2 and L3 instars has been performed resulting in an initial high detection and scoring accuracy. This has currently been expanded to assess already staged larval selections before further retraining and automated scoring.

Key issues to be addressed in the next year
All Scoring and data analyses need to be completed to finalise results.

Lead partner	JIC
Scientific partners	Rothamsted Research
Industry partners	Elsoms, Limagrain, RAGT, Bayer, LS Plant Breeding, KWS, DSV, BASF, AHDB
Government sponsor	BBSRC

Has your project featured in any of the following in the last year?	
Events	***Press articles***
JIC Breeders day 24 AHDB monitoring meeting presentation 24 Visit from industry partner Innolea 24 Visit from LSPB to trials 24 Visit by AIC (Agricultural Industries Confederation) board of directors - seed sector 24 Visit from the Defra AgriScience team 24 Frontier agriculture visit 24 HZAU phenotyping group visit 24 OSR Reboot development meetings 24 OSR Reboot Breeding, Policy and Agronomy meetings 24	

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Conference presentations, papers or posters	*Scientific papers*
International Conference of Entomology, Kyoto, Japan, Sept 24 **Brock R, Wells R, Ortega-Ramos P, Cook S** IOBC, Integrated Control in Oilseed Crops, Dresden, Sept 24 **Solomon E, Cook S, Ortega-Roamos P** Invited Speaker, University of Durham, Nov 24 **Wells R** AAB IPM and climate change conference, Nov 24 **Wells R**	
Other	
Royal Society of Biology Plant Health Undergraduate Studentship – Investigating genetic variation associated with cabbage stem flea beetle larval resistance in *Brassica napus* JIC Year 10 work experience student hosted in CSFB group JIC Training in cabbage stem flea beetle rearing and experimentation – commercial partner Provision of cabbage stem flea beetle to 3 commercial companies	