Legume
Generation

**Boosting innovation in breeding
for the next generation of legume crops for Europe**

Progressing the breeding of clover

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Legume Generation Report 14

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Contents

Summary

This report sets out the progress that the Legume Generation Clover Innovation Community made up until August 2025 in boosting the breeding of clovers (*Trifolium repens*) and red clover (*Trifolium pratense*). These are two key forage legumes in agricultural systems.

Selection of a white clover diversity panel from European collections held at the Aberystwyth University Seed Bio-bank supported the establishment of yield and persistency plot trials in Wales (UK) and Germany across four different sites. To date, one full harvest year of data has been collected on the two yield trials in Aberystwyth, Wales, revealing large variations in annual clover dry matter yields and mean percentage ground cover of clover. Notably, wild collections from northern Spain and southern France showed the best agronomic performance in both standard and low phosphate soils, with collections mostly from eastern Europe failing to establish or thrive. Contrastingly, yield data from plots established in Pulling, Germany revealed higher performance from collections originating from eastern Europe, where those which failed to thrive originated from Spain and Portugal. Data collected at German sites also revealed great variation in hydrocyanic acid production in leaf tissues, with numerous wild collections producing zero.

Whole genome short-read sequencing of 172 of the 204 white clover diversity panel was completed. Analysis of population structure via Admixture shows four distinct clusters, which reflect geographical differences in collection site of the highest and lowest yielding accessions. We expect that further phenotypic data will be collected on a spaced plant trial of the diversity panel to be established 2026 to improve capabilities of marker identification through utilisation of this genome sequencing.

A red clover semi-hybrid breeding population has been established, which has currently been taken to the F2 generation, where half-sib families will be produced over winter 2025/6, then assessed for agronomic performance in a replicated yield trial 2026-2028.

Drawing on results of previous projects, we have successfully identified high confidence self-incompatibility candidate genes for S-alleles, via fine mapping of the S locus in new long-read references of white clover. The location of the S locus has been cross validated by molecular and diversity analysis. The allelic diversity of the S locus in the panel will be validated experimentally in late 2025/6. S-allele work on red clover at USDA has comprised of the initial analysis of six commercial cultivars using previously identified SSR DNA markers, set to inform whether restriction of S-allele diversity in standard synthetic population cultivars is contributing to seed production difficulties experienced in red clover.

Methodologies for influencing factors affecting seed yield in the aforementioned cultivars have further been investigated via the application of boron to a spaced plant trial established in Bulgaria). Transcriptome analysis of mature inflorescences from these plants revealed elevated expression of transcription factor ARF17, an auxin response factor which is associated with anther development, dehiscence, pollen maturation and filament elongation. Increased expression of other transcriptomes was also observed, although this was not consistent across all cultivars. Additional florets from the same flowering heads have been collected to test for pollen viability and pollen count. In addition, analysis of leaf material revealed elevated nitrogen in boron treated plants.

Introduction

Forage legumes are a valuable resource in grasslands, providing ruminants with a highly digestible and protein-rich feed that boosts grassland productivity by reducing the need for fertiliser through nitrogen fixation, and improves soil architecture through extensive rooting structures. In Europe, white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) are amongst the most important forage species, for grazing and cutting, respectively.

The Clover Innovation Community (IC), established within the Legume Generation project, looks to address and investigate some of the current challenges which the crops face. The work supports 10 pre-breeding and 8 breeding programmes spread across three continents. Our plan to improve the breeding of clovers involves providing pre-breeding material from wild germplasm, investigating s-allelic diversity and methodologies from improving seed yield.[1] Activities undertaken in relation to this plan are outlined below.

The development of commercial white clover cultivars can be greatly improved by incorporating traits found in wild germplasm, such as higher yields, greater persistency under natural grazing, and adaptation to abiotic stresses. The work of the Clover Innovation Community assesses and utilises the breadth of genetic variation found in wild and semi-natural collections across Europe, through collecting data on the performance of these collections in contrasting environments with different climatic variables. Our focus is on the improvement of the existing breeding programmes at Germinal Horizon. Multi-environment field trials and whole genome sequencing facilitate potential regions under selection for such traits and will provide markers to assist the breeding programmes. The IC is also committed to the elucidation and development of an S-allele breeding system in white clover.

In red clover, producing sufficient seed for the market is one of the crops greatest challenges. We aim to further develop the use of S-alleles (self-incompatibility alleles) to improve hybrid seed production, to capture heterosis, and to investigate their use in improving seed yield. The genetic location of the S-alleles and their use in hybrid seed production have previously been described by Riday and Khron.[2] We are taking a second approach to improving this trait through the application of boron treatments on plants, to review the effect of boron on pollen fertility, seed yields and germination.

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[1] Jones, C., Anderson, S.U., de Vega, J., Griffiths, A., Hartmann, S., Howarth, C., Iantcheva, A., Lloyd, D. and Riday, H, 2024. Improvement plan for clover. Legume Generation Report 6. Available from www.legumegeneration.eu and www.legumehub.eu

[2] Riday, H. and Krohn, A.L., 2010. Genetic map-based location of the red clover (Trifolium pratense L.) gametophytic self-incompatibility locus. Theoretical and Applied Genetics, 121(4), 761-767. (Journal paper).

Progress

Establishment and continuation of the Clover Innovation Community

The Clover Innovation Community (IC) was successfully established with partners from Europe, New Zealand and the USA. Members of the IC gathered at the Legume Generation consortium in November 2023 at IPK, Gatersleben, Germany. The IC now meets monthly through online video conferencing, providing a platform to update members with progress across the project and discuss any emerging innovations or challenges.

Provision of pre-breeding material for clover breeders

White clover diversity panel phenotypic assessment

A total of 204 different accessions of white clover (*T. repens*) were selected for the diversity panel from 51 wild collections, 104 semi-natural collections, 11 breeder's lines and 38 advanced cultivars (Figure 1). Accessions of white clover were selected from the world's largest forage species biobank held at Aberystwyth, UK. These were chosen to best represent the breadth of biodiversity present in wild populations, giving the best opportunity to reveal novel sources of variation for stress tolerance, both biotic and abiotic. Sufficient germination rate and availability of seed were also used as a means of selection.

Four augmented randomised complete block (with repeated checks) yield trials and two unrandomised persistency plot trials with single entries have been established across four sites and two countries, encompassing different elevations above sea level, differing annual rainfall and soil phosphate indices (Table 1, Figure 2, Figure 3). Assessing the performance of the diversity panel in varying annual rainfall and soil phosphate indices could identify elite phenotypes for drought/flood tolerance and phosphate use efficiency. White clover is usually grown along with grasses, so plots were sown at the seed rate of 0.35g/m^2 (according to industry standards) along with companion diploid *Lolium perenne* (perennial ryegrass) at a seed rate of 2.5g/m^2. Trials at the sites in Aberystwyth were sown in the summer of 2023. Trials at Pulling and Triesdorf were sown in 2024. Fertiliser was applied in the spring as 40kg/ha N, 175kg/ha P_2O_5 and K_2O (depending on soil requirements), then as 40kg/ha N after each cut in yield trials.

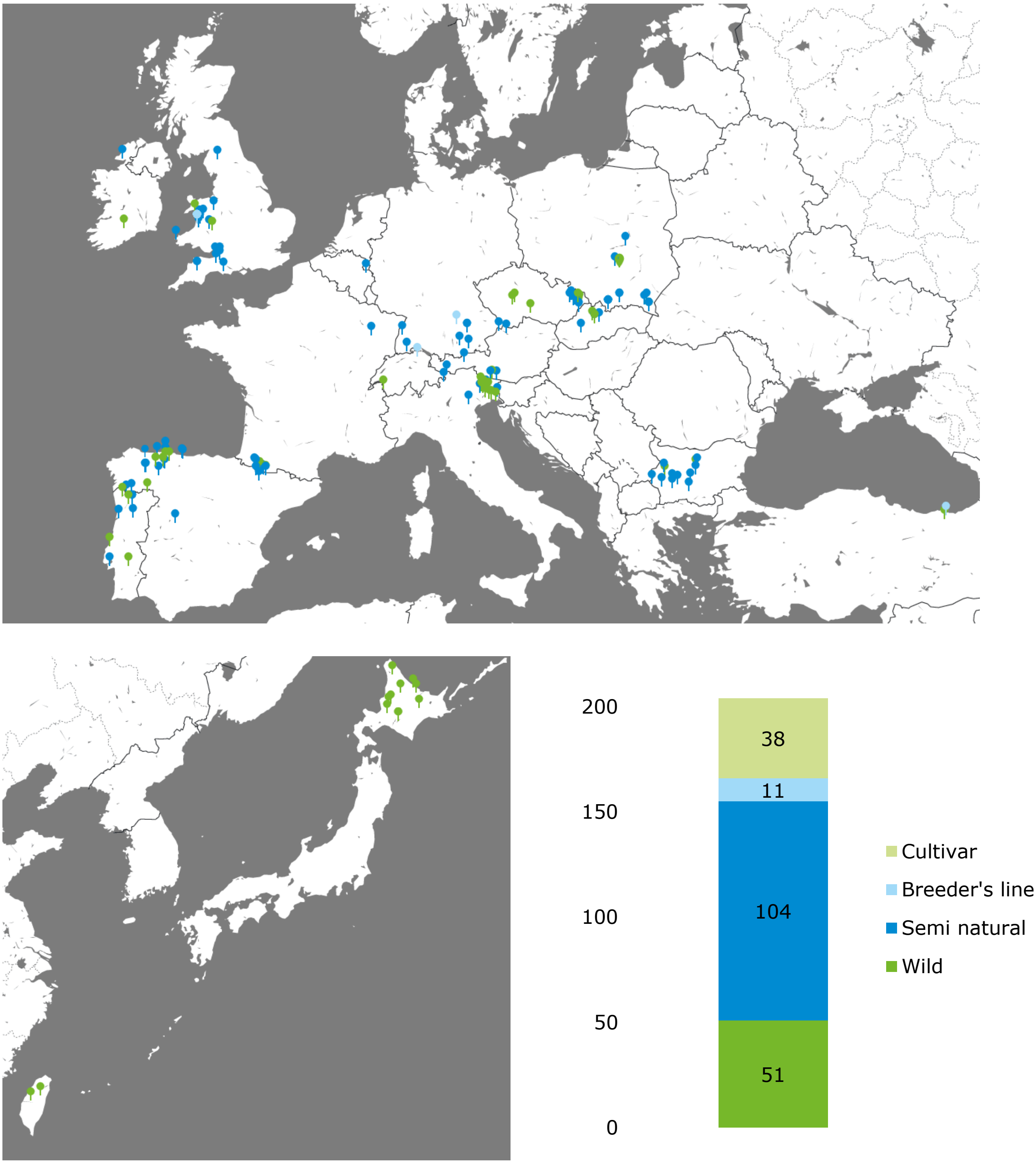


Figure 1. Origin of wild and semi-natural accessions used in the white clover diversity panel across Europe and Asia. One accession, not pictured, was collected in Iran. Images created using Snazzy maps, Atmist, Edmonton, Alberta, Canada.

Table 1. Details of trials used in assessing the white clover diversity panel.

Location	**Trial type**	**Plot size**	**Elevation (OS Terrain 50)**	**Soil P Index**	**Avg. annual rainfall (Met Office)**	**Phenotypic data**
Cae Glanyrafon, Germinal Horizon, Aberystwyth	Yield	1.2x2 m	18 m	2.8	1107 mm	DMY, clover percentage, herbage quality
Cae Glanyrafon, Germinal Horizon, Aberystwyth	Persistency	1x1 m	18 m	2.8	1107 mm	Clover percentage following simulated grazing
Cae Gwlyb, Germinal Horizon, Aberystwyth	Yield	1.2x2 m	50-55 m	1.8	1107 mm	DMY, clover percentage, herbage quality
Cae Gwlyb, Germinal Horizon, Aberystwyth	Persistency	1x1 m	50-55 m	1.8	1107 mm	Clover percentage following simulated grazing
Pulling, Freising, Germany	Yield	1.5x1.5 m	460 m	8	825 mm	DMY, clover percentage, herbage quality, HCN quantity
Triesdorf, Ansbach, Germany	Yield	1.5x1.5 m	443 m	No data	650 mm	DMY, clover percentage, herbage quality, HCN quantity

Up to August 2025, one full year and one-half year of plot harvesting was completed at the GER sites in Wales and one-half year at the LfL sites in Germany. Samples were only taken from yield trials, where persistency plots were cut every 10-15 days to 20 mm commencing at 60 mm sward height and stopping once growth ceased in the autumn. Yield plot sampling commenced at an approximate grass and clover content of 1500 kg/ha, estimated by visual assessment. The same criteria were used to inform timing of subsequent cuts. Prior to harvesting, plots were observed and scored where appropriate for percentage clover content, disease incidence and insect damage. Plots at LfL were sub-sampled and manual botanical separations were undertaken, compared to the GER sites where logistical challenges did not allow for this. Plots were cut, fresh weight recorded and sub samples taken using a Haldrup F55 harvester at all sites. Fresh weight of sub-samples was recorded before drying the samples at 80 °C for 48 hours to record dry weights. Total dry matter yield per plot was then calculated from these sub-samples and dry matter yield of clover calculated using the clover percentage scores. Harvest data from the field trials are analysed using R scripts embedded in Genovix (Agronomix, Winnipeg, Canada).

Yield sampling commenced at Aberystwyth in June 2024, where three sampling cuts were taken on the standard phosphate site and two cuts on the low phosphate site. The difference in elevation and exposure between these sites most likely resulted in reduced growth rate, with low phosphate levels impacting this also. Statistically significant differences were observed in clover, grass and combined dry matter yields at both Aberystwyth sites. Clover yields per sampling cut were generally greater at the standard phosphate site and lower at the low phosphate site, ranging from 0–4.8 t/ha and 0–2.2 t/ha, respectively. A similar trend was observed in mean percent clover per cut, reaching up to 88.2% at the standard phosphate site, but only 56.8% at the low phosphate site.

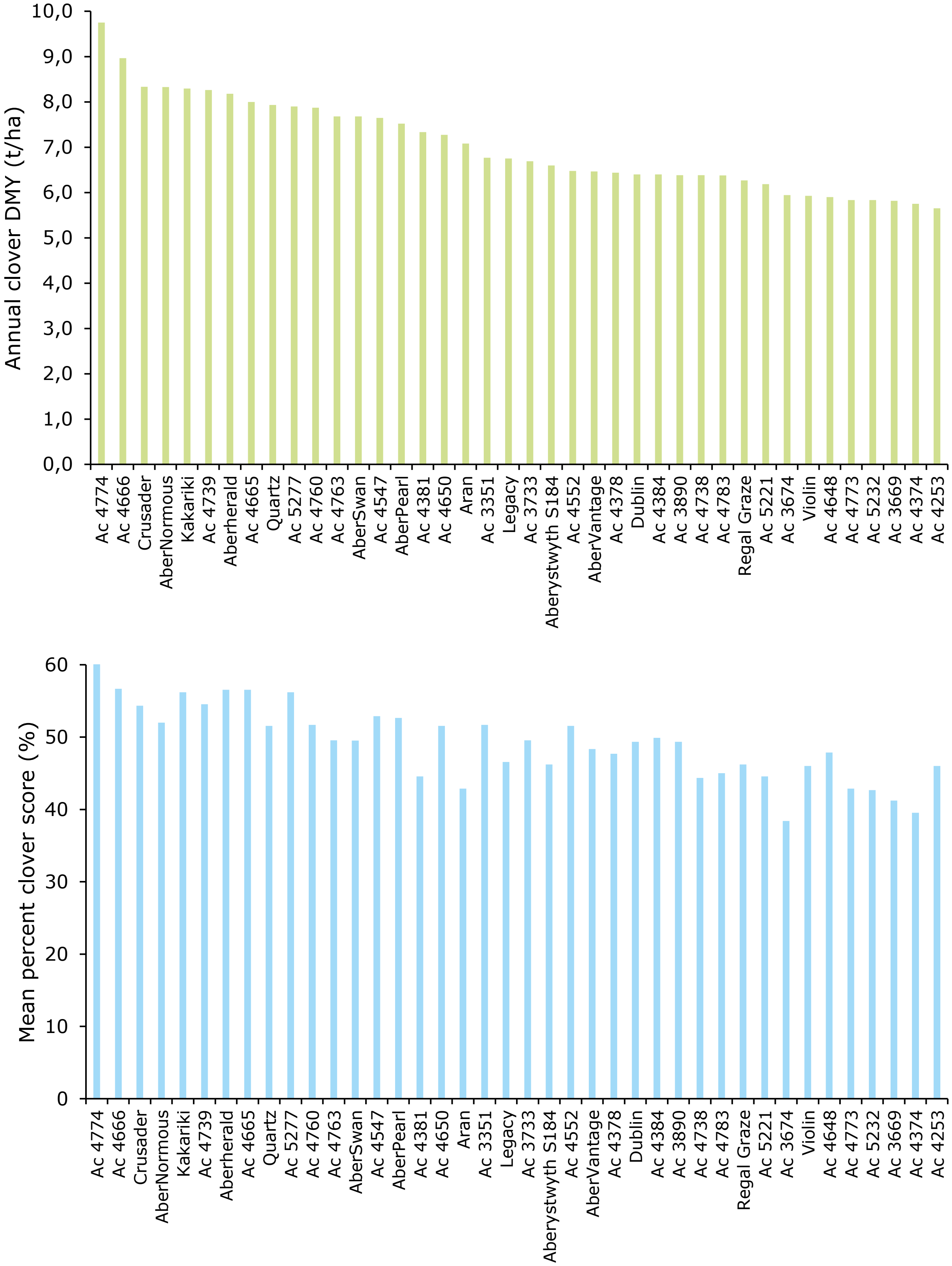


Figure 2. Annual clover dry matter yield (DMY) and mean percent clover score of the forty best performing entries in the trial at the standard soil phosphate trial, Cae Glanyrafon, Aberystwyth.

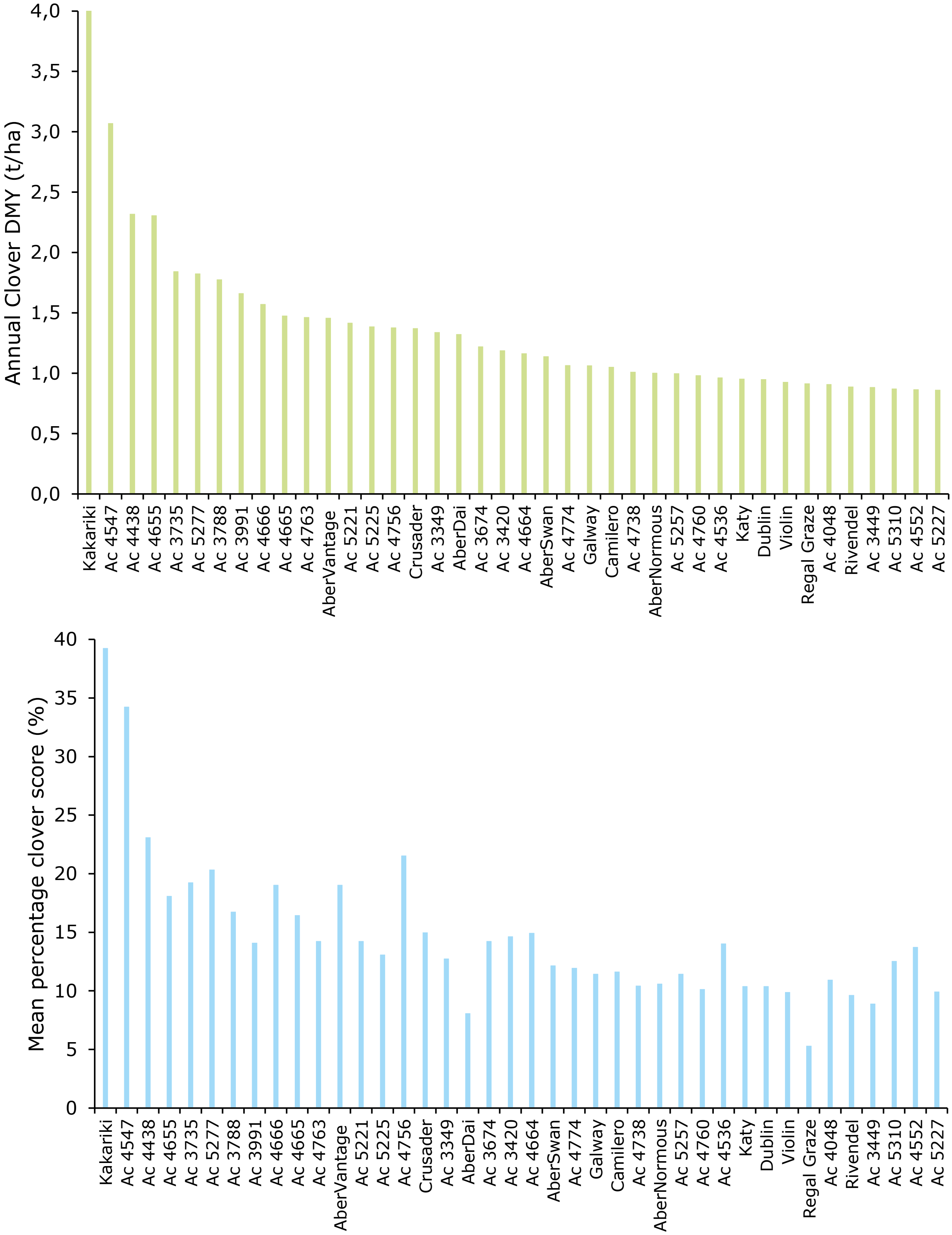


Figure 3. Annual clover dry matter yield (DMY) and mean percent clover score of the forty best performing entries in the trial at the low soil phosphate trial, Cae Glwyb, Aberystwyth.

A strong positive correlation was observed between annual clover yields and mean percent clover scores at both the standard and low phosphate site (0.93 and 0.96, respectively), indicating that the effect of variations in leaf size between accessions on clover yields was lesser than percentage clover within plots. Similar accessions/cultivars have been seen to produce the greatest annual clover dry matter yields at both Aberystwyth sites – wild and semi-natural collections Ac 4774, Ac 4666, Ac 4665, Ac 4760, Ac 4763, Ac 4547, Ac4552, Ac 4738, Ac 3674 (Figure 2, Figure 3). Namely, a number of these accessions exist from collection sites across northern Spain, southern France, and Portugal (Figure 4). Elevation at collection sites of these accessions varied from 3-1407 m and thus indicates this had little influence on clover yields. The best performing cultivars exist from the United Kingdom, Ireland and Western Europe – to be expected as these have been selectively bred for performance in temperate climates. However, New Zealand cultivar Kakariki produced exceptional clover yields in the low phosphate site, outperforming any of the aforementioned cultivars.

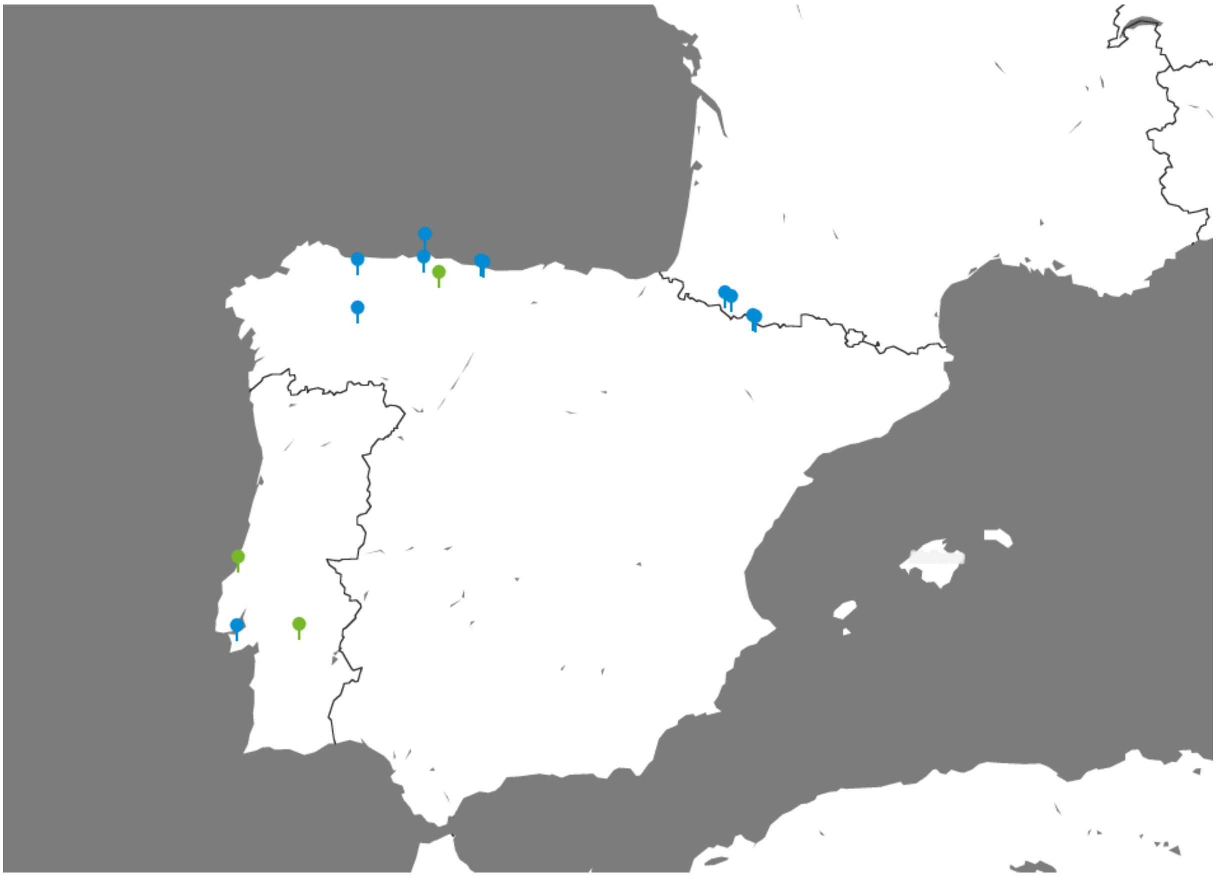


Figure 4. Collection sites of wild (green) and semi-natural (blue) white clover collections exhibiting the greatest clover dry matter yields at both the standard and low phosphate sites at Aberystwyth. Images created using Snazzy maps, Atmist, Edmonton, Alberta, Canada.

Several accessions showed poor establishment at both Aberystwyth sites; percentage clover scores in subsequent cuts revealed that these failed to thrive entirely (hereby defined as <5% clover within the plot consistently). Figure 5 illustrates the collection sites of these accessions, showing an eastern European demographic. As year of collection of these accessions ranged from 1982-2008, it can be said that the age of seeds sown was not a contributing factor to their failure to thrive.

Figure 5. Collection site of wild (green) and semi natural (blue) white clover collections which failed to thrive in yield trials sown at both standard and low phosphate sites in Aberystwyth. Image created using Snazzy maps, Atmist, Edmonton, Alberta, Canada.

Contrastingly, those which failed to thrive at Pulling (Germany) were markedly different to those at Aberystwyth (Wales). Specifically, two of the best performing accessions in the low phosphate trial in Aberystwyth were amongst those which failed to thrive in Pulling. Also, a number of other accessions which failed to thrive at this site originated from collections in Portugal and Spain, countries where some of the best yielding accessions from the Aberystwyth sites were collected. The distribution of accessions which failed to thrive at the site in Pulling are illustrated in

Figure 6. However, accessions which failed to thrive at the Triesdorf (Germany) site showed some similarities to those which failed to thrive at the Aberystwyth sites. Accessions originating from Italy which failed to thrive in Triesdorf were amongst the best yielding at the Pulling site.

Yield plot sampling commenced at both German sites in May 2025, where three sampling cuts have been taken to date on the trial in Pulling and only one cut on the trial in Triesdorf (Figure 8), due to insufficient summer growth resulting from prolonged dry weather.

Additional cuts are projected to be taken autumn 2025 where necessary. Statistically significant differences were observed in some cuts in clover, grass and combined dry matter yields (

Figure 7. Cumulative clover dry matter yield (DMY) and mean percent clover score of the forty best performing white clover diversity panel across 3 cuts, in the yield trial sown at Pulling, Freising, Germany.). These differences were mostly seen as lower clover yields and respective higher grass yields than control cultivar AberSwan. Whilst some of the highest clover yielding collections at the Pulling site also originated from northern Spain/southern France like those at Aberystwyth, they were distinctly not the same accessions. There tended to be a more eastern origin of the highest yielding collections, particularly from countries such as Czechia and Italy. Collections from Bulgaria were also amongst the highest yielding. Unlike clover yields at the Aberystwyth sites, there is evidence that leaf size influences yield, as ranking of highest yielding entries did not reflect that of percent clover within plots. Moreover, large leaf cultivars such as Bombus and Katy were amongst the highest yielding.

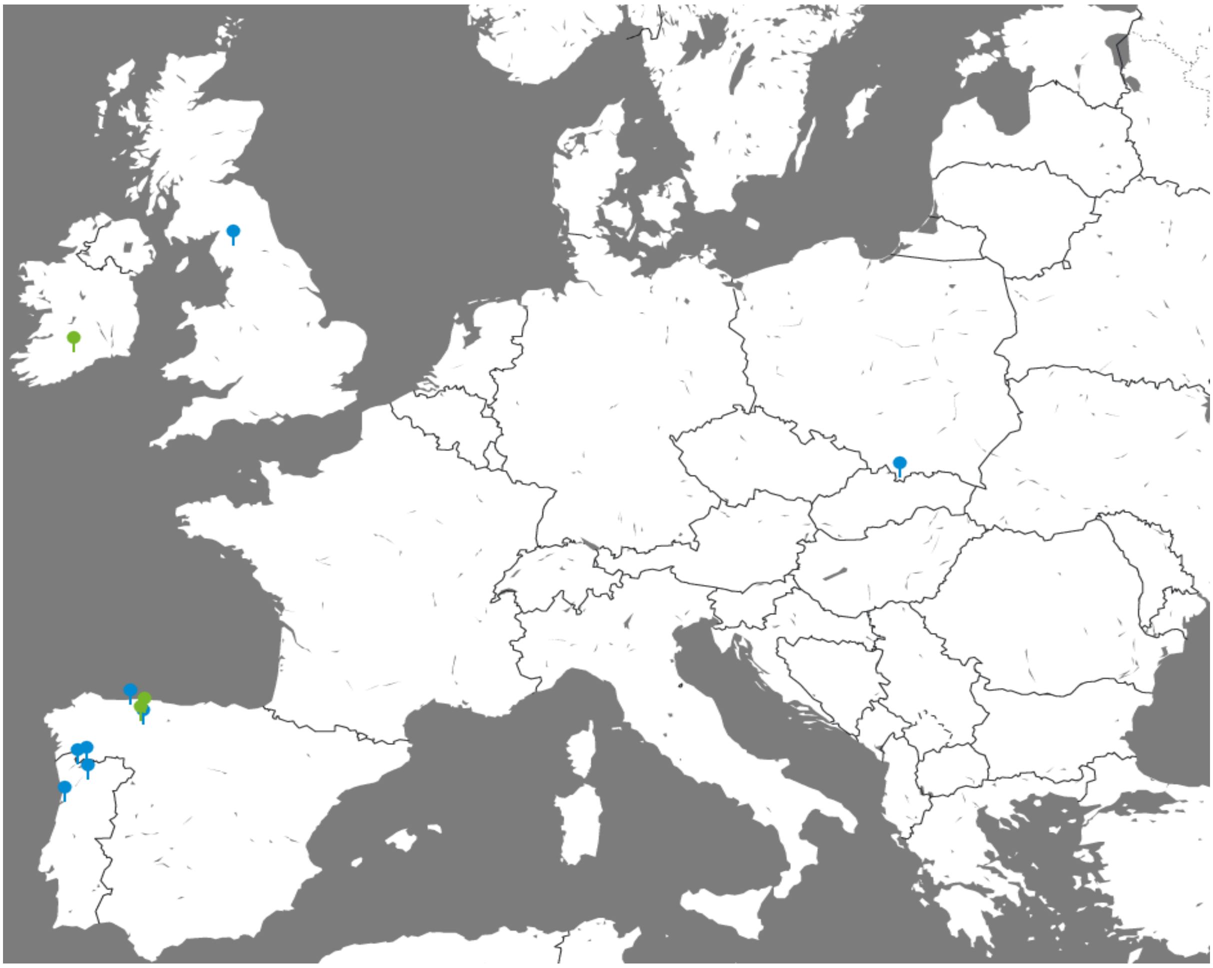


Figure 6. Collection sites of wild (green) and semi-natural (blue) white clover collections which failed to thrive in a yield trial in Pulling, Germany. Image created using Snazzy maps, Atmist, Edmonton, Alberta, Canada.

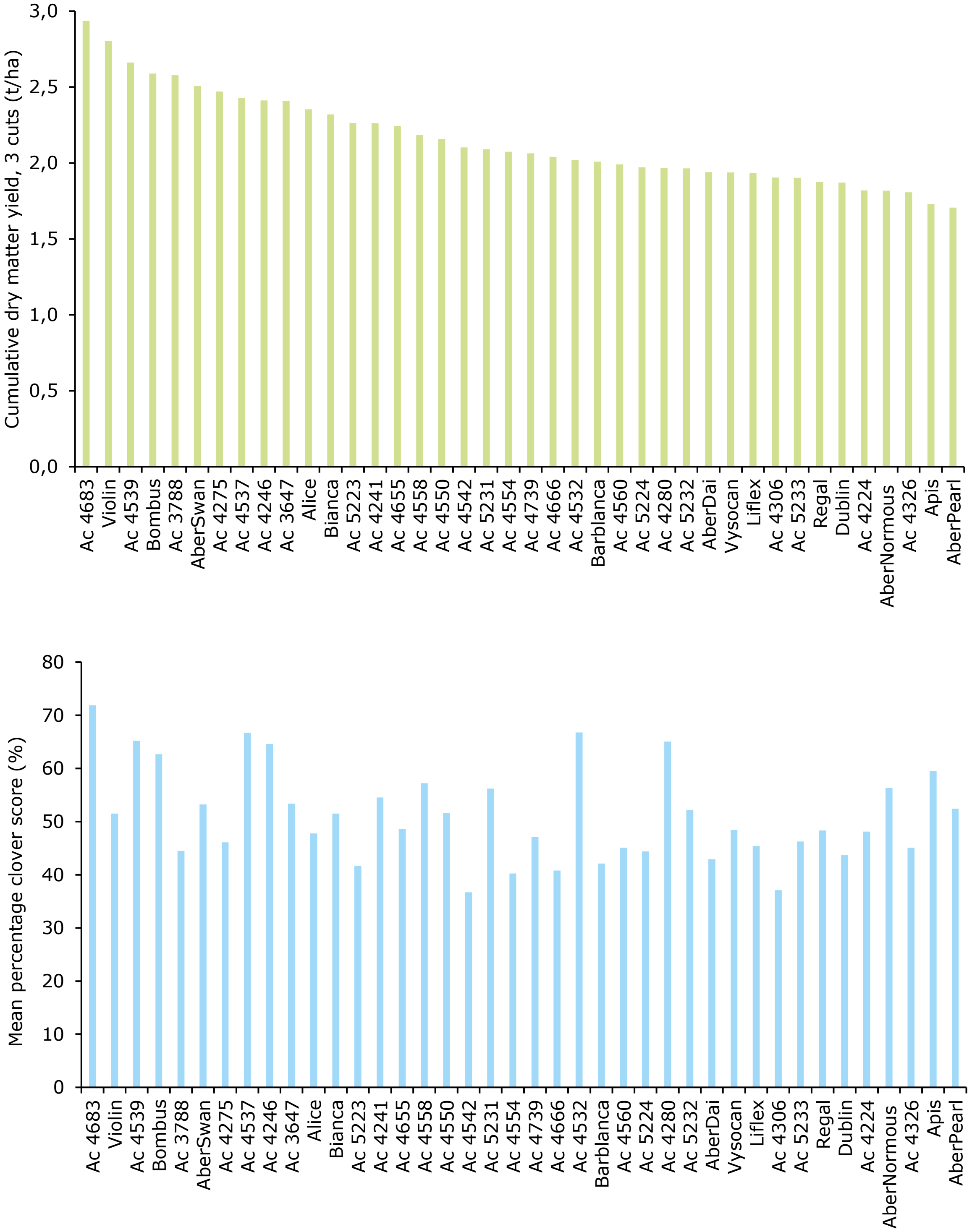


Figure 7. Cumulative clover dry matter yield (DMY) and mean percent clover score of the forty best performing white clover diversity panel across 3 cuts, in the yield trial sown at Pulling, Freising, Germany.

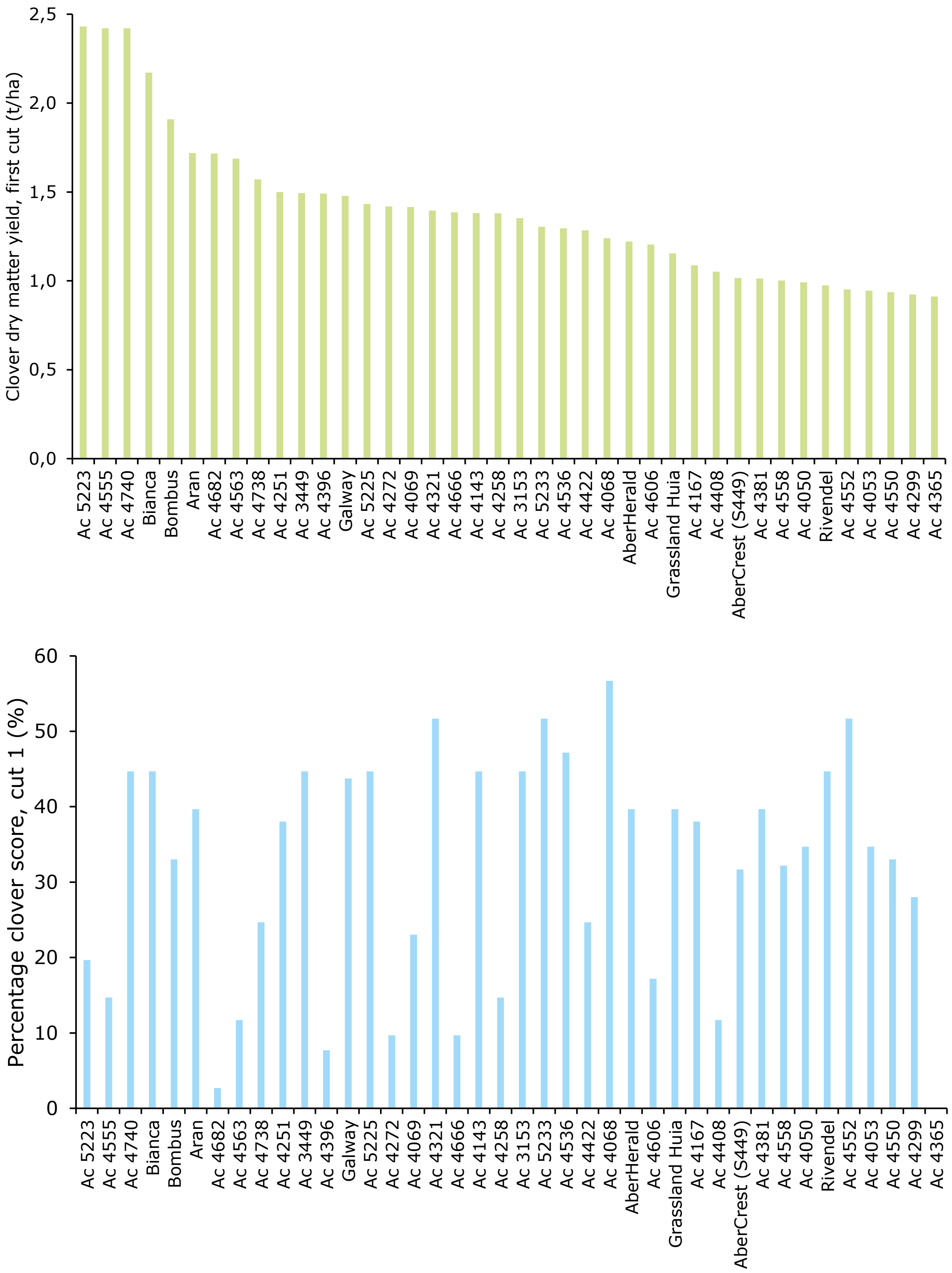


Figure 8. Cumulative clover dry matter yield (DMY) and mean percent clover score of the forty best performing white clover diversity panel across 3 cuts, in the yield trial sown at Triesdorf, Germany .

Infection on leaf tissues from fungal pathogens *Cymadothea trifolii* and *Stemphylium spp.* was observed at Pulling in August 2025, ahead of the third harvest. Infection on fungal pathogens in forage legumes can result in reduced forage quality, palatability and therefore voluntary uptake from livestock. Therefore, it is of importance to identify any variation within the diversity panel which could be utilised to selectively breed for tolerance to these pathogens. No significant differences were seen in infection severity of *C.trifolii*, and only one cultivar showed significantly worse infection from *Stemphylium spp.* Incidence of pathogens will continue to be monitored throughout the duration of the trials.

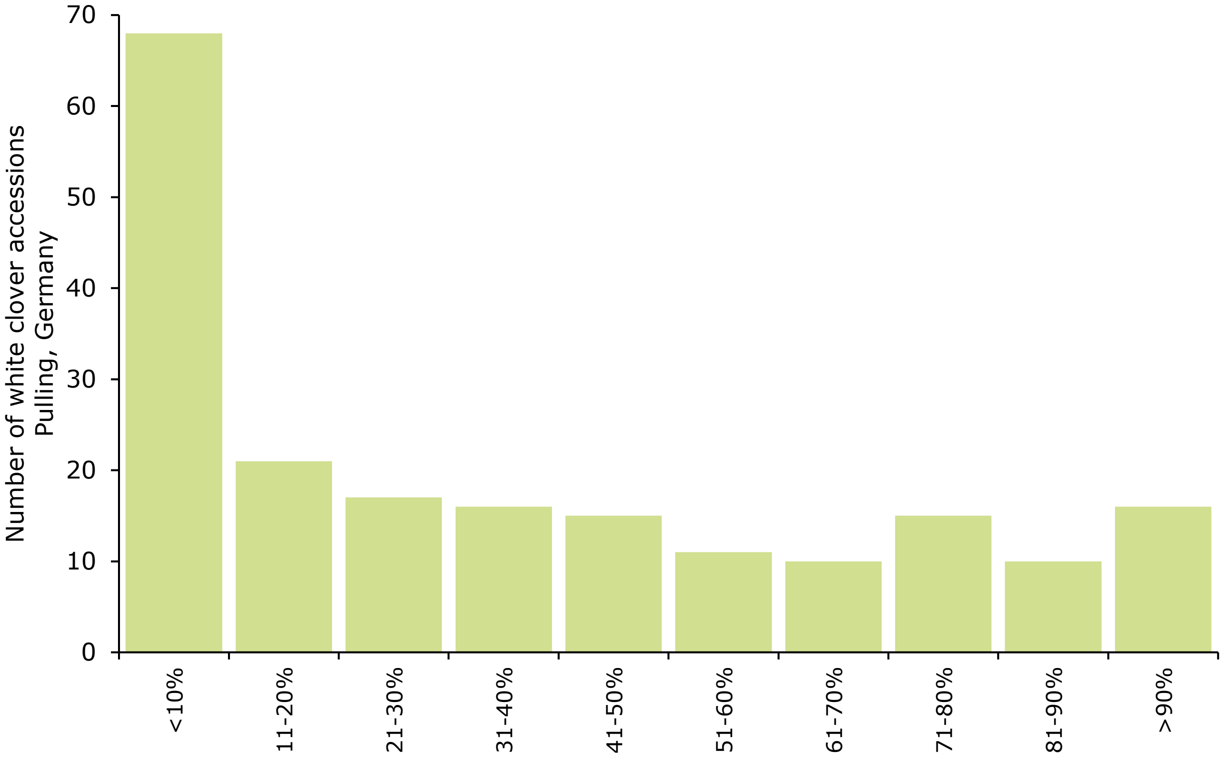
Quantification of hydrocyanic acid (HCN, commonly known as cyanide) in leaf tissues of the diversity panel was undertaken on plots at both the Pulling and Triesdorf sites. Hydrocyanic acid is known to be produced in the breakdown of cyanogenic glucosides (namely linamarin and lotaustralin) caused by disruption of membranes in leaf tissues during grazing from livestock. The content of cyanide within leaf tissues can vary greatly between genotypes and can reach toxic levels in some cases. Methodologies involved the collection and freezing (-80˚C) of 20 pinnate leaves from each plot, where later thawing of the leaf tissues at ambient temperate allowed for cells to burst, initiating the production of HCN. Cyantesmo qualitative test paper strips for cyanide (Klüver & Schulz GmbH) were then used to assess the number of pinnate leaves which released HCN.

Results revealed significant variation in HCN production across the diversity panel (Figure 9). At the Pulling site, 47 out of the 197 entries produced no HCN, with all but one of these existing from wild collections and not cultivars. A similar trend was seen at the Triesdorf site, with 43 entries producing zero HCN, yet there was very little correlation between the HCN production of accessions across the sites (R^2=0.09).

Due to required labour intensity and scale, assessments will focus on dry matter yield, clover percentage, disease/insect damage and persistency under simulated grazing. We intend to carry out further phenotypic analysis on replicated individual plants, both in the glasshouse and as a spaced plant field trial in 2026/2027 at an Aberystwyth site. The following characteristics are set to be measured on such plants/trial:

- Intensity of green colour
- Development of inflorescences before vernalisation
- Density of foliage
- Date of inflorescence flowering, number and size of inflorescences
- Plant height, width and growth habit
- Solon morphology
- Petiole and leaf morphology.

These characteristics align with those outlined in UPOV protocols. The addition of these data will greatly improve the capabilities of GWAS, when compiling this data with the genome sequencing being undertaken as part of this project.



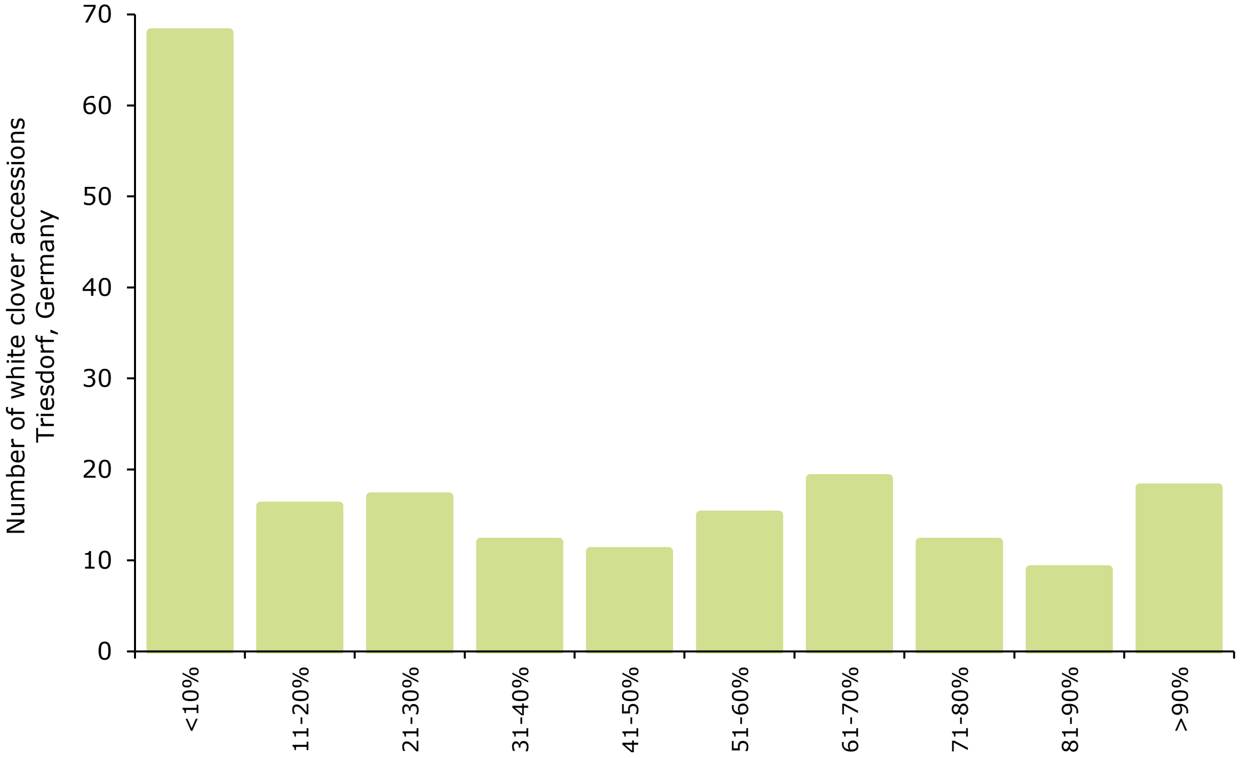


Figure 9. Frequency graph of accessions within the white clover diversity panel, showing percentage of trifoliate leaves collected from plots which produced hydrocyanic acid (HCN). Data presented are collected from the Pulling (above) and Triesdorf (below) sites in Germany.

White clover diversity panel genome sequencing

Whole genome short-read sequencing (Illumina, San Diego, USA) of 172 of the 204 white clover diversity panel (127 wild/semi-natural collections, 32 advanced cultivars, 12 breeders' lines and 1 landrace) was completed with approximately 5x coverage per haplotype. Each accession was analysed with pooled samples of 50 genotypes to adequately cover genetic diversity in the allogamous populations.

The genome reference, ACLI19, was re-assembled with HiFi and Hi-C reads to obtain high-quality haplotype-phased reference. Briefly, this was achieved by first using Hifiasm (v0.20.0) to generate primary contigs (hap1 & hap2), followed by scaffolding both haplotypes with HapHiC (v1.0.3) and curating assembling errors with PretextView (v0.1.9). The resulting phased genome shows high contiguity and completeness (Table 2), indicated by scaffold N50 (57.62 Mb) and a high rate of complete BUSCO markers (99.6%)

Table 2. Assembly statistics for haplotype-phased ACLI19 genome.

Statistics	**ACLI19**
Number of contigs	3801
Number of contigs > 50,000 bp	522
Longest contigs	67.95 (Mb)
Scaffold N50	57.62 (Mb)
Genome size	208.81 (Mb)
Total chromosome length	193.17 (Mb)
Complete BUSCOs (*Fabaceace*)	5343 (99.6%)

Using the hap1 from ACLI19 as a reference, around 84K high-quality, genome-wide SNPs have been obtained across the 172 accessions. With this SNP dataset, population structure was estimated using Admixture (v1.3.0). Admixture results suggest that the diversity panel likely represents four genetic groups (Figure 10). Accessions with more than 60% of genetic background from a single cluster were defined as those from pure population (n=86); otherwise, they were grouped into admixed (n=86). Based on the geographic map from Figure 11, population A comes mainly from the UK; population B is mostly distributed in Iberia (Portugal); population C is found mainly in Italy and northern Spain/southern France; population D is common in central/eastern Europe and Bulgaria. Most cultivars and breeders' lines either show admixed genetic background or come from population C.

These genetic clusters are apparent when comparing clover yields across the four trial sites. In the trials at the two Aberystwyth (Wales) sites, accessions from within clusters B and C produced the best clover yields, where most of those which failed to thrive are in cluster D. Accessions from cluster D showed some of the highest clover yields at the Pulling site (Germany), where those mostly from cluster B failed to thrive.

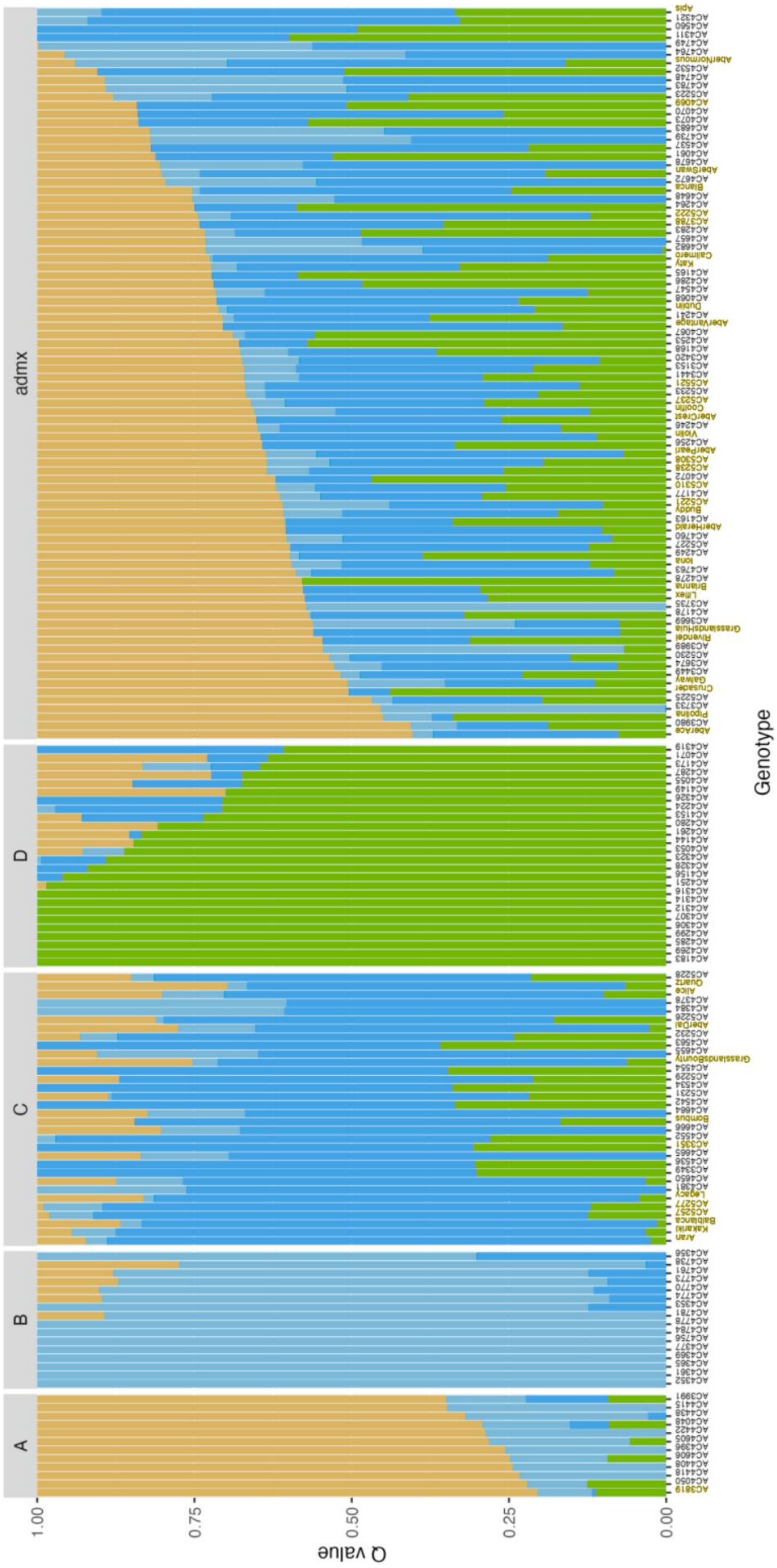


Figure 10. Population structure within the diversity panel. Four distinct genetic groups (A-D, n=86) alongside widespread admixture (n=86) were identified in the diversity panel. Q-value indicates the proportion of genetic background of each accession belonging to the inferred populations (A-D) based on genome-wide SNP markers. Accessions highlighted in yellow refer to cultivars or breeders' lines. (CV) errors of admixture with different number of assumed clusters (Ks) revealed K with the lowest CV (K=4) refers to the best possible number of genetic clusters

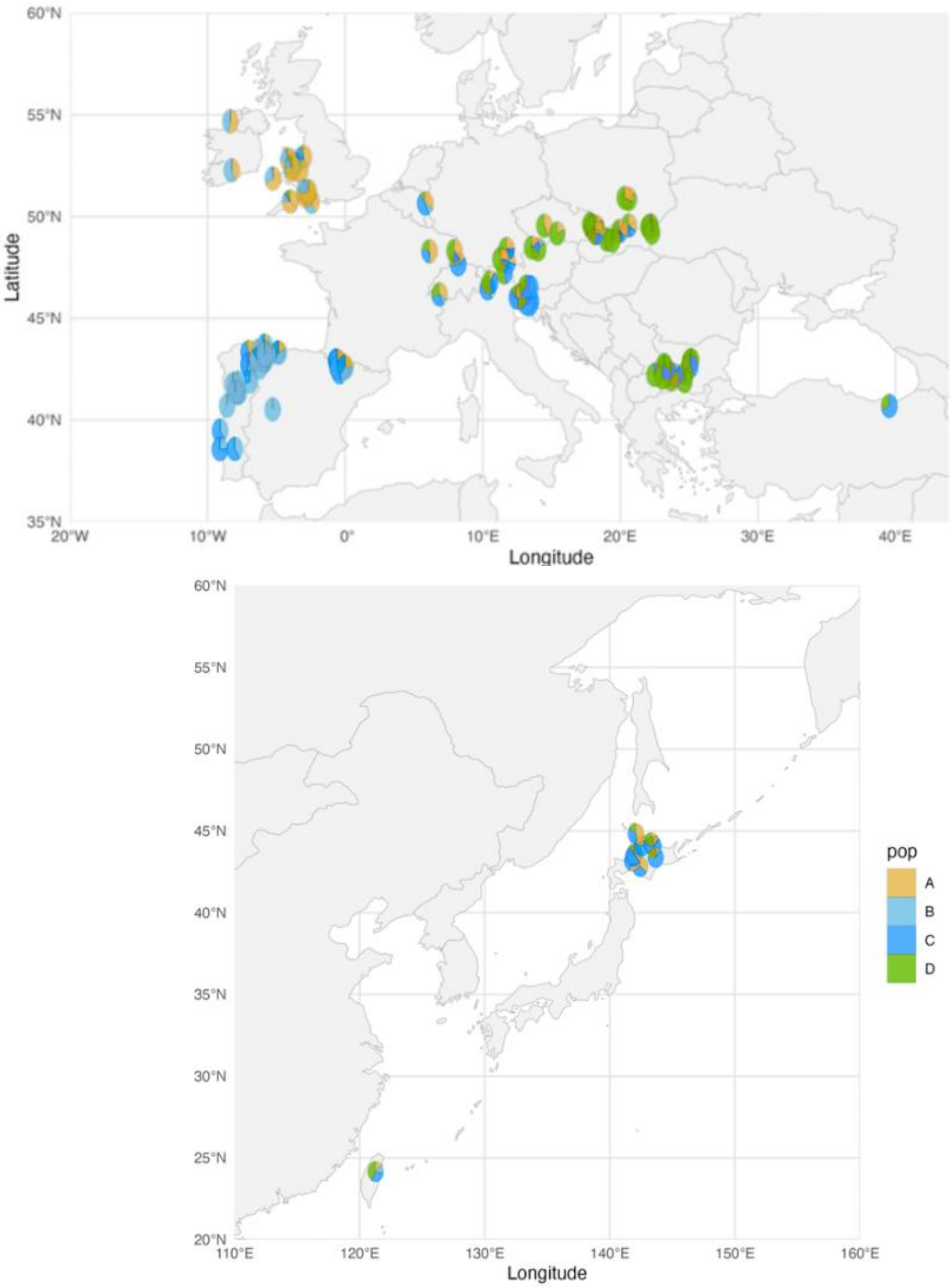


Figure 11. The geographical distribution of Admixture results from genome sequenced white clover entries within the diversity panel. Inferred population structure (K=4) is shown across Europe (above) and Asia (below).

Red clover semi-hybrid breeding system

An experimental recurrent selection breeding population has been constructed using highly diverse parental genotypes. This has been taken to the F2 generation, and half-sib families are currently in production. These families are projected to be produced in a glasshouse with controlled temperature and lighting over the winter of 2025/6, then assessed for agronomic performance in a replicated yield trial 2026-8. These are to be characterised according to SSR markers outlined in Riday and Khron[3] to assist with selection of the F2 mother plants for development of the F1 populations with restricted, divergent S-alleles.

Self-incompatibility-allelic diversity in clover and seed yield improvement

White clover S-allele identification

Previous work characterised the location of s-allele loci in white clover on Chromosome 1 of the *Trifolium occidentale*-derived sub-genome. They consist of numerous alleles expressed in pollen and stigma that influence self-incompatibility. We look to further this by identifying markers to use for selection. The adopted approach aims to achieve this via fine mapping of the SI locus and transcriptome analysis.

To create a mapping population, a self-compatible plant (previously identified at AgResearch in New Zealand) was pair crossed with a standard self-incompatible plant. Progeny resulting from this cross were then self-fertilised to produce an F2 mapping population, with an allele ratio of 3:1 (Self-compatible: self-incompatible) at the s-locus. F2 populations were then sequenced. This resulted in identification of a 1.5 Mb deletion in $Tr_{To}1$ of the self-compatible plant. Alongside this work, transcriptome analysis was undertaken using three individual plants: the self-compatible and self-incompatible parents of the F2 mapping population, and an unrelated self-incompatible plant. For each plant, RNA was extracted from anthers and stigma/styles from ~650 florets, leading to the identification of differentially expressed genes. Combined with assessment of diversity at the individual gene level, this led to identification of three neighbouring, high-confidence candidate genes within the region.

Red clover S-allele analysis

Germplasm of six red clover accessions with known variation in seed yield have been initially analysed for S-allele diversity. These comprise two UK cultivars (Aberystwyth S123 and AberClaret), one Swiss cultivar (Milvus), one German cultivar (Global) and two Bulgarian cultivars (Nika 11 and Sofia 52). These cultivars are also being investigated in boron application work carried out by in Bulgaria as part of this project. Initial analysis was carried out using simple sequence repeat (SSR) markers. This will inform whether restriction of S-allele diversity in standard synthetic population cultivars is contributing to seed production difficulties.

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[3] Riday, H. and Krohn, A.L., 2010. Genetic map-based location of the red clover (Trifolium pratense L.) gametophytic self-incompatibility locus. Theoretical and Applied Genetics, 121(4), 761-767. (Journal paper).

Boron application to red clover

A spaced plant field trial was established in Bulgaria in May 2024 at an experimental field in Negovan nr. Sofia (42.769455, 23.404125, altitude 549 m), to assess the effect of boron application on phenotypic characteristics and transcriptome expression related to seed yield in red clover.

The trial consisted of six commercial cultivars (Nika 11, Sofia 52, AberClaret, Milvus, Global and S123). From each cultivar, six plants were treated with “Lebasol” (11% active water-soluble boron eqv. 150g/l) at the stage of early bud formation, while six other plants from the same cultivar were left untreated as a control. Following this application, collection of flowers for pollen counting was not possible (except for a few flowers from S123) due grazing by rabbits. Therefore, the field was secured with metal netting in the autumn of 2024 to prevent this issue in the following year.

Soil samples taken next to boron treated plants, control plants and outside of the trial area, but within the same field, showed very little variation in boron content (6.68-7.18 mg/kg, Table 3). This indicates that boron application did not result in elevated boron within the soil.

In 2025, an investigation into transcriptomes related to pollen growth and development, and the effect of boron transport on transcript level and pollen viability was undertaken. Florets from both newly emerged and fully mature flowering heads were collected from the spaced plant field trial throughout May and June 2025 to isolate RNA for transcript analyses. Additional florets from the same flowering heads were collected to test for pollen viability and pollen count. Up to August 2025, transcript analyses were nearly completed, except for florets from S123, as flowering did not commence until the end of June. Pollen counts have been completed for each cultivar, but additional samples are still being processed to allow for a greater volume of data, and so data are not reported here.

Table 3. Boron indices in soils samples taken in various locations around the spaced plant trial in Negovan, Bulgaria.

	Boron (mg/kg)	
Field location	**Sample 1**	**Sample 2**
Near treated plants	7.17	6.92
Near control plants	7.18	6.68
Trial perimeter	6.91	6.73

Transcriptomes investigated via profile analyses are reported in Table 4. In the performed transcript profile analyses, florets from fully mature flowering heads that had been treated with boron showed higher expression of transcription factor ARF17 across all cultivars (Figure 12). ARF17 is an auxin response factor which is associated with anther development, dehiscence, pollen maturation and filament elongation. Increased expression of this gene in boron treated plants offers promising results in relation to improvements in seed yield. Expression level of WAT1 (influences auxin homeostasis) and NIP7.1 (Nodulin intrinsic protein, pollen specific aquaporins) were found to be correlated with ARF17 expression, although this correlation was only true for cultivars Nika 11, Sofia 52, Milvus and Global (Figure 13). Similarly, preliminary results from pollen count and viability were also correlated to transcript level gene expression but was also only observed in some cultivars and not others. Contrastingly, maturity of flowering heads, boron treatment nor cultivar did not influence the expression of APETALA 3 (homeotic gene related to petal and stamen development).

Table 4. Summary of transcript profile analyses on florets from spaced plants in trial at Negovan, Bulgaria.

Gene	**Purpose**	**Details**
Transcription factor ARF17 (Auxin response factor)	Anther development Regulates MYB108 expression	Auxin synthesized in anthers, acting through auxin receptors, coordinates anther dehiscence, pollen maturation, and filament elongation
APETALA 3	Petal and stamen development	Homeotic gene
WAT1 & WAT2	Auxin homeostasis	WAT 1 and ARF 17 expression level are linked
NIP4;2 & NIP 7;1 (Nodulin Intrinsic Protein)	Aquaporin	Pollen-specific aquaporin; expression peaks during pollen tube growth. characterized with transport of boron, anther specific.

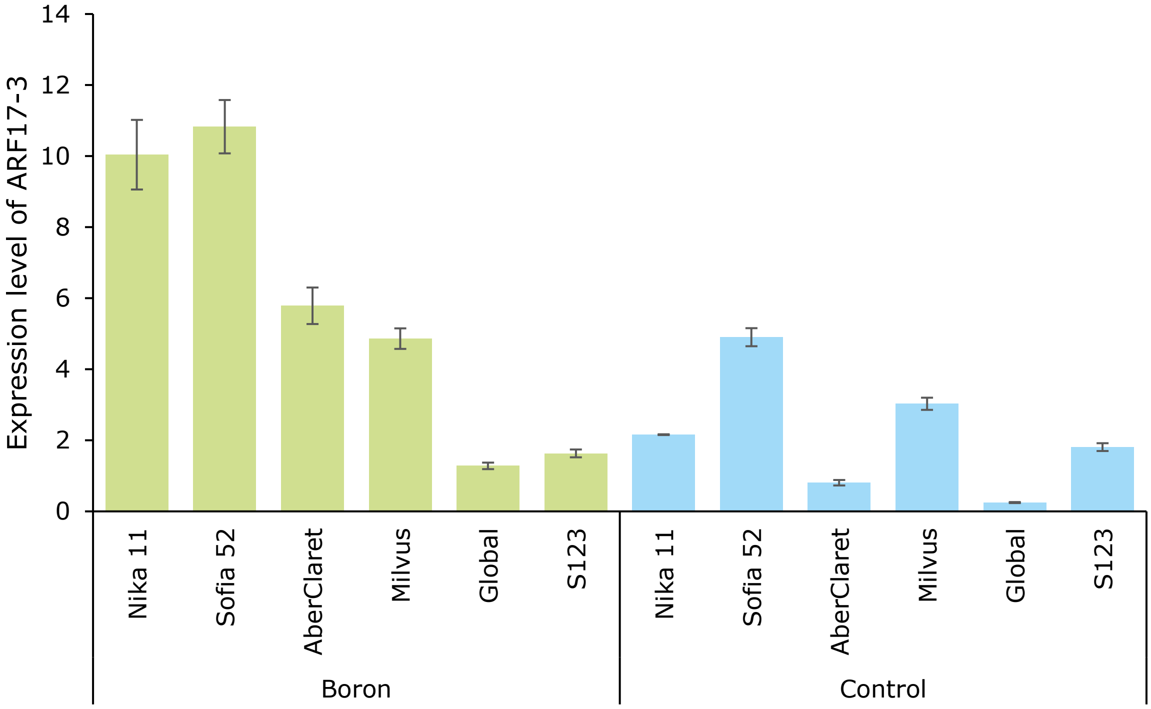

Figure 12. Expression level of transcriptome ARF17-3 in both boron treated and control plants within the spaced plant field trial at Negovan, Bulgaria. Error bars are given as SEM.

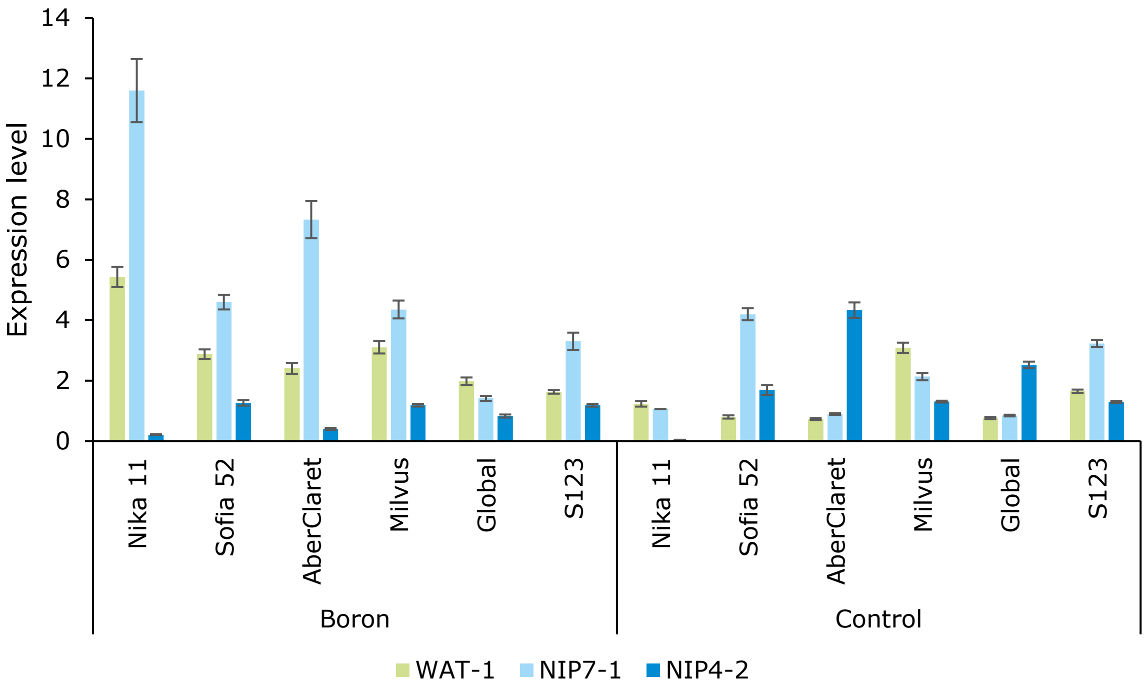

Figure 13. Expression level of transcriptomes WAT-1, NIP7-1 and NIP4-2 in both boron treated and control plants within the spaced plant field trial at Negaovan, Bulgaria. Error bars are given as SEM.

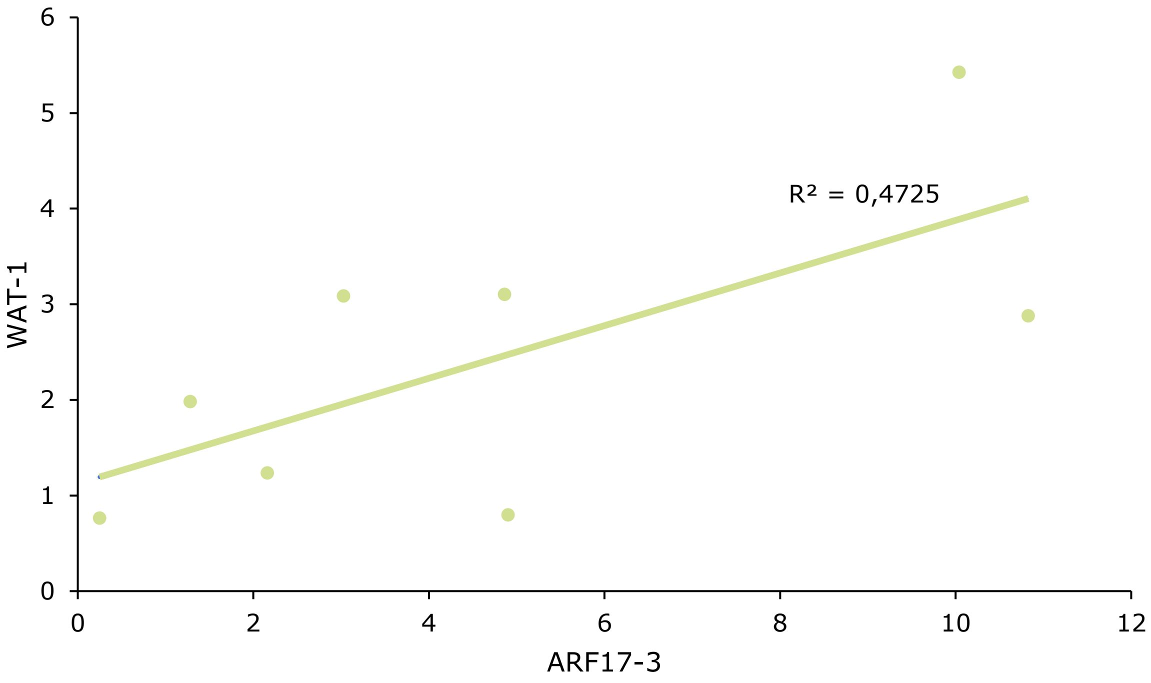

Figure 14. Correlation between expression level of transcriptomes ARF17-3 and WAT-1 in both boron treated and control plants within the spaced plant field trial at Negovan, Bulgaria. Data only represents cultivars Nika 11, Sofia 52, Milvus and Global, as S123 and AberClaret did not show any correlation.

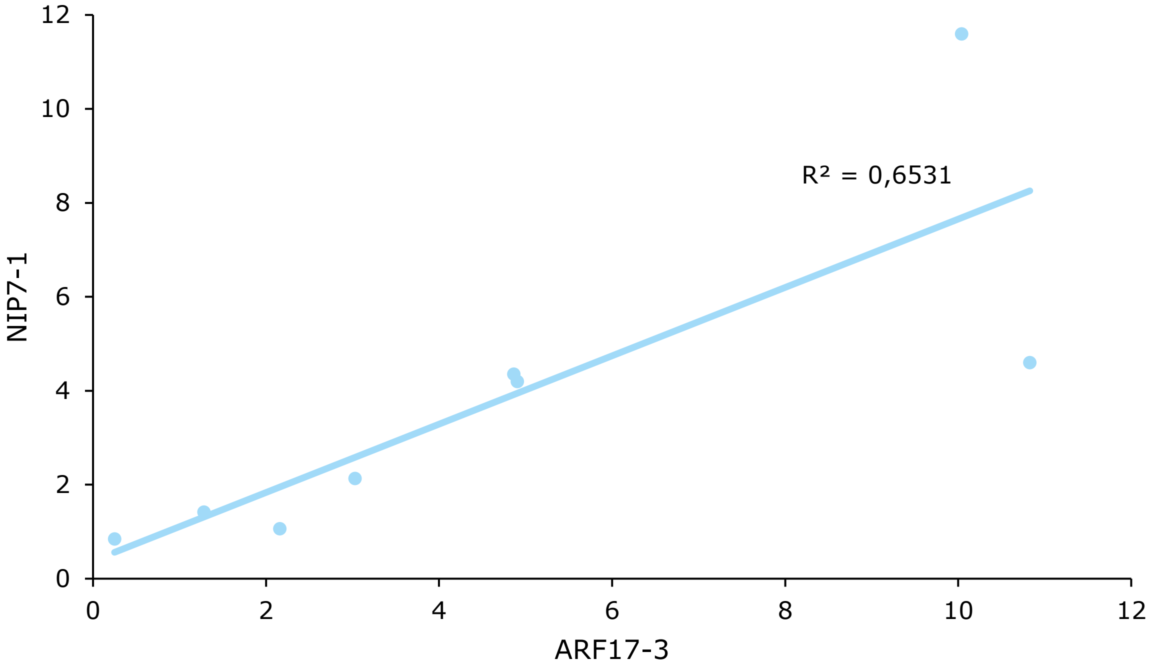

Figure 15. Correlation between expression level of transcriptomes ARF17-3 and NIP7-1 in both boron treated and control plants within the spaced plant field trial at Negovan, Bulgaria. Data only represents cultivars Nika 11, Sofia 52, Milvus and Global, as S123 and AberClaret did not show any correlation.

In June 2025, leaf material was collected across spaced plants within the field trial in Negovan. Initial observations of leaf mass, length and width indicate that boron application does not significantly affect leaf size or weight, when comparing treated plants to their respective controls. However, boron application did increase percentage nitrogen in leaves in all cultivars except Sofia 52, when analysed using a PrimacsSNC-100 (Skalar Analytical B.V) (Figure 16).

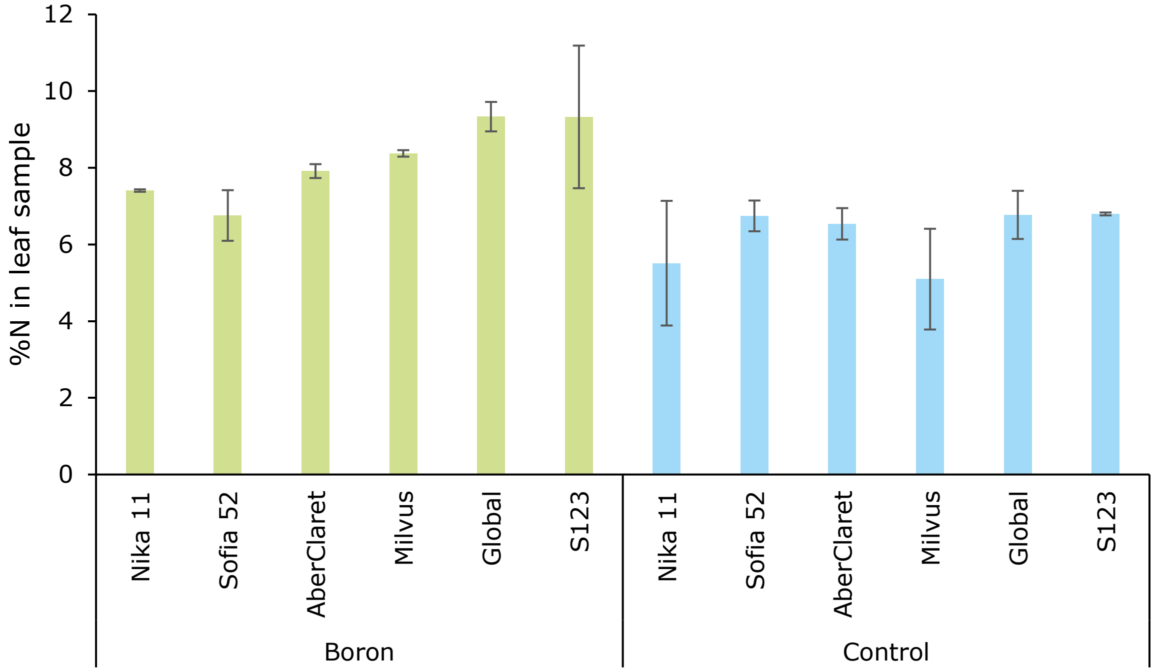

Figure 16. Percentage nitrogen in leaf samples 2 in both boron treated and control plants within the spaced plant field trial at Negovan, Bulgaria, sampled in June 2025.

Outreach and public engagement

Throughout 2024/5, numerous visitors including members of the British Grassland Society, AHDB, farming groups and students from Aberystwyth University visited the white clover diversity field trials at Aberystwyth, offering an opportunity to disseminate knowledge of the current work being undertaken by the Clover IC. Notably, activities undertaken at the EUCARPIA Fodder Crops and Amenity Grasses 36th section meeting held at Aberystwyth University in September 2025 included members of the IC giving presentations, plot tours of the diversity panel and general attendance of IC members at the conference.

Additionally, IC members from the AgroBioinstitute (Bulgaria) participated in the International Agricultural Exhibition Agra 18th-19th February 2025; where posters were presentations and promotional materials were given relating to this project. Additionally, staff partook in the Career Forum of the Faculty of Biology at Sofia University in March 2025, giving a presentation "The Importance of Legumes", as well as demonstration materials presenting work with model and cultivated legume plants (Figure 17).

Figure 17. Staff giving a poster presentation at the International Agricultural Exhibition Agra (left) and disseminating knowledge of work involved in the Legume Generation project at the Career Forum at Sofia University (right).

Conclusions

The Clover Innovation Community has been successfully established and continues to be a platform for collaborative research to better the breeding of both white and red clover. The collection of yield data on the white clover diversity panel and relative full genome sequencing has provided insight into natural genetic variation across the panel and how this is reflected in clover yields. These data will be valuable when selecting wild collections to introduce into breeding populations to improve the commercial breeding of white clover. Further data proposed to be collected 2026/7 will strengthen this, allowing for a GWAS. Additionally, the fine mapping of self-incompatibility s-allele loci in white clover could allow for selections to improve seed yield, improving the commercial viability of white clover. Developments in the utilisation of an SSR marker system in s-allele identification in red clover will continue to be explored within the IC. Results from boron application on individual red clover plants emphasises the potential that application of micronutrients may have on improving seed yield (and potentially nitrogen/protein content) in red clover.

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