Integrating the use of endophyte-infected grasses (Barrier *Festulolium*) **as a biological control method to combat aphids** (*Myzus persicae*) **and virus yellows in sugar beet**

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Project write up submitted to the University of Nottingham in fulfilment of the requirements for the degree of Master of Research in Plant and Crop Sciences

September 2024

Keywords: Sugar beet, Endophyte, Aphids, Biological control, Volatile Signalling, Lolines, Alkaloids

Total count: 17,940 words

Abstract

More than half of the UK sugar demand and 25% of the European sugar demand is met by indigenously grown sugar beet, making it one of the most economically important crops. The biggest threat to the sugar beet industry is virus yellows (VY) transmitted by peach potato aphids (*Myzus persicae*). The VY complex that consists of three different viruses, can cause yield losses ranging from 10 – 70% depending on the specificity and severity of the infection. Historically, neonicotinoids were used to control these viral vectors, however recent bans on these systemic pesticides owing to the harm they cause to beneficials has created a vacuum with no alternative available to combat the aphids and VY diseases (Francis *et al*., 2022). Thus, biological alternatives like endophyte-infected-infected grasses can play an important role in helping control pests and diseases. Barrier *Festulolium* is one such endophytic grass species developed by New Zealand based CropMark Seeds^{TM}. Barrier associates with the symbiotic fungal species *Epichloë uncinata*. This fungus lives within the grass tissues and when under stress it releases lolines, a class of alkaloid based volatile compounds, that in turn has been proven to keep pests away in grassland systems. This project aimed to try and integrate these grasses into sugar beet cultivation in the UK with the hypothesis being that if the sugar beet can take up some of these lolines secreted by the grass, it may help confer deterrence to aphids, potentially reducing virus incidence. As part of the investigation, Barrier *Festulolium* as a seed meal at four different doses (25%, 50%, 100% and 200%) was trialled in a controlled environment along with a field trial looking three different seed rates of the grass (10, 20, 30 kg/ha and control) and four different cultivation strategies (strip treated, shallow till, deep till and glyphosate) in each of the seed rates. The controlled environment results showed that the seed meal applications did not prevent VY infection, but induced beneficial responses in crop, helping keep the beet canopies greener for longer. Additionally, they also had a biostimulant effect on the sugar beet with increases in canopy and root biomass in the higher doses. The results from the field trial showed similar results – while the endophytic grass did not prevent aphids and virus yellow infections, it did seem to affect disease as there were more individual plants with yellowed canopies rather than large patches. There were also significant differences in leaf chlorophyll content at higher grass seed rates, with the glyphosate and strip treated plots showing the greenest canopies. However, the shallow till treatments at 20 and 30

kg/ha proved detrimental to sugar beet, with lowest plant establishment, more stressed canopies with higher temperatures and lowest leaf chlorophyll, proving how crucial it is to ensure that the grass is destroyed at the right time to ensure minimal competition or phytotoxic effects on the beet. Overall, while the endophytic grass doesn't prevent aphids/virus from affecting the beet, they are still a worthy biological alternative, as the beet show healthier canopies for longer and increased biomass accumulations, potentially translating to higher yields.

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Chapter 1 – Introduction and literature review

Sugar beet (*Beta vulgaris*) is a major source of sugar in Europe with sugar beet production in the UK meeting around 60% of indigenous sugar demand. As per British Sugar, the sole processor of sugar beet in the UK, it receives around 8 million tonnes of beet annually from growers, producing up to 1.2 million tonnes of sugar every year. 1n 2022, UK exported raw sugar worth $ 68.7 million (OEC, online). UK sugar beet is grown over approximately 100,000 hectares in eastern England encompassing East Anglia, East Midlands and Yorkshire. The beet is processed at four factories located within this region – Bury St Edmunds, Cantley and Wissington in East Anglia, and Newark in Nottinghamshire. After a severe decline in beet yields in 2020 owing to a virus yellows (VY) epidemic, UK sugar beet production recovered in 2021, with yields increasing by 26% to 7.4 million tonnes between 2020 to 2021, which led to an increase in overall production value by approximately 30% within the same period to £216 million (DEFRA, 2022). The UK sugar industry which continues to grow, will need to adapt to the changing government regulations and the challenges posed by the environment whilst making itself resilient to the future. As with any crop, warming environmental conditions and constantly evolving pathogens, mean that there is a growing risk of increased pest and disease incidence for sugar beet. Historically, the industry has always turned to chemical products, mainly neonicotinoids, to combat these challenges, but recent regulatory changes have left sugar beet farmers with barely a handful of options, with many of these once widely used chemical actives, being banned or highly restricted (Laurent *et al*., 2023). Neonicotinoids are the actives used in many systemic pesticides and are one of the most widely used products in the world (Bellis and Suchenia, 2022). They work by targeting the central nervous systems of insects causing paralysis and death. In sugar beet production, neonicotinoid seed treatments have been used to combat aphids and other pests during the initial few weeks of plant establishment. However, when used as seed treatments, 80 – 98% of these actives in the seed treatment leaches out into the environment and accumulates, causing contamination of groundwater, soil and posing threats to beneficials like bees and ladybirds (Slujis *et al*., 2013). The European Union (EU) had first banned neonicotinoids in 2018 (when UK was a member of the EU), with the UK subsequently banning it in 2020 (post Brexit). However, owing to the risk of significant aphid

pressures in 2022 and the potential incidence of virus yellows (transmitted by aphids), the UK government first granted an emergency use authorisation in January 2022 (Bellis and Suchenia, 2022) and emergency authorisations have been granted every year since then. This shows, how the lack of effective alternatives to these chemical measures can still lead to the use of products harmful to the environment and beneficials, in order to control pests and diseases.

Agronomy of Sugar Beet in the UK

Sugar beet is a biennial C3 crop that takes two years to complete its natural lifecycle. The first year is marked by vegetative growth and rapid canopy expansion resulting in high rates of photosynthesis to produce sucrose that is stored in a tap root. The crop then requires a period of cold-induced vernalisation (usually 40 days) at 5-10°C, to move into its reproductive phase in the second year (Sparkes, 2017). Once vernalisation requirements are met, the sugar beet 'bolts', elongating the stem into a tall shoot, with the auxiliary buds developing into a raceme-like inflorescence bearing flowers, which set seed once pollinated (Brar at al., 2015). If sugar beet is sown too early, and the vernalisation requirements are met, then even seedlings in the first year can bolt, which drastically reduces sugar content on the root. To avoid this, in the UK, sugar beet is sown usually only after mid-March. Preparation of good stale seedbed while sowing ensures good plant establishment, with reduced competition from weeds. Nitrogen availability directly affects photosynthesis, canopy growth and maintenance. Large amounts of nitrogen produce larger canopies but at the expense of root biomass, and also result in amino acid accumulation in the root that makes it difficult to extract sugar (Sparkes, 2017). Thus, it is important to ensure sufficient, but not excess nitrogen, is available early in the season. Sugar beet yield shows a direct linear correlation with the amount of light intercepted by the crop (Werker and Jaggard, 1998), thus rapid canopy establishment and closure is essential to ensure high yields (D. Sparkes, personal communication, 2023). The start of the season is marked by rapid canopy growth and increases in leaf area, with a transition towards increase in root biomass later in the season (Tiller *et al*., 2023). While incident radiation peaks in the UK in June, the canopy reaches closure a few weeks later due to the sowing prerequisites mentioned earlier, hence the goal is to ensure the canopy reaches closure as fast as possible in order to allow for maximum light interception. Once canopy closure is achieved, what matters the most is keeping the canopy green and actively

photosynthesising for as long as possible, to ensure high photosynthate production and translocation into the root. However, as shown in Figure 1, it is by this stage that the threat from aphids emerges – with virus symptoms starting to appear on infected plants soon after canopy closure. The canopy becomes increasingly yellow, and leaves start to senesce faster than newer leaves can emerge, thus significantly impacting radiation interception and yield. This is why it's crucial to help delay virus expression if it can't be prevented.

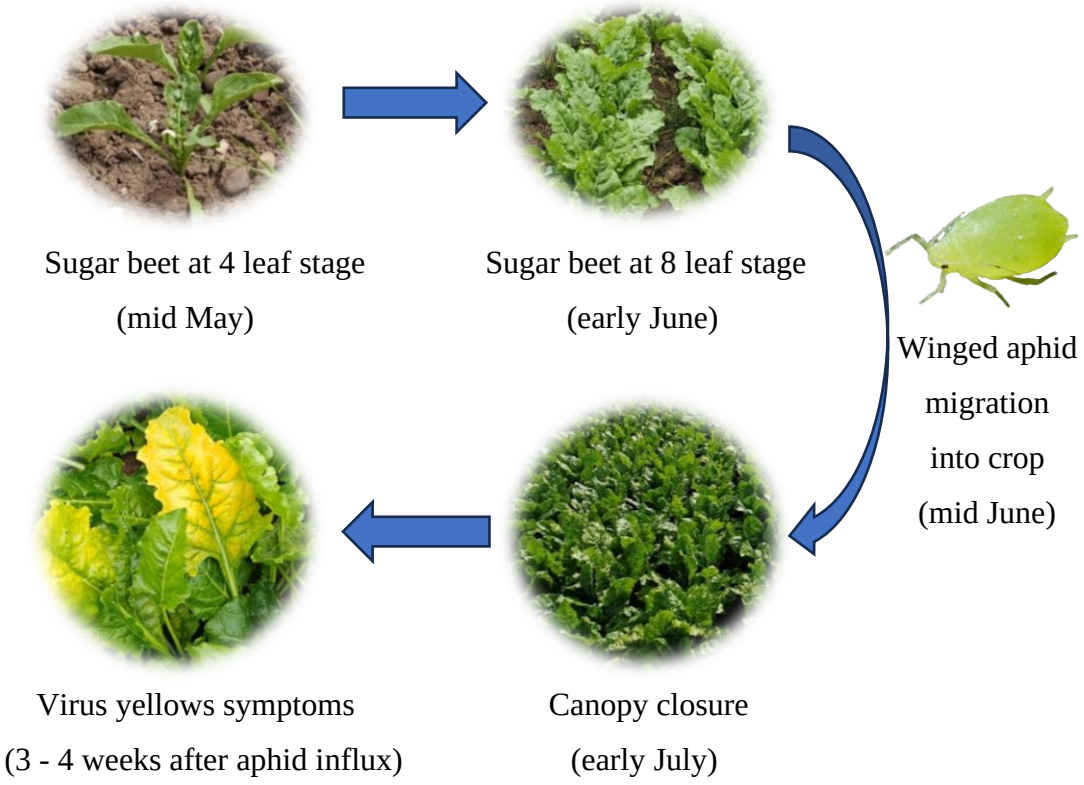


Figure 1. Normal life cycle of beet with aphid influx timeline

Pathology of sugar beet in the UK

Peach potato aphids (*Myzus Persicae*) are the primary vectors of the virus yellows (VY) complex that affect sugar beet. The VY complex that affects sugar beet consists of three main viruses in UK and Europe – Beet Yellows virus (BYV), Beet Mild Yellowing virus (BMYV), and Beet Chlorosis virus (BChV). BYV belongs to the *Closterovirus* genus and infection manifests as yellowish discoloration of leaves followed by reddish brown necrosis of leaf tissue (Hossain *et al*., 2020). BYV virions usually move via the phloem but are also capable of cell-to-cell transmission, crossing

the plasmodesmata and colonizing the mesophyll and epidermal cells (Dolija and Koonin, 2013). BMYV and BChV belong to the *Polerovirus* genus, with both viruses causing a yellowish-orange discoloration of the leaf tissue and premature leaf death (Hossain *et al*., 2020). Unlike BYV, *Poleroviruses* like BMYV and BChV are strictly found in the various phloem types – the phloem parenchyma and the companion cells where the virus replicates, and the sieve elements through which the virus travels to different parts of the plant (Boissinot *et al*., 2017). BChV is the least economically damaging causing 5 - 25% yield loss, whereas BMYV can cause a moderate yield loss ranging 20 - 40%. BYV is the most economically damaging virus to affect sugar beet and can lead to a massive 50 - 70% yield loss in the worst infections (A.Wright, personal communication, September 2023).

A study by Kozlowska-Makulska *et al.* (2009) sought to examine the transmissibility of European *polerovirus* isolates by four different aphid species - *M. persicae*, *Macrosiphum euphorbiae*, *Aphis fabae* and *Myzus ascalonicus* obtained from virus free French cultures. Results indicated that out of all four aphid species, *M. persicae* was the only one that could transmit both BMYV and BChV with 100% efficiency. While *M. euphorbiae* showed also showed 90 % transmission efficacy, both *A. fabae* and *M. ascalonicus* showed poor transmission of these *poleroviruses*. This helps highlight the major threat posed by *M. persicae*. The aphids and viruses can overwinter in bolting sugar beet, fodder beet, brassica crops, weeds and spoilage heaps (Dewar and Qi, 2020). When combined with a mild wet winter and a warm early spring, this can result in an early migration of aphids into the newly emerged sugar beet at the start of the season (Watson *et al*., 1975). In light of the restrictions imposed on neonicotinoids, the only option to control these aphids is though broadcast insecticide applications (Borgolete *et al*., 2024). However, owing to excessive use of chemical insecticides, *M. persicae* has become the most widely and strongly resistant pest species in the world, resistant to most classes of insecticides including pyrethroids, neonicotinoids and organophosphates (Bass *et al*., 2014) Thus, it is the need of the hour to find alternative control mechanisms for the aphids, that can reduce the risk of virus incidence.

Plant signalling and fungal symbiosis

Volatile organic compounds (VOCs) are chemical compounds that usually results in vaporization under normal environmental conditions (Dreher *et al*., 2019). VOC signalling is widespread in the plant kingdom and a large number of VOCs belonging to different classes have been identified so far shown in Figure 2 below. As part of their natural defences, it is widely known that plants use cocktails of VOCs as a mode to defend themselves from pressures of pests, diseases and herbivory. The production of these VOCs varies widely depending on the situation and can help to directly fend off herbivores but can also serve to indirectly attract beneficials and natural predators of pests (Michereff *et al*., 2011). For instance, when leaf beetle (*Diabrotica vigifera*) larvae feed on maize (*Zea mays*) roots, it induces maize to produce an indirect defensive terpenoid VOC called sesquiterpene (E)-β-caryophyllene, which in turn attracts an entomopathogenic nematode *Heterohabitis megidis* (Rasmann and Turlings, 2008).

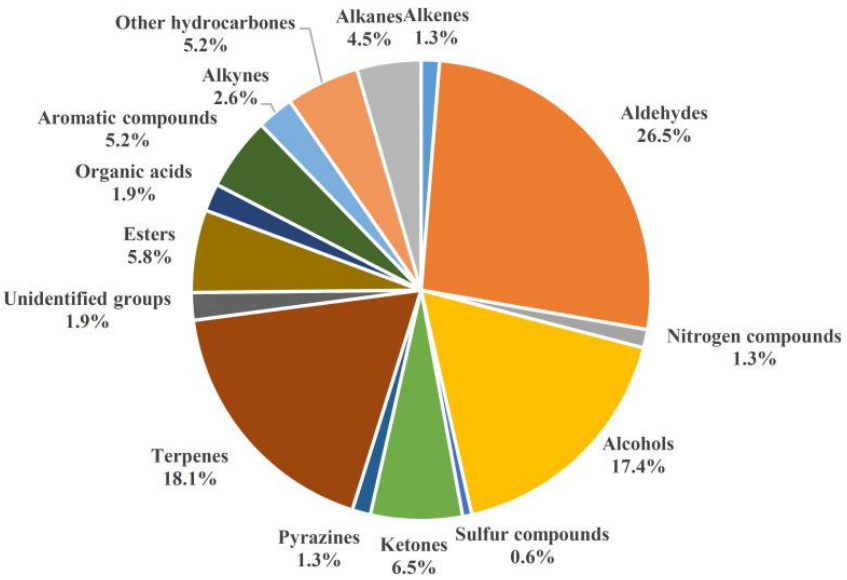


Figure 2. Diverse classes of VOCs identified so far. Figure by Duc *et al*. (2022).

Many plant-microbe symbiotic relations also arise from VOC signalling and form a crucial part of plant-environment interactions. Mutualistic root-associated fungal interactions as a result of this signalling plays essential roles in nutrient uptake, carbon cycling, plant growth and in some cases, increased resistance against pathogens and pests (Zuccaro *et al*., 2014). One example is plant root interactions with Arbuscular

Mycorrhizal Fungi (AMF). When plants starved for inorganic phosphorus release strigolactone molecules into the soil, this triggers the initial germination of AMF spores. These spores then release VOCs which further enhance strigolactone synthesis and root proliferation, playing a crucial role in aiding the plant to identify the AMF hyphae, allowing it to colonize the roots (Duc *et al*., 2022). The symbiotic AMF hyphal network help forage for water and nutrients, delivering it directly to the roots of plants. Another study by Sun *et al*. (2015) showed that VOC secretions by the AMF *Gigaspora margarita* trigger the expression of the gene LiCCD7 in *Lotus japonicus*, which is a crucial part of the strigolactone synthesis pathway affecting lateral root formation, and hence enhancing nutrient acquisitions.

Although the exact strategies are still not known, it has also been noted in several studies that plants can differentiate between signals produced by symbiotic and pathogenic fungi very early on, thus dictating their response accordingly. However, insects like aphids use these plant signals as olfactory cues to locate their preferred host plants or find a mate (von Arx *et al*., 2011).

Endophytes

Endophytes are an endosymbiont group of microorganisms (bacteria or fungi) that colonise inter or intracellular spaces in plant tissues, without causing any harm to the host species (Gouda *et al*., 2016). *Epichloë* endophytes are one such naturally occurring endophytic fungi species that are commonly found in pastoral grassland systems. Successful endophyte colonisation involves intimate plant microbe interactions and cross-talk of signalling molecules (Khare et al., 2018). An example of this is root exudates that are rich in biomolecules and metabolites such as flavonoids which serve as chemo-attractants and are recognised by endophytic fungi. A study conducted by Liu *et al*. (2022) on *Achnatherum inebrians* (drunken horse grass – commonly found in China) found that in endophyte-infected infected strains of *A. inebrians*, metabolites such as purine derivatives, indole derivates and phytoestrogens were upregulated. As a result, by modifying VOC composition, they help in providing resistance to biotic and abiotic stresses to their grass hosts with some species even offering resistance against pests like root aphids and mealybugs (Barker, 2016). Endophytic fungi asymptomatically colonise the intracellular and intercellular space

of the host plant forming a mutualistic relationship. These endophytes primarily help in plant defence by producing alkaloids and other metabolites that can deter pathogens and pests, but can also promote a growth response in their host by modulating biosynthesis of growth hormones and enhancing nutrient acquisition (Yan *et al*., 2019). Alkaloids produced by *Epichloë* endophytes can be grouped into four different classes– ergot alkaloids (eg chanoclavine), peramine, indole-diterpines (eg terpendole C) and lolines (N-acetylnorloline and N – formylloline) (Shyamanovich *et al*., 2015). The specificity of the alkaloid and the quantity of these volatile compounds is determined by specific situations and pressures on the host as well as the expansive variation in genome of the *Epichloë* species.

For this project, the species of grass being investigated is called Barrier *Festulolium*, containing the *Epichloë uncinata* (*E. uncinata*) U2 strain of endophyte-infected that produces **lolines** (Meyer *et al*., 2020). The structure of various loline isomers is given in Figure 3 below. *Festulolium spp*. hybrids are intergeneric crosses between *Festuca pratensis* (meadow fescue) and *Lolium perenne* (perennial ryegrass). This species is bred by the New Zealand based breeder CropMark Seeds^{TM} and have been extensively used in New Zealand's pastoral systems for grazing, with a track record of keeping pastures healthier by preventing pest infestations. Barrier is also proven livestock safe as, unlike its wild relatives that can produce toxic alkaloids, this species has been bred specifically for its loline producing endophyte *E. uncinata*. Unlike other toxic alkaloids such as ergot alkaloids produced by different endophyte species, lolines do not cause toxicity issues in grazing animals (Fletcher *et al*., 2017). While this specific species of endophytic grass has not been investigated for *M. persicae*, there have been some studies conducted with grass root mealybug and root aphid in Australia. Barker (2016) explored the effect of this Barrier U2 endophyte-infected grass on the infestation levels of these two pests compared to non-endophyte-infected Barrier *Festulolium* and found that post inoculations with both grass root mealybug and root aphids (common pests in Australia and New Zealand pastoral systems), the species containing *E. uncinata*, drastically reduced pest numbers as compared to the non-endophyte-infected grass. In fact, grass root mealybug infestations were reduced by 90% per gram of root and root aphid infestations reduced by 88% per gram of root, in the Barrier containing *E. uncinata* as compared to the non-endophyte-infected Barrier.

This provides good evidence that the lolines produced by the endophytic grass has played a role on conferring resistance to these root pests.

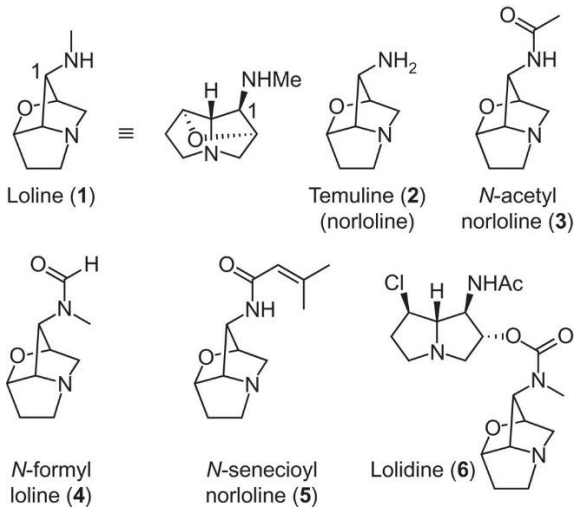


Figure 3. Structures of loline isomers. Figures by Cakmak *et al*. (2011)

The sufficient availability of these alkaloids and their potential exudation into the soil environment under different environmental conditions is a significant research gap, needing investigation (Mwangi *et al*., 2024). Additionally, studies on grass - endophyte interactions have mainly been under controlled environments, with barely any documented field trials on their efficacy. A study is currently being undertaken at Harper Adams University looking at the impact of the endophytic grass on free living nematode control in sugar beet. However, until now there has been no significant research done to investigate the effect of Barrier U2 on aphids, virus yellows and sugar beet physiology in field cultivations in the UK. In line with their Integrated Pest Management (IPM) strategy, the British Beet Research Organisation (BBRO) had conducted some observational strip trials in 2022, looking at the effects of the grass on sugar beet and virus yellows, when grown as intercropped strips in between rows of beet. The observations were interesting, as can been seen in figure 4 below where one can visually see the greener canopies and reduced yellow symptom expression where the endophyte-infected grass was present. This formed the foundation of the MRes project with the aim being to investigate the integration of endophytic grasses into sugar beet cultivation.

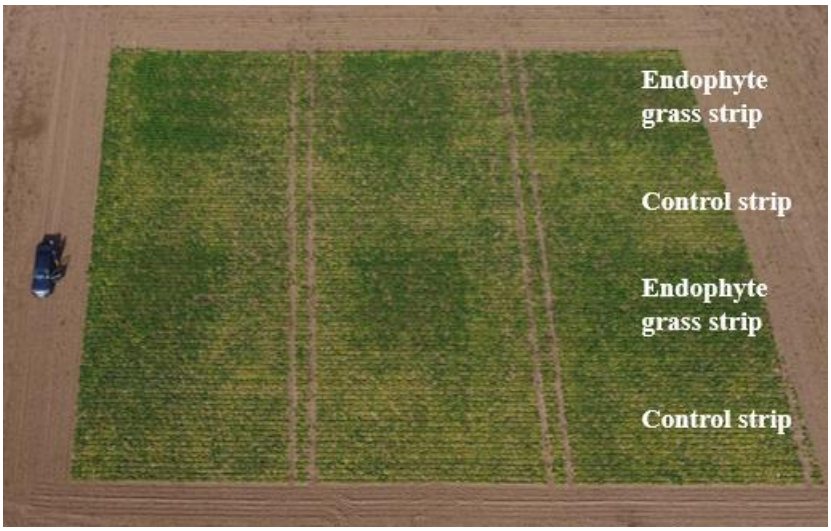


Figure 4. Drone shot of early endophyte-infected grass trials by BBRO at Morley in 2022 (A. Wright, personal communication, May 2023)

This project aims to answer the following research questions:

1) Do endophyte-infected grass species (U2 Barrier *Festulolium*) contribute to bio-stimulant effects on crops?
2) Which form of endophyte-infected grass (seed meal, intercrop, strip crop, cultivated) is the best for conferring induced aphid or virus yellows resistance in sugar beet, if any?
3) Does the endophytic grass result in healthier beet with greater biomass that can maintain a greener canopy for longer, potentially translating to higher beet yields?

Chapter 2 – Effect of endophyte-infected seed meal doses on sugar beet physiology and VY infections in controlled conditions

Introduction

Myzus persicae is the most economically important aphid species worldwide (Bass *et al*., 2014) and is highly damaging to the sugar beet crop as it is a vector of the VY complex, that can cause 10-70% yield loss depending upon severity of the infection. Neonicotinoids were commonly used as a seed coating to combat a wide range of arthropod pests including aphids and were very successful (Laurent *et al*., 2023). With the EU banning neonicotinoids seed treatments in 2018 (Gasparic *et al*., 2021) and the UK government banning them in 2020 owing to their harmful impacts on beneficials, the only option to control aphids was application of chemical insecticides, but their overuse has led to the aphids developing widespread resistance to them, rendering them ineffective. Hence, while the pest pressures continue to increase, sugar beet is one of the many crops that remain largely unprotected due to a lack of alternative crop protection strategies (Francis *et al*., 2022). Lack of immediate alternatives make it crucial to find IPM-based approaches to combat aphids. It is here where endophyte-infected grasses may prove a useful tool to protect sugar beet from virus yellows.

The loline alkaloids can make up, up to 2% of the dry weight of the grasses and the presence of the endophyte *E. uncinata* is essential to ensure loline production (Blankenship *et al*. 2001). Within the grasses, the concentration of lolines is usually highest in the seeds, followed by the stems and panicles with the least amount of loline alkaloids in the leaf blades (Mwangi *et al*., 2024). There have been some studies looking at the grass itself and its pest deterring ability in grassland systems. A study by Popay and Thom (2009) found that loline alkaloids produced by the grass were effective in deterring various pests like root aphid, pasture mealy bug, black beetle, grass grubs and Argentine stem weevils, all common pests in New Zealand grasslands. However, there haven't been any controlled environment studies to look at the potential impacts of the grass on *M. persicae* in sugar beet systems, in detail.

This chapter describes glasshouse experiments in which different scenarios of applications were tested to determine whether the seeds of the endophytic grass and its loline content can help keep aphids away from sugar beet and potentially reduce

virus incidence, whilst also investigating the effect it has on plant health. The glasshouse study was a seed meal dose response experiment to study virus infection at different doses and collect diagnostic data on sugar beet health and canopy response to the doses. The aim was to identify whether the seed meal affected virus infection whilst also examining what dose and application time has the most beneficial impacts on the sugar beet.

Experimental design

The glasshouse, located at the Sutton Bonington campus of the University of Nottingham, is a south facing Cambridge style climate-controlled glasshouse, heated through pipes with automated hydraulic vents for cooling and airflow purposes. Sandy loam field soil obtained from the Sutton Bonington farm was sieved and used in this experiment. Three litre deep pots were set up on benches corresponding to the blocks as per the experimental design shown in Figure 5. The trial had six blocks in a split plot design, each with 5 different rates of the endophyte-infected seed meal. The glasshouse had minute variations in temperature and lightning conditions in different sections that could affect the results and create outliers. Hence a split plot design was chosen, to account for the effect of these variations in the data and try and obtain a normally distributed dataset. Discards were the buffer between blocks and were treated with the Control dose.

For the sake of the experiment each dose was named based on a percentage mix of ryegrass seed meal and the endophytic grass seed meal, based on the composition breakup in Table 1, totalling to 10 grams (g) of seed meal in each pot. The grass seed was provided by CropMark Seeds^{TM}. While we didn't specifically test the seed for endophyte presence, the breeder confirmed that the Barrier U2 grass seed was harvested from a grass sward that showed positive endophyte presence and that the seeds contained highly concentrated amounts of loline approximating to 15,000 µg/g seed. As the lolines are most concentrated in the seeds, for the sake of this experiment, 10 g of seed meal was estimated to be sufficient to deliver enough lolines to allow for examination of any impacts it might have. Seeds were then weighed and ground to a semi-fine powder using a coffee grinder to make the seed meal. Thus, the lowest endophyte-infected dose was the 0% control and most concentrated endophyte-infected dose was 200%. The range of concentrations are named as Control (0%

endophyte seed meal), 25%, 50%, 100% and 200%. These specific doses were chosen to try and generate a normally distributed dataset covering the lowest to highest range. The highest dose was labelled as 200%, since it was hypothesised that this dose may be more than the optimum concentration that could be realistically feasible, with potential negative/phytotoxic impacts on the sugar beet.

Two additional treatments, involving different timings of seed meal application, each with all the five rates, were also investigated in the glasshouse and these were randomized throughout the trial. Treatment 1 involved applying and incorporating the seed meal when the sugar beet is sown, whereas Treatment 2 involved applying the seed meal without incorporation, just 3 days before aphid inoculation to simulate a surface dressing of the seed meal which could be applied in-field, when aphids are expected to begin their migration. Thus, the glasshouse trial involved 2 treatments, 5 seed meal rates and 6 blocks with 4 discards in each block as shown in Figure 1, for a total of 84 plants. The sugar beet variety used in this trial was '*Lightning*' bred by SESVanderHave, with three seeds planted per pot, thinned to one seedling per pot post germination.

Table 1. The composition of the five dose rates used in the glasshouse trial

Rate	**Endophyte-infected seed meal (g)**	**Ryegrass seed meal (g)**
Control (0%)	0	10
25%	1.25	8.75
50%	2.5	7.5
100%	5	5
200%	10	0

Figure 5. Endophyte-infected seed meal dose response trial plan. Both Treatment 1 and Treatment 2 were entirely randomised in the blocks and between both sides of the glasshouse. The arrow represents the entrance to the glasshouse. The pots were arranged on a bench 75cm above ground.

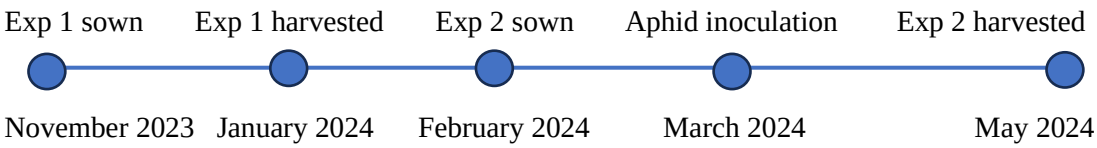
Experiment one - establishment

The first glasshouse experiment was sown on 17th November 2023, with three seeds sown in each pot, as per the trial plan in Figure 1, thinned to one plant post establishment. In accordance with the experimental design described above, in half (42) of the pots, the seed meal was applied as Treatment 1 prior to sowing the sugar beet. Endophyte-infected and normal Ryegrass seeds were separately ground down to a semi-fine powder using a coffee grinder. The doses, as described in Table 1, were then prepared in a porcelain crucible and weighed using a calibrated laboratory weighing scale, correct to 10 g (+/- 0.04 g).

Experiment two – establishment

Due to time constraints, the aphid inoculations and application of Treatment 2 could not be completed in the first experiment. Hence, a second experiment using the same experimental design was conducted, but this time looking specifically at crop physiology and virus symptoms. This second trial was sown on 5th February 2024, with three seeds sown in each pot, thinned to one plant post establishment. Just like experiment one, the seed meal was again applied to half the pots at sowing, in accordance with the experimental design.

A timeline of both the glasshouse experiments is given below:



BMYV aphid inoculation and Treatment 2

According to the timeline, the second seed meal treatment (Treatment 2) was applied on 1st March, three days before aphid inoculation in this second experiment. The *M. persicae* aphid culture for the same, carrying BMYV, was sourced from BBRO. Three wingless *M. persicae* aphids carrying BMYV were inoculated onto all the beet in the glasshouse on the 4th of March 2024. Following the inoculation, their numbers were monitored regularly for twelve days.

Experiment maintenance and conditions

Lighting, temperature control and watering

As this experiment ran through the winter of 2023/24 with lesser daylight hours, supplementary sodium vapour halogen lamps were used to provide lighting as shown in Figure 6. These lights were on for 16 hrs every day. This was a *TomTech microclimate* controlled glasshouse, with the day and night temperatures set to 22°C and 18°C respectively to speed up germination and growth. The glasshouse was set to vent at 25°C. The pots were watered with a hose once in three days until few weeks after germination and the watering frequency was gradually increased to once in two days post plant establishment.

Figure 6. The glasshouse experimental set up

Weed control and fertiliser

As field soil was used in the trial, weeds did occasionally appear and were manually weeded out. Thus, there were no herbicides applied in the trial. The beet were fertilised using a 15:7:30 (N : P : K) horticultural feed connected to the watering hose, once the beet reached the 4 leaf stage. These strategies served to minimise as much chemical inputs as possible, in order to obtain results that correspond more directly to the seed meal dose response. We also wished to keep within 'sustainable' agricultural practices as much as possible.

Assessments and data analysis

Photosynthesis measurements

A crucial part of the endophyte dose response trial was to understand the effect of the seed meal on plant health. To do this we studied whether there are any detrimental effects on canopy photosynthesis. A soil plant analysis development (SPAD) meter was used to measure chlorophyll content and a leaf porometer (LICOR 600 model) was used to collect plant diagnostic data: stomatal conductance, Photosystem II (PSII) chlorophyll fluorescence and leaf temperature after the beet reached the 4-leaf stage. The fully expanded leaf 4 was selected in each plant and measurements were taken on the leaf lamina avoiding the veins, centrally between the midrib and the leaf margin. SPAD measurements took about 5 seconds per plant. The LICOR 600 porometer was clamped, covering both sides of the leaf, and used to measure stomatal conductance ϕ PSII (Fq'/Fm') and leaf temperature which took about 10-12 seconds per plant.

SPAD readings in experiment two, were taken both before inoculation to represent pre-symptom expression values, and after symptom expression to help identify potential trends in symptom severity or virus infection based on the doses. The remaining photosynthetic data (same as experiment one) was collected approximately 6 weeks after aphid inoculation.

Biomass measurements

Experiment one was harvested on 30th January 2024 and harvest data encompassing leaf area, fresh and dry weights were analysed. Experiment two was harvested on 28th May (12 weeks after inoculation) following which harvest data encompassing leaf area, fresh and dry weights were analysed.

ELISA (experiment two)

For experiment two, leaf discs from leaf 6 were collected using a size 8 (13.75 mm diameter) cork borer, two weeks after aphid inoculation and an Enzyme-linked Immunoabsorbent Assay (ELISA) performed at BBRO, to confirm if the inoculation was successful and infer whether disease incidence severity differed with the different seed meal treatment. The ELISA is a biochemical analytical technique that uses antibodies to detect the presence of specific viral associated proteins in the sample. For these samples, a Beet Western Yellows Virus Triple antibody sandwich ELISA was performed. The process of the indirect ELISA is shown in Figure 7.

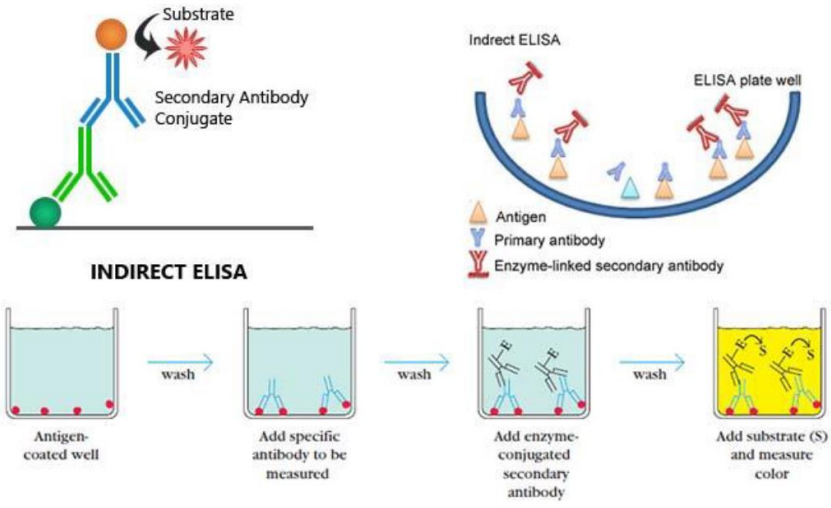


Figure 7. The process of indirect ELISA used to confirm VY infection and identify virus (Aryal, 2021)

For both experiments, after the data was collected, an ANOVA (Analysis of Variance) was performed using the statistical analysis software RStudio, to test for statistical significance. The least significant difference (LSD) values were then calculated for the results that were significant. All data obtained was found to be normally distributed and did not require any transformation.

Results - Experiment one

The beet grew much slower than expected in this first glasshouse experiment, as a result the aphid inoculations due at the four-leaf stage, could not be done. However, plant diagnostic data such as SPAD, stomatal conductance, fluorescence and leaf temperature were collected in order to gain an insight into how the seed meal doses affected plant photosynthesis and also to observe if there were any detrimental effects on plant health.

Additionally, the sugar beet were harvested at the start of February 2024 (at the 12 leaf stage) to observe impacts on leaf area, canopy fresh and dry weights and root fresh and dry weight, This helped provide an early insight into how the beet were performing and used to narrow down which dose may be having the best overall impact, the output of which is shown in table 2.

A significant difference in canopy and root weights and leaf area was observed compared to both control and untreated treatments. A general increasing trend across all endophyte-infected seed meal doses was observed for the above parameters with the 100% seed meal dose resulting in the highest canopy fresh weight and leaf area and the 200% dose resulting in the highest root weight. However, the 50% treatment showed a dip as it was skewed due to an outlier in the dataset. When this outlier was removed, it did increase the overall median and better fit the trends for some of the parameters, but this reduced the sample size, so for consistency, was still included in the results. Trends in leaf temperature (T leaf) are harder to interpret in the lower doses, but the 200% dose shows a significantly higher leaf temperature. No significant changes could be identified in the other photosynthesis parameters.

Table 2. Statistical analysis of experiment one showing impact of various endophyte-infected seed meal doses on canopy photosynthesis, canopy biomass and root weights. p values and LSDs show significance at 95% confidence. The entire dataset is attached in Appendix 1

Parameter	p value	LSD
SPAD (a.u.)	0.21	
Stomatal Conductance (mmol/ m^2/ s)	0.19	
Fluorescence (ϕ PSII)	0.18	
T leaf (°C)	**0.02**	**0.99**
Canopy Fresh Weight (g)	**0.0002**	**15.7**
Canopy Dry Weight (g)	**0.009**	**1.65**
Root Fresh Weight (g)	**0.02**	**3.79**
Root Dry Weight (g)	0.11	
Leaf Area (cm^2)	**0.006**	**190.98**

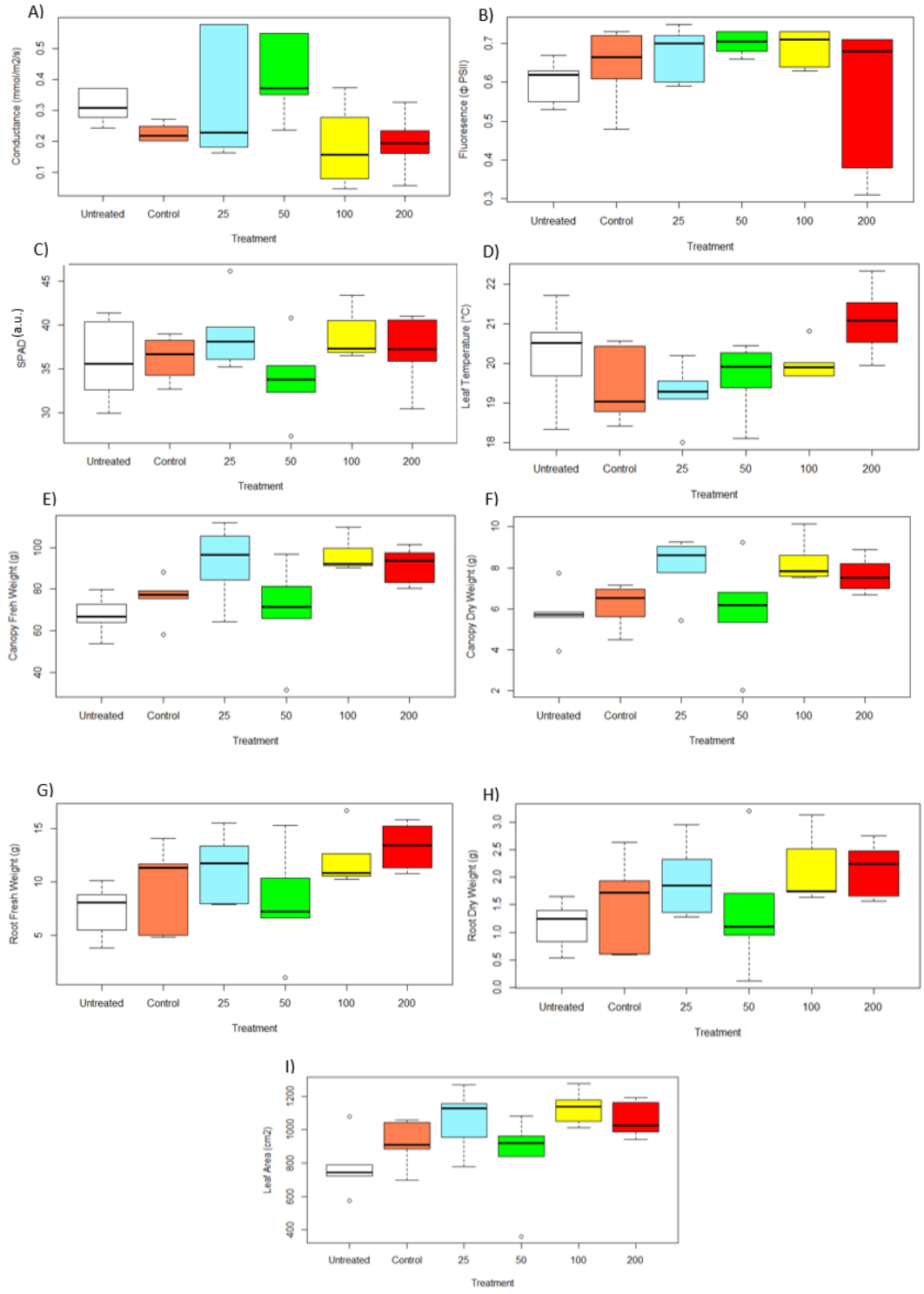


Figure 8. The effect of various endophyte-infected seed meal doses on Stomatal Conductance (A), Fluorescence (B), SPAD (C), Leaf Temperature (D), Canopy fresh weight (E), Canopy dry weight (F), Root fresh weight (G), Root dry weight (H) and Leaf area (I). The boxes represent all data within the first and third quartiles, thick black lines depict the median values, the whiskers depict the maximum and minimum values in the data and the hollow circles depict outliers.

SPAD data did not show any statistically significant differences ($p > 0.05$). However, some trends were apparent - the 100 % dose had a high SPAD value. The dip at 50 % dose can be explained by an outlier in the dataset that skewed the graph. However, any potential greening effect seems to plateau out at 100%.

Leaf temperature showed significant differences with higher temperatures compared to control when the dosage increased, which was also picked up on by the ANOVA ($p = 0.02$). Data obtained in this first experiment, as seen in Figure 8 (D) shows a steady rise in leaf temperature as the seed meal dose increases, with the highest dose 200%, recording the highest overall leaf temperature. LSD results indicated that the 200 % dose showed the highest overall mean temperature (mean = 21.09°C) and was significantly higher than all the other treatments except the Untreated (mean = 20.26°C). The 25% treatment (mean = 19.24°C) showed the lowest mean leaf temperature and was significantly cooler compared to the Untreated and 200%. There were no significant differences in leaf temperature between the Untreated, Control, 50% and 100% doses.

Stomatal conductance showed a gradual dropping trend as the dose increases, whereas fluorescence plateaued out at 100% dose. Canopy fresh and dry weights (Figure 8(E) and 8(F)) showed an increase compared to the untreated, suggesting enhanced plant vigour. There is a significant increase in the canopy ($p = 0.0002$) and root biomass ($p = 0.02$) with higher doses of the seed meal, with the highest mean root fresh weight being recorded in the highest dose at 200%. LSD results indicated that the 100% dose had the highest mean canopy fresh weight (mean = 95.93g) which was significantly higher than the Untreated (mean = 67.25g), Control (mean = 75.98g) and 50% (mean = 69.67g). This also held true for canopy dry weights. There were no significant differences between in the canopy fresh and dry weights between the 25%, 100% and 200% treatments, and between the Untreated and 50%.

Root fresh weight also showed a rise with increasing dose, with the highest root fresh weight seen at 200%, clearly indicating that the higher concentration of the endophyte-infected seed meal had a growth stimulating effect on the beet, also underscored by the significant ANOVA result ($p =0.02$) (Table 2). LSD analysis showed that the 200% dose had the highest mean (mean = 13.32 g) root fresh weight and was significantly

higher compared to the Untreated (mean = 7.38g) and 50% (mean = 7.96g). However, the 200% showed no significant differences against 25%, 100% and Control.

A similar trend was observed in the canopy leaf area, but here the highest mean leaf area is observed in the 100% dose (mean = 1113.11 cm^2). The leaf areas at 25% (mean = 1069.34 cm^2), 100% and 200% (mean = 1055.66 cm^2) weren't significantly different among themselves, but rather showed significantly higher leaf area when compared to the Untreated (mean = 774.95 cm^2), Control (mean = 916.26 cm^2) and 50% (mean = 847 cm^2) doses. This provides support that the seed meal does seem to have a positive effect on canopy vigour, photosynthate production and storage reserves. As can be seen in Figure 8(I), with increase in dose rate of the endophyte-infected seed meal, there is a good positive correlation to leaf area, which is also backed by highly significant result returned by the ANOVA ($p = 0.006$).

To check whether the endophyte-infected seed meal contained higher nutrients that could have explained the higher biomass compared to the standard ryegrass meal, samples of both seed meals were submitted to Cawood Scientific Ltd. for GrainCheck® elemental analysis. The analysis revealed no major differences in nitrogen, phosphorus, potassium, calcium, magnesium, manganese, copper, zinc iron and boron. There was a slightly higher sulphur composition in the endophyte-infected seed meal (Appendix figure 1) compared to normal ryegrass, but this wasn't considered significant to impact the trial.

Results - Experiment two

The main difference in this second glasshouse experiment was addition of the aphid inoculation and a second seed meal application (Treatment 2) just before inoculation. Beet were inoculated with aphids on 4th March 2024. Experiment two was completed and harvested on 28th May 2024. All the data shown below was collected post inoculation, but the SPAD was specifically collected pre and post inoculation, to account for changes in chlorophyll content when VY symptoms manifest.

Pre inoculation SPAD in Table 3, show that on average across the doses, the values were higher in Treatment 1 compared to Treatment 2. Since the seed meal was applied at sowing in treatment 1 as compared to just prior to inoculation in treatment 2, this

might suggest a higher nutrient uptake in the beet caused by longer exposure to the endophyte seed meal. This does match the findings in the first glasshouse experiment. Additionally, almost all the SPAD values across both treatments show a drop post VY symptom expression, which is to be expected as the yellowing reduces chlorophyll content in leaves following a successful infection.

Table 3. The differences in SPAD values pre and post inoculation, between both treatments and across all doses. Treatment 1 is seed meal applied at sowing and Treatment 2 is seed meal applied just before aphid inoculation.

Endophyte-infected seed meal dose	SPAD means (a.u.)			
	Pre inoculation		Post inoculation	
	Treatment 1	Treatment 2	Treatment 1	Treatment 2
Control (0 %)	34.7	33.3	33.1	32.8
25 %	36.6	33	34.2	31.8
50 %	35.8	33.9	33.5	29.3
100 %	34.9	36.6	35.1	36.8
200 %	37.4	36.1	35.1	33.7

When all the parameters were analysed, the seed meal doses were found to highly influence leaf temperature. Leaf temperature was overall significantly ($p < 0.001$) lower at higher doses of the seed meal. While this wasn't statistically significant when looking at the treatment timings (Treatment 1 and Treatment 2) individually, on interaction with the seed meal doses, the differences were highly significant ($p = 0.003$) as summarised in Table 4. This can also be clearly seen when comparing Figures 9D and 10D, which show a much sharper drop in leaf temperature in Treatment 2 (seed meal at inoculation) at the higher doses, compared to Treatment 1.

LSD analysis revealed the leaves in both 100 % (mean = 20.72°C) and 200% (mean = 20.52°C) doses were the coolest and were significantly cooler than the Control (mean = 24.62°C), 25% (mean = 25.1°C) and 50% (mean = 23.62°C) doses. This suggests that despite virus infection, the higher seed meal dose keeps the canopy cooler as a result of higher photosynthesis.

In Treatment 2, both stomatal conductance and leaf temperature trended to a sharp decline in the highest doses with an increase in PSII activity at the higher doses. Interestingly, both the application timings did not return a significant result for any biomass or leaf area parameters, in contrast to the first experiment. In treatment 1 no clear trend could be identified in any of the biomass measurements, but its worth noting that the median canopy and root weights (Figure 9 (E to H)) and leaf area (Figure 9 (I)) were the highest in the 200% dose. In treatment 2 there was a better trend of a slight increase in canopy and root weights and leaf area as the dosage increased, but again the differences weren't statistically significant.

Table 4. Two-way ANOVA results for experiment two. p values and LSDs show significance at 95% confidence. T1 treatment refers to seed meal applied at sowing and T2 treatment refers to seed meal applied at inoculation. 'Seed meal dose & treatment' column shows the interaction between both factors. The entire dataset and means are attached in Appendix 2.

Parameter	**p value**			**LSD**
	Seed meal dose	**Treatment (T1 or T2)**	**Seed meal dose & treatment**	
SPAD (a.u.)	0.172	0.285	0.598	
Stomatal Conductance (mmol/ m^2/ s)	0.397	0.923	0.232	
Fluorescence (ϕ PSII)	**0.034**	0.98	0.258	**0.085**
T leaf (°C)	**< 0.001**	0.205	**0.003**	**1.178**
Canopy Fresh Weight (g)	0.744	0.168	0.525	
Canopy Dry Weight (g)	0.705	0.215	0.355	
Root Fresh Weight (g)	0.338	0.175	0.213	
Root Dry Weight (g)	0.324	0.102	0.219	
Leaf Area (cm^2)	0.661	0.153	0.446	
ELISA	0.519	**0.013**	0.936	**0.124**

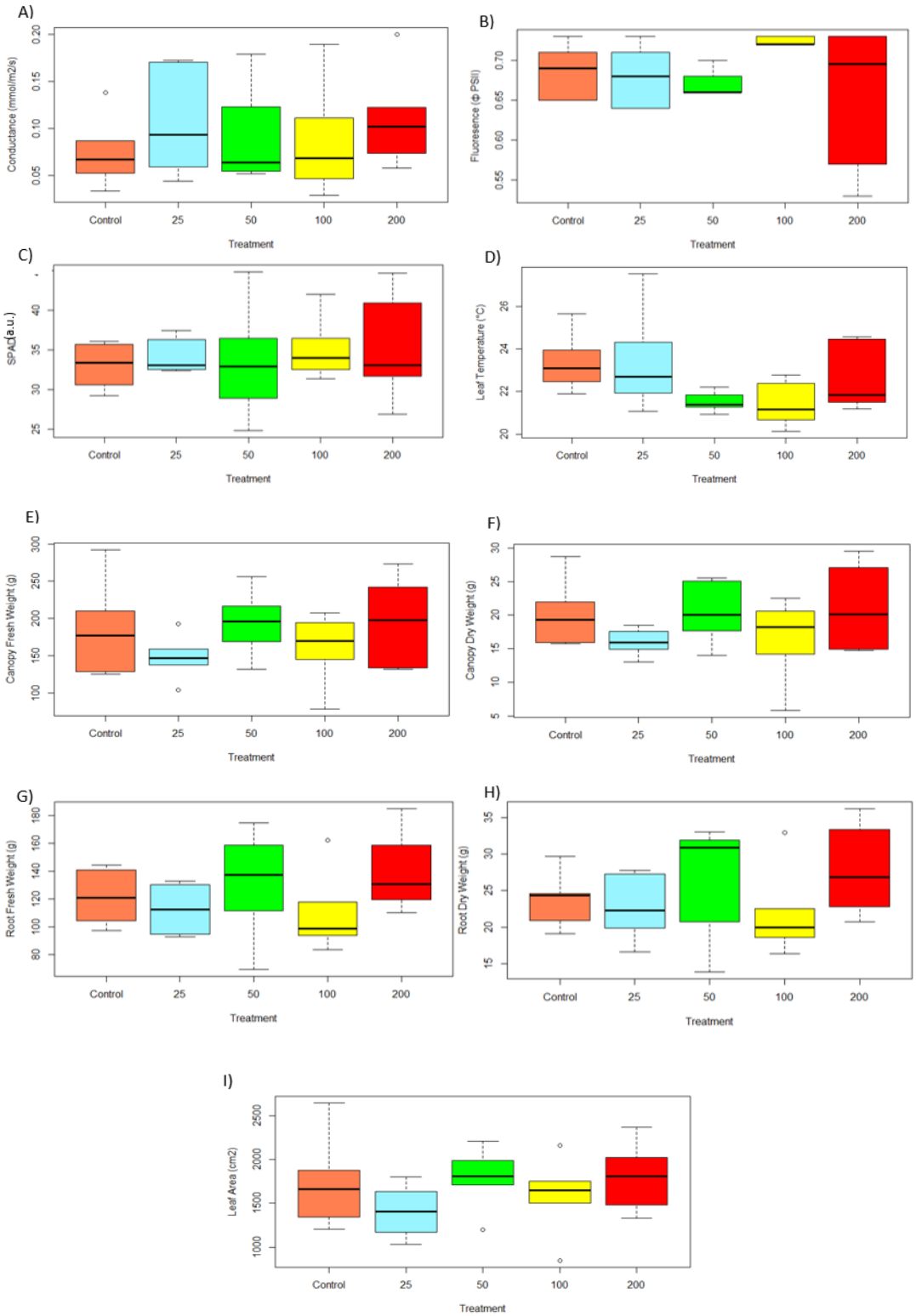


Figure 9. The effect of various seed meal doses applied **at sowing**, on Stomatal conductance (A), Fluorescence (B), SPAD (C), Leaf temperature (D), Canopy fresh weight (E), Canopy dry weight (F), Root fresh weight (G), Root dry weight (H) and Leaf area (I). The boxes represent all data within the first and third quartiles, thick black lines depict the median values, the whiskers depict the maximum and minimum values in the data and the hollow circles depict outliers.

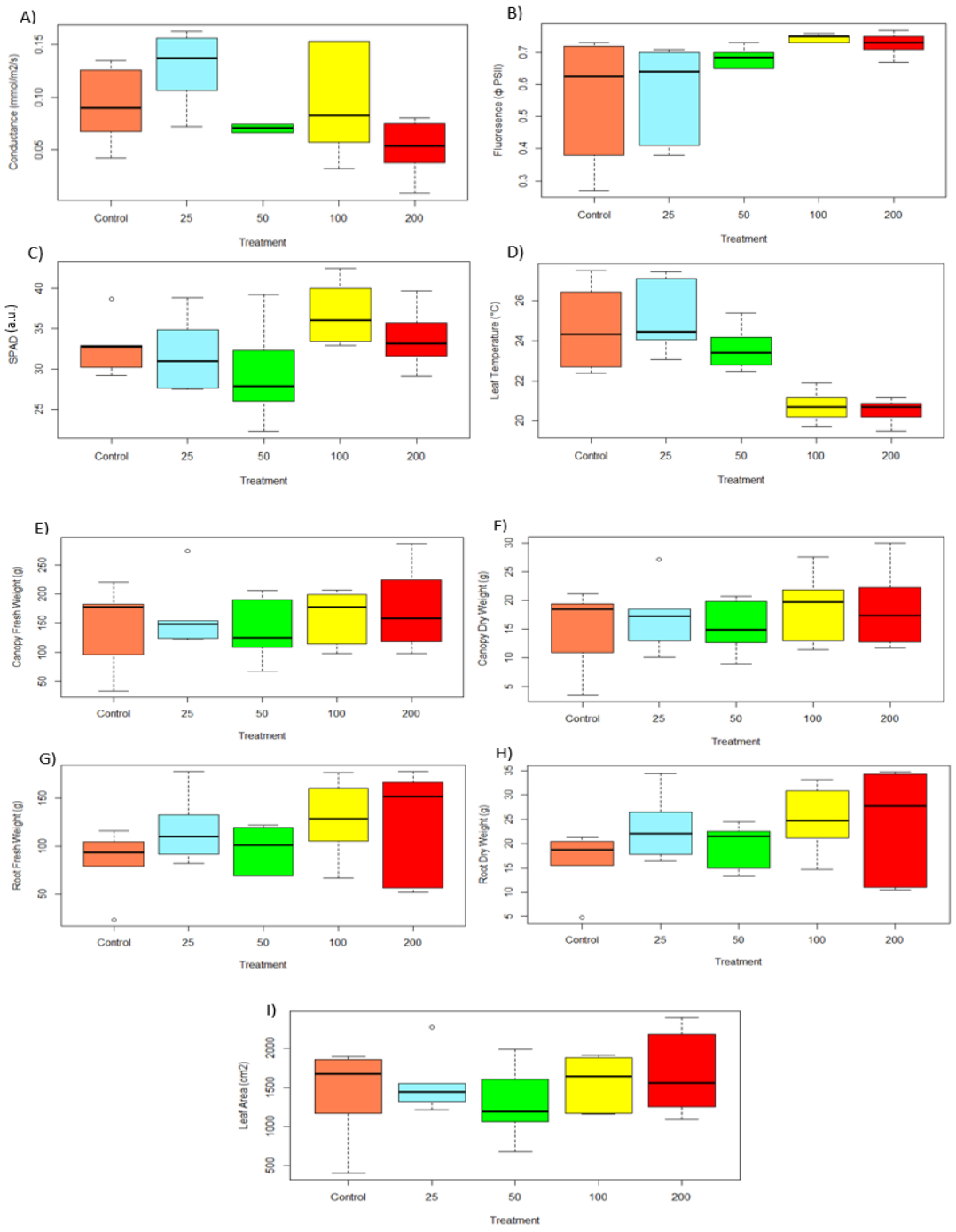
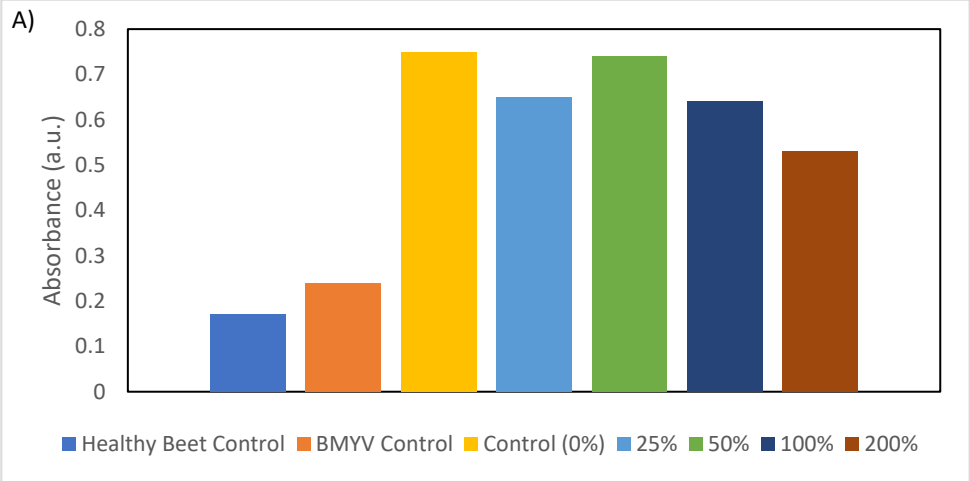


Figure 10. The effect of various seed meal doses applied **at inoculation**, on Stomatal conductance (A), Fluorescence (B), SPAD (C), Leaf temperature (D), Canopy fresh weight (E), Canopy dry weight (F), Root fresh weight (G), Root dry weight (H) and Leaf area (I). The boxes represent all data within the first and third quartiles, thick black lines depict the median values, the whiskers depict the maximum and minimum values in the data and the hollow circles depict outliers.

The photosynthesis data across treatment 1 (Table 4) showed there is no significant difference ($p > 0.05$) or trend in SPAD and conductance across the doses. However, ϕ PSII (fluorescence) shows a significant result ($p = 0.034$) which is also clearly visible in Figure 10(B) with the highest median fluorescence value in the higher doses. LSD analysis showed Control had the lowest mean ϕ PSII (mean = 0.56) which was significantly lower than both 100% (mean = 0.75) and 200% (mean = 0.73) doses. However, there was no significant difference observed between the Control, 25% and 50% doses.

While there were no statistically significant differences in plant weights, one trend that seems consistent across both the treatments is the highest doses showing increased plant biomass. The 200% seed meal dose results in the highest median canopy and root weights in Treatment 1, while in Treatment 2 the 100% dose shows the highest median canopy weight and 200% shows the highest median root weight.

Lastly, the ELISA results shown in Figure 11, clearly show that while the seed meal doesn't prevent virus infection (all absorbance values are above BMYV control), the lowest absorbance value in the highest (200%) dose across both treatments indicates a lower severity of infection.



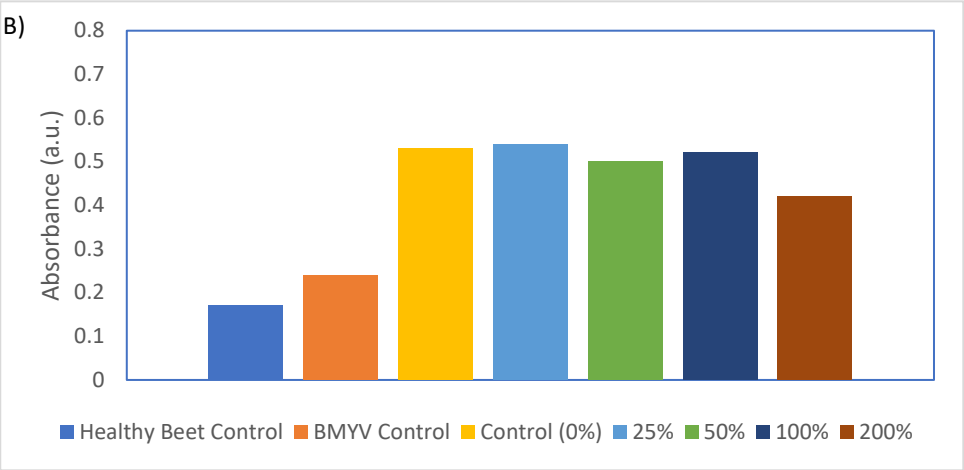


Figure 11. ELISA results (absorbance at 403nm) from glasshouse experiment two for seed meal Treatment 1 (A) and Treatment 2 (B). If the sample absorbance values at 403 nm are above the BMYV control value it indicates a successful infection. The higher the absorbance value, the greater the infection.

ANOVA performed on the ELISA output (Table 4) couldn't find a significant difference between seed meal doses, however it shows that the application of the seed meal at inoculation (Treatment 2) was significantly more beneficial ($p = 0.013$) in helping reduce severity of the VY infection. This can also be observed with Treatment 2 having lower absorbance values overall compared to Treatment 1. This seems to suggest a beneficial effect of the endophyte-infected seed meal, perhaps attributed to a kind of immune response in the beet against BMYV.

Discussion

In experiment one, the seed meal was applied only at sowing without any aphids throughout the experiment. It provided significant evidence of an increase in canopy and root weights as the dosage of the seed meal increased. The greatest canopy biomass and leaf area was at the 100% dose and the heaviest roots at the 200% dose. No significant conclusions could be drawn from the photosynthesis measurements but there were suggestions that the highest dose was resulting in a warmer canopy. Experiment two which explored the two different application times of the seed meal – at sowing (Treatment 1) and just before aphid inoculation (Treatment 2), showed contrasting results. Experiment two, unlike experiment one, neither showed a significant response for the canopy and root biomass nor photosynthesis measurements despite the same application time of the seed meal at sowing. Only leaf temperature

and fluorescence were found to be significantly affected. The coolest canopy and highest fluorescence value was found to be at the highest dose.

Stomatal conductance is a measure of the degree of stomatal opening and indicates rate of water lost via stomata in the leaves (Gimenez *et al*., 2013). As such it can be used to infer how active the plant is photosynthetically and whether there is a limitation or stress. Generally, a higher conductance value, means a greater gas exchange is taking place through the stomata in the leaf and indicates a higher rate of photosynthesis (E. Murchie, personal communication, March 2024). Experiment one results show no significant trend which is also backed by the ANOVA returning a non-significant output. Chlorophyll fluorescence is another parameter used to estimate the plant photosynthetic activity, especially that of Photosystem II (PSII), which is the primary complex that drives the electron transport chain. The parameter ϕ PSII is the operational quantum efficiency of PSII in light (Murchie and Lawson, 2013). The results obtained from experiment one, indicate a gradual rise in ϕ PSII, plateauing out at 100% dose. However, the ANOVA shows that the data is statistically not significant.

Leaf temperature is a measure that indirectly indicates the state of photosynthesis and gas exchange. A higher leaf temperature can indicate lower rates of leaf transpiration by closed stomata and lower photosynthesis. There are indications that a theoretically higher loline dose, shows greater stress on the beet when looking at experiment one. However, this inference can only be made if we look at leaf temperature in isolation. If we consider the other photosynthetic parameters, support for this is very limited. With the general increase in canopy fluorescence and SPAD as the dose rate increases, the higher leaf temperature could also be attributed to a higher chlorophyll concentration due to greening and a thicker leaf, potentially indicating higher photosynthesis in the higher doses. Fungal endophytes can trigger changes in chemical and physical properties of the host plant leaves resulting in higher cellulose content and a denser lamina (Khare *et al*., 2018). This helps provide a possible explanation to the higher SPAD and fluorescence values in higher doses.

Of all the aspects analysed in experiment one, the best relation between the seed meal and its effects on the sugar beet, is observed in the plant biomass and canopy measurements. This further supports the idea that higher seed meal doses are triggering

a biostimulant response in the beet and is along similar lines to the evidence for higher photosynthesis above, modulated by the impact of the endophyte. The same is also evident in the canopy and root fresh weights.

In the second glasshouse experiment, two different seed meal application times were investigated and their effect on aphid behaviour and virus expression was also explored. ELISA results from the leaf discs reveal an interesting trend. If we look at the Figure 11 of the virus infection in each dose, in both treatments, there is a marked trend that the highest seed meal dose has the lowest absorbance value in both treatments, indicating lower severity of infection. In fact, the significantly lower absorbance value in Treatment 2 of the second experiment, provides good evidence that applying the endophyte-infected Barrier seed meal at inoculation is more beneficial in reducing the impacts of the virus infection. While the seed meal does not prevent virus infection in the beet, the results suggest that perhaps the loline alkaloids present in the seed meal are being successfully taken up by the sugar beet and seem to provide a kind of resistance to BMYV, possibly delaying its progression. Several studies have provided evidence that endophytes can help enhance plant defences against phytopathogens by priming their immune systems. Endophytes can help in early detection of the pathogen through surface receptor and cytoplasmic kinases, and mediate defence responses by triggering ethylene and jasmonic acid pathways in the host plant (Khare *et al.,* 2018). This can also be further corroborated by the SPAD values. As the VY infection progresses, one would expect the beet canopy to become more yellow, thereby lowering the SPAD. But if we look at the results, it suggests that as the seed meal dose increases, the SPAD value also increases. This is along similar lines to the first glasshouse experiment and confirms that the seed meal helps the canopy stay greener for longer.

Stomatal conductance doesn't show a marked trend with the different doses, whereas a significant increase in ϕ PSII is observed. In fact, in contrast to experiment one, Treatment 2 in experiment two shows significant rise in ϕ PSII at both 100% and 200%, indicating higher activity in photosystem II as the seed meal dose increases. Leaf temperature also stands out in the second experiment. In the second treatment, there is a significant sharp fall in leaf temperature with higher doses of the endophyte-infected seed meal. This provides strong evidence that the higher seed meal doses,

especially when applied just before aphid inoculation, seem to be affecting beet photosynthesis, since a cooler canopy is evidence of greater photosynthesis. This would explain the higher SPAD values despite virus infection and can also explain the general uptick in canopy fresh weight.

For the plant weight and leaf area data, there was no statistical significance in experiment two, which unlike the results in experiment one, might be due to the added aphid pressure on the beet. However, some trends continue to indicate that a higher seed meal dose may be helping keep the canopy greener for longer and increase photosynthesis, translating to a higher beet yield. The graphs for the root fresh and dry weights as well as the means (Appendix 2), show trends toward a general increase in root biomass with the higher doses, albeit the results are mixed. The median values seem to indicate that this increase in root and canopy biomass is more pronounced at the 200% dose in Treatment 1 whereas for Treatment 2 it varies between both the 100% and 200% doses. While the seed meal application does seem to be boosting photosynthesis and possibly plant growth, perhaps also increasing resistance against BMYV, there are also no detrimental impacts from the highest dose which would be expected to have the highest concentration of the loline alkaloids.

Referring to the aims of this chapter and the evidence across both glasshouse experiments, it can be concluded that the endophyte-infected grass' seed meal application does not help prevent virus infection altogether but rather seems to maintain a greener canopy for longer and keep photosynthesis relatively high despite the virus infection. This is underscored by a higher root and canopy biomass when compared to the lesser concentrated doses. Additionally, there were no apparent detrimental impacts of the 10 g seed meal dose on the sugar beet and in some cases, it may have even helped the canopy stay cooler as evidenced by the lower temperatures. Finally, both 100% and 200% doses of the seed meal show strong positive impacts on the sugar beet. While there isn't enough evidence to conclude that one dose might be better than the other, the seed meal applied when beet were inoculated with the aphids, seems to elicit the best all round response. It is suggested that the loline alkaloids in the seed meal may have been taken up much later than the earlier application timing, thus may play a more direct role in crop health and immune response, especially under added aphid and virus pressure.

Chapter 3 – Effect of the endophyte-infected grass on sugar beet physiology and VY infection, when grown as a cover crop at different sowing densities and under various destruction methods, in field conditions

Introduction

As with any crop, sugar beet is vulnerable to many pests and diseases such as black root rot, *Cercospora* leaf spot, wireworms, free living nematodes, virus yellows and aphids to name a few (Yamane (2016) and British Beet Research Organisation (2022)). However, undoubtedly the biggest threat posed is by *M. persicae* aphids. Since the active ingredient imidacloprid was introduced in 1991, pelleted neonicotinoids seed treatments became the go to product to control aphids and became the most widely used systemic insecticide in the world (Jeschke and Nauen, 2008). A seed treatment is the process of applying fungicidal and /or insecticidal seed dressing products onto seeds to create a 'protective zone' of the active ingredient in the soil that gives protection against soil borne pathogens/insects in addition to early season pests and diseases (Nuyttens *et al*., 2013). However, the manufacturing, transport and storage and sowing itself can create fine dust of the active ingredient that gets released into the environment. In fact, in 2008 more than 11,000 bee colonies were damaged by insecticidal dust released during the sowing of neonicotinoid treated maize seeds in the Upper Rhine valley in Germany (Hauer *et al*., 2017). This brought into focus the harmful implications of using chemical insecticides. Additionally, the over-reliance and overuse on seed treatments since the late 20th century has resulted in aphids developing resistance against a large number of chemical actives, making it one of the strongly resistant species in the world (Bass *et al*., 2014).

With major changes in environmental policies there are now stricter curbs on use of plant protection products. The EU first banned imidacloprid and other seed treatments in 2018 (Gasparic *et al*., 2021), with the UK government following in 2020 (Bellis and Suchenia, 2022). The ban on the use of chemical insecticides and seed treatments has created a vacuum regarding control of virus vectors like aphids in sugar beet, with no alternate method available except foliar sprays, to fight against the different virus yellowing diseases (Fracis *et al*. (2022) and Gasparic *et al*. (2021)). Breeding new varieties that are resistant to the VY complex is an option widely being worked on (Francis *et al*., 2022), however it will take years to breed high yielding varieties

resistant to all the yellowing diseases (Borgolte *et al*., 2024). Moreover, with governmental schemes such as the EU Farm-to-Fork strategy that aims to halve the dependence on chemical plant protection products by 2030 (European Commission, 2020), there is an urgent need to move toward more biological, integrated and organic approaches to control pests.

The ability of the fungal symbiont endophyte *E. uncinata*, living within the endophytic grass, to defend itself using volatile signalling is a fascinating but relatively unexplored topic. The loline alkaloids released by the endophytic grass when under stress, are water soluble and what makes them special is their ability to move around within the plant tissues even to areas where the symbiont endophyte does not colonise the grass (Mwangi *et al*., 2024). This water solubility would theoretically make it mobile even when in the soil, potentially allowing it to be taken up by the crop.

To examine this principle, this field trial will focus on the impact the endophyte-infected grass would have on the sugar beet physiology and VY infection if grown as a cover crop in the winter prior the beet is due to be sown, under a plethora of different sowing densities and destruction techniques. While the controlled environment glasshouse experiments were a direct seed meal dose response trial, this chapter will explore the impacts of the grass under real environmental conditions and try to determine how might the grass best be optimised, to be implemented in real world sugar beet cultivations. The field trial built on from the results obtained in the glasshouse in the months prior and examined in depth the effects of different sowing densities and destruction methods of the Barrier U2 endophyte-infected grass, on sugar beet, throughout the growing season spanning May-August 2024. In particular, bio-stimulant characteristics, crop physiology, virus incidence and symptom expression were the main aspects focussed on in the field trial.

Trial site and experimental design

The field trial (Figure 12) was conducted at the University of Nottingham farm on Sutton Bonington campus, on an east west oriented field that had previously been used to grow Winter Oats. The trial consisted of four large blocks, each further subdivided into four main plots based on the different endophyte-infected grass' sowing densities per hectare (ha) – 10 kg/ha, 20 kg/ha, 30 kg/ha and an unsown Control. Each of the

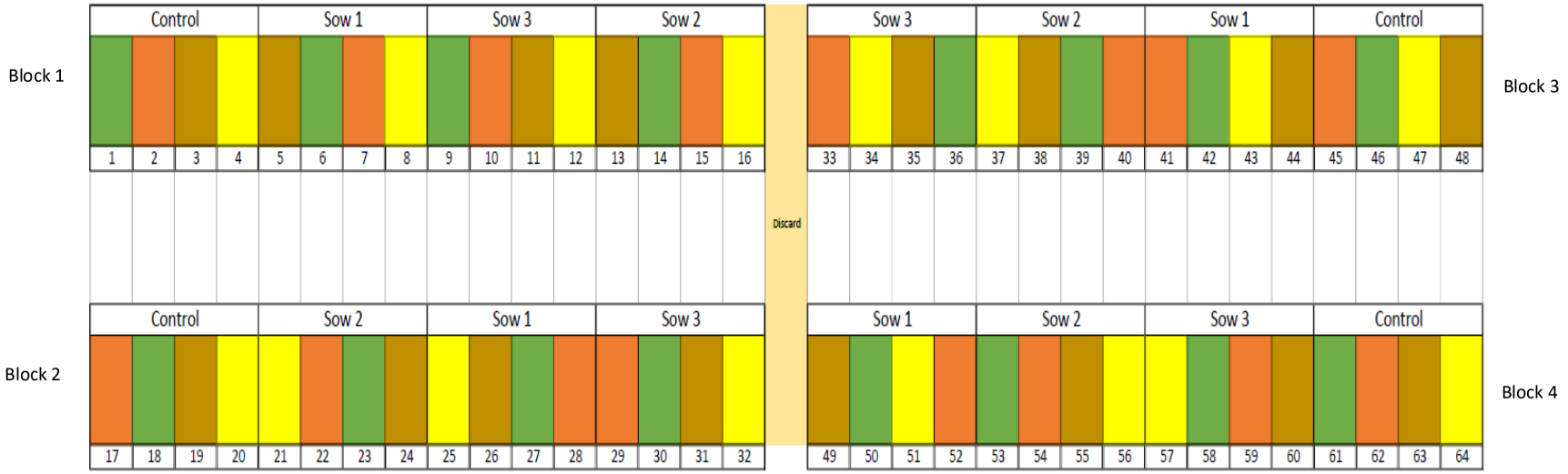
four plots within each block were further subdivided into four sub-plots based on the four different endophyte-infected destruction techniques. These destruction methods included Strip spraying, Deep tilled, Shallow tilled and Glyphosate and were randomised throughout the trial along with the sowing densities. A description of what each destruction method entailed is given in Table 5. The cultivations were done using an Opico-vari disc with leading tines. Discs were set at 10 cm, and tines were set at 25 cm.

Table 5. Details of the grass destruction methods in the field trial

Destruction method	Description
Strip treated (T1)	Strip spray grass & incorporate residues
Deep tilled (T2)	Thoroughly incorporate grass by running discs twice
Shallow tilled (T3)	Shallow incorporation of grass by running discs once
Glyphosate (T4)	Spray off all grass with glyphosate & shallow incorporation

The endophyte-infected grass seed sourced from CropMark Seeds™, was sown on 10th October 2023 and allowed to grow through winter and spring. In preparation for beet sowing, the Glyphosate treatment was applied on 1st May 2024. The incorporation treatments were done on 7th May 2024. Due to a very wet Spring in 2024, sowing of the sugar beet was delayed. The field trial was sown by BBRO on 10th May 2024 with 50 cm wide rows. Each plot was 7.5 metres long and 3 metres wide. 100 kg/ha of Nitrogen (N) was applied to the trial, split into two applications - 40 kg/ha of N was applied on 10th May and the remaining 60 kg/ha N was applied on 29th May.

The Sugar Beet variety *Wren* (bred by SESVanderHave) was chosen for this trial, owing to its relatively high susceptibility to virus yellows and corresponding symptom expression, making it easier to study the disease incidence. As this experiment aims to explore the biological impacts and resistance potentially offered by the grass, insecticides and fungicides were not applied, and herbicides were only applied when absolutely needed or as part of the glyphosate treatment. The beet was sown at a rate of 125,000 plants/ha which is the standard rate recommended by BBRO to achieve a target beet establishment of 100,000 plants/ha. Additionally, we also hoped this would account for any potential losses through the more environmentally friendly approach.



Plot Numbers 1 - 64 are main trial plots

Sow U2 Endophyte @ varied rates
Main plot = Seedrate
Sub plot = Destruction method

Sowing Treatements:
Control - Unsown
Sowing 1 - 10kg/ha
Sowing 2 - 20kg/ha
Sowing 3 - 30kg/ha

Destruction Methods:
Strip treated — Strip sprayed, Lightly incorporated grass
Glyphosate — Spray off, Lightly incorporated grass
Shallow till — Lightly incorporated grass
Deep till — Moderate incorporated grass

Sugar Beet Variety
Wren

Seedrate
1,25,000

Figure 12. Endophyte-infected grass field trial plan

Assessments and data analysis

Plant population protocol

On 10th June 2024 when the sugar beet were at 4 leaf stage, four rows each five metres long, were randomly measured using a tape measure for each plot, in each block. The beet in all four rows were added up to get a count of sugar beet in a 20m length of row, and multiplied by 1000, to get the plant population per ha for each plot.

Physiological measurements

Various plant photosynthesis and canopy measurements were performed to analyse the effects of the various treatment methods on sugar beet physiology. It took three consecutive days to collect all the data, and the measurements were planned on sunny/bright mornings to get the most appropriate readings. Apart from SPAD data, a LICOR 600 Porometer was used to gather stomatal conductance and PSII fluorescence data and a Kane-May thermal IR gun (KM823 model) was used to measure canopy temperature. For SPAD, fully expanded leaf 6 was selected for 10 plants in row 3 in each plot, and measurements were taken on the leaf lamina avoiding the veins, centrally between the midrib and the leaf margin. An average of the 10 readings was then noted for each plot. SPAD measurements took about 5 seconds per plant. The LICOR 600 porometer was clamped covering both sides of leaf 6 and used to measure stomatal conductance and ϕ PSII (Fq'/Fm') which took about 10-12 seconds per plant. Three random leaves spatially well distributed per plot were chosen for this and the readings averaged per plot. The thermal gun was randomly moved across a random section of each plot and the averaged reading noted as the canopy temperature for each plot. Finally, a tractor mounted Crop Circle ACS-430 (Holland Scientific) active canopy sensor was used to collect multi-spectral canopy reflectance data and calculate NDVI.

Virus yellows disease scoring

About five weeks after canopy closure, on 19th August 2024, a visual assessment of the beet canopies was conducted. The number of visually symptomatic beet that had developed yellow leaves as a result of VY infection, were counted in all 64 plots in the trial. Considering approximately 210 beet per plot, a percentage of symptomatic plants per plot was then calculated and averaged for each treatment across all the four seed rates. This percentage of disease incidence in the beet was then used to assign a disease score on a scale of 1 to 5 as per table 6 below.

Table 6. Procedure for scoring beet based on their visual symptom expression (leaf yellowing).

Average VY incidence (%)	Beet disease score
0 – 2	1
2 – 4	2
4 – 6	3
6 – 8	4
8 – 10	5

Data analysis

After the data was collected, a two-way ANOVA (Analysis of Variance) was performed using the statistical analysis software RStudio, to test for statistical significance and treatment interactions. The least significant difference (LSD) values were then calculated for the results that were significant. All data obtained including plant establishment, physiological measurements as well as the disease incidence was found to be normally distributed and did not require any transformation.

Results

Table 7. Visual assessment of virus yellow symptomatic beet. The destruction methods are as follows: Strip treated (T1), deep till (T2), shallow till (T3) and glyphosate (T4). The full dataset and means are attached in Appendix 3

Seed rate (kg/ha)	Destruction method	Average number of visually symptomatic beet	Average VY disease incidence (%)	Disease score
Control (0)	T1	12.75	6.1	4
	T2	4.75	2.3	2
	T3	4.75	2.3	2
	T4	15	7.2	4
10	T1	9.25	4.4	3
	T2	15.25	7.2	4
	T3	13.25	6.3	4
	T4	9.25	4.4	3
20	T1	8	3.8	2

	T2	9.5	4.5	3
	T3	12.75	6.1	4
	T4	10.75	5.1	3
30	**T1**	10	4.8	3
	T2	9	4.3	3
	T3	5.25	2.5	2
	T4	18	8.6	5

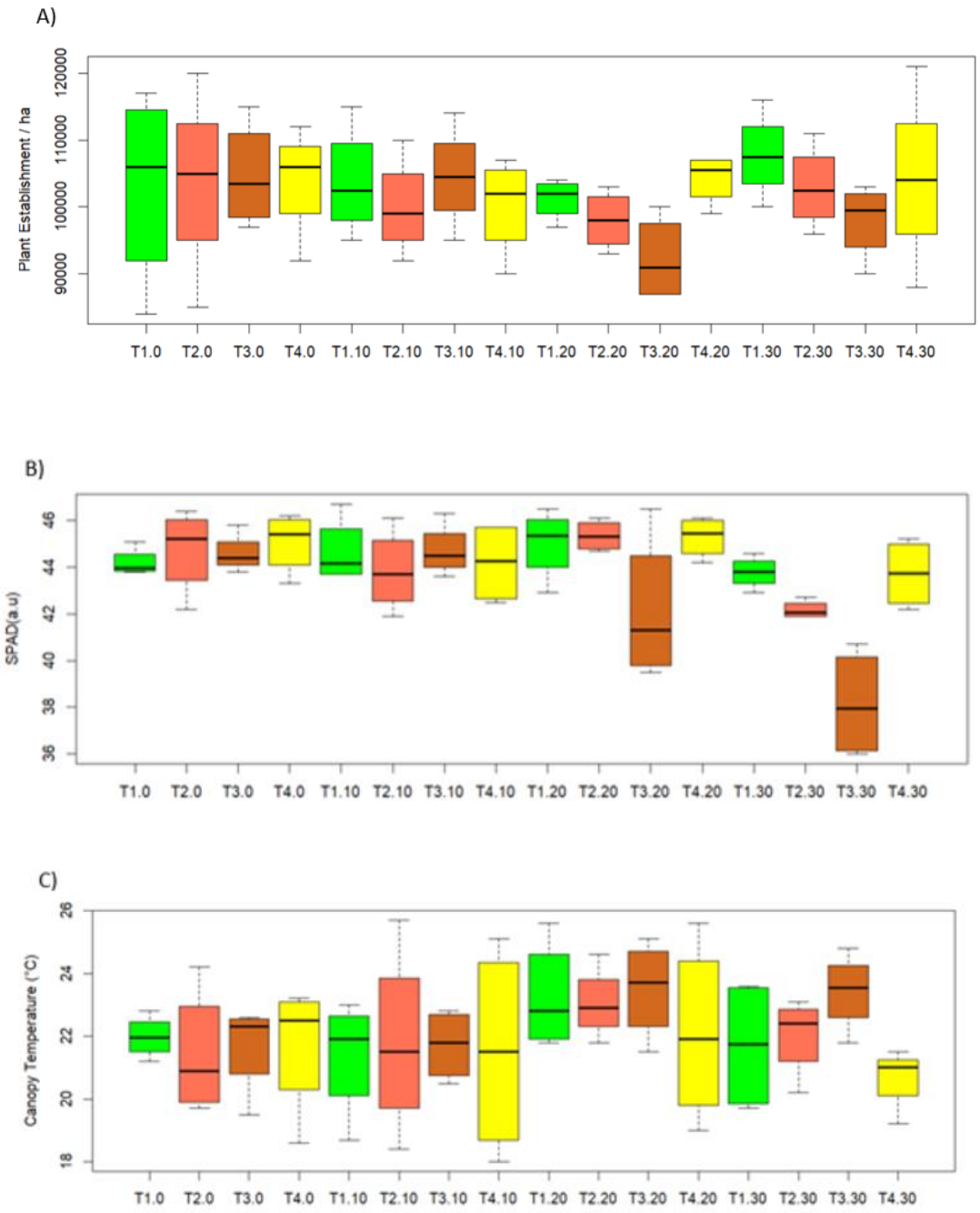
Table 8. Two-way ANOVA results for the field trial. p values and LSDs show significance at 95% confidence. 'Seed rate & discussion method' column shows the interaction between both factors.

Parameter	**p value**			**LSD**
	Seed rate	**Destruction method**	**Seed rate & destruction method**	
Plant establishment	0.37	0.52	0.77	
SPAD	**0.01×10^{-3}**	**0.0007**	**0.0038**	**1.4**
Canopy temperature	0.18	0.49	0.96	
Fluorescence	0.49	0.89	0.96	
Stomatal conductance	0.16	0.49	0.81	
NDVI	0.13	0.58	0.89	
VY incidence	0.86	0.45	0.35	

The different sowing densities of the endophyte-infected grass and its destruction method didn't have a significant impact on sugar beet establishment ($p > 0.05$) as shown in table 8. However, a trend that can be identified is that the shallow and deep till treatments at the higher grass seed rates of 20 and 30 kg/ha have the lowest plant establishment as can be seen in Figures 13(A) and 14(A).

There is however, a highly significant ($p < 0.05$) effect of the seed rates and treatment methods (Table 8) on the chlorophyll content of the sugar beet, as can be seen clearly in the SPAD data in figures 13 (A) and 14 (A). This is similar to the results of the glasshouse experiments, wherein the higher chlorophyll can be attributed to the role played by the endophyte in boosting photosynthesis and thickening of the leaf lamina, as past studies have shown. LSD analysis however, revealed that the shallow till (T3) treatment at higher seed rates (20 and 30 kg/ha) of the endophyte infected grass, significantly lowered the SPAD in the sugar beet. This is clearly noticeable in Figures 13(B) and 14(B). LSD analysis showed that when averaged across the whole trial, mean SPAD in the shallow till T3 treatment (mean = 42.41) was the lowest and significantly lower than Glyphosate (mean = 44.57), Strip treated (mean = 44.42) and Deep tilled (mean = 44.03) destruction treatments. There was no significant difference in SPAD between the glyphosate, strip treated and deep tilled beet. When looked at from a grass sowing density perspective, mean SPAD at the 30 kg/ha grass seed rate was the lowest with LSD results indicating that sugar beet SPAD values at 30 kg/ha (mean = 41.96) was significantly lower that the Control (mean = 44.66), 10 kg/ha (mean = 44.36) and 20 kg/ha (mean = 44.46).

There was no significant impact on the ϕ PSII activity as indicated by the fluorescence data and it is hard to interpret any trends. Canopy temperature also shows no significant patterns, although Figures 13 (C) and 14 (C) show a trend that the beet canopies of the shallow till treatments are generally the warmest and the beet in the glyphosate treatments are the coolest. While stomatal conductance does show a small decline at higher sowing densities of the grass (Figures 13 (E) and 14 (E)), the effect was not significant statistically. Finally, the various treatments also do not have a significant impact on NDVI. However, when looking a figure 13 (F), an identifiable trend is that as compared to the control, the shallow and deep till treatments in all the different seed rates of the grass have the lowest NDVI values.


A)
Plant Establishment / ha
90000
100000
110000
120000
T1.0
T2.0
T3.0
T4.0
T1.10
T2.10
T3.10
T4.10
T1.20
T2.20
T3.20
T4.20
T1.30
T2.30
T3.30
T4.30
B)
SPAD(a.u)
36
38
40
42
44
46
T1.0
T2.0
T3.0
T4.0
T1.10
T2.10
T3.10
T4.10
T1.20
T2.20
T3.20
T4.20
T1.30
T2.30
T3.30
T4.30
C)
Canopy Temperature (°C)
18
20
22
24
26
T1.0
T2.0
T3.0
T4.0
T1.10
T2.10
T3.10
T4.10
T1.20
T2.20
T3.20
T4.20
T1.30
T2.30
T3.30
T4.30

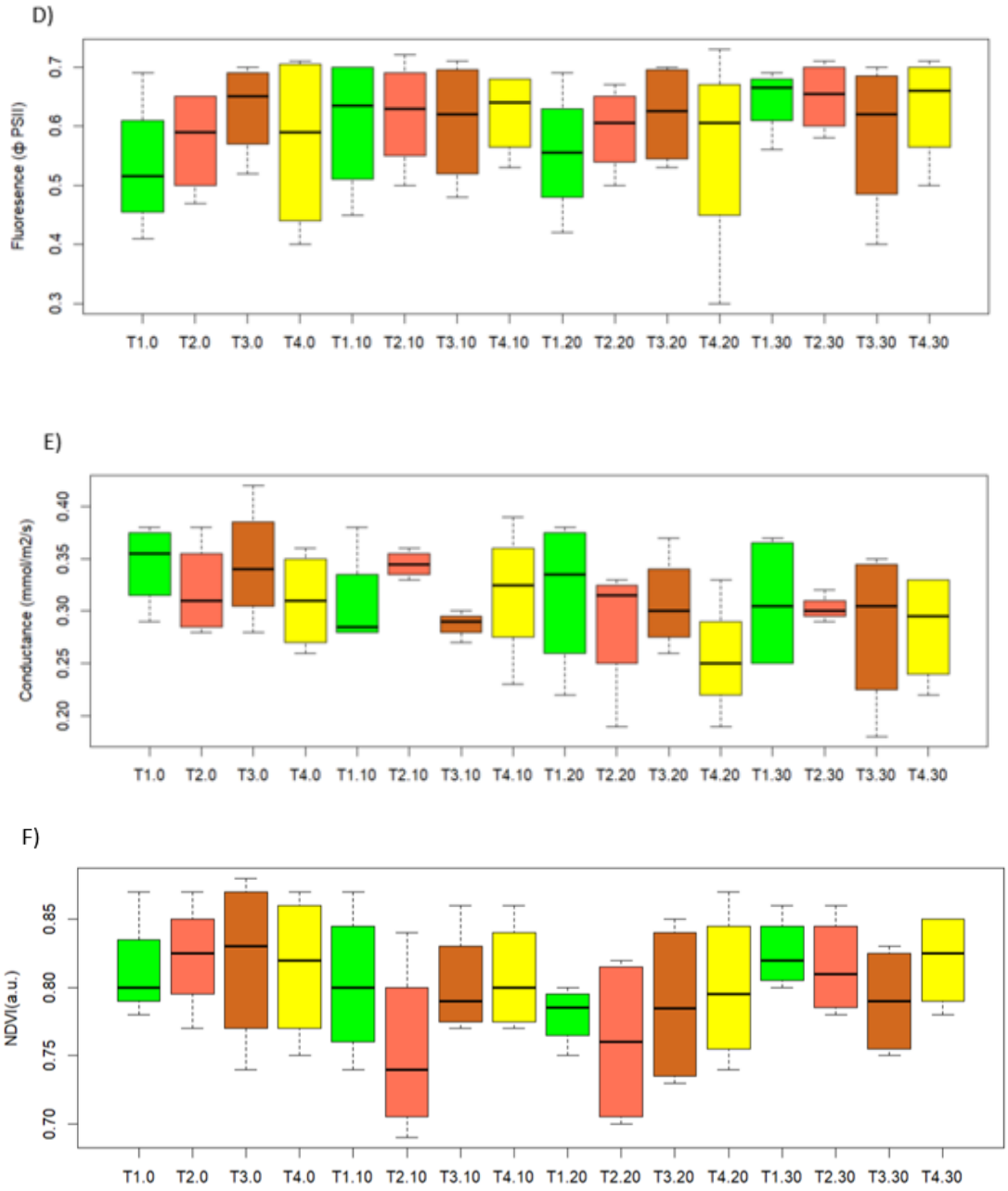
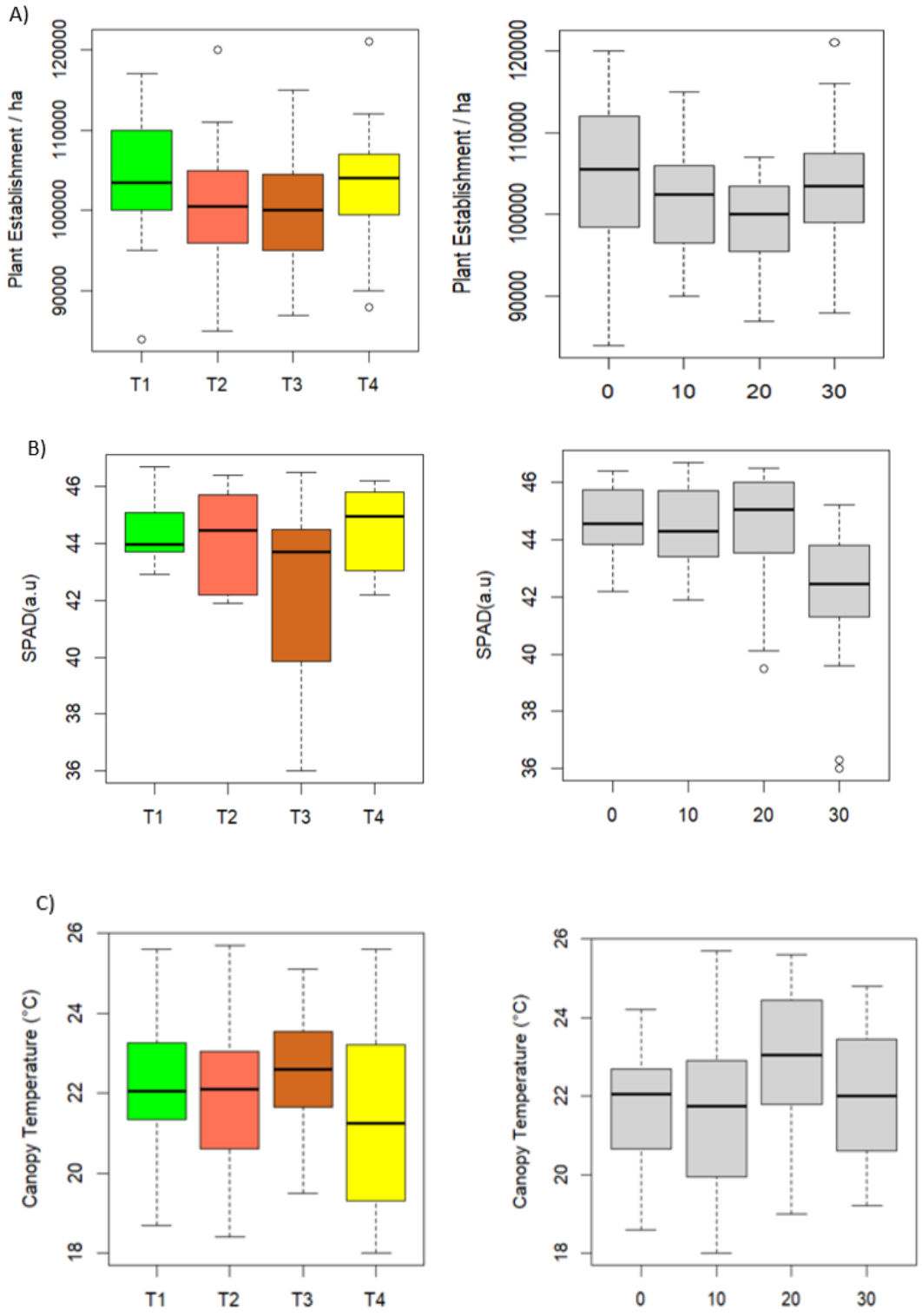


Figure 13. Combined effects of endophytic grass across four different seed rates (0,10,20,30 kg/ha) and four different treatments (Strip treated (T1), deep till (T2), shallow till (T3) and glyphosate (T4)) on plant establishment (A), SPAD (B), Canopy temperature (C), Fluorescence (D), Conductance (E) and NDVI (F). X-axes indicates the treatment number and seed rate. For example, T1.0 indicates Strip treatment for the 0 kg/ha (Control) seed rate and so on. Boxes represent data within first and third quartiles, thick black lines represent medians, and whiskers represent maximum and minimum values in the data.


A)
Plant Establishment / ha
90000
100000
110000
120000
T1
T2
T3
T4
Plant Establishment / ha
90000
100000
110000
120000
0
10
20
30
B)
SPAD(a.u)
36
38
40
42
44
46
T1
T2
T3
T4
SPAD(a.u)
36
38
40
42
44
46
0
10
20
30
C)
Canopy Temperature (°C)
18
20
22
24
26
T1
T2
T3
T4
Canopy Temperature (°C)
18
20
22
24
26
0
10
20
30

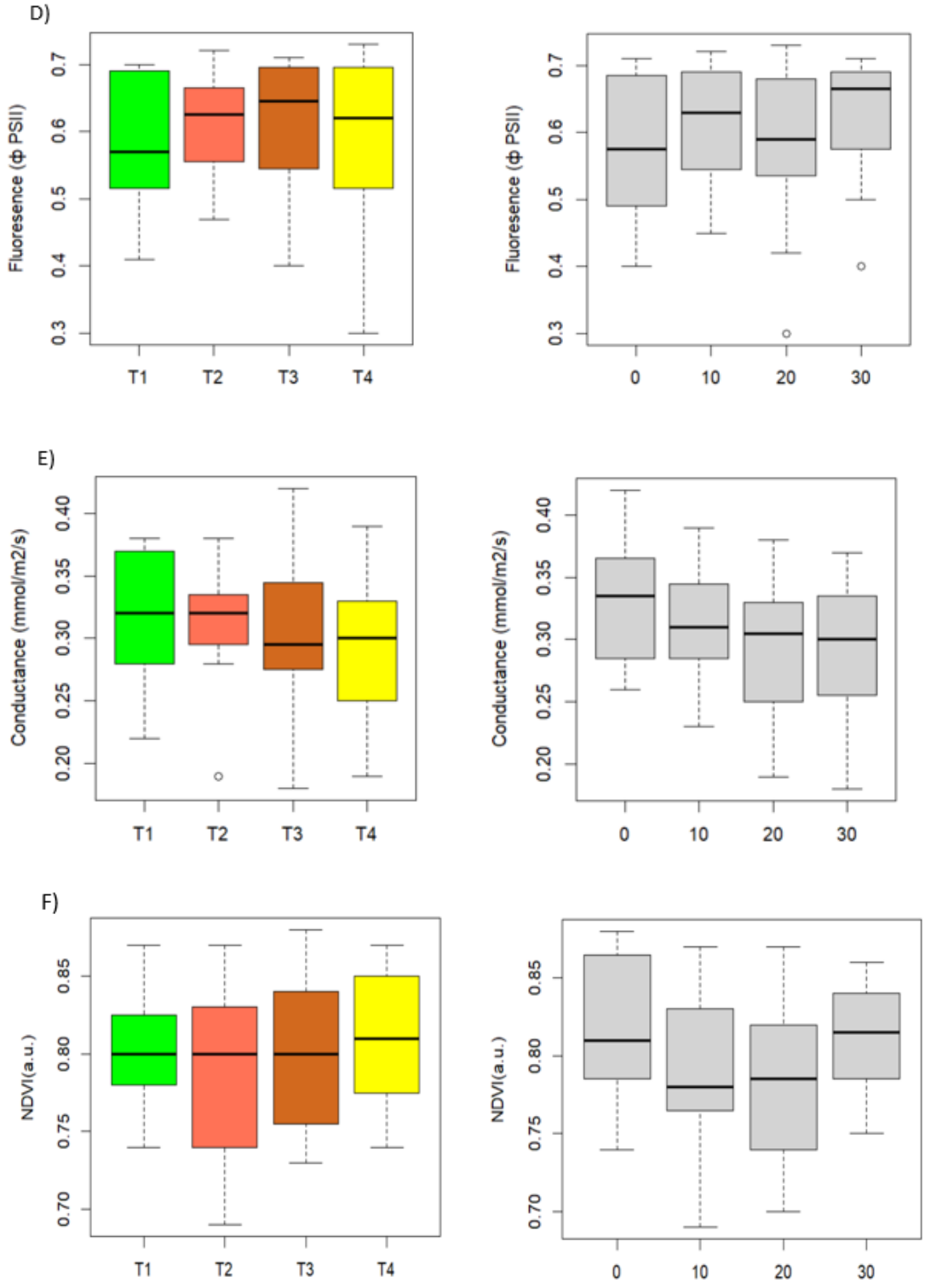


Figure 14. Individual impacts of the four different treatments (Strip treated (T1), deep till (T2), shallow till (T3) and glyphosate (T4)) and four different seed rates (0,10,20,30 kg/ha) on plant establishment (A), SPAD (B), Canopy temperature (C), Fluorescence (D), Conductance (E) and NDVI (F). Boxes represent data within first and third quartiles, thick black lines represent medians, and whiskers represent maximum and minimum values in the data. Hollow circles depict outliers.

Discussion

When BBRO had conducted the endophytic grass strip trials in 2022, they observed interesting trends and patterns in VY symptom expression as shown in Figure 4 in the first chapter. However, the main problem arose with the grass significantly outcompeting the sugar beet leading to major drops in yield (A.Wright, personal communication, September 2023). Thus, one of the main aims of the field trial was to try and optimise the destruction time of the endophyte-infected grass, so that competition with the beet is reduced but also to get the best possible effects of the loline released into the soil by the grass. The grass sown in early October 2023, was allowed to grow through the winter and spring without any herbicidal applications. An interesting observation made was where the grass was sown at progressively higher seed rates like 30 kg/ha, these plots seemed to show the least weed germination as compared to the control and 10 kg/ha. An image depicting the same is shown in Figure 15 below that was taken on 30th April. However, this herbicidal impact was not investigated in detail in this trial, so further studies are needed before arriving at a conclusion.

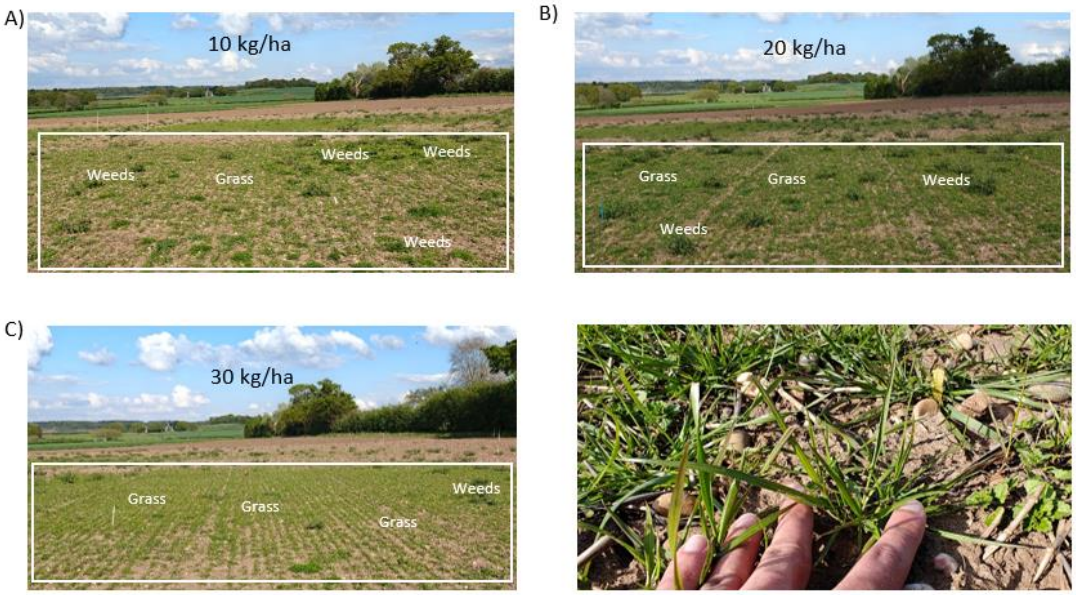


Figure 15. Growth of the endophytic grass Barrier *Festulolium*, at 10 kg/ha (A), 20 kg/ha (B) and 30 kg/ha (C) seed rates until April 2024, before being destroyed as per the treatments in the experimental design. Individual blocks have been outlined.

About 10 days before the sugar beet were due to be sown, we started spraying the herbicide Goltix, on the grass in all the destruction treatments except the glyphosate

treatment, where glyphosate was used. The intention was that as the beet would start emerging, the grass should slowly die back within a few weeks, reducing competition. However, even after two applications of the herbicide, the grass while pretty set back, still continued to grow in shallow cultivation (T3) plots, even after the beet had germinated, leading to competition with the beet. As a result, we decided to apply the graminicides (herbicides meant for grass weeds) Laser and Toil on 18th June 2024 to destroy the grass.

Since the grass couldn't be destroyed on time, sugar beet establishment was reduced and growth delayed by 3 - 4 weeks in shallow till plots at higher sowing densities of the grass like 20 and 30 kg/ha (Figure 16). This could be down to direct competition with the grass but might also be a side effect of the grass being under greater stress because of the herbicides acting on it, thus in theory, would result in greater loline secretion into the soil, which might have stressed the beet. In fact, even trends in canopy temperature provide some evidence that these beet in T3 were stressed, since on average they had the warmest canopies with temperatures exceeding 23°C (Appendix 3). This would explain the lower beet establishment in the shallow till plots at higher sowing densities. In both 20 and 30 kg/ha seed rates of the endophytic grass, the shallow till plots had the lowest mean sugar beet establishment at 92250 and 98000 plants /ha respectively. This also explains another trend where these same plots had the lowest overall NDVI values.

Figure 16. An example of the difference in sugar beet canopy establishment between shallow till (left) and strip treated (right) plots. Both these images were taken on 1st July 2024.

While the above observations did show consistent identifiable trends none of them were statistically significant. The only physiological measurement that showed very high significance were the SPAD values, which are indicative of chlorophyll content

of the leaves. This significance also held true when looking at the combined interaction of the seed rate and the destruction method of the grass. The shallow till plots with grass seed rates of 20 and 30 kg/ha, had the lowest SPAD values.

From the results discussed earlier, this suggests that the canopy chlorophyll content is severely reduced in T3 cultivation treatment of the grass and this effect is the most pronounced in T3 plots at the 30 kg/ha seed rate of the endophyte-infected grass. This is also in line with the effect the delayed destruction of the grass has had on the other parameters discussed. Thus, it can be inferred that the shallow till treatments (T3) are not a viable option, especially at higher sowing densities of the grass as it results in delayed plant establishment and reduced canopy cover. This also underscores how important it is to make sure that the grass is destroyed on time, preferably within few weeks after beet germination, before the sugar beet starts to establish its canopy.

The aphid migration arrived by 17th June 2024, about 3 weeks before canopy closure. The first virus yellow symptoms started appearing around 5th July, with more widespread symptoms appearing by 17th July. In order to study the potential effect of the lolines secreted by the grass and whether these alkaloid secretions affected virus expression, there were *no pesticides, fungicides or insecticides applied* in the entire field trial. However, towards end of July there was a period of unusually low rainfall for the time of year in the UK, that lasted more than a month. In fact, statistics from the Met Office show this was akin to drought like conditions.

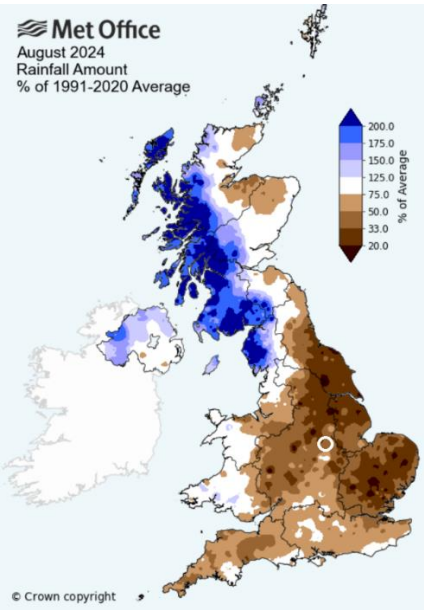


Figure 17. Distribution of rainfall in the UK during August 2024 showing large deficiencies in the southern and eastern parts of the country

Figure 17 above shows the location of our field trial at Sutton Bonington (white circle on the map) received just 30-50% of its monthly rainfall in August 2024. As the soil moisture dried up, this impacted the beet canopies causing wilting and senescing. An interesting observation noted was that the severity of canopy wilting differed across the different treatment methods. Most notably, the sugar beet in many of the deep tilled (T2) plots were severely wilted as compared to the other grass destruction treatments of strip, shallow till and glyphosate. However, beet in the shallow till plots that had been lagging in establishment and were the poorest performing beet so far, surprisingly showed very minimal wilting. An example of this is shown in Figure 18 below.

Figure 18. An example of canopy wilting severity observed in the deep till (left) and shallow till (right). These images are from Plot 40 (deep till) and Plot 31 (shallow till) specifically, taken on 19th Aug 2024.

On 19th August 2024, the sugar beet in the field were scored for VY disease incidence to try and identify any patterns or trends in yellowing such as the size and spread of the yellowed beet canopies and use them to infer disease severity. The results are shown in Table 7 above. The beet were scored on a scale of 1 – 4 based on the protocol shown in Table 6 earlier, with score of 1 being the least infected. A visual observation of the beet seemed to suggest that the symptomatic beet patches were smaller and more isolated in the strip treated and glyphosate treatments, perhaps indicating that the aphids spent lesser time feeding in these plots. However, there were no statistically significant relationships that could be identified in overall symptom expression of the infected beet across the grass sowing densities and destruction treatments.

Going back to the aims of this field trial chapter, it can be concluded that while the endophytic grass does not prevent aphids or virus yellows infection, it does help enhance the photosynthesis in the beet, evidenced by the SPAD results. This is also similar to the findings of the glasshouse experiments. The grass might play some role in minimising the severity of virus yellows. Visual observations and VY incidence scores suggest that aphids might be spending less time feeding on the glyphosate and strip treated endophyte-infected plots as these showed lesser and more patchier yellowing of the crop canopies infected with virus yellows. While this cannot be said with certainty as results were not statistically significant, several endophyte

pathosystems have shown evidence of resistance/deterrence to pathogens. For example, *Theobroma cacao* which is the source of cacao beans, extensively associates with the foliar endophyte *Colletotrichum tropicale. C. tropicale* occurs widely in the tropics and is abundant in healthy leaves of *T. cacao.* A study by Mejia *et al*. (2008) showed that *T. cacao* trees dominated with this endophyte showed lower incidence of black pod disease caused by *Phytophthora spp*. In fact, studies have also found that *C. tropicale* presence reduces herbivore pressure in several other host plants in the tropics (Mejia *et al*., 2014).

The shallow till field trial treatments do not seem to be a viable option especially at higher seed rates of 30 kg/ha as they cause a significant reduction in the canopy chlorophyll (SPAD) content compared to the control as well as the other treatment types. However, similar to the glasshouse experiments, there is a greening and growth boosting effect on the sugar beet under the other treatments, as evidenced by the SPAD charts. Visually this seemed to be very pronounced in the first 8 weeks, gradually tailing off as the canopy reaches closure. However, the shallow till plots continued to lag behind by about 3 - 4 weeks to reach closure. Thus, the destruction time of the endophytic grass is crucial to ensure that it does not compete with the sugar beet for resources. If not completely destroyed before the beet germinates, there may also be a potential phytotoxic effect of the alkaloids secreted by the dying grass on the beet, which results in a delay in canopy establishment.

Chapter 4 – Conclusions and future prospects

To conclude, the endophytic grass Barrier *Festulolium* containing the fungal endophyte *E. uncinata* does have significant positive impacts on the sugar beet performance in both its forms investigated in this project – as a seed meal and a cover crop. Looking back at the research questions, while it does not prevent virus yellows infection or keep aphids away completely, it could play a major role in deterrence. Further analysis, especially on the loline content in the different sugar beet tissues, will help give an idea of how much the plant is able to uptake. We didn't have enough time to include these results in the report due to timeline constraints.

The endophytic grass also seems to confer an immune response in sugar beet against virus infection. This is not a new concept and is similar to many studies that have shown endophytes proving beneficial to trigger defence responses in host plants. Mejia *et al*. (2014) found that when the foliar endophyte *C. tropicale* successfully symbiotically colonised *T. cacao* plants, it caused an upregulation of various host genes involved in defence against biotic stresses like pathogens and herbivores, most notably ethylene signalling and receptor kinases. Endophyte presence also upregulates genes involved in production of cellulose and lignin in the leaf cells, thus making the leaf lamina thicker and tougher for pests to feed on (Khare *et al*. (2018) and Mejia *et al*. (2014)).

The endophyte infected grass in our experiment also helped the sugar beet maintain a greener canopy for longer, helping increase crop photosynthesis and biomass accumulation. Interestingly, this is in complete contrast to the study by Mejia *et al*. (2014) that found the endophyte *C. tropicale* negatively affecting *T. cacao* photosynthesis. While each endophyte species may have a different impact on the host plant, in our experiment, the *E. uncinata* endophyte is affecting the beet indirectly and hence the impacts might have more to do with the loline amounts being potentially taken up by the beet. However, further research is needed to help narrow down the best dose and concentration of the lolines secreted by *E. uncinata* infected grass. We didn't have enough time to include the final harvest data as the field trial outran the duration of the MRes course. At this point, it is also not known what exactly is causing this boost in plant photosynthesis and canopy sustenance, making it an important factor

to identify in future studies. What we know is there are no major nutritional differences in the endophyte-infected grass seed compared to regular grass. More importantly, there were no detrimental impacts that we could find in the sugar beet when the loline, theoretically, might be provided at much higher concentrations via a seed meal. With that said, when grown as a cover crop pre-season it is essential that the grass be destroyed thoroughly before the beet are sown in the field to avoid any competition or possible phytotoxic issues. As evidenced by the results, if the grass is not destroyed on time, then it can severely delay crop establishment, especially at higher seed rates. However, if destroyed on time, then even at higher sowing densities of the grass there are no setbacks to plant health that could be observed.

Regarding what may be the best form of the *E. uncinata* endophyte-infected grass to implement in the UK, there isn't sufficient evidence yet to pick a specific strategy. While both the seed meal and grass itself as a cover crop show beneficial impacts like maintaining a greener canopy and seem to be inducing some form of immune response, additional studies need to be conducted to examine in detail the pattern of aphid feeding behaviour and virus yellows infection in sugar beet under different loline concentrations. As the seed meal was trialled in controlled conditions, it would need to be trialled in the field to check the impacts under real world scenarios. Results from the field trial show that if the endophyte-infected grass is used as a cover crop, the strip treated and glyphosate treatment at the higher sowing densities (20 and 30 kg/ha) are the best option, since they ensure the grass is destroyed and its remains cultivated back into the soil on time, before the beet begins to establish.

Interestingly, there were also suggestions of a herbicidal effect observed in this experiment, since both the seed meal and grass seems to supress weed germination, at higher concentration and sowing densities respectively. Further research is needed however to determine if this is yet another factor influenced by the alkaloid, or just a physical smothering effect of the grass in the field. Further research could also be conducted to examine if the grass shows similar impacts on crop vigour and biomass accumulations when used as amendments like dried hay or green mulch.

It is important to note that as with any biological control method, the efficacy may not be hundred percent just like this investigation in which the results vary with each

treatment that was investigated, likely being modulated by differing environmental stresses and other factors. Overall, through this investigation, we have proven that endophyte-infected grasses are a strong contender in future IPM strategies as they seem to show some deterrence against virus yellows, with the lolines secreted by them having potentially beneficial impacts on crop growth. This shows they could potentially act as an effective biological alternative to chemical insecticides and pesticides. If we can find ways to ways to fully optimise the integration of the endophytic grasses into sugar beet cultivations in the UK, it could very well be one of the best measures of an IPM agricultural system.

Acknowledgements

This MRes project would not be possible without the funding provided by the British Beet Research Organisation and The Morley Agricultural Foundation. Huge thanks go to Dr. Alistair Wright and Professor Mark Stevens from BBRO, Professor Erik Murchie and Professor Debbie Sparkes at the University of Nottingham.

Special thanks also go to Laura Briers at the university for assisting with data collection, to John Alcock and Matthew Tovey for managing the field trials.

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Appendix

Appendix Table 1. Full set of photosynthesis and sugar beet biomass measurements (including means) from the first glasshouse trial under seed meal dose (Untreated, Control (0% endophyte-infected), 25%, 50%, 100% and 200%)

Block	Treatment	SPAD (au)	Conductance (mmol/m^2/s)	Fluorescence (ϕPSII)	T leaf (°C)	Canopy Fresh Weight (g)
1a	Untreated	29.9	0.277	0.63	20.59	68.06
1a	Control	34.3	0.228	0.69	18.42	88.14
1a	25	38.3	0.162	0.59	19.1	105.34
1a	50	32.3	0.237	0.68	20.45	81.24
1a	100	37.3	0.209	0.7	19.8	93.02
1a	200	37.7	0.161	0.71	19.95	101.3
1b	Untreated	41.4	0.335	0.61	20.46	63.9
1b	Control	36.8	0.271	0.72	18.85	79.28
1b	25	35.2	0.22	0.6	20.2	64.43
1b	50	27.3	0.549	0.66	19.38	65.88
1b	100	36.9	0.277	0.73	19.69	91.38
1b	200	30.4	0.2	0.66	21.46	91.53
2a	Untreated	32.6	0.28	0.53	21.72	65.25
2a	Control	36.5	0.248	0.48	20.43	76.2
2a	25	36.1	0.182	0.72	19.35	97.85
2a	50	35.4	2.081	0.73	18.11	31.45
2a	100	43.4	0.373	0.73	19.68	91.5
2a	200	41	0.056	0.31	21.54	80.43
2b	Untreated	34.8	0.242	0.63	19.68	79.86
2b	Control	39	0.207	0.61	20.56	58.25
2b	25	46.2	1.295	0.71	18	95.06
2b	50	35	0.386	0.72	20.26	75.7

2b	100	36.5	0.046	0.63	20.02	99.67
2b	200	40.6	0.186	0.7	20.7	95.9
3a	Untreated	40.4	0.37	0.55	20.79	72.78
3a	Control	32.7	0.201	0.73	18.79	78.7
3a	25	39.8	0.578	0.75	19.21	112.08
3a	50	32.5	0.351	0.69	19.89	96.74
3a	100	40.5	0.08	0.64	20.81	109.82
3a	200	36.8	0.234	0.71	20.53	97.4
3b	Untreated	36.3	0.578	0.67	18.33	53.66
3b	Control	38.3	0.108	0.64	19.23	75.3
3b	25	37.9	0.235	0.69	19.55	84.35
3b	50	40.8	0.356	0.73	19.94	67.03
3b	100	37.3	0.106	0.72	19.99	90.2
3b	200	35.9	0.326	0.38	22.34	83.13

Block	Treatment	Root Fresh Weight (g)	Leaf Area (cm^2)	Canopy Dry Weight (g)	Root Dry Weight (g)
1a	Untreated	3.8	721.58	5.6	0.53
1a	Control	11.7	1043.2	6.69	1.93
1a	25	15.5	1152.17	8.98	2.32
1a	50	10.36	960.91	5.35	1.71
1a	100	10.21	1119.17	7.68	3.13
1a	200	11.32	942.72	8.22	2.48
1b	Untreated	5.5	1078.05	7.73	0.83
1b	Control	11.6	885.28	7.15	1.73
1b	25	13.38	780	5.43	1.55
1b	50	7.02	358.02	6.81	1.07

1b	100	12.62	1177.8	8	1.74
1b	200	13.16	988.03	6.69	2.38
2a	Untreated	10.1	789.24	5.78	1.25
2a	Control	4.8	1056.44	6.95	0.6
2a	25	10.9	1106.5	7.78	2.15
2a	50	1.03	839.64	2.03	0.12
2a	100	10.98	1011.1	8.61	2.51
2a	200	15.8	1191.6	7	2.75
2b	Untreated	8.3	731.91	5.65	1.24
2b	Control	14.1	903.86	5.61	2.63
2b	25	7.87	1154.9	9.04	1.28
2b	50	15.3	931.98	6.43	3.2
2b	100	10.51	1161.28	7.57	1.73
2b	200	13.65	1003.08	7.08	2.1
3a	Untreated	8.8	754.01	5.85	1.65
3a	Control	5	697.3	4.49	0.61
3a	25	7.95	1267.53	9.26	2.95
3a	50	6.63	1080.66	9.25	1.13
3a	100	10.74	1277.62	10.15	1.74
3a	200	10.78	1046.78	8.9	1.57
3b	Untreated	7.8	574.93	3.95	1.4
3b	Control	11	911.47	6.35	1.71
3b	25	12.53	954.94	8.26	1.36
3b	50	7.42	910.83	5.9	0.95
3b	100	16.68	1051.73	7.52	1.64
3b	200	15.21	1161.74	7.97	1.66

Parameter	Means for each endophyte-infected dose					
	Untreated	**Control (0%)**	**25%**	**50%**	**100%**	**200%**
SPAD (a.u.)	35.9	36.3	38.9	33.9	38.65	37.07
Stomatal Conduct. (mmol/ m^2/ s)	0.347	0.21	0.445	0.66	0.182	0.194
Fluorese. (ϕ PSII)	0.603	0.645	0.677	0.702	0.692	0.578
T leaf (°C)	20.26	19.38	19.24	19.67	20	21.09
Canopy Fresh Weight (g)	67.25	75.97	93.19	69.67	95.93	91.62
Canopy Dry Weight (g)	5.76	6.21	8.13	5.96	8.26	7.53
Root Fresh Weight (g)	7.38	9.7	11.36	7.96	11.96	13.32
Root Dry Weight (g)	1.15	1.54	1.94	1.36	2.08	2.16
Leaf Area (cm^2)	774.95	916.26	1069.34	846.96	1133.12	1055.66
Root:Shoot Ratio	0.2	0.25	0.24	0.23	0.25	0.29

End Appendix 1

Appendix Table 2. Full set of photosynthesis and sugar beet biomass measurements from the second glasshouse trial when seed meal doses (Untreated, Control (0% endophyte-infected), 25%, 50%, 100% and 200%) are applied at two different times – at sowing (T1) and at inoculation(T2)

Block	Treatment	SPAD T1	SPAD T2	Conductance T1	Conductance T2	Fluorescence T1	Fluorescence T2	T leaf T1	T leaf T2
1	Control	35.7	32.9	0.138	0.042	0.71	0.69	23.55	22.7
1	25	32.9	38.8	0.067	0.163	0.47	0.7	27.54	23.08
1	50	30.5	22.3	0.052	0.066	0.66	0.69	21.52	23.54
1	100	33.6	33.4	0.047	0.057	0.72	0.75	20.67	21.15
1	200	33.7	31.6	0.102	0.037	0.68	0.73	21.18	20.81
2	Control	36.1	29.2	0.034	0.067	0.65	0.72	23.95	22.38
2	25	32.5	34.9	0.059	0.106	0.64	0.68	24.32	24.05
2	50	24.8	39.2	0.055	0.042	0.7	0.7	21.84	22.49
2	100	36.5	37.9	0.029	0.032	0.73	0.76	21.04	21.88
2	200	31.7	35.7	0.074	0.075	0.53	0.73	24.47	20.21
3	Control	29.2	32.8	0.056	0.087	0.71	0.73	22.66	23.12
3	25	32.4	27.5	0.172	0.072	0.71	0.71	22.73	24.1
3	50	28.9	26	0.123	0.074	0.66	0.65	22.21	25.39
3	100	34.4	34.1	0.071	0.101	0.72	0.75	20.13	20.86
3	200	32.4	32.8	0.102	0.008	0.73	0.75	21.49	21.15
4	Control	33.1	38.7	0.079	0.126	0.73	0.38	21.91	26.45
4	25	33.3	30.9	0.17	0.156	0.66	0.41	21.93	27.13
4	50	35.3	26.7	0.179	0.165	0.66	0.68	20.92	23.3
4	100	42	42.5	0.066	0.153	0.5	0.73	22.79	20.51
4	200	26.9	29.1	0.2	0.08	0.71	0.67	21.99	19.48
5	Control	33.7	30.2	0.087	0.092	0.67	0.27	22.48	27.53
5	25	36.3	27.7	0.044	0.144	0.7	0.38	22.66	27.45
5	50	44.9	29.1	0.06	0.069	0.68	0.65	21.27	24.18

5	100	31.4	40	0.111	0.064	0.72	0.75	22.38	19.74
5	200	40.9	33.5	0.122	0.055	0.73	0.77	24.56	20.6
6	Control	30.6	32.8	0.053	0.135	0.35	0.56	25.65	25.56
6	25	37.5	31.1	0.12	0.13	0.73	0.6	21.08	24.8
6	50	36.5	32.3	0.068	0.072	0.62	0.73	21.28	22.79
6	100	32.5	32.9	0.189	0.305	0.73	0.73	21.3	20.19
6	200	44.7	39.7	0.058	0.052	0.57	0.71	21.7	20.88

Block	Treatment	Canopy Fresh Weight T1	Canopy Fresh Weight T2	Root Fresh Weight T1	Root Fresh Weight T2
1	Control	166.45	182.76	104.55	79.35
1	25	192.41	274.3	121.7	177.8
1	50	256.1	123.8	154.13	91.91
1	100	207.25	198.93	117.9	160.87
1	200	242.54	287.82	110.5	178.22
2	Control	187.38	178.82	144.36	105.12
2	25	137.72	153.84	130.2	92.1
2	50	216.8	190.24	158.65	119.6
2	100	78.2	197.8	94.1	128.32
2	200	226	97.41	158.7	51.8
3	Control	125.6	177.27	113.08	91.82
3	25	158.9	154.55	133.15	132.86
3	50	180.3	108.4	120.7	69.5
3	100	194.6	158.6	162.5	128.95
3	200	273.65	187.24	185.21	144.9
4	Control	292.68	95.55	140.93	95.48
4	25	103.75	144.15	103.65	127.25

4	50	168.68	67.4	174.82	69.3
4	100	192.66	97.29	103	66.84
4	200	169.44	129.4	119.6	166.64
5	Control	209.7	220.8	128.7	116
5	25	152.27	121.75	92.92	82.13
5	50	131.83	206	69.3	122.26
5	100	147.07	207.18	94	176.53
5	200	133.42	224.53	130	158.34
6	Control	128.26	32.89	97.3	23.44
6	25	141.63	124.36	94.88	93.76
6	50	212.5	126.91	111.7	110.84
6	100	145.25	114.6	83.7	105.29
6	200	131.47	117.87	131.46	57

Block	Treatment	Leaf Area T1	Leaf Area T2	Canopy Dry Weight T1	Canopy Dry Weight T2	Root Dry Weight T1	Root Dry Weight T2
1	Control	1513.9	1672.71	17	19.4	19.15	15.58
1	25	1800.28	2269.7	18.46	27.1	22.1	34.4
1	50	2207.8	1232.78	25.54	15.16	30	20.6
1	100	2156.3	1878.5	22.5	21.8	22.56	30.8
1	200	2023.65	2394.3	23.1	30.03	20.76	34.8
2	Control	1810.4	1674.02	22	18.86	29.7	20.5
2	25	1420.95	1550.73	15.9	18.43	27.26	17.84
2	50	1986.2	1600.6	25.06	19.83	31.86	24.5
2	100	845.5	1711.96	5.8	19.73	21	23.66
2	200	1965.7	1093.35	27.1	11.76	33.35	10.6
3	Control	1206.98	1858.88	15.9	18	24.2	17.03

3	25	1637.7	1501	17.6	17.04	27.8	24.87
3	50	1707.83	1057.37	19.46	12.64	31.8	13.3
3	100	1715.08	1566.63	20.22	19.6	32.9	25.8
3	200	2366.63	1842.8	29.56	19.42	36.2	26.67
4	Control	2646	1167.5	28.73	10.9	24.62	20.4
4	25	1033.15	1391.1	13	17.35	22.56	26.5
4	50	1728.57	674.7	17.68	8.9	33	15
4	100	1750	1163.15	20.61	11.45	18.95	14.75
4	200	1643.05	1274.56	17.1	15.33	22.8	34.27
5	Control	1873.5	1895	21.54	21.12	24.46	21.33
5	25	1391.4	1321.8	14.9	13	16.68	16.5
5	50	1195.77	1983.9	14	20.67	13.9	22.4
5	100	1503.16	1912.6	16.2	27.56	18.62	33.2
5	200	1331.8	2174.6	14.74	22.2	25.9	28.85
6	Control	1343.15	400.87	15.8	3.46	20.9	4.8
6	25	1169.26	1210.05	16	10.1	19.9	19.4
6	50	1883.7	1149.19	20.61	14.6	20.75	22.5
6	100	1578.4	1163.87	14.2	13	16.38	21.18
6	200	1485.1	1251.5	14.96	12.78	27.7	11

Parameter (T1)	Means for each endophyte-infected dose				
	Control (0%)	**25%**	**50%**	**100%**	**200%**
SPAD (a.u.)	33.1	34.2	33.5	35.07	35.1
Conductance ($mmol/m^2/s$)	0.08	0.11	0.09	0.09	0.11
Fluorescence (ϕ PSII)	0.09	0.65	0.66	0.69	0.66
Leaf temperature (°C)	23.37	23.38	21.51	21.39	22.57
Canopy Fresh Weight (g)	185	147.78	194.37	160.84	196.09
Canopy Dry Weight (g)	20.16	15.98	20.39	16.59	21.09
Root Fresh Weight (g)	121.5	112.75	131.55	109.2	139.25
Root Dry Weight (g)	23.84	22.72	26.89	21.74	27.79
Leaf Area (cm^2)	1732.32	1408.79	1784.98	1591.41	1802.66

Parameter (T2)	Means for each endophyte-infected dose				
	Control (0%)	**25%**	**50%**	**100%**	**200%**
SPAD (a.u.)	32.8	31.8	29.3	36.8	33.7
Conductance ($mmol/m^2/s$)	0.09	0.13	0.08	0.12	0.05
Fluorescence (ϕ PSII)	0.56	0.58	0.68	0.75	0.72
Leaf temperature (°C)	24.62	25.1	23.62	20.72	20.52
Canopy Fresh Weight (g)	148.015	162.16	97.24	162.4	174.05
Canopy Dry Weight (g)	15.29	17.17	15.3	18.86	18.59
Root Fresh Weight (g)	85.2	117.65	97.24	127.8	126.15
Root Dry Weight (g)	16.6	23.25	19.72	24.89	24.37
Leaf Area (cm^2)	1444.83	1540.73	1283.09	1566.12	1671.85

End Appendix 2

Appendix Table 3. Full set of sugar beet establishment, photosynthesis and virus yellow data from the field trial looking at three sowing densities of endophytic grass (Control (0), 10, 20 and 30 kg/ha) and four different destruction techniques (Strip treated (T1), Deep till (T2), Shallow till (T3) and Glyphosate (T4))

Plot	Block	Grass Seed rate (kg/ha)	Destruction Treatment	Establishment (plants/hectare)	SPAD (au)	Canopy Temp (°C)
1	1	0	T1	117000	45.1	22.1
2	1	0	T2	105000	42.2	21.7
3	1	0	T3	107000	44.4	22.6
4	1	0	T4	106000	43.3	23
5	1	10	T3	114000	46.3	21
6	1	10	T1	115000	43.7	21.5
7	1	10	T2	98000	46.1	22
8	1	10	T4	100000	45.7	23.6
9	1	30	T1	108000	43.7	23.5
10	1	30	T2	111000	42.2	22.6
11	1	30	T3	101000	40.7	23.7
12	1	30	T4	104000	44.8	21.5
13	1	20	T3	100000	42.5	25.1
14	1	20	T1	103000	46.5	23.6
15	1	20	T2	100000	46.1	23
16	1	20	T4	107000	46.1	23.2
17	2	0	T2	120000	46.4	24.2
18	2	0	T1	112000	44	22.8
19	2	0	T3	115000	45.8	22.5
20	2	0	T4	112000	44.9	23.2
21	2	20	T4	107000	45.9	25.6
22	2	20	T2	103000	44.9	24.6
23	2	20	T1	97000	45.1	25.6

24	2	20	T3	87000	39.5	24.3
25	2	10	T4	104000	45.7	25.1
26	2	10	T3	104000	44.6	22.8
27	2	10	T1	104000	46.7	23
28	2	10	T2	110000	41.9	25.7
29	2	30	T2	96000	42.7	23.1
30	2	30	T1	116000	42.9	23.6
31	2	30	T3	90000	36	24.8
32	2	30	T4	121000	42.2	21
33	3	30	T2	101000	41.9	22.2
34	3	30	T4	104000	42.7	21
35	3	30	T3	98000	36.3	23.4
36	3	30	T1	107000	44.6	19.7
37	3	20	T4	104000	45	19
38	3	20	T3	95000	46.5	23.1
39	3	20	T1	101000	45.6	22
40	3	20	T2	96000	45.7	21.8
41	3	10	T2	100000	43.2	21
42	3	10	T1	101000	44.6	18.7
43	3	10	T4	107000	42.8	18
44	3	10	T3	95000	43.6	20.5
45	3	0	T2	85000	44.7	19.7
46	3	0	T1	84000	43.8	21.2
47	3	0	T4	92000	45.9	22
48	3	0	T3	100000	44.4	22.1
49	4	10	T3	105000	44.4	22.6
50	4	10	T1	95000	43.7	22.3
51	4	10	T4	90000	42.5	19.4

52	4	10	T2	92000	44.2	18.4
53	4	20	T1	104000	42.9	21.8
54	4	20	T2	93000	44.7	22.8
55	4	20	T3	87000	40.1	21.5
56	4	20	T4	99000	44.2	20.6
57	4	30	T4	88000	45.2	19.2
58	4	30	T1	100000	43.9	20
59	4	30	T2	104000	41.9	20.2
60	4	30	T3	103000	39.6	21.8
61	4	0	T1	100000	43.9	21.8
62	4	0	T2	105000	45.7	20.1
63	4	0	T3	97000	43.8	19.5
64	4	0	T4	106000	46.2	18.6

Plot	Block	Fluorescence (PSII)	Conductance ($mmol/m^2/s$)	NDVI (au)	VY Symptomatic plants	% symptomatic (VY/210*100)
1	1	0.53	0.38	0.87	13	6.2
2	1	0.65	0.33	0.87	4	2
3	1	0.62	0.35	0.86	6	2.9
4	1	0.71	0.34	0.85	43	20.5
5	1	0.71	0.3	0.86	30	14.3
6	1	0.7	0.28	0.87	10	4.8
7	1	0.72	0.36	0.84	7	3.3
8	1	0.68	0.39	0.86	7	3.3
9	1	0.69	0.36	0.83	6	2.9
10	1	0.62	0.32	0.86	7	3.3
11	1	0.7	0.34	0.83	10	4.8
12	1	0.71	0.33	0.85	14	6.7

13	1	0.7	0.29	0.73	30	14.3
14	1	0.57	0.3	0.8	18	8.6
15	1	0.63	0.19	0.82	8	3.8
16	1	0.73	0.33	0.77	5	2.4
17	2	0.53	0.38	0.82	8	3.8
18	2	0.5	0.29	0.78	22	10.5
19	2	0.68	0.33	0.74	6	2.9
20	2	0.4	0.26	0.75	11	5.2
21	2	0.6	0.25	0.74	10	4.8
22	2	0.58	0.33	0.71	6	2.8
23	2	0.42	0.22	0.75	2	1
24	2	0.56	0.26	0.74	8	3.8
25	2	0.6	0.32	0.77	10	4.8
26	2	0.56	0.29	0.78	8	3.8
27	2	0.57	0.29	0.78	11	5.2
28	2	0.6	0.34	0.69	20	9.5
29	2	0.69	0.29	0.78	20	9.5
30	2	0.66	0.25	0.8	13	6.2
31	2	0.57	0.27	0.75	4	2
32	2	0.5	0.33	0.78	14	6.7
33	3	0.71	0.3	0.83	2	1
34	3	0.63	0.22	0.85	16	7.6
35	3	0.67	0.18	0.76	1	0.5
36	3	0.67	0.25	0.86	7	3.3
37	3	0.61	0.25	0.87	21	10
38	3	0.69	0.37	0.83	4	2
39	3	0.69	0.38	0.79	6	2.8
40	3	0.67	0.32	0.7	10	4.8

41	3	0.66	0.35	0.76	12	5.7
42	3	0.7	0.38	0.82	6	2.8
43	3	0.68	0.23	0.82	2	1
44	3	0.68	0.29	0.8	6	2.8
45	3	0.65	0.28	0.83	4	2
46	3	0.69	0.37	0.8	12	5.7
47	3	0.7	0.36	0.87	4	2
48	3	0.7	0.28	0.88	2	1
49	4	0.48	0.27	0.77	9	4.3
50	4	0.45	0.28	0.74	10	4.8
51	4	0.53	0.33	0.78	18	8.6
52	4	0.5	0.33	0.72	22	10.5
53	4	0.54	0.37	0.78	6	2.8
54	4	0.5	0.31	0.81	14	6.7
55	4	0.53	0.31	0.85	9	4.3
56	4	0.3	0.19	0.82	7	3.3
57	4	0.69	0.26	0.8	28	13.3
58	4	0.56	0.37	0.81	14	6.7
59	4	0.58	0.3	0.79	7	3.3
60	4	0.4	0.35	0.82	6	2.8
61	4	0.41	0.34	0.8	4	2
62	4	0.47	0.29	0.77	3	1.4
63	4	0.52	0.42	0.8	5	2.4
64	4	0.48	0.28	0.79	2	1

Seed rate (kg/ha)	Destruc. method	Means					
		Sugar beet estab. (per ha)	SPAD	Canopy temp (°C)	Fluorescence (ϕ PSII)	Stomatal conductance (mmol/m^2/s)	NDVI
Control (0)	T1	103250	44.2	22	0.53	0.35	0.81
	T2	103750	44.75	21.4	0.58	0.32	0.82
	T3	104750	44.6	21.68	0.63	0.35	0.82
	T4	104000	45.07	21.7	0.57	0.31	0.82
10	T1	103750	44.67	21.37	0.61	0.31	0.8
	T2	100000	43.85	21.78	0.62	0.35	0.75
	T3	104500	44.7	21.7	0.61	0.29	0.8
	T4	100250	44.2	21.5	0.62	0.32	0.81
20	T1	101250	45	23.25	0.56	0.32	0.78
	T2	98000	45.35	23	0.60	0.29	0.76
	T3	92250	42.2	23.5	0.62	0.31	0.79
	T4	104250	45.3	22.1	0.56	0.26	0.8
30	T1	107750	43.78	21.7	0.65	0.31	0.83
	T2	103000	42.67	22	0.65	0.30	0.82
	T3	98000	38.15	23.43	0.59	0.29	0.79
	T4	104250	43.73	20.88	0.63	0.29	0.82

End Appendix

Appendix Figure 1. Seed meal analysis report by Cawood Scientific Ltd.

nrm part of Cawood

ANALYTICAL REPORT

Report Number	31848-24	L935	JOHN ALCOCK
Date Received	12-APR-2024		PLANT SCIENCES
Date Reported	17-APR-2024		UNIV OF NOTTINGHAM
Project	SEED MEAL		SUTTON BONINGTON CAMPUS
Reference	ATHREYA SHETTY		LOUGHBOROUGH
Order Number	462236		LEICS LE12 5RD

Laboratory Reference		FORA286410	FORA286411								
Sample Reference		ENDOPHYTE SEED MEAL	RYEGRASS SEED MEAL								
Determinand	**Unit**	**SEEDMEAL**	**SEEDMEAL**								
Total Nitrogen DUMAS	% w/w	2.18	2.36								
Total Phosphorus	% w/w	0.45	0.39								
Total Potassium	% w/w	0.69	0.62								
Total Calcium	% w/w	0.31	0.21								
Total Magnesium	% w/w	0.16	0.14								
Total Sulphur	mg/kg	2217	1944								
Total Manganese	mg/kg	37.8	53.8								
Total Copper	mg/kg	5.7	5.1								
Total Zinc	mg/kg	35.4	33.1								
Total Iron	mg/kg	89.8	75.7								
Total Boron	mg/kg	2.8	1.1								

Notes

Analysis Notes: The sample submitted was of adequate size to complete all analysis requested.
The results as reported relate only to the item(s) submitted for testing.
The results are presented on a dry matter basis unless otherwise stipulated.

Document Control: **This test report shall not be reproduced, except in full, without the written approval of the laboratory.**

Reported by *Teresa Clyne*

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