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Management of Chocolate Spot in Norwegian Faba Bean Production and Isolate Characteristics of Causal Organisms

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Abstract

Faba beans and other cool climate legumes are well suited for cultivation in Vestfold and Østfold in the Norwegian south-east because of their requirement for long growing seasons and are desired due to their high protein content and beneficial biological nitrogen fixation properties. Including such crops in rotations is an advantage due to the subsequent reduction in costs and CO_2 emissions from fertilizer production. Additionally, their presence in rotations could be a tool for improving integrated pest management in cereals by reducing disease pressure.

A challenge specifically related to the management of faba bean crops is the disease chocolate spot (cs) caused by pathogen species in the genus *Botrytis*, typically *Botrytis fabae* Sardiña. and *Botrytis cinerea* Pers.: Fr. Management of chocolate spot epidemics is limited by the number of fungicides available to commercial growers, and the development of fungicide resistance is a challenge currently being investigated.

A randomized factorial split-plot field trial with 3 replicates was set up in at Vollebekk research farm in Ås in the spring of 2023 and separated by early and late varieties. For each section three seed rates, two cultivars and four fungicide treatments were used.

The severity of disease was scored, the developmental stages of the crops were recorded, and the resulting yield was dried and weighed. By collecting diseased leaves and making single spore isolates, the pathogens available in the field were sequenced using a NEP2 primer and tested against the active compounds in the currently utilized fungicide Signum®. Causal organisms were *B. fabae* and *B. cinerea,* there was no relationship between severity and fungal species, and no noteworthy signs of resistance to fungicide compounds were found. Results showed significant differences in chocolate spot levels between treated and untreated plots in early and late varieties, and the severity was lowest in plots treated with Elatus® Era, a fungicide currently unavailable for use in faba beans. Yield and chocolate spot correlated negatively, and the yield was highest in plots treated after the first symptoms appeared. The difference in yield between this treatment and untreated plots was significant in late varieties. Canopy density measured by sowing rate had no significant effect on disease severity in either early or late varieties, although the correlation was positive in both.

Sammendrag

Åkerbønner, erter og andre vekster i erteblomstfamilien med høyt proteininnhold egner seg godt for klimaet på Sørøstlandet på grunn av deres krav til lang vekstsesong. De siste årene har etterspørselen etter plantebaserte produkter med høyt proteininnhold økt og disse vekstene er i tillegg en kilde til nitrogen uten bruk av mineralgjødsel, noe som er gunstig både for bonden og miljøet. Dette kan gagne vekstskifter med blant annet korn både når det gjelder forgrødeeffekten og som bidrag til å holde sykdomspresset nede.

Det finnes likevel ulike utfordringer knyttet til dyrking av åkerbønner, blant andre soppsykdommen sjokoladeflekk forårsaket i hovedsak av artene *Botrytis fabae* Sardiña og *Botrytis cinerea* Pers.: Fr. Grunnet begrensningene i antall tilgjengelige preparater for produsenter kan håndteringen av sjokoladeflekk-epidemier bli krevende dersom resistens mot virkemåtene i disse utvikler seg. Dette er et aktuelt tema i utviklingen av integrert plantevern i åkerbønne.

Et randomisert faktorialt split-plot forsøksfelt ble anlagt på Vollebekk forsøksgård i Ås våren 2023, delt inn i tidlige og sene sorter. For hver del inngikk tre såmengder, to sorter og fire soppmiddelbehandlinger som faktorer i forsøket.

Sykdomsregistreringer, notering av utviklingsstadier i plantene og avling ble tørket og innveid. Bladprøver ble hentet ut fra smittede planter og enkeltsporeisolater fra disse ble artssekvensert og testet in vitro mot de aktive stoffene i soppmiddelet Signum® som brukes i dag, men ingen tydelige tegn til resistens ble påvist. Artene *B. fabae* og *B. cinerea* ble identifisert, og det fantes ingen sammenheng mellom disse og sykdomspress i feltene de ble hentet fra. Forskjellen i sykdomsnivå mellom sprøytede og ubehandlede felter var signifikant i både sene og tidlige sorter, og nivået var lavest i felt behandlet med Elatus® Era, et preparat ikke godkjent til bruk i åkerbønner. Avling korrelerte negativt med sykdomsnivå, og den var høyest i felt behandlet ved første tegn til sjokoladeflekk. Forskjellen mellom avling i sistnevnte og ubehandlede felt var signifikant i sene sorter.

Plantetetthet målt i såmengde hadde ingen signifikant effekt på sykdomspress verken i tidlige eller sene sorter, men korrelasjonen var positiv hos begge.

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Introduction

The Norwegian cultivation of protein rich seed crops has been one of many agricultural initiatives in recent years for realizing the potential for increased food security and decreasing the food productions' impact on several environmental factors/indicators (Svanes et al., 2024). The incorporation of more of these high protein crops (HPC) into crop rotations has been suggested as a counter to the monocropping of cereals that has traditionally been established in the comprehensive arable areas around the Oslo-fjord. A large portion of the wheat grown for food (spring wheat) has been located here as they require a longer growing season than winter wheat used as feed, hence these large and level areas are well suited for production of food crops that can include HPCs. As suggested in a previous study by Abrahamsen et al. (2019), the production of peas and faba beans could be increased by 380% in Østfold and Vestfold and 724% overall throughout other regions of Norway. Increasing the volume of crops like faba beans (*Vicia faba* Linnaeus), peas (*Pisum sativum* Linnaeus), turnip rapeseed (*Brassica rapa* var. *rapa* Linnaeus), and rapeseed (*Brassica napus* Linnaeus) here would decrease the frequency of cereal crops in each given field which has the potential for reducing the general disease pressure on the crops. Using more diverse crops in rotations has been shown to strengthen cropping systems as the inoculum sources like survival structures of pathogens that mainly persist on monocot plants like barley and wheat will be gradually reduced when using dicots as break crops in a monocereal rotation (Uhlen et al., 2017).

Plant species of the family *Fabaceae* have biological nitrogen fixation (BNF), which can reduce the need of N fertilization, a perk both for the farmer in terms of cost and to the environment in terms of lessened risk of nitrous oxide (N_2O) gas release, leaching, and surface runoff with its consequent eutrophication. (Wang & Li, 2019) A surplus of up to 10-20 kg/daa of fixed nitrogen can result from the inclusion of faba beans according to Jensen et al. (2010).

Faba beans have the highest BNF efficiency of the *Fabaceae* cultigens (Peoples et al., 1995) relevant for Norwegian growers due to the size and multitude of nodules produced. The plant roots form symbioses with a variety of *Rhizobium* strains, including but not limited to the *Rhizobium leguminosarum* symbiovar *viciae*, and the species *Rhizobium fabae* and *Rhizobium etli* (Argaw & Mnalku, 2017). Inoculation is usually not necessary for faba beans since neither yield nor protein content have been shown to increase with inoculation (Serikstad et al., 2013).

The requirement of faba beans for P and K is usually provided by reserves in the soil, although this varies with the soil type, production system as well as different cultivation regions (Øverland, 2023). Faba beans have a higher protein content than most legumes of around 30% and they are less prone to lodging than peas due to their shorter and more robust growth.

A larger share of plant-based foods incorporated into the Norwegian diet in exchange for animal-based products, in particular red meat, might improve public health outcomes by reducing risk of lifestyle diseases such as cardiovascular disease (Bouvard et al., 2015; Schwingshackl et al., 2017). Moreover, a diet shift towards more plant-based food for the

Norwegian population was suggested as one of the most efficient measures to reduce environmental impacts of the food system and climate gas emissions in particular (Mittenzwei et al., 2020). In such a scenario, utilizing the potential in Norway to produce HPCs is important to maintain or even increase self-sufficiency (Svanes et al., 2024).

The faba bean crop has a large potential to solve several of the important future challenges in Norwegian agriculture, but growers have encountered problems yet to be solved. One of the most important issues concerning plant protection in faba bean is the fungal disease chocolate spot mainly caused by the species *B. fabae* and *B. cinerea*. Epidemics can occur with the presence of the pathogen, high rainfall and humidity, and temperatures of around 20ºC and develop fast, potentially causing large yield reduction in Norwegian fields.
For producers to obtain their goals of high legume yields in the coming years, developing and improving the knowledge basis for integrated pest management (IPM) is crucial. Plant breeding to develop more resistant faba bean cultivars is currently underway, and although these are yet to become readily commercially available for Norwegian growers multiple qualitative trait loci have been identified and are promising candidates for future cultivars that can be used as the primary line of defence against chocolate spot disease (Webb et al., 2024).

Chocolate spot - symptoms & disease development

As primarily necrotrophic but weak parasites, fungus species of the *Botrytis* genus will most often infect a canopy of faba beans through infected seeds or from survival structures such as sclerotia in the soil (Harrison, 1988). No regulations currently exist to limit infected seed in distribution, but practises such as seed dressing are under investigation to limit seed infection (Aamot et al., 2024). Devastating losses have been attributed to chocolate spot epidemics, destroying whole crops of faba beans, but attacks can also develop only to a non-aggressive stage that allow for acceptable yields.
Symptoms include brown speckles or spots of sizes between 1-10 mm, often with a lighter centre. As they expand these spots can develop concentric circles outward from the centre, or several spots can combine and create larger lesions. Further along in disease development older leaves may become necrotic and fall off if covered with small spots, or large brown blotches may appear on leaves caused by infection from abscised of infected flowers. Signs can include mycelium in the necrotic tissue inside the spot borders or sclerotia on infected pods, but mycelium isn't necessarily visible to the naked eye (Harrison, 1988).
When the weather is warm and dry, even though pathogen spores might be present in the surrounding area, infection will not take place. The pathogen group has an optimal temperature for development and infection of 20-23ºC (Benzohra et al., 2017; Tupenevich & Kotova, 1970; Wilson, 1937), disseminates by wind and rain and infects easily in relative humidity (RH) of >90% and on leaves left wet for longer periods such as in the bottom of canopies (Bankina et al., 2021).

Causal organisms

Since 1946 *B. fabae* has been considered to be the main causal agent for chocolate spot in faba beans, although *B. cinerea* can also be isolated from chocolate spot lesions (Harrison, 1988). *B. cinerea* can have both sexual and asexual stages – the survival structures sclerotia can germinate and develop (2-6) sexual reproductive structures called apothecia opening on the sclerotia surface to release fertilized spores or conidiophores in surface cracks that release asexual conidia spores. The former can happen only in the field after a resting period of several months brought on by the winter, but not all *Botrytis* species (for example *B. fabae*) have a known teleomorph stage (Harrison, 1988; Jarvis, 1977). Conidia spores produced from branching conidiophores can germinate in nutrient media or in free water and spread with wind or by water-splash when temperatures are between 5ºC and 25ºC, although different experiments have seen germination at temperatures as low as 1ºC and as high as 34ºC.
The species *B. fabae* will usually infect faba beans or other species in the *Fabaceae* family whereas *B. cinerea* has a broader range of host plants (Harrison, 1988) that includes important crops like berries, vegetables and high protein crops (Hermansen, 2023). This may increase the need of control to protect surrounding fields if *B. cinerea* is the main chocolate spot causing pathogen in the faba bean field. In addition to *B. cinerea* and *B. fabae* there have been observations of *Botrytis fabiopsis* (Zhang et al., 2010) and *Botrytis pseudocinerea* (Plesken et al., 2015) as a causal agent of chocolate spot in faba beans.
Earlier findings by Sundheim (1973) have shown that *B. fabae* may cause larger lesions and may be more aggressive than *B. cinerea*, but this in an area of research currently lacking in certainty (Aamot et al., 2023). It has been found that spore production in *B. fabae* happens during the night when temperatures are mild and RH is high, usually at between 92% and 100% (Harrison, 1988). Harrison (1984) found that the highest number of spores are produced at a temperature of 20ºC.
The life cycles of *B. fabae* and *B. cinerea* are similar. They have the same survival structures, both spread by conidia spores and their life cycles follow the same patterns except that their host plants differ.
B. cinerea sclerotia produce conidiophores when exposed to light in and the subsequent spore production happens at a wide range of temperatures, from 3ºC to 27ºC (Harrison, 1988). Its substantially wider range of host species than *B. fabae* means that there may be more widespread areas and plant material for the pathogen to survive on throughout the winter and disseminate from.

Current protocol for dealing with fungal disease in conventional Norwegian production includes fungicide applications up to twice in the flowering period with 7-14 days between treatments, using the approved compound Signum®. It contains the active ingredients boscalid and pyraclostrobin at percentages of 26.7% and 6.7%, respectively. The allowed dosage of Signum is 100g/30 L water per decare (daa), or 3.33 g/L, which comes out to 0.89 g/L boscalid and 0.22 g/L pyraclostrobin. It is regulated for use as a preventative fungicide. (BASF, 2023)
Together they have both preventative and curative properties, and each have different modes of action: Pyraclostrobin is classed as a strobilurin or Quinone outside Inhibitor (QoI), which

means it targets a specific site of the cytochrome b complex III along the mitochondrial electron transport chain of fungal cells by binding to it. This inhibits normal respiration of the pathogen, thereby rendering it unable to undergo crucial processes for survival through the production of ATP (Xiong et al., 2020). Since pyraclostrobin targets a specific site and is in FRAC group 11, pathogens have a high risk of developing a gene-mutation and become resistant to it. Boscalid is classed as a broad spectrum carboxamide or succinate dehydrogenase inhibitor (SDHI) (Stammler et al., 2008). The succinate dehydrogenase enzyme is crucial to the TCA/Krebs cycle, which brings about the energy release from nutrition sources consumed by the fungi, thereby impacting the pathogen cells' ability to produce ATP and other intermediate compounds necessary for synthesizing fatty acids and amino acids. It has both systematic and translaminar effects, which in effect means that it works both inside the plant after being taken up through the leaves and inhibits appressorium formation. (BASF, 2016) It is in FRAC group 7, which places it at a medium to high risk of acting as selective pressure for the evolution of resistant pathogen strains.
The broad-spectrum systemic fungicide Elatus® Era contains two active ingredients, the first being benzovindiflupyr (Solatenol™), which is a pyrazole 4-carboxamide and like boscalid is classed as a SDHI. The other is prothioconazole, which is a triazole (Syngenta, 2024b). Prothioconazole is a demethylation inhibitor and sterol biosynthesis inhibitor (SBI), which prevents the production of sterols, the function of which is essential for the structural integrity and function of fungal cell membranes. With a FRAC code of 3, it is rated as an active ingredient with a medium risk of developing resistant strains due to it not having any cross-resistance potential with any other SBI-classed components. (FRAC, 2024)
Resistance of *B. cinerea* to Signum has been known to occur in the past few years, showing growth at high concentrations both of boscalid and pyraclostrobin. Moderate resistance to boscalid was also recorded in *B. fabae* isolates, but not to pyraclostrobin (Aamot et al., 2023). The only other fungicide available for use in Norwegian faba beans is Amistar® (Syngenta, 2024a), another strobilurin in FRAC group 11 that when used repeatedly increases the risk of resistance developing towards other compounds in the same group such as pyraclostrobin.

The research project 'Increased and market-adapted production of grain legumes in Norway to increase self-sufficiency of proteins for food and feed' or 'FutureProteinCrops' (Norsk forskningsråd (NFR) project no. 326701) includes a section on developing IPM strategies specifically to improve control of chocolate spot. We wish to improve our general understanding of how the fungal disease chocolate spot can be best controlled, and which factors influence yields. Developing these strategies could strengthen the argument for lower fungicide dosages within an acceptable yield penalty and contribute to positive environmental impacts on SDGs 3, 6, 12, 14 and 15. (UN, 2015)
The research field at Vollebekk Research Farm in Ås, Akershus, that was established in the 2023 growing season, was used as the basis of this thesis which is a part of this wide-ranging project.

The thesis objectives are to I. investigate in what ways plant density impacts the disease severity of chocolate spot in faba bean, II. compare the current fungicide in commercial use

today (Signum®) to the effects of the fungicide Elatus® Era, how they each fare in terms of efficiency in controlling chocolate spot, and what the optimal timing for application of Signum is, III. determine which species of *Botrytis* are most prevalent in the faba bean field, and how the two active ingredients in Signum affect these pathogen species in vitro and IV. determine whether there is any indication of which causal organism is more aggressive.

Materials and methods

Field trials

Layout and experimental design

The field trial was set up in a randomized split-plot with three factors: fungicide treatment regimen, cultivar, and sowing rate. Two experiments were laid out with early and late varieties, respectively, and placed adjacent to each other in the field at 59º39'23.3"N latitude and 10º45'26.2"E longitude in Ås. A detailed map of the field is shown in Figure 1.

The early cultivars 'Louhi' (A), 'Sampo' (B) and late cultivars 'Vertigo' (A) and 'Tiffany' (B) were used. Sowing, as well as the rest of the activities essential to the field trial, was carried out by professionals at SKP, NMBU using a plot sowing machine (Wintersteiger Plotseed S) on May 7th, and sowing rates were set at 60 seeds/m^2 (1), 80 seeds/m^2 (2), and 100 seeds/m^2 (3) for the early varieties, and 40 seeds/m^2 (1), 60 seeds/m^2 (2), and 80 seeds/m^2 (3) for the late varieties.

The applied fungicide treatments were as follows:
I: untreated
II: 0.075 kg Signum applied when plants were at BBCH 60-61
III: 0.075 kg Signum applied when plants started showing symptoms of chocolate spot
IV: 0.06 l Elatus Era applied when plants were at BBCH 60-61

Fungicide treatments were applied on the main plots, and all combinations of variety and seed rate on sub-plots. The size of the sub-plots was 1.5m*6m, reduced to 1.5m*5m before harvest to avoid border inconsistencies.
Thus, each main plot comprised 6 sub-plots. The main plots were separated by border plots to facilitate precise application of the fungicides. (Labelled "Vern" in Figure 1) The subplots with combinations of variety and seed rate were randomized within the main plots, and the main plots were randomized within the three replicates.

Harvesting of early varieties was done by SKP using a plot combiner on September 4th, and late varieties were harvested on October 5th. Seed yield from each plot was collected and dried to below 15% water content, and then weighed by NIBIO at Apelsvoll. The seed yields in kg/daa were calculated at 15% water content.

Additional crop protection measures

Due to an occurrence of pea leaf weevil (*Sitona lineatus*) in the entire field, 15 ml/daa of the pesticide Karate® 5 CS was applied on May 27th, followed by the application of 15 g of the herbicide Basagran to combat emerged weeds in and between the plots on June 5th. Another combined application of 25 g/daa Basagran and 15 ml/daa of Karate® 5 CS was administered on June 12th because new weevils had since flown in, likely from surrounding areas (Schøll, 2021).

The drought and warm weather in May and throughout June (Figure 2) led to SKP watering the field on three dates: June 2nd, 10th, and 23rd.

Rute	Rk1.	Rk2.	Rk3.
1	Vern (101)	Vern (201)	Vern (301)
2	IA2 (102)	IIIA1 (202)	IIA3 (302)
3	IA1 (103)	IIIA3 (203)	IIA2 (303)
4	IA3 (104)	IIIA2 (204)	IIA1 (304)
5	IB2 (105)	IIIB3 (205)	IIB2 (305)
6	IB3 (106)	IIIB1 (206)	IIB3 (306)
7	IB1 (107)	IIIB2 (207)	IIB1 (307)
8	Vern (108)	Vern (208)	Vern (308)
9	Vern (109)	Vern (209)	Vern (309)
10	IIA2 (110)	IVA3 (210)	IIIA2 (310)
11	IIA1 (111)	IVA2 (211)	IIIA1 (311)
12	IIA3 (112)	IVA1 (212)	IIIA3 (312)
13	IIB3 (113)	IVB2 (213)	IIIB1 (313)
14	IIB1 (114)	IVB3 (214)	IIIB2 (314)
15	IIB2 (115)	IVB1 (215)	IIIB3 (315)
16	Vern (116)	Vern (216)	Vern (316)
17	Vern (117)	Vern (217)	Vern (317)
18	IIIA1 (118)	IA3 (218)	IVA2 (318)
19	IIIA3 (119)	IA2 (219)	IVA1 (319)
20	IIIA2 (120)	IA1 (220)	IVA3 (320)
21	IIIB2 (121)	IB3 (221)	IVB1 (321)
22	IIIB1 (122)	IB2 (222)	IVB3 (322)
23	IIIB3 (123)	IB1 (223)	IVB2 (323)
24	Vern (124)	Vern (224)	Vern (324)
25	Vern (125)	Vern (225)	Vern (325)
26	IVA3 (126)	IIA2 (226)	IA3 (326)
27	IVA2 (127)	IIA1 (227)	IA2 (327)
28	IVA1 (128)	IIA3 (228)	IA1 (328)
29	IVB2 (129)	IIB3 (229)	IB1 (329)
30	IVB1 (130)	IIB2 (230)	IB3 (330)
31	IVB3 (131)	IIB1 (231)	IB2 (331)
32	Vern (132)	Vern (232)	Vern (332)

Rute	Rk1.	Rk2.	Rk3.
1	Vern (101)	Vern (201)	Vern (301)
2	IA1 (102)	IIIA2 (202)	IIA1 (302)
3	IA2 (103)	IIIA3 (203)	IIA2 (303)
4	IA3 (104)	IIIA1 (204)	IIA3 (304)
5	IB2 (105)	IIIB3 (205)	IIB2 (305)
6	IB3 (106)	IIIB1 (206)	IIB3 (306)
7	IB1 (107)	IIIB2 (207)	IIB1 (307)
8	Vern (108)	Vern (208)	Vern (308)
9	Vern (109)	Vern (209)	Vern (309)
10	IIA2 (110)	IVA3 (210)	IIIA2 (310)
11	IIA3 (111)	IVA1 (211)	IIIA3 (311)
12	IIA1 (112)	IVA2 (212)	IIIA1 (312)
13	IIB2 (113)	IVB1 (213)	IIIB2 (313)
14	IIB1 (114)	IVB3 (214)	IIIB1 (314)
15	IIB3 (115)	IVB2 (215)	IIIB3 (315)
16	Vern (116)	Vern (216)	Vern (316)
17	Vern (117)	Vern (217)	Vern (317)
18	IIIA1 (118)	IA2 (218)	IVA3 (318)
19	IIIA3 (119)	IA1 (219)	IVA2 (319)
20	IIIA2 (120)	IA3 (220)	IVA1 (320)
21	IIIB2 (121)	IB3 (221)	IVB2 (321)
22	IIIB3 (122)	IB1 (222)	IVB3 (322)
23	IIIB1 (123)	IB2 (223)	IVB1 (323)
24	Vern (124)	Vern (224)	Vern (324)
25	Vern (125)	Vern (225)	Vern (325)
26	IVA3 (126)	IIA2 (226)	IA3 (326)
27	IVA1 (127)	IIA3 (227)	IA1 (327)
28	IVA2 (128)	IIA1 (228)	IA2 (328)
29	IVB3 (129)	IIB1 (229)	IB2 (329)
30	IVB1 (130)	IIB2 (230)	IB3 (330)
31	IVB2 (131)	IIB3 (231)	IB1 (331)
32	Vern (132)	Vern (232)	Vern (332)

Figure 1 The randomized, split-plot design of the two fields. Early (left) and late varieties (right).

Weather conditions

From the time of sowing on May 7th until June 17th, there was almost no rainfall. The temperatures early in the season were at their highest throughout the whole season, which combined with the low levels of precipitation led to the need of several waterings of the trial fields. Later on, from mid-July until the end of September, there was frequent rainfalls with high amounts of precipitation. The weather data for the season was obtained by the faculty of Science and Technology at NMBU from the meteorological data at the BIOKLIM field station (BIOKLIM, 2023) can be seen in Figure 2.

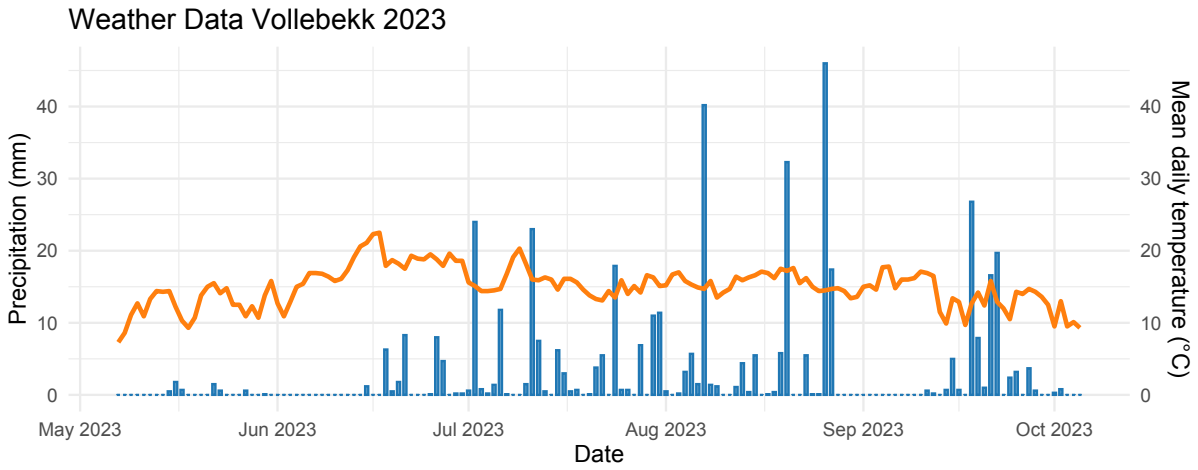

Figure 2 Weather data represented in bars (precipitation) and a line (mean daily temperatures) collected at NMBU's weather station BIOKLIM in Ås.

BBCH- developmental stages

Registrations were made roughly every week since emergence by using a key developed for faba beans (Lancashire et al., 1991), judging the developmental stage of each plot and noting the stage of the majority of the plants. The weeks when the replications appeared to be even in terms of development, only one or two repetitions were recorded.

Fungicide treatments

The fungicide treatments were performed by NLR, using a modified experimental version of the Oxford sprayer Norsprøyta, manufactured by Langmyr (1999) with the nozzle Hypro ULD 02-120. Sprayer pressure was set at 1.5 bar and 25 litres/daa was applied.

Fungicide application dates of early varieties:
Treatment II: 23.06.2023 (at BBCH 60-61)
Treatment III: 07.07.2023 (at first symptoms, plants were at BBCH 65-70)
Treatment IV: 23.06.2023 (at BBCH 60-61)

Fungicide application dates of late varieties:
Treatment II: 28.06.2023 (at BBCH 60-61)
Treatment III: 14.07.2023 (at first symptoms, plants were at BBCH 65-70)
Treatment IV: 28.06.2023 (at BBCH 60-61)

Disease registrations and sampling

The disease severity in the plants was visually determined at three levels in the plant canopy by looking at the leaves of a cluster of 3-4 plants at six points in each plot. The lower, middle and upper leaves of the plants in each cluster were scored separately. A disease scoring key was used, (Plant Pathology Laboratory at the Agricultural Development Advisory Service, 1976) and an example of field scorings can be seen in images A through F in Figure 3. Leaf samples with ample amounts of chocolate spots (at least 10% diseased area) were taken from each plot in the field. Adjoining plots with identical treatments except seed rates were grouped and named for the first plot in the group of three (e. g. samples from plots 105, 106 and 107 were grouped and named "105").

Figure 3 Individual leaves scored as 1 (A), 3 (B), 5 (C), 10 (D), 20 (E) and 35 (F). Images: Nora Steinkopf

Laboratory experiments

Preparation of fungal cultures and single spore isolates

Plating leaf samples

Leaf samples from three sub-plots containing all three sowing rates (e. g. 102, 103, and 104 in one bag labelled 102) were combined to decrease the number of isolates. The diseased leaves were cut into approximately 1x1 cm pieces and separated into trays of either large (labelled "STORE") or small (labelled "SMÅ") chocolate spots. A rim of healthy plant material was included in each piece to ensure living fungal cells, and 5-6 pieces were prepared for each tray to safeguard against loss in the straining process.
Using sterile techniques inside a sterile bench, samples were placed in a strainer and submerged in 70% ethanol for 10 seconds, followed by 1 minute 30 seconds in 0,5% NaClO. Directly after NaClO soaking, the sample pieces were spaced out on filter paper. 4-5 pieces were positioned onto a potato dextrose agar (PDA) plate, 8 of which were placed in perforated 20x40 cm plastic bags. The plates were incubated at 20ºC with UV + white light for 12h, for around 14 days or until the plates had sufficient growth for further transfer.
After incubation, the plates were visually examined to distinguish between fungal genera or other organisms. Each plate was marked with the number of colonies, and each colony with "–" (no fungal or bacterial growth), "B" (*Botrytis*), "S" (saprophyte), "A" (*Ascochyta*), "F" (*Fusarium*) and "U" (unknown). The colonies selected for transfer were marked with an accompanying number, e.g. B1, and if two *Botrytis* colonies from one plate were chosen: with B1 and B2. Only cultures of *Botrytis* species were further investigated after this categorization was done.

Transfer of pure fungal cultures

For every colony transferred, the new agar plate was marked with the same information and the number. A plug of sclerotia or hyphae was cut from the original plate, those from the outer border of each colony were selected to avoid contamination from other colonies.
New plates were incubated in aerated plastic bags under white light + UV for 14 days.

Spore transfer

The plates were examined in a light microscope to make sure that enough spores were present in the plates. A sterile swab was rolled over the area of a plate with visible spores, and carefully tapped on the edge and spread out on a water agar plate. All plates were put into zip-lock bags and incubated at room temperature in the dark for 24 hours.

Making single spore isolates

The microscope was set to the 10x objective, and a small cut was made on the surface of the plate with a sterilized dissection needle to facilitate localization of the surface on each plate. Next, the plate was searched for germinated spores surrounded by an area of clear agar. When a good candidate was found, the agar cutter was sterilized and dipped in the new agar plate. Any pieces of agar stuck to it was removed. Holding the agar plate containing the spore in place, the cutter was used to cut out the germinated spore and surrounding area, making sure to not include any surrounding hyphae germinated from other spores. The plug was lifted out

and placed upright onto a new potato dextrose agar with low pH (PDAs) plate marked on the underside.
The plates were incubated at room temperature in daylight until sufficient growth for further transfer was achieved. (~5-14 days)

Figure 4 Example of a single spore on agar and a transferred plug of one on PDAs. Images: Nora Steinkopf

Transfer of single spore isolates

Using a sterilized dissection needle, a ~0.5 cm plug of the mycelium from the edge of each plate was cut out and transferred onto new plates as seen in Figure 4. They were incubated at room temperature in daylight for 1-2 weeks. From here, plugs with mycelium from the outer edge of the plates was transferred onto new PDA. When these had grown sufficiently as to coat the surface of the agar with mycelium, plugs from each were transferred further for DNA extraction and spore production onto PDA, PDA+/Mung bean agar (MBA)/PDA*, respectively. The PDA+ medium was prepared with 39 g agar/L water (3.9%), 30 g $NaNO_3$/L water (3%) and 200 g sucrose/L water (20%).
*The media selected here was expanded to include MBA because of some of the isolates' difficulties sporulating.

DNA extraction and sequencing

Collection & disruption of tissue samples:

Removing any excess agar medium, two strips of fungal material ~0.5x3 cm were cut from the surface of each single spore isolate (SSI), placed in Eppendorf tubes, and set in an ice box until storage at -20ºC.
Each frozen sample was submerged in liquid nitrogen and crushed to a powder with a pestle and mortar. The resulting disrupted sample was then stored at -20ºC until extraction.

DNA extraction, PCR and sequencing

Using the DNEasy® Plant Mini Kit Quick-Start Protocol (QIAGEN, 2016) and forward + reverse NEP2-primers encoding genes for necrosis and ethylene producing proteins, DNA was extracted from all 42 samples.

Following extraction, the DNA amplification was done by PCR in a BIO-RAD T100™ Thermal cycler using the following program settings: 5 minutes at 94ºC, then 34 cycles á 30 seconds at 94ºC, 30 seconds at 55ºC and 1 minute at 72 ºC. After 34 cycles the program maintained 72ºC for 7 minutes, ending at 4ºC.
Gel electrophoresis was done by preparing 1.2% agarose gels and placing them in a gel box with enough buffer solution to cover its surface. 3µl of DNA template and 12µl of SYBR Green were mixed by pipette, and 12µl of each sample was placed in each gel well. As the *Botrytis* species' fragments present when using the NEP2-primer tend to be ~800-900 basepairs (bp) long, a 100 bp ladder was placed in the first well for size comparison (Figure 5). The electrophoresis was run at 100V for 45 minutes, followed by UV-imaging and visualization using the BIO-RAD ChemiDoc™ Imaging System.

Purified genomic DNA was sent to Eurofins for sequencing. The forward and reverse sequences were made into contigs, aligned, and trimmed for errors using the CLC Main Workbench (Qiagen). Blastn searches were carried out of the sequences using the NCBI nucleotide database, and isolate sequences were identified as the species match closest to 100%. No further testing of any isolates using different primers was carried out.

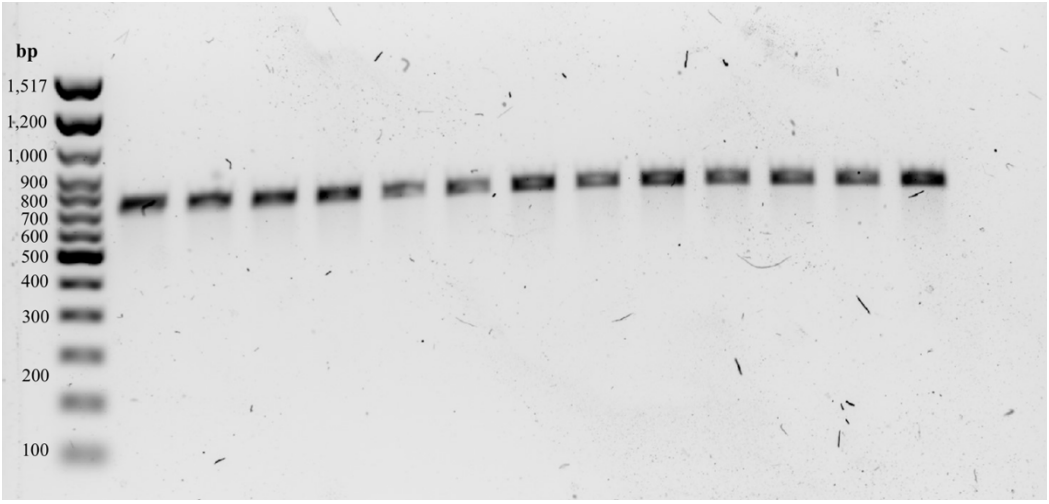

Figure 5 Visualized products of PCR analysis. DNA segments settled at ~700-800 base pairs, normal for Botrytis species.

Bioscreen

The number of isolates and fungicide active ingredients (boscalid and pyraclostrobin) we wished to test, lead to the decision of running four cycles. 15 isolates were selected to test based on the field plot the sample came from so that most treatments were well represented. Each Bioscreen cycle run contained 2x trays á 100 wells and two reference isolates were included as controls, as well as a negative sample with sterile, deionized water instead of spore solution. A dilution series of each active ingredient (AI) was prepared to determine the concentration at which fungal growth stalled.

Preparation of spore solutions:

Including all field treatments and every *Botrytis* species identified during sequencing, 16 isolates were selected for fungicide screening. Spores were removed by scraping a wet scalpel along the agar surface and depositing them in 1ml of sterile water. Using KOVA® Glasstic Slide 10 with Grids under a light microscope at 400x magnification and a mechanical tally counter, solutions were diluted or supplied with additional spores to obtain a concentration of 10^4. Finished spore solutions were vortexed and pipetted into 3-4 1.5 ml Eppendorf tubes of á 1-1.2ml and stored at -20ºC.

Preparation of AI media, dilution series and tray loading

Mixing magnets were put in all flasks to allow for mixing.

Boscalid: A stock solution of yeast broth (YB) medium was prepared by mixing 1.33 g yeast extract, 1.33 Bacto™ Peptone, 2.66 g sodium acetate and 100 ml sterile distilled in each. An additional 100 ml of YB was prepared. Semi-liquid medium (YBA) was made by mixing 0.25 g powdered agar, 1.33 g yeast extract, 1.33 Bacto™ Peptone and 2.66 g sodium acetate in 100 ml sterile distilled water.

Following the manufacturers' precautions and the protocol developed by NIBIO, 0.2 g powdered boscalid was weighed out and mixed into 100 ml YB stock solution to obtain a concentration of 1000 mg/litre. Ten 50 ml centrifuge tubes were marked from 1-10, 2000 µl of stock solution was pipetted into tubes 2-10, 2700 µl in tube "1". 300 µl boscalid solution was pipetted into the one marked "1", and a dilution series was made by pipette mixing and transferring 1000 µl between tubes in ascending order. No boscalid solution was added to the negative control "10". Concentrations of 100, 33, 11, 4, 1, 0-4, 0.1, 0.05, 0.02 and 0 mg/L were used to cover a broad range above and below the point at which isolates usually stop growing. The middling concentration of 1 was set as a threshold to identify signs of resistance in later analyses.

Pyraclostrobin: A stock solution of malt extract medium (ME) was prepared by mixing 2.667 g in 100 ml sterile distilled water. An additional 100 ml of ME was prepared for later addition.

Semi-liquid medium (MEA) was made by mixing 0.25 g powdered agar and 2.667 g malt extract in 100 ml sterile distilled water. 502 µl of the active ingredient (AI) Comet® Pro (pyraclostrobin) was pipetted into 100 ml of ME stock solution to obtain a concentration of 999 mg/litre. After labelling 10x 50 ml centrifuge tubes from 1-10, 2000 µl of ME stock solution (without AI) was pipetted into tubes 2-10, 2700 µl in tube "1". 300 µl AI solution was pipetted into the tube marked "1", and a dilution series was made by 1-5 ml pipette mixing and transferring 1000 µl between tubes in ascending order. No AI solution was added to the negative control "10". The end concentrations of 25, 8.25, 2.75, 1, 0.25, 0.1, 0.025, 0.0125, 0.005 and 0 mg/L were used to the get a wide spectrum of concentrations above and below the point at which isolates usually stop growing. The middling concentration of 0.25 was set as a threshold to identify signs of resistance in later analyses.

Immediately preceding loading of the trays, a SHAM-solution was prepared in an extractor hood by adding 0.1 g salicyl hydroxamid acid to 1 ml methanol in a glass vial. Due to the

fungal cells' potential ability to circumvent the inhibition of respiration normally caused by pyraclostrobin by using an alternative pathway via the enzyme alternative oxidase, SHAM was added because it inhibits this enzyme and ensures that the effect seen is only caused by the pyraclostrobin and isn't confounded by this resistance mechanism.
The wells were filled using sterile techniques inside a sterile bench, by first using a stepper pipette and one tip to distribute 100µl agar media in each well. 50µl of fungicide solution was added to each well, working from low to high concentrations so that the same tip could be used. Finally, 50µl of vortexed spore solution was added to each well with an automatic pipette, 9 isolates to 90 wells, one tip per isolate, working from low to high AI concentrations. The control (autoclaved, distilled water) was placed in the remaining 10 wells. In addition to the isolates we were testing, isolates previously determined to be sensitive (58/19-96) and medium resistant (58/19-55A) from storage were included for comparison.
The Bioscreen Cº Pro program settings for both runs were set at 18ºC, with OD_{405} and OD_{600} readings every 6h for seven days without shaking. Readings at both 405 and 600 nm were included to get a fuller picture of both the total fungal biomass present (600 nm) and the metabolic products and enzymes produced in the culture (405 nm).

Data analysis

Using R version 4.3.2 (R Core Team, 2024) and RStudio, bivariate correlation analyses, analyses of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) tests were run on the field data parameters. End-point disease levels were tested against yield at 15% relative water content (RWC), end-point disease levels against treatments, and yield against treatments. These were visualized using scatter- and box plots, adding significance letter groupings to the latter based on the results from the post-hoc analyses. BBCH stages were plotted over time separated by replicates, and against end-point disease levels using scatter plots. ANOVA and Tukey's tests were used to determine significance in disease x sowing rate, disease x pathogen species, sowing rate x yield, RWC at harvest x treatments separated by strobilurin content, and yield x sowing rate. Box plots were created for visualization.
Fisher's Exact Tests was carried out to determine the contingency between *Botrytis* species and sclerotia size and lesion size, respectively. Pearson's Chi-square tests were carried out to determine whether there was any association between species and sclerotia size, species and lesion size, and between lesion size and sclerotia size. The level of confidence was set to 95% for all analyses.
After loading the output from Bioscreen into a Microsoft Excel template spreadsheet with preconfigured formulas, the OD-values of the negative controls were subtracted in each repetition and cycle and the OD-data for all isolates was analysed and compared to the reference isolates. Plotting graphs of OD at 405 and 600 nanometres, they were examined for traces of resistance. The fungicide concentration at which the OD_{600} readings indicated no growth were used because the measured opacity at 600 nm closely reflects the mass of the fungal growth contained in the wells.

As the OD values are impacted by the presence of the active ingredient itself because of its high opacity in high concentrations, all negative control well OD measurements were subtracted from the OD measurements of wells containing spore solutions before analysing growth rates.

Results

Field trials

The results from the scoring of chocolate spot severity in the untreated plots over time are presented in Figure 6 and Figure 7. They represent the untreated plots, which had the most symptoms compared with the others, which overall had lower disease scores. In early varieties (Figure 8) the untreated plots had significantly higher disease levels compared to all other treatments (p-value range: 0.0000003-0), and the plots with variety 'Louhi' treated with Elatus Era had significantly lower disease rates compared to 'Sampo' treated with Signum at BBCH 60-61 (p: 0.0008).

In late varieties (Figure 9) only the untreated 'Vertigo' plots had significantly higher disease severity than all other treatment groups (p-value range: 0.002-0) except for the untreated 'Tiffany' plots. The untreated 'Tiffany' plots also had a significantly higher percentage of chocolate spot cover than 'Tiffany' plots treated with Signum at the first symptoms (p: 0.024) and all plots treated with Elatus Era (p-value range: 0.00005-0.000006). Boxplots that include sowing rates as well as fungicide treatment and cultivar can be seen in Appendix 1 and Appendix 2.

An ANOVA analysis showed no significant difference between disease severity and the *Botrytis* species sampled Appendix 7.

BBCH at the point of the last disease scorings against disease levels had no correlation of note in either early (Appendix 8) nor late (Appendix 9) varieties.

ANOVA analysis showed a nonsignificant correlation (p: 0.543) between seed rates and disease severity in early varieties (Figure 10) and the result from the ANOVA analysis in late varieties (Figure 11) was also not significant (p: 0.684), although positive correlations (seen in Appendix 12 and Appendix 13) were found to be $R^2 = 0.1146$ in early varieties and $R^2 = 0.1025$ in late varieties. The size of the harvested beans from the early and late plots were negatively and positively correlated ($R^2 = 0.104$ and $R^2 = 0.004$, respectively) with disease severity (Appendix 10 and Appendix 11). No noteworthy flower infection was recorded throughout the growing season.

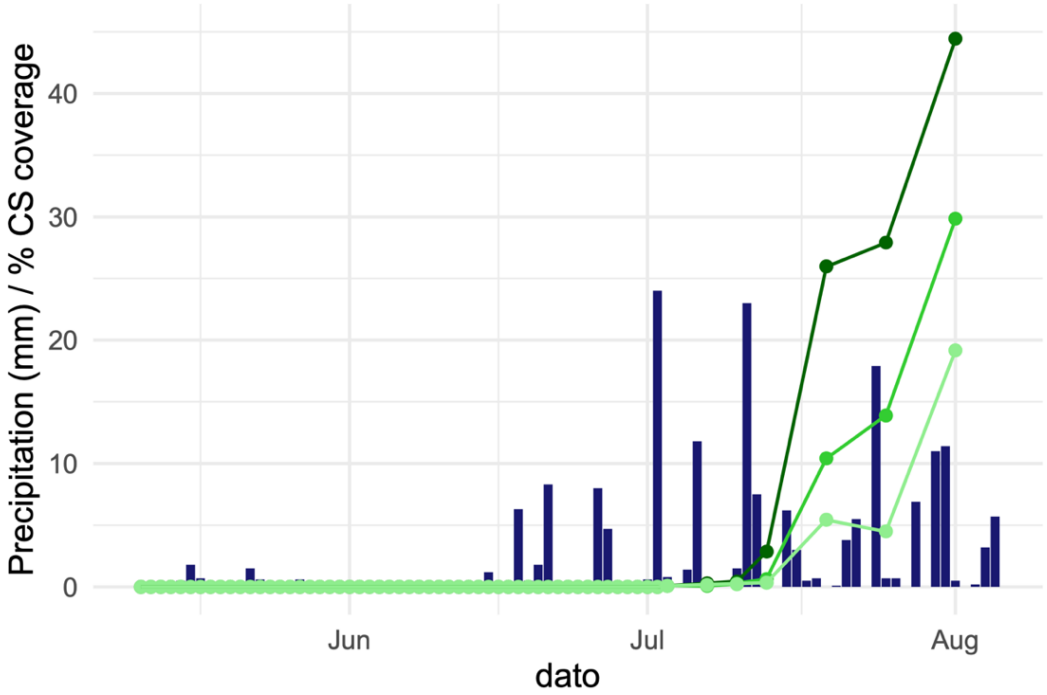

Figure 6 Precipitation and disease development in the untreated early varieties at three levels of the canopy (dark green = lower leaves, green = middle leaves, light green = top leaves) of the untreated plots.

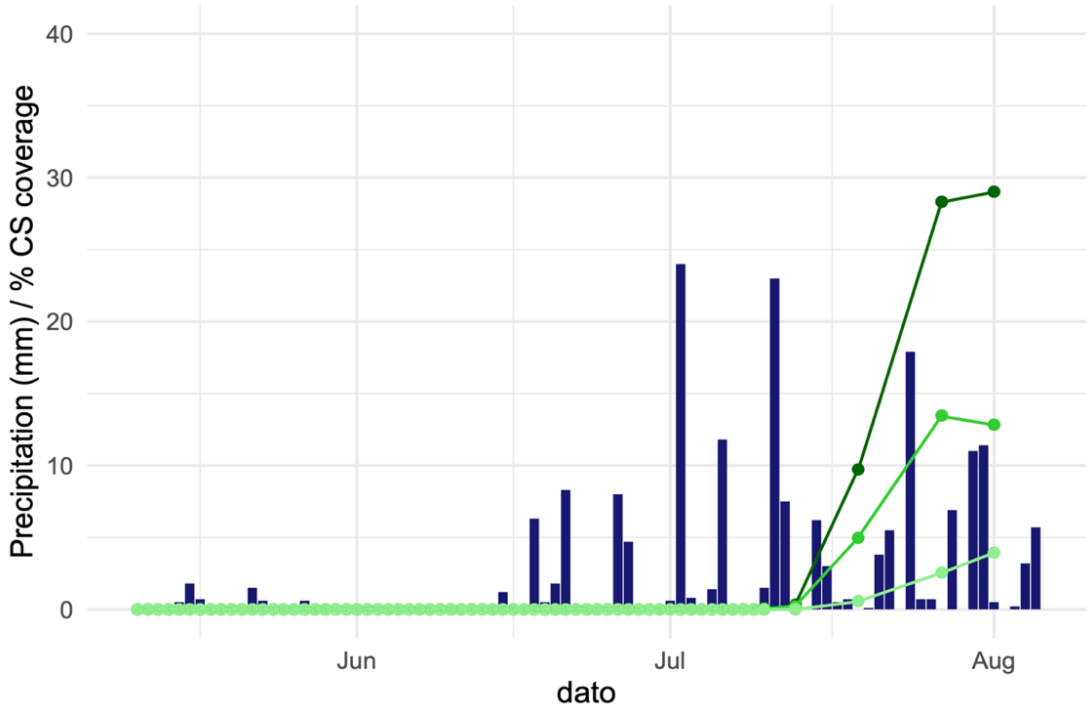

Figure 7 Precipitation and disease development in the untreated late varieties at three levels of the canopy (dark green = lower, green = middle, light green = top) of the untreated plots.

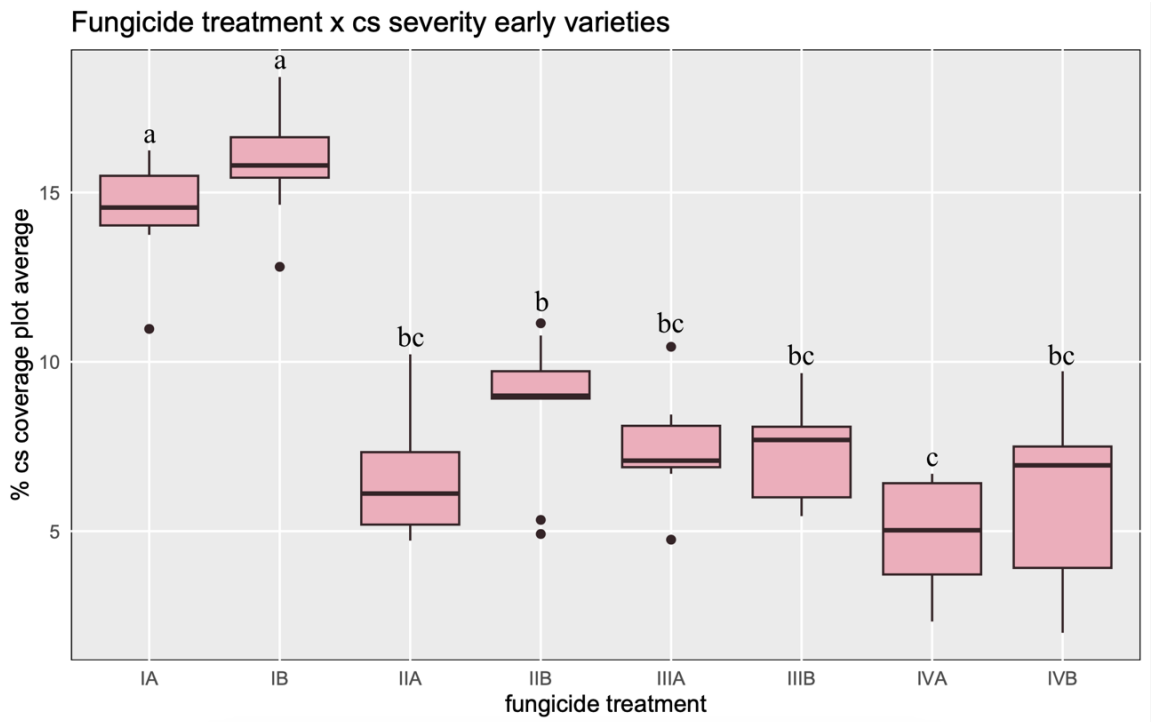

Figure 8 Shows the end-point disease severity of early varieties against treatments with sowing rates grouped together, with letter groupings representing significance.

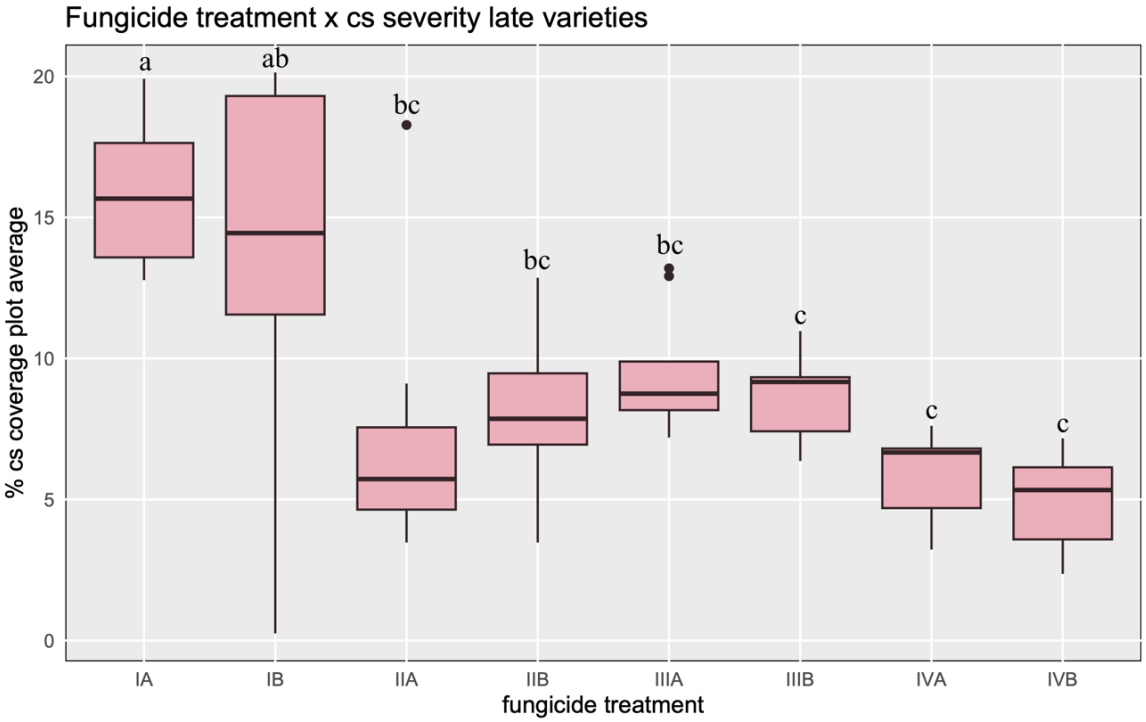

Figure 9 Shows the end-point disease severity of late varieties against treatments with sowing rates grouped together, with letter groupings representing significance.

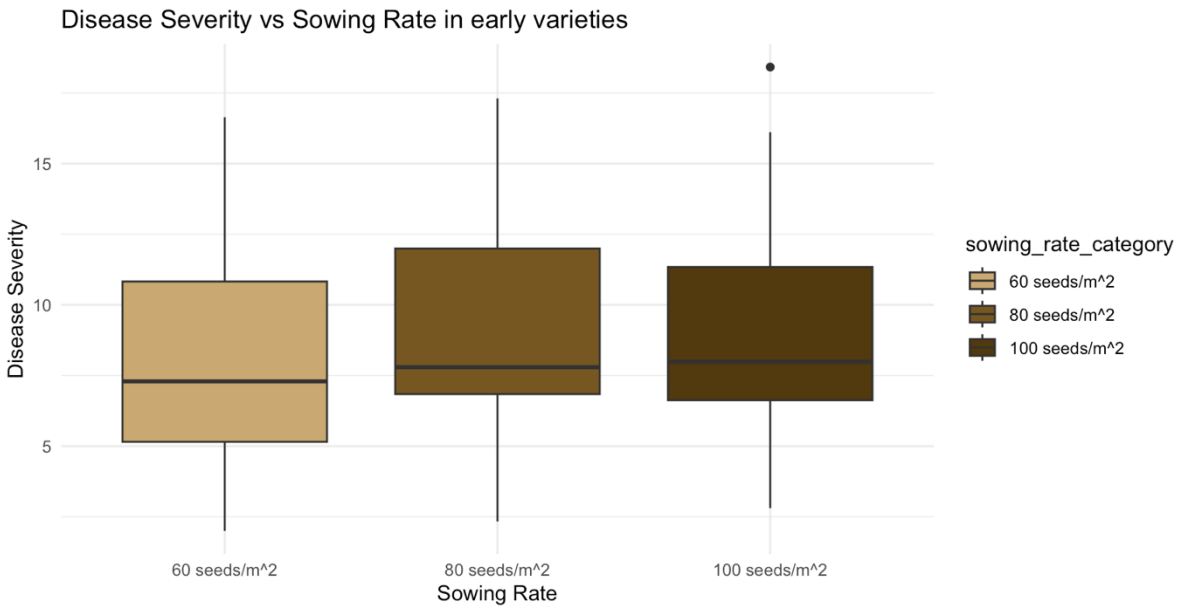

Figure 10 Shows a boxplot of early variety sowing rates against disease severity.

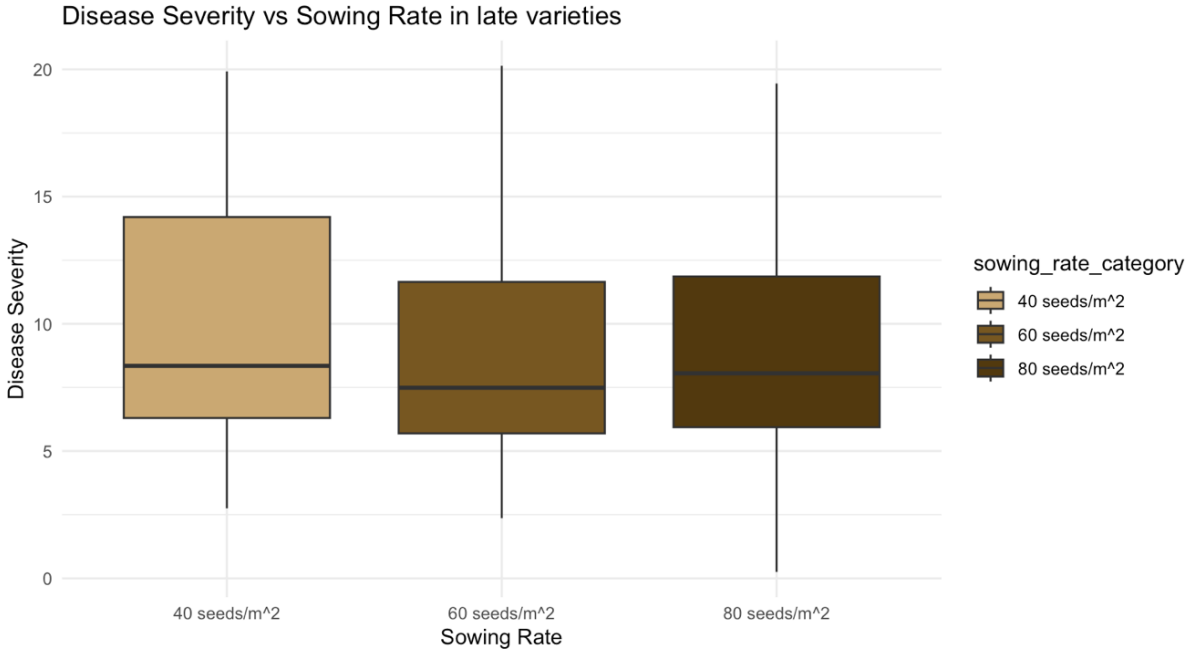

Figure 11 Shows a boxplot of late variety sowing rates against disease severity.

Yields ranged from 131 to 378 kg/daa in early varieties and 185 to 596 kg/daa in late varieties. In the early varieties (Figure 12) when combining all sowing rates within each trio of subplots with the same fungicide treatment and variety, ‘Louhi’ (A) achieved yields significantly higher than that of in Sampo across all treatments (p-value range: 0.047-0.0002). The bivariate correlation analysis in early varieties indicated a negative correlation between yield and chocolate spot severity of $R^2 = 0.036$ (Figure 16) and visualizes the fact that ‘Sampo’ has a lower yield level than ‘Louhi’. In late varieties (Figure 13) the untreated plots and ‘Tiffany’ treated with Signum and Elatus Era at BBCH 60-61 had yields significantly lower than those treated with Signum at the appearance of the first chocolate spot symptoms. Boxplots that include sowing rates as well as fungicide treatment and cultivar can be seen in Appendix 3 and Appendix 4.
Cultivars within the same treatment did not differ significantly from each other. When combining early varieties (Figure 14), there was no significant difference between treatments, but in the combined late varieties (Figure 15) the yield of the plots treated with Signum at the onset of the first symptoms was significantly higher than all other treatments. The bivariate correlation analysis in late varieties indicated a negative correlation between yield and chocolate spot severity of $R^2 = 0.104$, as seen in Figure 17.

ANOVA analysis did not show a significant difference (p: 0.217) between sowing rates and yield in early varieties, but there was a positive correlation of 0.04 in the bivariate correlation analysis Appendix 14. The ANOVA analysis and Tukey’s test showed a significant difference (p: 0.0144) in late variety yield between 40 and 80 seeds/m^2 (Figure 18) and a positive bivariate correlation coefficient of $R^2 = 0.11$ (Appendix 15).

The results from a Welch two sample t-test showed that the mean water content was significantly higher (p: 0.001537) in late variety plots (Figure 19A) treated with Signum compared to the untreated plots and plots applied with Elatus Era. The same test showed no significant difference (p: 0.0654) in early varieties (Figure 19B).

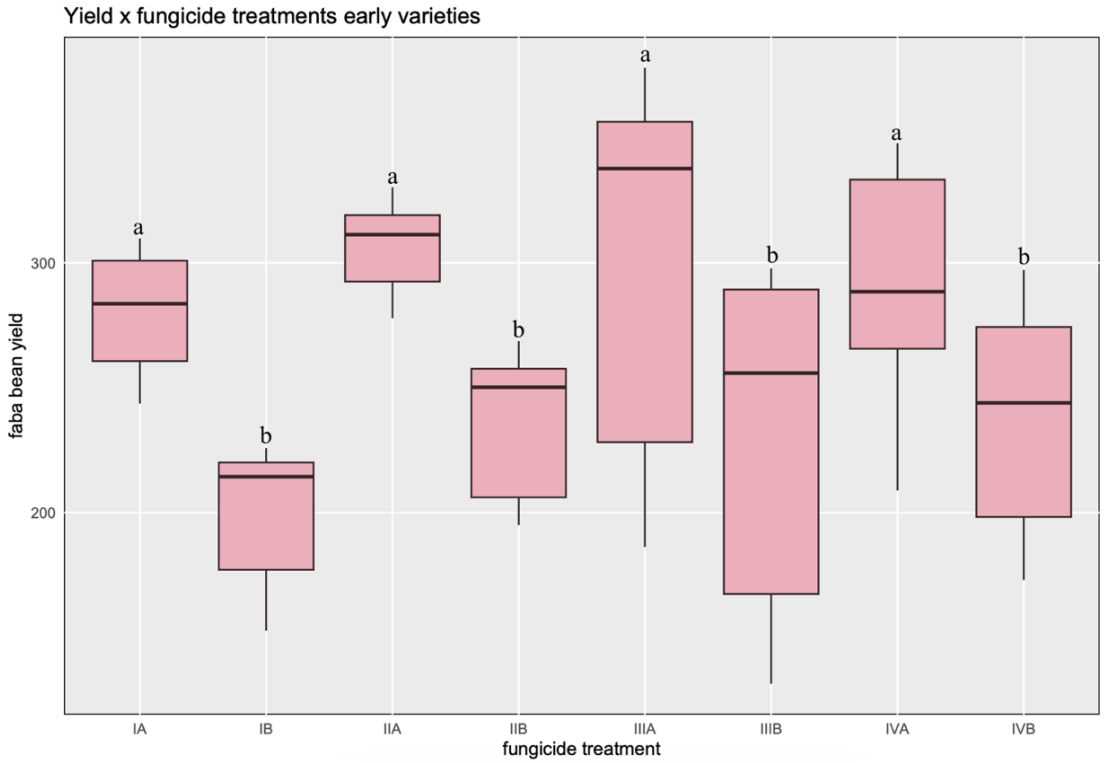

Figure 12 The yield in kg/daa at 15% RWC from the early varieties grouped into simplified treatments where the seed rates have been combined within cultivar and fungicide treatments, with letter groupings representing significance.

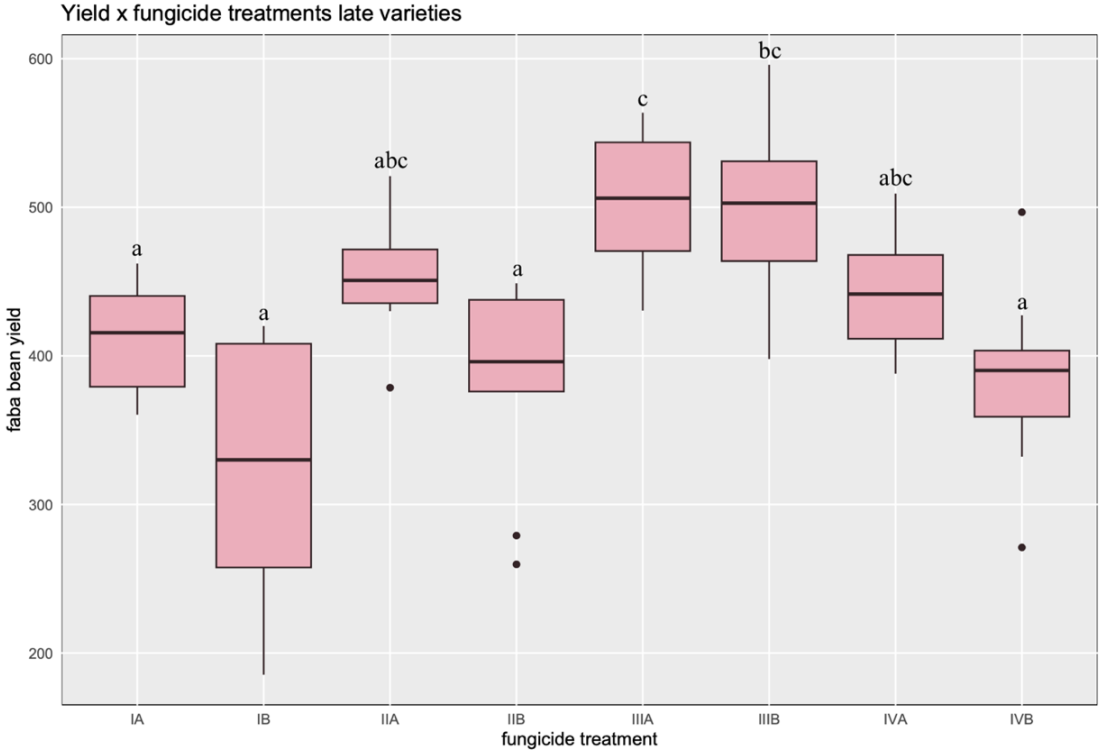

Figure 13 The yield in kg/daa at 15% RWC from the late plots grouped into simplified treatments where the seed rates have been combined within fungicide treatments, with letter groupings representing significance.

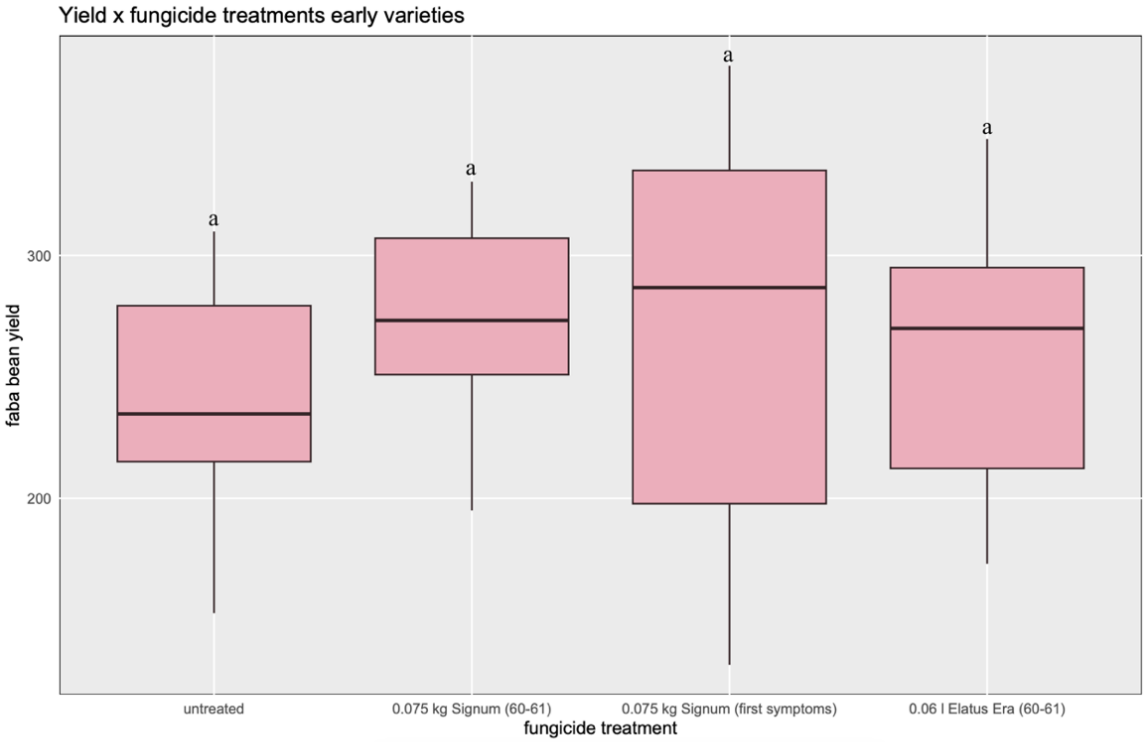

Figure 14 The yield in dry weight grouped into simplified treatments where the two cultivars and seed rates have been combined within fungicide treatments, with letter groupings representing significance.

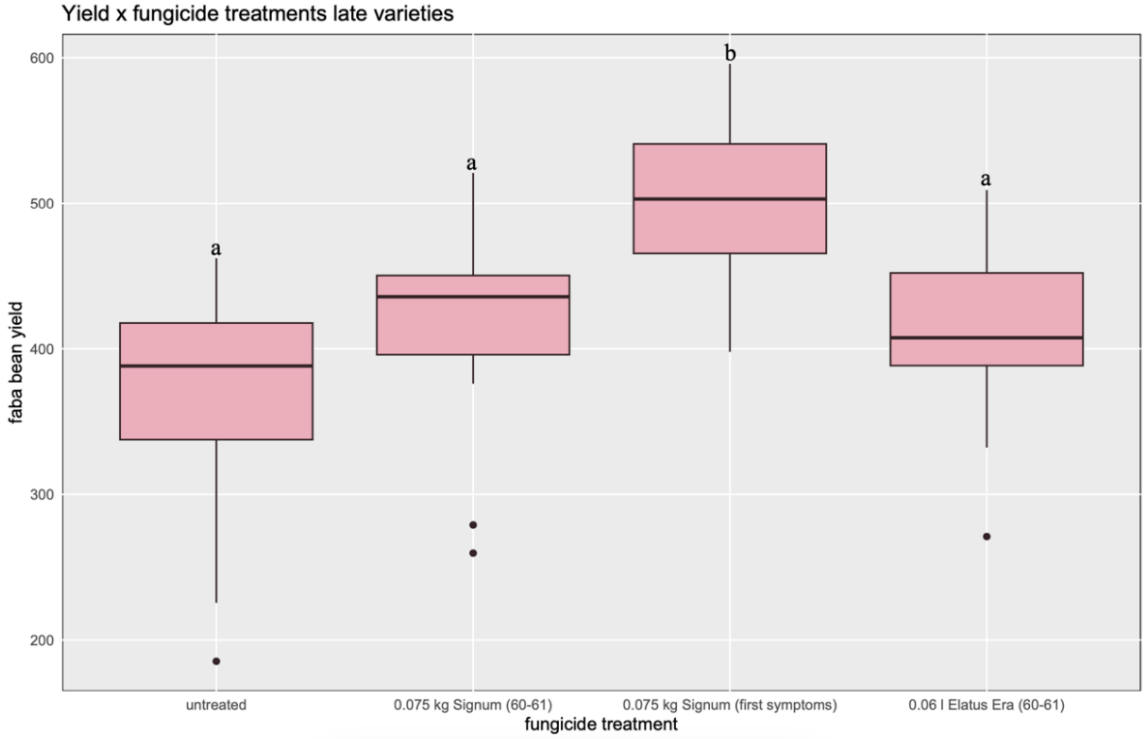

Figure 15 The yield in dry weight grouped into simplified treatments where the two cultivars and seed rates have been combined within fungicide treatments, with letter groupings representing significance.

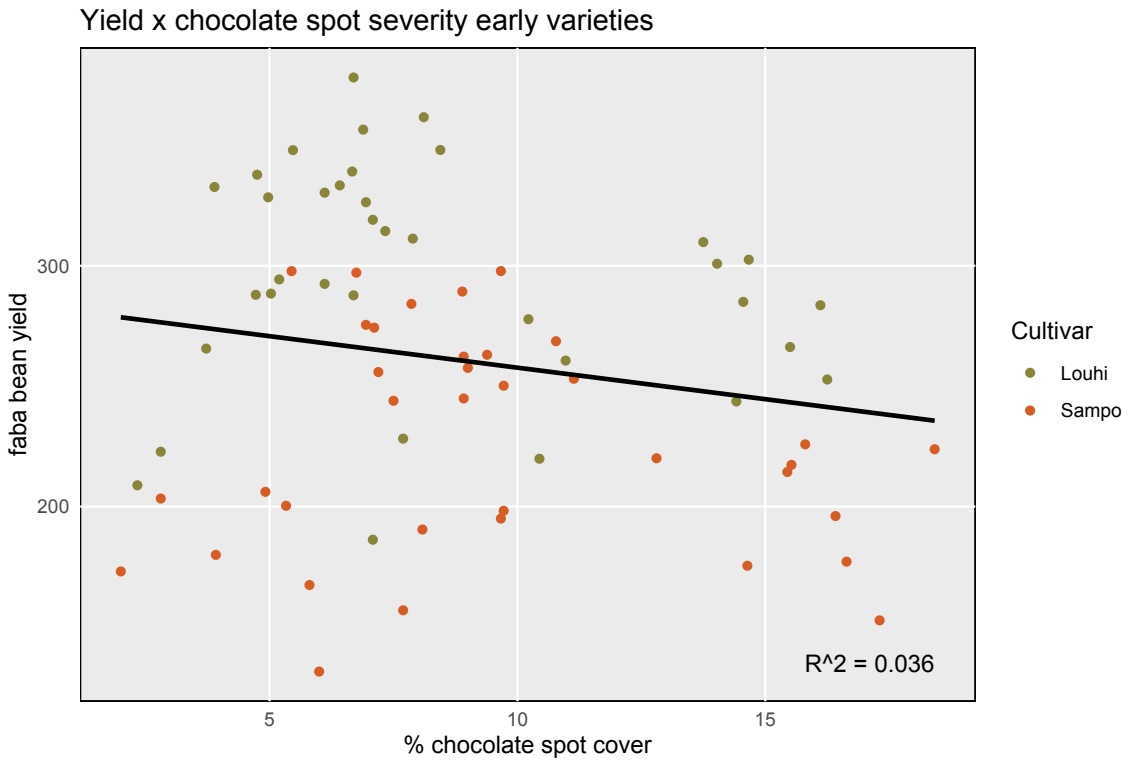

Figure 16 Pairwise correlation plot of the early variety yield against end-point disease severity.

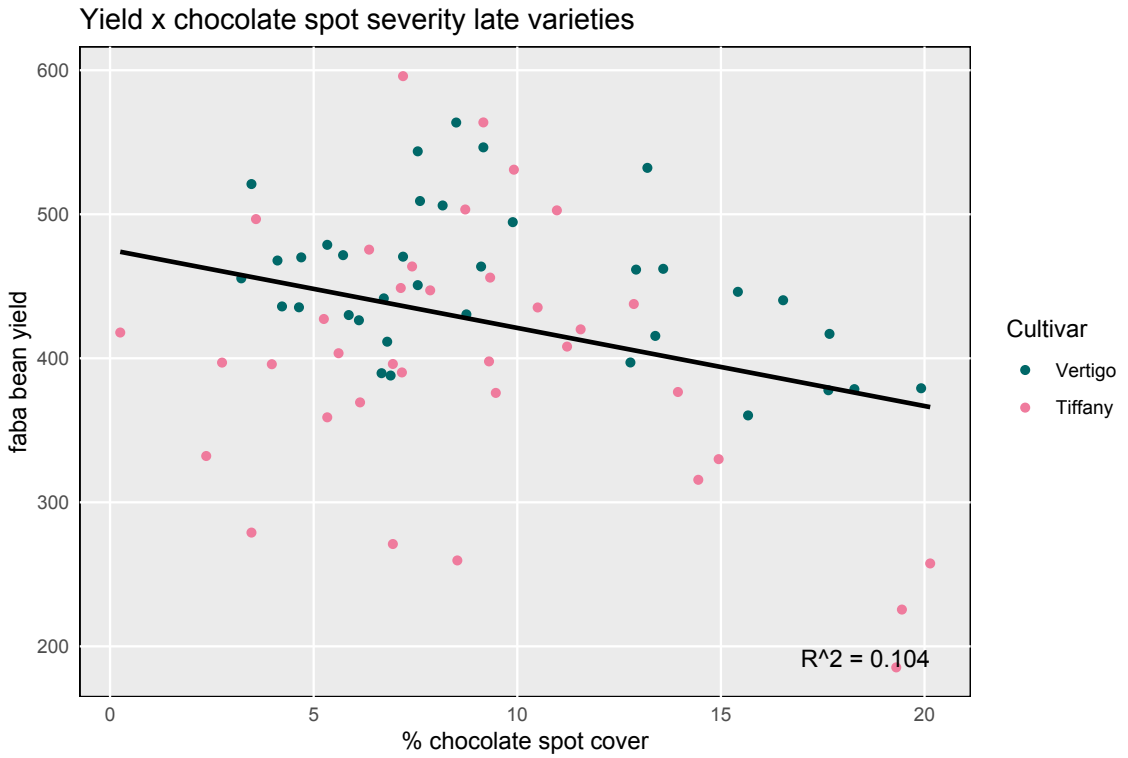

Figure 17 Pairwise correlation plot of the late variety yield against end-point disease severity.

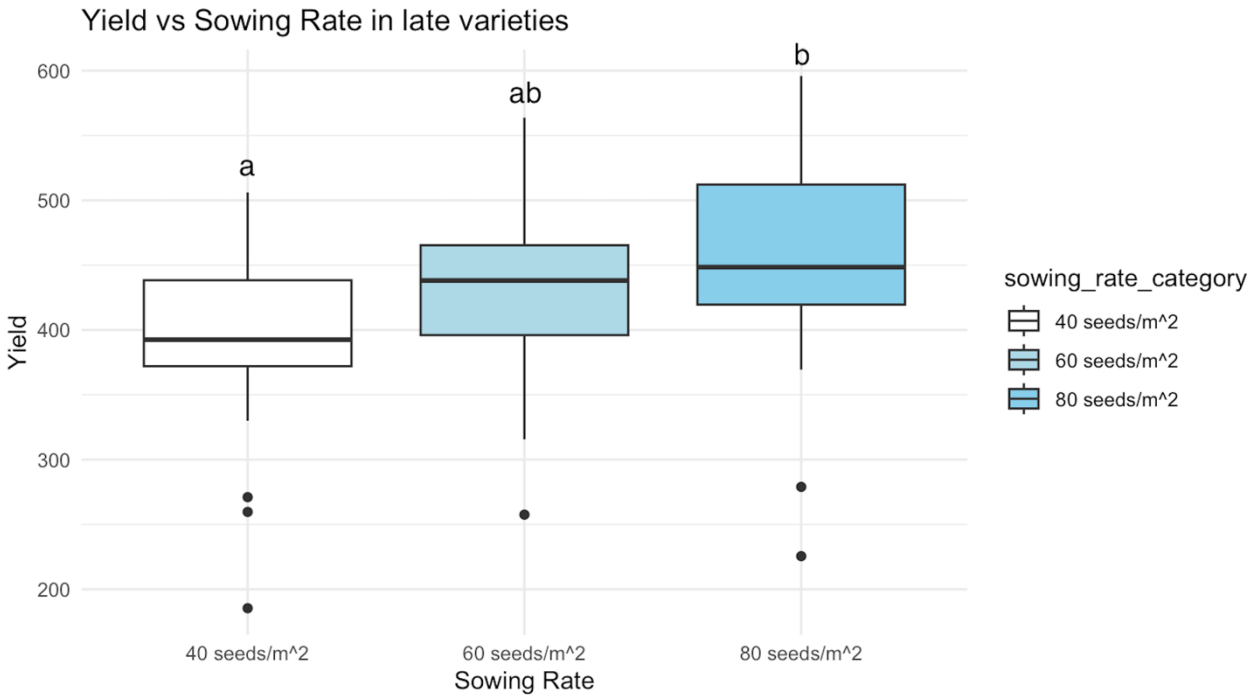

Figure 18 Shows the differences in late variety yield between sowing rates with letter groupings representing significance.

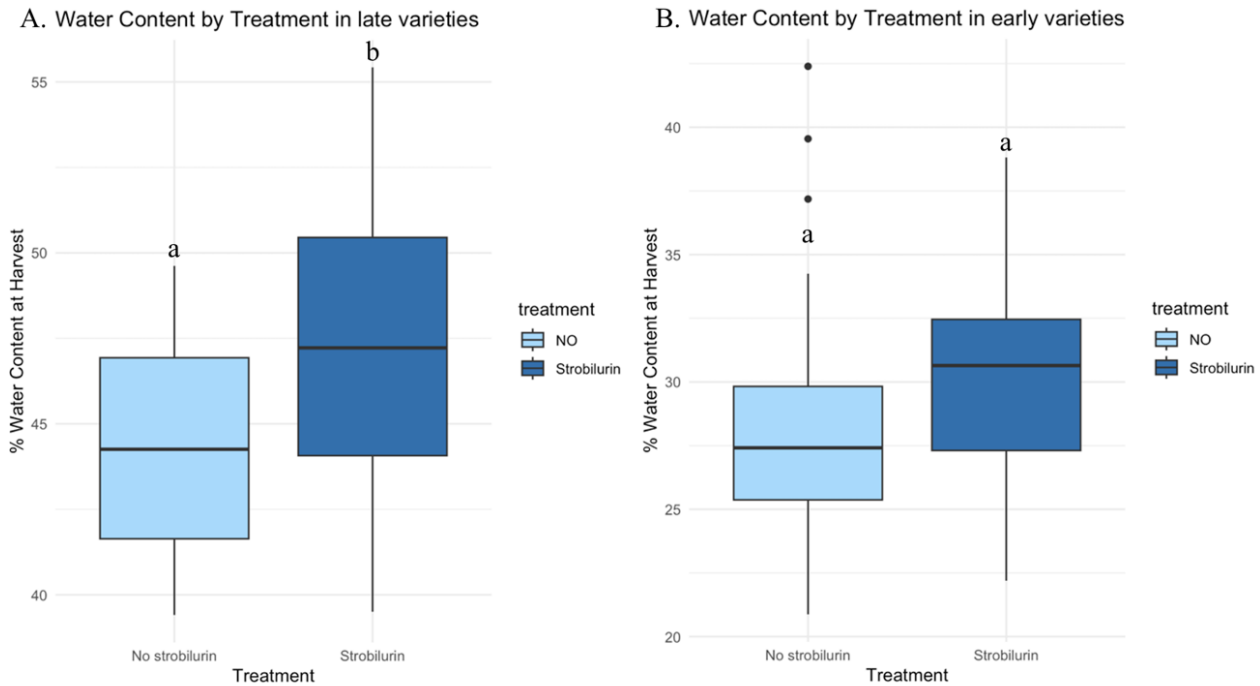

Figure 19 Shows boxplots of RWC at harvest against plots treated with and without strobilurin in late varieties (A.) and early varieties (B.) with letter groupings representing significance.

Laboratory experiments

Sequencing

The best matched species resulting from the blastn search of the NEP2 sequence were found to be *B. fabae*, *B. cinerea* and *B. eucalypti*. The latter is a novel species (Liu et al., 2016) not yet identified in faba beans in Norway and its NEP2 sequence very closely resembles that of several *B. cinerea* strains. Therefore, it is likely that it was misidentified, and the finds of *B. eucalypti* were thus categorized as *B. cinerea* in the analyses.

Morphology

The morphology of the isolates ranged widely in terms of sclerotia size, arrangement and spore density but only sclerotia size was recorded. They were determined to be either small, intermediate or large as shown in Figure 20 and Figure 21. Pearson's Chi-square tests were performed and the relationship between sclerotia size and species was significant (p: 0.000063). The relationship between lesion size and species was not significant (p: 1), and neither was the relationship between sclerotia size and lesion size (p: 0.3536).

Bioscreen

The results of the bioscreen analyses were evaluated by determining the fungicide concentration at which the fungal growth of each isolate stopped. The isolates were separated by *Botrytis* species and plotted on a grid (Figure 22) to visualize how they responded to boscalid and pyraclostrobin. Thresholds representing the middling concentrations tested for each of the AIs were added to the grid to separate the isolates into two degrees of sensitivity. The majority of isolates were sensitive to both compounds, one *B. fabae* isolate indicated weak resistance to boscalid and one to pyraclostrobin, and one *B. cinerea* isolate showed some resistance to pyraclostrobin.

Boscalid

The isolates tested against boscalid were similar in which concentration was needed to stop fungal growth, but there was some variability between isolates. Most growth curves (example given in Figure 23) except for the resistant reference isolate (Figure 24) levelled out at boscalid concentrations of 1 mg/L. Isolate T313 ST B1 stopped growing at the low dose of 0.1 mg/L as shown in Figure 25, T318 SM B2? stopped growing at 0.4 mg/L, and isolate S118 ST B1 only stalled at 11 mg/L (Figure 26).

Pyraclostrobin

The sensitive control isolate showed fungal growth up until pyraclostrobin concentrations of 2.75 mg/L. The resistant isolate continuous growth at all concentrations. Most isolates from the field trial stopped growing at AI concentrations of between 0.1 and 0.25 mg/L.

*Table 1 Shows all isolate names, their accompanying treatments and codes, the cultivars they are sampled from, cultivar earliness, sequenced species, sclerotia size and the boscalid and pyraclostrobin concentrations required to stop the fungal growth. *isolate not included in the pyraclostrobin analyses.*

Isolate name	Treatment code	Fungicide treatment	Cultivar	Earliness	Species	Boscalid concentration to stop growth [mg/L]	Pyraclostrobin concentration to stop growth [mg/L]	Sclerotia size
VBS 326 SMÅ B1	IA	Untreated	Vertigo	Late	*Botrytis fabae*			small
VBT 102 SMÅ B1	IA	Untreated	Louhi	Early	*Botrytis fabae*	1 (normal)	0.25	small
VBT 218 STORE B1	IA	Untreated	Louhi	Early	*Botrytis fabae*			small
VBT 326 SMÅ B1	IA	Untreated	Louhi	Early	*Botrytis fabae*			small
VBT 329 SMÅ B1-B	IB	Untreated	Sampo	Early	*Botrytis cinerea*	1 (normal)	2.75	large
VBS 105 SMÅ B1	IB	Untreated	Tiffany	Late	*Botrytis fabae*			small
VBS 329 SMÅ B1	IB	Untreated	Tiffany	Late	*Botrytis fabae*			small
VBT 105 SMÅ B1	IB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis fabae*	1 (normal)	0.25	large
VBT 105 SMÅ B2	IB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small
VBT 329 SMÅ B1-A	IB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small
VBT 226 SMÅ B1	IIA	Signum (BBCH 60-61)	Louhi	Early	*Botrytis eucalypti (99%)*			large
VBS 110 SMÅ B1-A	IIA	Signum (BBCH 60-61)	Vertigo	Late	*Botrytis fabae*	1 (normal)	0.25	intermediate
VBS 110 SMÅ B1-B	IIA	Signum (BBCH 60-61)	Vertigo	Late	*Botrytis fabae*			intermediate
VBS 302 SMÅ B1	IIA	Signum (first symptoms)	Vertigo	Late	*Botrytis fabae*			small
VBT 110 SMÅ B1	IIA	Signum (first symptoms)	Louhi	Early	*Botrytis fabae*	1 (normal)	0.1	small
VBT 302 SMÅ B2	IIA	Signum (first symptoms)	Louhi	Early	*Botrytis fabae*			small
VBT 113 SMÅ B1	IIB	Signum (first symptoms)	Sampo	Early	*Botrytis eucalypti*			intermediate
VBS 113 SMÅ B1	IIB	Signum (first symptoms)	Tiffany	Late	*Botrytis fabae*			small
VBS 113 SMÅ B2	IIB	Elatus Era (BBCH 60-61)	Tiffany	Late	*Botrytis fabae*	>1 (normal)	0.1	small
VBS 229 SMÅ B1	IIB	Elatus Era (BBCH 60-61)	Tiffany	Late	*Botrytis fabae*			small
VBT 113 SMÅ B2	IIB	Elatus Era (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small
VBT 113 STORE B1?L	IIB	Elatus Era (BBCH 60-61)	Sampo	Early	*Botrytis fabae*	1 (normal)	8.25	large
VBT 118 SMÅ B1	IIIA	Elatus Era (BBCH 60-61)	Louhi	Early	*Botrytis cinerea*			small
VBS 118 SMÅ B1	IIIA	Untreated	Vertigo	Late	*Botrytis fabae*			small
VBS 118 STORE B1	IIIA	Untreated	Vertigo	Late	*Botrytis fabae*	11 (semi-resistant)	0.25	intermediate
VBS 202 SMÅ B1	IIIA	Untreated	Vertigo	Late	*Botrytis fabae*			small
VBT 310 SMÅ B1	IIIA	Signum (BBCH 60-61)	Louhi	Early	*Botrytis fabae*			small
VBT 313 STORE B1	IIIB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis cinerea*	0.1 (sensitive)	0.1	large
VBS 313 SMÅ B1	IIIB	Signum (BBCH 60-61)	Tiffany	Late	*Botrytis fabae*	0.4 (semi-sensitive)	0.1	small
VBT 121 SMÅ B2	IIIB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small
VBT 313 SMÅ B1	IIIB	Signum (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small
VBT 126 SMÅ B1	IVA	Signum (BBCH 60-61)	Louhi	Early	*Botrytis cinerea*	>1 (normal)	*	large
VBT 318 SMÅ B2?	IVA	Signum (first symptoms)	Louhi	Early	*Botrytis cinerea*	0.4 (sensitive)	0.1	large
VBS 126 SMÅ B1	IVA	Signum (first symptoms)	Vertigo	Late	*Botrytis fabae*			small
VBS 210 SMÅ B1	IVA	Signum (first symptoms)	Vertigo	Late	*Botrytis fabae*			small

VBS 318 SMÅ B1	IVA	Signum (first symptoms)	Vertigo	Late	*Botrytis fabae*			small
VBS 318 SMÅ B2	IVA	Elatus Era (BBCH 60-61)	Vertigo	Late	*Botrytis fabae*	1 (normal)	0.25	small
VBT 318 STORE B1?	IVA	Elatus Era (BBCH 60-61)	Louhi	Early	*Botrytis fabae*			small
VBS 129 SMÅ B1	IVB	Elatus Era (BBCH 60-61)	Tiffany	Late	*Botrytis fabae*	<1 (normal)	0.25	small
VBS 129 STORE B1	IVB	Elatus Era (BBCH 60-61)	Tiffany	Late	*Botrytis fabae*			small
VBT 129 SMÅ B1	IVB	Elatus Era (BBCH 60-61)	Sampo	Early	*Botrytis fabae*	1 (normal)	0.25	small
VBT 321 SMÅ B1	IVB	Elatus Era (BBCH 60-61)	Sampo	Early	*Botrytis fabae*			small

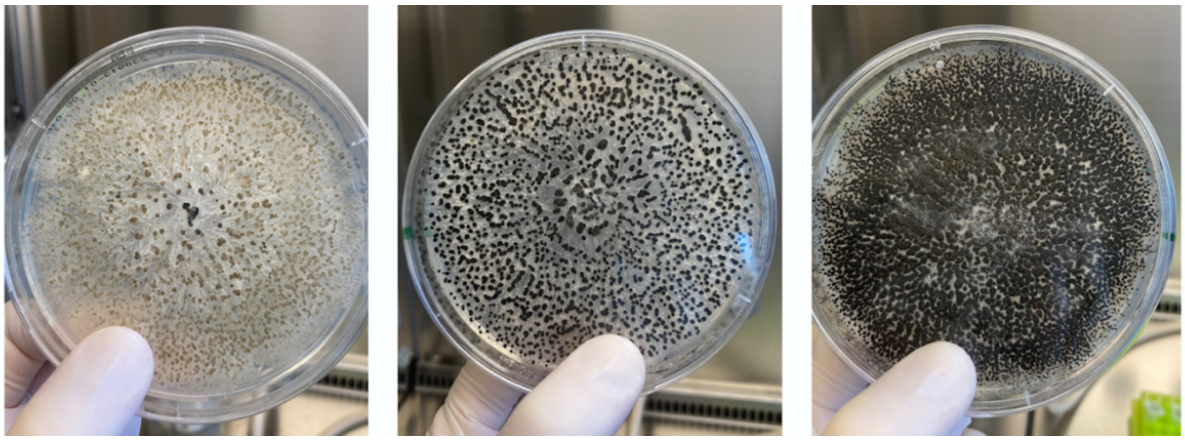

Figure 20 Morphology of isolates in which sclerotia were categorized as small. (All isolates pictured were identified as B. fabae).

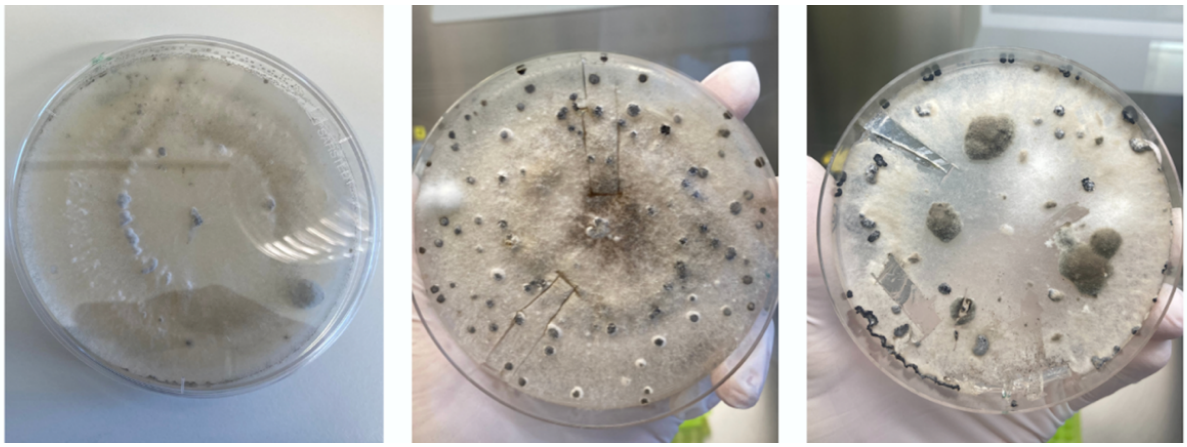

Figure 21 Morphology where the sclerotia were categorized as large. (All isolates pictured were identified as B. cinerea.)

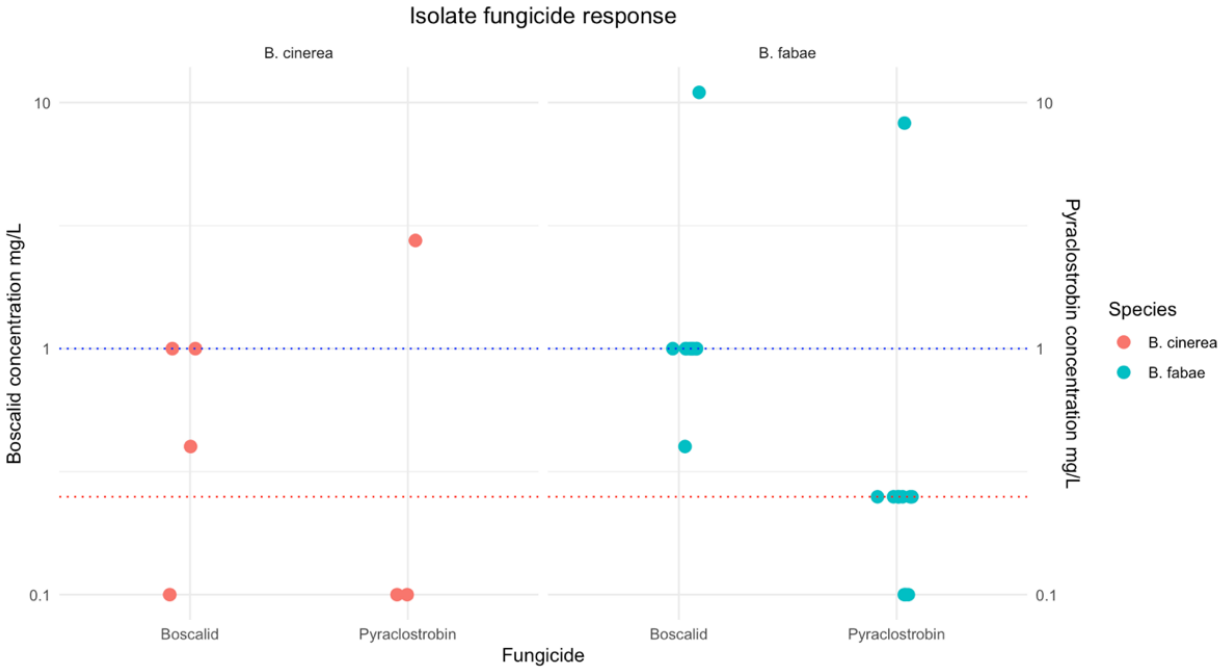

Figure 22 Shows a scatter plot of all isolates and their respective response to the two fungicide active ingredients boscalid and pyraclostrobin. The blue dotted line represents the middling boscalid concentration, the red dotted line the middling pyraclostrobin concentration.

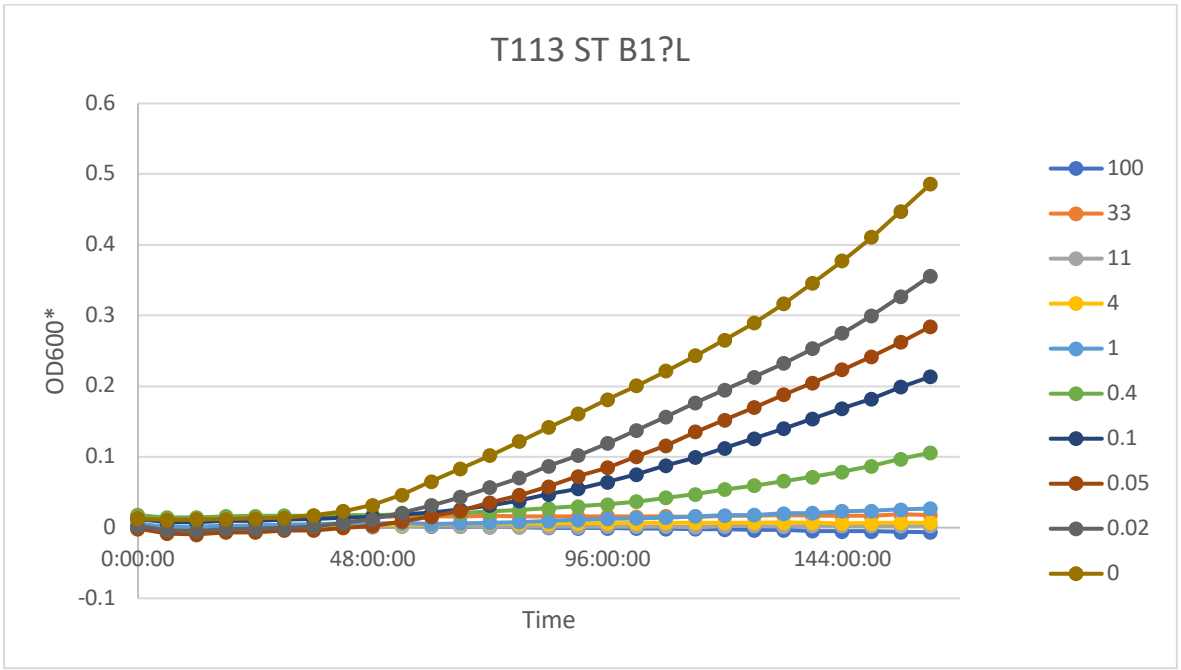

Figure 23 Example of the typical isolate growth of the samples. The growth curve in most isolates, at both wavelengths, started flattening out at a boscalid concentration of around 1 mg/L, as shown here.

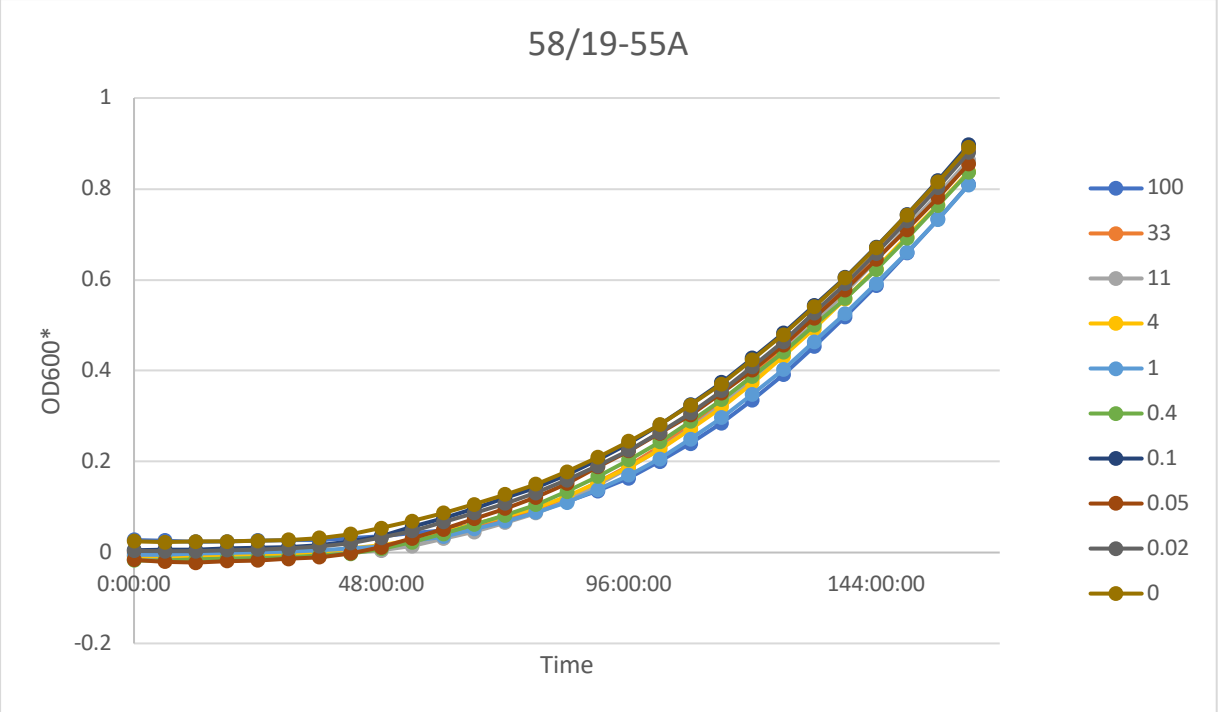

Figure 24 Example of the medium resistant reference isolate. Fungal growth was not hindered by the presence of Boscalid at any concentration, as predicted.

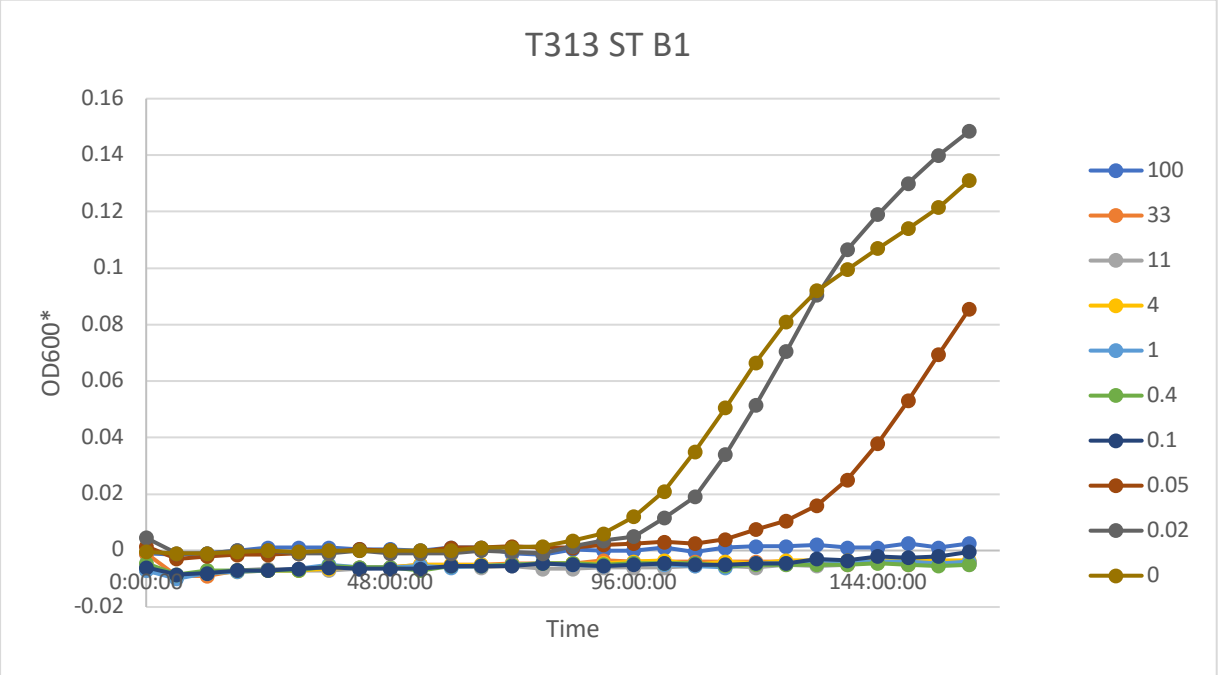

Figure 25 Shows the growth curves of isolate T313 ST B1 and is an example of one of the more sensitive isolates, as its growth was entirely inhibited by a boscalid dose of 0.1 mg/L.

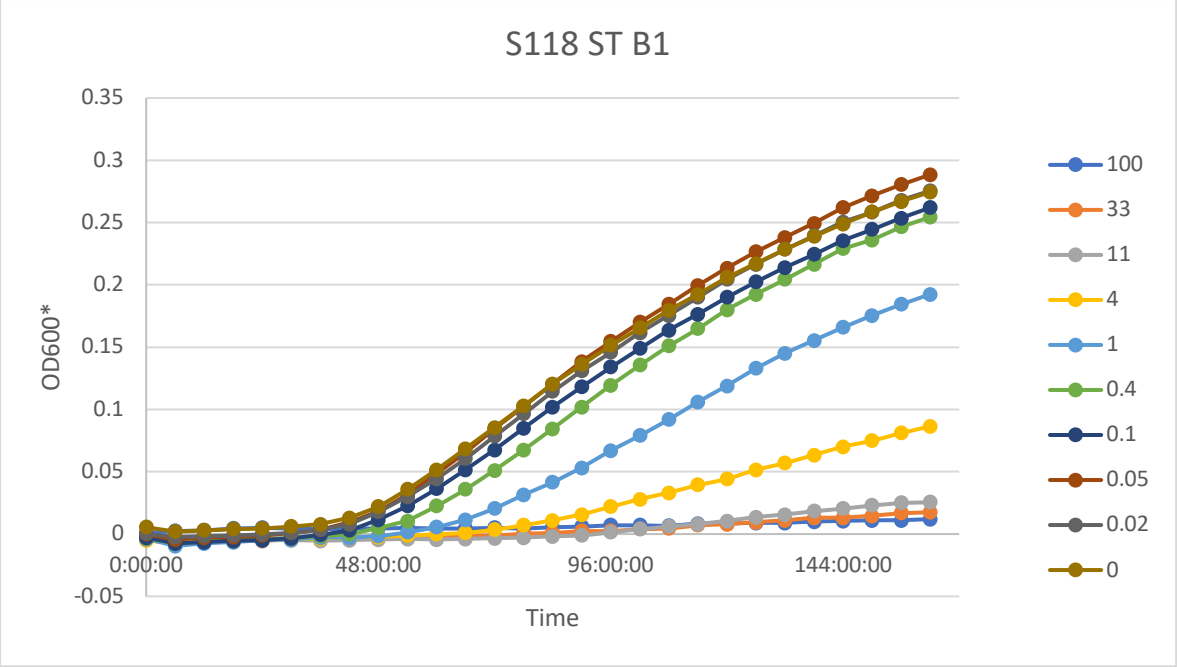

Figure 26 Shows the growth curve of S118 ST B1, one of the more resistant isolates sampled. Its growth was only inhibited at a boscalid concentration of 11 mg/L.

Discussion

Because of the high temperatures in mid to late June and the low precipitation level throughout the spring until the start of July, the BBCH development of the plants in both fields had a rapid progression.

The temperature was at its highest point of the season in the 8 days between and including June 12th and 19th as seen in Figure 2, at a mean of 19.9ºC (BIOKLIM, 2023). Due to the resulting rapid accumulation of growing degree days (GDD) this week, the development of the whole crop was accelerated, which likely is the reason behind the jump in BBCH stages between these dates (Appendix 5 and Appendix 6). The dry conditions in this same time period might have compelled the plants to mature even quicker than they usually would have done as well, despite the field being thoroughly irrigated at three points during the weeks of no rainfall. Further on in the season, there was a considerably higher amount of rainfall which accelerated the spread of the chocolate spot. Severity was highest in the lower plant parts in all plots due to the higher canopy density and moisture levels, as seen in Figure 6 and Figure 7.

The flowering stage (BBCH 60) was reached between the middle of June and middle of July across all cultivars and lasted until mid-July to start of August, and the onset of disease in the untreated plots only started at the end of July after a sufficient period of precipitation. This minimal cross-over between flowering and spore dissemination may have been the reason for the minimal to non-existent infection of flowers. As flower infection is more often the cause of the most destructive yield losses following chocolate spot epidemics (Aamot et al., 2023), the absence of it this season may have been one of the main reasons for the acceptable yield levels achieved.

Treatment had a larger impact on disease severity than cultivar (Figure 8 and Figure 9), which reflects the lack of available cultivars with any meaningful resistance to chocolate spot.

Since pea weevils infested the entire field at the same time points by grazing on leaf margins, no analyses were done to determine whether damaged plants were more susceptible to infection of *Botrytis* and the development of chocolate spot disease after weevil attacks. They will usually cause most harm by feeding on the nitrogen fixing nodules in legumes (Hermansen & Aamlid, 2019), something that was not recorded in this experiment.

Yield varied between treatments, and the results showed that untreated plots had the lowest yields out of all treatments in both early and late varieties. The pathogens' ability to limit photosynthesis in unsprayed plots significantly impacted the plants' capacity to produce photosynthates and further transport them to the pods for seed filling.

The yield of the late varieties treated with Elatus Era was significantly higher than other treatments, which may indicate some evolution of resistance to Signum in the inoculum in the area.

The yield of the late varieties seem to be more negatively correlated with disease severity (Figure 17), whereas the yield of the early varieties seems to have a weaker negative correlation with disease. (Figure 16) The fact that early varieties were more developed than the late varieties at the time of the June heat, may be explained by the fact that early varieties

have a lower GDD requirement than late varieties and will yield lower in seasons with excessive heat. This could have led to accelerated pod setting early on, which in turn may be the reason why yields did not correlate so heavily to the outbreak of chocolate spot, which came later.

Strobilurin compounds have been known to have a greening effect in faba bean crops (Øverland & Grieu, 2024), which can delay its maturation. The results seen in Figure 19 indicate that the late variety plots treated with fungicide containing strobilurin had a significantly higher compared to those not treated with strobilurin, at least in the varieties tested in this experiment. It suggests that the greening effect caused by strobilurins played a role in the late varieties and resulted in delayed plant senescence and the consequent late maturation of seeds. Early varieties did not have this significance, which might have been due to the confounding factor of higher coverage of chocolate spot which is known to hasten the maturation of the crop prematurely. After the faba beans are at a RWC of around 50% the dry matter has reached its potential (Stensrud, 2021), so no noteworthy yield loss was attributed to this effect in this experiment.

In the field trial carried out, only two time points of fungicide application were included, which might not be sufficient for ascertaining optimal timing. This being said, the plots treated after visible symptoms appeared had higher yields than the ones sprayed at the beginning of anthesis, at least in ‘Louhi’, ‘Tiffany’ and ‘Vertigo’ (Figure 12 and Figure 13), which could be a useful finding. Further investigation into additional times of application timing would need to be done to establish optimal control of chocolate spot. In addition, different fungicide dosages would be illuminating to implement in studies alongside timing to discover which combination is ideal.
Higher sowing rates have previously been shown to increase yield (Grieu et al., 2021), although a denser canopy with an accompanying microclimate with higher humidity might result in more rapid spreading of causal pathogens of chocolate spot (Stoddard et al., 2010). However, no significant effects of plant density on chocolate spot were seen in this experiment and further studies could help illuminate this effect.

There was no correlation between how developmentally advanced the plants were and the severity of their attack, only a slight positive correlation between BBCH and percentage of diseased tissue in the early varieties as seen in Appendix 8, and in the late varieties (Appendix 9).

Earlier faba bean varieties tend to produce smaller seeds than late varieties. The 1000 kernel weight from early and late plots were negatively and positively correlated with disease severity (Appendix 10 and Appendix 11) respectively, although this was likely only due to the fact that the two early cultivars varied more in size and the plants with larger seeds had lower levels of chocolate spot. The late varieties produced seeds more homogenous to each other in size and approached no correlation at all with disease severity. Thus, probably no noteworthy causal relationship exists between harvested seed weight and disease pressure.

Results from the fungicide screenings indicated little tendency toward development of resistance in both *B. cinerea* and *B. fabae* isolates, as seen in Figure 22, and some were even more sensitive than the sensitive control isolate. All isolates exhibiting growth at heightened AI concentrations were sampled from untreated plots (

Table 1). Since our sampling was not comprehensive, these outcomes may have been coincidental.

Although *B. fabae* has been shown to be more aggressive than *B. cinerea* (Mansfield & Deverall, 1974), our sampling method did not reflect any significant relationship. If it had however, we would still not be able to conclude that the species had anything to do with disease severity because our sparse sampling method could hide unseen diversity within each plot that wasn't reflected in the few selected isolates per treatment. A much higher number of isolates would need to be extracted for anything to be determined about the actual effects of the different causal organisms on disease severity.

Conclusions

I will now conclude by addressing the objectives outlined in the introductory chapter and highlighting the findings most relevant to each of them.
The results I. did not indicate any significant relationship between canopy density and disease severity. II. The time of Signum application affected both disease levels and yields in a positive direction, leading to a lower severity of chocolate spot and significantly higher yield in late varieties. In terms of yield, Elatus Era performed similarly to Signum applied at the start of flowering. Chocolate spot severity was at its lowest when treated with Elatus Era, and a little higher when Signum was applied after symptoms arrived. III. Of the 42 single spore isolates sequenced from leaf samples in the field, 35 (83%) of them were identified as *Botrytis fabae* and 7 (17%) were *Botrytis cinerea* (including 2 incidences of likely misidentified *Botrytis eucalypti* that were counted and analysed as *B. cinerea*). IV. No significant association between pathogen species and disease severity was found in the analyses, neither was there any significant relationship between lesion size and species.

The findings in this thesis will be useful in the further development of management strategies in faba bean and can be built upon with the goal of achieving successful high protein harvests with reduced costs for workers and the environment. The accomplishment of such goals related to self-sufficiency, environmental and population health is incumbent upon studies like these as necessary tools for furthering the much-needed knowledge of plant-pathogen interactions.

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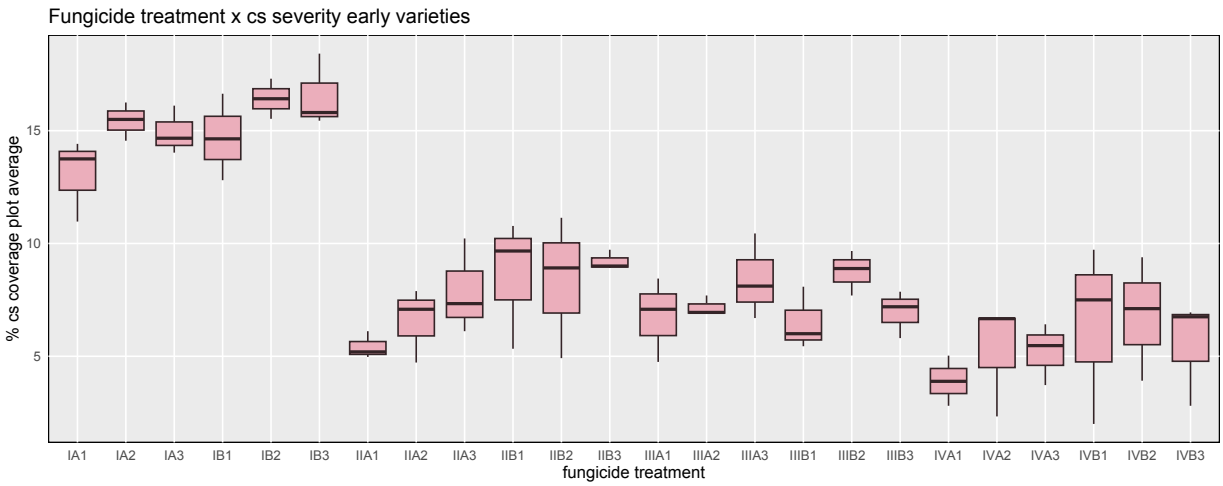
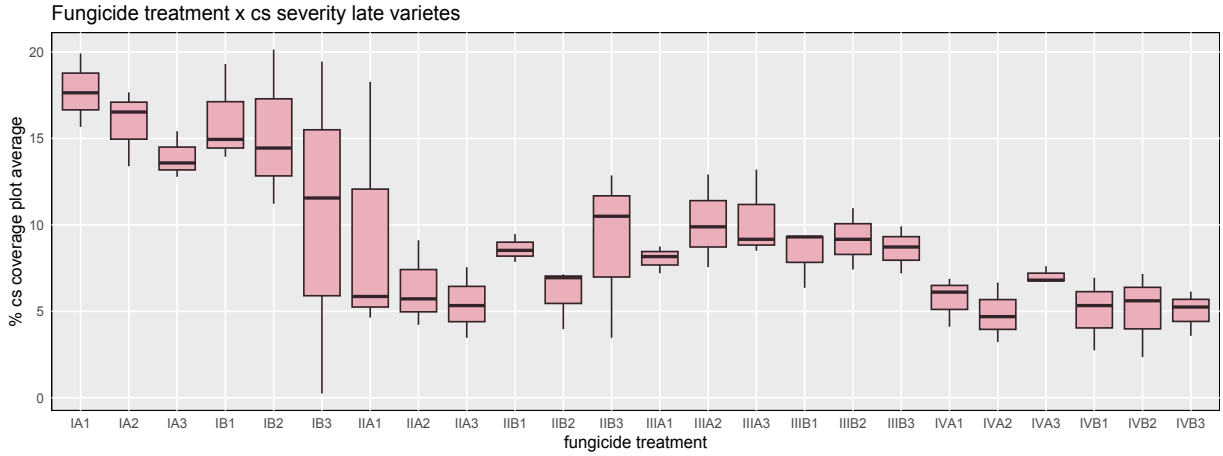
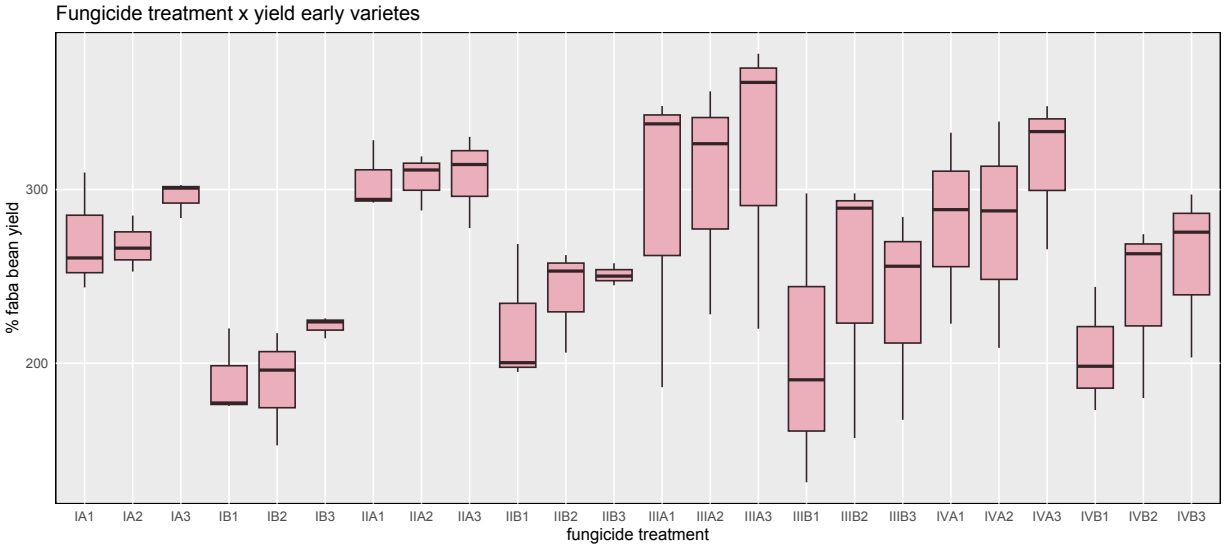
Appendix



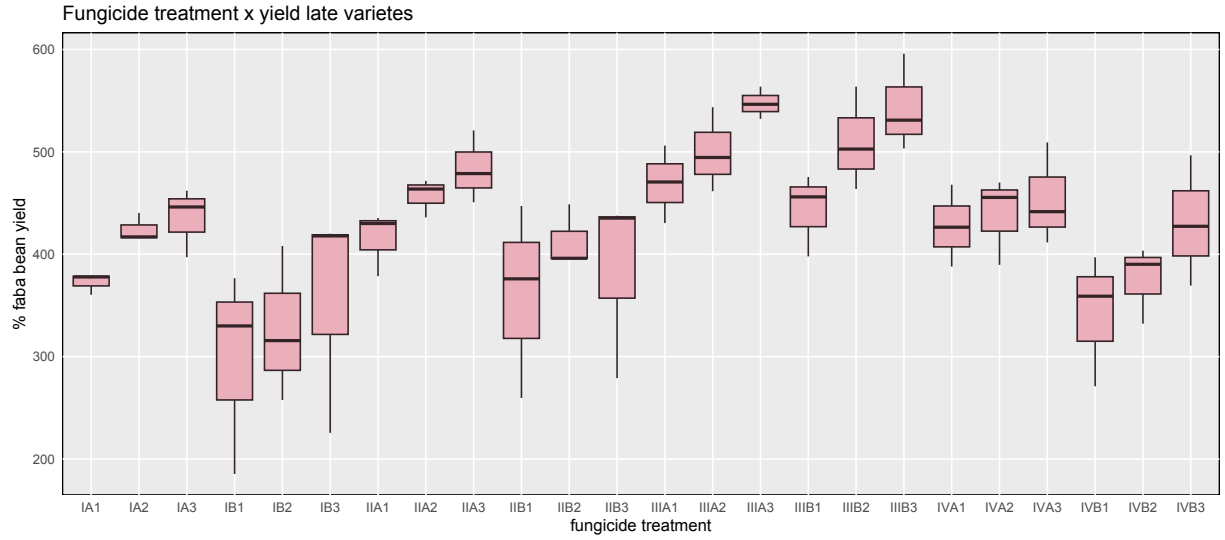
Appendix 1 Visualization of the chocolate spot coverage in early variety plots with all sowing rate and fungicide treatments at the last disease scoring date. registration. Means within replicates are represented here.



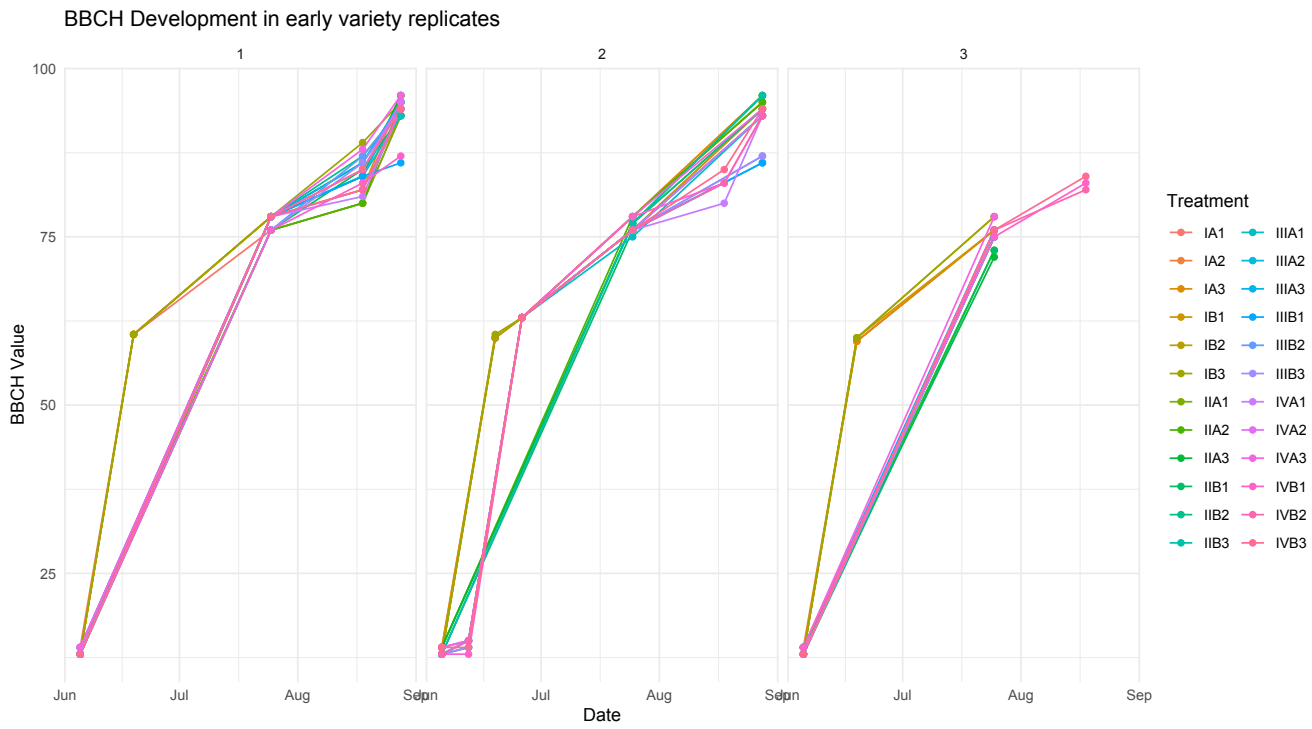
Appendix 2 Visualization of the chocolate spot coverage in late variety plots with all sowing rate and fungicide treatments at the last disease scoring date. Means within replicates are represented here.



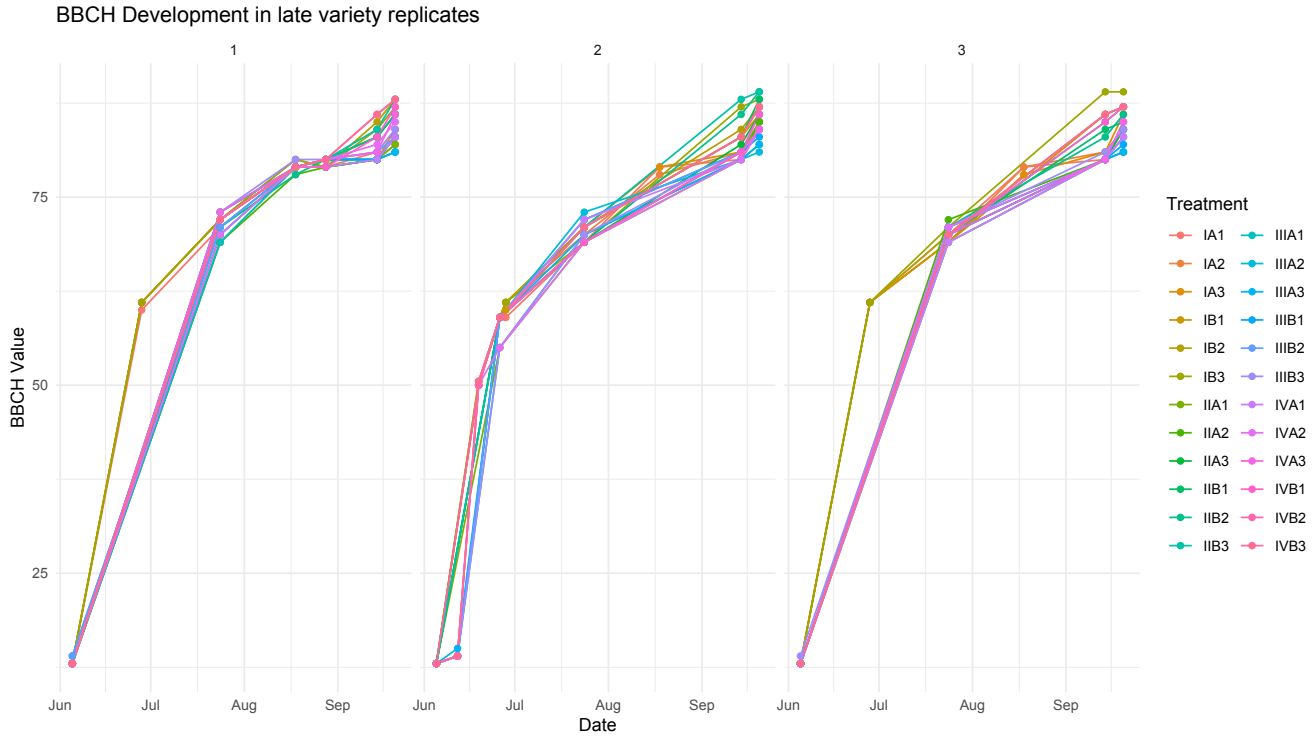
Appendix 3 Visualization of the faba bean yield of early varieties across all treatments. Means within replicates are represented here.



Appendix 4 Visualization of the faba bean yield of late varieties across all treatments. Means within replicates are represented here.

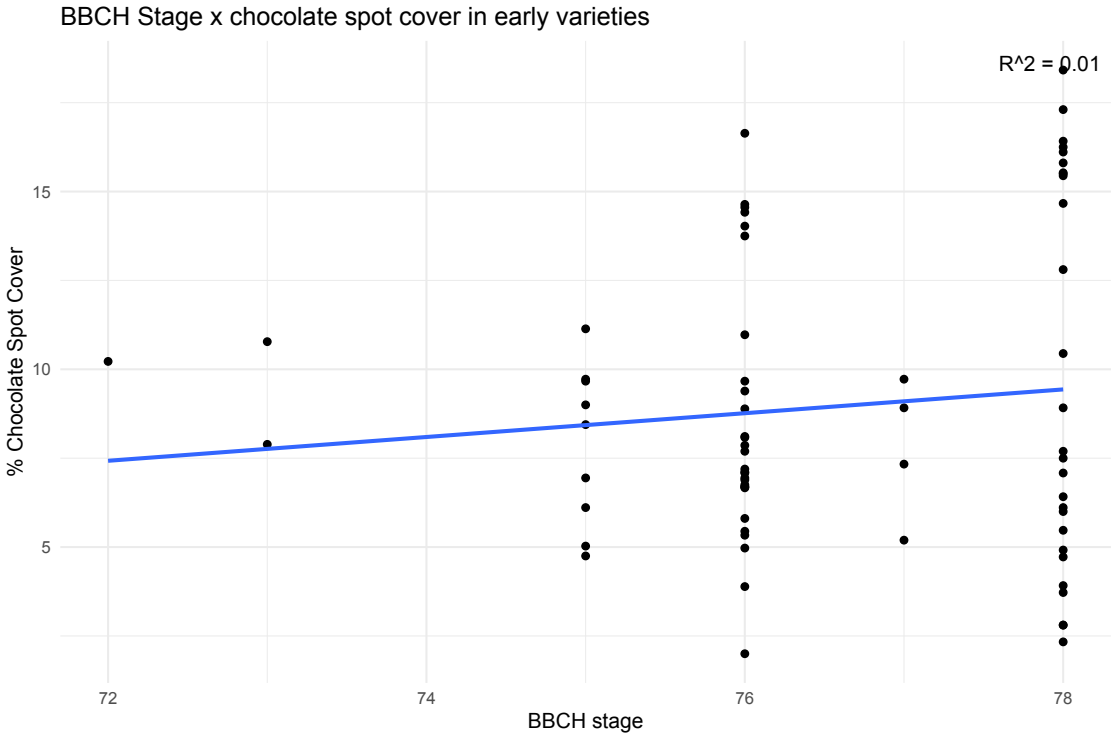


Appendix 5 Shows the three replicates and the developmental stages of the treatment plots along time in early cultivars.

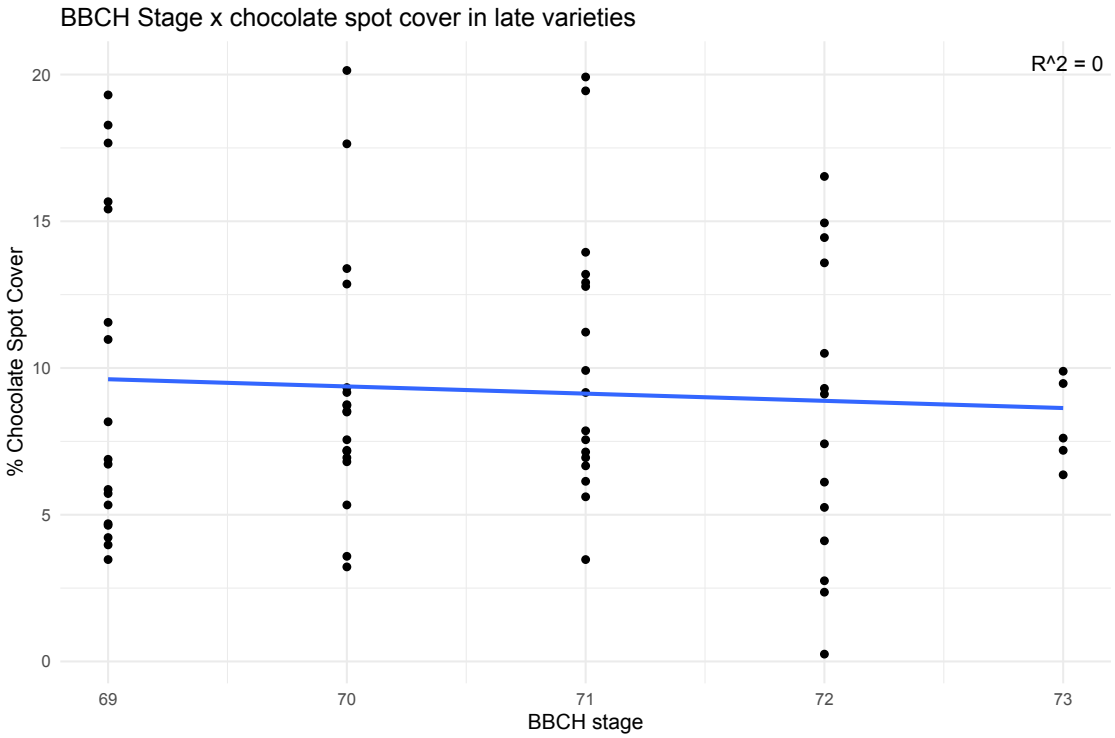


Appendix 6 Shows the three replicates and the developmental stages of the treatment plots along time in late cultivars.

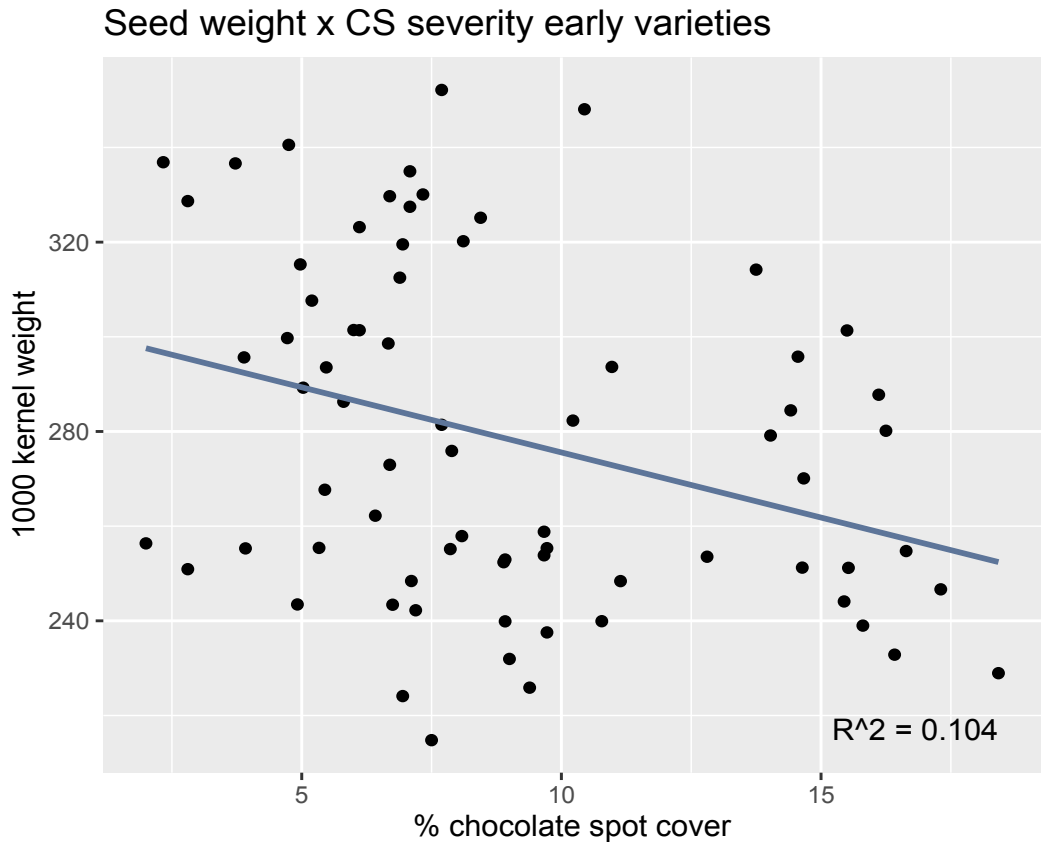
Appendix 7 Shows the disease severities of the plots where infected leaf samples were collected and their associated Botrytis species. The relationship between species and disease severity in the plots was not significant (p: 0.9858831)



Appendix 8 Shows the percentages of chocolate spot cover against BBCH stages at the time of the last disease scoring. A very low positive correlation was found.

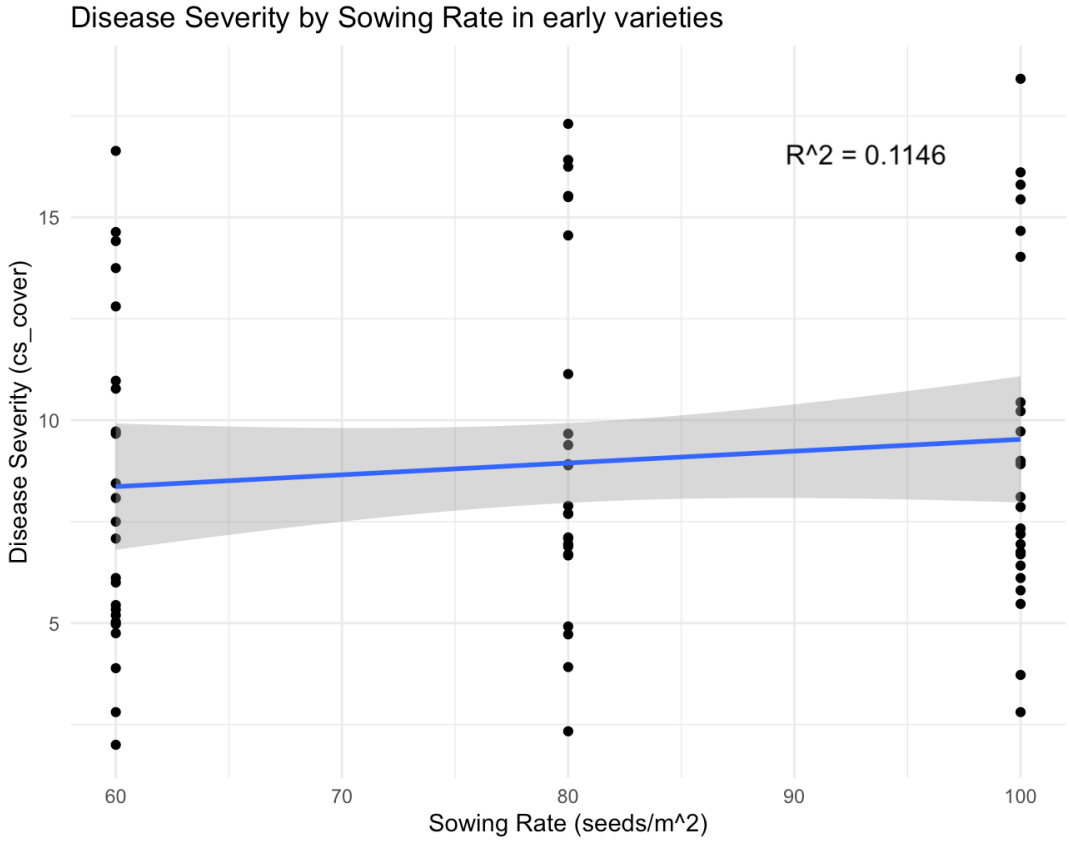


Appendix 9 Shows the percentages of chocolate spot cover against BBCH stages at the time of the last disease scoring in late varieties. No correlation was found.

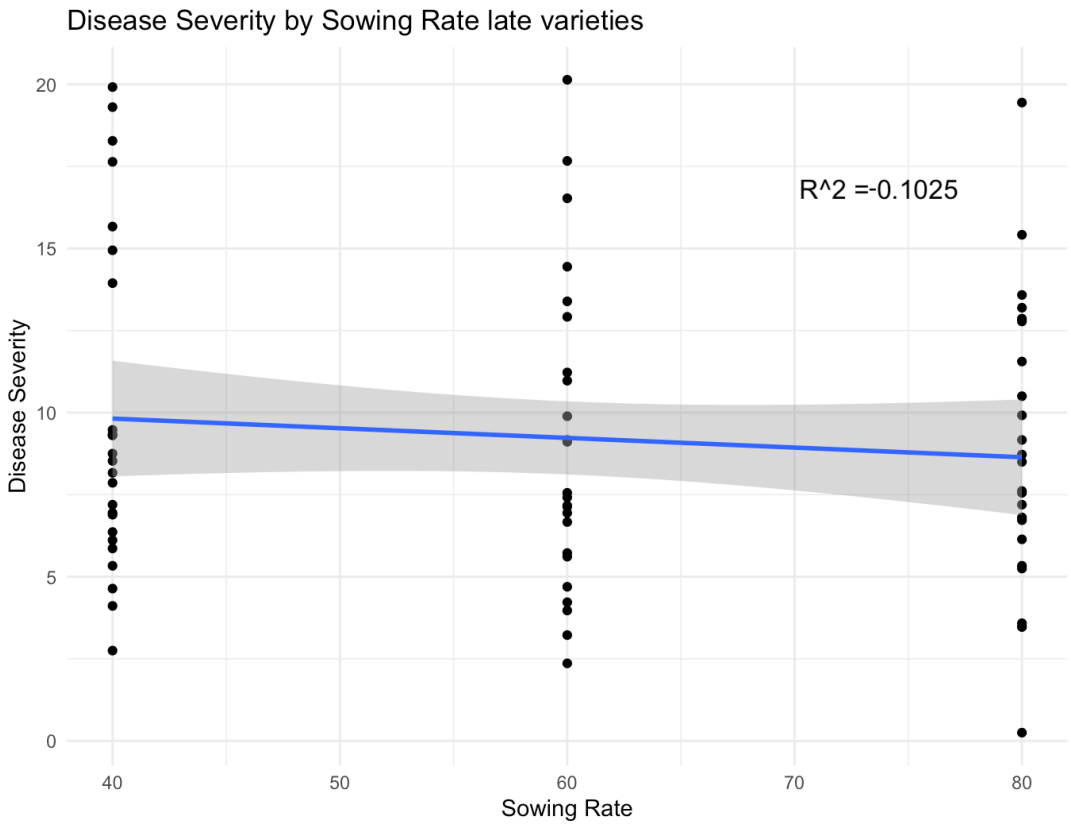


Appendix 10 Shows a pairwise plot of seed size of early varieties against severity of disease with a negative correlation of R^2 =0.104.

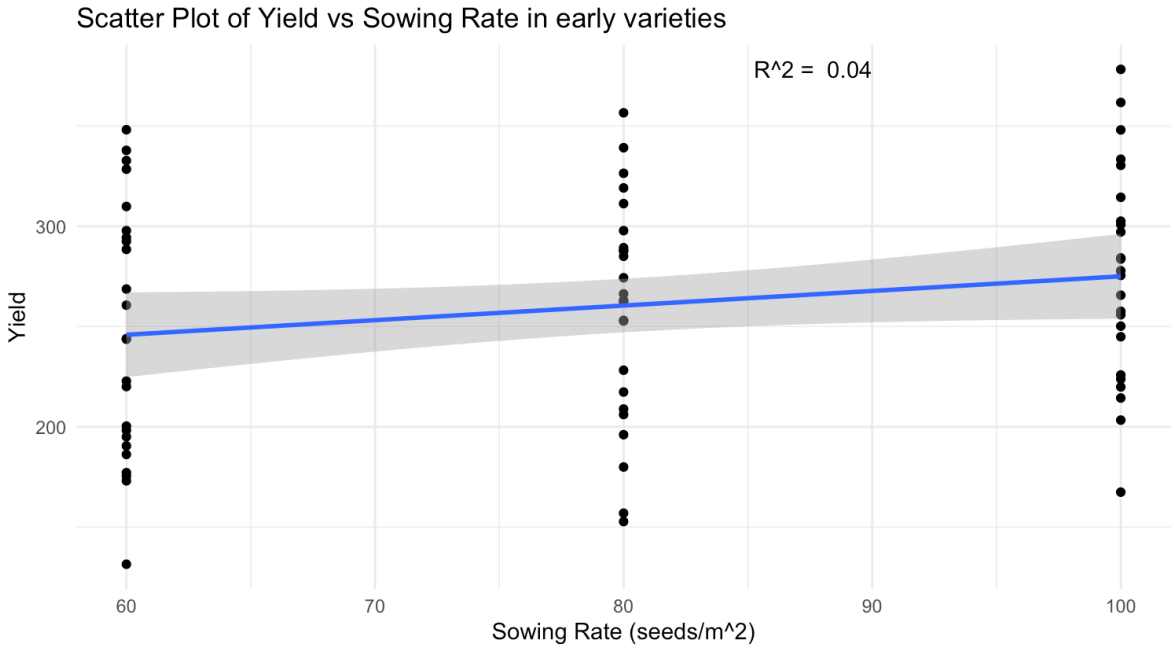
Appendix 11 Shows a pairwise analysis with seed size of late varieties plotted against disease severity with a slight positive correlation of $R^2 = 0.004$.



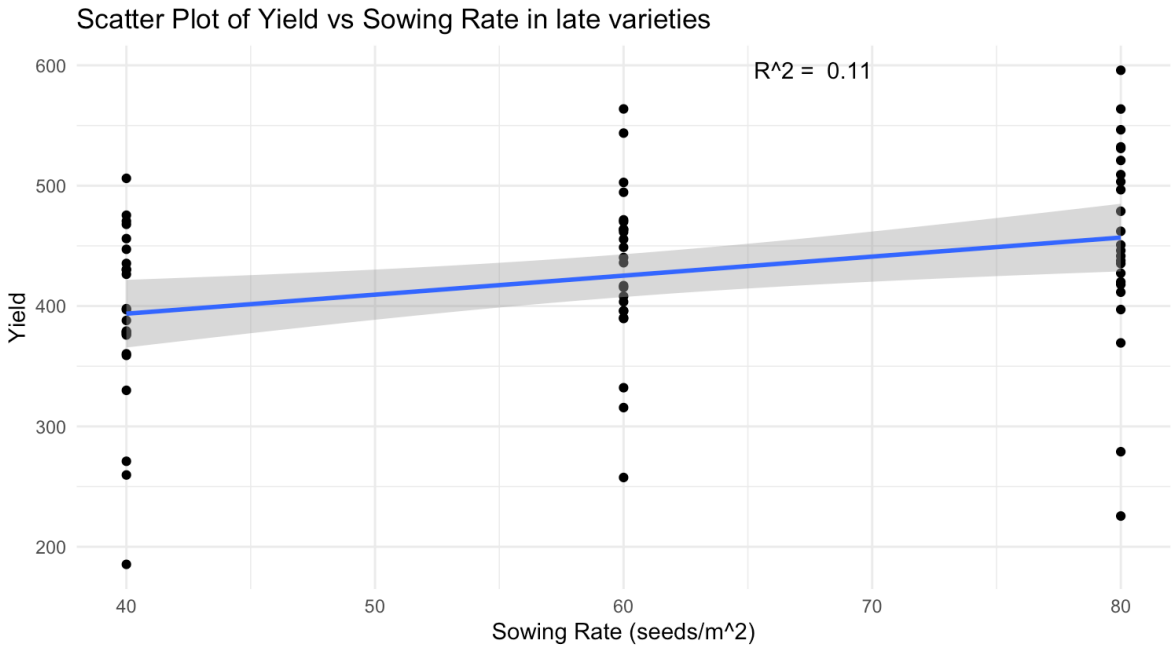
Appendix 12 Shows a scatterplot of early variety seed rate against percentage chocolate spot with a positive correlation coefficient of $R^2 = 0.1146$



Appendix 13 Shows a scatterplot of early variety seed rate against percentage chocolate spot with a negative correlation coefficient of $R^2 = 0.1025$.



Appendix 14 Shows the scatterplot of sowing rate and yield showing a positive bivariate correlation coefficient of $R^2 = 0.04$ in early varieties.



Appendix 15 Shows the scatterplot of sowing rate and yield showing a positive bivariate correlation coefficient of $R^2 = 0.11$ in late varieties.

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