Towards understanding the role of Neighbor-Modulated Immunity in the wheat response to Fusarium Head Blight

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Abstract

Wheat, a crucial global staple, faces significant yield threats from Fusarium Head Blight (FHB), primarily caused by *Fusarium graminearum.* Although integrated efforts like crop variety mixtures are expected to improve FHB management, their effectiveness varies often due to a limited understanding of how different plants in these mixtures interact. A promising yet underexplored strategy is Neighbor-Modulated Immunity (NMI), where interactions between neighboring plants can naturally boost disease resistance. Understanding and leveraging NMI could improve the effectiveness of variety mixtures in combating FHB. This study aims at developing the tools to deepen our understanding of how plant-plant interactions influence agronomic traits in cultivated crops, with a focus on exploring the concept of NMI in the wheat response to *F. graminearum.* We have optimized a pipeline to investigate NMI, and used it as a proof-of-concept to assess the effectiveness of the strategy. Resistance to leaf infections caused by *F. graminearum* was evaluated using a coleoptile stage infection assay in several small screenings. Results indicate that the emmer wheat accession MG5323, when grown alongside the bread wheat cultivar Bob-white, exhibited enhanced immunity to *F. graminearum.* This suggests a potential relationship between MG5323 and Bob-white, providing evidence of below-ground interactions among these genotypes. This finding highlights the need for further research to uncover the molecular mechanisms underlying NMI. To investigate these putative interactions, a root phenotyping cassette was developed and optimized to examine root interactions and identify traits associated with disease resistance. While this initial study did not reveal significant findings, the successful development of this tool for analyzing root characteristics offers a valuable resource for future research.

Keywords: Wheat, Fusarium Head Blight (FHB), Neighbor-Modulated Immunity (NMI), Leaf infection assay , BobWhite, MG5323, Root phenotyping cassette

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NMI	Neighbor Modulated Immunity
SLU	Swedish University of Agricultural Sciences
PDA	Potato Dextrose Agar

1. Introduction

1.1 Wheat as a crop

Wheat is one of the most important cereal crops globally due to its high yield, adaptability to various climates, excellent storage capacity, and unique processing characteristics (de Sousa, Telma et al., 2021). It serves as a staple food for over 2.5 billion people, contributing to 20% of human calorie intake (Brenchley R, Spannagl M, Pfeifer M, et al. 2012). Besides being a significant source of calories, wheat provides essential dietary fibre components crucial for the human diet (Shewry, P. R., & Hey, S. J., 2015).

The two most significant modern wheat species are hexaploid bread wheat (*Triticum aestivum* L.) and tetraploid durum wheat (*Triticum durum*) (Dubcovsky, J., & Dvorak, J et al., 2007). Common wheat (hexaploid) makes up about 95% of the world's wheat production and is primarily used for baking bread, cookies, and pastries. The remaining 5% consists of durum wheat (tetraploid), used to produce pasta and other semolina-based products (Nesbitt, M., & Samuel, D. 1996). Despite being developed with specific characteristics to maximize yield, modern wheat varieties show productivity reductions under biotic and abiotic stresses (Arzani & Ashraf, 2017). With an increasing population and food security becoming increasingly critical, boosting the wheat yield potential in the developing world remains a high priority (Duveiller et al.2007). Addressing the issues that threaten wheat productivity is crucial for ensuring future food security.

1.2 Fusarium Head Blight (FHB)

Wheat production faces significant economic losses from various diseases caused by different pathogens, with FHB being one of the most devastating (Dean *et al*., 2012). Primarily caused by the fungus *F. graminearum* (anamorph: *Gibberella zeae*), FHB leads to severe yield reductions and poor grain quality, resulting in substantial economic losses (Wu, Lei et al., 2022). The impact of FHB extends beyond yield loss. The disease contaminates grains with harmful mycotoxins such

as deoxynivalenol (DON) and nivalenol, posing serious health risks to humans, and animals and further degrading the market value of wheat (Alisaac, E., & Mahlein, A. K., 2023). These mycotoxins can make the grain unsafe for consumption, leading to stricter regulations and additional costs for testing and remediation. Altought several species of the genus *Fusarium* are known to cause FHB, the most significant one is *F. graminearum*. This fungus primarily enters the plant through the spike, particularly targeting the floral organs (Duveiller et al., 2007). Initially, subtle water-soaked spots emerge on the spikelets, then gradually progress, leading to tissue necrosis and a characteristic bleaching of the entire spikelet. As the disease advances, it compromises grain development, resulting in shrunken, chalky kernels with reduced weight and quality. It is also remarkable that, despite this main source of infection in the plant spikelet, some results suggest that *Fusarium* species typically implicated in FHB, could be isolated from wheat leaves with blotch disease symptoms, suggesting the coexistence of *Fusarium* species as endophytes with leaf pathogens such as *Zymoseptoria tritici* (Kaur and Vilvert, 2024).

Efforts to combat FHB include developing resistant wheat varieties, implementing effective crop rotation and tillage practices, and utilizing fungicides. However, no single control measure has provided complete resistance to FHB (Zhanwang Zhu et al., 2019). Due to the complexity and variability of the disease, integrated management strategies are essential for minimizing its impact (Buerstmayr et al., 2020). In comparison with *F. graminearum, F. oxysporum* is a common soil-borne pathogen that causes vascular wilt in many different plants, including wheat (Zuriegat et al., 2021). These pathogens persist in the soil for long periods, resist chemical treatments, and rapidly evolve to overcome resistance (Zuriegat et al., 2021).

1.3 Cultivar mixtures

Focusing solely on high-yielding monoculture varieties can increase their susceptibility to pests and diseases, thus presenting a challenge to sustainable crop production. With the expectation of more restrictive pesticide regulations in the European Union over the next years, maintaining the current wheat production will be even more challenging. Utilizing cultivar mixtures is a promising strategy to overcome this trade-off (Wuest, Samuel E., et al., 2021). In principle, mixtures involve using agronomically compatible cultivars, without requiring additional breeding for disease resistance (Mundt, C. C. 2002). Actually, evidence clearly indicate that such approach can enhance disease resistance, increase productivity, and promote environmental sustainability (Mundt, C. C. 2002). Furthermore, cultivar mixtures with greater functional trait diversity can yield about 5-6.2%

more than monocultures (Borg, J., et al., 2018). However, randomly selecting cultivar mixtures does not always ensure effective disease control. In fact, it is crucial to choose cultivars that are specifically effective against the targeted pathogen (Mundt, C. C., 2002).

Cultivar mixtures work by combining different genotypes with varying levels of resistance and susceptibility to pests and diseases (Bancal, M. O., 2021). Mixtures with functionally different characteristics contribute to overcome functional redundancy and achieve positive effects (Barot, S. et al., 2017). Resistant cultivars help protect the more susceptible ones, thus reducing the spread and severity of outbreaks. This complementary effect enhances the overall health and productivity of the crops (Bancal, M. O., 2021). Despite the evidence in favor of a larger adoption of cultivar mixtures, active breeding for such traits has been largely neglegted. In fact, the effectiveness of a mixture is often considered an additional aspect rather than a primary objective of a breeding program (Wuest, Samuel E, et al., 2021). Historically, breeding programs have prioritized performance in a monoculture, thus contributing to the reducing genetic diversity in many crops. Given these concerns, there is now a growing interest on identifying and maintaining genetic diversity, especially in wheat. Wheat breeders are aware of the role that genetic diversity plays within crop species and are incorporating a range of traits into new commercial varieties while continuing to improve yields (Baniszewski, Julie, et al., 2021).

1.4 Neighbor-modulated immunity

Recent, though still limited, evidence suggests that plant-plant interactions can significantly influence plant immunity and resistance, affecting how plants defend against various pathogens. Original work in the model plant *Arabidpsis thaliana* showed that the ability of two genotypes to positively interact can be controlled by simply mendellian traits (Wuest et al, 2022). Such trait, referred to as genotype per individual (GxI) interactions, was further studied by Pelissier et al (2021) leading to the observation that neighboring genotypes can negatively affect the resistance levels in a mixtures and was coined out Neighbor-Modulated Susceptibility. Taken together, this work and additional studies shows that GxI can lead to both enhanced susceptibility or enhanced resistance which has led to the concept of Neighbor-Modulated Immunity formulated by Bourras et al. (in preparation).

Research has largely overlooked the potential role of plants as neighbors in modulating each other's immunity and pathogen susceptibility. This indicates a gap in our understanding of how plant-plant interactions might enhance natural

defences and resistance against diseases. Thoroughly studying these interactions could unveil new strategies for improving plant health and disease resistance in agricultural systems. (Pélissier et al., 2021). Plants possess a basal immune system expressed at low levels but get activated by both biotic and abiotic cues, resulting in varying degrees of resistance to pathogens and pests (Pélissier, Rémi et al.,2021). In their natural habitat, plants interact with neighboring plants, which can alter their physiology and gene expression. These plant-plant interactions also influence their relationship with the microbiome, potentially enhancing immunity.

There are several mechanisms that could potentially trigger NMI:

Soil microbiome modification: Plant neighbors can modify the microbiome composition of the soil, rebounding in a different response of the plant to the pathogen throughout the activation of genes related to plant defence mechanism (Yuan, J. et al., 2009), (Rolfe, S. A. et al., 2019).
Root direct contact: There are a few studies suggesting that plant density affects key elements of plant immunity but the effect of these alterations on plant susceptibility to biotic stress is still poorly understood (Zheng, M. et al., 2017), (Kula, A. A. R. et al., 2020).
Root exudates: There are several studies investigated how root exudates from neighboring plants influence a plant's response to pathogen attack (Yuan, J. et al., 2009), (Rolfe, S. A. et al., 2019). Despite this interesting effect, the majority of these studies have been performed in the model plant *A. thaliana* (Biedrzycki, M. L. et al., 2011), while very few have addressed this effect in crops (Pélissier et al., 2023), (Pélissier, Ballini, et al., 2023), (Zhu et al., 2019). Moreover, the molecular mechanisms underlying this interesting interaction are elusive, with limited knowledge about the genes implicating in the regulation of such traits (Pélissier et al., 2023).

There are also intriguing questions about the genetic distance between plants and their role in NMI, as it seems that the genetic distance between neighbor plants also modifies the interaction (Pélissier, Ballini, et al., 2023). Moreover, it appears that NMI does not have a fitness cost in rice (Pélissier et al.,2023), implying that such interactions can increase crop resistance without a decrease in productivity. This is of high interest for resistance breeding, where often, the introduction of resistance gene has a negative impact on growth or yield. It has also been demonstrated that genotype mixtures can increase the yield (expressed as biomass) in *Arabidopsis thaliana* (Wuest et al., 2023), suggesting that there is a diverse range of agricultural traits that can be positively affected by neighboring interactions.

1.5 Objectives

This lack of knowledge of the mechanisms underlying NMI restricts its potential application. To establish a comprehensive framework for studying NMI against FHB in wheat, we aimed at designed experimental to further our understanding if such complex trait, using wheat-*F.graminearum* as a pathosystem. Thus, he objectives of this MSc work were:

1. To develop a protocol to study NMI in the wheat-*F. graminearum* pathosystem, including:
    a) Developing and optimizing a pipeline based on wheat coleoptile infections.
    b) Testing the sensitivity of the pipeline to detect meaningful phenotypic changes in plant morphology.
    c) Testing the sensitivity of the pipeline to detect changes in plant response to *F. graminearum* triggered by neighbouring cultivars.
    d) Evaluating the impact of different neighbouring cultivars on the phenotypical characteristics of MG5323, including root length, shoot height, and leaf length, to establish a baseline for comparison.

2. Develop a phenotyping cassette to monitor and analyze the interactions between *F. graminearum* and wheat during root infections.

2. Materials and methods

2.1 Plant material

A diverse selection of wheat genotypes was chosen for the study, ensuring representation tetraploid and hexaploid species. This included reference hexaploid wheat cultivars Chinese Spring, Fielder, and Bob-white, as well as parental breeding lines: Agadir, Artico, and Victo (*Triticum aestivum* spp. *aestivum*), Latino (a *T. turgidum* spp. *durum* cultivar), MG5323 (a *T. dicoccum* accession), and Zardak (an old cultivar of *T. turgidum* spp. *turanicum*). Commercial spring wheat cultivars were sourced from the Swedish Agricultural Cooperative Lantmännen Lantbruk (Sweden). To ensure consistency in the experimental setup, only healthy and intact seeds were selected. MG5323 was specifically designated as the "probe cultivar" for measuring phenotypic responses, while the other cultivars served as neighboring cultivars. Additionally, MG5323 was grown as a monoculture to establish a baseline comparison as the control.

2.1.1 Growth conditions

Plants were cultivated under controlled environmental conditions within a growth chamber. The temperature was set to 21°C, humidity at 70%, and light intensity at 300 µmol, with a photoperiod of 18 hours light and 6 hours dark, providing ideal conditions for wheat growth. Subsequently, plants designated for inoculation with *F. graminearum* were placed in a closed infection chamber within the growth chamber with the same conditions. The infection chamber was supplemented with additional LED light sources to enhance the light spectrum, focusing on wavelengths of 380 and 700 nM.

2.1.2 Soil preparation

To establish an optimal growing condition, we prepared a substrate mixture comprising 50% regular soil and 50% perlite soil, supplemented with a solution containing 75% sterile distilled water and 25% standardized nutrient solution provided by the growth facility at the SLU Biocentrum. The mixture was further examined to remove any unwanted solid particles. For the experimental setup, a

green plastic tray containing 40 wells, measuring 7.6 cm in height, 35.5 cm in length and 22cm in width, was employed. Initially, the trays were filled with the substrate mixture, and then, three seeds of each neighbouring cultivar were sown in individual wells in a randomized manner, and appropriately labelled.

2.1.3 Pathogen material and inoculum preparation

F. graminearum was grown in Müesli medium for 1 month at 25°C under a cycle of 8 hours of light and 16 hours of darkness according to a propotol shared by Dr. Dimitar Douchkov (Leibniz Institute of Plant Genetics and Crop Plant Research, Germany). After the incubation period, the medium was soaked with 0.05% TWEEN and filtered using a Miracloth filter. *F. graminearum* spore concentration was adjusted in 1 ml vials at 250 spores/µL and stored at -20 C. In cases where the concentration needed to be increased, light centrifugation was then employed. Four fungal vials were centrifuged at 4000 rpm for 3 minutes. The supernatant was gently pipetted out, leaving a pellet with a higher concentration of spores. These pellets were then carefully transferred to a new 1.5 mL Eppendorf tube, resulting in a final spore concentration of 1000 spores/ µL.

2.2 Experimental design

2.2.1 Plant-Plant interactions in response to *Fusarium graminearum*

To study potential plant-plant interactions that could modify the response to *F. graminearum*, we developed a system based on the work of Wuest et al (Wuest et al. 2023). In our experimental setup, the MG5323 accession was used as a 'Probe' cultivar. The latter was planted alongside other ’Test’ cultivars to investigate if any of these can modulate the response of the probe to *F.graminearum.* Three seeds of each neighboring cultivar were sown in individual wells in a randomized manner. After 7 days of germination of the test cultivars, any additionally grown plant was removed and then the ‘probe’ was sown, ensuring that each pot had one probe accession plant and two test accessions. An incision was performed on the 4-day-old probe MG5323, followed by the insertion of a cotton plug. Subsequently, 50 µL of spore solution at a concentration of 1000 spores/uL was inoculated onto the cotton plug. As a negative control, we used 0.05% Tween solution, and as a positive control, we inoculated the incision with hyphae from *F. graminearum* grown on Potato Dextrose Agar (PDA) plates at 24°C in the dark.

To create favourable conditions *F. graminearum*, two litres of hot water were poured in the trays to increase humidity, then the infection chamber was covered

with a black cloth for a period of 24 hours. The entire infection experiment was conducted in a growth chamber to minimize the risk of pathogen spread and ensure consistent experimental outcomes. Once the experimental setup was established and potential errors were ruled out, we conducted three different assays using the established protocol.

- The first assay was performed to analyze various phenotypic effects on the focal cultivar when grown alongside neighboring cultivars (Fielder, Bob-white, Chinese Spring, and MG5323). Ten replicates were generated for each neighboring cultivar, with seeds sown in a randomized manner. The focal cultivar was sown 7 days after the germination of the neighboring plants, and phenotypes such as root length, stem length, and leaf length were analyzed after 14 days of growth.
- In the second assay we performed an infection assay which involved a set of biological replicates, including Fielder, MG5323, Bob-white, and Chinese Spring to examine the effect of NMI in genotype mixtures and monoculture. Each cultivar had four biological replicates, and a positive control. The focal plants were infected four days after germination, and disease progression was analyzed 12 days post-infection.
- Finally, we performed an assay specifically examining NMI in MG5323 when grown with a larger diversity of wheat accessions including GTC 1846, Latino, Artico, Agadir, Zardac, Victo, 249, Spada, and MG5323. For each cultivar, three biological replicates were included, along with positive and negative controls. The probe cultivar was infected with *Fusarium* using the cotton plug method four days after germination. Pathogen progression was analyzed 12 days post-infection.

2.2.2 Disease Assessment

The assessment was conducted 12 or 21 days after infection (depending on the assay) to allow for sufficient disease progression (see previous section). In order to standardize the assessment, all scores were based on a 10 cm segment cut from all infected leaves. Delicate cleaning of the necrotic area was carried out using a q-tip to remove epiphytically grown fungal hyphae. This is important to ensure clarity and precision during subsequent scanning and image analysis procedure. Each cleaned leaf section was then placed on a piece of paper and labelled accordingly to differentiate among wheat genotypes. Following this, the leaf sections were digitalized using a high-resolution A3 format scanner, and the images were subsequently analysed using the ImageJ software to precisely measure the leaf area undergoing necrosis.

2.3 Root Phenotyping cassette

2.3.1 Cassette design and infection assay

To investigate the root interactions in response to *F. graminearum,* we developed a phenotyping cassette as follows. Polycarbonate plastic sheets with dimensions (Length: 300mm, Width: 300mm Thickness: 3mm and Density: 1.2g/cm^2) were utilized due top their high durability. Two sheets were stacked on top of one another, with PLUS PLUS® blocks serving as a divider to create space in between and split the cassette into two compartments. Bottom and side edges were sealed using masking tape, forming a cassette structure (*figure 5*). Agar media was prepared at a concentration of 0.8% (6.4 grams in 800 ml of distilled water), sterilized by autoclaving, and then poured inside the prepared cassette, allowing it to solidify. Wheat seeds were surface sterilized in 70% ethanol for 20 seconds, followed by three serial rinses with distilled water, each lasting 20 seconds. The seeds were then carefully placed inside the cassette, and a light mist of water was sprayed to maintain moisture for germination. Then, the top of the cassette was covered with masking tape. Finally, the cassette was transferred to the growth chamber and covered with a black cloth for a period of 24 hours to induce germination.

2.3.2 Root phenotyping assessment

Plants grown in the cassette designed at the lab were carefully removed from the agar and cleaned. Roots were then placed on a flatbed scanner to obtain high-quality scans. Root images were processed with ImageJ, initially to distinguish the roots from the background. Then, a specialized ImageJ plugin called Smart Root, was used to trace and measure root length. A scale setting was applied to convert pixel measurements to metric units, where 780 pixels equaled 32 mm in our experiment. After analysis, data was exported for further statistical analysis in the R statistical software.

2.4 Statistical analysis

The statistical analysis was performed using R (version 4.2.2) in the R studio environment (R Core Team, 2022) using the phenotypic data obtained from each experiment. The assumptions of normality was tested systematically for all datasets using the Shapiro test and homoscedasticity with the Score test for non-constant error variance. Once these assumptions were verified, appropriate

parametric tests were employed to test the hypothesis and statistically validate the results. Specifically, the t-test (Student's t-test) was used for comparing the means between two groups, while ANOVA (Analysis of Variance) was applied to compare means across multiple groups.

A non-parametric alternative to ANOVA, the Kruskal-Wallis Rank Sum Test, was employed to further assess the significance of the data. This test is particularly well-suited for small sample sizes and is robust against outliers. Therefore, it was used to compare the medians across multiple groups, as it does not rely on assumptions of normality or equal variances, providing a more reliable analysis under these conditions.

3. Results

3.1. Development of a protocol to study Neighbor Modulated Immunity (NMI)

3.1.1 Analyzing the Phenotypic Variation in MG5323 Traits Influenced by neighboring genotypes

Previous work by Wuest et al (2022) suggested that NMI could be associated with root morphology. Considering that the roots are a possible interface for signal exchange between cultivars in a mixture, we decided to examine the root system of MG5323 when grown both in mixtures and in monoculture. In doing so, we have also examined the shoots. With that aim, we measured a range of relevant morphological features, including root length, shoot height, and the plant's leaf length.

This assay was conducted with two replicates, each consisting of ten biological samples per genotype mixture and monoculture. The probe cultivar was not subjected to any infection, allowing us to focus on potential influences from neighboring cultivars under non-stress conditions. By maintaining consistent experimental procedures across replicates, we ensured that any observed effects were due solely to interactions within the plant mixtures rather than external stressors. After completing the experiments, data from both replicates were merged for interpretation of the probe cultivar performance in three different genotype mixtures and monoculture.

For root length, we found that all genotype mixtures resulted in reduced root growth compared to the monoculture group. Among these mixtures, the BobWhite combination showed root lengths most similar to the monoculture. In contrast, the Chinese Spring genotype mixture significantly inhibited the root development of MG5323. These result suggest that BobWhite has a neutral effect while Chinese spring has a negative effect on the root development of MG5323.

For stem length we found that all genotype mixtures exhibited enhanced stem growth compared to the monoculture group, with the BobWhite mixture showing the most significant increase in stem length, suggesting this genotype has a strong

positive effect on stem development compared to all other mixtures. The shorter stem length observed in the monoculture could suggest a lack of competition. In contrast, the increased stem length in genotype mixtures could reflect competition for light as it is a crucial resource for photosynthesis.

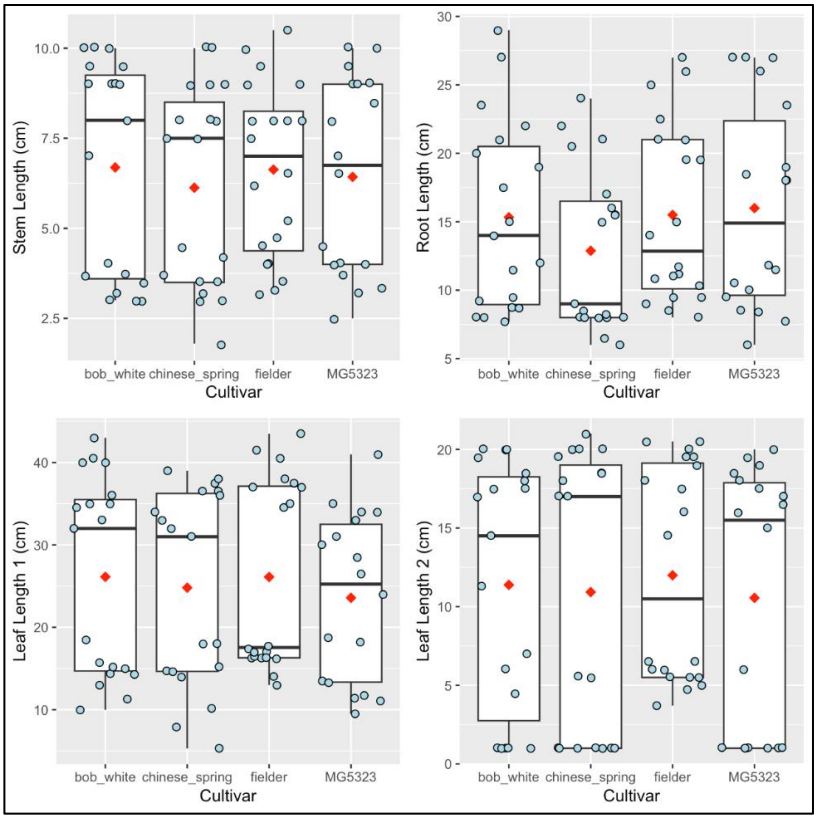

Figure 1: Development dynamics of MG5323 cultivar in mixed crops with four different wheat lab standards. (A) Stem length of MG5323, (B) Root length, (C) leaf length 1 and (D) leaf length 2, X-axis indicates cultivars and Y-axis represents the respective lengths in cm. Red dots indicate the average length in each treatment, and the blue dots represent each measurement. The size of the sample was n = 20.

For leaf length, considering both leaf length 1 and leaf length 2 collectively, we found that MG5323 exhibited the least leaf growth when grown alongside Fielder compared to all other genotype mixtures. This suggests a negative influence of Fielder on leaf development in MG5323. In contrast, mixtures with BobWhite and Chinese Spring as neighbors demonstrated relatively increased leaf length, suggesting a potentially positive impact on leaf growth. However, the differences in leaf length among the various genotype mixtures were not as pronounced as compared to the clear negative effect of Fielder. While Fielder consistently suppressed leaf growth in MG5323, the influence of other cultivars like BobWhite and Chinese Spring is less consistent but tends toward enhanced leaf growth.

When comparing the phenotypic variation in MG5323 influenced by different genotypes to that of the monoculture, we observed distinct trends. Chinese Spring negatively affected root length but promoted minimal stem and leaf growth, indicating a mixed influence. Fielder had a negative impact on leaf growth, with likely reductions in root growth, and a possible moderate increase in stem length. BobWhite maintained root growth similar to the monoculture, while significantly enhancing stem length and moderately increasing leaf growth, making it the most beneficial genotype in mixed cultivation, at least based on this defined set of criteria.

Although the data showed variability, suggesting that the genotypic effects were not entirely consistent, we observed potential trends in how different genotype mixtures may influence trait development in MG5323. To evaluate these observed differences, we applied various statistical tests as outlined in the methods. Despite the large sample size, the data did not meet the assumptions of normality (Shapiro-Wilk test) or homoscedasticity (non-constant variance score test). So we employed the Kruskal-Wallis test to compare the medians across genotype mixtures without relying on the assumptions of normality. The analysis did not reveal any statistically significant differences, suggesting that none of the tested cultivars had a significant impact on MG5323 phenotypes.

However, our system allows us to detect differences in plant growth and its response to pathogens within mixed crop systems. These results strongly suggest that our pipeline could be used in large screening to study NMI.

3.1.2 Experimental proof-of-concept for NMI

We performed leaf infections with the aim of testing our setup with various hexaploid lab standard cultivars (test) and the genotype of interest (probe), the tetraploid wheat cultivar MG5323. The experimental set up was employed as described in the methods. Briefly, MG5323 was grown alongside two plants of each test genotypes being either BobWhite, Chinese Spring, or Fielder. The coleoptiles of MG5323 were cut, and the infection was performed by adding 50 µl of *F. graminearum* spore suspension (250 spores/µl) as described in Methods. Disease severity was evaluated at 21 dpi by measuring the length of the necrotic lesion on the MG5323 probe in the presence of the three hexaploid test genotype mixtures, and when grown as monoculture.

We observed no significant differences in necrosis development on MG5323 when grown alongside Chinese Spring and Fielder as compared to the monoculture. Although the median values of necrosis in these genotype mixtures

are similar, variability becomes more apparent when considering the average values, indicating subtle differences in the response to these genotype mixtures (Figure 2).

However, a reduction of necrosis development was observed in MG5323 when grown in mixture with BobWhite as compared to the monoculture control. Together, these results suggest that BobWhite may modulate the immune response of MG5323 to *F. graminearum*. Thus, providing a proof-of-concept observation of NMI between two wheat genotypes. Additionally, the failure to observe such an effect with Chinese Spring and Fielder indicates that the expression of NMI is genotype specific, further supporting the hypothesis that NMI only operates between 'compatible' genotypes.

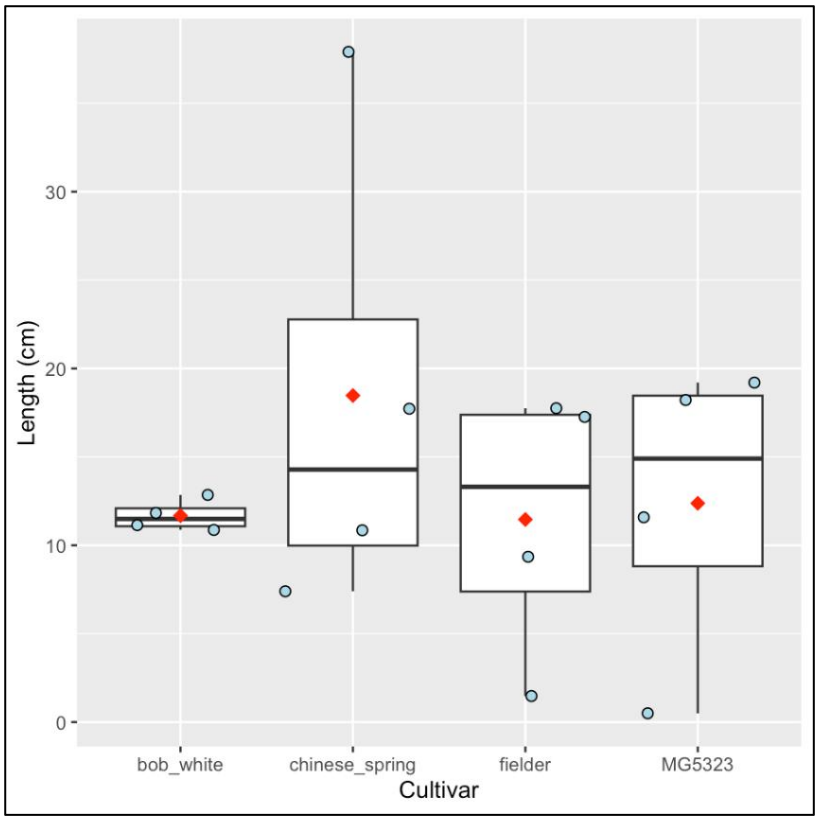

Figure 2. Disease susceptibility of Emmer wheat MG5323 accession with several neighboring cultivars: The X-axis indicates the neighbouring cultivars while the Y-axis represents the length of necrotic region in cm. Red dots indicate the average of necrotic length, and the blue dots represents each sample. Necrotic lesions were measured at 21 dpi, each treatment has a n = 4.

We then decided to replicate the assay using a higher dose of inoculum (1000 spores/uL) in order to reduce the latency period and hopefully minimize variability. First, we found that susceptibility in the monoculture control was more marked with higher doses of inoculum demonstrating a clearer disease response. Furthermore, a marked trend for less necrosis development was now observed in

all three genotype mixtures as compared to the monoculture. Of these, MG5323 grown alongside BobWhite exhibited a significant reduction in the disease severity compared to the control, strongly suggesting that BobWhite potentially enhances the resistance of MG5323 against *F. graminearum* (Figure 2).

While MG5323-BobWhite genotype mixtures exhibit increased resistance against *F.graminearum*, the Chinese spring and Fielder genotype mixtures also showed a trend towards reduced necrosis, although with considerable variability. This suggests that while these genotypes may have some influence on MG5323 resistance, their effects are less consistent or pronounced compared to BobWhite. Overall, these results indicate that the BobWhite cultivar consistently enhances the resistance of MG5323 to *F. graminearum*. The differences in necrosis between the mixtures are reflected in both the median and average values, highlighting the stronger resistance in the BobWhite-MG5323 combination, with the monoculture control remaining the most susceptible.

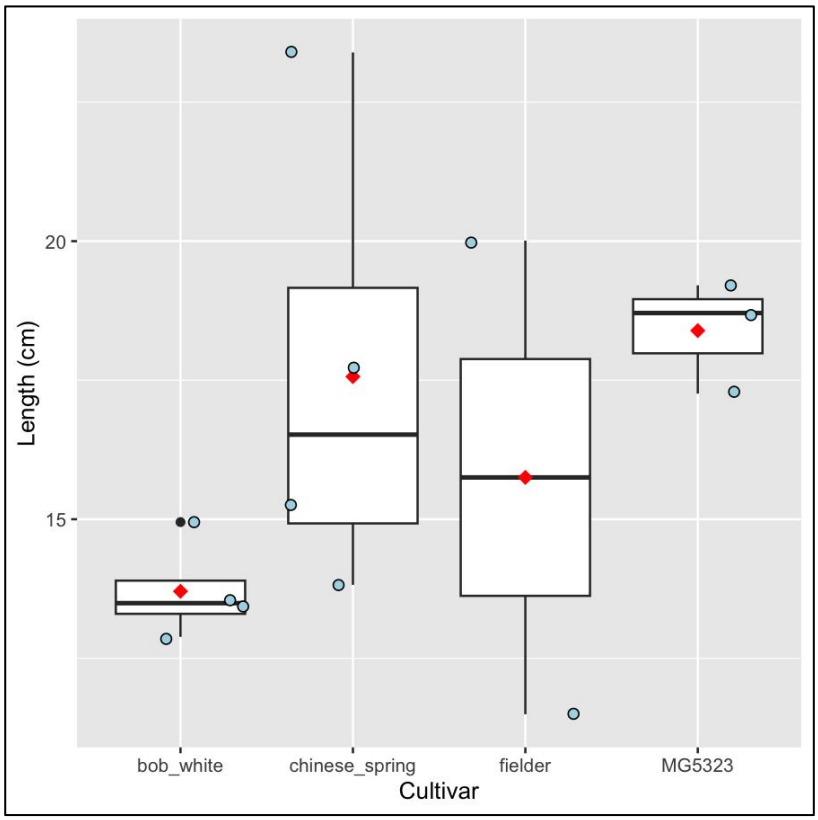

Figure 3. Disease susceptibility of Emmer wheat MG5323 accession with several neighboring cultivars. The X-axis indicates the neighboring cultivars while the Y-axis represents the length of necrotic region in cm. Red dots indicates the average necrotic length, and the blue dots represents each sample. Necrotic lesions were measured at 12 dpi, each treatment has a n = 4.

To further assess the significance of the observed differences and quantify associations within our data, we conducted several statistical tests. Specifically,

we analyzed the normality and homoscedasticity of the data using the Shapiro-Wilks test and a score test for non-constant error variance. In the first replicate with low spore concentration, the data did not pass the assumptions of normality and homoscedasticity as the obtained P-value was 0.02636 (α=0.05). This outcome is not entirely unexpected given the small sample size (n=5), which makes it challenging for the data to meet these assumptions. Despite this, we proceeded under the assumption that our data were sufficient for the application of parametric methods. In the second replicate with an increased dose of inoculum, the data was tested as previously mentioned and the Shapiro-Wilk test yielded a p-value of 0.04701(α=0.05). Unlike the first replicate, the score test for non-constant error variance in the second replicate produced a p-value of 0.50472. To further confirm the statistical significance we conducted an analysis of variance (ANOVA) on our data and obtained a P-value of 0.02785, indicating that the data is normally distributed and did not pass the assumptions of normality and homoscedasticity. However, similar to the first replicate, the small sample size likely contributed to the challenges in meeting these assumptions.

Although neither replicate provided statistically significant results to strongly support our hypothesis, we observed a consistent trend: replicates with MG5323 grown alongside BobWhite exhibited reduced necrosis compared to the monoculture group. This trend persisted across both replicates, suggesting that the BobWhite genotype may have a potential neighbor-modulated immunity effect on MG5323, even though the small sample size limited the statistical power of our tests.

3.1.3 Investigating NMI to *F. graminearum* in a larger diversity of wheat species

In this assay, we further examined the NMI by selecting a diverse set of wheat cultivars as neighbors , including Agadir, Artico, Victo, Latino, Zardak, GTC 1846, GTC 1863, 249, Spada, and MG5323 (as control) to analyse their influence on the resistance of the probe against *F.graminearum.* The experiment followed the same protocol and twelve days after infection, disease severity was assessed by measuring the length of the necrotic region on the leaves. To ensure consistent and reliable results, cultivars that did not germinate were excluded from the statistical analysis.

The results indicate that the probe grown alongside Artico exhibited the least resistance on average to *F. graminearum* with high variation in the size of the necrotic area (Figure 3). In contrast, all other test cultivars (Agadir, GTC1846, GTC1863, and Latino) reduced disease severity in MG5323. Notably, the control

monoculture exhibited the highest necrosis length compared to these cultivars. By looking at the average necrotic length, no clear difference was observed between the GTC1846 or Latino mixtures and the control Monoculture. However the GTC1863 and Agadir mixtures resulted in slightly lower disease severity in the MG5323 probe, although mixtures with Artico still had the highest necrosis length. The variation observed with Artico as a test genotype may be attributed to outliers in the data. To assess the normality and homoscedasticity of the data we employed statistical analysis as described in the methods, The Shapiro-Wilk test resulted in a p-value of 0.7026, and the score test for non-constant error variance produced a p-value of 0.059927.

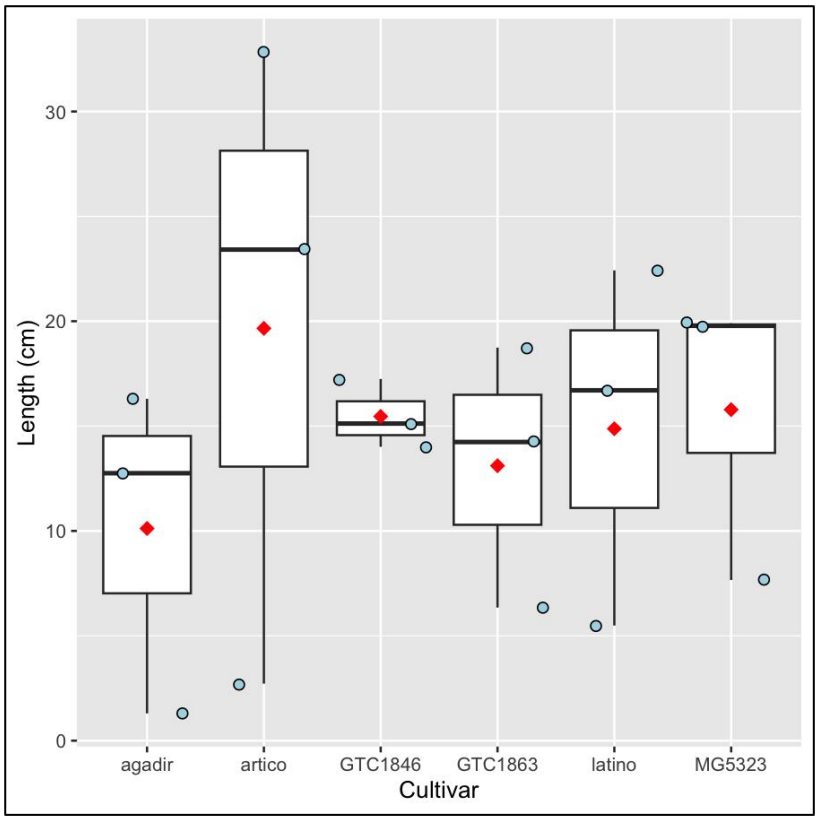

Figure 4. Disease susceptibility of Emmer wheat MG5323 accession with diverse wheat cultivars as neighboring cultivars: The X-axis indicates the neighboring cultivars while the Y-axis represents the length of the necrotic region in cm. Red dots indicates the average of necrotic length, and the blue dots represents each sample. Necrotic lesions were measured at 12 dpi, each treatment has a n = 3.

At the conventional significance level (α=0.05) the obtained P-value suggests that the data is not normally distributed. The observed trends do not strongly suggest that the tested genotype mixtures have a significant influence on NMI. However, With a p-value (0.059927) slightly above the conventional significance level,

there is some evidence (though not strong) to suggest that there is variance. Given the smaller sample size in this assay, further investigation with a larger sample size could reveal the full potential of the tested genotype mixtures to enhance the disease resistance of MG5323 against the fungal pathogen *F. graminearum*.

3.2 Development of new tools for root infection phenotyping

3.2.1 Root Phenotyping of wheat cultivars

In order to further investigate how NMI may affect the root morphology of a cultivar in presence of another nearby genotypes, we designed a transparent cassette system that would allow us to monitor such interactions in presence and absence of a fungal infection (see Methods). In this first assay, we simply conducted a test to evaluate the extent and quality of the phoentypic data we can extract from the system. Three sterilized seeds of each genotype (BobWhite, Fielder, MG5323 and Chinese Spring) were sown on the surface of the medium.

The transparent nature of the cassette allowed for clear observation and daily tracking of root growth. Each cultivar was spaced evenly to maintain uniform growth conditions, ensuring accurate and reliable results for our root phenotyping experiments.

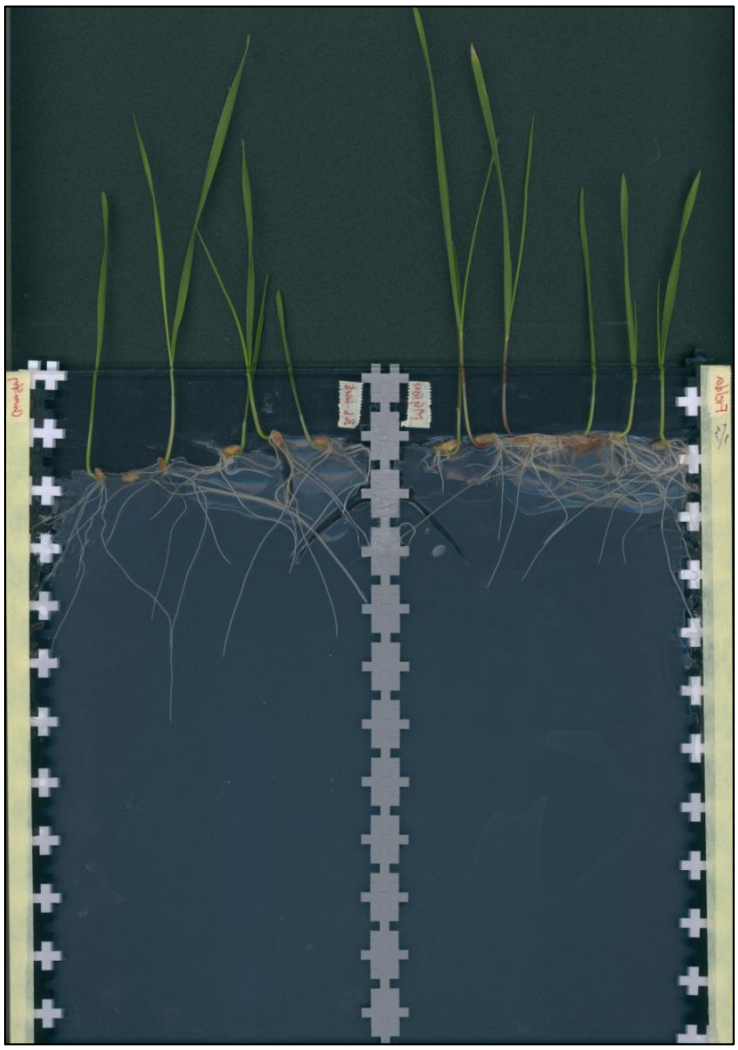

Figure 5. Lab designed Cassette with four standard wheat cultivars (Fielder, MG5323, BobWhite and Chinese Spring) grown in media.

The cassette in (Figure 5) represents the developed system, showing that our pipeline and experimental approach towards examining the root phenotype is functional. The plants were grown for 15 days after which the cassette was scanned using a high-resolution scanner. The resulting images were assessed using the ImageJ software, allowing us to measure various root characteristics such as length, surface area, volume, direction, and diameter. These data enabled us to analyze various morphological features successfully, demonstrating the efficiency of our pipeline in measuring roots.

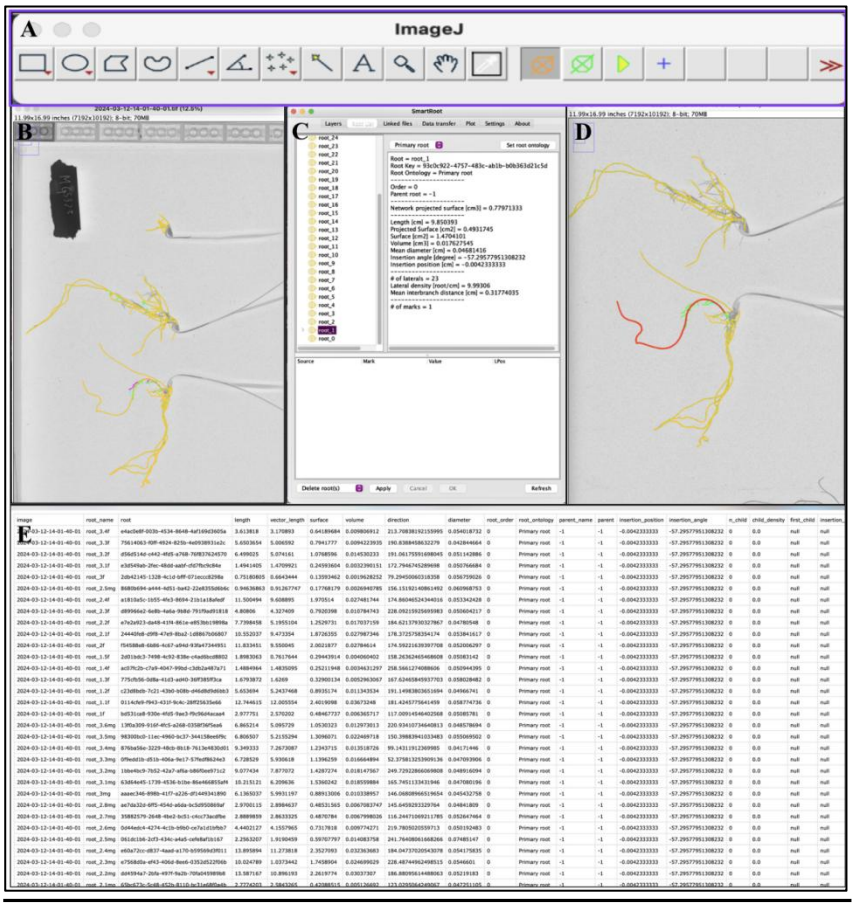

Figure 6: SmartRoot ImageJ plugin interface used to trace, measure and analyse various root characteristics.

To perform a comprehensive analysis of root systems, we used the ImageJ toolbar (Figure 6.A) to measure different parameters such as root length, surface area, volume, and root diameter. The imported images were enhanced (inverted) to improve root visibility and distinguish the roots from the background. Then, the SmartRoot software tool was used for automated tracing of root system architecture (RSA), providing a detailed list of traced roots and their measurements (Figure 6.C). Yellow traces highlighted the primary roots, while green traces highlighted the lateral roots (Figure 6.B-D). The list of root measurements from SmartRoot including the root volume, direction and surface area was reviewed and analyzed (Figure 6.E). The intuitive interface of both ImageJ and SmartRoot allowed us to easily configure the parameters and evaluate the root system.

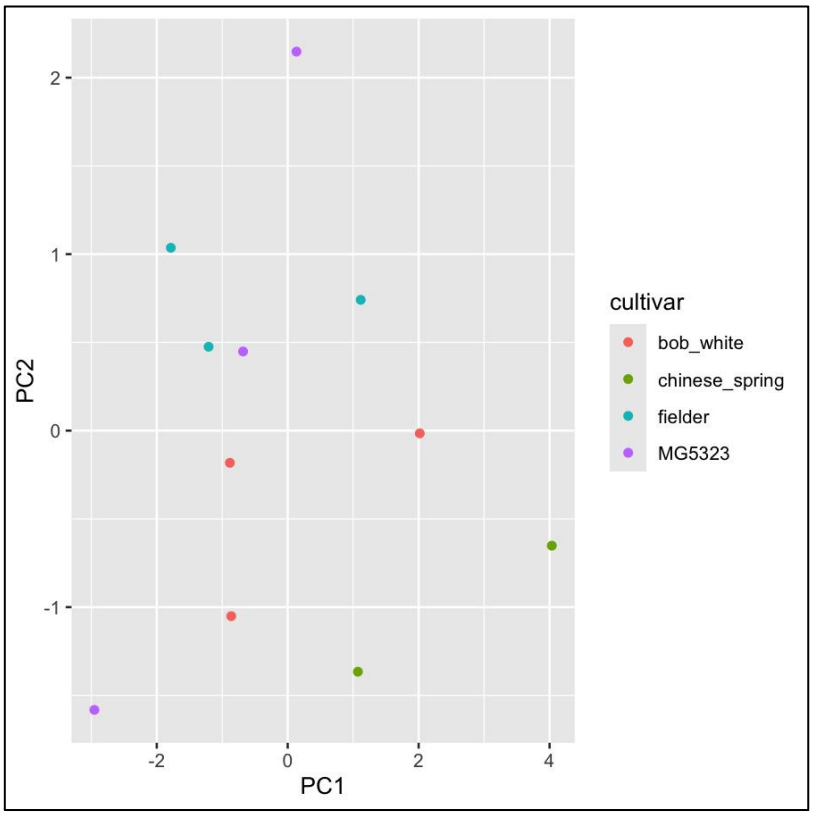

Figure 7: Principal component analysis (PCA) of the root measurements. Dots represent root measurements of one plant. Colours indicate cultivar (red, BobWhite, green, Chinese Spring, blue, Fielder, purple, MG5323).

As we obtained several measurements of different root characteristics, we performed a PCA to observe the differences and similarities among the cultivars. We can differentiate the samples clearly from cultivars Chinese Spring (lower right corner), BobWhite (centre) and Fielder (above). MG5323 roots show high variability and can not be clustered easily, although the first dimension (PC1) can separate MG5323 roots from Chinese Spring roots. We therefore conclude that our system is at least capable of differentiating gentotype specific morphologies, and satisfactorily distinguish between the test cultivars.

3.2.2 Exploring Root-Fungal Interactions in Wheat Cultivars: A Transparent Cassette System for Root Infection Phenotyping

To test the performance of our pipeline phenotyping root infections, we prepared a new cassette containing six seeds of the wheat cultivars Fielder and BobWhite. We injected *F. graminearum* into the cassette at a concentration of 250 spores/µl using a pipette. To ensure thorough documentation, we captured images of the cassette every day to record and monitor root growth. Despite our continuous monitoring, we observed no signs of disease proliferation or symptoms, either in the cassette or on the roots. This lack of visible infection suggests that the initial

concentration and application method of *F. graminearum* were insufficient to establish infection in the root systems.

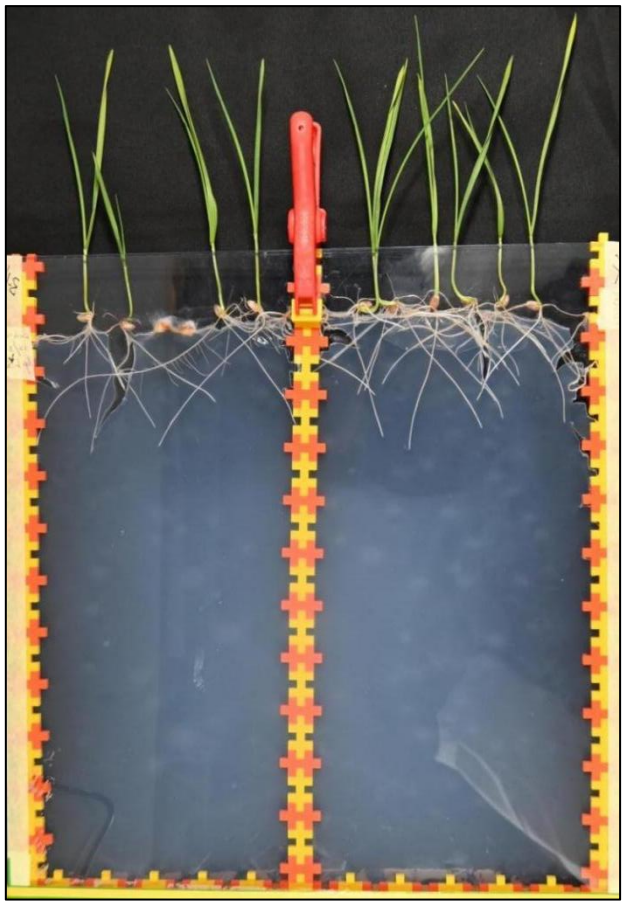

Figure 8: Cassette containing seeds of two standard wheat cultivars (Fielder and BobWhite) subjected to F. graminearum, white spots indicate the spread of the fungus throughout the medium.

To further explore the infection strategy, we conducted another experiment with an increased concentration of 1000 spores/µl of *F. graminearum* in a new batch of cassettes. Surprisingly, even with this higher fungal concentration, the roots remained unaffected. Subsequently, we modified our approach by first dipping the seeds, which had been germinated on Petri dishes for three days, into a *Fusarium* suspension before planting them in the cassette. This adjustment allowed the fungus to spread throughout the medium (Figure 8), yet the roots continued to show no signs of infection. This series of experiments emphasizes the difficulties encountered in developing a reliable root infection model for *F.graminearum*. It underscores the need for continued optimization of the infection strategy to deepen our understanding of the pathogen's interactions with wheat roots. However, the established cassette-based pipeline enables systematic investigation and documentation of dynamic root growth under controlled conditions.

4. Discussion

The need for more sustainable agriculture practices to reduce crop loss due to disease has become increasingly important in recent decades. With the European Union moving towards stricter regulations on pesticide use, maintaining current wheat production levels may become more challenging. As a result, We must explore alternative methods to enhance wheat yield and resilience, rather than relying solely on traditional breeding techniques. One promising yet underexplored approach is neighbor-modulated immunity (NMI), which examines how interactions with neighboring plants can influence a crop's resistance to diseases such as Fusarium head blight (FHB), mainly caused by *Fusarium graminearum*. While most research on NMI has focused on *Arabidopsis thaliana* as a model plant, our study has developed and refined a specific protocol to investigate NMI in the wheat - *F.graminearum* pathosystem.

In our study, we evaluated both the technological aspect (sensitivity and possible applications of the pipeline) and the biological aspect (putative cultivars which could develop a plant-plant interaction). One key optimization of the pipeline was the infection strategy, using a cotton plug on the incision proved to be a significant advancement in the infection process as the cotton plug effectively adhered to the coleoptile, ensuring that the *Fusarium* spores remained in contact with the plant tissue, resulting in more uniform disease progression. We observed a delayed progression of the disease, which may be associated with the fact that *F. graminearum* initially exhibits a biotrophic lifestyle before transitioning to a necrotrophic phase, producing mycotoxin deoxynivalenol (DON) which facilitates its spread and ultimately triggering cell death, (Molemi et al.,2020, Zongyi, et al.,2014). To address this, we optimized the sensitivity of the pipeline by increasing the spore concentration from 250 spores/µl to 1000 spores/µl. This adjustment reduced the disease progression time from 21 days to 12 days, ensuring consistent pathogen exposure across all treatments and allowing for a faster assessment of disease severity. Consequently, this method ensured a more accurate assessment of the plant response to the pathogen.

A comparison was made between different genotype mixtures with the monoculture control group. We observed trends suggesting that plant-plant interactions can modulate plant immunity. The results indicate that various

combinations of genotype mixture can affect the disease resistance of the probe MG5323 against *F. graminearum.* Plants of MG5323 grown alongside the BobWhite cultivar exhibited a notable trend towards reduced necrosis compared to other combinations, suggesting that BobWhite potentially enhances the resistance of MG5323 to the fungal pathogen. The observed effects are likely due to several factors. Plant neighbors can produce volatile organic compounds (VOCs) that spread through the community inducing changes in plant physiology, a phenomenon known as "eavesdropping" (Pélissier et al., 2021; Rolfe et al., 2019; Rebolleda-Gómez et al., 2019). However, plant cultivars were kept in a close environment and despite the short distance among cultivars only the BobWhite-MG5323 combination show changes in the probe plant response to *F. graminearum.* In case that this response would be triggered by VOCs other cultivar combinations would show the same response, but it is not the result obtained in our experiments. For this reason, it seems more probable that this improvement in *F. graminearum* response triggered in MG5323 by BobWhite could be caused by soil microbe modifications or interactions between the roots of both cultivars through direct contact or root exudates. Previous experiments have shown that this mechanism could develop an enhanced response of the probe cultivar to the pathogen (Pélissier et al., 2023; Rolfe et al., 2019; Yuan et al., 2009) or improvements in plant productivity (Wuest & Niklaus, 2018) but in durum wheat-durum wheat combinations, rice or *A. thaliana* models (Pélissier et al., 2023; Wuest & Niklaus, 2018). The observed changes might also be a result of transcriptional changes induced by BobWhite that lead to a change in the expression of the defence-related genes in MG5323. Pélissier et al. (2021), have demonstrated that in rice, interactions between intraspecific neighboring plants can trigger changes in the expression of defense genes, effectively priming the plants to better respond to pathogen attacks. In any case, it is necessary to conduct more experiments to reveal the exact mechanism that enhances the response of MG5323 to *F. graminearum* through root interaction with the BobWhite cultivar.

In contrast, the control group (MG5323/MG5323) experienced the highest disease severity, indicating poor resistance when MG5323 is grown as monoculture. This result is supported also by an extensive bibliography that suggests that crop genetic biodiversity increase plant productivity and resistance to several pathogens (Smith et al., 2015; Smithson & Lenné, 1996). Chinese Spring and Fielder, as neighboring cultivars displayed inconsistent effects on necrosis and had a weaker impact on MG5323 resistance to *F. graminearum*. As we achieved to phenotype the plant-plant interactions and it is effect in the plant response to a necrotroph pathogen *(F. graminearum)*, our results demonstrate the efficacy of our developed pipeline. In conclusion, we have developed an effective methodology that allow us to identify and quantify neighbor-modulated immunity between phenotypes consistently.

Although we have explored the effect of plant-plant interaction on various phenotypical traits, more research in this area is necessary. There are several evidences that some plant-plant interactions could lead to improved yield productivity (Wuest et al., 2022; Wuest & Niklaus, 2018). A potential next step would be to examine how different neighboring cultivars affect different phenotypic traits of the probe cultivar. For example, Kula et al. (2020) demonstrated that increased cultivar density can reduce several measures of plant growth due to intraspecific competition. Our results, which revealed trend variations in phenotypes when the probe cultivar was grown alongside Chinese Spring and Fielder, provide further evidence of such competitive effects. In our results, we observed that the MG5323 cultivar tends to show increased stem and leaf length when grown alongside the BobWhite. Although the impact was not statistically significant or consistent, the trend suggests that BobWhite may positively influence the groth of MG5323 and exhibit low competition for resources. This suggests that the system could be valuable for larger-scale screenings and further studies into how neighboring plants influence the trait development and, in last instance, yield productivity. To classify and understand the functional traits of different cultivars, studying their root traits is crucial. This research will help design crop variety mixtures that enhance nutrient absorption and maintain trait diversity. For example, mixtures with deeper roots can better handle suboptimal conditions like low nutrient availability and drought. Barot et al. (2017) emphasize that a trait-based approach, which combines complementary beneficial varieties, can significantly boost productivity and resilience.

As our results suggest a root-root interaction as a potential cause of enhanced plant resistance to *F. graminearum,* future experiments could focus on root phenotyping, characterizing changes in root length, ramification, or diameter. In fact, the development of a tool for root phenotyping is a mandatory step to continue researching in this idea. The key advantage of our system is its ability to track and observe root growth daily without disturbing the root environment. By providing clear visibility and ease of monitoring root interactions, our design proved both functional and efficient in root phenotyping. Additionally, the use of high-resolution scanning combined with ImageJ and SmartRoot software allowed us to obtain detailed and accurate measurements of root characteristics. This allows us to solve usual problems in these approaches as separating roots from substrate or characterize root phenotypes without damaging them.

Although we have proved the value of our cassette system to phenotype roots, we have faced different difficulties in performing *F. graminearum* root infection. Despite using higher fungal concentrations and methods like dipping seeds in a fungal suspension, the roots remained unaffected. The lack of successful infection underscores the need for further optimization. Among future experiments we

could try to perform infections with *F. oxysporium* isolates, increase the agar concentration of the substrate to avoid the fall of the spores throughout the gel and test the approach in different pathosystems.

Traditional root phenotyping methods such as Soil Core Method, Trench Method, Mesh Bag Method and Shovelomics involves destructive sampling techniques, losing a part of plant root and provide limited view of the roots (Li, Anchang et al.2022). In our system, these limitations have been addressed as the cassette approach allows us to separate the roots without any damage, which makes it possible to track how quickly roots grow, known as root elongation rates.

In modern techniques like hydroponics, tracking root orientation and making repeated measurements is difficult because the roots are in a nutrient solution instead of soil. Handling the roots, particularly during nutrient solution changes, can cause damage to the roots, especially smaller lateral roots. Our cassette approach addresses these challenges by allowing daily root observations and avoiding damage while handling. Our Root phenotyping cassette design works well alongside other methods and complements other modern techniques like gel-filled observation chambers. Especially the one developed by (Futsaether et al. 2002) which also focuses on improving root observation and measurement.

5. Conclusion

- BobWhite cultivar interacts with MG5323 cultivar reducing the necrosis caused by *Fusarium graminearum* in the MG5323 cultivar.

- Plant-plant interaction of selected cultivars could lead to improved resistance against pathogens. The results are a proof-of-concept of Neighbor modulated immunity (NMI) could be applied to wheat in order to improve plant resistance against pathogens.

- The pipeline successfully identified differences in plant traits and responses to pathogens, showing its potential use for large-scale screening to study NMI.

- The root phenotyping cassette system for root infection phenotyping accurately measured root traits in various wheat cultivars, indicating its potential application in future research on below-ground plant-plant and plant-microbe interactions.

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Popular Science Summary

Wheat, a staple food crop worldwide, is under constant threat from Fusarium Head Blight (FHB), a disease primarily caused by the fungus Fusarium graminearum. However the current control measures are not proven effective in controlling FHB. Although planting different wheat varieties together (variety mixtures) has been explored as a solution, the results have been inconsistent, often due to a lack of understanding of how plants interact with one another.

A promising and a new concept called Neighbor-Modulated Immunity (NMI) suggests that plants can naturally enhance each other's resistance to disease through their interactions. Our study aimed to explore how NMI might help wheat resist FHB by developing tools and protocols to study this phenomenon in the wheat-F. graminearum system.

We created a pipeline to observe differences in the physical traits (phenotypes) of various wheat varieties. This pipeline was tested using infection assays on wheat seedlings (coleoptiles) exposed to *F. graminearum*, with disease symptoms tracked through image analysis. Additionally, we developed a tool to monitor root growth and below ground interactions without disturbing the plants. as this is key to understanding how plants might influence each other's health.

Overall we found that when the emmer wheat variety MG5323 was grown next to a common bread wheat variety called Bob-white, the bread wheat showed improved resistance to FHB. This suggests that the two wheat types may be interacting below ground, influencing each other's disease resistance.

Thus follow up experiment is needed to understand the mechanisms behind these interactions, but our experiments open up possibilities for new strategies that could lead to better disease management for wheat.

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