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Impact of Fusarium Crown Rot on Root System Area and Links to Genetic Variation within Commercial Wheat Varieties

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Abstract: Fusarium crown rot (FCR), caused by the fungal pathogen *Fusarium pseudograminearum* (*Fp*), is a major constraint to cereal production worldwide. The pathogen restricts the movement of solutes within the plant due to mycelial colonisation of vascular tissue. Yield loss and quality downgrades are exacerbated by this disease under water stress conditions. Plant root systems are adaptive and can alter their architecture to optimise production in response to changes in environment and plant health. This plasticity of root systems typically favours resource acquisition of primarily water and nutrients. This study examined the impact of FCR on the root system architecture of multiple commercial bread and durum wheat varieties. Root system growth was recorded in-crop in large transparent rhizoboxes allowing visualization of root architecture over time. Furthermore, electrical resistivity tomography was used to quantify spatial root activity vertically down the soil profile. Results demonstrated a significant reduction in the total root length and network area with the inoculation of FCR. Electrical resistivity measurements indicated that the spatial pattern of water use for each cultivar was influenced differently from infection with FCR over the growing season. Specifically temporal water use can be correlated with FCR tolerance of the varieties marking this investigation the first to link root architecture and water use as tolerance mechanisms to FCR infection. This research has implications for more targeted selection of FCR tolerance characteristics in breeding programs along with improved specific varietal management in-crop.

Keywords: fusarium crown rot; root system architecture; wheat; rhizobox; electrical resistivity tomography; water use efficiency

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1. Introduction

Fusarium crown rot (FCR) caused by the fungal pathogen *Fusarium pseudograminearum* (*Fp*) is a major disease of wheat that can cause significant wheat production losses through both yield reduction and grain quality downgrades worldwide [1,2]. This stubble borne disease reduces not only yield and quality but also nitrogen use efficiency [3–5]. Multiple studies have investigated the effects of FCR on growth, yield and grain quality of infected wheat plants above the soil surface; however, limited research has been conducted into the effects of this disease on plant root systems and their architecture.

Root system structure and function are important characteristics and are responsible for nutrient and water acquisition [6–8]. However, it is often a poorly studied component of plant interactions due to the difficulty of observing root systems in soil [9,10]. Hydroponic studies increase the ease of quantifying root system functions and are commonly used to analyse whole root systems [11,12]. However, these studies are often not reflective of highly dynamic plant: soil interactions, and this artificial environment is a poor surrogate for natural in-field conditions [10,11]. Some root system studies use soil as a growth medium

for the plants, but conclusions are limited by the difficulty in recovering total root biomass from the soil as well as observing root distribution [13]. These studies are also commonly restricted in their ability to measure temporal changes in root system morphology and often assess only one time point in the growth of a crop [10,11,14].

Genetic differences in resistance to FCR, measured as the severity of basal browning, have been studied for decades, but, currently, only limited partial resistance genes have been identified amongst bread and durum wheat cultivars worldwide [15]. Differences in genotypic tolerance (i.e., reduced yield loss in the presence of infection) to FCR do exist [15,16], yet tolerance mechanisms driven by physiological traits are poorly understood. Genotypic variation amongst root systems may help explain wheat varietal differences in tolerance to FCR. For example, root growth angle variation might allow plants to forage deeper in the soil profile [17] reducing the acuity of drought stress and, therefore, the severity of FCR expression. This study was undertaken to determine the impact of FCR infection on root system architecture (primarily exploration as quantified by the root area) and root activity (measured using spatial water uptake). The research also sought to investigate the links between genetic variation in FCR tolerance and root system traits that may explain the reduced relative yield loss of specific wheat varieties under FCR pressure.

2. Materials and Methods

2.1. Soil, Rhizobox Design and FCR Treatments

Clear perspex rhizoboxes with soil dimensions of 950 mm width × 900 mm depth × 20 mm breadth were used for plant growth and to allow root activity to be measured and visualised over time (Figure 1a). Rhizoboxes were seated in racks of 6, oriented at an angle of 20° and were individually wrapped in reflective aluminium sarking to reduce light exposure and prevent overheating and microbial proliferation. Grey Dermosol soil [18] was used with a plant available water-holding capacity (PAWC) of 202 mm/m and available N concentration of 36 mg N/kg soil as nitrate and 4 mg N/kg soil as ammonium. A bulk density of 1.1 g cm^{-3} was established for the entire profile. Two FCR treatments were imposed: uninoculated and inoculated. The inoculated treatment contained a 20 mm band of *Fp* inoculated soil placed in the 10–30 mm depth range. This was prepared by evenly mixing ground wheat seed (0.5–2 mm fraction) that was colonised by 5 different isolates of *Fp* throughout soil at rates of 1 g inoculum/100 g of soil [19]. The uninoculated treatment had 20 mm of soil mixed in a similar manner with ground grain that had been sterilised in an autoclave (60 min at 123 °C). Ten millimetres of soil was added above both treatments to minimise *Fp* colonisation across the soil surface during the experiment.

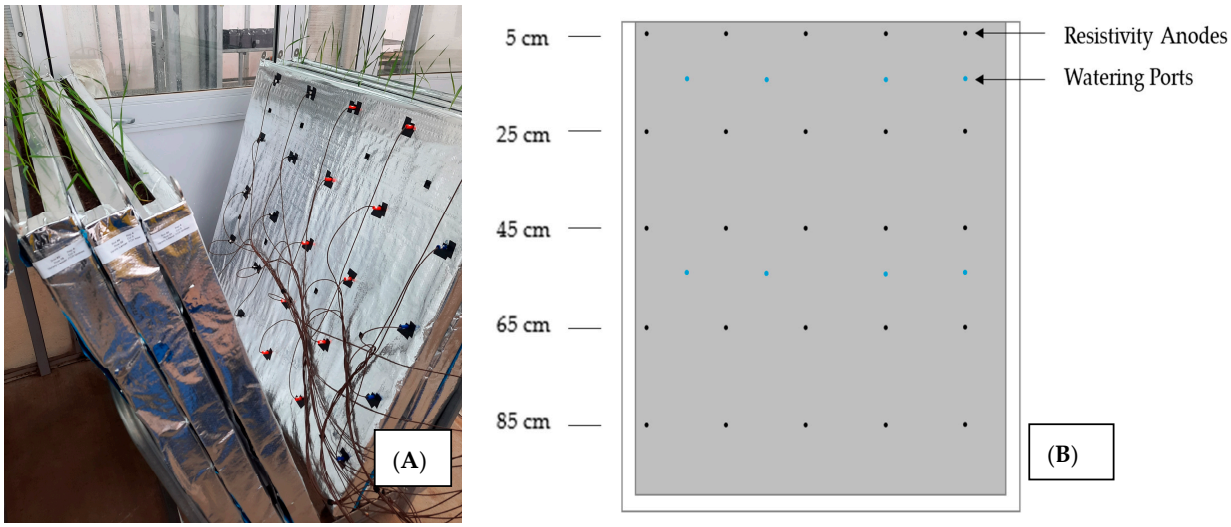

Figure 1. (**A**) Rhizobox configuration in rack with anodes connected. (**B**) Diagrammatic representation of rhizobox anode and watering port configuration.

2.2. Plant Materials and Growing Conditions

Three spring bread wheat varieties (LPRB Lancer, LPRB Hellfire and Suntop) and three durum wheat varieties (DBA Lillaroi, DBA Aurora and Jandaroi) were grown over a six-month period. Variation in tolerance of these varieties to FCR has been previously explored [4,5], and, currently, there are no industry tolerance ratings of wheat and durum varieties to FCR. However, there is no separation in industry FCR resistance ratings between the bread wheats with all three varieties rated as moderately susceptible to susceptible (MS-S). In the durum varieties, DBA Lillaroi is rated as susceptible to very susceptible (S-VS) and the other two varieties as very susceptible (VS) [20]. Bread wheat or durum varieties with the same or very similar resistance ratings were used in this study as the focus of this study was on tolerance mechanisms against FCR. Seed was treated with Vibrance® (Syngenta, BASEL, Switzerland) and Emerge® (Syngenta) at rates of 360 mL/100 kg and 240 mL/100 kg, for standard bunt and smut control and early protection against aphids, respectively. Vibrance® seed dressing also prevented seedling blight in the presence of *Fp* inoculation [21] without preventing FCR infection of plants. Ten seeds of each cultivar were sown below the *Fp* inoculum layer approximately 30 mm below the soil surface and thinned to a population of eight plants per box upon establishment. There were four replicates of each cultivar and treatment combination spatially distributed in a randomised complete block design. The experiment was conducted in an air-conditioned polyhouse complex at Tamworth Agricultural Institute (TAI), Tamworth, NSW from 20 May to 4 November 2022 with a 25 °C day and external ambient night temperature regime. One replicate of rhizoboxes was individually weighed fortnightly to assess water use of the experiment because manual handling of boxes was difficult and risked damaging plants. Rhizoboxes were watered through eight ports distributed across the face of the rhizoboxes, where each port had flow valves to evenly distribute water ingress. This method was designed to reflect dryland conditions where summer-dominant rainfall results in minimal in-crop winter rainfall and where plants predominantly rely on stored soil moisture. Three weeks prior to harvest, rhizoboxes were not watered to dry the soil profile down for soil and root extraction.

2.3. Fertiliser

At planting, rhizoboxes were amended with KCl and urea to ensure that K and N were not limiting, equivalent to 25 kg K/ha and 80 kg N/ha, respectively, evenly mixed in the top 300 mm of soil on the basis of mg/kg for the top 300 mm. This was provided to rectify a known soil K deficiency issue with the soil used and provide adequate N for crop growth.

2.4. In-Crop FCR Measurements

Plants were visually scored for the severity of FCR infection based on the extent of browning of stem bases using a 1–3 scale at full flag leaf emergence (GS 39) with values presented as a crown rot index [19]. This reflected whether all the FCR inoculated treatments visually displayed signs of FCR infection.

2.5. Root Image Acquisition

Roots were imaged approximately every two weeks from establishment until the onset of flowering and then once more at harvest. Images were taken at square inspection sites 100 mm × 100 mm at three fixed locations vertically aligned down the profile at 100 mm, 300 mm and 500 mm. The location of inspection sites avoided watering ports and permanent electrodes. A custom light box was used to remove glare on the perspex caused by sunlight and to provide artificial LED purple and UV light to enhance the roots' contrast against the soil medium (Figure 2) [22]. The camera used was a Sony Alpha a6400 fitted with 16–50 mm lens (Sony Corporation®, Tokyo, Japan). All images were manually focused taken at a distance of 160 mm from the perspex surface.

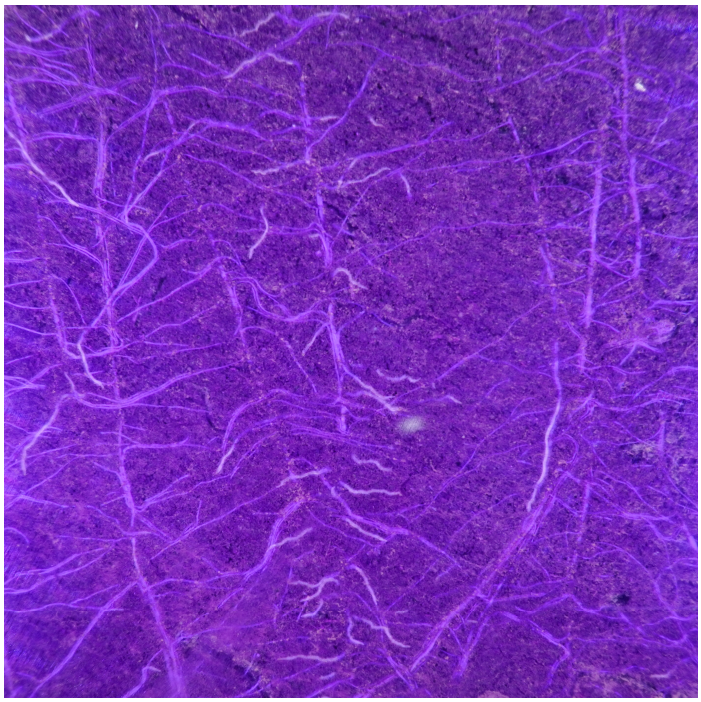

Figure 2. Raw image taken of roots from inspection site using custom LED lightbox.

2.6. Root Image Processing

Using the image processing software Fiji® V2.9.0, each spatially calibrated image was cropped to 90 × 90 mm square [23]. Roots were then segmented using a custom macro, which extracted the blue colour channel subtracting the red colour channel to remove non-root material from the image. The resultant greyscale image was then manually thresholded to extract root matter whilst minimising non-root material. The finished file contained only the visible root matter and minimal non-root material. Rhizovision Explorer® V2.0.3 was used to analyse the root images exported from the image processing step [24]. The root area (mm^2) was a more accurate representation of root architecture compared with the total root length (mm). This is likely due to the software not being able to separate close parallel aligned roots resulting in the underestimation of the total root length. Root morphology data were analysed using mixed models with box and spatial location included in the model as outlined under statistical analysis.

2.7. Electrical Resistivity Tomography Measurements and Analysis

An electrical resistivity tomography (ERT) profile was constructed through the upward facing side of the rhizobox. Resistivity was measured between 25 evenly spaced electrodes on each rhizobox at emergence, GS 30 and GS 65. Seven days prior to analysis, rhizoboxes were watered to field capacity, and the resultant 2D distribution of resistivity was interpreted as a measure of root activity where dry soil (relative to field capacity) indicated greater root uptake of water and therefore water removal. The stainless-steel electrodes (10 mm diameter) were arranged in matrix formation at 200 mm isotropic intervals resulting in 100 observations per rhizobox per time point (Figure 1b). Apparent electrical resistivity was measured with a SuperSting R8IP (Advanced Geosciences, Inc., Austin, TX, USA) using a dipole–dipole array. Apparent resistivity was calculated from measurements of current and the voltage drop [25]). Earth Imager 2D (Advanced Geosciences, Inc.) inversion software was used to interpret collected resistivity readings creating 2D models of the estimated resistivity. Modelled resistivity values were calibrated against measured gravimetric water contents in test boxes based on a calibration of measured resistivity and known volumetric water contents conducted on an unplanted rhizobox. The result of the

ERT data represented gravimetric water content removal after seven days of growth at the three different crop growth stages.

2.8. Harvest Assessments

Plant, tiller and head numbers were immediately counted prior to harvest. Heads from each plant were removed at the base of the peduncle followed by stems keeping the main stem separate from the secondary tillers cut at the soil surface. Both heads and stems were dried at 40 °C for 72 h prior to weighing. Grain was threshed from the collected heads from each rhizobox; the total grain weight (yield) was recorded along with the mass of 500 seeds. Yield was presented as $g \cdot box^{-1}$ as scaling this to standard units t/ha is fraught and misleading. The visual severity of FCR infection on the main stems was assessed at harvest as outlined [4]. Tiller weights and threshed chaff weights were also recorded.

2.9. Statistical Analysis

2.9.1. Harvest Data

The statistical software package R version 4.1.0.143. 2020 [26] was used to fit linear models to the datasets, and analysis of variance (ANOVA) was performed. Model diagnostics were checked, and, where necessary, data were transformed to uphold model assumptions. Post hoc multiple comparisons were performed using Tukey's method (package: lsmeans).

2.9.2. Electrical Resistivity Tomography and Root Data

To determine the variation in apparent resistivity (from ERT) with depth, a linear mixed-effects model was fitted to the inverted data. In this model, depth, variety and FCR were fitted as fixed terms with all interactions. Since variety and FCR were fitted as factors, a fitted line (ERT vs. depth) was given for each of the 12 combinations of variety by FCR. To model the curvature in the data, the inclusion of a spline term as a random effect and its interactions with variety, FCR and variety:FCR provided a curve for each treatment. Factors for replicate (block), box (rhizobox) and line and their interaction with a spline term were also added to reflect the statistical design of the experiment. These account for the blocking effects of Rep, Box and Line. The model was used to predict values of water content (as determined by calibration to be proportional to resistivity) along a depth plane allowing comparison between inoculated and uninoculated treatments.

Linear mixed-effects models were fitted to root area data at each of three depths (100, 300 and 500 mm) to predict the root area over time using the statistical software asreml [27]. Days after sowing (DAS), variety, FCR and all their interactions were fitted as fixed terms. A spline term was added as a random effect in the model, and its interactions with variety, FCR and variety:FCR produced a curve for each treatment. Terms for replicate and box (rhizobox) and their interaction with the spline term were added. Wald test of the fixed terms showed that variety was not significant at any depth ($p = 0.954$). Dropping random terms involving variety showed no significant change in the fit of the model, using log likelihood as a metric, for any term at any depth. Similarly, there was no significant effect of FCR on the root area at a depth of 50 cm. The models were refitted dropping all terms involving variety and dropping all terms involving FCR from the model for the 50 cm depth.

3. Results

3.1. Harvest Measurements

3.1.1. Inoculation Response

Infection severity (measured as a crown rot index through visual browning) increased within a range of 0–28% in the uninoculated treatments to a range of 57–96% in the inoculated treatments (Figure 3) ($p < 0.001$) [19]. There was no significant interaction in visual browning between FCR and variety ($p < 0.494$), indicating that all cultivars had increased infection in response to *Fp* inoculation.

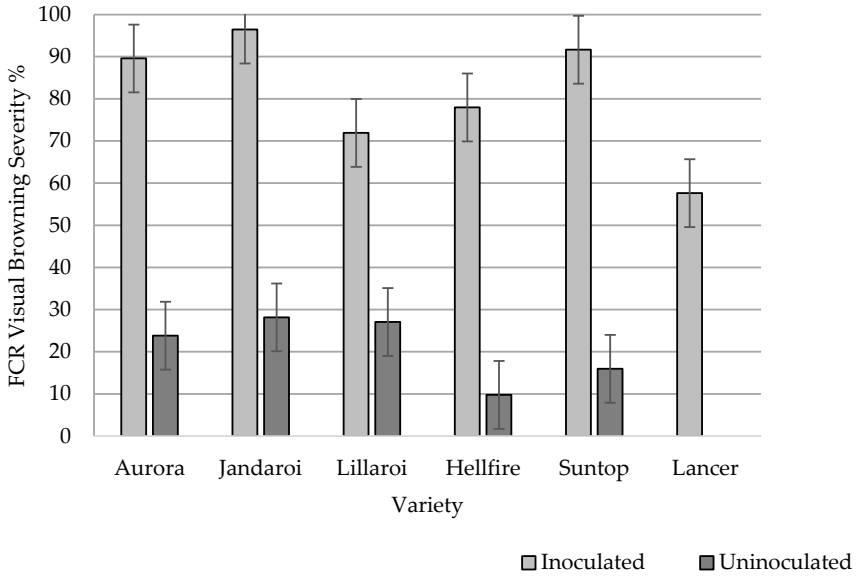

Figure 3. Fusarium crown rot (FCR) visual browning severity of three bread *Triticum aestivum* L. (LRPB Lancer, Suntop and LRPB Hellfire) and three durum *Triticum durum* Desf. (DBA Lillaroi, Jandaroi and DBA Aurora) wheat varieties when inoculated or uninoculated with *Fusarium pseudograminearum*. Error bars represent standard error.

3.1.2. Yield and Grain Quality

Fusarium crown rot (*Fp*) inoculation significantly reduced yield in two bread wheat varieties (LPRB Lancer 11% and LPRB Hellfire 12%) and one durum variety (DBA Aurora 30%). There was also a numeric decrease in yield in the durum variety DBA Lillaroi by 18%; however, this was not statistically significant (Figure 4). No significant yield penalty was observed in Suntop and Jandaroi varieties in response to *Fp* inoculation (Figure 4). The average grain weight was significantly reduced with the inoculation of FCR by 9.6% across all varieties from 28.9 g per 500 grains to 27.6 g per 500 seeds ($p = 0.049$). The durum varieties had significantly higher grain protein contents (GPCs) at 10.5% as compared with 9.1% for bread wheat ($p < 0.0001$); there was no significant effect from FCR inoculation on GPC.

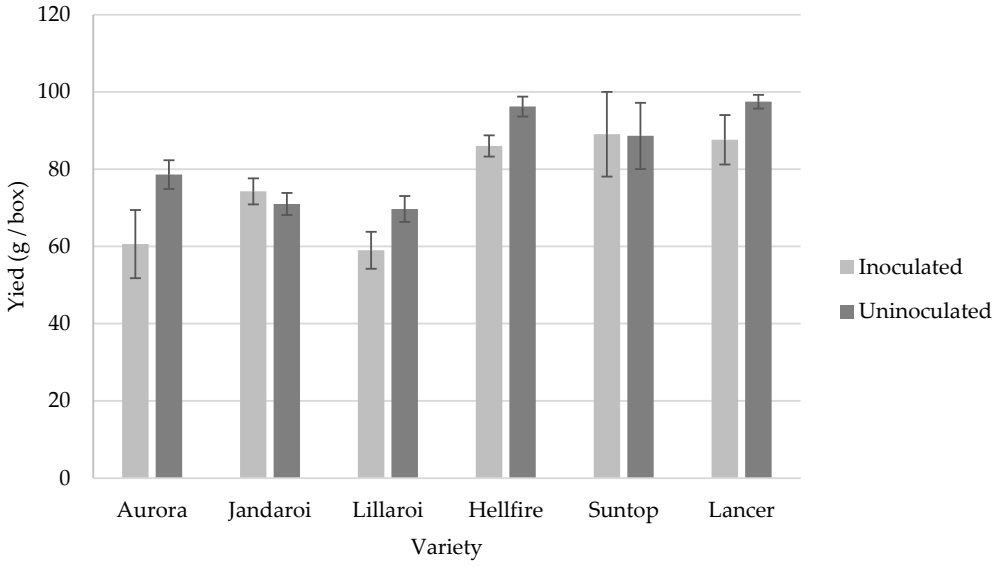

Figure 4. Grain yield per box of three bread (LRPB Lancer, Suntop and LRPB Hellfire) and three durum (DBA Lillaroi, Jandaroi and DBA Aurora) wheat varieties when inoculated or uninoculated with Fusarium crown rot. Error bars represent standard error.

3.2. Water Measurements

3.2.1. Water Measurements at Stem Elongation (GS 30)

The pattern of early season (GS 30) water use was significantly different between FCR inoculation treatments within varieties ($p < 0.05$). LPRB Lancer was the only variety in which the inoculated treatment used significantly shallower (>40 cm) soil water as compared with the uninoculated treatment (Figure 5A). The other varieties, with the exception of Jandaroi and Suntop, did not show a significant reduction in water use following *Fp* inoculation. Jandaroi and Suntop behaved contrary to the other varieties recording an increase in water use in the inoculated compared with the uninoculated treatment where significant differences in water use was observed below 60 cm and 70 cm of depth for the two cultivars, respectively (Figure 5B,C).

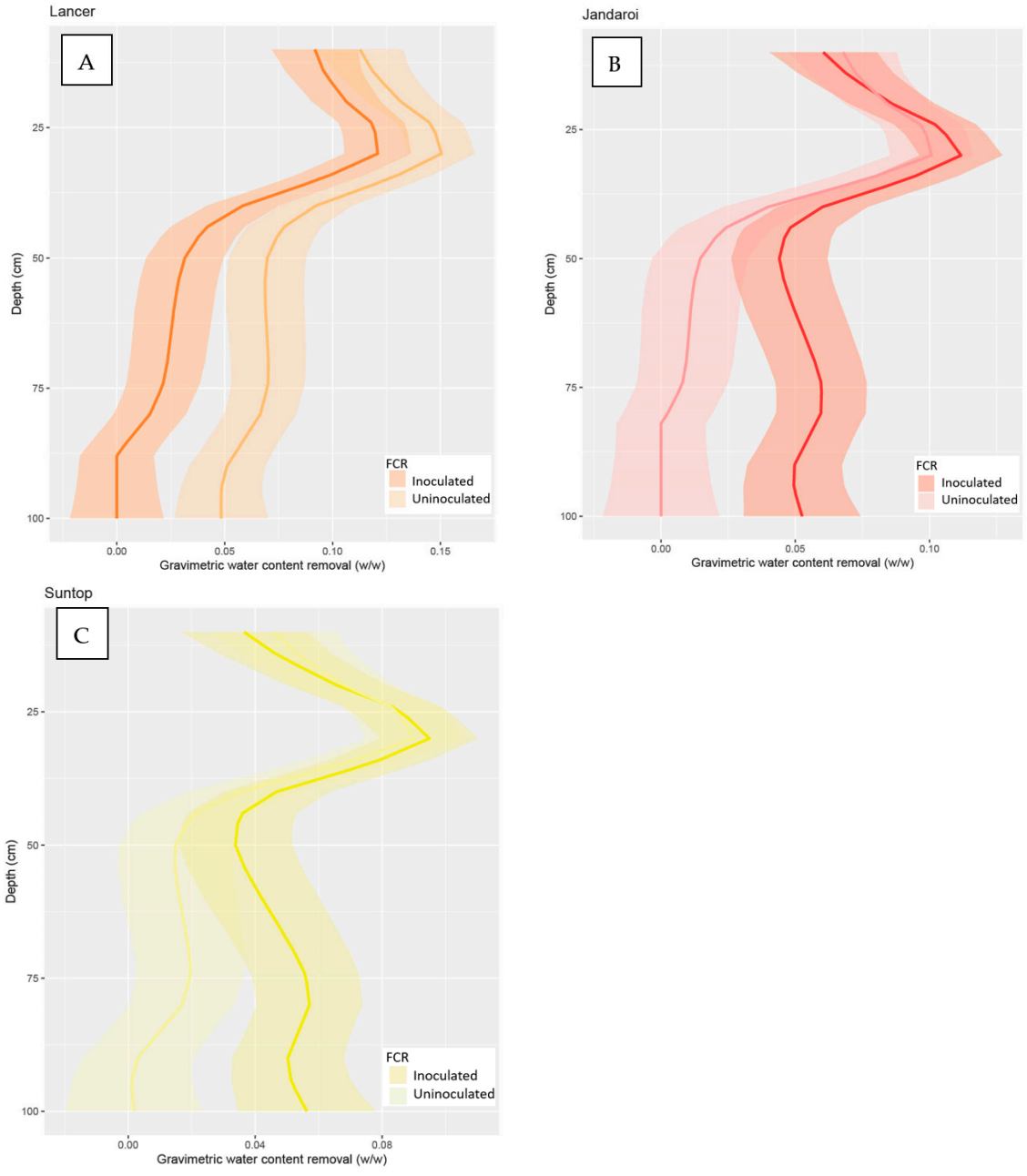

Figure 5. (**A**) LRPB Lancer, (**B**) Jandaroi and (**C**) Suntop wheat at GS 30 gravimetric water content removal (w/w) after seven days from full soil water profile. Lines indicate mean response, and shading indicates FCR inoculation status; error is depicted as 95% confidence limits shading extent.

3.2.2. Water Measurements at Flowering (GS 60)

The pattern of late season (GS 60) water use was significantly different between FCR inoculation treatments within varieties ($p < 0.05$) and was greater than earlier growth stage measurements. All varieties excluding LRPB Lancer and DBA Lillaroi suggested an increase in water use in the *Fp* inoculated compared with the uninoculated treatment, but these differences were rarely significant (Figure 6A,C). LRPB Hellfire below 55 cm of depth and DBA Aurora at 75–100 cm of depth were the only points where patterns of water use significantly diverged (Figure 6A,B). By contrast, LRPB Lancer and DBA Lillaroi indicated a decrease in water use in the *Fp* inoculated compared with the uninoculated treatment; however, significance was only observed between 80 and 90 cm of depth in LRPB Lancer (Figure 6C).

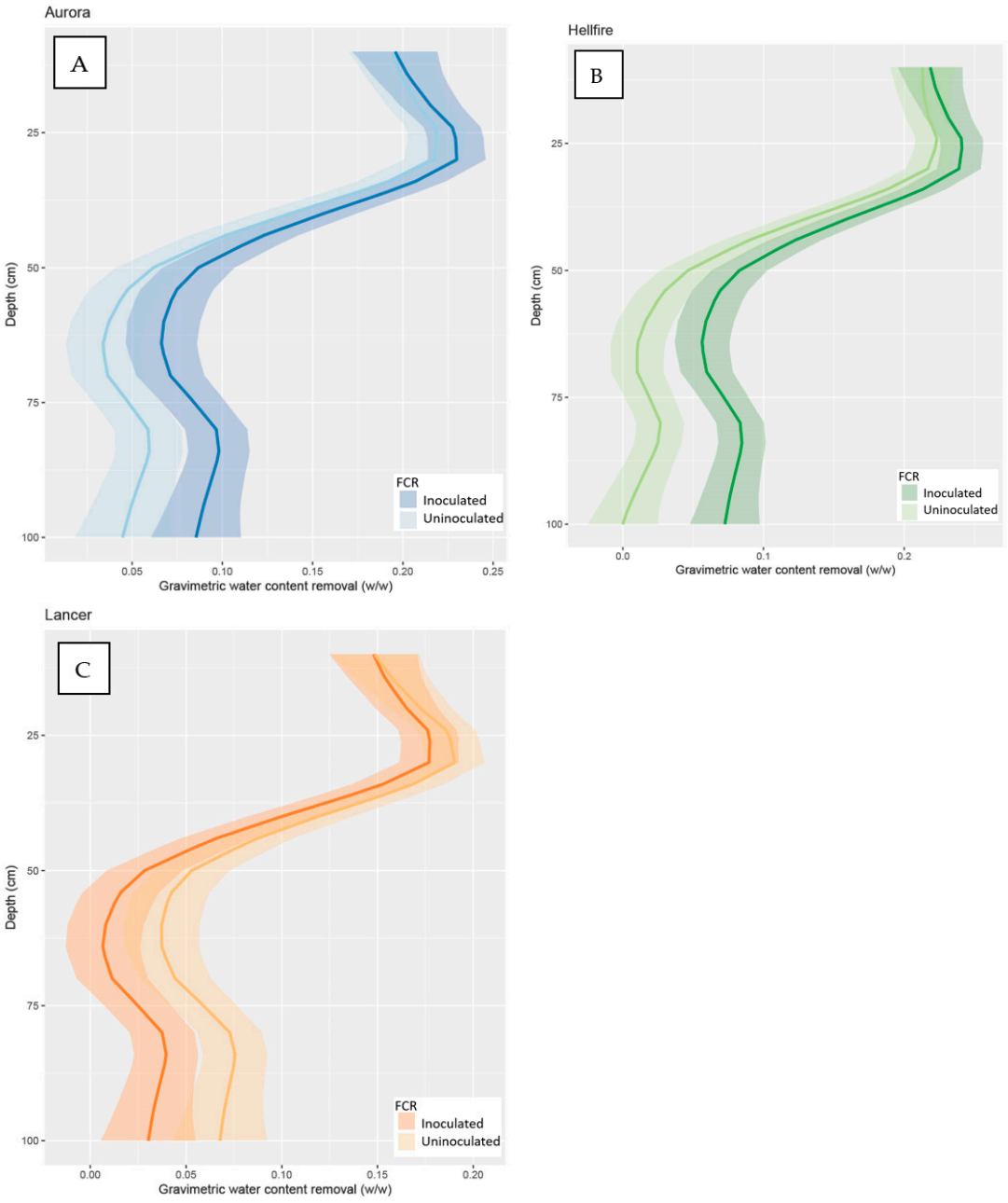

Figure 6. (**A**) DBA Aurora, (**B**) LRPB Hellfire and (**C**) LRPB Lancer wheat at GS 60 gravimetric water content removal (w/w) after seven days from full soil water profile. Lines indicate mean response, and shading indicates FCR inoculation status; error is depicted as 95% confidence limits shading extent.

3.3. Root Architecture Measurements

Inoculation with FCR significantly reduced the root area at depths of 10 cm and 30 cm throughout the experiment when averaged across six varieties ($p = 0.031$; Figure 7). No significant difference was observed at a depth of 50 cm ($p > 0.05$; hence was not presented). No significant difference was observed between varieties at any of the observed depths ($p > 0.05$).

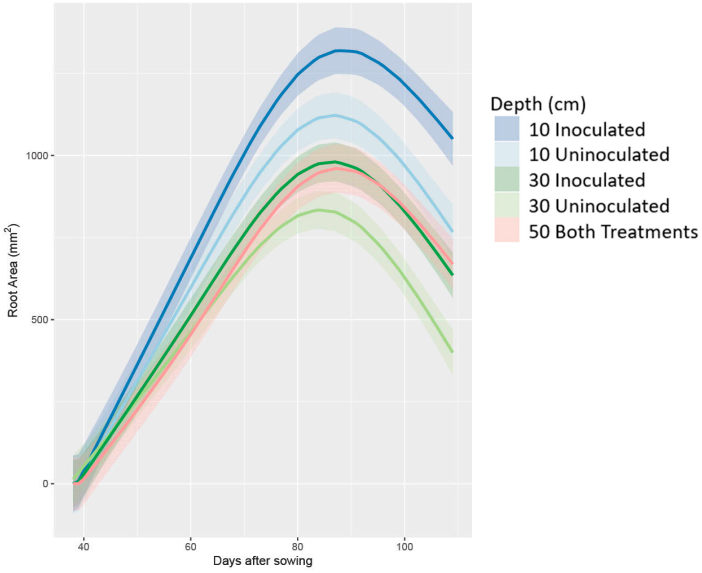

Figure 7. Mean root area recorded from 35 d after sowing to harvest averaged across three bread (LRPB Lancer, Suntop and LRPB Hellfire) and three durum (DBA Lillaroi, Jandaroi and DBA Aurora) wheat varieties when inoculated or uninoculated with Fusarium crown rot. Lines indicate mean response, and shading indicates FCR inoculation status; error is depicted as 95% confidence limits shading extent.

4. Discussion

4.1. Electrical Resistivity Tomography and Spatial Water Use

We hypothesised that variability in FCR tolerance within current commercial varieties of bread and durum wheats can be related to and dependent on rooting characteristics. Due to FCR being a vascular restrictive disease [28], an increase in adventitious root systems might allow greater tolerance due to increased access to soil moisture and nutrients [14,29], though hydraulic conductivity would still presumably be constrained. Resistivity measurements demonstrated very low variability in water use within the topsoil (top 30 cm) between both variety and FCR infection at all time points. However, much greater variability was observed between all six varieties in the subsoil (below 30 cm) indicating differences in subsoil root activity. It has previously been demonstrated that FCR infection reduces overall water use [4,30]; however, it appears that reduction in water use is not constant for the duration of the season with greater differences observed early in the season.

Four patterns of water use were identified. Firstly, Suntop is the only variety that has demonstrated tolerance to FCR [20]. This is further supported in this experiment where no significant yield loss was observed despite clear differences between infection levels. Interestingly, root activity (water usage at depth) appeared to be stimulated in both early and late growth stages in the FCR inoculated treatment, a result also observed in Jandaroi. This appears to represent a potential tolerance mechanism being exhibited by Suntop (and Jandaroi). Water and solute transport from the root system is inhibited by the higher FCR infection, and the root system may compensate with additional root activity by increasing either the tissue osmotic potential or the root length. While differences were not observed in the root area intercepted on the rhizobox surface, it remains possible that undetectable

differences in the root length can have substantial impact on water uptake (given inherent root system variability and relatively low numbers of replicates, meaning detection limits of analysis remain high). The stimulation of plant growth has been observed in response to pythium [31] and plant growth promoting fungi in fusarium [32] infection in other studies, and it is possible that this instance provides further evidence for some stimulation effect in response to low levels of FCR infection. The lack of similar response on other genotypes might indicate genotypic variation in plant responses to the pathogen and suggest different potential tolerance strategies that can be further investigated for breeding targets should they prove to be related.

The second pattern of water use was observed in LRPB Lancer, which depicted the largest significant reduction in water use compared with all other varieties at both time points. Water use was reduced in early and late stages in the presence of FCR infection. In this cultivar, inoculation treatments imposed clear differences between infection levels with 58% infection achieved in inoculated treatments, and uninoculated treatment control displayed 0% infection. Correspondingly, yield was also reduced in the inoculated treatment by 11%. Water use both at early and late growth stages was suppressed by the presence of *Fp*, particularly at depth >30 cm. We hypothesize that the greater differences observed in deep water use are due to the relatively lower root length density at depth; however, significant differences in the root area were not seen in this experiment. Surface layers with more dense roots allow comprehensive drawdown of soil water by neighbouring root systems even when the water uptake rate is compromised by limited hydraulic conductivity of the root system caused by FCR inhibition of water transport. This is consistent with previous studies that have identified the suppression of transpiration, particularly in early growth stages in the presence of FCR [4].

The third pattern of water use was observed in DBA Aurora and LRPB Hellfire, which demonstrated a decrease in yield associated with *Fp* inoculation. Low to moderate infection rates were observed in the uninoculated treatments and high levels in the *Fp* inoculated treatment. The pattern of water use for these cultivars was slightly different with no significant difference observed between early water use but trending down with later season water use being increased. This pattern may conform to the previously described pattern with Jandaroi and Suntop, where later season expression of upregulation of root activity occurs.

The fourth pattern of water use was observed in the durum variety DBA Lillaroi. DBA Lillaroi displayed no significant effects between *Fp* inoculation treatments in water use or yield as all treatments were suppressed by FCR. This cultivar is highly susceptible (S-VS) to FCR and achieved 72% infection, but the uninoculated control also had 27% infection. This susceptibility is supported by its total average yield, which was amongst the lowest of the cultivars selected, which may not be the case in the absence of FCR. While the infection rate is not the highest, the influence on yield appears to be severe suggesting that there might be genetic differences associated with cultivar susceptibility and that some cultivars have more limited tolerance to low levels of infection. In this instance, DBA Lillaroi did not demonstrate any significant variation in the pattern of water use between inoculated and uninoculated treatments at any time point, which might indicate that even at the relatively low levels of infection, DBA Lillaroi had highly suppressed water use.

These results align with previous research [4,30] and provide strong evidence for associated interactions between FCR tolerance and rooting architecture, particularly subsoil activity. There appears to be multiple different patterns of response amongst different bread and durum wheat varieties to FCR and water use, which indicates that potential tolerance mechanisms through root architecture are not uniform across the cultivars. However, lack of consistency in rooting responses between varieties to FCR infection should be welcomed as an opportunity as multiple rooting mechanisms exist to potentially be exploited to build greater tolerance.

4.2. Spatial Root Area and Its Importance in Tolerance

Observed changes in spatial water use are only indicative of an internal physiological change. At present, no major genes are linked to either tolerance or resistance to FCR in wheat [16]. Simpfendorfer, McKay [33] highlight that there is still a large limitation to effectively breeding for resistance and/or tolerance to FCR as there remains lack of knowledge concerning the underlying mechanisms and their interactions with both biotic and abiotic factors. Quantifying root architectural characteristics allows for the first-time validation of differing tolerance to FCR based on plant physiological characteristics. Observations from this study highlight the importance of productive root systems particularly in their ability for not only spatial exploitation but temporal resource acquisition as well. The results from this study demonstrated a significant reduction in the rooting area from 10 cm to 30 cm of soil depth with FCR inoculation. The magnitude of separation between inoculated and uninoculated treatments increased over time, indicating that early infection compromises the plant's roots system and has long-term effects on growth and development. Furthermore, this observation highlights the significant effect of FCR on rooting systems even in non-water-limited scenarios where only relatively small reductions in yield are observed similar to our previous study [4].

Unlike results captured in the ERT measurements, no statistical significance was observed between the varieties tested. This is likely a result of not having the sensitivity in the measurements taken across the course of this experiment. The method used was similar to that of Schmidt, Lowry [34] where observations were made in situ through a two-dimensional pane of the roots. This method, although improved by growing the rhizoboxes on a 20° angle to aid in gravitropism maximizing roots on the visible pane, still did not manage to capture the entire root system. However, if the aim is to observe roots over time, in a true soil base medium, it would be difficult to improve upon this method. Increased observation points where roots can be imaged at more growth stages may improve sensitivity.

5. Conclusions

This is the first study to demonstrate the impact of FCR on spatial water use of bread and durum wheat plants as well as the first application of ERT to determine root activity through time. This study investigated the impact FCR has on water dynamics in the soil and observed that a wide range of varietal rooting responses exist in response to *Fp* inoculation. The results suggest that there can be an upregulation of root activity and solute transport to compensate for reduced hydraulic conductivity caused by FCR infection. Furthermore, multiple observed patterns of responses in water use between genotypes may provide an insight to tolerance mechanisms that can be investigated in further studies.

Author Contributions: Conceptualization, M.B., R.J.F. and S.S.; methodology, M.B., R.J.F. and S.S.; software, M.B. and R.J.F.; validation, M.B.; formal analysis, M.B., S.H. and R.J.F.; investigation, M.B.; resources, M.B.; data curation, M.B.; writing—original draft preparation, M.B.; writing—review and editing, M.B., R.J.F., M.S., S.H., C.G. and S.S.; visualization, M.B.; supervision, R.J.F., M.S., C.G. and S.S.; project administration, R.J.F. and S.S.; funding acquisition, R.J.F., M.S., C.G. and S.S. All authors have read and agreed to the published version of the manuscript.

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