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Aerenchyma Formation in Adventitious Roots of Tall Fescue and Cocksfoot under Waterlogged Conditions

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Abstract: The formation of aerenchyma in adventitious roots is one of the most crucial adaptive traits for waterlogging tolerance in plants. Pasture grasses, like other crops, can be affected by waterlogging, and there is scope to improve tolerance through breeding. In this study, two summer-active cocksfoot (*Dactylis glomerata* L.) cultivars, Lazuly and Porto, and two summer-active tall fescue (*Lolium arundinaceum* Schreb., syn. *Festuca arundinacea* Schreb.) cultivars, Hummer and Quantum II MaxP, were selected to investigate the effects of waterlogging on root growth and morphological change. Cultivars were subjected to four periods of waterlogging treatments (7, 14, 21 and 28 days), while comparable plants were kept under free drained control conditions. The experiment was arranged as a split–split plot design, with waterlogging treatments (waterlogged, control) considered as main plots, time periods (days of waterlogging) as subplots and cultivars as sub-subplots. Plants began to show signs of waterlogging stress 14–21 days after the onset of waterlogging treatments. There were no significant differences in shoot biomass between the waterlogged and control plants of any cultivar. However, waterlogging significantly reduced root dry matter in all cultivars, with greater reduction in cocksfoot (56%) than in tall fescue (38%). Waterlogging also led to increased adventitious root and aerenchyma formation in both species. Cocksfoot cultivars showed a greater increase in adventitious roots, while tall fescue cultivars had a greater proportion of aerenchyma. Both cultivars within each species showed similar responses to waterlogging treatments. However, an extended screening program is needed to identify whether there are varietal differences within species, which could be used to discover genes related to aerenchyma or adventitious root formation (waterlogging tolerance) for use in breeding programs.

Keywords: aerenchyma formation; waterlogging tolerance; tall fescue; cocksfoot; grasses

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1. Introduction

Waterlogging is a productivity constraint for many temperate agricultural regions of southern Australia, where it can reduce yields of crops, including maize (*Zea mays* L.) [1], wheat (*Triticum aestivum* L.) [2–4], barley (*Hordeum vulgare* L.), field peas (*Pisum sativum* L.), lupins (*Lupinus albus* L.) and canola (*Brassica napus* L.) [2,5]. The losses in crop production invariably result in lower returns and profit [6–8]. Waterlogging has been estimated to cause an annual agricultural production loss of more than 71 billion USD worldwide [9].

One serious consequence of waterlogging is the rapid reduction in oxygen availability within soil [10], where gas exchange between the roots and the atmosphere is limited due to saturation of soil pores [6,11]. As a result, the energy produced by root respiration is restricted, leading to a substantial decrease in plant growth [12,13]. While the application of management approaches [14,15] has been shown to mitigate waterlogging effects, the development of waterlogging-tolerant varieties is considered as the most economical way to reduce losses [16]. Waterlogging-tolerant plants adapt to waterlogging stresses through changes in anatomical [17,18], metabolic [19] and/or morphological [20] processes and mechanisms. However, tolerance is variable among species [9].

The formation of aerenchyma in adventitious roots is a common morphological response of plants under waterlogging stress [16,21]. Adventitious roots can develop as a part of the root system, but differ from primary roots in that they emerge from locations such as stem nodes and hypocotyls [22]. Under waterlogged conditions, adventitious roots support and replace primary roots in order to improve gas diffusivity along and across the roots [23,24] via gas spaces called aerenchyma. "Aerenchyma" is a term used to describe plant tissues containing larger gas spaces than those commonly seen in intracellular spaces [25]. It forms in the shoots and roots of wetland species and in some dryland species under oxygen stress [25]. There are two basic types of aerenchyma: lysigeny and schizogeny. Lysigeny aerenchyma is formed by cell death, which produces gas spaces, whereas schizogeny is formed by cell separation without cell death [26]. The formation of root aerenchyma produces an internal pathway to transport gases, which enables roots to grow in saturated soils by providing more internal oxygen diffusion to the root tips and to the rhizosphere [11].

In addition to perennial ryegrass (*Lolium perenne* L.) and halaris (*Phalaris aquatica* L.), cocksfoot (*Dactylis glomerata* L.) and tall fescue (*Lolium arundinaceum* Schreb. Syn. *Festuca arundinacea* Schreb.) are the most widely sown, productive, and persistent introduced temperate perennial grasses grown for livestock production in Australia. Tall fescue is known for its tolerance of waterlogging, while cocksfoot is suited to well drained soils [27]. Variation in the soil type within fields poses challenges for selecting the species to sow, and mixes of species are often used. Improving the tolerance of species to waterlogging through breeding is a potential way to mitigate its effects. Identifying the mechanisms for tolerance and inter- and intra-specific variation in tolerance is the first step in a strategic breeding effort. There have been many glasshouse and field studies focusing on the effects of waterlogging on grass growth and biomass production [28–30], but there has been little research on the root morphological response, which has been shown as a crucial adaptive trait for waterlogging tolerance [13,30]. Changes in root morphological responses is a likely mechanism for tolerance. This study aimed to investigate the root morphological response of two perennial grass species (cocksfoot [*Dactylis glomerata* L.] and tall fescue [*Lolium arundinaceum* Schreb.]) in response to waterlogging.

2. Materials and Methods

2.1. Seed Materials and Preparations

A pot experiment was conducted at the Tasmanian Institute of Agriculture's Mt. Pleasant Laboratories in Launceston, Tasmania from May to August 2020. Two continental cultivars of tall fescue (TF) (*Lolium arundinaceum* Schreb.), cv. Hummer and cv. Quantum II MaxP, and two continental cultivars of cocksfoot (CF) (*Dactylis glomerata* L.), cv. Lazuly and cv. Porto, were used in this study (Table 1). Seeds were germinated in plastic containers on filter paper with distilled water for 4 days. Seedlings were then transferred to pots (70 mm diameter × 210 mm deep) filled with potting mix with one plant per pot. Pots were placed under glasshouse conditions and positioned in a shallow tray of water, which was maintained at 30 mm depth throughout the 5 weeks of establishment. All pots were then moved to tanks outside of the glasshouse for 1 week before implementing waterlogging treatments.

2.2. Experimental Design and Treatments

The experiment was arranged in a split–split plot design with treatments (waterlogging and control) considered as main plots, time periods (days of waterlogging) as subplots, and cultivars (2 cultivars of TF and 2 cultivars of CF) as sub-subplots. The experiment was conducted in four main plot tanks considered as four blocks. Each tank was divided into two sub-tanks for waterlogging and control conditions. Water in the control treatments was maintained at 30 mm above the bottom of the tanks (the base of the pot) for the whole period of the experiment. In the waterlogged treatments, water was raised up to the potting mix surface of the pots for the duration of the waterlogging treatments. Plants were sampled just before waterlogging (day 0), and at 7, 14, 21 and 28 days after waterlogging.

Table 1. Seed materials used in the experiment.

Common (Genus Species)	Cultivar	Reference
Cocksfoot (*Dactylis glomerata* L.)	Lazuly	[31]
	Porto	[32]
Tall fescue (*Lolium arundinaceum* Schreb.)	Hummer	[33]
	Quantum II MaxP	[34]

2.3. Measurements

For each treatment, two pots of each cultivar in each tank were randomly chosen for sampling (8 pots per cultivar) at each harvest time (days 0, 7, 14, 21 and 28).

2.3.1. Shoot and Root Measurements

The number of live tillers was measured in-situ before harvest. Plants were then removed to collect shoot and root data. The roots of each plant were carefully washed using a low-pressure hose. The number of adventitious roots were counted, and root length was measured from the surface of the soil to a depth which the majority of the root tips had reached (both primary and adventitious). Shoot herbage and roots were then separated and dried in a fan-forced oven at 60 °C for 48 h to collect dry matter (DM) biomass.

2.3.2. Chlorophyll Content (SPAD Reading)

Leaf greenness/chlorophyll content was measured on the second youngest fully expanded leaf of each plant prior to destructive measurements using a portable chlorophyll meter (SPAD-502 Plus; Konica Minolta Sensing, Osaka, Japan). Three measurements were taken from each plant, one at 1/3, one at 2/3 and one in the middle of the leaf length, from which the mean was then calculated.

2.3.3. Aerenchyma Formation

Two roots with approximately the same diameter from the mature root zone of the sampled plants (50–60 mm from the root tips) were cut into transversal sections using a razor blade. The root transversal sections were then observed and captured under a bright field light microscope (Figure 1a). Subsequently, the captured images were analyzed by ImageJ software based on the method described by Schindelin et al. [35]. The percentage of aerenchyma was calculated by dividing the number of pixels in aerenchyma spaces (Figure 1d) by the number of pixels in the total root section area (Figure 1c).

2.4. Statistical Analysis

Each output variable was analyzed using generalized linear mixed model procedures in PROC GLIMMIX (SAS software version 9.4. Copyright (c) 2002–2012 by SAS Institute Inc., Cary, NC, USA). The main effects of treatment, species and days of waterlogging were all treated as fixed effects. The effect of the cultivar was nested within species. The model had a split–split plot design, and to achieve this, the following were included as random effects in the model: Tank Treatment*Tank Day*Treatment*Tank. Denominator degrees of freedom were estimated using the between–within (bw) method. The slice option in lsmeans statements was used to specify pre-determined subsets of interactions, and Tukey's statistic was used to test differences ($p < 0.05$) among means. Graphs were made with R-language software version 3.6.2 [36].

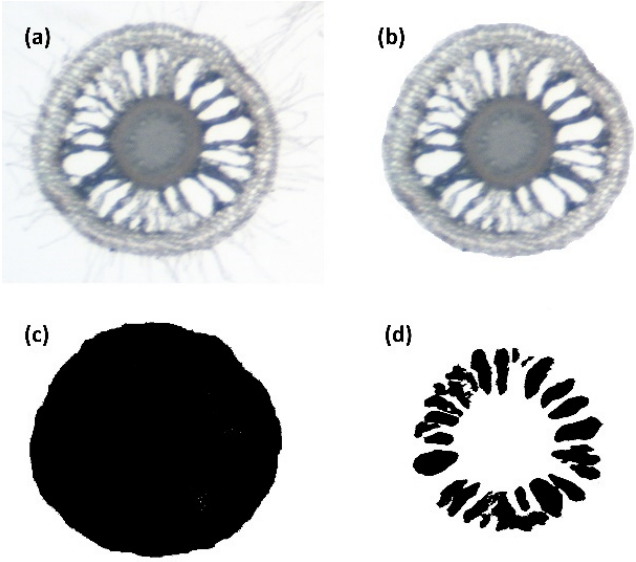


Figure 1. Representative images used ImageJ software to determine aerenchyma area. (**a**) Original digital image of root cross section, (**b**) cleaned image of root cross section after removing root hairs, (**c**) total root cross sectional area, (**d**) aerenchyma spaces. The percentage of aerenchyma was calculated by dividing the number of pixels in (**d**) by the number of pixels in (**c**).

3. Results

3.1. Waterlogging Induced the Formation of Aerenchyma in Adventitious Roots of Tall Fescue and Cocksfoot Plants

Waterlogging significantly ($p < 0.05$) increased the formation of adventitious roots in both CF and TF species (Figure 2). For CF, the significance between control and waterlogged treatment was observed from day 14 onwards, and it was observed in day 28 of TF plants (Figure 2). From 14 days onwards, waterlogging stress resulted in a far greater number of adventitious roots in CF, which was 100% higher than control CF plants, while the number of roots in TF was approximately 17% higher in waterlogged plants compared to controls. Adventitious roots also formed in control plants of both species, but to a greater extent in TF plants following 28-day waterlogging (Figures 2 and 3).

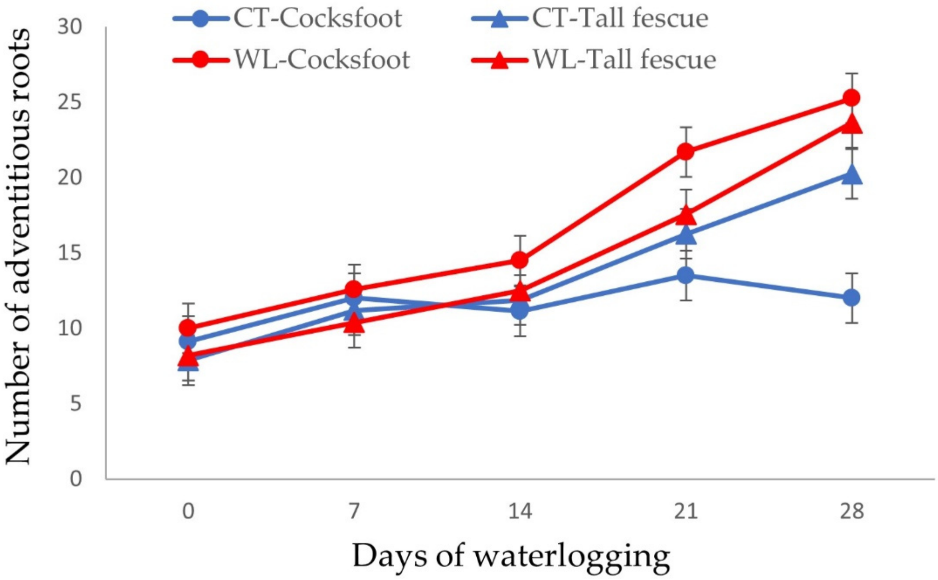
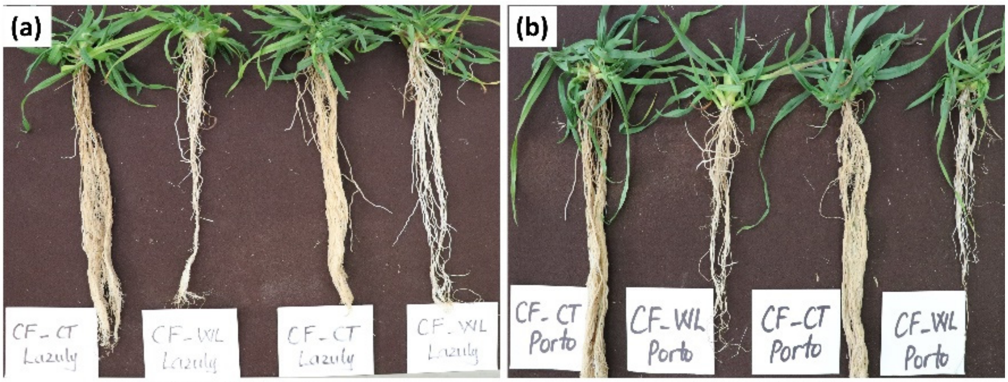
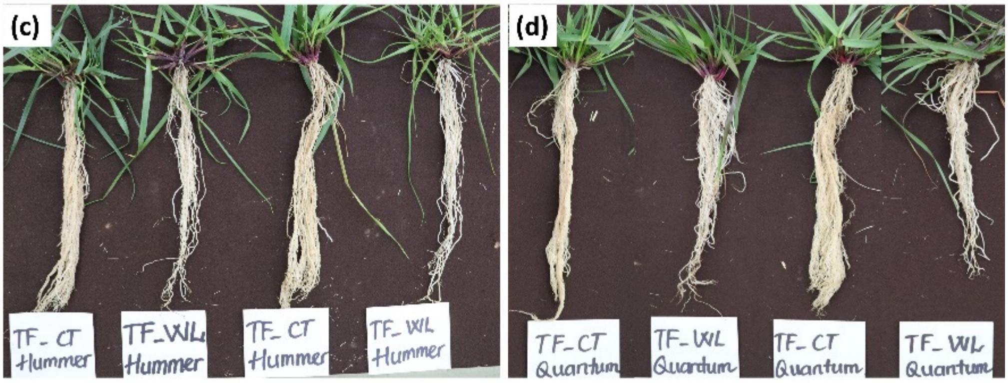


Figure 2. Number of adventitious roots of cocksfoot and tall fescue at different stages of treatment under both control (CT) and waterlogging (WL) conditions. Values given are least squares means of four replicates and error bars indicate 95% confidence intervals of the least squares means.



Cocksfoot cv. Lazuly Cocksfoot cv. Porto



Tall fescue cv. Hummer Tall fescue cv. Quantum II

Figure 3. Adventitious roots of 4 cultivars following 28-day waterlogging: a) Lazuly, b) Porto, c) Hummer and d) Quantum II; CF-CT and CF-WL indicate cocksfoot–control and cocksfoot–waterlogging, respectively; TF-CT and TF-WL indicate tall fescue–control and tall fescue–waterlogging, respectively. White-colored roots indicate adventitious roots, whereas darker roots are primary roots.

Waterlogging significantly ($p < 0.05$) induced the formation of aerenchyma in the adventitious roots of both CF and TF (Figure 4). It also formed in plants under control conditions, with TF having a slightly higher proportion than CF (Figure 4). This significant difference was evident after 14 days of waterlogging and continued throughout the treatment period. Waterlogging-induced aerenchyma increases were significantly ($p < 0.05$) greater in TF than CF (Figure 4). By the end of the experiment, the aerenchyma area increased from 5% in the control to 13% in the waterlogged CF plant roots, and from 9% in the control to 20% in the waterlogged TF plant roots.

3.2. Morphometric, Chlorophyll and Biomass Measurements

There was no significant ($p > 0.05$) effect of waterlogging on tillering for the first 14 days of treatment (Table 2). However, by day 28 of continuous waterlogging, tiller numbers were significantly ($p < 0.05$) lower for waterlogged plants than comparable control plants in both species, with TF showing a greater reduction (28%) than CF (19%) (Table 2).

Waterlogging had significant effects on leaf chlorophyll content (measured by SPAD readings) beyond 14 days of waterlogging, with SPAD readings being lower in waterlogged plants of both species. At the end of the treatment periods (day 21 and 28) SPAD readings were significantly ($p < 0.05$) lower in waterlogged CF plants compared to waterlogged TF plants, whereas there were no significant ($p > 0.05$) differences in the control plants of both species (Table 2).

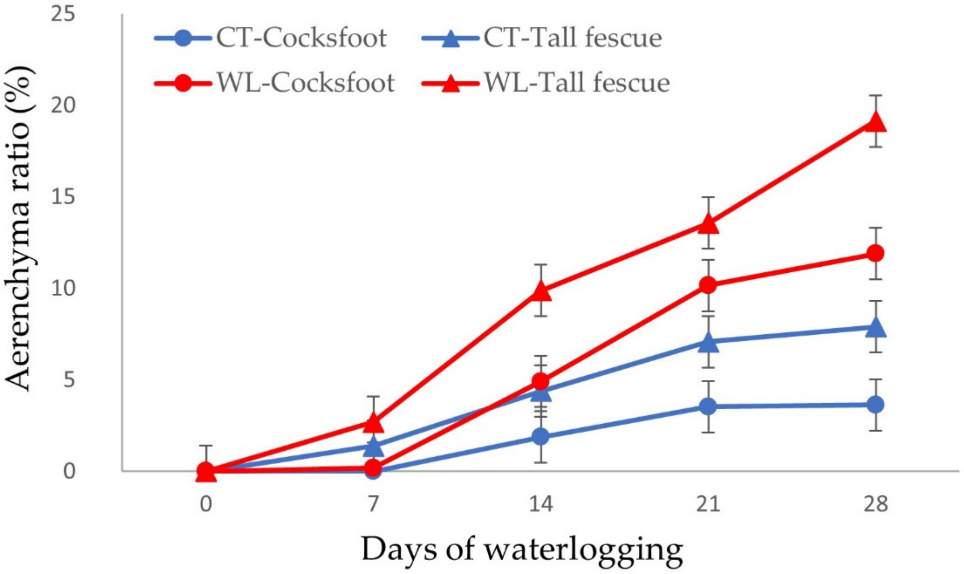


Figure 4. Formation of aerenchyma in the adventitious roots of cocksfoot and tall fescue at different stages of treatments under both control (CT) and waterlogging (WL) conditions. Values given are least squares means of four replicates and error bars indicate 95% confidence intervals of the least squares means.

Table 2. Morphometric, biomass and chlorophyll measurements of tall fescue and cocksfoot in response to waterlogging. Values presented are least squares means of 4 replicates.

Species	Treatment	Day-0	Day-7	Day-14	Day-21	Day-28	Reduction Ratio * (%)
			Number of live tillers				
Cocksfoot	Control	3.50	7.56 ^{ab}	10.44 ^{ab}	13.88 ^{b}	14.56 ^{b}	
	Waterlogging	3.56	6.63 ^{b}	9.25 ^{b}	11.75 ^{c}	11.81 ^{c}	−18.9
Tall fescue	Control	4.31	9.25 ^{a}	11.94 ^{a}	17.75 ^{a}	22.81 ^{a}	
	Waterlogging	4.13	8.50 ^{ab}	11.25 ^{a}	14.81 ^{b}	16.31 ^{b}	−28.5
			SPAD reading				
Cocksfoot	Control	26.07 ^{b}	36.17 ^{b}	41.13 ^{a}	40.43 ^{a}	38.38 ^{a}	
	Waterlogging	27.21 ^{ab}	36.30 ^{b}	36.47 ^{b}	32.02 ^{b}	30.02 ^{c}	−21.8
Tall fescue	Control	29.57 ^{a}	39.84 ^{a}	41.14 ^{a}	41.61 ^{a}	39.99 ^{a}	
	Waterlogging	29.19 ^{a}	37.49 ^{ab}	37.68 ^{b}	35.26 ^{c}	33.50 ^{b}	−16.2
			Root length (cm)				
Cocksfoot	Control	18.84	23.03	22.81 ^{a}	24.47 ^{a}	26.75 ^{a}	
	Waterlogging	18.66	22.72	18.59 ^{b}	19.88 ^{b}	18.53 ^{c}	−30.7
Tall fescue	Control	18.00	21.59	21.75 ^{a}	24.63 ^{a}	24.94 ^{ab}	
	Waterlogging	17.00	21.03	18.91 ^{b}	22.63 ^{a}	24.22 ^{b}	−2.9
			Shoot dry matter (g/plant)				
Cocksfoot	Control	0.08	0.25	0.44	0.61 ^{c}	0.83 ^{b}	
	Waterlogging	0.12	0.27	0.49	0.63 ^{c}	0.83 ^{b}	0.0
Tall fescue	Control	0.14	0.33	0.50	0.84 ^{a}	0.98 ^{a}	
	Waterlogging	0.14	0.33	0.49	0.73 ^{b}	0.91 ^{ab}	−7.1
			Root dry matter (g/plant)				
Cocksfoot	Control	0.04	0.16	0.30 ^{a}	0.44 ^{b}	0.68 ^{b}	
	Waterlogging	0.06	0.12	0.19 ^{b}	0.24 ^{d}	0.30 ^{d}	−55.8
Tall fescue	Control	0.05	0.17	0.29 ^{a}	0.49 ^{a}	0.74 ^{a}	
	Waterlogging	0.05	0.13	0.19 ^{b}	0.30 ^{c}	0.46 ^{c}	−37.8

^{abcd} Least squares means of each day sharing the same letter are not significantly different. * The reduction ratio was calculated between waterlogging values and control values within the same species for the 28-day waterlogging period.

Waterlogging treatments showed no significant ($p > 0.05$) effects on the root length of TF, but for CF, continuous waterlogging beyond 14 days led to significantly shorter roots in waterlogged plants than in control plants. After 28 days, the root length of waterlogged CF plants was 30.7% shorter than the controls (Table 2).

Shoot dry matter was not significantly ($p > 0.05$) affected by waterlogging (Table 2). In contrast, root dry matter of both CF and TF was significantly ($p < 0.05$) affected (Table 2). After 14 days of waterlogging, root dry matter in waterlogged CF and TF was 36% and 34% lower than those of comparable control plants, respectively. These differences increased with the extension of waterlogging time, with root dry matter after 28 days of waterlogging being 55% and 37% lower in CF and TF plants, respectively, compared to control plants (Table 2).

3.3. Shoot and Root Growth Rate

Waterlogging had more adverse effects on root growth rate (GR) than shoot GR. In comparison between the two species, the root GR of CF was more affected by waterlogging (49%, 55%, 59% and 57% of controls) than TF (70%, 68%, 72% and 76% of controls) (Figure 5a). Waterlogging significantly affected the root growth rate of CF in all periods of waterlogging but did not affect TF plants (with the exception of 7 days of waterlogging) (Figure 5).

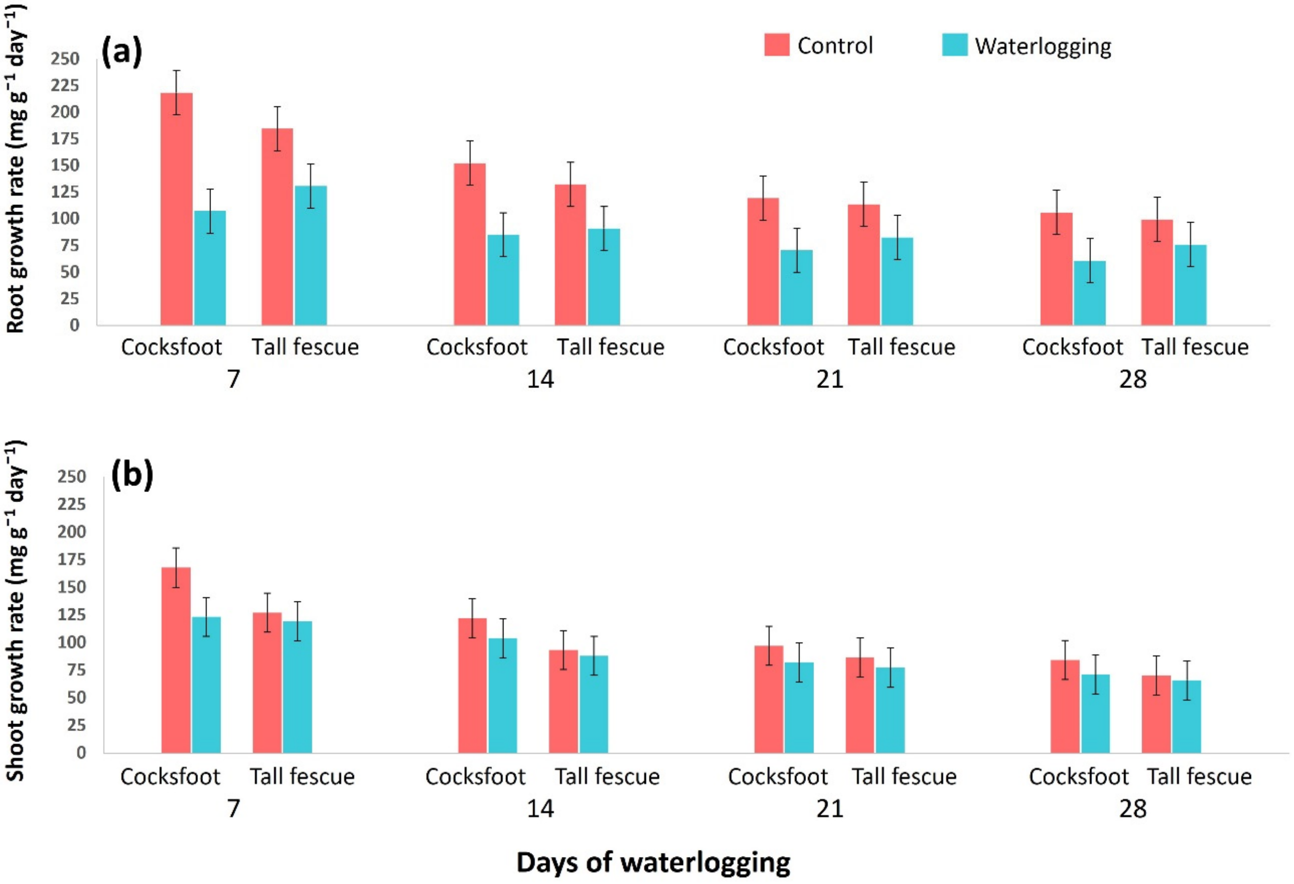


Figure 5. Root (**a**) and shoot (**b**) growth rate of tall fescue and cocksfoot at different stages of treatment under both control and waterlogging conditions. Values given are least squares means of four replicates and error bars indicate 95% confidence intervals of the least squares means.

3.4. Root Responses among Cultivars following 28-Day Waterlogging

Waterlogging significantly ($p < 0.05$) induced the formation of adventitious roots (Figure 6a) and reduced root length (Figure 6b) of cvs. Lazuly and Porto (both CF plants). By day 28, there were 27 and 22 roots/plant in waterlogged plants of cvs. Lazuly and Porto compared to 10 and 13 roots per plant, respectively, in controls.

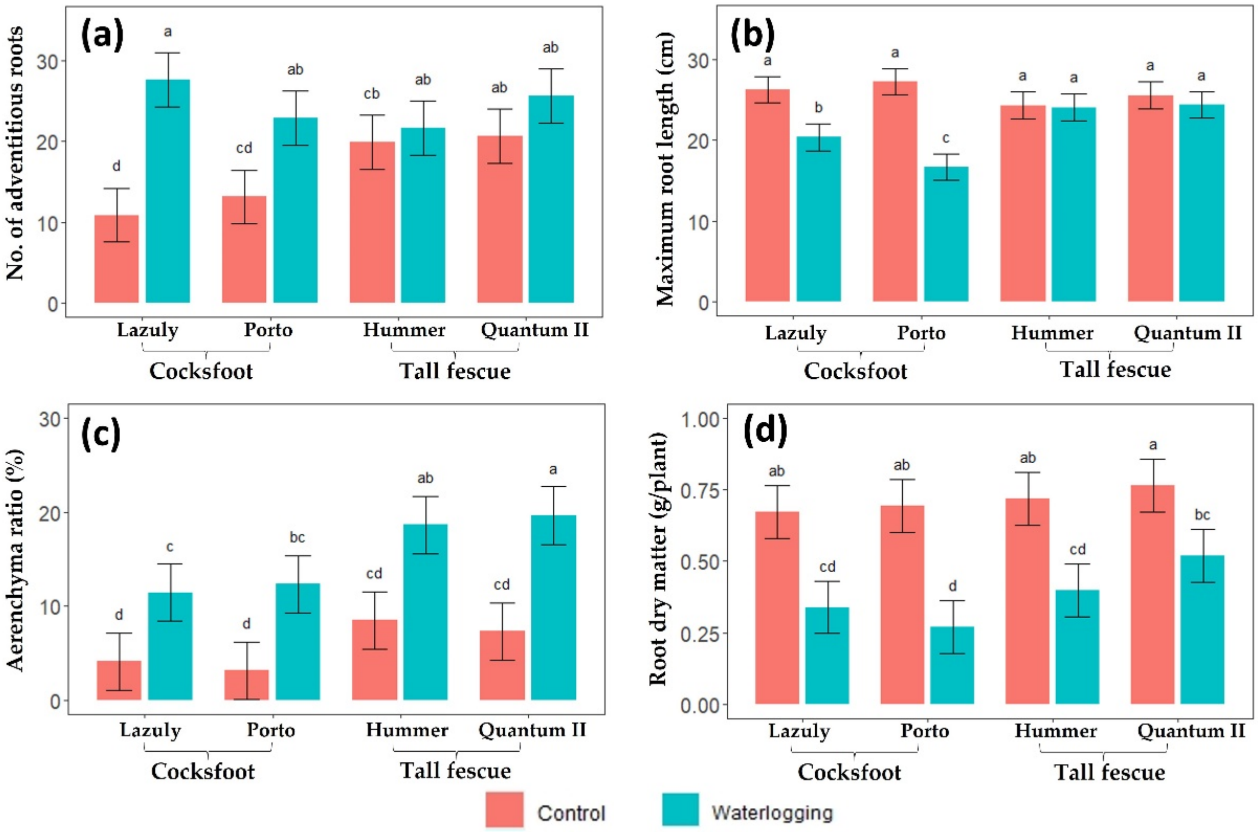


Figure 6. Responses of four cultivars (Lazuly, Porto, Hummer and Quantum II) to 28-day waterlogging via below-ground variables: (**a**) number of adventitious roots, (**b**) maximum root length, (**c**) aerenchyma ratio and (**d**) root dry matter. Values given are least squares means of four replicates and error bars indicate 95% confidence intervals of the least squares means. For each variable, least squares means sharing the same letters are not significantly ($p > 0.05$) different.

Waterlogging significantly ($p < 0.05$) increased the formation of aerenchyma in the adventitious roots of all four cultivars examined, irrespective of species. However, it formed a higher proportion in waterlogged TF plants (Figure 6c). The aerenchyma ratio in waterlogged plants of cvs. Lazuly, Porto, Hummer and Quantum II MaxP was approximately 64%, 74%, 54% and 62% higher, respectively, than those in control plants.

Waterlogging reduced root dry matter of all four cultivars by day 28; it was significantly ($p < 0.05$) higher in control plants of each cultivar (Figure 6d). Root dry matters for control plants of cvs. Lazuly, Porto, Hummer and Quantum II MaxP were 49%, 61%, 44% and 32% higher, respectively, than those for waterlogged plants of the same cultivar.

4. Discussion

This study provides a valuable insight into possible mechanisms involved in the development of waterlogging tolerance in CF and TF plants. The formation of adventitious roots containing aerenchyma was identified as an important trait influencing the waterlogging tolerance of both species examined. The root system of TF and CF formed more adventitious roots under waterlogging conditions. This trait is a common adaptive change in morphology in waterlogged plant roots [24,37], as it is a strategy to improve access to oxygen at or above the saturated soil surface to provide oxygen to the shoots and roots [38,39]. However, oxygen transport efficiency depends on the production of adventitious roots containing aerenchyma volume [40]. The newly formed roots of more waterlogging-tolerant species often contain more aerenchyma than less tolerant species [38]. Compared to TF, the root system of waterlogged CF plants formed more new adventitious roots, but the amount of aerenchyma was higher in waterlogged TF plants. The higher aerenchyma formation of TF plants may improve waterlogging tolerance by enhancing internal aeration within or between roots and shoots via gas pathways to increase gas diffusion, in line with the findings of Colmer [11] and Takahashi et al. [41]. Aerenchyma may not typically form under well-drained soil conditions in some plant species such as maize, barley and wheat, but it may be induced by poor aeration [41,42]. In this study, aerenchyma formation was observed in control conditions in both TF and CF plants, with a greater ratio in TF plants. The formation of aerenchyma in the roots of TF plants under control conditions has also been reported by Liu et al. [29], whereas there was no or less area of aerenchyma found in control CF plants reported by Wang et al. [43]. This finding again confirms the waterlogging tolerance of TF plants, and it may be useful in screening genetic resources for the breeding of waterlogging-tolerant TF cultivars.

We hypothesize that the formation of adventitious roots, combined with higher aerenchyma formation, may also have assisted in the maintenance and development of root length in waterlogged TF plants (cvs. Hummer and Quantum II MaxP), whereas waterlogging significantly reduced the root length of CF (cvs. Lazuly and Porto). Previous studies have shown that waterlogging promotes the formation of adventitious roots, but it restricts the development of root length [44,45]. The root growth of waterlogging-intolerant species was rapidly suppressed by hypoxic or anoxic conditions, whereas waterlogging-tolerant species had the ability to maintain their root growth to some extent [40]. Although waterlogging did not restrain the development of root growth in TF plants (cvs. Hummer and Quantum II MaxP), it significantly reduced root dry matter in plants of both CF and TF species (cvs. Lazuly, Porto, Hummer and Quantum II MaxP). Many primary roots [46] and old roots [47] die under waterlogging conditions, as root tip death is accelerated by waterlogging [48]. Our visual observations suggested that the reduction of total root dry matter under waterlogging conditions was attributed to the loss of primary roots, which led to significant reduction in root dry matter. Similar findings have been reported in perennial ryegrass (*Lolium perenne* L.) [49], Koronivia grass (*Brachiaria humidicola* Schweick.) [18] and wheat (*Triticum aestivum* L.) [44], where root mass reduction was among the first impacts of waterlogging on the root system. In this study, we assumed that the death of primary roots was to prioritize energy for the development of new roots close to the soil surface, where they might access more oxygen diffusion. The reduction of root mass might then be beneficial to reduce oxygen demand under oxygen-deficient conditions, as each root needs oxygen for maintenance and growth [18]. Additionally, the death/loss of primary roots was likely due to the lack of oxygen availability, which is typically supported via aerenchyma under waterlogging conditions.

The duration of a waterlogging event can have significant impacts on grass growth [20,49,50]. In this study, the examined grasses started to exhibit negative effects on root function and the structure of root systems between 14 and 21 days of continuous waterlogging. Waterlogging significantly reduced SPAD reading values, root length and root dry matter of CF and TF plants following 14 days of exposure to waterlogging, whereas the impact of waterlogging on tiller development indicated significant impacts after 21 days of waterlogging exposure. McFarlane et al. [49] reported a similar finding for perennial ryegrass subjected to transient waterlogging, where plants showed a decrease in root and shoot biomass after 14 and 21 days of waterlogging. Likewise, Hare et al. [50] reported more than 50% mortality in some tropical grasses, Purple guinea (*Panicum maximum* cv. purple) and Ruzi (*Brachiaria ruziziensis* Germ. & C.M.Evrard), after 20 days of waterlogging. An explanation for this finding may be the quickly reduced levels of oxygen availability in waterlogged soils as oxygen concentration in water used for saturating soil can decrease

100-fold compared to its initial value in 75 min [51], and disappear entirely within 10 h of submergence [52,53].

5. Conclusions

The development of aerenchyma in adventitious roots was a major mechanism involved in the waterlogging tolerance of cocksfoot and tall fescue plants. Although waterlogging induced the formation of adventitious roots in both waterlogged cocksfoot and tall fescue plants, it promoted higher aerenchyma development in waterlogged tall fescue plants. This may have assisted the root growth of tall fescue and enabled it to not be affected by waterlogging, whereas more severe impacts were recorded in root systems of waterlogged cocksfoot plants. In this study, although waterlogging did not induce significant reductions in biomass, it showed serious impacts on the root system, which may have long-term effects for plant growth if waterlogging lasts longer. As only two cultivars were tested for each species, further studies are required to identify whether there is in-species variation with regards to waterlogging tolerance and whether the results hold true for the species as a whole future and identify germplasm that could be used in breeding efforts.

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