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*Published in:*  
*Functional Plant Biology*

*DOI:*  
10.1071/FP20200

*Publication date:*  
2021

*Document version*  
Early version, also known as pre-print

*Document license:*  
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*Citation for published version (APA):*  
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Citation:


Publisher’s DOI:

https://doi.org/10.1071/FP20200

Access through CIAT Research Online:

https://hdl.handle.net/10568/111275

Terms:

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Root length is proxy for high-throughput screening of waterlogging tolerance in *Urochloa* grasses

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Running Head: Root traits for waterlogging tolerance in Urochloa
Abstract

C4 perennial *Urochloa* grasses are widely planted in extensive areas in the tropics. These areas are continuously facing waterlogging events, which limits plant growth and production. However, no commercial cultivar combining excellent waterlogging tolerance with superior biomass production and nutritional quality is available. The objective of this study was to identify root traits that can be used for selecting waterlogging tolerant *Urochloa* species. Root respiration, root morphological, architectural and anatomical traits were evaluated in eight contrasting *Urochloa* genotypes grown under aerated or deoxygenated stagnant solutions. Moreover, modelling of internal aeration was used to relate differences in root traits and root growth in waterlogged soils. Increased aerenchyma formation in roots, reduced stele area and development of a fully suberized exodermis are characteristics improving internal aeration of roots and therefore determining waterlogging tolerance in these C4 forage grasses. Waterlogging-tolerant genotypes had steeper root angles and greater root lengths than the waterlogging-sensitive genotypes. In stagnant conditions, waterlogging-tolerant genotypes which had a greater proportion of aerenchyma and reduced stele area in root cross-sections also had deeper roots, steeper root angle and larger root biomass, which in turn, allowed for greater shoot biomass. Total root length had the strongest positive influence on shoot dry mass and can therefore be used as proxy for selecting waterlogging tolerant *Urochloa* genotypes.

**Keywords:** Root anatomy, tropical grasses, abiotic stress, root angle, root respiration, suberin, lignin, radial oxygen loss, root internal aeration, root length modelling.

Introduction

Waterlogged soils affecting forage production are considered as one of the major constraints impeding livestock intensification in the American tropics (Rao et al. 2011). During waterlogging, the porous space in soil is filled with water and O$_2$ diffusion is highly restricted (Armstrong 1979). Due to a lack of O$_2$, root respiration is impeded and nutrient uptake is restricted (Colmer and Greenway 2011), resulting in reduced growth and sometimes mortality. The frequency of heavy rainfall events is likely to increase in several regions of the tropics (Hirabayashi et al. 2013),
therefore, the development of tropical forages adapted to soil waterlogging conditions should be a priority for the productivity of these areas.

C4 grasses from the genus *Urochloa* have been extensively planted in the tropics to sustain livestock production (Miles et al. 2004). Among the *Urochloa* grasses, *U. humidicola* genotypes have been identified as superior to other varieties when challenged by soil waterlogging (Dias-Filho and Carvalho 2000; Cardoso et al. 2013; 2014; Jiménez et al. 2015a; Supplementary Table 1). The tolerance of *U. humidicola* to waterlogging (based on the evaluation of several intraspecific genotypes) is associated with traits improving internal aeration of roots, including greater aerenchyma development, reduced stele proportion to the cortex, and a barrier to impede radial O₂ loss (ROL) to the rhizosphere via deposition of suberin and lignin in the outer part of the roots (Cardoso et al. 2013; 2014; Jiménez et al. 2015b; 2019). The enhanced O₂ transport from shoots to roots in plants under waterlogging conditions allows a greater root growth and higher uptake and translocation of nutrients (Armstrong 1979; Armstrong et al. 1983; Gibbs et al. 1988; Armstrong and Drew 2002; Colmer and Greenway 2011).

The root growth into a waterlogged substrate is constrained by the internal capacity to transport O₂ from shoots to the root tip (Armstrong 1979). The effectiveness of the internal longitudinal O₂ transport into and along the roots depends on the longitudinal O₂ diffusion path-length, the amount and distribution of pore space resistance, the formation of barriers to impede ROL to the rhizosphere and the O₂ demand by tissues along the path (Armstrong et al. 1983). Waterlogging tolerant genotypes (with greater capacity for internal O₂ movement) usually have longer roots and faster root growth under anoxic soil conditions than sensitive genotypes. Mathematical models computing root anatomical characteristics have provided the theoretical basis of internal aeration processes and its relation to the maximum root length attained in waterlogged soils (Armstrong 1979). These models have recently been modified to compute variations in the proportion and respiratory activities of the stele and cortex, which substantially impacts the internal capacity to transport O₂ and therefore the accuracy of models (Pedersen et al. 2020). The direction of the root elongation into the soil is determined by the basal root angle and thus, steeper root angles allow greater rooting depth. This has been shown in non-waterlogged conditions (e.g., wheat, Oyanagi 1994; Wasson et al. 2012; Anzooman et al. 2019; rice, Uga et al. 2013; Kato et al. 2016;
Ramalingam et al. 2017; maize, Liakat et al. 2015), but variation in root angle and its influence on root length in plants grown under low-O$_2$ conditions remain largely unexplored.

The evaluation of root anatomical traits improving internal aeration is a time-consuming and technically specialized activity, thus, limiting their use for phenotyping large breeding populations of plants. Therefore, the objective of this study was to identify root traits that can be used as proxies for waterlogging tolerance in *Urochloa* grasses, one of the most important tropical forage grasses. Root and shoot dry mass, root morphological and architectural traits (root length, root cross-sectional area, root angle) and root anatomical traits (stele area and aerenchyma percentage, suberin and lignin deposition) were evaluated in eight contrasting *Urochloa* genotypes grown for two weeks under aerated or stagnant solutions. In addition, the maximum root length achieved in waterlogged conditions was modelled using the root respiration rates measured in two contrasting *Urochloa* genotypes. We hypothesized that longer roots is a trait that can be used as proxy for waterlogging tolerance. This knowledge will extend our understanding of plant responses to low-O$_2$ conditions and will serve as a basis for improving phenotyping in tropical grass breeding programs.

**Materials and Methods**

*Plant material and growing conditions*

Seeds for different accessions (Supplementary Table 1) of the waterlogging-sensitive species *U. brizantha* (cv. Marandú “CIAT6294”, cv. Toledo “CIAT26110”, CIAT26124), waterlogging-sensitive hybrid Mulato II (*U. ruziensiis x U. decumbens x U. brizantha*, “CIAT36087”) and waterlogging-tolerant species *U. humidicola* (cv. Tully “CIAT679”, cv. Llanero “CIAT6133”, CIAT16888, CIAT26570), were sown in sterilized river sand. Seeds were irrigated daily with DI water. Seeds of *U. humidicola* genotypes (slower germination) were sown three weeks before being transplanted into 3.7 L pots filled with nutrient solution, and seeds of the other four genotypes 2 weeks before transplanting. Four plants were transplanted into individual pots and were held by foam in holes in the lid of each pot. Plantlets were grown in a nutrient solution known to maximize the phenotypic differences in growth of *Urochloa* plants under stagnant compared to aerated solutions (Jiménez et al. 2019). The nutrient solution contained (in μM): 5000 NO$_3^-$, 500
Plantlets were grown with the roots in aerated nutrient solution for one week to allow recovery after transplanting. Pots were covered with Al foil to reflect sunlight and minimize changes in the nutrient solution temperature. After one week of regrowth, plants were exposed to either aerated or stagnant root-zone treatments during 2 weeks. Aerated solutions were continuously bubbled with air. Stagnant solutions were made by dissolving agar at 0.1% (w/v) in the nutrient solution and pre-flushing it with high purity N₂ gas to purge out O₂. This stagnant solution was continuously flushed with N₂ when syphoning stagnant solutions from preparation tanks into pots so as to avoid any O₂ mixing into the solution. The higher viscosity of the 0.1% agar solution prevents convection, so that it mimics the changes in gas composition (low O₂, but increased ethylene in roots) which occur in waterlogged soils (Wiengweera et al. 1997) since any O₂ entry is greatly impeded. Oxygen concentrations in deoxygenated stagnant solutions remain very low (c. 0 – 0.2 mg L⁻¹; Kotula et al., 2009; 2015). Nutrient solutions were renewed every week. The experiment was run in a completely randomized design with two treatments (aerated and stagnant), eight genotypes and four replications. Plants were grown in a greenhouse at 30/19 °C day/night air temperatures; 12 hours daylight; located at CIAT, Cali, Colombia and during the months of March to and April, 2018.

Harvest

The harvest was conducted after 2 weeks of treatments. Two out of four plants per pot were separated into shoots (stems and leaves) and roots (the remaining two plants were used for root architecture and root anatomy determination, see below). The number of main roots (‘nodal’ or ‘adventitious’ roots) per plant were counted for these two plants. Dry weights were measured after oven drying tissues at 60 °C for three days. Data for two plants from each pot were pooled and the mean, expressed on a per plant basis, was used to provide one replicate. There were 4 replicate pots for each treatment x genotype combination.

Root architecture

The *Urochloa* root system consists of one seminal root and several adventitious (nodal) roots, each having several lateral roots. The seminal root is small and typically dies back a few days after the
adventitious root system is established (4 to 7 adventitious roots) and therefore it was not used for analysis. Root extension was determined by measuring the length of an individual adventitious root (initial length of 6-10 cm, one plant per pot previously marked with a cotton thread) using a ruler at 0 and 14 days after treatments commencement, and is expressed in cm per day. One of the other two remaining plants per pot was removed, laid down on a protractor and photographed from a nadir view at 20 cm height using a 13 megapixels digital camera (Nikon, Coolpix, P6000, Japan). The root angle was determined by measuring the angle of the basal 5 cm formed relative to the vertical axis (Supplementary Fig 1), thus, small angles indicate roots growing downwards. After photographing, all roots from each plant were separated and scanned at a resolution of 300 dpi in a flatbed scanner (EPSON Expression 1680, Japan). The total length of main and laterals roots were measured using the scanned images and the WinRhizo software (Regent Instruments, Canada). Main axes and lateral roots were differentiated based on their diameter ranges that were determined both microscopically (see details below) and using the WinRhizo software in random samples.

**Root anatomy**

One root (~100 mm in length) was excised from the remaining plant in each pot (same plant used for root extension rate). Roots were fixed in 1.6% (v/v) paraformaldehyde in phosphate-buffered saline, pH 7.4 and stored at 4 °C until required. Segments of 10 mm length were excised at distances of c. 50 mm behind the root tip and were embedded in 5% (w/v) warm agar. Cross sections were obtained by cutting solid agar blocks using a vibrating blade microtome (VT 1000S Leica, Wetzlar, Germany). Adhered agar was removed by clearing root sections with 85% lactic acid saturated with chloride hydrate for 1 h at 70 °C (Lux et al. 2005) and washing several times with DI water. Suberin was visualized by green-yellow colour after staining cross-sections with 0.01% (w/v) Fluorol Yellow 088 in polyethylene glycol glycerol for 1 h (Brundrett et al. 1991) and viewed under UV light (Axioscope2 plus, Zeiss, Oberkochen, Germany; Excitation G365, Emission LP397). Root sections containing suberin were photographed with a Zeiss AxioCam Digital Camera. Lignin was visualized by brown colour after treating root cross-sections successively with 1% (w/v) KMnO4, 12% HCl and a concentrated solution of ammonia for the Mäule reaction (Kutscha and Gray 1972). These cross-sections were viewed and photographed under white light microscope (AxioCam ERc5s, Zeiss, Oberkochen, Germany; software ZEN 2012). The root cross-sectional area, aerenchyma percentage (% gas-filled large spaces in the root
cortex) and the stele area at c. 50 mm behind the root apex were determined using white light images and the ImageJ software (National Institutes of Health, Bethesda, USA). The ratio (area) of each root tissue was calculated using the cross-sectional areas.

O₂ consumption of root segments
The purpose of these measurements was to obtain data on root respiration for use in modelling of possible maximum root lengths based on internal O₂ movement to the apex (next section). Seeds of waterlogging-sensitive Urochloa hybrid cv. “Mulato II” (CIAT 36087) and waterlogging-tolerant Urochloa humidicola cv. “Tully” (CIAT 679) were sown in sterilized sand and then transferred to deoxygenated stagnant solutions as explained above but in a constant temperature room at 30 °C, with PAR at shoot height of 150 μmol m⁻² s⁻¹, 12 h light/dark at the University of Copenhagen. After two weeks of growth in stagnant conditions, rates of O₂ consumption by excised root segments were measured following the procedure described in Pedersen et al. (2013) using a MicroResp system (Unisense A/S, Aarhus, Denmark).

Root segments of 10 mm length (45-55 mm behind the root tip) were excised and inserted in 4-ml glass vials containing nutrient solution (same as used to grow plants, see above, but lacking agar) at O₂ in equilibrium with air. Each vial contained a glass-coated magnetic stir bar and the stirring rate was set to 600 rpm using the stirrer controller unit (MR2-St-Co, Unisense A/S). The vials were placed in a rack and submerged into a constant temperature bath (30 °C) and left to stabilize for c. 15 min. Oxygen consumption by the root segment was measured in each vial using an O₂ optode (OP-MR, Unisense A/S); O₂ in the medium declined from air equilibrium (20.6 kPa) to no less than 16 kPa as O₂ was consumed by the root segments. Vials without tissue served as blanks. The volume of each vial and the fresh mass of the root segment were determined immediately after finishing the O₂ measurements. Experiments were run for 1.5 – 2.5 h, depending on how quickly O₂ was depleted from the vials.

Oxygen consumption (root respiration; ‘Resp’, nmol O₂ g⁻¹ FM s⁻¹) rates were calculated using Rate (SensorTrace Suite version 2.3.100, Unisense A/S) as follows:

\[
Resp = \frac{(C_2 - C_1) \times Vol}{(t_2 - t_1) \times FM}
\]

Eqn (1)

Where \(C_2 - C_1\) (μmol O₂ L⁻¹) is the difference in O₂ concentration in the solution within the vial at two time points, \(t_1\) and \(t_2\) (s) is the time between time points, \(Vol\) (L) is the volume of the vial and
$FM$ is the fresh mass (g) of the root tissue. Respiration of root segments for both genotypes was measured several times ($n = 3$ to $6$) on different roots. In addition, for root segments of waterlogging tolerant $U.\ humicola$ cv. Tully that constitutively form a barrier to radial $O_2$ loss at $50$ mm behind the root (i.e., also restricting $O_2$ consumption from the external medium, cf. Jiménez et al. 2019), the root segments were sliced opened (to allow $O_2$ consumption by root tissue) and the $O_2$ consumption rate was measured as explained above. Small differences in initial respiration rates possibly due to wounding (cf. Gronewald and Hanson 1982) were not included in the calculations; as indicated by linear regressions of the slope among sequential $O_2$ consumption measurements had stabilized during the measurements which lasted 1.5-2.5 h and a period of 30-45 mins within this was used to calculate the respiration rate.

**Modelled maximum root lengths**

The maximum length of adventitious roots when growth is supported by internal $O_2$ diffusion from the shoot into and along the roots as the only $O_2$ source to the growing apex, was calculated using the model proposed by Armstrong (1979). Based on the assumption that there is no lateral diffusion of $O_2$ to the waterlogged rhizosphere (i.e. no ROL) and that respiration is homogenous across the root cross-section, the maximum aerated path length equals the maximum length of adventitious roots in waterlogged soils and can be calculated from the following equation (Armstrong 1979):

$$L = \sqrt{\frac{2CoD\epsilon\tau}{Mt}}$$  \hspace{1cm} Eqn (2)

where $L$ is the maximum aerated path length, $Co$ is the cortex $O_2$ status at the root-shoot junction, $D$ is the diffusion coefficient of $O_2$ in gas phase, $\epsilon$ is the fractional root porosity (assuming uniformity along the entire length of the root), $\tau$ is the tortuosity factor (assumed to be 1.0) and $Mt$ is total root tissue respiration (assuming constant respiration along the root).

In addition, the maximum length of adventitious roots was calculated using a modified version of Armstrong’s model which includes also the variation in the respiratory activities of the stele and cortex tissues, as well as the proportions of these tissues in roots (Pedersen et al. 2020). This modified model incorporates the fractions of cortex respiration ($Mc$; respiration corrected for volume-based as tissue porosity changes) and stelar respiration ($Ms$) as components for the
respiration of the root as a whole, which enables assessment of the influence of the stele size as a root trait influencing internal aeration (Pedersen et al. 2020).

Maximum root lengths were calculated for all eight genotypes using the root respiration rates measured for waterlogging-sensitive *U.* hybrid cv. Mulato II and waterlogging-tolerant *U.* *humidicola* cv. Tully as follows: 1) averaging the respiration rates of both sensitive and tolerant genotypes and using the mean respiration for all genotypes and; 2) respiration rates from sensitive and tolerant genotypes were used for the modelling of maximum root length of all sensitive and tolerant genotypes, respectively.

**Statistical analyses of data**

Statistical differences between data values for treatments and genotypes were evaluated through two-way ANOVA. The multiple comparison Tukey test was run further to separate differences between means for each variable analyzed. Pearson’s correlation coefficient between different traits were calculated. Statistical analyses were run in the software R (R core team 2012), using the library Agricolae (Mendiburu 2014).

**Results**

**Growth of Urochloa genotypes in aerated or stagnant conditions**

In aerated conditions, shoot dry mass was similar between the eight different genotypes evaluated, averaging 1.77 and 2.14 g per plant for sensitive (Marandú, Toledo, CIAT26124, Mulato II) and tolerant (Tully, Llanero, CIAT16888, CIAT26570) genotypes, respectively. Average shoot dry mass under stagnant conditions, expressed as a percentage of controls (aerated treatments), decreased 41% in sensitive and 26% in tolerant genotypes (Supplementary Table 2). Sensitive genotypes all showed similar root dry mass in aerated conditions, and had on average 53% lower root dry mass in stagnant compared to aerated conditions (Supplementary Table 2). In contrast, the root dry mass of the tolerant genotypes was not significantly reduced under stagnant conditions, except for the cv. Tully. Under stagnant conditions, the average root dry mass of the tolerant genotypes was 1.6-fold higher than that of the sensitive genotypes (Supplementary Table 2).

**Root architecture of Urochloa genotypes in aerated or stagnant conditions**
The average number of main roots in plants grown in stagnant solutions (for all genotypes) was 1.2-fold higher than that of aerated controls. However, the average number of main roots was only significantly higher in stagnant in comparison to aerated treatments for the tolerant genotypes, except for cv. Llanero (Table 1). The stagnant treatment resulted in slower root extension rate for all genotypes. The average root extension rate in stagnant treatments as percentage of controls was 12% for sensitive and 35% for tolerant genotypes (Table 1). In aerated conditions, the average root extension rate of the sensitive genotypes was 1.5-fold higher than that of tolerant genotypes. On the contrary, in stagnant conditions, the average root extension rate of the tolerant genotypes was 1.9-fold higher than that of sensitive genotypes (Table 1). The root angle relative to the vertical axis was steeper in the tolerant genotypes (ranging from 17 to 27°) than in the sensitive genotypes (ranging from 31 to 48°). This trait was not influenced by the aeration treatment, except for Toledo where the root angle was shallower in the stagnant treatments (Table 1).

The total length of both main axes of adventitious roots and their lateral roots was significantly lower in stagnant in comparison to aerated treatments for all genotypes, except for Llanero and CIAT 16888 (Table 1). In stagnant conditions, the average total length of main axes of roots as percentage of controls was 31% for sensitive and 66% for tolerant genotypes (Table 1). The average total length of lateral roots in stagnant treatments as percentage of controls was 38% for sensitive and 72% for tolerant genotypes (Table 1).

**Root anatomy**

The root cross-sectional area at 50 mm behind the root tip significantly increased under stagnant conditions in comparison to aerated treatments in three of the eight genotypes, being on average (for all genotypes) 1.4-fold greater in stagnant than in aerated conditions (Table 2). This increase in the root cross-sectional area is driven by a larger cortex which contains root aerenchyma (Table 2) and had greater elongation of the cortical cells which remained intact under stagnant conditions (see Supplementary Fig 2). The average percentage of root aerenchyma significantly increased from 3.2 to 13.3% and from 9.8 to 23.1% from aerated to stagnant treatments for sensitive and tolerant genotypes, respectively (Table 2). The aerenchyma formation is a constitutive trait for the tolerant genotypes but it is not or barely present in sensitive genotypes in aerated conditions. The percentage of aerenchyma in the root cross section of the tolerant genotypes was 3.7- or 1.8-fold greater than in sensitive genotypes for aerated or stagnant conditions, respectively. Under stagnant
conditions, the stele area at 50 mm behind the root tip was reduced for cv. Marandú and increased for cv. Mulato II. No significant changes in stele area were found for the waterlogging tolerant genotypes (Table 2). The average (for all genotypes) cortex to stele ratio (CSR) in stagnant was 1.5-fold higher than in aerated controls. However, the CSR was only significantly higher in stagnant in comparison to aerated treatments for the tolerant genotypes (Table 2).

The outer part of the root of all genotypes was characterized by a multi-seriate layer composed by one epidermal cell, one or two exodermal cells and one or two cells of sclerenchyma. Tolerant genotypes exhibited one layer of bigger exodermal cells while sensitive genotypes exhibited two but smaller exodermal layers (Fig 1). Deposition of lignin, was evident in the sclerenchyma of all genotypes evaluated under both treatments, except for the hybrid Mulato II (Fig 1). All of the tolerant genotypes exhibited two layers of lignified sclerenchyma, except for the genotype CIAT16888 which only exhibited one. In contrast, sensitive genotypes only exhibited one layer of lignified sclerenchyma, except for Mulato II which did not exhibit sclerenchyma at all.

At a distance of 50 mm behind the root tip, deposition of suberin was exhibited in some cells of the exodermis in the sensitive genotypes, indicating a patchy suberin development (Fig 2). In contrast, suberin lamella was exhibited in all exodermal cells in tolerant genotypes (Fig 2). The suberin deposition was not affected by the aeration versus stagnant treatments.

The correlation between total root length and dry mass of shoots was positive and significant for aerated (0.44, $P \leq 0.1$) and stagnant treatments (0.94, $P \leq 0.001$). In aerated conditions, the root angle measured relative to the vertical axis was neither related to dry mass of roots (0.22, $P = 0.23$) nor related to the dry mass of shoots (0.16, $P = 0.39$). However, in stagnant conditions, plants with steeper root angle had higher dry mass of roots (-0.53, $P \leq 0.01$) and dry mass of shoots (-0.53, $P \leq 0.01$; Fig. 3). The relationship between the percentage of root aerenchyma and the dry mass of shoots was strong for stagnant (0.66, $P \leq 0.001$) but not for aerated treatments (0.22, $P = 0.24$). Similarly, in stagnant conditions, the relationship between root extension rate and both dry mass of roots (0.73, $P \leq 0.001$) and dry mass of shoots (0.59, $P \leq 0.001$) was stronger than in aerated conditions with a correlation of 0.35 ($P \leq 0.5$) for dry mass of roots and no correlation of 0.02 ($P = 0.91$) for dry mass of shoots (Fig 3). In stagnant conditions, total root length had the strongest positive influence on shoot dry mass (Supplementary Table 3).

$O_2$ consumption of root segments
The average $O_2$ consumption (respiration rate) of roots from waterlogging-sensitive cv. Mulato II grown for two weeks in deoxygenated stagnant solutions was 2.94 nmol $O_2$ g FW sec$^{-1}$ (SE= 0.6, n=4). The average $O_2$ consumption rate of roots of waterlogging-tolerant *U. humidicola* cv. Tully was 0.91 and 2.22 nmol $O_2$ g FW sec$^{-1}$ for intact (SE= 0.3, n=6) and sliced opened (SE= 0.2, n=4) root segments, respectively. There were not significant differences between the respiration rates of intact segments of the waterlogging-sensitive and sliced opened segments from the waterlogging-tolerant genotypes ($P=0.29$). In the waterlogging-tolerant cv. Tully, the average $O_2$ consumption of sliced opened root segments was 2.4-fold higher than that of intact root segments, highlighting the strength of a barrier to ROL to impede radial $O_2$ diffusion across the outer tissues (inwards as well as outwards) and thus also impeding $O_2$ consumption from the medium. The rate from the sliced opened root segments (and not the rate from the intact root segment) was used for modelling maximum root lengths in waterlogging-tolerant cv. Tully, as this rate would better represent $O_2$ consumption by tissues when $O_2$ is available internally via aerenchyma. For the waterlogging-sensitive cv. Mulato II, the average $O_2$ consumption rate of intact root segments was used, as this genotype does not form a barrier to ROL (Supplementary Fig 3) and so $O_2$ could enter the tissues and was assumed to represent the rate likely also with $O_2$ available internally via aerenchyma.

*Modelled maximum root lengths*

The predicted maximum length of adventitious roots when using the original model (Armstrong 1979) and assuming similar respiration rates among genotypes (i.e., the average respiration rate assessed for all genotypes) averaged (for four genotypes) 144 mm for waterlogging-sensitive and 208 mm for waterlogging-tolerant genotypes (Table 3). The predicted maximum length of adventitious roots, however, decreased 8 mm for sensitive (average of 136 mm) and increased 14 mm for tolerant genotypes (average of 221 mm) when assuming different respiration rates (i.e., the respiration of sensitive cv. Mulato II assumed equal to all sensitive genotypes and the respiration rate of tolerant cv. Tully assumed equal to all tolerant genotypes). Moreover, these predicted values of maximum root length attained in waterlogged soils, decreased for the sensitive (average 82 mm) and increased (average 360 mm) for the tolerant genotypes when including differences in tissue respiration based on the differences in the proportion of the stele in these roots (smaller stele in tolerant genotypes, Pedersen et al. 2020; Table 3). The correlations between modelled and observed maximum root lengths were all positive ($P<0.05$) and increased as based...
on the Pearson correlation coefficient from an average of 0.63 from calculations using the original model (Armstrong 1979) to 0.70 from calculations using the model including variation in respiratory activity of stele and cortex (Pedersen et al. 2020; Supplementary Fig 4).

Discussion

This study documents the responses of eight Urochloa genotypes (four considered to be waterlogging-sensitive and four waterlogging-tolerant, see Supplementary Table 1) to low O₂ in the root-zone. Root anatomical and architectural characteristics improving internal aeration represent major changes determining waterlogging tolerance in these C₄ forage grasses. Likewise, results indicated that genotypes with longer roots produce greater biomass under stagnant conditions. This trait can therefore be used as a proxy for selecting waterlogging tolerant Urochloa genotypes.

All genotypes evaluated in this study increased the percentage of root aerenchyma under stagnant conditions (Table 2). However, the percentage of aerenchyma was higher for the waterlogging-tolerant U. humidicola genotypes. Aerenchyma is a constitutive trait found in U. humidicola genotypes under aerated conditions that can be further increased under low-O₂ conditions (Cardoso et al. 2013, 2014; Jiménez et al. 2015b, 2019; this study). Many wetland species have roots with constitutive aerenchyma (Justin and Armstrong 1987). The presence of constitutive aerenchyma in roots is advantageous to plants when a soil is initially flooded, as O₂ can diffuse from shoots into and along the roots to allow root respiration and growth in anoxic substrates (Colmer and Voesenek 2009; Yamauchi et al. 2019b). Moreover, the amount of aerenchyma increases further during waterlogging, so-called ‘inducible’ aerenchyma, both in wetland species and in many non-wetland species (Justin and Armstrong 1987; Colmer and Voesenek 2009). In addition to a higher root aerenchyma percentage, tolerant genotypes in stagnant conditions had also higher cortex to stele ratio (CSR) than sensitive genotypes (Table 2). The stele is a low porosity tissue of high diffusive resistance to O₂, with a higher O₂ consumption rate than the cortical tissue (Armstrong and Beckett 1987; Armstrong et al. 1991; Aguilar et al. 2003). Therefore, higher percentage of aerenchyma and higher CSR are traits acting together to improve root aeration of waterlogging-tolerant genotypes (cf. Yamauchi et al. 2019a).
Under stagnant conditions, major differences in root growth were apparent between sensitive and tolerant genotypes. In stagnant conditions, the total length of main axes of adventitious roots was 1.8-fold greater in tolerant genotypes than in sensitive genotypes (Table 1). This greater total root length (main axes) in tolerant genotypes was influenced by a higher number of longer main roots developed in stagnant conditions, than for the sensitive genotypes (Table 1). In most, if not all species, the length of roots is severely reduced under waterlogging conditions (clover, Gibberd et al. 2001; wheat, Wiengweera and Greenway 2004; Haque et al. 2012; barley, Kotula et al. 2015). The reduction of root growth in stagnant conditions is attributed to O$_2$ concentrations approaching zero in the root apical zone since this tissue becomes more distant from the O$_2$ source as roots grow further into an anoxic medium (Armstrong 1979; Kotula et al. 2015); root extension is reduced below a threshold level of O$_2$ (the critical O$_2$ partial pressure for root extension, Armstrong and Webb 1985). The apical O$_2$ concentration in the root declines as roots grow and the distance from the root-shoot junction to the root tip increases (Armstrong 1979), when the only source of O$_2$ is supplied via internal gas-phase diffusion for roots in an anoxic medium (Armstrong et al. 1983). Therefore, an improved internal O$_2$ movement driven by an increased aerenchyma and higher CSR in roots of tolerant genotypes (Table 2) allows greater root growth than that of sensitive genotypes. This conclusion was supported by mathematical modelling that predicted longer roots in tolerant in comparison with sensitive genotypes (even when using similar respiratory O$_2$ consumption for calculations, Table 3). The correlation between modelled and observed maximum root lengths increased when using the mathematical model that includes the variation in respiratory consumption in cortex and stele tissues (Pedersen et al. 2020) in comparison to the original model that does not (Armstrong 1979; Supplementary Fig 4); highlighting the importance of an improved internal aeration to sustain root growth into anoxic substrates.

The total length of lateral roots under stagnant treatments was on average 1.7-fold higher in tolerant than in sensitive genotypes (Table 1). Greater lateral root formation has previously been associated to waterlogging-tolerant but not -sensitive genotypes of pasture legumes (Gibberd et al. 2001). The production of lateral roots can have a significant influence on the O$_2$ regime of the primary root. Lateral roots are reliant on the O$_2$ available from the aerenchyma within the main root, thus, an increase in the number of laterals reduces the apical O$_2$ concentration in main roots, as demonstrated experimentally by an increase of main root O$_2$ upon excision of the lateral roots (Armstrong et al. 1983; Sorrell et al. 2000). Greater aeration capacity of the main axes of
adventitious roots of tolerant genotypes allows greater lateral root formation than in sensitive genotypes with poorer aeration capacity (cf. Sorrell et al. 2000). This root architecture in waterlogging-tolerant *U. humidicola* genotypes, resembles the basic architecture of wetland rice root systems which consist of aerenchymatous primary roots with barriers to impede ROL, conducting O$_2$ down to short, fine and gas-permeable laterals. This system provides a compromise between the need for internal aeration and the need for a large nutrient absorbing surface per unit root mass (Kirk 2003).

The outer part of the main axes of adventitious roots of all *Urochloa* genotypes evaluated here, were characterized by a multiseriate band of cells. This characteristic has been suggested to increase mechanical strength of roots with higher porosity under low-O$_2$ conditions (cf. Striker et al. 2007). Tolerant genotypes had one layer of bigger exodermal cells whereas sensitive genotypes exhibited two smaller exodermal layers. These cell layers would determine the ‘strength’ (i.e. degree of impedance) of the barrier to ROL in these roots. A stronger O$_2$ diffusion impedance is determined by the cell wall composition (see next paragraph), as well as the path-length across this tissue and O$_2$ consumption rates of these cells. Further studies are needed to clarify the relation between cell size and number and the respiratory consumption of O$_2$ by epidermal/hypodermal cells in roots of plants growing in low-O$_2$ conditions.

Lignified sclerenchyma and suberized hypodermis/exodermis in roots are both found in roots with barriers to ROL (Armstrong 1979; Kotula et al. 2009; Abiko et al. 2012; Jiménez et al. 2019). However, detailed studies comparing both suberin and lignin deposition in the outer part of the root and O$_2$ profiles across the tissues of roots have indicated that suberisation rather than lignification is responsible for restricting ROL (De Simone et al. 2003; Shiono et al. 2014; Kotula et al. 2017). Therefore, complete suberin lamellae formation in waterlogging-tolerant but not -sensitive *Urochloa* genotypes (Fig 2) is very likely contributing to restrict ROL and thus improve root longitudinal O$_2$ transport under low-O$_2$ conditions in the root zone.

*Urochloa humidicola* waterlogging-tolerant genotypes had steeper root angles and greater root lengths than the waterlogging-sensitive genotypes (Table 1). Plants with steeper root angles had a greater number of roots, greater dry mass of roots and greater dry mass of shoots in stagnant treatments but not in aerated conditions (Fig 3). Moreover, in stagnant conditions, genotypes with higher root extension rate had more dry mass of roots and more dry mass of shoots; this trend was
not similar in aerated conditions in which there was a weak relation between root extension rate and dry mass of roots (0.35, \( P \leq 0.5 \)) and no relationship between root extension rate and dry mass of shoots (0.02, \( P = 0.91 \)). The root extension into a waterlogged soil is largely determined by the internal movement of \( O_2 \) from the atmosphere into and along the root axis and to the root apex (Armstrong 1979). Therefore, the improved aeration efficiency (higher aerenchyma, higher CSR and a full suberin lamella in the hypodermis/exodermis) in tolerant genotypes allows greater root growth to explore for resources and therefore greater shoot biomass.

In conclusion, in stagnant conditions, waterlogging-tolerant \( U. \) humidicola genotypes which had a greater proportion of aerenchyma and higher CSR also had deeper roots, steeper root angle and larger root biomass, which in turn, presumably provided for a greater leaf biomass (Tables 1 and 2, Fig 3). These findings suggest that rooting depth may be used as a proxy for aerenchyma formation and root internal aeration efficiency in \( Urochloa \) grasses grown under waterlogged soil conditions. Deeper roots (longer roots with steeper angles) are particularly important for transient waterlogging after the water level recedes, as these roots growing downwards should be able to take up more water and resources to support recovery while the upper soil layers dry out. Substantial variation in root anatomy and root morphology traits were found among the 8 genotypes evaluated in this study, so characterization of more \( Urochloa \) genotypes under waterlogged conditions could reveal additional genotypes showing desirable characteristics upon which, together with other agronomic characteristics, a breeding program could be designed.

Conflicts of interest
The authors have no conflicts of interest to declare.

Acknowledgements
We thank Dr. Luis Guillermo Santos from the CIAT’s Genetic Resources Unit for providing seeds and Valheria Castiblanco and Luis Miguel Hernandez for allowing access to greenhouse facilities. We are grateful to Alfer Valencia for assistance during the experiment set-up and harvest process. JCJ is grateful to COLCIENCIAS (Colombian government) for a PhD scholarship grant and to The University of Western Australia for a reduced-fees scholarship. We thank ICESI University for the use of the vibrating microtome. The authors acknowledge use of the Microscopy Australia
facilities at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments. JCJ thanks the R.N. Robertson Travelling Fellowship, the Australian Society of Plant Scientists and the University of Western Australia for a travel award to visit the University of Copenhagen. This work was partially undertaken as part of the CGIAR Research Program on Livestock. We thank all donors that globally support work through their contributions to the CGIAR system.

List of Figures

Figure 1. Development of lignified sclerenchyma of genotypes Marandú (a, i), CIAT26124 (b, j), Toledo (c, k), Mulato II (d, l), Llanero (e, m), Tully (f, n), CIAT 16888 (g, o) and CIAT 26570 (h, p) grown in aerated (left column: a – h) or deoxygenated stagnant solutions (right column: i – p) for 2 weeks. Cross-sections were made at 50 mm behind the apex and syringyl groups of lignin were stained orange/brown with KMnO$_4$ and HCl, see black arrowheads pointing to examples. Abbreviations: Ep, epidermis; Ex, exodermis; Sc, Sclerenchyma Co, cortical cells. Scale Bar = 200 µm.

Figure 2. Development of suberized exodermis of genotypes Marandú (a, i), CIAT26124 (b, j), Toledo (c, k), Mulato II (d, l), Llanero (e, m), Tully (f, n), CIAT 16888 (g, o) and CIAT 26570 (h, p) grown in aerated (left column: a – h) or deoxygenated stagnant solutions (right column: i – p) for 2 weeks. Cross-sections were made at 50 mm behind the apex and suberin deposition was stained yellow-greenish with Fluorol Yellow 088 and viewed under UV illumination. See white arrows pointing to examples of suberin and arrowheads pointing to ‘passage cells’ without suberin deposition. Abbreviations: Ep, epidermis; Ex, exodermis; Sc, Sclerenchyma; Co, cortical cells. Scale Bar = 200 µm.

Figure 3. Binary relationships and Pearson’s correlation coefficients between root architectural and anatomical traits and growth of eight waterlogging contrasting Urochloa genotypes grown in aerated conditions (upper right, empty black dots) or deoxygenated stagnant solutions (lower left, gray dots). RAer= root aerenchyma (%), RExt= root extension rate (cm per day), TRL= Total root length including both main and lateral roots (cm), RAngle= root angle measured to the Y-axis,
NoRoots= number of main roots, DMR= dry mass of roots (g per plant), DMS= dry mass of shoots (g per plant). n= 32 for each biplot. Pearson’s correlation coefficients are indicated with their statistical significance as follows: $P \leq 0.5$, $*P \leq 0.1$, $**P \leq 0.01$, $***P \leq 0.001$.

References


cortex with radial losses to the stele, the wall layers and the rhizosphere. *New Phytologist* **105**, 221-245.


Uga Y, Sugimoto K, Ogawa S et al. (2013). Control of root system architecture by *DEEPER ROOTING 1* increases rice yield under drought conditions. *Nature Genetics* 45, doi:10.1038/ng.2725


Table 1. Number of main (adventitious) roots, root extension rate, root angle, length of the main axes of adventitious roots and length of the lateral roots of eight *Urochloa* genotypes after 2 weeks of growth in aerated or stagnant deoxygenated nutrient solutions. Different letters indicate statistically significant differences (*P* < 0.05, Tukey test). The root angle was determined by measuring the root angle of the basal 5 cm of the first adventitious root formed relative to the vertical axis. Initial lengths of roots used for extension rate measurements were 6 to 10 cm.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Tolerance to waterlogging</th>
<th>Number of roots</th>
<th>Root extension rate (cm day(^{-1}))</th>
<th>Root angle (°)</th>
<th>Main roots length (cm)</th>
<th>Lateral roots length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerated Stagnant</td>
<td>Aerated Stagnant</td>
<td>Aerated Stagnant</td>
<td>Aerated Stagnant</td>
<td>Aerated Stagnant</td>
</tr>
<tr>
<td>Marandú</td>
<td>Sensitive</td>
<td>12 def 9 f</td>
<td>2.4 ab 0.3 g</td>
<td>31 bcde 36 bc</td>
<td>8362 a 2522 ef</td>
<td>1429 ab 470 ef</td>
</tr>
<tr>
<td>CIAT26124</td>
<td>Sensitive</td>
<td>11 ef 11 ef</td>
<td>2.4 ab 0.2 g</td>
<td>32 bcd 39 ab</td>
<td>9149 ab 2232 f</td>
<td>1223 a 385 f</td>
</tr>
<tr>
<td>Toledo</td>
<td>Sensitive</td>
<td>9 f 11 ef</td>
<td>2.7 a 0.3 g</td>
<td>36 bc 46 a</td>
<td>8501 ab 2658 ef</td>
<td>1322 ab 533 ef</td>
</tr>
<tr>
<td>Mulato II</td>
<td>Sensitive</td>
<td>16 bcd 14 cde</td>
<td>2.1 bc 0.4 g</td>
<td>40 ab 38 ab</td>
<td>7587 ab 2917 def</td>
<td>1262 abc 586 ef</td>
</tr>
<tr>
<td>Llanero</td>
<td>Tolerant</td>
<td>13 cdef 16 bc</td>
<td>1.4 de 0.4 fg</td>
<td>20 f 22 def</td>
<td>5315 cd 4231 cde</td>
<td>885 cd 767 def</td>
</tr>
<tr>
<td>Tully</td>
<td>Tolerant</td>
<td>15 cde 19 ab</td>
<td>1.5 cd 1.0 ef</td>
<td>18 f 17 f</td>
<td>8585 a 4621 cd</td>
<td>1430 ab 859 def</td>
</tr>
<tr>
<td>CIAT16888</td>
<td>Tolerant</td>
<td>12 def 20 a</td>
<td>1.9 bcd 0.3 g</td>
<td>21 ef 25 def</td>
<td>6188 bc 4698 cde</td>
<td>1014 bcd 775 de</td>
</tr>
<tr>
<td>CIAT25670</td>
<td>Tolerant</td>
<td>17 bc 21 a</td>
<td>1.5 cde 0.6 fg</td>
<td>27 cdef 20 f</td>
<td>8956 a 5495 bc</td>
<td>1400 a 1026 cd</td>
</tr>
</tbody>
</table>
Table 2. Root cross-sectional area, aerenchyma percentage of root cross-section, stele area of adventitious roots and cortex to stele ratio at 50 mm behind the root tip of eight *Urochloa* genotypes grown in aerated or stagnant deoxygenated solutions for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$, Tukey test).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Tolerance to waterlogging</th>
<th>Root cross-sectional area (mm$^2$)</th>
<th>Aerenchyma (%)</th>
<th>Stele area (mm$^2$)</th>
<th>Cortex/Stele (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerated  Stagnant</td>
<td>Aerated  Stagnant</td>
<td>Aerated  Stagnant</td>
<td>Aerated  Stagnant</td>
</tr>
<tr>
<td>Marandu</td>
<td>Sensitive</td>
<td>4.04 ab  3.59 abc</td>
<td>3.2 de  13.3 c</td>
<td>0.79 a  0.59 bc</td>
<td>2.69 g  3.75 fg</td>
</tr>
<tr>
<td>CIAT26124</td>
<td>Sensitive</td>
<td>2.19 de  2.61 bcd</td>
<td>1.5 e  13.0 c</td>
<td>0.39 def  0.38 def</td>
<td>3.25 g  4.36 defg</td>
</tr>
<tr>
<td>Toledo</td>
<td>Sensitive</td>
<td>2.06 de  4.52 a</td>
<td>1.5 e  5.1 d</td>
<td>0.48 cde  0.59 bc</td>
<td>3.05 g  4.99 cdefg</td>
</tr>
<tr>
<td>Mulato II</td>
<td>Sensitive</td>
<td>2.70 bcd  4.07 a</td>
<td>6.4 d  10.3 c</td>
<td>0.50 cd  0.67 ab</td>
<td>3.07 g  3.95 efg</td>
</tr>
<tr>
<td>Llanero</td>
<td>Tolerant</td>
<td>1.29 e  2.43 cde</td>
<td>11.2 c  23.1 a</td>
<td>0.16 h  0.22 gh</td>
<td>4.34 defg  6.81 bc</td>
</tr>
<tr>
<td>Tully</td>
<td>Tolerant</td>
<td>2.52 cde  3.76 cde</td>
<td>12.5 c  17.3 b</td>
<td>0.28 fgh  0.29 fgh</td>
<td>5.62 cde  9.31 a</td>
</tr>
<tr>
<td>CIAT16888</td>
<td>Tolerant</td>
<td>1.81 de  2.46 cde</td>
<td>9.8 c  19.0 b</td>
<td>0.23 gh  0.28 fgh</td>
<td>4.94 cdefg  6.08 cd</td>
</tr>
<tr>
<td>CIAT25670</td>
<td>Tolerant</td>
<td>2.49 cde  3.92 ab</td>
<td>12.9 c  17.4 b</td>
<td>0.28 fgh  0.34 efg</td>
<td>5.56 cdefg  8.09 ab</td>
</tr>
</tbody>
</table>
### Table 3. Modelled maximum lengths of adventitious roots attained in waterlogging conditions of eight *Urochloa* genotypes (four sensitive and four tolerant).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tolerance to waterlogging</th>
<th>Original model*</th>
<th>Model including stele proportion**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Maximum root length (mm)</td>
<td>Maximum root length (mm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Averaged resp. rates¥</td>
<td>Sensitive vs tolerant resp.Φ</td>
</tr>
<tr>
<td>Marandú</td>
<td>Sensitive</td>
<td>167</td>
<td>158</td>
</tr>
<tr>
<td>La Libertad</td>
<td>Sensitive</td>
<td>165</td>
<td>156</td>
</tr>
<tr>
<td>Toledo</td>
<td>Sensitive</td>
<td>99</td>
<td>93</td>
</tr>
<tr>
<td>Mulato II</td>
<td>Sensitive</td>
<td>144</td>
<td>136</td>
</tr>
<tr>
<td>Llanero</td>
<td>Tolerant</td>
<td>234</td>
<td>249</td>
</tr>
<tr>
<td>Tully</td>
<td>Tolerant</td>
<td>195</td>
<td>208</td>
</tr>
<tr>
<td>CIAT16888</td>
<td>Tolerant</td>
<td>206</td>
<td>220</td>
</tr>
<tr>
<td>CIAT26570</td>
<td>Tolerant</td>
<td>196</td>
<td>209</td>
</tr>
</tbody>
</table>

* This model was calculated using equation (2) and assuming constant respiration.
** This model also uses equation (2) but differences in respiration rates among cortex and stele tissues are computed.
¥ The respiration rates were averaged between both sensitive and tolerant genotypes and the mean was used for calculations.
Φ Respiration rates from waterlogging-sensitive cv. Mulato II and waterlogging-tolerant cv. Tully were used for the modelling of maximum root length of all sensitive and tolerant genotypes, respectively.

The presence of a tight barrier was computed for tolerant genotypes while no barrier was assumed for sensitive genotypes, based in suberin depositions in exodermal layers. The numerical values used in the models are as follows: Cortex O$_2$ status at the root-shoot junction (Co): 2.7 $10^{-4}$ g cm$^{-3}$; diffusion coefficient of oxygen in air (D): 0.258 cm$^2$ s$^{-1}$ (at 30 °C); fractional root porosity (ε): different values summarised in Table 2 but expressed as proportion (i.e., 0.13 instead of 13%); Tortuosity (τ): 1.0; tissue respiration (M): different values of root respiration were used for comparison: 1) the average values between the two genotypes evaluated (i.e., 2.63 nmol O$_2$ g FW sec$^{-1}$) and 2) the respiration rate of waterlogging-sensitive cv. Mulato II (i.e., 2.94 nmol O$_2$ g FW sec$^{-1}$) assumed equal for all sensitive genotypes and the respiration rate of waterlogging-tolerant cv. Tully (i.e., 2.31 nmol O$_2$ g FW sec$^{-1}$) assumed equal for all tolerant genotypes. For the model calculations root respiration values were expressed based in volume (according to porosities in Table 2 and expressed as ng O$_2$ cm$^3$ s$^{-1}$). Respiration values for stelar tissues are assumed to be 3-fold higher than those of cortical tissues (Pedersen et al. 2020).