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BRIEF COMMUNICATION

Evaluating root characteristics under field conditions in perennial ryegrass for potential application in commercial breeding programmes

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Abstract

Perennial ryegrass (PRG; *Lolium perenne*) remains the backbone of grass swards in Northern Ireland due to its improved digestibility persistence, and ease of management compared with other grass species. However, innovative breeding approaches are needed that include positive environmental outcomes, as well as improved productivity in ruminants. The objective of this study was to evaluate the feasibility of root-trait screening and selection using the *in situ* coring method under commercial grass breeding field conditions. 108 root cores were sampled over a 2-year period from a field trial sown in autumn 2021. Root cores were washed, scanned, and analysed using the open-access root scanning platform *Rhizovision*. A seasonal effect was noted whereby significant differences were detected in October for root volume, network area, and surface area, but no significant differences for any root parameter were detected in April. No association was observed between root volume, network area, or surface area at the October sampling with either dry matter (DM) yield at the 4th cut (October) or annual DM yield. These results suggest that this method may be useful for identifying improved germplasm in PRG for root characteristics; however, being comparatively labour and time intensive this method may not be practicable for large-scale breeding programmes.

Keywords: *Festulolium*, grass breeding, *Lolium perenne*, perennial ryegrass, *Rhizovision*, root samples, root washing.

The livestock sector in Northern Ireland (NI) is underpinned by a successful and high performing grassland base of which perennial ryegrass (PRG; *Lolium perenne*) is a dominant species, benefitting from a temperate climate and high yields under high N fertiliser usage whether managed for conservation or grazing (Cyriac *et al.* 2018). Agriculture accounts for 28% of NIs greenhouse-gas (GHG) emissions, totalling 6.2 million tonnes of carbon dioxide equivalent, and NI will require a reduction in GHG emissions of at least 35%, with a strong focus required on nitrous oxide, by 2050 (Northern Ireland greenhouse gas statistics 1990-2021). Climate change is forecast to affect NI with an estimated increase in mean annual temperature of between 1 - 1.6°C by 2050 and an increase in the number of unfavourable weather events such as drought and heavy rainfall (O'Brien and Nolan

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Abbreviations: AFBI - Agriculture Food and Biosciences Institute; CF - commercial *Festulolium*; CT - commercial tetraploid; cv - coefficient of variation; DAERA - Department of Agriculture, Environment, and Rural Affairs; DM - dry matter; DOMD - digestibility of organic dry matter; GHG – greenhouse gas; IT - intermediate tetraploid; LED - light emitting diode; NI - Nothern Ireland; NUE - nutrient use efficiency; PRG - perennial ryegrass; REML - restricted maximum likelihood; SOC - soil organic carbon.
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2023). Future government policies across the world will challenge ryegrass breeders to target a more sustainable, 'green' future, and adaptations such as increased nitrogen use efficiency (NUE) and resilience to climate change.

Scientific interest in ryegrass root systems is high because of the importance of roots in NUE, drought tolerance, and in the soil organic carbon (SOC) content captured by pastures (Wedderburn *et al*. 2010, Paez-Garcia *et al*. 2015). Breeding for improved root traits, such as depth and volume, therefore has the potential to improve NUE, resilience to abiotic stresses such as drought, and increase carbon sequestration in soils (Kell 2011). Deeper penetrating roots can also improve soil structure by improving aeration and drainage, ultimately improving soil fertility and reducing erosion (Marshall *et al*. 2016, Pierret *et al*. 2016).

Selecting for increased root production in PRG is possible, including a greater root depth after several generations of selection (Bonos *et al.* 2004). Houlbrooke *et al*. (1997) reported evidence of genetic diversity within breeding populations with, for example, differences in sensitivity to soil bulk density noted between breeding lines and a registered variety, while Matthew *et al*. (1998) found differences in root nodal production frequency between two ryegrass breeding populations. However, it was noted that environmental factors can have a significant impact on the expression of any root mass-mediated NUE or carbon sequestration.

A number of studies have investigated the effect of drought on PRG under controlled environmental conditions. For example, Tozer *et al*. (2017) investigated the effect of drought on root mass and depth on four PRG cultivars under greenhouse conditions, whereas growing systems using rhizotrons and boroscopes have been used to examine differences in root growth under both watered and drought conditions (Wedderburn *et al*. 2010). Crush *et al.* (2010) also successfully used growth tubes filled with sand and irrigated with a nutrient solution, to accurately measure root:shoot ratio, with roots assessed using image analysis software. This process has been further expanded to include computer-based learning methods to generate an automated segmentation system for root analysis of plants grown under experimental conditions in rhizotrons (Smith *et al.* 2020). These experiments allow for direct control of growth conditions for the entirety of the study, as well as analysis of the complete root system.

Poor correlations have been shown, however, between controlled condition systems and the field environment, due to large genotype × environment interactions and density effects (Hakl *et al*. 2017, Rich *et al*. 2020). From a breeding perspective, the ideal screening method would combine measurement of root systems '*in-situ*' in experimental field plots or established swards with above-ground yield data to provide an overall view of the performance of varieties in trial both above- and below-ground. The aim of this study was therefore to evaluate the feasibility of root-based evaluation methods under field conditions that could be combined with above-ground trait measurement under commercial grass breeding conditions.

The study was conducted on an established intermediate tetraploid PRG trial (sown October 2021) at AFBI Loughgall (54°27'N, 6°04'W). The trial consisted of 18 entries replicated 3 times in an incomplete block design (6 m^2 plots), including 1 commercial *Festulolium* control variety, 7 of the top-performing commercial intermediate tetraploid varieties (from the Recommended List for England and Wales in 2021), and 10 perennial ryegrass synthetic populations derived from the AFBI ryegrass breeding programme. A high-nitrogen fertiliser regime was applied across the growing season (total of 472 kg ha^{-1} N; 127 kg ha^{-1} P_2O_5; 359 kg ha^{-1} K_2O) to reflect typical industry practice for PRG breeding plots in the United Kingdom.

Plots were managed under a simulated conservation management, with 4 sampling dates for herbage yield assessed during the growing season, taken from mid-May to mid-October, with harvest dates determined by sward height (target of 60 mm above-ground level for first 3 cuts and 40 mm above-ground level for the fourth cut in October). Herbage was defoliated to 5 cm above-ground level using a plot harvester (*Haldrup F55*). Regrowth intervals ranged from 4 to 6 weeks. Random herbage samples of approximately 300 g were dried at 80°C for 16 h to determine dry matter (DM) content and adjust fresh yield to DM yield per plot. DM yields were summed across all harvests to produce annual DM yield.

Root sampling was carried out at two sampling dates (October 2022 and April 2023), with 54 root core samples taken on each date (108 total). The hydraulic arm of a digger was used to press a steel corer into each plot to collect a 30 cm deep core from each plot (Chimento and Amaducci 2015) which were labelled and stored at 4°C until washing. Each sample was submerged in water for 1 h before being washed through a 3.5-mm sieve to remove excess soil, grass, and grit. The roots were then carefully cut at the collar/crown and washed again through a 2.5-mm and 1.8-mm sieve. The clean roots were transferred using tweezers into a clean 200-ml container and suspended in water for storage at 4°C until scanning.

Five intact roots from each sample were scanned individually (submerged in water in an acrylic tray) using an *Epson* flatbed scanner with a mounted light emitting diode (LED) light above. A sheet of paper was placed over the scanning tray to diffuse the light. Each image was saved before being analysed using the open-access root scanning platform *Rhizovision Explorer* (Seethepalli *et al.* 2021). *Rhizovision Explorer* was used as an analysis tool *via* the 'broken' root system mode to produce data for number of root tips, number of branch points and branching frequency, total root length, diameter, volume, surface area, network area, and perimeter. A scientific illustration of a typical *Rhizovision Explorer* output image of a processed root from variety intermediate tetraploid 8, IT 8, is presented in Fig. 1. Root washing and separation plus scanning required approximately a further 45 min per sample.

DM yields were analysed by analysis of variance using *TrialWizard* statistical analysis software designed for grass

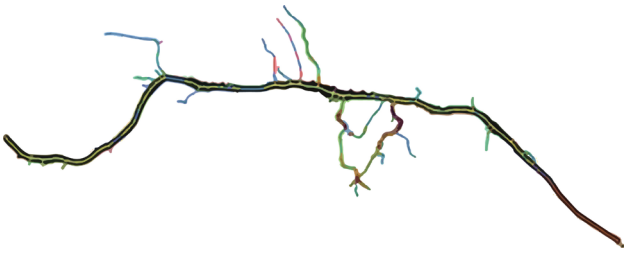

Fig. 1. Scientific illustration of a typical *Rhizovision Explorer* output image of an example root scanned from an experimental population of perennial ryegrass (IT8). Sample taken in 2022 from a plot-based field trial under conservation management. Colours denote sections of root at defined diameters (data not shown). IT8, Intermediate Tetraploid population 8 bred in 2020 at the AFBI perennial ryegrass breeding programme pipeline at Loughgall, NI.

breeding use. Randomization was designed to enable Nearest Neighbour analysis to adjust for environmental variability in the field. Root data were analysed using linear mixed model methodology, restricted maximum likelihood (REML) estimation method using *Genstat v.21.1*. As the varieties were laid out in a row/column design, row and column were fitted as random effects while variety was fitted as a fixed effect. For all data, the significance of variety was determined by comparing an *F*-statistic against the corresponding *F*-distribution. If this was significant ($P<0.05$), then *Fisher*'s Least significant difference test was used to compare the pairwise differences between the levels of variety. The adequacy of the models fitted was assessed by visual examination of the appropriate residual plots.

There was a significant difference between the populations assessed for root volume, network area, and surface area ($P<0.05$; Table 1) in the October 2022 sampling. The commercial *Festulolium* produced higher root volume, network area, and surface area compared with the commercial tetraploid PRG varieties and most other breeding populations included in the trial. One breeding population, however, produced root volumes, network areas, and surface areas that were as high as the *Festulolium* control – intermediate tetraploid 8 (IT8), and higher than most, although not all other populations tested as part of the study. There were no significant differences observed for any root trait assessed from the samples taken in April 2023 (data not presented). There was no observable trend between root volume, network area, or surface area at the October sampling with either dry matter (DM) yield at the 4th cut (October) or annual DM yield. IT8 produced significantly higher DM yield for both the 4th cut and for annual yield than

Table 1. Yield, root volume, root surface area, and root network area for commercial and experimental populations of perennial ryegrass and *Festulolium* taken in 2022 from a plot-based field trial under conservation management. * - Varieties sharing a letter are not significantly different according to *Fisher*'s least significant difference test ($P<0.05$). CF - commercially available *Festulolium*; CT - commercially available intermediate tetraploid; cv - coefficient of variation; DM - dry matter; IT - intermediate tetraploid populations 1 - 6 (IT 1 - 6) bred in 2020 at the AFBI perennial ryegrass breeding programme pipeline at Loughgall, Northern Ireland.

Variety	Volume [mm^3]	Surface area [mm^2]	Network area [mm^2]	DM yield Cut 4 (October) [t ha^{-1}]	Annual DM yield [t ha^{-1}]
CF	46.65^{a}	285.3^{a}	81.01^{a}	3.36^{g}	15.91^{ef}
IT 8	42.17^{ab}	255.0^{ab}	72.51^{ab}	4.27^{abc}	18.60^{bc}
CT 4	33.45^{abc}	210.6^{abc}	59.31^{bc}	3.71^{efg}	15.73^{ef}
CT 1	31.22^{bcd}	198.5^{bcd}	56.32^{bcd}	3.63^{fg}	17.88^{cd}
IT 4	27.98^{bcd}	182.4^{bcd}	51.81^{bcd}	4.38^{ab}	19.67^{ab}
CT 5	27.84^{bcd}	171.5^{cd}	48.41^{cd}	3.56^{fg}	17.34^{cde}
IT 7	27.30^{cd}	176.9^{cd}	50.93^{cd}	4.11^{bcde}	18.42^{bc}
IT 10	26.91^{cd}	164.0^{cd}	46.24^{cd}	4.15^{abcd}	16.99^{cdef}
IT 5	25.02^{cd}	165.2^{cd}	46.60^{cd}	4.31^{ab}	20.20^{a}
CT 3	23.30^{cd}	159.0^{cd}	45.23^{cd}	3.67^{fg}	17.34^{cde}
CT 7	22.93^{cd}	152.3^{cd}	44.06^{cd}	3.79^{defg}	16.45^{def}
CT 6	22.63^{cd}	163.4^{cd}	46.05^{cd}	3.87^{cdef}	16.99^{cdef}
CT 2	22.54^{cd}	155.5^{cd}	44.45^{cd}	3.63^{fg}	15.38^{f}
IT 1	22.31^{cd}	141.0^{cd}	39.83^{cd}	4.31^{ab}	18.42^{bc}
IT 2	21.52^{cd}	143.9^{cd}	40.96^{cd}	3.87^{cdef}	19.67^{ab}
IT 6	21.52^{cd}	143.4^{cd}	40.14^{cd}	4.27^{abc}	18.60^{bc}
IT 9	19.92^{cd}	140.0^{cd}	39.37^{cd}	4.58^{a}	19.85^{ab}
IT 3	18.79^{d}	130.1^{d}	37.00^{d}	3.71^{efg}	18.60^{bc}
cv	3.60	4.27	4.26	6.80	5.30
LSD*	14.55	77.21	21.34	0.43	1.61
P-value	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.001$	$P<0.001$

the commercial *Festulolium* (4.27 t ha^{-1} and 18.6 t ha^{-1} *versus* 3.36 t ha^{-1} and 15.91 t ha^{-1}, respectively), but both IT8 and the commercial *Festulolium* produced equal root volumes, network areas, and surface areas.

Previous research has indicated that the investigation of root systems of field-grown plants with extensive rooting systems is labour intensive and time consuming (Freschet *et al*. 2021), requiring the collection of a large number of soil cores, a rigorous root washing process and further analysis of the collected root samples (Rinehart *et al.* 2022). This study confirms this finding, with each sample requiring a processing time of approximately 105 min in total for processing following removal from the field. Compared with processing time required for above-ground sampling (for example, fresh yield), which can be accomplished in seconds using machinery with on-board weighing devices, this represents a considerably higher investment in labour and time requirements. Thus, the method outlined in this paper is likely not suitable for use as part of routine screening at commercial grass breeding programmes, although the methodology could be useful for related research projects and for the advancement of novel high-throughput root-based technologies.

Seasonal patterns of root growth have been identified in PRG, with root growth being greatest in autumn and least in summer, when there is more vegetative growth (Wedderburn *et al.* 2010). This suggests that autumn sampling may be more likely to identify subtle differences in rooting traits in a field-based trial. In addition, seasonal growth patterns are also known to result in increased foliage production in the spring, while root biomass is more likely to increase during the autumn (Robin *et al.* 2018). This study supports these conclusions, with significant variation observed only in autumn. It is likely that regrowth after cutting in October utilized energy reserves stored in the roots stunting root growth and reducing variability at the April sampling date. Further work is underway to evaluate rooting under field conditions in ryegrass over the entire growing season and over multiple seasons.

Festuloliums are created by targeted cross-breeding of ryegrass and fescue species, and are known to combine the stress resistant genes of fescue with the yield and digestibility of ryegrass, often producing the deeper and larger root structures associated with the fescue species (Humphreys *et al.* 2014). In this study, a commercially available *Festulolium* derived from a cross between *Festuca mairei* and *Lolium perenne* was included to test the hypothesis that *Festuloliums* could be useful as controls in this type of root screening work. The *Festulolium* included produced significantly higher root volume, network area, and surface area than all other populations tested with the exception of one AFBI-bred ryegrass pre-commercial population, supporting this hypothesis.

Although variation between populations of elite germplasm was detected in this study, no correlation was observed between any root-based trait and above-ground yield. These results therefore support previous work which detected differences between specific varieties of PRG for root depth and mass, but similar root:shoot ratios for these varieties (Sokolovic *et al*. 2013). It has been suggested that investment by the plant into dry matter in the root does not automatically lead to reduced shoot yield, supporting the premise that PRG can be improved for both root and shoot-based traits simultaneously. Further work is required to determine the heritability within ryegrass for root-based traits at field level in association with above-ground traits that impact productivity. This data will be critical for a full evaluation to be made by breeders' regarding the feasibility of breeding for root-based traits in PRG alongside above-ground parameters.

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