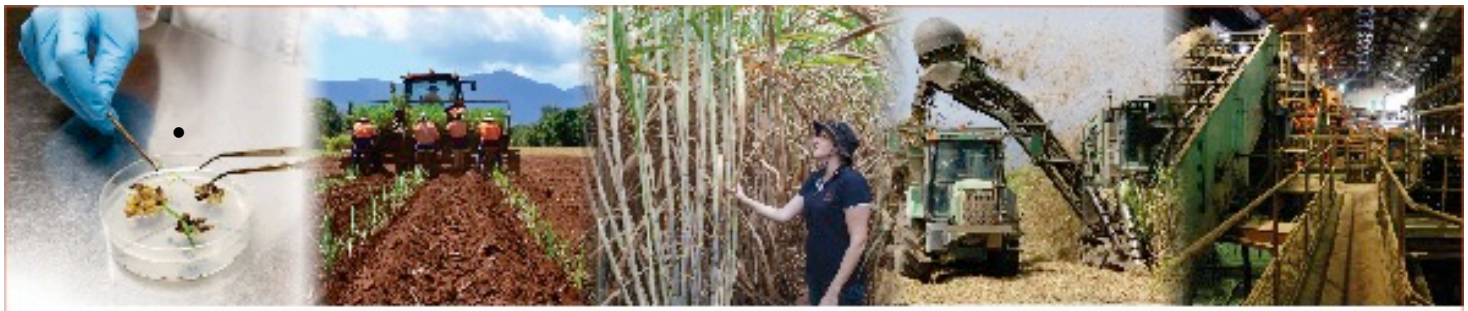

FINAL REPORT 2015/002

Sugarcane root systems for increased productivity; development and application of a root health assay

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ABSTRACT

A better understanding of the sugarcane root system has the potential to improve productivity and overcome soil constraints. By adapting the digital methods that have been developed in other crops, we have developed a toolkit of reliable methods that enable analysis of large numbers of root samples. These methods have been used to provide a baseline understanding of the range and variation of root parameters for sugarcane, including root/shoot ratios, root opening angle, root length, proportion of fine roots, branching density, average diameter and diameter in each size class. We found a consistently high proportion of fine roots, but there was genetic variation for many other key traits amongst current commercial lines. Importantly, there were no significant reductions in root system size or quality in modern varieties compared to older varieties. The methods and baseline were then applied to test the response to stresses encountered in Australian growing environments. When comparing plants with or without YCS symptoms, we found no differences in root system structure, despite significant reductions in shoot mass. Limiting growth by removal of tillers or by restricting nitrogen availability identified plasticity in specific root traits that enabled the plants to adapt to the restrictions. Root system distribution by depth, and relative allocation of resources to the root system showed adaptations to stress while root angle appeared to be stable. With the new methods and knowledge of trait plasticity, we can now start to test which traits provide a benefit in various agronomic situations and develop an integrated understanding of root health which can be used to monitor soil health and promote the adoption of better agronomic practices.

EXECUTIVE SUMMARY

The root system plays a key role in determining yield by controlling nutrient and water uptake during crop production. As root systems are the interface between the plant and soil microorganisms, they are also important for resistance to soil-borne diseases. Root systems in sugarcane also have a structural role in anchorage, preventing lodging and loss of plants at harvest, which has a major impact on ratoon yields. Finally, the root systems provide resources for the growth of the following season's ratoon crop. Hence, a better understanding of root systems has the potential to improve productivity and overcome soil constraints. Studying roots in soil has always been challenging and is especially complicated in sugarcane because of the size of the plant and the long growing season. The manual measurement methods that were previously available were laborious and prone to error. As a result, our knowledge of sugarcane root system was based on a small number of varieties and samples. Without reliable methods that can be applied at scale, it was difficult to develop a baseline definition of the healthy root system that would enable meaningful comparisons of the responses to biotic and abiotic stresses. This project has capitalised on the advances in digital analysis methods that were being developed in other crops to adapt and apply the new method to sugarcane. The goal was to deliver new methods for analysis, provide a baseline understanding of the range and variation of root parameters for sugarcane, and identify key traits that respond to stresses.

The project was conducted as a collaboration between CSIRO and SRA, and was initially managed as part of the SRA Yellow Canopy Syndrome Investigation. Root system samples were harvested from plants grown in large planter bags or tall pots in the glasshouse, or from blocks of soil collected underneath field-grown plants. We tested methods for characterising root system architecture using images of intact root systems, and methods for comparing root structural features, using images of individual roots. Digital image analysis successfully identified key traits, including root opening angle, root system total length, average diameter, proportion of roots in each size class, nodal root number, specific root length (m per g of root), and root branching density. These methods were used to compare the early root systems of 20 modern and historical varieties, providing a baseline definition of healthy root systems in sugarcane, including the extent of variation for each trait. This baseline then provided an opportunity to test how sugarcane root systems from a subset of varieties respond to constraints that are likely to occur in Australian growing regions, specifically, shoot growth restriction by removing tillers, nitrogen limitation and Yellow Canopy Syndrome (YCS).

In addition to defining new methods that can be applied to large numbers of samples, the results showed that there is genetic variation for key traits amongst current commercial lines, such as shoot/root ratio. Current varieties have a consistently high proportion of fine roots when grown in optimal conditions, and there is a strong overall relationship between above-ground mass and root volume. Compared to other crops, sugarcane roots are very efficient in terms of the energy cost to maintain a large root system. Importantly, analysis of modern varieties compared to older varieties showed that there have been no consistent trends towards particular root features over a 50-year timeline of released varieties.

The new methods and baseline were first applied to test the role of root systems in Yellow Canopy Syndrome (YCS). There was no correlation between root system structure and the propensity of a variety to display YCS symptoms. Furthermore, in samples from field grown plants with or without YCS symptoms, we found no differences in root system structure, despite significant reductions in shoot mass.

Key traits that allow sugarcane root systems to overcome challenging environments were identified. Removal of tillers tested how limitation to carbon supply impacts the allocation of resources between shoot and root. Nitrogen limitation showed adaptations to the root system structure, architecture and anatomy to enable a larger volume of soil to be explored. Overall, changing root angle does not appear to be a mechanism used by sugarcane to adapt to stress. However, plasticity in the distribution by depth, and relative allocation of resources to the root system are traits used to adapt to challenging environments.

These results are a substantial increase in our knowledge of the “typical” sugarcane root system. The immediate outcome of this project is that more rigorous methods for comparing between genotypes for root system traits are now available for researchers to use. This has already enabled us to show the impact of breeding and selection on the root systems of modern varieties and will also benefit investigations into pathogen resistance, ratoon vigour and other traits. The outcome of this approach will be an integrated understanding of root health which can be used to monitor soil health and promote the adoption of better agronomic practices. In addition, the implications of the present results are that there are genetic differences which could be exploited to improve performance. With the knowledge of trait plasticity, we can now start to test which traits provide a benefit in various agronomic situations. This information can add to the variety improvement program, unlocking the potential of below-ground traits for improved productivity.

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1. BACKGROUND

1.1. Role of the root system

The root system plays a key role in determining nutrient and water uptake. During crop production, inadequate root systems lead to inefficient water and fertiliser use, limiting yields and increasing nutrient run-off into waterways. As root systems are the interface between the plant and soil microorganisms, low resistance to soil-borne diseases can lead to significant yield losses. In sugarcane, root systems also play an important role in anchorage. Poor anchorage leads to lodging and loss of plants at harvest, contributing to declining ratoon yields. Uprooting of stools at harvest increases impurities delivered to mills and causes damage to the rollers. Finally, the perennial nature of sugarcane means that root systems are an important reservoir of resources for the growth of the following season's ratoon crop. Hence productivity gains across multiple sectors could be achieved through better understanding of root systems.

Unfortunately the opaque and solid nature of the soil has always been a major limitation for the study of root systems and this is complicated in sugarcane by the size of the plant and the length of the growing season. Consequently, our knowledge of sugarcane roots is sparse and plant breeders lack the knowledge and tools to select for root traits either directly or indirectly (Smith et al. 2005). There is a clear need to develop better methods for analysing sugarcane root systems and to understand their diversity and adaptation to soil conditions

1.2 Root system traits for improved productivity in grass crops

In other crops, root phenotypes that contribute to increased productivity have been identified. For example, vigorous early root growth in wheat enables efficient uptake of nitrogen (Liao et al. 2006). Recently, the impact of root architecture on efficiency of water and nutrient capture has been recognised. A number of root architectural properties influence the depth and volume of soil explored including angle of emergence, total length and branching. In maize, studies of root architecture enabled the definition of an ideotype for the optimum root system architecture, summarised by Lynch (2013) as "steep, deep and cheap". Modelling and field experiments have demonstrated that deeper roots improve growth in dry conditions by accessing subsoil moisture (Kamoshita et al. 2002; Lilley and Kirkegaard 2011; Wasson et al. 2014). In wheat this can be achieved by faster root elongation and/or a steeper angle of growth (Wasson et al. 2014). In sorghum, genetic variation in root angle has been shown to be a highly heritable trait which contributes to water use efficiency (Mace et al. 2012).

Root hairs and mucilage secretions have been shown to improve nutrient uptake from dry soils by stabilising a "rhizosheath" of soil surrounding the roots (Watt et al. 1994). Features of the root internal anatomy can also influence productivity. One of the best known examples focussed on the diameter of xylem vessels in wheat roots. Modelling studies had predicted that reduced vessel diameter would restrict the uptake of water in drying soils so as to maximise water use throughout the season. A breeding program selecting for this trait was able to produce varieties with up to 11% higher yields in dry environments (Richards and Passioura, 1989).

Recently, root anatomical traits have been identified that reduce the metabolic cost of soil exploration by changing the proportions of living to non-living tissue (Lynch 2014). For example, shoot biomass under drying conditions was increased in maize lines that had either larger root cortical cells or fewer cortical cell files (Chimungu et al. 2014a, 2014b). Similar gains were achieved by increasing the proportion of aerenchyma, a modified root cortical tissue with large airspaces between living cells (Zhu et al. 2010). It is likely that the reduced number of living cells in the tissue

results in higher allocation of resources to root extension and permits a greater volume of soil to be explored (Chimungu et al. 2014a, Lynch et al. 2014). Substantial variation in this trait has been identified and efforts are now underway to select optimum varieties for tropical maize production.

1.3. Structure of sugarcane root systems

Most previously available descriptions of sugarcane root systems have been derived from excavation of a small number of plants. Plants are generally established vegetatively from setts, short segments of stalk including one or more buds and bands of root primordia located at nodes. The studies showed that sett development was initially sustained by very fine and highly branched sett roots which arose from the band of root primordia adjacent to the sett nodes (Glover 1967). About 7-14 days after the initiation of sett roots, shoot nodes developed roots that formed the main root system. These roots arise from the band of primordia on the unexpanded internodes of the new shoots (Glover 1967, Spaull 1980). The rate of elongation of shoot roots was found to be higher than that of sett roots with rates of between 28-80 mm/day reported (summarised in Smith et al. 2005). The sett roots eventually declined in both number and total mass but their longevity varied between genotypes (Spaull 1980). Smith et al. (2005) reported that the majority of roots remained close to the surface (top 60 cm) while root length densities varied between 1.3 - 5.3 cm cm^{-3} in different environments. Root hairs were found on both sett roots and shoot roots, but their abundance as a function of total root length declined over time (Spaull 1980). The role and function of the different root types during the crop cycle are not well characterised (Smith et al. 2005).

1.4. Variation in sugarcane root systems

Root system architecture shows some plasticity and is influenced by both genetics and environment (Robinson et al. 2008). Glasshouse experiments showed that there were specific varietal differences in shoot-root ratios (Magarey et al. 1999) and there was a tendency to maintain these ratios. Interestingly, young plants were able to regenerate their entire root systems to reach a consistent mass within 8 weeks after removal of roots and replanting (Spaull 1980). Branching frequency also appears to be under genetic control. There were strong genotypic influences on the ratios of primary: secondary: tertiary root lengths, which remained constant under a range of treatments (Magarey et al. 1999). Root systems are also strongly responsive to environmental conditions. For example Otto et al. (2009) found a higher proportion of roots in the upper soil layer in fertilised plots compared to unfertilised plots. In irrigated fields, the majority of roots were found within 60 cm of the surface, compared to rainfed sites where 90% of the roots were found within 90 cm of the surface (Magarey et al. 1999, Reghenzani 1993). Application of water and nutrients through a surface drip feed system also resulted in a higher proportion of roots in the top 50 cm of soil (Evensen et al. 1997). Studies of small numbers of genotypes have confirmed that there is also genetic variation in the way that cultivars respond to environmental influences (Robinson et al. 2008). There appears to be "spare capacity" in sugarcane roots enabling functional compensation for damage to the root system or variability in the soil. Plants were able to tolerate the loss of up to 50% of the root system with little impact on growth rates in some circumstances (Smith et al. 2005).

1.5. Root system traits and sugarcane productivity

There are very few studies of the impact of particular root traits on productivity in sugarcane. Anatomical characteristics of sugarcane roots have been shown to affect hydraulic conductance (Saliendra and Meinzer 1992). The proportional area of the stele and the xylem vessels was correlated with increased capacity for water uptake.

The rate of root turnover and the fate of the root system between each ratoon are largely unknown (Smith et al. 2005). Glover (1968) showed that root systems remain alive after harvest and contribute to the early growth of the ratoon crop, although new roots are eventually produced by the new shoots. Research in other perennial grasses suggests that the below-ground organs contribute to performance in subsequent seasons by acting as storage organs for water, carbohydrates and nutrients, especially nitrogen. Root and rhizome traits were shown to be associated with the performance under drought stress in bermudagrass genotypes (Cynodon spp.) (Zhou et al. 2014). In the temperate perennial, switchgrass, efficient withdrawal of nitrogen into the root system at the end of the growing season is an important element of the economic system for biofuel production (Wayman et al. 2013).

1.6. Overcoming phenotyping limitations

Developing healthy root systems is now recognised in many crops as essential to closing the yield gap through management or genetic solutions. Researchers have been working on innovative solutions to overcome the phenotyping bottleneck (Gregory et al. 2009; Zhu et al. 2011), specifically to (i) develop phenotyping techniques in controlled conditions that will mimic the field situation; (ii) develop high throughput field phenotyping techniques to translate laboratory observations to the field. As a result, new techniques have been developed for maize (Grift et al. 2011), wheat (Rich et al. 2013), rice (Nagel et al. 2012) and sorghum (Singh et al. 2011).

Improvements in digital technology underpin new methods for using digital cameras and flat bed scanners to assess dynamic aspects of root development (Adu et al. 2014; Ingram et al. 2012; Nagel et al. 2012). As these techniques restrict the study to young seedlings, end-point methods have also been developed for pots where the entire root system is extracted and scanned (Poire et al. 2014). Automatic image processing can then extract and classify individual roots according to more than a dozen characteristics. This technique allows the root system to develop in a 3D space under a set of conditions which can be altered to test a range of constraints relevant to field conditions. Other laboratory based techniques have been developed to analyse roots in-situ (X-ray tomography, NMR) but are still experimental and cannot be applied to large numbers of plants.

Previously, root colour has been the major way of estimating root viability, since darker colours indicate cell degradation. Recently, more precise methods for quantitating the proportion of living cells have been developed including DNA-based methods (Haling et al. 2012) and high-throughput assays using cell viability dyes (Luce et al. 2014, Ruf and Brunner 2003).

For field phenotyping the leading techniques are: (i) mechanical excavation of the top 50 cm of root systems by a large diameter soil corer (Wu and Guo 2014); (ii) a 2 m long auger fixed on a tractor that will extract a narrow soil core beneath the stalk (Wasson et al. 2014). For both techniques the subset of root system is analysed by automatic image processing and the measured root traits are correlated to yield components. Minirhizotrons, consisting of a transparent tube containing a camera have been used to monitor root growth in the field but may not produce reliable results (discussed in Wasson et al. 2012). For high-throughput phenotyping of below-ground traits, techniques that may be valuable in the future include ground-penetrating radar (Zenone et al. 2008) and electrical resistance tomography Amato et al. (2009), but these are still at an experimental stage.

1.7. Gaps and opportunities in sugarcane research

In sugarcane, as in other crops, there is likely to be variation in root traits and some traits will be beneficial in overcoming constraints to growth. Reliable methods for analysing sugarcane root

systems are lacking. This limits progress towards defining the features of a healthy root system and investigating how the root system is influenced by genotype, crop stage and environmental factors.

Better trait knowledge offers several advantages to a variety improvement program (Jackson et al. 1996). Understanding the key physiological constraints to plant performance leads to more effective design of selection trials, which may benefit from inclusion of a particular environment or particular developmental stage. By identification of key traits and development of screening methods, the research also enables assessment of variation amongst parental lines to determine whether introgression of new germplasm would be beneficial. With the progress on root phenotyping in other crops, there is now an opportunity to adapt these methods for sugarcane and unlock the potential of below-ground traits for improved productivity.

2. PROJECT OBJECTIVES

The overall objectives of this project were to provide a baseline description of healthy sugarcane root systems and to identify specific constraints that affect root development and root health. Specific research objectives were:

- Establish a tall pot growth system and develop digital methods that can be used widely in pot and field trials to standardise the measurement of root architecture traits.
- Establish a set of parameters to measure root cell viability and integrity to assess root function and turnover.
- Provide a baseline description of a healthy root system, in terms of root architecture and function, based on plants grown in pots with non-limiting supplies of water and nutrients. This will form the standard for assessing root systems in field conditions.
- Assess the diversity of sugarcane root systems amongst commercial varieties and near-to-commercial lines.
- Define the effect of specific factors on root architecture and function in a range of genotypes in both pot and field situations.
- Assess changes to root architecture and function in plants affected by Yellow Canopy Syndrome.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

The major outputs delivered by the project are:

(i) Baseline description of sugarcane root systems.

- New methods and key parameters for assessing sugarcane root system traits in plants grown in controlled environments and in the field were developed.
- Application of these methods demonstrated that there is genetic variation for root system traits amongst current commercial lines.
- Analysis of root system traits in modern varieties compared to older varieties showed that there have been no consistent trends towards particular features over a 50-year timeline of released varieties.

(ii) Effects on root systems of specific environmental constraints which are likely to occur in Australian growing regions.

- Structural, architectural and anatomical changes in root systems in response to carbon limitation, nitrogen limitation and YCS were defined, compared to the baseline for healthy roots.
- Genetic variation in response to abiotic stress was identified.
- Traits that show plasticity and potential to adapt to stresses were measured.

3.2. Outcomes and Implications

The outputs are relevant to the research, extension and breeding sectors. The immediate outcome of this project is that more rigorous methods for comparing between genotypes for root system traits are now available for researchers to use. Previously, the sparse knowledge of root systems prevented accurate comparisons, as manual methods for measuring root system traits were laborious and prone to error, such as loss of fine roots. Furthermore, without a meaningful baseline for comparisons, it was difficult to score the effects of treatments on root systems. The methods and baseline presented here can now be used as a framework for comparison of variety performance under biotic and abiotic stresses. One application that may benefit from improved root characterisation is screening for pathogen resistance. For example, the soil-borne pathogen Pachymetra is known to cause damage to root systems, but until now, it has been difficult to test for a correlation between Pachymetra population size and degree of root damage. In a similar way, the effects of soil physical, chemical and biological factors on root growth can now be measured. The outcome of this approach will be an integrated understanding of root health which can be used to monitor soil health and promote the adoption of better agronomic practices.

Root systems are likely to play a role in ratoon vigour and yields, but this has been difficult to quantify. The implications of the present results are that there are genetic differences which could be exploited to improve performance. With the knowledge of trait plasticity, we can now start to test which traits provide a benefit in various agronomic situations. For example, we can now test whether early vigorous growth is an advantage for establishment or regrowth, or whether deeper roots resist stool tipping. If optimal traits are not already present in the breeding populations, target traits can be included in progeny screens in the introgression program, using methods developed in this project.

Finally there are implications for the breeding program. Once varieties are released, their performance can be tracked by yields and this has been an effective tool to monitor genetic gain. However, other feedback on variety performance tends to be anecdotal. This particularly affects the aspects of plant growth that are hard to visualise, either because they are hidden, such as root systems, or complex networks, such as ratoon performance. This is the first time that sound scientific data on the root systems from a timeline of commercial varieties has been available. By showing that there has been no decline in root systems due to genetic selection, industry effort can be re-focussed on agronomic influences on root growth. This information can contribute to grower knowledge and is also valuable feedback for the breeding program on the changes achieved by breeding and selection.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

Communication of objectives and results has occurred throughout the course of the project.

4.1.1. Presentations to industry and research community

- YCS Program meetings in November 2015, June 2016 and November 2016.
- Annual Meeting of the Burdekin Productivity Services Group, Ayr, August 2016.
- SRA Ratooning Workshop, Brisbane, August 2016
- ISSCT conference in Chiang Mai, Thailand, Dec. 2016 (poster presentation)
- SRA Grower Update workshop in Mackay in March 2017.
- SRA Grower Update workshop in Proserpine in April 2018 (invited but cancelled due to a cyclone)
- ASSCT conference in Mackay in April 2018.
- ISRR10th conference in Jerusalem, Israel, July 2018 (Oral presentation)

4.1.2. Other meetings with industry and research community

- NQ Dry Tropics in Townsville in August 2016
- Meeting with Graham Stirling, June 2017 in Brisbane
- Meeting with Davey Olsen (project leader, Soil Health) Jan. 2018 at SRA Brandon.
- Meeting with Paul Nelson, James Cook University, Cairns, Feb. 2018.
- Collaboration with Kirsten Verburg and Zhigan Zhang (CSIRO, Canberra) to add parameters for early root growth in APSIM in a project testing N uptake scenarios.

4.1.3. Industry publications

Brad Pfeffer from the PEC unit interviewed Anne Rae and Johann Pierre and wrote an article about the project that was published in the Cane Connections magazine:

- “Digging down into the function of sugarcane roots”, CaneConnection, Spring 2016.

4.1.4. Journal publications

- Pierre JS, Giblot-Ducray D, McKay AC, Hartley DM, Perroux JM and Rae AL. DNA based diagnostic for the quantification of sugarcane root DNA in the field. Revised for Scientific Reports.
- Pierre JS, Perroux JM and Rae AL. A sugarcane variety screening for root phenes reveals that reducing tillering does not lead to an increased root mass fraction. Submitted to Frontiers in Plant Science.
- Pierre JS, Perroux JM and Rae AL. From root system architecture to root anatomy; adaptation of sugarcane root systems to low nitrogen environments. Manuscript in preparation.

4.1.5. Industry conference papers

- Rae AL, Pierre JS, Olsen D and Perroux JM. (2016) Approaches to analysis of root system traits in sugarcane. Proceedings of the International Society of Sugar Cane Technologists Congress 29. Chiang Mai, Thailand.
- Pierre JS, Rae AL, Olsen DJ, Perroux JM. 2018. Sugarcane Root Systems: Developing a Toolkit of Methods to Understand What’s Going on Below Ground. Proceedings of the Australian Society of Sugar Cane Technologists. Mackay.

4.2. Industry communication messages

Key communication points are as follows:

- We have developed a toolkit of reliable methods that enable analysis of large numbers of root samples.
- Our new methods for assessing root system structure and architecture compare key features, including root/shoot ratios, root opening angle, root length, proportion of fine roots, branching density, average diameter and diameter in each size class.
- We found significant differences between varieties but there were no significant trends towards particular features in our varieties over time.
- Compared to other crops, sugarcane roots appear to be very efficient in terms of the energy cost to maintain a large root system.
- Current varieties have a consistently high proportion of fine roots when grown in optimal conditions, and there is a strong overall relationship between above-ground mass and root volume. These relationships were maintained when the shoot growth was artificially restricted.
- When comparing KQ228 plants with or without YCS symptoms, we found no differences in root system structure, despite significant reductions in shoot mass.
- Insufficient nitrogen supply caused stunted shoot growth but directed a larger proportion of resources into the root systems. To maximise the root system length, plants decreased the root average diameter and tended to decrease branching.

5. METHODOLOGY

5.1. Pot trials

5.1.1. Soil Type

After examining the options for soil types or soil substitutes, the University of California (UC) soil mix Type C was selected, consisting of 50% sand and 50% fine peat bark. This mix mimics field root growth because it provides some resistance to root penetration, compared to soil substitutes such as perlite or vermiculite.

5.1.2. Planting bags

An initial experiment to characterize and compare the early root systems from 20 sugarcane varieties was set up in planting bags. The purpose of the experiment was to test the methodology and to assess the extent of variation amongst a wide set of genotypes. Published information was used to select the size of the planting bags and the timeframe of the experiment. Results suggested that shoot roots emerged after 4 to 6 weeks (Magarey, 1999; Smith, 2005) and had an average descent rate of 1.4 cm per day. Using bags of 30 cm diameter and 35 cm depth, the plants would be able to grow for 8 to 9 weeks before becoming pot bound.

Twenty varieties representing a 50-year span of release dates were selected and treated single eye setts were supplied as planting material by SRA Meringa (Table 1). The setts were initially germinated in small pots and were then transferred to the planting bags before shoot roots emerged. The bags contained the UC soil mix supplemented with Osmocote slow-release fertiliser (5g/L) and were watered manually daily. Three replicate plants from 20 genotypes were planted and

grown for approximately 8 weeks. Glasshouse conditions were 30°C/13 h and 24°C/11h with natural lighting (825°Cd day since planting, assuming a base temperature of 15°C).

Plants were removed from the bags and gently washed with water on a mobile root washing station consisting of a series of mesh screens to retain as much fine root material as possible. Washed root systems were photographed immediately (see section 5.3) and were then stored for later analysis at 4°C in sealed plastic buckets containing 50% aqueous ethanol. The above-ground organs were also characterised by measuring maximum shoot height, number of stalks and leaves, length and area of each leaf, total shoot fresh weight and dry weight.

Table 1. Sugarcane varieties grown in a glasshouse pot trial for analysis of variation in early root growth.

	Variety	Year first planted as seedling		Variety	Year first planted as seedling
1	NCo310	1937	**11**	Q234	1988
2	Empire	1937	**12**	Q200	1989
3	Pindar	1937	**13**	MQ239	1993
4	Q117	1963	**14**	Q232	1994
5	Q124	1969	**15**	Q238	1997
6	Q138	1975	**16**	Q242	1997
7	Q167	1977	**17**	KQ228	1998
8	Q151	1981	**18**	Q252	2000
9	Q188	1982	**19**	SRA1	2005
10	Q208	1987	**20**	QN04-668	

5.1.3. Tall pots

Based on the results of the first experiment, six varieties with contrasting root system size and structure were selected for detailed analysis. The selected varieties were:

- High shoot/root ratio: SRA1, MQ239, Q242
- Low shoot/root ratio: Q208, KQ228, Q151

Treated setts were kindly supplied by SRA Meringa and were germinated in seedling trays in the glasshouse. For each experiment, twelve replicate plants of each variety were planted in tall cylindrical plastic pots, 1.2 m in height and 22 cm in diameter. The interior of the pot was lined with a transparent polyethylene film sleeve 200 µm thick, pierced at the bottom to allow free drainage of water. Pots were filled with UC soil mix and maintained in a glasshouse at 30 °C/24 °C day/night temperatures, with natural lighting and an automatic watering system. Extra pots were set up to monitor growth rate and the experiment was harvested when roots appeared at the base of these pots. Approximately 13 weeks or 1700°Cd after planting, the plants were removed from the pots and root systems were washed out. Growth parameters were measured in the shoot and root systems.

5.1.4. Tiller restriction method

In this experiment, the resilience of the shoot/root ratios for each variety was tested by imposing a treatment that removed part of the shoot mass. For each variety (as listed above), six of the twelve replicate plants growing in the tall pots were restricted to a single tiller. New tillers were removed by

hand when they appeared above the soil. The remaining six replicates were allowed to grow without restriction.

5.1.5. Nitrogen restriction method

Based on the results of a pilot study to define appropriate nitrogen concentrations, the following treatment was applied to six sugarcane varieties (Q208, KQ228, Q151, SRA1, MQ239, and Q242). Six replicate plants of each variety were planted in tall cylindrical plastic pots, as above, filled with the modified UC mix containing micronutrients but lacking the macronutrients. Before planting, columns were soaked with their respective nutrient solutions and then subsequently fertigated, in excess, to prevent salt build up, via dripping irrigation for 10 min every morning (approx. 2 L daily per pot). The concentrated nutrient solutions were mixed to the water in irrigation lines with an Aquablend pump 0.2-2% (Hydro Systems Company, Cincinnati, USA).

Plants were treated with either a high (10 mM) or low (1 mM) nitrogen solutions in the form of ammonium nitrate (n=3 for each treatment). Solution formula for the 200x concentrate was the following: 1 M/0.1 M of ammonium nitrate for high or low nitrogen respectively, 0.2M potassium sulphate, 0.4 M magnesium sulphate hepta-hydrate, 0.091 M monopotassium phosphate and 0.009 M dipotassium phosphate. Solutions were kept in the dark in the glasshouse to prevent any bacterial contaminations and were changed weekly. Nitrate concentration in the solutions at the dripper level and from the pot flow-through were regularly checked using a colorimetric assay (Miranda *et al.*, 2001). Effectiveness of the treatment during the experiment was controlled with regular SPAD measurements on the youngest mature leaf. Glasshouse conditions were 30°C/13h day, 24°C/11h night with natural lighting, and plants were harvested 14 weeks or 1790 °Cd after planting. The entire experiment was conducted twice.

5.2. Field sampling

Roots from field sites were collected using a sampling procedure based on the “shovelomics” method developed for maize (Trachsel et al., 2011). Each sample consisted of a block of soil of 30 cm diameter directly under the stool to a depth of 30 cm. The blocks of soil were soaked overnight in buckets containing sodium polyphosphate solution and were then gently rinsed with water to remove soil from the roots. The intact root systems were photographed immediately and were then transported to the laboratory in Brisbane and stored in the same conditions as for glasshouse samples.

Samples were collected from a field displaying symptoms of Yellow Canopy Syndrome (YCS). The sampled field contained a plant crop of variety KQ228 near Ayr, where YCS symptomatic and asymptomatic plants could both be identified in the same field, and the characteristic symptoms had recently appeared. The criteria for YCS symptoms were yellowing of the upper leaves, consistent with the criteria applied to other YCS investigations (Marquardt et al., 2016). Root systems were collected from 25 symptomatic and 25 asymptomatic plants. Photographs of the intact root systems were collected and roots were then transported to Brisbane, stored in 50% ethanol at 4^{o}C as above, until further analysis.

5.3. Digital imaging and analysis of root system architecture and structure

Digital images of the root systems were captured using two methods:

(i) The intact root system was photographed as soon as possible after washing by suspending it inside a “black box”. Initial tests showed that the photographs need to have a uniform dark background for the optimal performance of the analysis software. The box (106 cm x106 cm x106 cm) was constructed with a uniform black surface with two lateral LED lights (Ledgo 150 LED, Ledgo) and a tripod- mounted camera (Powershot SX60HS, Canon, Tokyo, Japan) that are controlled remotely. A marker of a known size was suspended next to the root system. The digital images were analysed with the automated DIRT (Digital Imaging of Root Traits, Das et al., 2015) and semi-automated REST (Root Estimators for Shovelomic Traits, Colombi et al., 2015) programs.

(ii) The same root systems, after storage in 50% ethanol, were subsequently divided into portions and images were collected by scanning on a flatbed scanner. These images were analysed with the WinRhizo program (Regent, Montreal, Canada), which was able to measure cumulative root length and length in each root size class as well as root volume and diameter.

After all imaging was completed, roots and shoots from each plant were dried at 65°C to obtain their dry weights. These were used to calculate the root mass fraction (RMF) that represents the proportion of the total weight of the plant comprised by the root system, and the corresponding shoot mass fraction (ShMF) representing the proportion of the total weight of the plant comprised by the above-ground parts (StMF, stalk mass fraction and LMF, leaf mass fraction).

5.4. Digital imaging and analysis of root anatomy

Root anatomy was examined in the six varieties used for the structural analysis (SRA1, MQ239, Q242, Q208, KQ228 and Q151). To obtain images with sufficient quality for digital analysis, development and optimisation of a tissue fixation protocol was required. Initial methods that were tested using aldehyde solutions for fixation were found to produce distortion and shrinkage of the cell layers, which would have affected the accuracy and reproducibility of the measurements. This was particularly a problem when the roots developed internal air spaces (aerenchyma), a focus of the analysis because it is a key feature of efficient roots.

The optimal protocol was determined as follows. Roots were cut into pieces of no more than 1 cm long with a sharp blade. Samples were transferred into 15 ml tubes containing the fixative solution (50% ethanol, 2% acetic acid, 2% Tween20). In order to remove air bubbles within the sample to allow penetration of the fixative, tubes were placed in a vacuum desiccator and a gentle vacuum was applied (no more than 0.4 MPa) for about 20-30 seconds then released slowly. This operation was repeated five times. Tubes were then placed on a rotator at room temperature for a minimum of two days to allow complete fixation of the sample. Each sample was placed in an individual histology cassette and the cassettes were placed into the Leica TP20 automatic tissue processor (Leica, Wetzlar, Germany) for a gradual dehydration in ethanol and infiltration with xylene and paraffin. Samples were then embedded in paraffin and the blocks were hardened on a cold plate for 2-3 hours and cooled on dry ice before sectioning. Sections of 6-8 µm were cut with a Leica RM2235 microtome (Leica, Wetzlar, Germany), transferred to microscope slides and dried overnight at 50°C. Sections were dewaxed, rehydrated and stained with Toluidine Blue (2% aqueous). Sections were examined with a Leica MZ16FA dissecting microscope using bright field illumination and digital images were captured using a 5x objective.

For each embedded sample, the image of the best and most representative section was selected for image analysis. Samples were discarded if no cross-section was good enough for image analysis. Images were analysed using the RootScan program (Burton et al., 2012), an open-source software

for root cross section analysis developed by the Lynch group at the University of Pennsylvania. This semi-automated program enables analysis of many aspects of root cross sections including: number of cells in files, number of cortical cells, number of aerenchyma lacunae and their respective proportion relative to the root cross section.

5.5. Statistical analysis

Data processing, visualisation as well as analysis of variance and Tukey post-hoc test were done in R (R_Core_Team, 2017) using the tidyverse (Wickham, 2017) and agricolae (de Mendiburu, 2017) packages.

6. RESULTS AND DISCUSSION

6.1. Measurement methods and key parameters for assessing root system traits

In this report, root systems will be described in terms of architectural, structural and anatomical traits. Architectural features are characteristics of the whole root system, including root system opening angle and length of the projected structure. These features define different soil exploration strategies, as they report on the volume of soil that is accessed by the plant. Root structural features are characteristics of the combined individual roots, including total length, surface area, and size classes. These features can be used to discriminate between plant varieties and report on adaptation to soil constraints as they are often strongly responsive to treatments. At the microscopic level, root anatomy contributes functional information by defining uptake vs. transport roles, and the energy cost of maintaining the root system.

Root system architecture was assessed by imaging the intact root systems after harvest. For each root system, images were collected from a number of different angles. Analysis of the set of images allowed identification of the best program to use and of the parameters that provided the greatest discrimination between the samples. Initial tests showed that the DIRT software was not able to process the images as it was designed for plants that have a single main stalk, such as maize. The multiple stalk structure of sugarcane meant that the software was unable to identify the point of origin of the root system. The REST software allowed us to define manually the origin of the root system and was able to process the images successfully and enabled the measurement of the following parameters: (i) root opening angle which corresponds to the opening angle between the left and right outermost angles to the horizontal along an arc of 10 cm; (ii) area of the convex hull that describes the size of the root system in the image; and (iii) the projected length of the total structure calculated as the sum of the length of root-derived structures and the number of background patches within the convex hull.

For analysis of root system structural parameters, digital images were collected on a large (A3 sized) flatbed scanner. Although this process was tedious, requiring 40-50 separate scans for large root systems, it yielded extremely high quality data. The automatic identification of roots by the WinRhizo software was checked manually and the parameters were adjusted to improve recognition of the roots. The best discrimination was obtained for total root length, total root volume and average root diameter. A greyscale thresholding of 200 was selected and objects with a length:width ratio <4 and/or smaller than 4 cm^2 were filtered out. The results can be further divided into size classes (based on diameter) to compare the percentage of roots in each size class as a proportion of length or volume. In this study, the size classes ranged from [0-0.25] mm to <2.5 mm diameter with 0.25 mm increments.

Root branching percentages in entire root systems were difficult to estimate with WinRhizo due to the size and complexity of the root systems which lead to frequent false positive identifications by the software. Instead, a standardised sub-sample was designed to allow an estimate of root branching for each genotype and treatment. Six 10 cm root segments were cut 30 cm from the base of the crown using 6 randomly selected nodal roots for each plant and the number of lateral roots in each segment was manually counted. The number of lateral roots was then divided by 10 and averaged for each plant to obtain the average branching density per cm of root.

Anatomical traits were measured using images of root cross sections. Images were analysed with the RootScan program which automatically detected the various tissue types and calculated the areas of stele and cortex and the numbers of each cell type. Some manual correction was required to check the automatic detection and correct where necessary. Initial experiments established the pattern of development in the sugarcane roots and defined the best position for sampling in future experiments to test for differences between samples. Close to the root tip, the stele was surrounded by concentric layers of cube-shaped cortical cells arranged in radial files with only small intercellular air spaces. This anatomy is characteristic of roots that function in absorption, as there is an intact cell-cell pathway for radial transport of water and nutrients. Further from the tip, as the root matured, the cortical cells became enlarged and some radial files began to collapse in the early stage of lysigenous aerenchyma formation. This continued in older roots with large air spaces separated by narrow radial files of cortical cells so that the structure resembled a bicycle wheel. As a consequence of aerenchyma formation, mature roots contain fewer living cells per unit area than young roots and there is a reduced pathway for radial transport, indicating that the major function of these roots is in long-distance transport. It is important to note that these roots may be brown in colour and may eventually shed the cortical layer, but they remain functional and alive.

6.2. Features of the early root systems of twenty sugarcane varieties

To test the methodology and to assess the extent of variation amongst a wide set of genotypes, root systems from 20 sugarcane varieties grown for approximately 8 weeks in large planter bags were assessed.

Amongst these young plants, imaging of the intact root systems was not able to distinguish any differences in root system architecture between varieties. While small variations were measured in root system opening angle, these were not significant. It is likely that the root systems of young plants were relatively non-lignified and did not retain initial root system angles after washing. In other crops, researchers have used methods such as a pin-board during washing of root systems to maintain the initial root angle and distribution (Singh et al. 2010). The drawback of the pinboard method is that it requires the roots to be grown in a narrow “rhizobox” which may not be representative of the architecture in a three-dimensional soil space.

Analysis of the root system structure using images from the flatbed scanner was very successful and allowed comparison between varieties for total root length, root volume, average diameter and proportion of total root length in each size class (Table 2).

Table 2. Parameters measured by WinRhizo for the root systems of young sugarcane plants from 20 varieties grown in pots in the glasshouse. Values displayed are the means of three replicate plants for total root length, total root volume, average root diameter and percentage of roots in each size class.

cultivar	Average total root length (cm)	Average total root volume (cm^3)	root average diameter (mm)	Roots by size class (%)		
				< 0.25 mm	0.25-0.5 mm	> 0.5 mm
EMPIRE	23512	24.0	0.36	66	20	14
KQ228	56008	59.8	0.36	63	20	17
MQ239	40448	36.5	0.34	64	23	13
NCO310	16887	17.7	0.37	65	20	15
PINDAR	12978	16.9	0.42	56	26	18
Q117	27267	30.3	0.38	65	17	18
Q124	30633	31.6	0.38	63	21	16
Q138	23946	25.0	0.36	65	22	13
Q151	42164	45.3	0.37	61	24	15
Q167	25068	20.5	0.33	69	19	12
Q188	27369	32.2	0.39	58	24	18
Q200	19493	24.6	0.41	61	20	20
Q208	38452	44.6	0.39	61	21	18
Q232	45513	49.2	0.37	63	21	16
Q234	34569	33.7	0.37	65	21	14
Q238	37700	46.3	0.39	58	25	17
Q242	13273	13.4	0.36	61	25	14
Q252	24453	30.5	0.41	59	22	18
QN04	6549	8.3	0.41	59	21	20
SRA1	20277	26.5	0.41	56	24	19

6.2.1. Root system total length

The total length of roots per plant varied between less than 100 m and more than 700 m (Fig. 1, Table 2).

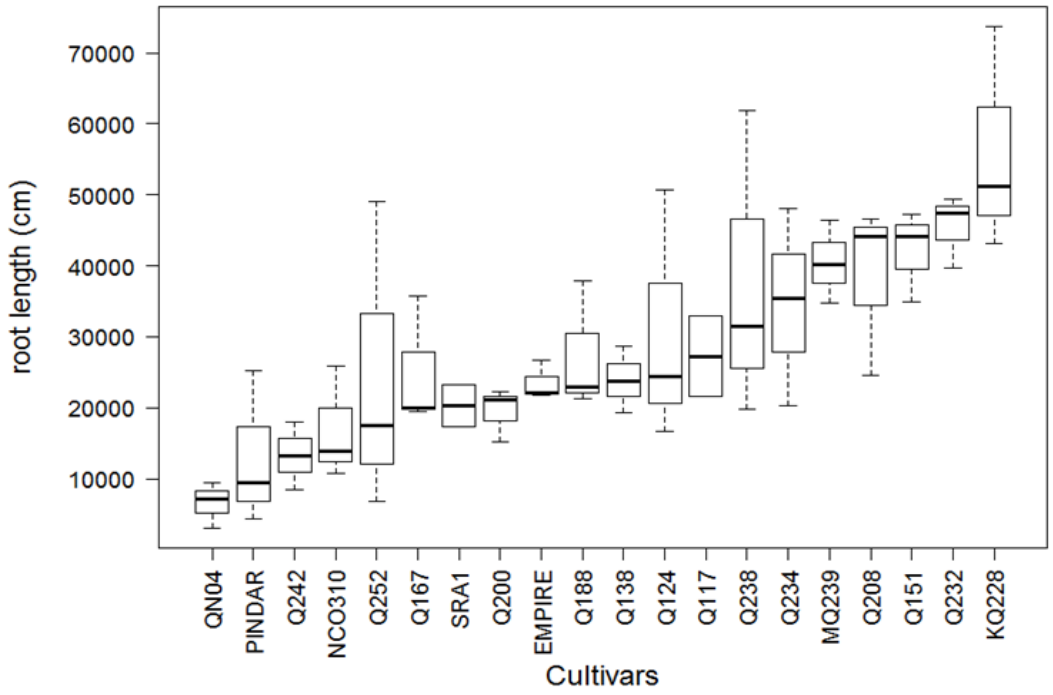

Figure 1. The total length of roots for young sugarcane plants from 20 varieties grown in pots in the glasshouse.

6.2.2. Proportion of fine roots

For each pot, the ratio of coarse to fine roots was calculated. The fine roots comprised a large proportion of the total root length. The results showed that the class of roots with the smallest diameter (less than 0.25 mm) comprised between 56% and 69% of the total root length, but only 6% to 14% of the total root volume. The average across all varieties was approximately 62% (Table 3). There did not appear to be any consistent varietal differences in the ratios of coarse to fine roots.

Table 3. Proportion of roots by length in each size class from young root systems of 20 varieties.

Size class	Proportion by length
< 0.25 mm diameter	61.9 ± 0.8 %
0.25 – 0.5 mm diameter	21.8 ± 0.5 %
> 0.5 mm diameter	16.3 ± 0.5 %

It has been assumed that fine roots, which constitute the major absorptive regions, comprise a high proportion of the root system as a whole, but until now it has been difficult to quantitate and compare this proportion, as fine roots are easily lost during washing and are difficult to measure manually. These results confirm that more than half of all roots measured less than 0.25 mm in diameter and interestingly, this seems to be fixed across all varieties tested.

6.2.3. Shoot/root ratios

Analysis of the above-ground parts of the plants enabled comparisons with the root parameters. Across all samples, there was a strong correlation between above-ground biomass and the total length of roots, with r=0.87 overall (Fig. 2).

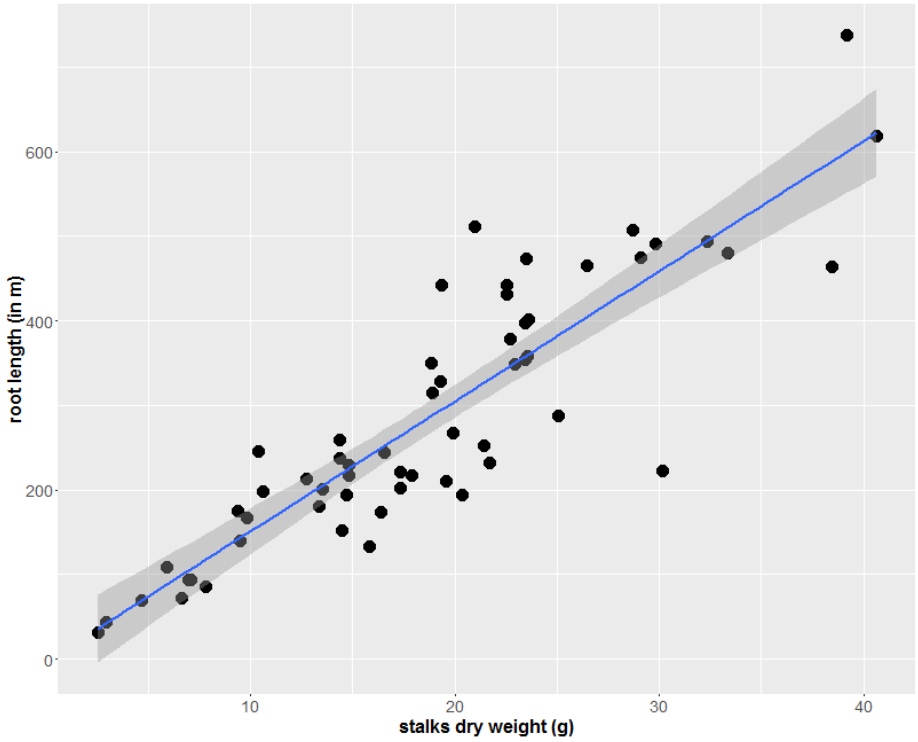

Figure 2. Correlation between total cumulative root length and above-ground biomass (as dry weight) for all samples from the pot trials.

However, despite this overall correlation, there were significant genotype differences in the ratio of shoot dry mass to total root volume. When shoot/root ratios were compared for individual varieties, the differences between varieties were highly significant with a p value of 2×10^{-9} (Fig. 3). These results are consistent with previous studies on a smaller number of varieties which showed distinct varietal differences (Magarey et al. 1999). The large variation observed in some varieties in the present study (e.g. Q138 and Q200) is likely due to the small number of replicates used in this experiment.

Varieties such as Q242 and MQ239 produced more shoot biomass per unit of root volume (g shoot dry weight per cm^3 of root) than varieties such as KQ228 and Q208. It is important to note that these results reflect the early allocations of resources to shoot and root for each genotype and ratios may change later in development. For example, shoot emergence in variety Q208 is known to be slower than in some other varieties (Bonnett et al. 2016); our results suggests that root growth is greater in Q208 at this stage.

Variations in early root growth rates may be important for improving establishment of plant cane. In some situations, establishing a strong root system may improve growth by enabling the plants to access water and nutrients deeper in the soil profile.

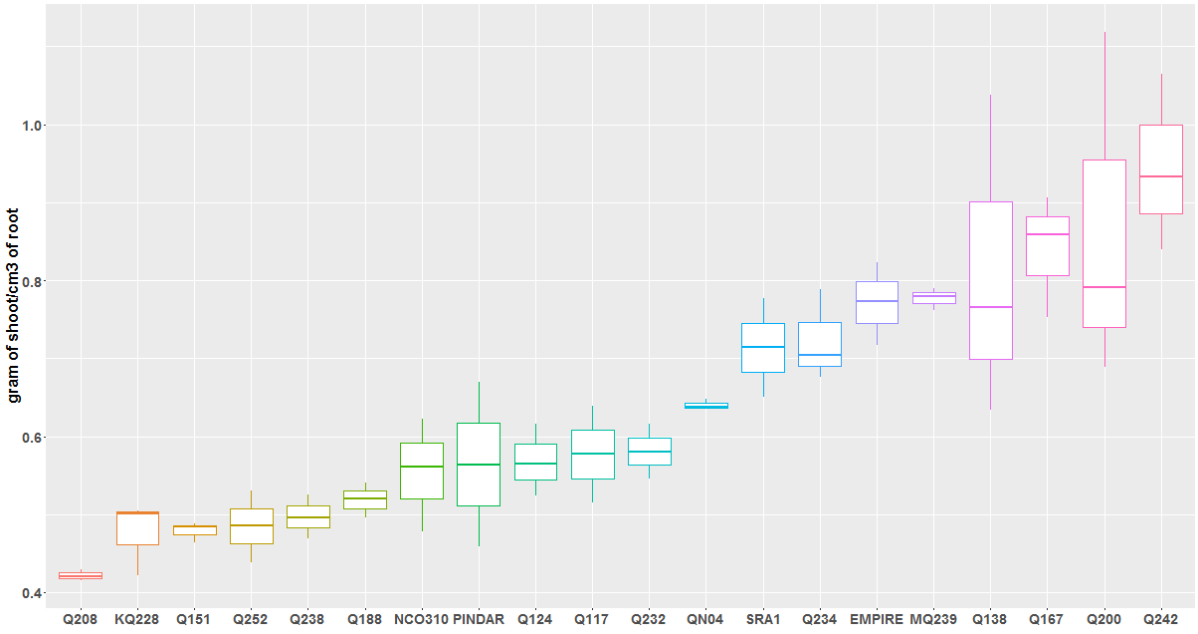

Figure 3. Ratio of shoot biomass to total root volume for the root systems of young sugarcane plants from 20 varieties grown in pots in the glasshouse. Varieties are ordered from low to high shoot/root ratio.

The varieties used in the pot trial represented a historical timeline of release dates over more than 50 years, allowing us to test whether there have been changes in root system size and structure over this time period. Based on the data and varieties used here, we found no consistent change in root systems over time. There was no relationship between seedling date and root system length or proportion of fine roots. Both older and newer varieties appeared at both ends of the spectrum of shoot/root ratios. For example, varieties Q151 and Q252, which had similarly low shoot/root ratios were first grown as seedlings in 1981 and 2000 respectively. Varieties Q138 and SRA1, which were first grown as seedlings in 1975 and 2005 respectively, both had high shoot/root ratios. This suggests that ongoing selection by sugarcane breeders for above-ground yield has not biased the plant architecture towards smaller root systems. In contrast, studies of historical and modern wheat varieties have shown that selection for yield has favoured an increase in shoot/root ratio over the last 100 years (Aziz *et al.*, 2017).

In summary, the comparisons of early root systems from 20 sugarcane varieties showed that:

- Methods have been developed that are able to discriminate between root systems and capture the diversity of structures.
- Within an overall correlation between shoot and root size, there were significant varietal differences in shoot/root ratios, suggesting that this trait is under genetic control.
- Some varieties were characterised by early vigorous root growth.
- The proportion of fine roots was consistently high, with little variation between varieties when plants were grown in unrestricted soil/water conditions.
- There have been no significant trends towards particular root traits or shoot/root ratios in Australian varieties over time.

6.3. Impact of reduced tiller number on root system structure and architecture

One of the major goals of the project was to test the response of root systems to growth constraints. The first of these experiments was designed to test the resilience of the shoot/root ratios. As described above, early vigorous root growth, resulting in an increased root mass fraction (i.e. the proportion of the plant's total mass that is allocated to roots) may be an advantage in some environments. A better understanding of the shoot/root ratio resilience could help in designing screens for varieties with more vigorous root growth and may also identify treatments to increase the root mass fraction.

Previous studies used root trimming to test this and found that varieties showed a tendency to revert to their original variety-specific shoot/root ratios within 8 weeks after treatment (Spaull 1980). They also found that the proportion of roots in each size class remained constant (Magarey et al. 1999). In wheat, increases in the root mass fraction have been achieved by restricting tillering, either by genetic manipulation of tillering genes or by removal of tillers (Duggan *et al.*, 2005, Palta *et al.*, 2007, Hendriks *et al.*, 2016). In the present study, we tested this approach in sugarcane by measuring the root system traits in plants where the shoot growth was restricted by artificial removal of tillers. These experiments were performed on six varieties with contrasting root mass fractions that were selected from the preliminary screen of 20 varieties. Three varieties with a high shoot/root ratio (SRA1, MQ239, Q242) and three with a low shoot/root ratio (Q208, KQ228, Q151) were grown in tall pots for approximately 13 weeks. For each variety, six of the 12 replicate plants were restricted to a single tiller (*till-* treatment) while six were allowed to tiller freely (*till+* treatment). The Q208 plants germinated extremely poorly resulting in a wide variation in results and consequently these plants were removed from the analysis. The five remaining varieties were analysed for the diversity of shoot traits, root structural traits and root architectural traits.

6.3.1. Shoot parameters

Amongst the control free-tillering treatment (*till+*), the average number of tillers ranged from 5.8 for MQ239 to 13.8 for Q151 (Fig. 4A) and was significantly different between varieties ($p<0.001$). The average dry weight of individual stalks from the *till+* plants was 13 g and was not significantly different between varieties (Fig. 4B). However, the average stalk weight amongst plants from the *till-* treatment was 85% greater (93 g *vs*. 13 g) compared to the control and was highly significantly different ($p<0.001$) between the treatments (Fig. 4B). Furthermore, within the *till-* treatment the individual stalk weights were significantly different between varieties ($p<0.001$) with MQ239 and Q242 at each end of the spectrum (70.5 g *vs*. 119.9 g) (Fig. 4B). Overall, the stalk mass fraction (StMF) increased by 31% in the *till-* treatment compared to the *till+* treatment (Fig. 4C).

Consistent with the increase in individual stalk weight in the *till-* treatment compared to the *till+* treatment, differences were also observed for the number of leaves per stalk and the individual leaf weights (Fig. 4D & E). The number of leaves per stalk increased by 50% from 5.8 in the control *till+* to 11.7 for the *till-* treatment on average. The dry weight of individual leaves increased by 30% (6.7 g *vs.* 4.6 g) in the *till-* treatment compared to the control *till+* treatment. While this increase was statistically significant overall when comparing varieties ($p=0.007$) and treatments ($p<0.01$), the individual leaf dry weight was not different between treatments for the varieties KQ228 and Q242. Although the single stalks in the *till-* treatment bore larger numbers of leaves per stalk, overall the leaf mass fraction (LMF) decreased on average by 24 % in the *till-* treatment compared to the control (Fig. 4F), most likely due to the larger total number of leaf-bearing nodes in the *till+* plants.

Whilst the individual stalk weight, leaf number per stalk and individual leaf weight increased in *till-* treatment compared to *till+* control, it is important to note that the overall shoot dry biomass was on average 50% higher ($p<0.001$) in the *till+* treatment compared to the *till-* treatment (182 g vs. 370 g) (Fig. 4G).

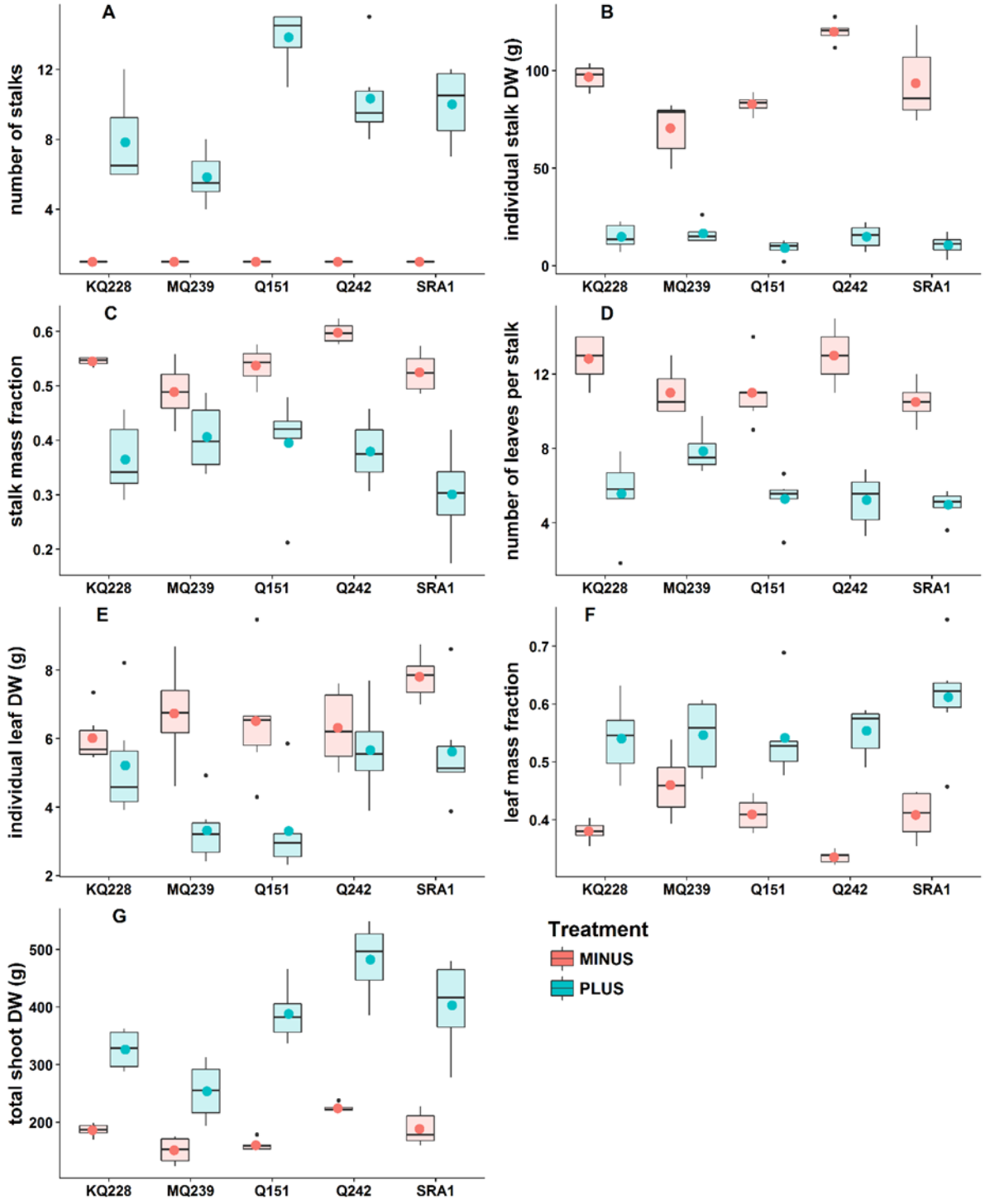

Figure 4. Variation in shoot traits between free (blue) and restricted (red) tillering plants. In each boxplot, the horizontal line represents the median and the coloured dots (red or blue) represent the average (n=6). The traits measured were (A) number of stalks, (B) dry weight of individual stalks (g), (C) stalk mass fraction, (D) number of developed leaves per stalk, (E) dry weight of individual leaves (g), (F) leaf mass fraction, (G) total shoot dry weight (g).

6.3.2. Root system structure

Individual root traits were measured to quantify phenotypic changes resulting from the restricted tillering treatment as well as the variation due to the differences in genetic background. The traits assessed were: total root length, nodal root number at 30 cm below the crown, specific root length (m per g of root), average diameter, and root branching density.

In the tall pot experiment, the total root length, for the control plants, ranged, on average, from 945 to 1721 m with a significant decrease ($p<0.001$) in the average total root length of 39% in the *till-* treatment compared to the *till+* control (Fig. 5A). This difference was more pronounced for variety Q151, which had a 44% decrease in the root system length, and less pronounced for KQ228 which showed only a 32% reduction.

Total nodal root number was also significantly lower in the restricted tiller treatment (39% lower than in the *till+* controls, $p<0.001$) and no differences were observed between varieties (Fig. 5B). Amongst the *till+* controls, there were few varietal differences and only Q151, with an average total nodal root number of 110, was significantly different from the other varieties. While the total nodal number of roots was higher in the *till+* treatment, when normalizing this figure to the number of stalks, the number of nodal roots per stalk was 81% higher for the *till-* treatment with an average of 52 nodal roots per stalk (Fig. 5B). For the *till+* controls, this figure was on average only 10 nodal roots per stalk.

The specific root length (SRL) ranged from 31.9 m g^{-1} to 77.3 m g^{-1} for the *till+* and was on average, 24% smaller than the *till-* treatment ($p<0.001$) (Fig. 5C). Within each treatment significant differences for SRL were observed between varieties; this was particularly noticeable for Q151 in the *till-* treatment, where SRL was significantly different from all the other varieties.

Field studies reported mean SRL down to 1 m in the field ranging between 17.6 m g^{-1} to 26.7 m g^{-1} (Magarey *et al.*, 1999, Battie Laclau and Laclau, 2009). Sugarcane crop models use specific root length values, derived from field studies, of 5 m g^{-1} in DSSAT (Hoogenboom *et al.*, 2010) or 18 m g^{-1} in APSIM (Keating *et al.*, 2003). In the current study, the average SRL was much higher at 54 m g^{-1} and 71 m g^{-1} for *till+* and *till-* respectively. While it is hard to compare the SRL of plants grown in the glasshouse under ideal conditions, to plants grown in the field that could encounter water and nutrient deficit, it seems likely that these differences between field and pot SRL values could be related to the proportion of fine roots which are usually lost and therefore underrepresented in field studies (Roumet *et al.*, 2016). Moreover, water and nutrient limited environments have been shown to favour an increase in SRL (Aerts and Chapin, 1999), hence this tends to suggest that previously published SRL could have been underestimated.

Amongst the *till+* controls, the root average diameter ranged from 0.46 mm to 0.63 mm with Q151 and SRA1 representing the two extremes (Fig. 5D). Overall, the imposed restriction on tillering significantly decreased root diameter by 7% ($p<0.001$) compared to the *till+* treatment. However, for MQ239 and Q242 the average root diameter was not different between treatments.

The average root branching density (RBD) ranged from 5.3 to 7.8 lateral roots per cm in the control plants and was not impacted by the restriction on tiller number (Fig. 5E). There were small but significant ($p<0.01$) differences between varieties for RBD.

When comparing between the results from small and large pot experiments, we found that less variation in root system traits was observed when plants were grown in small pots even though the plants were not root bound. Although this coul partially be explained by ontogeny, using small pots may also have a negative effect on root system development. For example, root diameter was very

different between the two experiments and by far larger and more variable in the large pot experiment. Knowing the importance of root diameter on the ability to penetrate compacted soils (Clark *et al.*, 2003) and on water and nutrient uptake (Rieger and Litvin, 1999), this reinforces the idea that large PVC columns like the ones used in our study are more suitable for root phenotyping in controlled environment conditions.

6.3.3. Biomass allocation to the root system

In addition to measuring the individual root traits, the proportion of the overall plant weight consisting of roots was measured. The root mass fraction (RMF) highlighted significant differences between varieties ($p<0.001$) with MQ239 and KQ228 sitting at both ends of the spectrum of the *till*+ control plants (Fig. 5F). More importantly there was no difference between treatments for this trait, so that within each variety, plants maintained a consistent RMF when tillering was restricted.

This result tends to agree with the conclusions from two meta-analyses conducted on a large number of species (Poorter and Nagel, 2000a, Poorter *et al.*, 2012b) that demonstrate that, outside of nutrient, temperature and compaction stress, for a given ontogenic stage, biomass allocation patterns tend to be constant. This is also consistent with previous observations in sugarcane from an experiment where above-ground biomass was restricted by defoliation but not removal of entire tillers (Smith *et al.*, 2005). Although this treatment would have reduced the plant photosynthetic capability, because the tillers were not removed, the entire root system was still present, which may have explained the contrast with wheat. Our results now confirm that the difference between wheat, where RMF increased and sugarcane, where RMF remained constant was not dependent on the additional roots associated with multiple tillers in sugarcane. RMF in sugarcane appears to be an extremely stable trait under strong genetic control. This stability of the shoot/root ratio to pruning has also been observed in other grass crops such as barley, where it was demonstrated that the allometric relationship is re-established 7-10 days after removal of 35% of shoot or 48% of roots. (Poorter and Nagel, 2000b).

Although the RMF remained the same, substantial changes were seen in both the shoot and root structure when tillering was restricted. As described above, the single stalk of the *till*- plant was seven times heavier than the average individual stalk in multi-tiller plants. In order to maintain the RMF in *till*- plants, the number of nodal roots per stalk increased by 81% and the average root diameter decreased, while specific root length (SRL, length per unit mass) increased. This demonstrates that while on a limited carbon budget, sugarcane will maintain its shoot/root ratio as well as maximise, as much as possible, its root length. Similar increases in SRL have been observed in plants on low root carbon budget resulting from nutrient-poor or dry environments (Aerts and Chapin, 1999). In such cases, the increase in SRL is a means for the plant to exploit its available resources as much as possible to mine the soil for nutrients and water. For sugarcane, retaining a large root system is also important for anchorage, and this would be particularly important for the large and heavy stalks in the *till*- treatment. In maize it was shown that nodal root number is highly correlated to resistance to lodging (Bruce *et al.*, 2001)

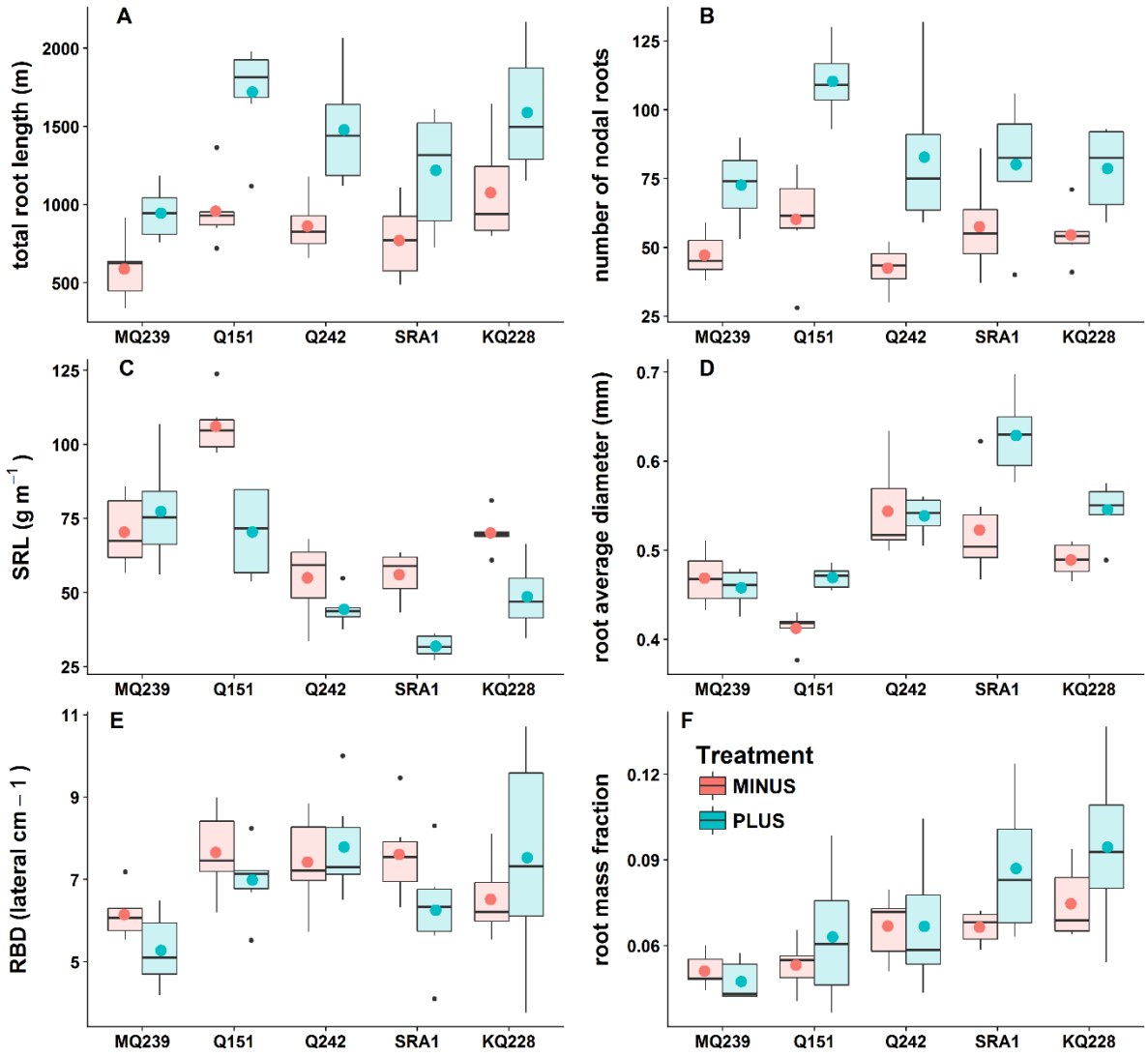

Figure 5. Variations in root traits between free (blue) and restricted (red) tillering plants. In each boxplot, the horizontal line represents the median and the colour dots (red or blue) represent the average (n=6). The traits measured were: (A) total root length, (B) shoot root number, (C) specific root length, (D) root average diameter, (E) root branching density, (F) root mass fraction.

6.3.4. Root system architecture

The spatial arrangement of roots is as important for the crop productivity as the individual root traits, and therefore we measured the root system architecture phenotypes as another approach to assess the impact of tillering restriction in the different genetic backgrounds.

A selection of representative root system architectures is presented in Figure 6. Whilst some visual differences were apparent for certain traits comparing between variety and treatment (e.g. root opening angle for Q242 or root system density for MQ239), a simple visual inspection did not reveal

any consistent differences between variety or treatment. Therefore, all the root system images were analysed with the REST software to measure root system architectural traits.

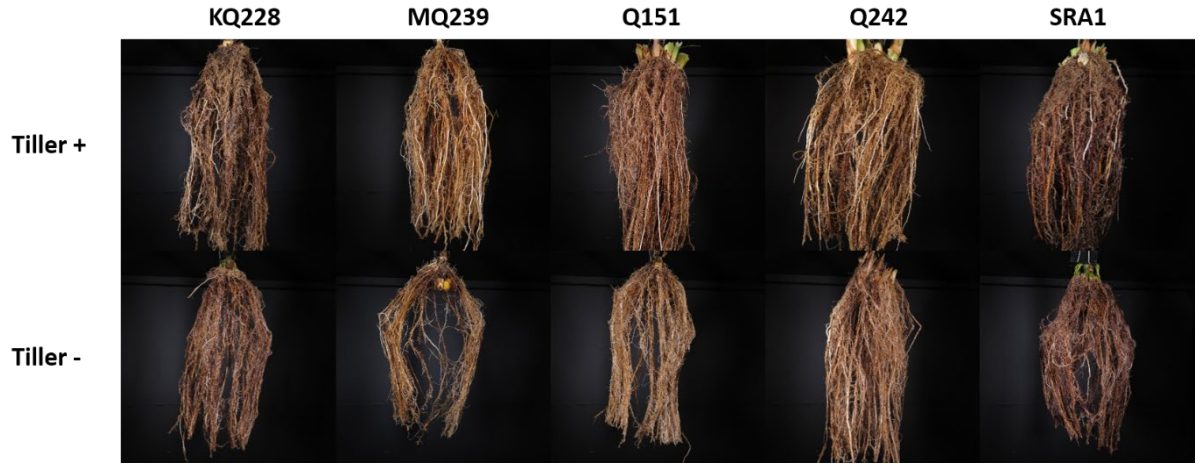

Figure 6. Variations in spatial arrangement of nodal roots between free (*tiller+)* and restricted (*tiller-)* tillering plants. Pictures are a selection of the root system architecture (0 to 30 cm from the crown) of each variety for the two treatments.

Figure 7 presents the results for three root system architecture traits: (i) the root opening angle (Fig. 7A) that measures the spatial ability of the root system to explore the soil profile; ii) the projected length of the total structure (Fig. 7B) that describes the size of the root system in the image; and (iii) the area of the convex hull which is both a measure of root system size and lateral extension (Fig. 7C).

The root system opening angle (Fig. 7A) was significantly different between varieties ($p<0.001$) with KQ228 and Q242 having the narrower and the larger root angles respectively. Overall, plants with restricted tillering had a significant ($P<0.001$) decrease in root opening angle. The projected length measured with REST (Fig. 7B) was a good trait to discriminate the varietal and treatment effects ($p<0.001$ for both). Q242 and MQ239 were the two cultivars that had a significantly larger and smaller projected root system length respectively. Finally, the area of the convex hull (Fig. 7C) highlighted significant differences for both treatment and variety ($p<0.001$) but was less discriminant than projected length for highlighting differences between varieties.

In wheat, the lateral spread angles of nodal roots is highly negatively correlated to the lodging rate (Pinthus, 1967). In our experiment, while the root system size was smaller for the single stalk plants, the 81% increase in nodal root number led to root system opening angles that were only 10° narrower on average than the angle in the free tillering plants. This ability to maintain root opening suggests that root system opening angle is highly genetically controlled and that the number of nodal roots is plastic enough to help to maintain this angle.

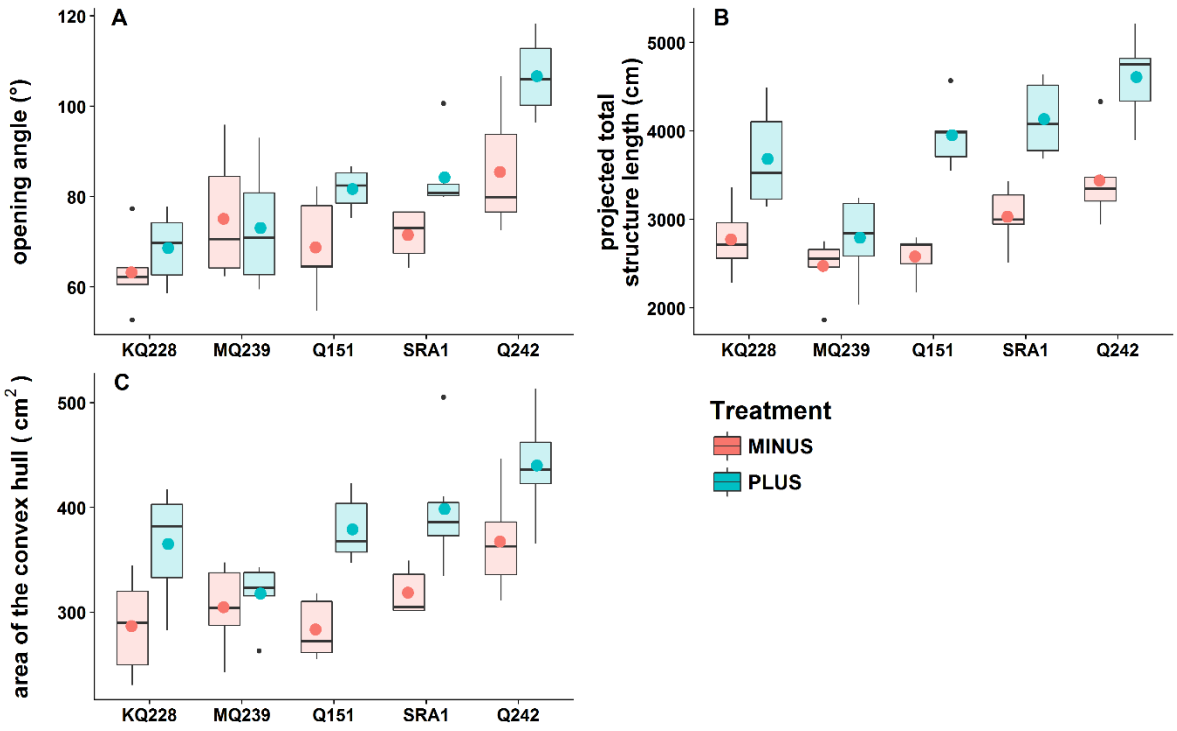

Figure 7. Variations in root system architecture between free (blue) and restricted (red) tillering plants. In each boxplot, the horizontal line represents the median and the coloured dots (red or blue) represent the average (n=6). The traits measured were (A) root system opening angle (°), (B) projected total structure length (cm), (C) area of the convex hull (cm^2).

In summary, the experiments with replicated plants grown in tall pots with or without restriction of shoot growth have added new information on root system traits and their resilience, including:

- Demonstrated that the sugarcane root mass fraction was extremely resilient to drastic reduction in tiller number.
- Restricted plants effectively maintained their root system configuration (opening angle) by dramatically increasing the number of nodal roots produced per tiller as well as maximizing root system length by increasing the SRL.
- The root parameters measured in this study, such as SRL, are likely to be more accurate than the parameters used currently in crop models and should be tested to see if they perform better to predict crop growth dynamics and yield.

6.4 Impact of nitrogen limitation on root system structure and architecture

Nitrogen is one of the major factors limiting plant growth but nitrogen fertilizer is a significant input cost and run-off of excess nitrogen has a negative impact on waterways. Optimizing the application rates, the composition of the fertilizer and the use of nitrogen by the plant are all important research targets. A review of nitrogen use efficiency in sugarcane (Bell, 2014) commented that "little is known about the contribution of root traits to N acquisition and crop productivity" and recommended targeted research into the amount of N accumulated by roots during a crop cycle and the impact of subsoil constraints on crop vigour and N accumulation. In view of the importance of nitrogen use efficiency and the potential for research synergies with other studies, we used the tall pot system to test the effect of nitrogen limitation on root systems. Six replicate plants of six varieties were used, with three plants receiving the high nitrogen treatment (HN) and three receiving low nitrogen (LN). The entire experiment was conducted twice.

6.4.1. Shoot parameters

The shoot biomass of the low nitrogen (LN) plants was significantly smaller for all varieties in the two experiments when compared to the high nitrogen (HN) plants (Fig.8, $p<0.001$). Nevertheless, in the second experiment, the shoot biomass of the HN plants was significantly smaller than for the first experiment ($p<0.001$), while LN shoot biomass remain unchanged ($p=0.141$). This reduction in growth was probably due to shorter day lengths experienced during the growth period.

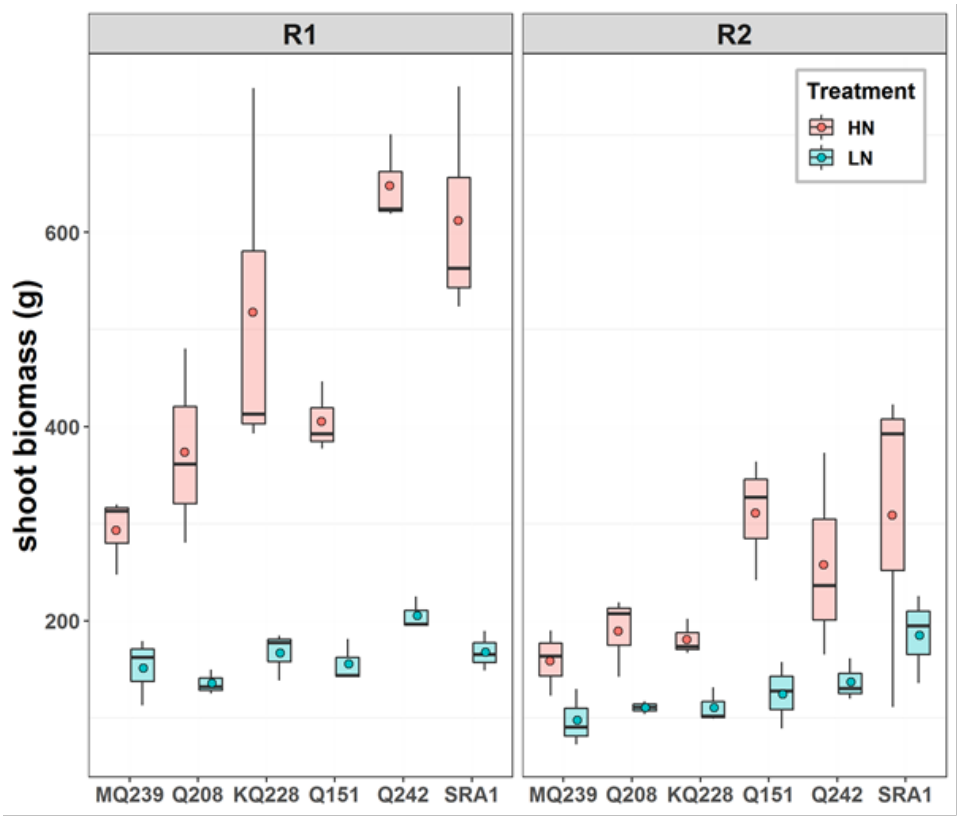

Figure 8. Shoot biomass for plants treated with high (red) and low (blue) levels of nitrogen in the two replicate experiments. In each boxplot, the horizontal line represents the median and the coloured dots (red or blue) represent the average (n=3).

In order to test for interactions between treatment and experiments on the plant biomass distribution, results were analysed by calculating the shoot mass fraction (ShMF) which is the sum of stalk mass fraction (StMF) and leaf mass fraction (LMF) (Table 4). When performing an ANOVA on the effect of variety, treatment and experiments on the ShMF there was a significant effect for both variety and treatment ($p<0.001$) as well as experiment ($p= 0.001$) (Table 4). Nevertheless there was no interaction between experiments with treatment or cultivar ($p=0.069$ and $p=0.057$) which indicates that while the shoot mass fraction tended to be lower in the second experiment, the ShMF of the LN plants was always significantly lower than that of HN plants. Because of the consistent rankings, we considered that the two experiments could be analysed together.

The converse of the lower shoot mass fraction (ShMF) in the low nitrogen treatment is that the root mass fraction (RMF) was always significantly higher ($p<0.001$) in the LN plants (Table 4). This result is consistent with previous studies conducted on various crops under nitrogen limitation (Poorter *et al.*, 2012) and it shows that while plants under N stress are smaller overall, they allocate a high proportion of their available biomass to the root system. In previous sections, we showed that shoot/root ratios were strongly variety specific and that plants tended to return to the same ratio, even when shoot mass was severely restricted. The results of nitrogen limitation suggest there is some plasticity in the ratios and that resource allocation can be modified under extreme stress. In the following section, we describe the root structure and architectural adaptations that underpin this plasticity.

Table 4. Variation in shoot and root mass fraction between high and low nitrogen conditions for plants from the two experiments R1 and R2. Value represent the mean ± two standard deviation (n=3). The lower table shows results from the ANOVA showing the variety, treatment and experiment effect and their interaction.

		HN		LN	
		R1	R2	R1	R2
Shoot Mass Fraction	KQ228	0.96 ±0.01	0.95 ±0	0.92 ±0.01	0.90 ±0.01
	MQ239	0.93 ±0.01	0.94 ±0	0.91 ±0.02	0.87 ±0.03
	Q151	0.97 ±0.01	0.96 ±0	0.94 ±0.01	0.94 ±0.01
	Q208	0.95 ±0.01	0.94 ±0	0.91 ±0.02	0.89 ±0.01
	Q242	0.95 ±0.01	0.96 ±0	0.92 ±0.01	0.94 ±0.01
	SRA1	0.96 ±0.01	0.94 ±0.01	0.91 ±0.02	0.91 ±0.03
Root Mass Fraction	KQ228	0.04 ±0.01	0.05 ±0	0.08 ±0.01	0.10 ±0.01
	MQ239	0.07 ±0.01	0.06 ±0	0.09 ±0.02	0.13 ±0.03
	Q151	0.03 ±0.01	0.04 ±0	0.06 ±0.01	0.06 ±0.01
	Q208	0.05 ±0.01	0.06 ±0	0.09 ±0.02	0.11 ±0.01
	Q242	0.05 ±0.01	0.04 ±0	0.08 ±0.01	0.06 ±0.01
	SRA1	0.04 ±0.01	0.06 ±0.01	0.09 ±0.02	0.09 ±0.03

Source of Variation	DF	F	P
variety	5	13.583	<0.001
treatment	1	127.331	<0.001
experiment	1	11.547	0.001
variety x treatment	5	1.418	0.235
variety x experiment	5	2.331	0.057
treatment x experiment	1	3.471	0.069

6.4.2. Root system architecture

We measured the root system opening angle as a first approach to assess the impact of nitrogen limitation in the different genetic backgrounds. The root system opening angle (Fig.9) was significantly different between varieties ($p<0.001$) with KQ228 and MQ239 having the narrower and Q242 the larger root opening angle of the varieties tested. Overall, plants treated with a sub-optimal level of nitrogen did not have a root opening angle significantly different from plants treated with a high level of nitrogen ($p=0.817$). It appears that the plants were not able to adjust their opening angle in response to nitrogen limitation.

Comparing the angles measured here in the control plants from the HN treatment with the angles measured previously in the control (unrestricted tillering) plants (section 6.3.3) showed very similar results. The ranking was preserved for the two extremes (KQ228 and Q242) and for these two varieties, the same angles were observed in both experiments. The other varieties fell between these two extremes in both experiments. In the previous section (section 6.3.3), we showed that although plants with severe tillering restriction had narrower angles, the difference was quite small, and the plants tended to return to the original angles through massive increases in root number per tiller. The results from the nitrogen experiment add weight to the observation that root system opening angles are a strongly conserved trait in sugarcane and varieties tend to maintain consistent angles even under stress. Consequently, there may be little plasticity in this trait to respond to challenges.

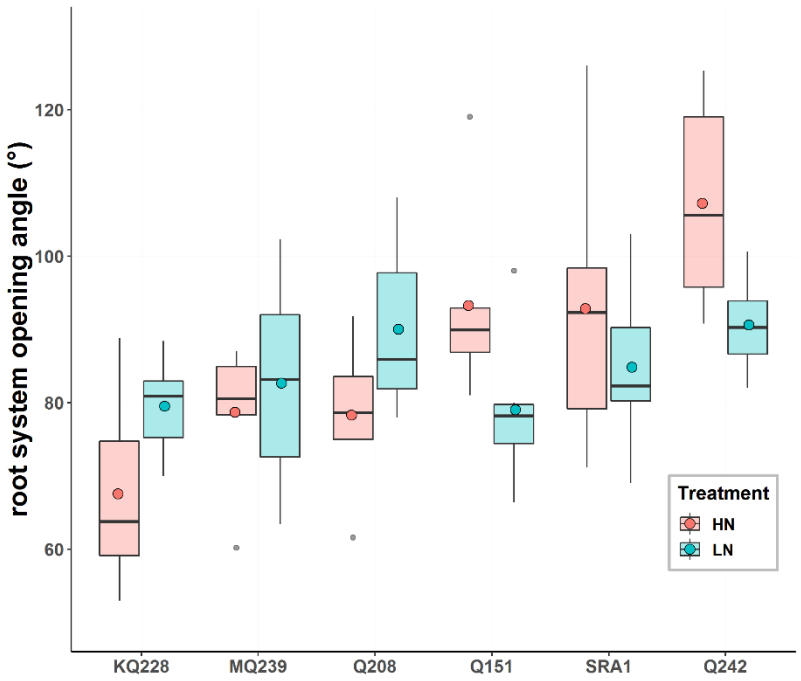

Figure 9. Root system opening angle in plants treated with high (red) and low (blue) level of nitrogen. In each boxplot, the horizontal line represents the median and the coloured dots (red or blue) represent the average (n=6).

6.4.3. Root system structure

Individual root traits were then measured to quantify phenotypic changes resulting from sub-optimal nitrogen treatment as well as the variation due to the differences in genetic background. The traits assessed were: total root length, total root volume, root average diameter and root branching density. In these experiments, we divided the root systems into three soil-depth segments before scanning so that the depth distribution could also be analysed in addition to the total structure.

Overall, the total root length was significantly slightly larger for the HN plants (1047 ± 499) compared to the LN plants (859± 239) (p=0.013) (Table 5) but no significant differences between treatment were observed for individual varieties.

Table 5. Variation in root traits between high and low nitrogen conditions. Value represent the mean ± two standard deviations (n=3). Superscript letters indicate significant differences between plants cultivated with either high or low nitrogen condition (P<0.05, Holm_Sidak).

	Total root system length		Average root diameter		Average root branching density	
	HN	LN	HN	LN	HN	LN
KQ228	1284 ±775[a]	958 ±227[a]	0.44[a]±0.01	0.41[b] ±0.02	9.7 ±1.9[a]	6.5 ±1.6[b]
MQ239	773 ±259[a]	812 ±220[a]	0.53[a] ±0.03	0.48[b] ±0.03	3.1 ±0.6[a]	3.5 ±0.3[a]
Q151	959 ±171[a]	748 ±254[a]	0.43[a] ±0.03	0.39[b] ±0.02	8.4 ±0.7[a]	6.6 ±1.2[b]
Q208	1005 ±315[a]	834 ±116[a]	0.46[a] ±0.02	0.42[b] ±0.02	4.9 ±1.4[a]	4.1 ±1[a]
Q242	1118 ±734[a]	825 ±372[a]	0.52[a] ±0.04	0.42[b] ±0.02	8.8 ±0.8[a]	8.1 ±1.1[a]
SRA1	1141 ±475[a]	979 ±186[a]	0.55[a] ±0.04	0.44[b] ±0.02	8 ±1.2[a]	5.7 ±0.8[b]

The distribution of the root volume according to depth was markedly different between treatments (p<0.001). Figure 10 shows the striking difference in distribution of roots in the deepest layer in LN conditions compared to the control which was well supplied with N. Nearly half of the root system volume of the LN plants (0.48 for LN *vs* 0.39 for HN) was in the deeper layer while HN plants tended to have a higher proportion of their root volume in the two top layers (0.61 for HN *vs* 0.51 for LN). Comparing between varieties, Q208 showed the greatest contrast in root volume distribution between treatments (Fig. 3), showing that it was highly adaptable.

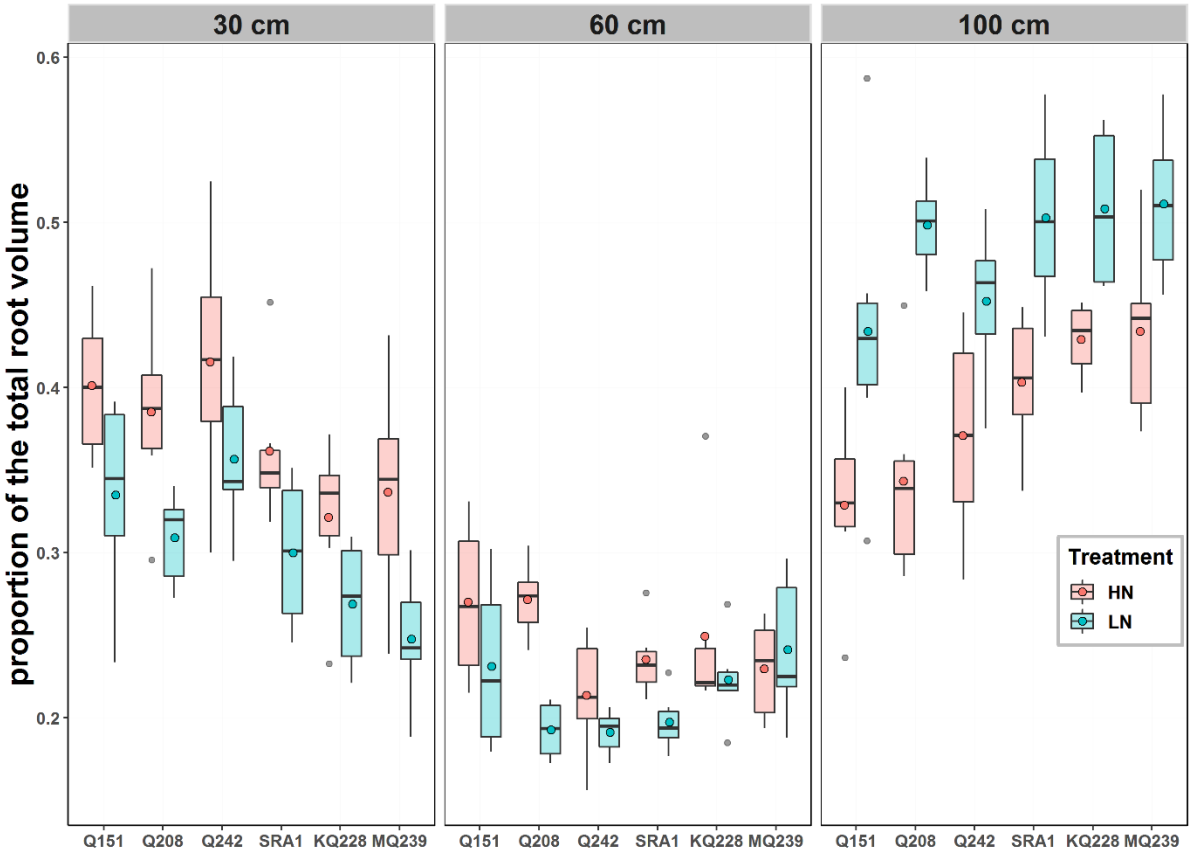

Figure 10. Variations in root system volume at three different soils depth (0-30 cm, 30-60 cm and 60-100 cm) between plants treated with high (red) and low (blue) levels of nitrogen. In each boxplot, the horizontal line represents the median and the coloured dots (red or blue) represent the average (n=6).

The root system average diameter was strongly contrasted between treatment ($p<0.001$) and varieties ($p<0.001$) (Table 5). Plants grown with the sub-optimal level of nitrogen showed a decrease in the average diameter across the root system. Q151 and KQ228 were the two varieties with the smallest root diameter while SRA1 and MQ239 had the largest diameter roots (Table 5). Reduced average diameter was also observed in the restricted tillering experiment, suggesting that some common mechanisms are used to adapt to stress.

The root branching density (RBD), for the control (HN) plants, ranged, on average from 3.1 lateral roots per cm to 9.8 lateral root per cm for MQ239 and Q242 respectively, and was highly statistically different between varieties (Table 5). There was a strong statistical effect of the nitrogen treatment on RBD ($p<0.001$) but the decrease was observed only on Q151, SRA1 and KQ228 with average reductions of 1.8, 2.3 and 3.2 lateral roots per cm (Table 5).

6.4.4. Root functional attributes

Root segments were harvested along selected shoot-borne roots to examine the evolution of the root structure as the root matures and the impact of nitrogen limitation. The root segments were taken from the first of the two replicate nitrogen experiments only. Both apical segments representing the younger part of the developing root, and medial parts representing a more mature and branched part of the root were collected for each variety in the HN and LN treatment at the conclusion of the experiment. For each plant 6 to 9 root segments were collected at both positions. After processing and quality control of the analysed pictures, a total of 482 samples were retained which corresponds to an average of seven root cross-sections analysed for each plant at each location. Metaxylem vessel number was determined on cross sections originating from the nodal root mid-point (n=249). Nodal root parameters related to the cortical burden (cortical cell file number, cortical cell number and cortical cell median size) were quantified using apical nodal cross sections where less than 5% of the cortex surface area was aerenchyma (n=157). The aerenchyma quantification was done with the 482 samples.

The number of cortical cells is an important indicator of the metabolic burden of maintaining a root system due respiratory carbon demand. The development of aerenchyma has been proposed to reduce the metabolic cost by reducing the number of living cells in the cortex (Zhu et al. 2010). In addition, aerenchyma is thought be important for oxygen supply to roots in anaerobic conditions and in some species, such as rice, development of aerenchyma is induced by waterlogging. Similarly, xylem vessel number and diameter can affect the water channelling capacity of the root (Richards and Passioura, 1989). Testing for changes in these parameters under nitrogen stress may indicate the capacity of the sugarcane plant to adapt its internal anatomy to overcome limitations by using carbon resources more efficiently.

Table 6. Nodal root internal structure near the apex and at the root mid-point. Metaxylem vessel number was determined using the medial root sections (n=249). Nodal root parameters related to the cortical burden (cortical cell file number, cortical cell number and cortical cell median size) were quantified using apical root cross sections where less than 5% of the cortex surface area was aerenchyma (n=157). Values represent the mean ± two standard deviation. Superscript letters indicate significant differences between plants cultivated with either high or low nitrogen condition (P<0.05, Holm_Sidak).

	Average metaxylem vessel number		Average cortical cell file number	
	HN	LN	HN	LN
KQ228	11.9 ±1.0[a]	10.6 ±1.4[a]	19.4 ±3.3[a]	18.9 ±2.8[a]
MQ239	14.14 ±2.7[a]	11.5 ±1.5[b]	26.3± 4.7[a]	19.9 ±3.4[b]
Q151	9.8 ±1.9[a]	8.5 ±1.1[b]	11.9 ±3.5[a]	12.6 ±2.4[a]
Q208	13.4 ±2.2[a]	12.5 ±2.3[a]	22.7 ±5.3[a]	21.7 ±5[a]
Q242	13.9 ±2.6[a]	12.2 ±2.2[b]	21 ±6.9[a]	20.3 ±4.9[a]
SRA1	13.9 ±1.8[a]	12.4 ±2.0[b]	24.7 ±6.2[a]	22.3 ±7.7[a]
	Average cortical cell number		Cortical cell median size	
	HN	LN	HN	LN
KQ228	2642 ±614[a]	2636 ±799[a]	406.3 ±87.5[a]	399.3 ±96.4[a]

MQ239	4634 ±1376[a]	2684 ±765[b]	478.7 ±121.7[a]	501.1 ±125.2[a]
Q151	998 ± 446[a]	1115 ± 378[a]	376.6 ±96.1[a]	311.9 ±87.3[a]
Q208	3358 ± 1248[a]	3315 ±840[a]	430.2 ±59[a]	365.4 ±38.9[a]
Q242	3006 ±1545[a]	2963 ± 1215[a]	506.3 ±150.8[a]	433.4± 157.8[a]
SRA1	3715± 847[a]	2860 ±1120[a]	492 ±84.3[a]	459.3 ±89.6[a]

In the control condition where sufficient nitrogen was available, significant differences in internal anatomy were noted between varieties, for example in cortical cell number and cell file number (Table 6). Figure 11 illustrates the differences between SRA1 and Q151, which showed contrasting root anatomies.

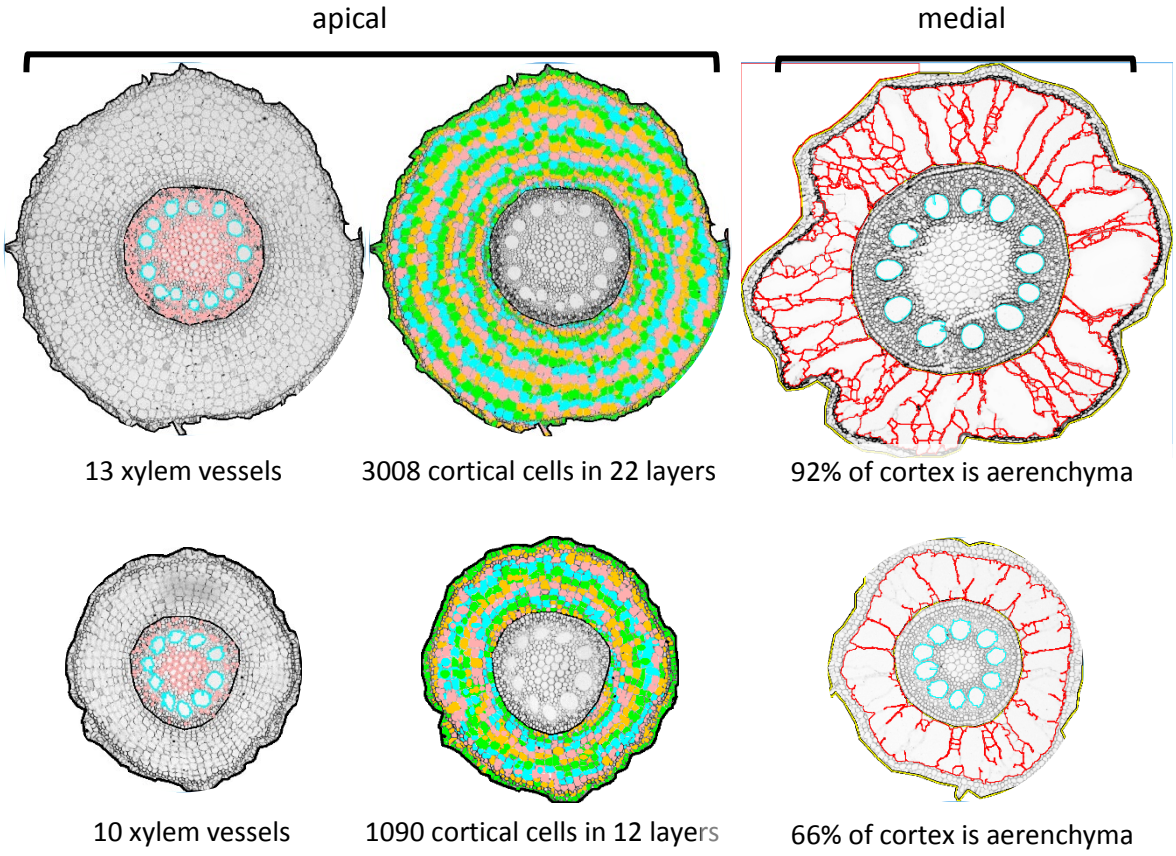

Figure 11. Comparison of root anatomy in apical and medial sections from two different varieties, SRA1 (upper row) and Q151 (lower row). All sections are shown at the same magnification and colours represent automated identification of features by the RootScan program.

Under low nitrogen conditions, the number of metaxylem vessels was significantly reduced (Table 6, $p<0.001$) and resulted in 1.5 fewer metaxylem vessels on average in the LN plants ($p<0.001$) except for KQ228 and Q208. MQ239 was the variety with the greatest contrast between treatments with an 18% decrease in metaxylem vessel number (Table 6).

The number of cortical cell files and number of cortical cells near the root apex were not significantly different between treatments for all varieties expect MQ239 (Table 6). In this variety, the cortical cell number and cell file number were significantly decreased by 42% and 24% respectively under the low nitrogen conditions (Table 6). Under sufficient nitrogen conditions, MQ239 was also the variety with the highest number of cortical cells (4634 ±1376) while Q151 was the variety with the lowest cortical cell number (994 ±446) (Table 6). No significant differences in cortical cell median size was observed for all varieties.

The cortical metabolic burden of the sugarcane nodal root was assessed by quantifying the percentage of the cortex that comprised aerenchyma (Fig. 12). Close to the root tip, the median value for the percentage of the cortex that is aerenchyma (perCisA) was relatively low ranging from 0 % to 4.1 %. In this apical location, the percentage of root cortical aerenchyma was not significantly higher in the low nitrogen plants ($p=0.768$). In the medial sections, the area of aerenchyma increased dramatically with median value for perCisA ranging from 64.7% to 75%. Similarly, the propensity of the nodal root to develop aerenchyma in the mid-section was not significantly affected by the nitrogen treatment ($p=0.267$). The percentage of the cortex that is composed of intact cortical cells at the root mid-point ranged from 10% to 16% (data not shown), the remainder of the surface area being cell wall**.**

The high proportion of aerenchyma was a very consistent trait across all varieties and it appeared that there was no further ability to increase this under stress. These results suggest that the sugarcane root is already a very efficient structure, with maximum aerenchyma development expressed constitutively. Unlike maize where there was variation in aerenchyma proportions, this may not be a trait that can be manipulated in sugarcane. However variation in the number of cortical cells was observed and this trait was also exploited to improve maize root efficiency (Chimungu et al. 2014b). It is interesting to note that the sugarcane variety with the largest number of cortical cells in the control HN condition (MQ239), also showed that largest reduction in cortical cell number in response to N stress and was also the most responsive in terms of xylem vessel number.

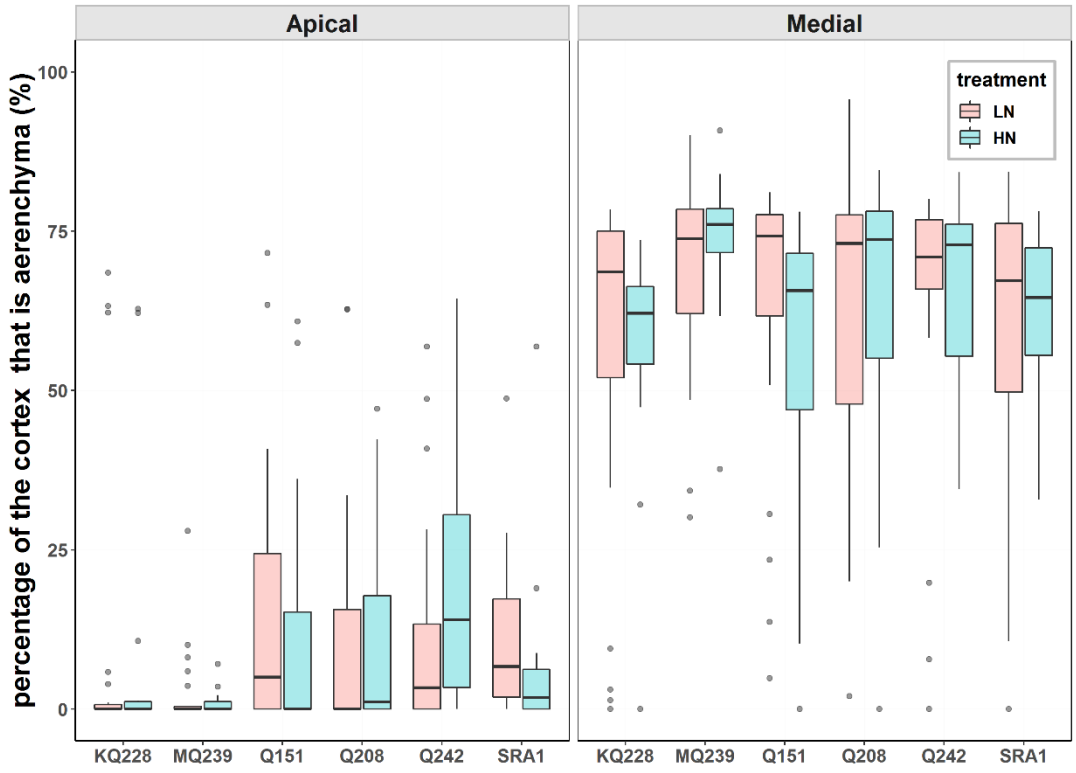

Figure 12. Variation in the percentage of the cortex of nodal root occupied by aerenchyma near the root apex or at the root mid-point for plants treated either with a high (red) and low (blue) level of nitrogen. 233 and 249 cross sections were analysed for the apical and medial sections respectively which represent an average of n=13 and n=14 cross sections per boxplot for the apical and medial sections respectively.

These experiments have added significantly to our knowledge of how nitrogen limitation impacts root systems and the plasticity of root growth to adapt to this stress. In summary the results showed that:

- While N limitation lead to a reduction in shoot and root growth overall, the relative allocation of available resources to the root system increased in most varieties, shifting the conserved shoot/root ratios.
- Under N stress, the distribution of the root volume in the soil profile was modified, so that a larger proportion of roots was found in the deeper zone compared to the control with sufficient nitrogen
- Plants maximised the volume of the root systems by producing narrower roots on average and by reduced branching frequency.
- Root opening angles were consistent for each variety and were maintained under N stress, with little adaptation to alter root architecture.
- Sugarcane varieties constitutively develop extensive and constitutive aerenchyma with little ability to increase the metabolic efficiencies through further decreases in cortical cell number.
- There is genetic diversity in root anatomical structure, for example in the area of the cortex in young roots.

6.5 Impact of YCS on root system structure and architecture

Yellow Canopy Syndrome (YCS) is the subject of intensive study and some observations have suggested that root systems of affected plants are impacted. Although there is anecdotal evidence, there have been no previous systematic studies and the difficulty of analysing root systems and the lack of a baseline description for a healthy root system have made this question challenging. As some varieties appear to be more likely to display YCS symptoms than others, one hypothesis was that that particular root system structures can predispose a variety to YCS. We were able to address this by examining the results of root system diversity in the 20 varieties described in section 6.2. There was no consistent relationship between any of the root traits measured and the propensity for YCS. For example, varieties KQ228 and Q208 display contrasting responses to YCS, but had very similar low shoot/root ratios and similar proportions of fine roots. The results suggest that root system structure and architecture are not determinants of YCS response.

The second hypothesis was that YCS causes poor root systems. To address this, we sampled a set of YCS-symptomatic and asymptomatic KQ228 plants from a field site in the Burdekin. The samples were analysed for root architecture and root structure parameters (Fig. 13). The root parameters measured were total root length (Fig. 13A), average root diameter (Fig. 13B), total length of fine roots (Fig. 13C), percentage of fine roots relative to total root length (Fig. 13D) and length of the projected structure (Fig. 13E). There were no statistically significant differences in any of the root parameters between asymptomatic and symptomatic plants. There was a highly significant 23% reduction in average shoot weight ($p<0.001$) between asymptomatic and symptomatic plants and consequently the root/shoot ratio was significantly higher in the symptomatic plants (Fig. 13F).

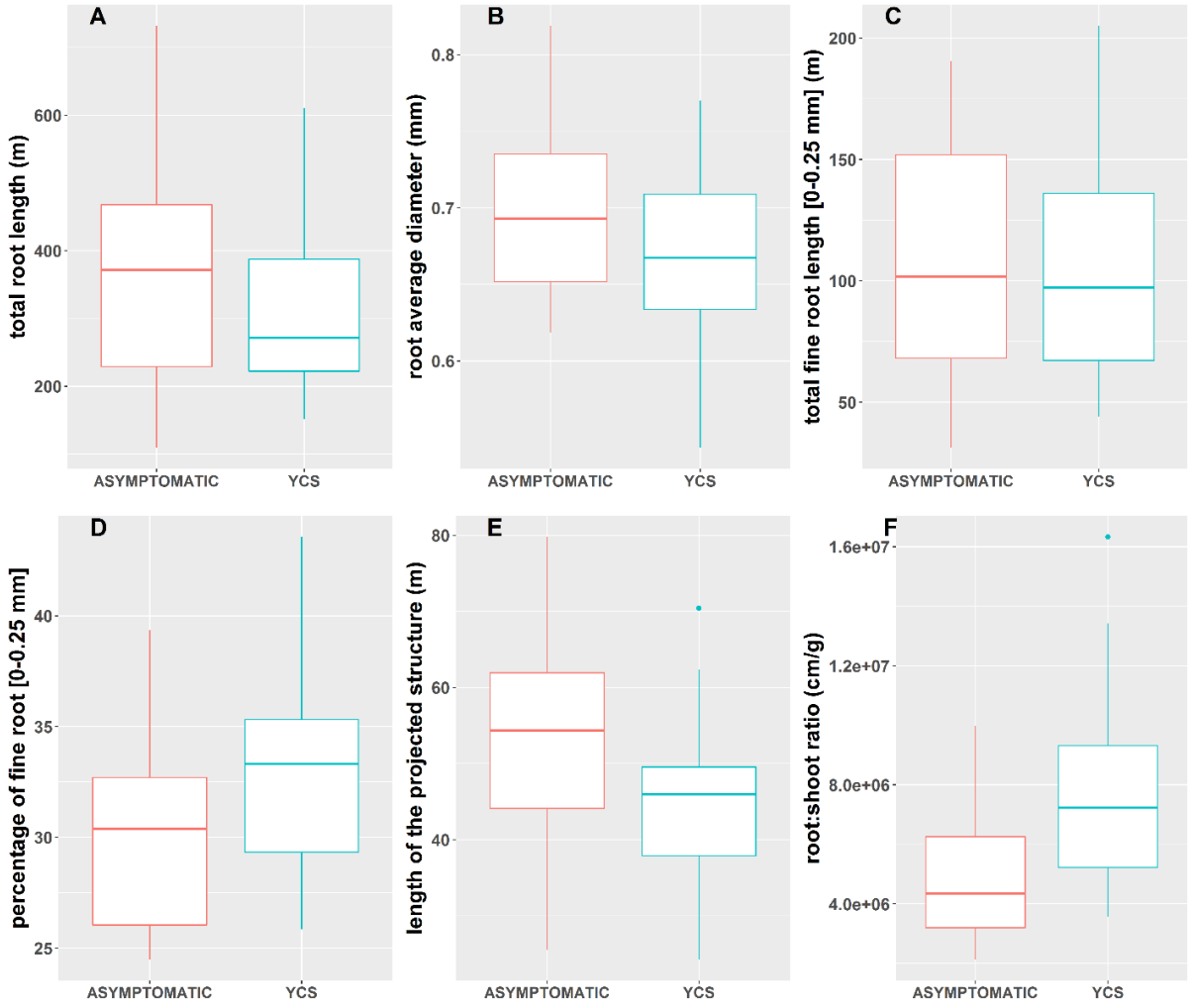

Figure 13. Comparison of asymptomatic (red) and YCS symptomatic (blue) plants of variety KQ228 (n = 25 for each set). The parameters compared were (A) total root length, (B) root average diameter, (C) total fine root length, (D) percentage of fine roots, (E) length of the projected structure, and (F) root:shoot ratio.

The only significant difference observed was an increased root/shoot ratio and this was due to lower shoot dry weights. Root system parameters, including the proportion of fine absorptive roots, appeared to be unchanged in the YCS symptomatic plants. These results were obtained from the block of soil directly under the stool to a depth of 30 cm in plants that had only recently begun to display symptoms (<2 weeks). It is possible that deeper sampling or sampling of a crop that had been affected for longer would reveal differences in root structure. As shown earlier, there is a tendency for varieties to maintain consistent shoot/root biomass ratios, so any condition that affects shoot growth would likely result in reductions to root growth over time. However, initial conclusions are that there was no change to the root system associated with YCS.

When the root architecture (whole root system) parameters were compared with the root system structural (WinRhizo) parameters for the field samples, some correlations between the two methods were observed. The total projected structure, based on the intact root system, and the total root length measured on individual roots showed a good correlation (r=0.77) (Fig. 14). This is useful because the whole root system imaging is much faster and could be used as a screening tool for field samples, allowing a much larger number of samples to be processed.

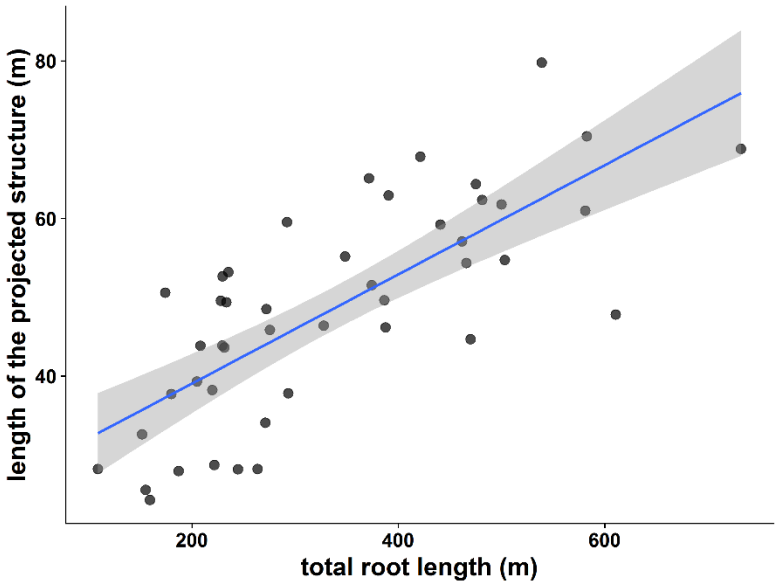

Figure 14. Correlation between the total root length, measured with WinRhizo, and the length of the projected structure, estimated with REST on samples from the field.

In summary, the roots from YCS affected plants have been examined for the first time, showing that:

- The "shovelomics" method for field sampling can be applied to sugarcane and enable meaningful comparisons between treatments.
- There was no apparent link between YCS susceptibility and root system traits.
- Despite a significant reduction in shoot mass, there were no differences in the root traits in YCS affected plants, including total root length, average diameter, total length, % of fine roots, and area of soil explored.

7. CONCLUSIONS

In this study, methods have been developed that are able to discriminate between sugarcane root systems and capture the diversity of structures. Using plants grown in large planter bags or tall pots in the glasshouse, we tested methods for characterising root system architecture using images of intact root systems, and methods for comparing root structural features, using images of individual roots. Digital image analysis successfully identified key traits, including root opening angle, root system total length, average diameter, proportion of roots in each size class, nodal root number, specific root length (m per g of root), and root branching density.

By applying these methods to compare the early root systems of 20 modern and historical varieties, we showed that there was significant genotype variation in many traits. A subset of these varieties was subsequently grown to a more mature stage in larger pots confirming that varietal specific differences in root system structure and architecture can also be observed at an older stage of plant growth. Within an overall correlation between shoot and root size, there were significant varietal differences in shoot/root ratios with some varieties investing relatively more resources into root system growth at the early stages. The proportion of fine roots was consistently high, with little

variation between varieties. As the varieties used in these experiments represent over 50 years of variety release by the Australian breeding program, we were able to examine whether the root system features have changed over this period. We found that there were no consistent trends towards particular root traits or shoot/root ratios in Australian varieties over time. This contrasts with the situation in some crops, such as wheat, where selection for yield has caused changes to the shoot/root ratios.

The descriptions of the root traits from the set of 20 varieties provided a baseline definition of healthy root systems in sugarcane, including the extent of variation for each trait. This is a substantial increase in our knowledge of the "typical" sugarcane root system, as previous descriptions were derived from a limited number of varieties due to the labour-intensive manual analysis. This baseline then provided an opportunity to test how sugarcane root systems respond to constraints, specifically, shoot growth restriction, nitrogen limitation and Yellow Canopy Syndrome (YCS).

Six varieties with contrasting shoot/root ratios were grown in tall pots and either allowed to tiller freely or restricted to a single tiller per plant. This treatment limits the carbon supply to the plant and is a good test of how this constraint impacts the allocation of resources between shoot and root. In wheat, artificially reducing the shoot mass has led to increases in the proportion of biomass allocated to the root system, known as the root mass fraction. In contrast, our results showed that the root mass fraction of sugarcane varieties was extremely resilient and that plants tended to return to their variety-specific shoot/root ratios, even with drastic changes to tiller number. Restricted plants effectively maintained their root system configuration (opening angle) by dramatically increasing the number of nodal roots produced per tiller. Although the total root length was reduced under the restricted tillering treatment, the plants modified the root structures to maximise the root system length for the available resources, by decreasing root diameters and increasing the specific root length.

The same six varieties were then grown with a limited supply of nitrogen and compared to control plants with sufficient nitrogen. Nitrogen limitation lead to a reduction in shoot and root growth overall, but in contrast to the reduced tillering treatment, reduced nitrogen availability was able to alter the conserved shoot/root ratios. In this situation, the relative allocation of available resources to the root system increased in most varieties, leading to a higher root mass fraction. Changes in both the distribution of the root volume in the soil profile and the structure of the roots were also observed, suggesting that the plants attempted to increase the volume of soil explored under nitrogen stress, so that a larger proportion of roots was found in the deeper zone compared to the control with sufficient nitrogen. We found no changes in the root opening angles when plants were nitrogen limited. Taken together with the results from the tillering restriction, we infer that changing root angle is not a mechanism used by sugarcane to adapt to stress. In contrast, plasticity in the distribution by depth, and relative allocation of resources to the root system are traits used to adapt to challenging environments.

The third growth constraint examined was Yellow Canopy Syndrome. Although it is not yet clear whether the causal agent of YCS is biotic, abiotic or a combination, there is good evidence that carbon flow from the photosynthetic tissues is affected. The root systems of plants displaying the symptoms of YCS were examined using a modified technique that could be applied to field samples and enabled meaningful comparisons between treatments. Although the shoot mass showed a significant reduction, we did not detect any differences in root system parameters, including total root length, average diameter, total length, % of fine roots, and area of soil explored. The results suggest that root system traits and root health were not a predisposing factor in YCS. However, it is

likely that prolonged deficit in carbon supply resulting from the reduced canopy would eventually lead to reductions in root mass, as we observed in the experiments where carbon supply was restricted by removing tillers.

The root internal anatomy of the same six varieties was examined to evaluate root energy efficiency. In other crops, the internal anatomy of the root has been modified to bring gains in energy efficiency, for example, by decreasing the proportion of living cells through mechanisms such as increased aerenchyma development. The digital microscopy revealed that aerenchyma in sugarcane roots was both extensive and constitutive, with little capacity to increase beyond this high level. However, there was genetic diversity in the area of the cortex in young roots, which may provide capacity to increase the metabolic efficiency.

The results from these studies have greatly expanded our knowledge of root system architecture and structure in Australian sugarcane varieties. As well as providing a baseline definition of a healthy root system, with mean and variation across a range of genotypes, we also defined the effect of stress on root systems and identified traits with the plasticity to adapt to the environment. This knowledge will underpin future studies to measure root system efficiency in the field and to identify traits that can contribute to better yields.

8. RECOMMENDATIONS FOR FURTHER RD&A

The project has: (i) developed digital methods and key parameters for comparing large numbers of root systems; (ii) defined baselines and diversity for current varieties; and (iii) shown how root systems respond to YCS, low N supply and shoot growth restriction. The results include messages for growers, data to assist ongoing research, and methods to underpin new research directions.

Key findings about varieties, such as the importance of fine roots and the similarities between historical and modern varieties should be communicated widely, to assist with grower assessments. This provides sound scientific data to balance against anecdotal reports of variety performance.

Nitrogen use efficiency research can benefit from better knowledge of root systems. SRA has significant investment in this research area. Optimizing the application rates, the composition of the fertilizer and the use of nitrogen by the plant are important research targets. The crop simulation models that are used to predict yields, such as APSIM, include root system parameters that are derived from a very small number of studies. There are significant gaps in the available root parameters, for example, the growth rate in the early ratoon crop, which makes it difficult to model demand for nitrogen in this period. The root parameters measured in the current study, such as SRL, are likely to be more accurate than current model parameters and should be tested to see if they perform better to predict crop growth dynamics and yield. In addition to improving the accuracy of growth rates and carbon demand, there is the potential to update the models to measure the value of particular root traits. In other crops such as wheat, modelling the effect of specific root parameters on productivity has also been a useful approach to identifying beneficial root traits.

Deterioration of the physical, chemical and biological qualities of soils, known collectively as soil health, has been identified as a major factor in declining productivity. The Sugarcane Yield Decline Joint Venture (SYDJV) investigated declining productivity in the Australian industry in three project stages from 1993-2005. The researchers found a complex pattern of degradation resulting from long term monoculture and poor agronomic practices. Root health is known to respond to soil conditions and can therefore be a useful barometer for soil health. Using the methods and baseline

descriptions from the current work, there is now an opportunity to develop diagnostic methods for root health that can define the impact of agronomic practices and soil-borne pathogens. Monitoring soil health has the potential to provide practical information that supports adoption of improved farming practices.

Soil constraints, especially compaction, remain a major problem for sugarcane production. Frequent waterlogging or rapid leaching of nutrients in high rainfall zones also pose challenges to plant growth. It is likely that specific root system adaptations will be important in improving productivity in these situations, and the present study has shown that there is genetic variation in these traits. In other crops, traits that offer advantages in challenging environments have been identified, including early vigorous root growth, increased root depth and root/shoot ratios, and increased root diameter. Deeper root systems increase the recovery of leached nutrients and also improve the physical and chemical properties of deep soils by returning more carbon to deep soil layers. The methods and critical parameters for root system analysis from the current work provide the basis for identifying root system traits that contribute to productivity in marginal soils. Assessment of variation amongst current varieties can allow informed choice of varieties suited to soil and/or climatic conditions by updating variety recommendation guides. In the longer term, target traits or surrogate markers can be included in progeny selection and introgression programs. Follow-up work to enable this includes tests of trait heritability and feasibility of screening. With further testing of genetic correlations across populations, the trait markers could become part of a selection program. The move in the SRA breeding program to implement molecular technologies, including genomic selection, now means that multiple traits can be included.

9. PUBLICATIONS

Journal papers:

Pierre JS, Giblot-Ducray D, McKay AC, Hartley DM, Perroux JM and Rae AL. DNA based diagnostic for the quantification of sugarcane root DNA in the field. Revised for Scientific Reports.

Pierre JS, Perroux JM and Rae AL. A sugarcane variety screening for root phenes reveals that reducing tillering does not lead to an increased root mass fraction. Submitted to Frontiers in Plant Science.

Pierre JS, Perroux JM and Rae AL From root system architecture to root anatomy; adaptation of sugarcane root systems to low nitrogen input. Manuscript in preparation.

Refereed conference papers:

Rae AL, Pierre JS, Olsen D and Perroux JM. (2016) Approaches to analysis of root system traits in sugarcane. Proceedings of the International Society of Sugar Cane Technologists Congress 29.

Pierre J, Rae A, Olsen D, and Perroux J. (2018) Sugarcane root systems: developing a toolkit of methods to understand what's going on below ground. Proceedings of the Australian Society of Sugar Cane Technologists.

10. ACKNOWLEDGEMENTS

Many of the results reported here have been published in the journal and conference papers listed above, and are reproduced here with permission.

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APPENDIX

11.1. Appendix 1 METADATA DISCLOSURE

Table 7 Metadata disclosure 1

Data	Data from analysis of root traits
Stored Location	CSIRO secure database
Access	Restricted
Contact	Dr Anne Rae, Anne.Rae@csiro.au