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Delivering a novel DNA-based diagnostic for root health to the sugar industry

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ABSTRACT

Plant root systems play many key roles including nutrients and water uptake, interface with soil microorganisms and resistance to lodging. Unfortunately, as for any other crop, large and systematic studies of sugarcane root systems have always been hampered by the opaque and solid nature of the soil. In recent years, methods for efficient extraction of DNA from soil and for species-specific DNA amplification have been developed. Such tools could be adapted for sugarcane and have the potential to greatly improve root phenotyping and health diagnostic capability in sugarcane. In this report, we present a fast and efficient method for the quantification of live sugarcane root mass in soil samples. First we demonstrated that this test is sensitive and specific to sugarcane. Then we established a universal calibration for the test to convert root DNA quantity to live root mass. Finally we validated our test on field samples and used it to answer the question of the fate of the root system after harvest. There we demonstrated that, two weeks after harvest, the sugarcane roots are still alive. It raises the question of the role that the former root system plays in the performance of the following crop and demonstrates how this test can be used to answer research question or to monitor crop root health.

EXECUTIVE SUMMARY

Plant root systems play many key roles as in nutrients and water uptake, interface with soil microorganisms and resistance to lodging. Unfortunately, like for any other crop, study of sugarcane root systems has always been hampered by the opaque and solid nature of the soil. Many critical questions about root systems in the field remain unanswered, hence plant breeders lack the knowledge and tools to select for root traits and growers lack a root diagnostic tool that will assist them with monitoring their crop's below ground development and health.

In recent years molecular biology tools have been developed and routinely used to quantify target organisms in soil samples in a fast, efficient and robust manner. Until now these tools have been mainly used for routine quantification of crop pathogens and we wanted to adapt these tools to allow for the quantification of sugarcane root in soil samples.

In partnership with the South Australia Research and Development Institute (SARDI), we worked toward the development of an assay for the quantification of live sugarcane root in field soil samples that would meet the standards required by SARDI and could be adapted to their high-throughput analytical service. The assay development was done in three main steps. First, adapting the existing molecular tools to be specific to sugarcane by targeting specific sugarcane DNA sequence. Then calibrating the assay to obtain the relationship between sugarcane DNA quantity measured in samples and sugarcane live root mass. Finally, validating the assay on field soil samples was also completed.

The diagnostic is now ready to be delivered. It will be provided as a commercial service by SARDI, a trusted organisation with a solid track record for these types of analyses, for a relatively low cost when compared to the cost of a soil sample analysis.

In order to be used widely by the industry as a diagnostic as well as a research tool, this diagnostic needs to complete an additional development phase that will focus on developing field sampling strategy and methodologies to greatly reduce the variability of assay results. A cost-benefit analysis could then determine the optimum sampling protocol for routine use in the sugar industry.

The delivery of a robust, fast and efficient method to quantify live sugarcane roots in the soil represents a *technical jump* for the whole Australian sugarcane industry. The diagnostic tool developed by this project could contribute to robust and practical decision-making for the sugar industry in the high priority area of soil and root health. It will advance sugarcane root phenotyping capabilities to a high throughput level as well as providing a tool to monitor crop underground health to growers

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1. BACKGROUND

Plant root systems play a key role in determining nutrient and water uptake and are also the interface between soil microorganisms and plants. In addition to these roles, the sugarcane root system plays an important part in anchorage of the plant to provide resistance to lodging during the cropping season and at harvesting. Sugarcane roots are also important for the succession between ratoon cycles, at least at the start of the next crop since the fate of sugarcane root systems between crop cycles is unclear (Otto et al., 2009, Smith et al., 2005).

Unfortunately, as for any other crop, the opaque and solid nature of the soil has always been a major limitation for the study of sugarcane root systems. The size of the plant as well as the length of the cropping season also limit the possibility of large scale experiments in a controlled environment. Consequently, our knowledge of sugarcane roots is sparse.

Characterizing roots in the field is a tedious task where the root system needs to be excavated, thoroughly washed and then measured (Pierret et al., 2005). Outside of the challenge posed by the root system excavation, the washing step is a critical process which in addition to being slow, could lead to the substantial loss of fine roots (Ø< 2mm) (Pierret et al., 2005). These fine roots make up around 90% of the root system total length and are the elements responsible for water and nutrient absorption (Pierret et al., 2005, McCormack et al., 2015). Measurement of root system basic descriptors such as root length, volume and diameter is also a slow process (up to 3 hours per plant) that does not give any information regarding whether a root is dead or alive. Due to these limitations most of the analyses done so far on sugarcane have been restricted to a limited number of varieties and replicates using small soil samples to monitor sugarcane root distribution in the ground (Ball-Coelho et al., 1992, Battie Laclau and Laclau, 2009, Chopart et al., 2008, Otto et al., 2009). Many critical questions about root systems, including inter-varietal variation and dynamic changes in the field remain unanswered. Hence plant breeders lack the knowledge and tools to select for root traits and growers lack a root diagnostic tool that will assist them with monitoring their crop's below ground development and health.

With the advent of molecular biology for diagnostics, methods for efficient extraction of DNA from soil and for species-specific DNA amplification have been developed for routine use (Ophel-Keller et al., 2008). These tools have been mainly used for research and for crop soil pathogen detection (Ophel-Keller et al., 2008) but have also been applied to quantify DNA from seed and roots in soil (Riley et al., 2010) and more recently to study variety differences in root growth in glasshouse and field trials (Haling et al., 2012, Haling et al., 2011, Huang et al., 2013, Mckay et al., 2008, Bithell et al., 2014). The techniques have the advantage of reporting DNA concentration in soil associated with living roots and are compatible with the high-throughput system developed at SARDI. Such tools could be adapted for sugarcane and have the potential to greatly improve root phenotyping and health diagnostic capability in sugarcane.

In this report we present the results of the development of a TaqMan assay for the quantification of sugarcane root DNA in the soil, its calibration to report the assay results as root mass, and the test validation on field samples.

2. PROJECT OBJECTIVES

The overall objective was to develop a high throughput method that could be used to unlock root phenotyping capability for the sugarcane research as well as providing a diagnostic tool that can be routinely and easily used for the monitoring of sugarcane crop root health status. Research objectives were the following

1- Screen sugarcane genome for ITS sequence and single copy genes and sequence ITS regions.
2- Develop a robust and sugarcane specific TaqMan assay.
3- Calibrate the root DNA assay with known quantities of roots.
4- Validate the root DNA assay on field samples.
5- Ensure adoption of the assay.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

The project outputs comprise a new diagnostic tool and new knowledge.

The major output is the new DNA-based method for accurate measurement of root biomass from field soil samples. The assay is specific for live roots and can discriminate sugarcane from a background of common weed species. It has been calibrated for modern sugarcane varieties and typical root/soil ratios, and it has been validated with field soil samples.

As part of the method development, the project has also produced molecular information on the sequence of the internal transcribed spacer (ITS) gene in sugarcane and the number of copies present in a range of modern varieties.

Validation of the method with field samples has provided new information on root system development, including profiles of live root mass to 60 cm depth and the rate of root turnover following harvest.

Researchers, consultants, extension advisors and growers will be able to use the new assay to examine root health through a commercial service provided by the Molecular Diagnostics Centre at SARDI. The project was designed and conducted in collaboration with SARDI to ensure that the assay would meet the standards required and could be adapted to their high-throughput analytical service. The Molecular Diagnostics Centre has the capacity to extract DNA from soil samples using a proprietary method and then apply the new DNA diagnostic developed by this project to report on the root mass. Implementing the diagnostic at SARDI will make it freely available to a broad range of potential users.

In future additional calibration of the DNA diagnostic could be performed to increase the value of the results reported. At present the assay reports soil sugarcane DNA concentration. This could be investigated further to determine varietal differences in root DNA:mass:length ratios for various functional classes of root. For application of the DNA diagnostic to monitor root health at the scale of a whole crop, further work will be required. Field sampling design, including spatial distribution and density, will need to be optimised to deliver a reliable protocol for estimating live root distribution at reasonable cost.

3.2. Outcomes and Implications

The diagnostic tool developed by this project will contribute to robust and practical decision-making for the sugar industry in the high priority area of soil and root health. This will occur through benchmarking and monitoring for soil health and management practices, and specific troubleshooting for problems in crop growth, such as pathogen damage. In the short term, the information can be used by growers to guide pest/pathogen treatments, variety planting choices, or plough-out and replant decisions. In the longer term, the information will support practice change as the industry moves to increase the adoption of improved farming techniques. Poor soil quality is a major limitation to industry productivity, but despite the scientific evidence, adoption of measures to restore soil health and improve productivity have been slow. Since it can take many years to see benefits in yield gain, tools to monitor improvements to soil health during the transition will be valuable. The root diagnostic assay, with further development for crop-scale reporting, will form a component of a soil health toolkit, contributing to increased productivity and sustainability of yields.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

The project aims, results and outcomes have been communicated in the following ways:

- An article was published in *CaneConnection* spring 2016 edition: Developing techniques for field analysis of roots.
- A meeting was held in Adelaide with Alan McKay and Daniele Giblot-Ducray from SARDI to use their expertise for the test development.
- The root diagnostic was mentioned in Alan McKay's ASSCT (McKay et al., 2017) presentation and then discussed with industry members during the question session.
- A meeting was organized with Davey Olsen who is leading project SRA2017/005 on soil health to discuss how the test can be used to demonstrate the impact on best practices on root health and distribution.
- A meeting with Harjeet Khanna and Felice Driver, project managers at SRA, was organised to discuss the future development of the test as well as its use as a research and diagnostic tool.
- A meeting was organized with Jason Eglinton, leader of the Australian sugarcane breeding program at SRA, to discuss the potential applications of the diagnostic in sugarcane as well as sharing his experience as he used to work with the same technology for barley root phenotyping.
- Plans were presented at meetings of the YCS integrated research programs in June and November 2016 to members of the scientific reference panel and research teams

4.2. Industry communication messages

We developed a robust, specific and rapid assay to test for the quantity of live sugarcane roots in soil samples. This assay will be used both as a diagnostic tool to test for root health as well as a research tool to unlock sugarcane root phenotyping capability for crop improvement. In order to be used widely by the industry, this test needs to complete an additional development phase that will focus on developing field sampling strategy and methodologies.

5. METHODOLOGY

5.1. Sequencing of the sugarcane Internal Transcribed Spacer 1 and 2 regions

5.1.1. Data mining for sugarcane ribosomal DNA region

To design universal primers for the cloning and sequencing of the ITS region, 184 sequences of the ITS1-5.8S-ITS2 region from sugarcane and its relatives were retrieved (Table 1) from the NCBI database. A well annotated sequence of *Lecomtella madagascariensis*, a grass, was also retrieved and used to delineate the boundary between ITS1, 5.8S and ITS2. An alignment of the 185 sequences was performed in CLC Main Workbench7 and PCR primers were designed based on the consensus sequence. To account for sequence polymorphism, two forward primers targeting ITS1 and two reverse primers targeting ITS2 were designed to amplify the entire ITS1-5.8S-ITS2 sequence (Table 2).

Table 1 Origin and number of ITS sequences retrieved from NCBI nucleotide database from sugarcane and its relatives.

Species	N° of sequence
Saccharum spontaneum	104
Saccharum sinense	9
Saccharum robustum	5
Saccharum officinarum	10
Saccharum longesetosum	2
Saccharum hybrid cultivar	22
Saccharum giganteum	2
Saccharum fulvum	3
Saccharum fallax	1
Saccharum brevibarbe var. contortum	1
Saccharum barberi	6
Saccharum baldwinii	2
Saccharum arundinaceum	17

Table 2 Forward primers targeting ITS1 region and reverse primers targeting ITS2 region used for the cloning of the ITS1-5.8S-ITS2 sequence in Australian sugarcane cultivars.

name	function	sequence (5'3')	length	Tm (°C)
ITS1-F1	forward primer	TCGTGACCCTTAAACAAAACAGACCGC	27	60
ITS1-F2	forward primer	TCGTGACCCTTAAACAAAACAGACCGT	27	58
ITS2-R1	reverse primer	AGCGGCTATGCGCTGCGG	18	57
ITS2-R2	reverse primer	AGCGGCTATGCGCTACGGT	19	55

5.1.2. Cloning and sequencing ITS 1 and ITS2 of Australian varieties.

Eighteen Australian sugarcane varieties were selected from amongst the most widely cultivated varieties (SRA, 2015) for the sequencing of ITS1 and ITS2. Genomic DNA (gDNA) was extracted from freeze dried leaves using Qiagen DNeasy plant mini kit according to the manufacturer's instructions. The ITS1-5.8S-ITS2 amplified DNA fragment was cloned in PGEM-T easy vector. The vector was then

used to transform JM109 competent cells that were then plated on LB/ampicillin/IPTG/X-gal medium in petri dishes. After 24 h at 37 °C, two positive colonies (white) were picked for each transformation and cultivated in liquid LB culture overnight. Plasmid DNA purification was done using the QIAprep Spin Miniprep Kit and the presence of the insert was tested using restriction digest with *Not1*. Thirty-six positive plasmid samples were sent to AGRF for Sanger sequencing.

5.2. TaqMan assay design

5.2.1. Assay design

qPCR primers and 6-FAM-labelled TaqMan Minor Groove Binding (MGB) probes were designed using the Primer Express 3.01 program. Primers and probes were tested for homology against plant DNA sequences from the NCBI database using Blastn to check for any cross reactions of the assay with ITS sequences from any of the major Australian weeds of sugarcane cultivation. qPCR reactions to test TaqMan assay were performed with an ABI ViiA7 instrument using, unless otherwise stated, the Qiagen QuantiTect probe PCR kit master mix according to the manufacturer's instructions. Briefly 10 µL reaction mixes contained 5 µL of 2x Qiagen QuantiTect Probe PCR kit Master Mix; 0.16 µL of water; 0.22 µL of 18 µM forward primer; 0.22 µL of 18 µM reverse primer; 0.4 µL of 5 µM probe and 4 µL of DNA template. Thermal cycling conditions were an initial step at 95°C for 15 min followed by 40 cycles of; melting step at 95°C for 15 s and then a combined annealing and extension step at 60°C for 1 min. For each PCR reaction, the sugarcane DNA concentration was calculated using a calibration standard and results reported as 'Number of copies of the target DNA sequence per gram of sample'.

5.2.2. Assay specificity

The specificity of the assay toward sugarcane was tested using 8 major weeds of Australian sugarcane growing regions (Table 4). Weed DNA was extracted from freeze dried leaf samples (kindly provided by Emilie Fillols, SRA) using a Qiagen DNeasy plant mini kit. Real-time PCR reactions were conducted, in triplicate, with a quantity of either 20 ηg of weed DNA or 25 ηg of sugarcane DNA per well from Q208 and MQ239.

5.3. ITS copy number quantification

In the absence of a single copy reference gene in sugarcane, the ITS copy number was quantified using digital droplet PCR (ddPCR). Six sugarcane varieties (KQ228, MQ239, Q151, Q208, Q242 and SRA1) were selected and for each variety, three separate DNA extractions were done from freeze dried leaves with a Qiagen DNeasy plant mini kit. DNA concentrations of the samples were determined using Qubit dsDNA HS assay and for each sample, three separate dilutions were made to a concentration of 30 ρg.µl^{-1}. Final concentrations were checked using the Qubit dsDNA HS assay. The ddPCR reactions were performed using the ITS2-pb1 assay (table 3), according to manufacturer's instructions, on a Biorad Qx200 system equipped with an automatic droplet generator. Each 25 µL reaction well contained 5 µL of DNA, 12.5 µl of ddPCR Supermix for Probes (without dUTP), 1.25 µL of ITS2-pb1 , 1 µL of the restriction enzyme *Alu*I and 5.3 µL of water. The ITS copy number, assuming an average 2C value of 12 pg for sugarcane, was calculated as follows:

ITS copy number per ρg of DNA = [(copies nb. µL^{-1} x reaction volume /volume of DNA in reaction)/sample concentration] x 2C value.

5.4. Assay calibration based on root weight

5.4.1. Growing conditions and harvesting

Three replicate plants of each of the six previously used varieties were grown from single-eye setts in tall PVC pots (22.5 cm x 100 cm) for about 110 days (30°C/16h day, 24°C/8h night) in UC soil mix (50% sand, 50% peat) in non-limiting water and nutrient conditions. At this stage, roots had reached the bottom of the pot and the estimated total root system length was between 0.7 km and 2.0 km with a dry weight ranging from 13 to 56 g. At harvesting, after being washed thoroughly, root systems were split in two parts (0-50 cm and 50-100 cm from the crown) and then dried for at least 24 h at 45°C in a large desiccating oven.

5.4.2. Weight range for calibration

Samples were coarsely blended and carefully weighed to obtain standard ranges from 60 mg to 600 mg with 60 mg increments as well as two additional points at 900 and 1200 mg. A total of 144 calibration data points were generated from these samples. Samples were then sent to SARDI DNA extraction commercial service where, prior to extraction, each sample was mixed with 200 g of sand. The qPCR reactions with the TaqMan assay were also conducted at SARDI.

5.5. Assay validation

The assay was validated on field samples to assess: (i) the effect of controlled release fertiliser compared to urea on root systems; and (ii) the root system viability after harvest.

5.5.1. Effect of nitrogen regime on root mass in the soil

Soil cores were collected from a field trial managed by HCPSL and located near Ingham, QLD (S18°36’11.0, E146°03’01.0). This was a second ratoon trial of Q208 that was established to test the effect of controlled released fertiliser compared to conventional fertiliser on sugarcane performance.

Soil core samples were collected for three different experimental treatments: 140 kg of N/ha as urea, 140 kg of N/ha as 75% polymer-coated urea and 25% urea, and 0 N kg/ha. Each experimental treatment was replicated four times in the field. Soil sampling was done using a Dig Stick Soil Probe (Ø15mm x 1100 mm) hammered down to the depth of 45 cm. After retrieval, soil cores were divided into 15 cm segments. Each sample was formed from a composite of 12 cores, from the same depth, for a total maximum weight of 500 g. For each treatment and replicate and depth, two samples were collected for a total of 72 samples. During the collection, samples were maintained on ice and then transferred to an oven to be dehydrated at 45°C for a week before being sent to SARDI for DNA extraction and analysis.

5.5.2. Root turnover after harvest

Soil cores were collected from a second ratoon field trial managed by CSIRO located near Gatton (S27°32'23.7, E152°20'21.0). Samples were collected from the following sugarcane varieties: MQ239, Q151, SRA1 and Q240. Half of the Q240 plants were not harvested until the last sampling date and were therefore used as a positive uncut control. For each variety, two 60 cm cores were collected per plant on four different plants. The same plants were used for the duration of the experiment. Each soil core was divided into 15 cm segments. Each composite sample was made up of eight soil core segments. Cores were collected on the day of harvest (24/08/17), one week after harvest (31/08/17) and 2 weeks after harvest (08/09/17). After collection, samples were kept on ice until transfer to a

dehydrating oven at 45°C for at least 48 h. Samples were then sent to SARDI for DNA extraction and analysis.

5.6. Statistical analysis

All the statistical analyses were done using R (R_Core_Team, 2017) with tidyverse package (Wickham, 2017).

6. RESULTS AND DISCUSSION

6.1. Sugarcane ITS sequences

In order to develop a robust TaqMan assay targeting one of the two sugarcane ITS sequences, ITS regions of 18 Australian sugarcane varieties were sequenced to obtain a consensus sequence.

The ITS1-F1/ ITS2-R2 primer pair was used to amplify the ITS1-5.8S-ITS2 DNA fragment of 583 bp for the 18 varieties. After cloning and sequencing, 16 complete sequences of good quality out of the 18 varieties were obtained. The sequences from Q200 and Q231 were incomplete and of poor quality and were therefore discarded. The consensus sequence obtained (Fig. 1) revealed a higher level of single nucleotide polymorphisms (SNPs) for ITS1 (12/203) compared to ITS2 (3/12).

>ITS1 _consensus

ACCCTTAAACAAAACAGACCGCGAACGCGTCCCTCGTNNCGCCGGGCTTCGGCNCGGCACGAGGTNCCCGAG
CTCCGTNCNGGAGCGGAGGGGCCACAACAGAACCNACGGCGCCTTAGGCGTCAAGGAACACCTANATTGCC
TTGCNCGGTGGAGCGGTCGGCCCGCCTTCCGCNCCCCNNGCAGCGATGATATCTTAATCG

>ITS2 _consensus

CAAAAGACACTCCCAACCCACCCGAGGGGGAGGGACGTGGTGTTTGGTCCCCCGTGCCGCAGGGCGCGGTG
GGCCNAAGTTGGGGCTGCCGGCNAATCGTGTCGGGCACAGCACGTGGTGGGCGACACTCAGTTGTTCTCGGT
GCAGCGCCCCGGCACGCGGCCGGCGCATCGGCCCTAAGGACCCANGGAGCACCGTAGCGCATAGCCGCT

Figure 1: Saccharum spp. ITS1 and ITS2 consensus sequences obtained from the sequencing of 16 of the most widely grown sugarcane varieties in Australia. N represents single nucleotide polymorphism between the varieties at this site

6.2. TaqMan assay design and specificity test

ITS1 was too rich in SNPs and therefore not suitable for the design of a robust and specific TaqMan assay; ITS2 was preferred instead. Two probes were developed targeting the ITS2 region: ITS2-pb1 and ITS2-pb2 (Table 3). ITS2-pb1 was able to amplify the region effectively with the ThermoFisher qPCR master mix (data not shown) but performed poorly with the Qiagen QuantiTect Probe kit master mix, which is used by the SARDI commercial DNA extraction and quantification services. ITS2-pb1 was therefore not used for the assay development.

Table 3 TaqMan assay primers and probe sequences.

assay	name	function	sequence (5'3')	length	Tm (°C)
ITS2-pb1	Fp_A1	forward primer	GAGGGACGTGGTGTTTGGTC	20	59
	Rp_A1	reverse primer	CACCGAGAACAACTGAGTGTCG	22	59
	Pb_A2	MBG TaqMan probe	CGGGCACAGCACGT	14	69
ITS2-pb2	Fp_A5	forward primer	AAAAGACACTCCCAACCCAC	20	60
	Rp_A5	reverse primer	ACCGAGAACAACTGAGTGTC	20	60
	Pb_A2	MBG TaqMan probe	CCGGCGAATCGTGT	14	69

ITS2-pb2 was tested with three different primer combinations (data not shown), for its specificity toward sugarcane ITS sequence using sugarcane DNA and DNA from eight Australian sugarcane weeds. Out of the three primer combinations, the one that yielded the best specificity was ITS2-Pb2 with FpA5 and RpA5.

The assay detected sugarcane DNA efficiently as evidenced by Ct (Cycle threshold) values ranging from 16.6 to 19.2 for 22.8-26.4 ng sugarcane DNA per reaction (Table 4). In contrast, the level of amplification with 20 ng of weed DNA matrix per PCR was very low, with Ct values ranging from 33.5 to 37.8 Ct. These levels of cross-reactions are not considered concerning as they reflect a sensitivity difference of 100,000 to 1,000 000 fold.

Table 4 TaqMan assay specificity test using four sugarcane varieties and eight common weeds of sugarcane cultivation. Cycle threshold mean values were calculated based on 3 replicates.

	matrix quantity (ng)	Ct mean
Sugarcane variety		
Q208	26.4	16.6
MQ239	23.6	19.2
KQ228	23.2	17.9
Q242	22.8	17
Weed		
Brachiaria subquadripara	20	33.5
Chloris gayana	20	35.7
Cynodon dactylon	20	35.7
Digitaria ciliaris	20	37.5
Echinochloa colona	20	36.5
Eleusine indica	20	34.8
Panicum maximum cv *guinea*	20	35.1
Panicum maximum cv *nami*	20	37.8

6.3. ITS copy number

ITS copy number determination for a given genetic background is an important step of the development of the assay. Since the assay relies on the amplification, in real time PCR, of the ITS

sequences, differences in ITS number will impact the interpretation on the PCR results. These results need therefore to be normalised to account for ITS copy number variation.

In previous studies, for example with wheat (Huang et al., 2013), ITS copy number has been quantified by using a known single-copy gene as a reference. In the absence of a single-copy gene in sugarcane it was not possible to quantify ITS copy number by the conventional means. An alternative way to quantify has been trialled with the use of ddPCR. This technology allows for the absolute quantification of the copy number of a DNA fragment of interest in a sample. As it is a very sensitive technique there was a need for a large number of replicates and a high degree of accuracy in the execution of the protocol to reduce variability.

ITS copy number in the tested varieties ranged from 4421 ± 557 for Q151 to 6942 ± 337 for Q208 (Fig. 2) which corresponds to 1.57x difference in ITS copy number in Q208 compared to Q151 (Table 5). While we observed some variations in ITS copy number within a variety, ITS copy number was not significantly different when comparing extraction methods or degree of dilution ($p=0.075$ and $p=0.267$ respectively) while ITS copy number was significantly different between varieties ($p<0.001$).

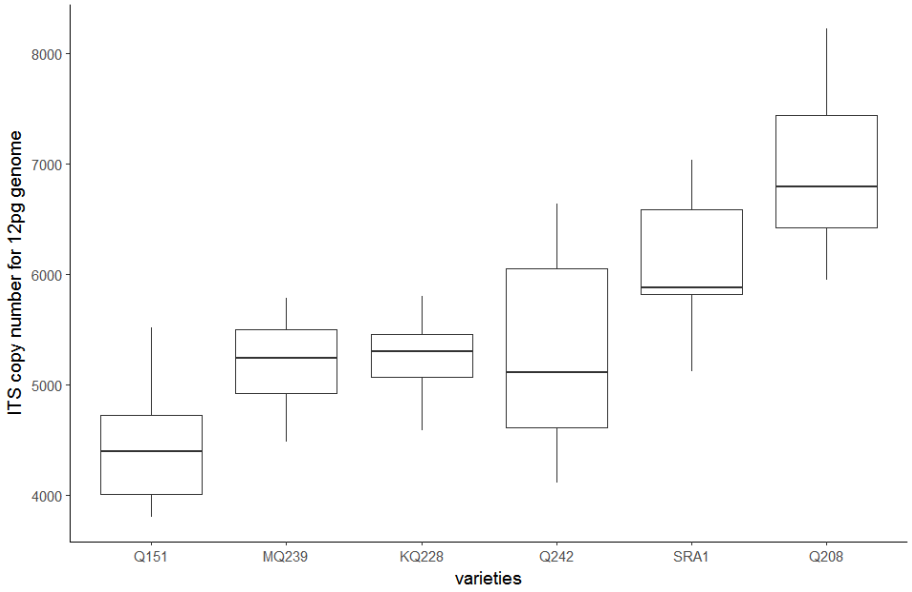

Figure 2 Estimation of the ITS copy numbers for a six sugarcane varieties using an average 2C value of 12 pg. For each variety the absolute number of ITS copies per pg was quantified using digital droplet PCR.

Table 5 Scaling factor to account for ITS copy number differences between six sugarcane varieties. Scaling factors were calculated for each variety using Q151 ITS copy number as reference.

Variety	Scaling factor
KQ228	1.18
MQ239	1.17
Q151	1
Q208	1.57
Q242	1.19
SRA1	1.36

Whilst ITS copy number was different between varieties, the extent of the variation was relatively small compared to other species. When ITS quantification was done in wheat using 20 cultivars, the

ITS copy number variation ranged from a factor 2 to 6 (Huang et al., 2013). Therefore it appears that for the 6 sugarcane varieties tested, it is not necessary to use a scaling factor to normalize the real time PCR results. If this test was to be extended to more varieties in the future, it may be important to check that ITS copy number is conserved, especially if the varieties had a different genetic background.

6.4. Assay calibration

The aim of the assay calibration was to obtain a general linear equation quantifying the relationship between root mass and the TaqMan assay results that are expressed as DNA copy number. In order to capture variation in this relationship due to diversity in root system morphology, six sugarcane varieties were selected and grown for their root system. These six varieties have been previously identified in project SRA2015/02 due to their contrasted root system morphology, especially in terms of root average diameter.

Assay calibration results are presented in Figure 3. For all varieties and depths tested, the correlations between root dry weight and DNA copy number were excellent with r^2 above 0.95 for all except Q242 at 100 cm. Based on these correlations, the assay efficiency was calculated to be on average 97%. Depth had a significant effect on the slopes of the linear regression ($p<0.001$). For a given variety and weight, the DNA copy number obtained was higher for the lower part of the root system (50-100 cm) compared to the upper part (0-50 cm). There was also a variety effect ($p<0.001$) with MQ239 and Q151 root systems yielding less DNA per mass unit of root. Finally the calibration for Q208 at 100 cm was significantly different and it appears to be an outlier, it was then discarded for any further analysis. All the data points were combined to obtain the linear regression presented in Figure 4 that represents a “universal” calibration for the test.

The depth effect we observed could be explained by changes to the root anatomy, particularly the increasing proportion of root cortical aerenchyma. As the crown roots age, live cortical cells are progressively being lysed until no cortical cells are left except for some cell wall bridges to maintain root structural integrity. The only living cells at this stage come from the epidermis and the stele. This structure indicates that older roots primarily function in transport and not in uptake of nutrients. The lower part of the root system, being richer in crown root apical segments with intact cortex, has a higher proportion of living cells and consequently the DNA content is higher there. This explains why, for an identical mass, there is more DNA in the lower part of the root system. Regarding the particularly high DNA content in the lower part of the Q208 root system, it is possible that root system growth delay for Q208 compared to the other varieties led to a higher proportion of younger root that had not developed aerenchyma yet.

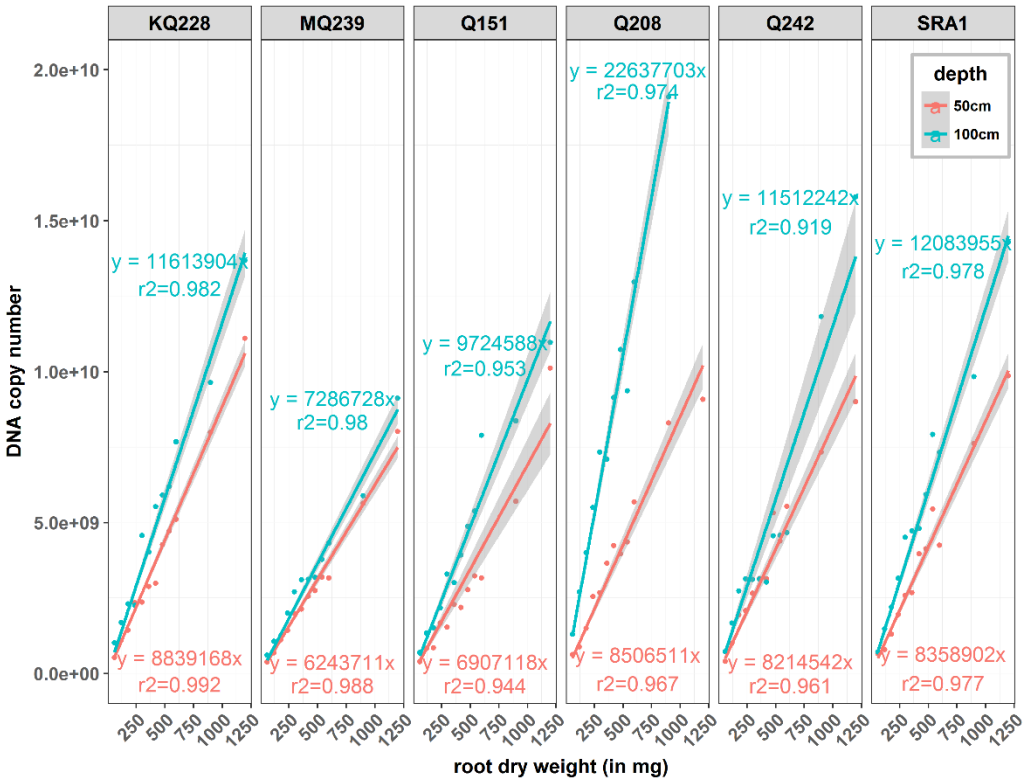

Figure 3 Root DNA assay calibration. Determination of the linear relationship between root mass and DNA copy number was done using roots from six sugarcane varieties with contrasting root system morphologies. For each variety, two linear regressions are presented, representing the relationship between root dry weight and DNA copy number for either the upper or the lower half of the root system.

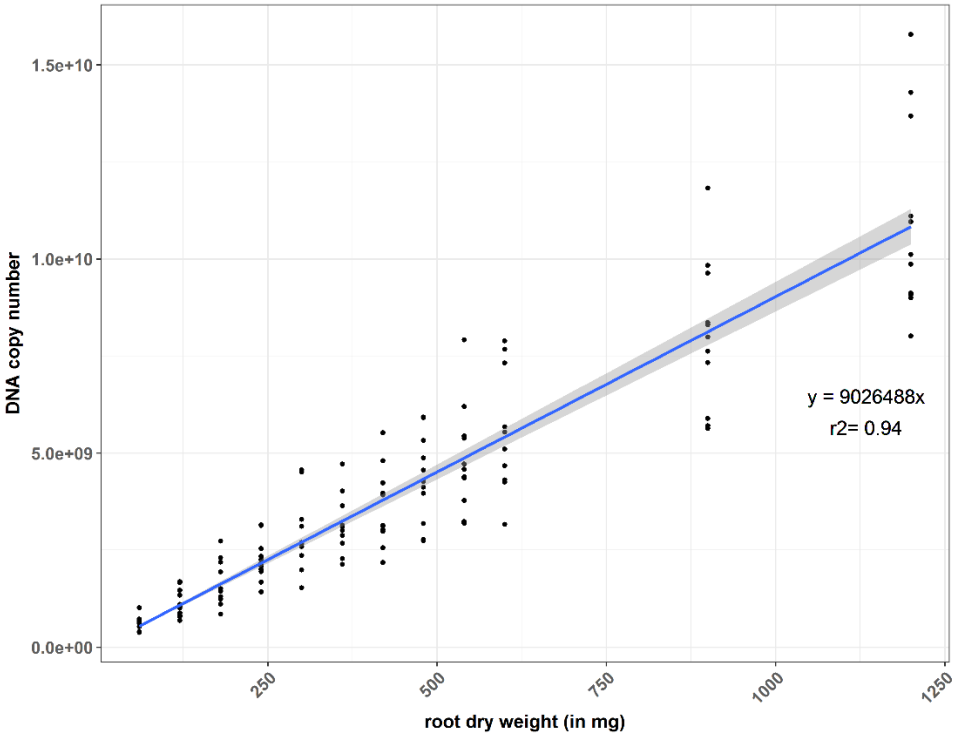

Figure 4 Consensus relationship between DNA copy number and root dry weight. Linear regression was obtained using all the data points from the calibration experiment with the exception of Q208 100 cm data. This regression was used as the "universal" calibration for the analysis.

6.5. Effect of nitrogen fertiliser on root system

Our test was evaluated using soil core samples from a field trial on the effect of different types of nitrogen input on sugarcane yield. The aims of this experiment were: (i) to validate the test on field samples; (ii) to describe sugarcane root profile under different nitrogen regimes; and (iii) to test the effect of sampling methods on experiment outcome.

In this experiment, results expressed as the number of copies of the target DNA sequence per gram of sample were converted to root dry mass using the Q208 linear regression obtained from the calibration experiment. The root quantities per g of soil ranged from 0.005mg to 0.67mg of root per gram of soil or 2.4 mg to 275 mg of root per 500 mg pooled soil core sample (Fig 5.).

From the results presented in Figure 5, we can see a sharp decrease of the root mass down the soil profile for the three different growing conditions. There was on average a 55 % and 84 % decrease in root quantity when comparing the 0-15 cm cores with the 15-30 cm and 30-45 cm soil cores respectively. Interestingly, while there was a depth effect on root quantity ($p<0.001$), there was no treatment effect on root quantities ($p=0.724$). For a given set of conditions the variability was important with a relative standard deviation of 57 % on average.

Our results confirm that the assay is extremely sensitive and can detect small amounts of root in soil samples. Our results described well the decrease in root mass from the surface down the soil profile. It was interesting to see that no differences in root mass were observed between the three treatments. The intra-sample variability was quite large and highlights the need to further develop the test toward developing sampling strategies which would be reliable enough to reduce sampling error.

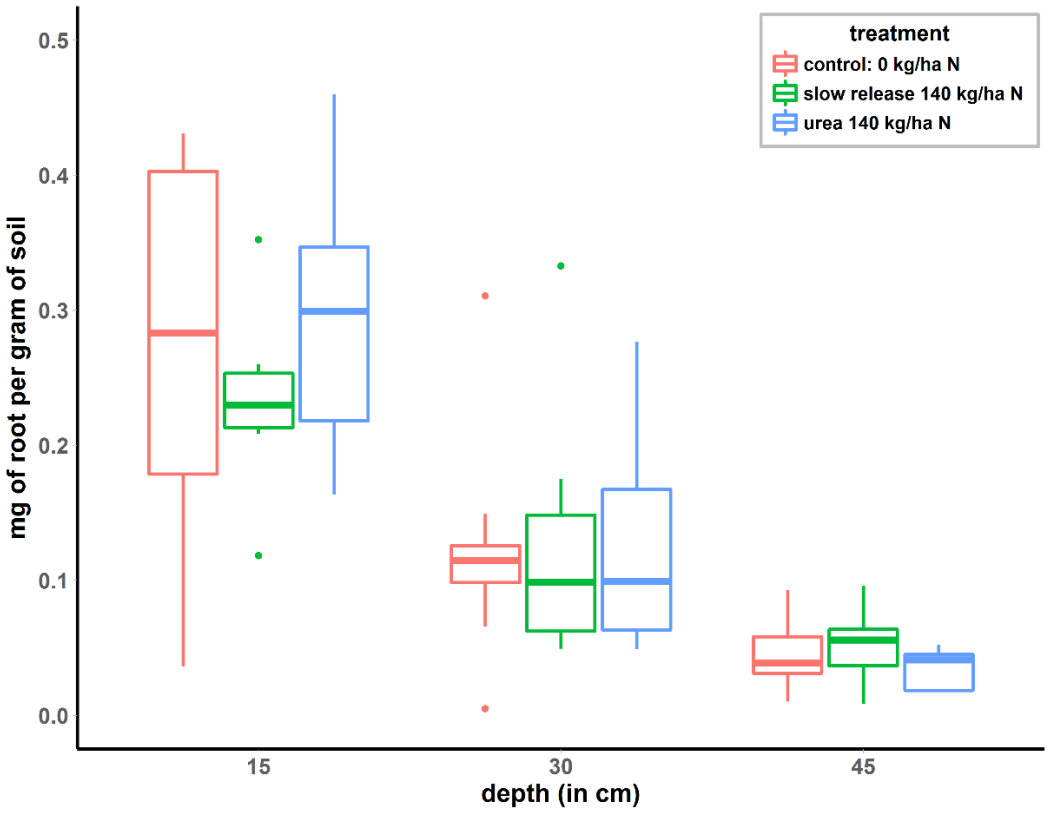

Figure 5 Field validation of the root DNA assay. Test results (DNA copy number) were converted to root dry mass using the Q208 linear regression obtained from the calibration experiment. Each boxplot (n=8) is the combination of results from four biological repetitions with two technical repetitions each.

6.6. Root viability after harvest

After calibration of the test using glasshouse grown samples and validation on field samples, we used it to answer a physiological question about the fate of sugarcane roots after harvest. Due to the perennial nature of sugarcane and its crop cycle, understanding the fate of the roots after harvest is an important research question to understand the ratoon performance. Several studies have been conducted on field grown sugarcane to answer this research question (Ball-Coelho et al., 1992) and results from these are currently used to quantify root dieback after harvest in the APSIM sugarcane crop model (Keating et al., 2003). In these studies, root viability was determined based on the root physical appearance, principally colours. There are two pitfalls with these types of assessments. First, washing roots out of soil cores is a slow and inefficient process that leads to the partial loss of the fine roots which are the absorptive part of the root system. Secondly, determining root viability based on their colours is operator dependant and subjective. We demonstrated previously in project SRA2015/002 that although dark-coloured primary roots underwent a pattern of programmed cell death to produce aerenchyma, nevertheless these roots are alive and functional. With the use of the DNA test we were able to overcome the first pitfall by avoiding root washing as well as the second one where, in our test roots, viability was purely defined in terms of DNA presence or absence. Using the same technique in ryegrass and phalaris (Mckay et al., 2008, Haling et al., 2011) it has been shown that the DNA test was able to discriminate between live and dead roots and that the test was able to detect root mortality when a visual assessment could not. We conducted a preliminary experiment to determine the rate of root die back after harvest using four varieties from the Gatton field trial.

Results from the monitoring of the changes to the sugarcane viable root mass in the soil after harvest are presented in Figure 6. For all varieties, the decline in root mass was positively and significantly correlated with the depth of the soil core samples ($p=0.003$) with the exception of MQ239 and SRA1 where there seemed to be an increase of root mass between the 45 cm cores and the 60 cm cores. The root mass of the uncut control plants significantly increased between T0 and T2. Concerning the rate of root die back after harvest, it appeared that time does not have an effect ($p=0.653$) on the quantity of viable sugarcane root in the soil after harvest, at least over this period.

Results from this experiment are another validation of the test and the observed decline in root mass down the soil profile is a good representation of root distribution in the soil, confirming the distribution found in the first set of field samples. Regarding the observed increase in root mass between at 60 cm depth compared to 45 cm depth for MQ239 and SRA1, and knowing the variability around the results that we observed in the field validation experiment, in the absence of further replicates it is not possible to determine whether this is a true increase or not. Nevertheless for MQ239 this increase is visible both in the T0 and T1 samples.

Regarding the significant increase in root mass for the uncut control plants, this variation is probably more to do with the sampling methods rather than a physiological cause. While for all the other varieties the samples were collected close the stool for the three time points, for the control, due to the size of the stool and the number of stalks it was hard to collect samples close to the stool when the plants were still standing. At T2 the control plants were cut and the sampling was done closer to the stool which may explain this sudden increase in root mass measured at T2 for the control. This change in root mass due to variations in the sampling distance from the plant reinforces the need to develop a robust field sampling method adapted to sugarcane.

Finally, we did not observe any dieback up to two weeks after harvest for the four varieties we sampled. As this was a preliminary experiment, it will be necessary to conduct a follow up experiment using a longer time course as well as increasing the number of replicates to reduce the variability

around the results. Our initial hypothesis was that after harvest the existing root system would die and be replaced quickly by new roots from developing tillers; it appears that this is not the case. While our results need to be confirmed, this raises the question of the role that the former root system plays in the performance of the following crop as well as how the root system is maintained in the absence of a supply of photo-assimilate during re-sprouting.

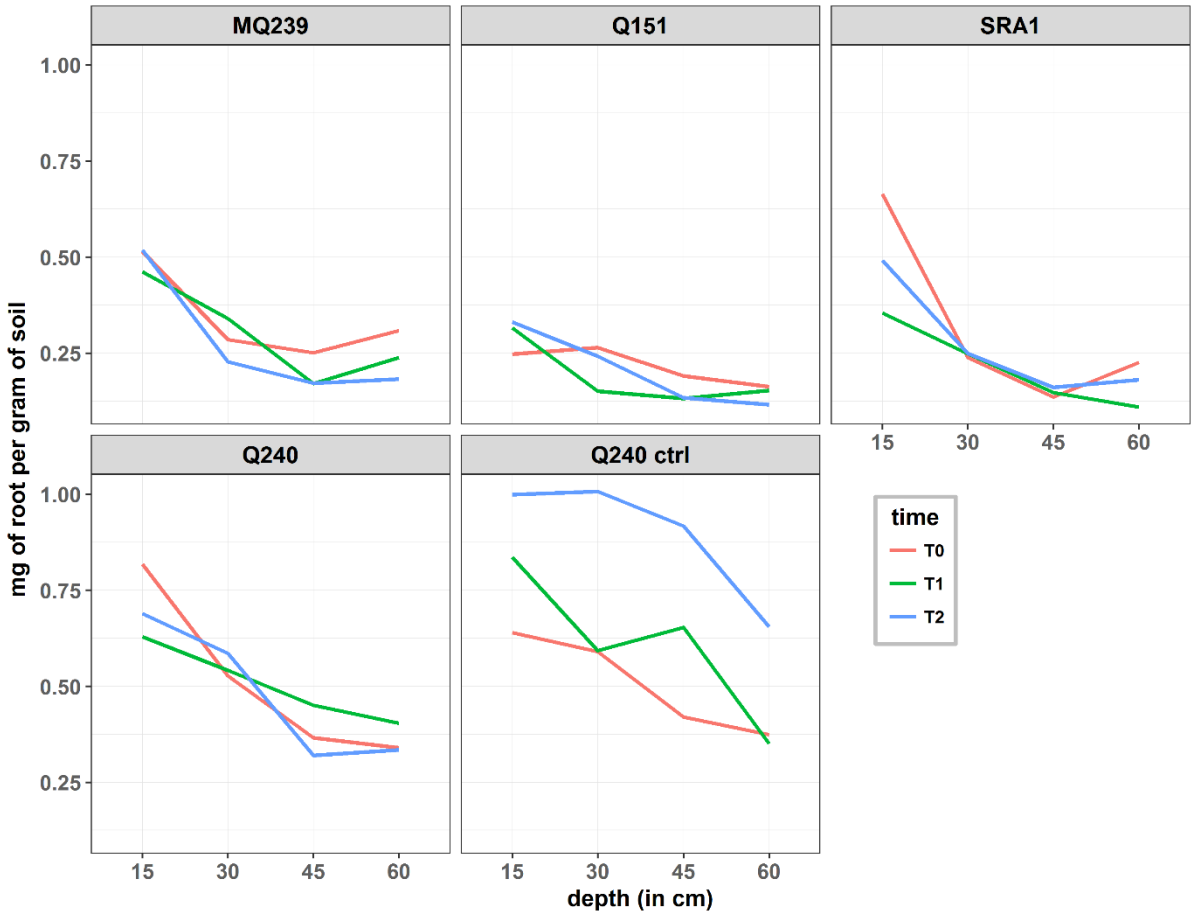

Figure 6 Root turnover after harvest. Root mass was measured at the time of harvest (T0, red), one week after harvest (T1, green) and two weeks after harvest (T2, blue) for five varieties at increasing depths in the soil profile.

6.7. Effect of *calibration* on calculated root mass quantities

Three of the varieties used in the field root turnover experiment are varieties that were also used for the test calibration (MQ239, Q151, and SRA1). We therefore decided to use the results from these three varieties to compare the three different methods of calibration to calculate root mass and to determine whether the results obtained with each method were significantly different from each other and in such case, which method was the best to select.

Root biomass calculated with the three different methods are presented in Figure 7. The three calibration methods tested were: (i) individual variety calibration; (ii) calibration to a representative variety (Q151) with ITS copy number correction for each variety; and (iii) a “universal” calibration derived from pooling all calibration data points. Even though MQ239 root mass calculated with the individual calibration methods seemed to give higher root mass compared to the two other methods, it was not the case, as the difference was not significant ($p=0.085$). Overall, the root mass calculation methods did not have an impact on the root mass result ($p=0.329$)

Conversion of the DNA copy number into root mass was not impacted by the methods of calculation. Since the individual calibration method and the ITS corrected method both rely on a new calibration for every new variety to be used in the assay, the universal calibration calculation methods would be preferable as it could be used with any number of new varieties without any prior calibration. The results confirm that the universal calibration would give sufficient accuracy across a broad range of varieties.

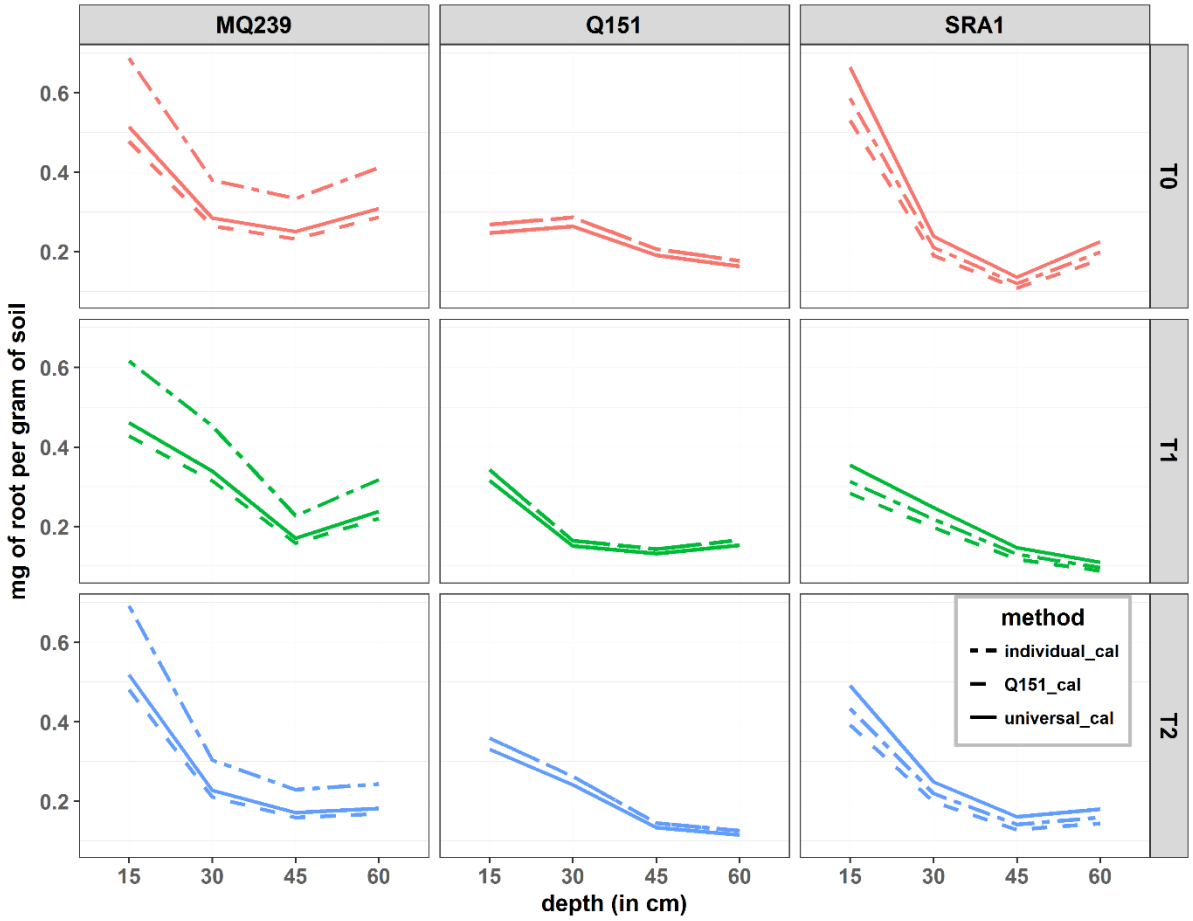

Figure 7 Comparison of root quantities obtained with three different calibration methods of the assay. The results shown in Figure 6 were re-calculated using individual variety calibrations (dashed line), a representative variety (Q151) calibration with ITS copy number correction for each variety (dot-dash line) and the universal calibration (solid line). Each panel shows the root depth profile for a single variety at a single time point calculated using the three different methods.

6.8. General conclusion and perspective

This report presents the successful development of a rapid, reliable and sensitive method to quantify viable sugarcane root mass in soil core samples. This is a technical jump that will advance sugarcane root phenotyping capabilities to a high throughput level. The results from the field validation and the root turnover experiment have shown that there is an important intra-sample variation that seems to be explained partly by: (i) the low number of soil core samples used; and (ii) a lack of spatial reference to achieve a consistent sampling along a row.

In order to be used widely by the industry as a diagnostic as well as a research tool this test needs to complete an additional development phase that will focus on developing field sampling strategy and methodologies. This sampling strategy would reduce the error associated with difference in

sampling positions and would determine the relationship between the number of samples and the accuracy of measurement. Cost-benefit analysis could then determine the optimum sampling protocol for routine use in the sugar industry.

7. PUBLICATIONS

DNA-based methods for root mass quantification to enable high throughput field phenotyping of sugarcane root system. *In preparation.*

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