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TITLE:

Measuring Stolons and Rhizomes of Turfgrasses Using a Digital Image Analysis System

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SUMMARY:

A software-based image analysis system provides an alternative method to study the morphology of stoloniferous and rhizomatous species. This protocol permits measurement of the length and diameter of stolons and rhizomes and can be applied to samples with a large amount of biomass and to a wide variety of species.

ABSTRACT:

Length and diameter of stolons or rhizomes are usually measured using simple rulers and calipers. This procedure is slow and laborious, so it is often used on a limited number of stolons or rhizomes. For this reason, these traits are limited in their use for morphological characterization of plants. The use of digital image analysis software technology may overcome measurement errors due to human mistakes, which tend to increase as the number and size of samples also increase. The protocol can be used for any kind of crop but is particularly suitable for forage or grasses, where plants are small and numerous. Turf samples consist of aboveground biomass and an upper soil layer to the depth of maximum rhizome development, depending on the species of interest. In studies, samples are washed from the soil, and stolons/rhizomes are cleaned by hand before analysis by digital image analysis software. The samples are further dried in a laboratory heating oven to measure dry weight; therefore, for each sample, the resultant data are total length, total dry weight, and average diameter. Scanned images can be corrected before analysis by excluding visible extraneous parts, such as remaining roots or leaves not removed with the cleaning process. Indeed, these fragments normally have much smaller diameters than stolons or rhizomes, so they can be easily excluded from analysis by fixing the

minimum diameter below which objects are not considered. Stolon or rhizome density per unit area can then be calculated based on sample size. The advantage of this method is quick and efficient measurement of the length and average diameter of large sample numbers of stolons or rhizomes.

INTRODUCTION:

The study of plant morphology is largely addressed in all disciplines of plants science including ecology, agronomy, biology, and physiology. The plant root system is widely studied for its importance in stress tolerance, soil stability, plant growth, and productivity. Stolons and rhizomes are also widely studied for their role in plant propagation strategies, recuperative ability, and carbohydrate storage. Stolons and rhizomes are modified stems that grow horizontally, either above-ground (stolons) or below-ground (rhizomes). Stolons and rhizomes also contain regularly-spaced nodes and internodes, as well as meristematic nodes that are capable of giving rise to new roots and shoots¹. There have been a wide number of studies on different topics investigating roots, stolons, and rhizomes of various plants²⁻⁸. Root systems, stolons, and rhizomes of turfgrasses are studied because of their importance in turf quality⁹, spring green-up after winter dormancy¹⁰, and wear tolerance and recuperative ability¹¹. Furthermore, these organs are also studied in other crops, turfgrasses such as rice¹², soybean⁴, and maize¹³, and pastures where lateral stems play a key role in soil erosion control⁵.

Root length density (root length per soil volume) and average diameter are commonly measured using scanning software^{3-5,9,14-18}. Conversely, the length and diameter of stolons or rhizomes are usually measured with a ruler and caliper^{3,19,20} and require significant time and labor²¹⁻²⁴. Hence, they are often measured in a restricted number of stolons or rhizomes^{11,20,25} and are often limited to the morphological characterization of spaced plants only. The study of stolon and rhizome traits in a mature canopy involves sampling a large amount of biomass so that usually only stolon and rhizome dry weight density (dry weight per unit of surface) are determined^{7,11,26,27}. Stolon dry mass, in fact, can be more easily measured than stolon length and diameter by drying samples in an oven. However, stolon length is an important species and varietal character that is not well-related to dry mass. A recent study on creeping perennial ryegrass (*Lolium perenne*) demonstrated that samples with high stolon length density did not necessarily have high stolon weight density⁶.

Image-analysis systems make the analysis of roots faster^{28,29}, more accurate, and less prone to human error^{30,21} than traditional, manual methods³¹⁻³³. Moreover, these systems provide high flexibility and easy-to-use tools including the light, optical setup, and resolution, which are often calibrated for each scanned image³⁴. Pornaro *et al.*²⁴ demonstrated that the WinRHIZO system, an image analysis system specifically designed for measurement of washed roots, may provide an alternative method to analyze stolon and rhizome traits more completely than current methods by overcoming measurement errors caused by human mistakes. For a morphological description and quantitative information on stolon and rhizome growth, image-analysis systems can be used to analyze a large number of samples quickly, even with a large amount of biomass, allowing increased statistical precision. Therefore, root analysis software packages provide an alternative, reliable, and fast method for studying the growth and morphology of stolons and

rhizomes of different plant species²⁴.

We present an experiment carried out in northeastern Italy to study stolon and rhizome development of four cultivars of bermudagrass (*Cynodon* spp.). The study aimed to increase knowledge on the development of stolons and rhizomes in seeded ("LaPaloma" and "Yukon") and vegetative ("Patriot" and "Tifway") cultivars of bermudagrass. The experiment was established in May 2013, and turf samples were collected over three sampling dates per year, from autumn of 2013 to summer of 2015 [March (before the green up), July (full growing season), and October (before winter dormancy)]. For the description and explanation of this method, we used samples collected in the summer of the second growing season (July 2014), as the large biomass of the samples at this time justified the need for a rapid analysis. The WinRHIZO, a digital image-analysis software tool specifically designed for washed root measurements, was used to determine stolon length density and average diameter.

PROTOCOL:

1. Collection of Biomass Samples

1.1 Collect samples including the aboveground biomass and a soil layer with an appropriate depth depending on species (for turf species, a 15 cm depth is generally sufficient) to ensure collection of both stolons and rhizomes.

Note: Overall plot size must be considered prior to initiating the study, since destructive samples will be taken. In general, the longer the experiment is conducted, the larger the required plot size.

1.2 Check soil conditions before sample collection: if the soil is too dry, especially in heavy soils, it may be difficult to collect samples. In this case, irrigate the plots before collection to soften sample layers.

1.3 Collect samples using a soil core sampler (≥ 8 cm diameter) or define the surface area to collect with a frame ($\geq 10 \times 10$ cm), and collect the samples with a spade. Label each sample with laboratory tape.

1.4 Collect several random samples per plot so they are representative of the plant population.

1.5 Use the same sampler for the entire experiment and record the area that each sample represents to calculate stolon and rhizome density.

Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than -18 °C.

2. Cleaning the Biomass Samples

2.1 Place the sample in a large sieve with 0.5-1.5 mm openings depending on stolon or rhizome size. The openings should be small enough to retain all stolons and rhizomes, but large enough to allow soil particles to be removed. For sandy soils, two sieves with different openings, placed one on top of the other, may allow for better precision and efficiency.

2.2 Clean the samples with a stream of water with enough power to remove soil particles without damaging the plants.

2.3 Retrieve the cleaned samples and place them in a tray with paper towels, taking care to label the trays appropriately.

Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than -18 °C.

2.4 Further clean the samples by removing roots and leaves with scissors. During this process, separate stolons and rhizomes, if needed, and record additional information such as the numbers of plants, tillers, and stolons per plant.

Note: Removing all root and leaf tissue from stolons and rhizomes will improve precision. Fine roots are difficult to remove; however, through the digital image analysis, it is possible to omit them from the analysis using a software application that excludes organs with a diameter less than a chosen value (see step 5.1), which is defined fairly accurately based on observations of images reproduced onscreen.

2.5 Place stolons and rhizomes in paper labeled bags.

Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than -18 °C.

3. Scanning and Image Analysis of Samples

3.1 Place the sample on a transparent plastic tray of the WinRHIZO standard scanning equipment. Manually place the stolons and rhizomes using laboratory forceps to minimize overlapping. Large samples may need to be split into subsamples.

3.2 Do not add water to the tray (as recommended for roots), because stolons and rhizomes have sufficient rigidity to avoid excessive proximity of organs which may cause reading errors, which usually happens with fine roots.

3.3 Place the tray on the scanner surface.

3.4 Turn on the scanner and start running the program.

3.5 Check image dpi in the **Image** menu, command **Image acquisition parameter**, for a possible further control in the saved image.

3.6 Check threshold in **Analysis**, command **Root & Background Distinction**, for good classification of pixel belonging to the scanned organs.

3.7 Check that the whole tray surface will be scanned in the **Image** menu, command **Image acquisition parameter**.

3.8 Check diameter class displayed for organs distribution per diameter, in the graphic area above the scanned image. Select 20 equal-width classes with 0.1 mm intervals by clicking on the horizontal axis of the graph. This function allows exclusion of data belonging to roots or small organs, when stolons or rhizomes were not perfectly cleaned. The literature reports that most roots of turf species have diameters lower than 0.2 mm.

Note: Width and number of classes can be modified while taking into account the average diameter of stolons and rhizomes for the analyzed samples and variability around this mean. A control should be conducted in some samples to determine the minimum diameter to be excluded.

3.9 Run the first sample scanning and check that the edit allows for a good analysis.

3.10 Follow the software instructions for saving the image and processed analysis. Label the image and analysis with the sample label.

3.11 Proceed with the scanning of all samples.

Note: The protocol can be paused here, and the samples can be stored in plastic bags and preserved at a temperature less than -18 °C.

4. Measurement of Dry Weight

4.1 Using a precise electronic balance, place the scanned samples in a tared aluminum tray.

4.2 Repeat step 4.1 for all scanned samples.

4.3 Insert all the samples into an oven set to 105 °C and dry them for 24 h.

4.4 Remove the samples and wait until the tissue weight has stabilized.

4.5 Weigh all the samples with their tare.

4.6 Subtract the tare from the recorded weight to obtain the net weight of each sample.

5. Correction of Data and Calculation of Length and Weight Density

5.1 Correction of length and averaged diameter

5.1.1 Convert the .txt file resulting from the analysis with WinRHIZO to a .csv file.

5.1.2 Use the results grouped for diameter classes to exclude data of organs smaller than 0.2 mm (roots, part of leaves, or scratches on the tray).

5.1.3 For each WinRHIZO reading (rows of .txt file) sum all the lengths recorded for diameter classes greater than 0.2 mm. The length calculated with this correction is the effective length to be used for further data processing.

5.1.4 For each WinRHIZO reading, sum the projection areas recorded for diameter classes more than 0.2 mm. The proportion between length and projection area gives the average diameter corrected for exclusion of organs with diameters less than 0.2 mm.

5.2 If the sample has been split into subsamples, calculate the final length as the sum of all subsample lengths, and calculate the final average diameter as the proportion between the sum of all subsample lengths and sum of all subsample projection areas.

5.3 When necessary, calculate the length and weight density per unit surface area based on sample size.

5.4 Use the data obtained for statistical analysis.

REPRESENTATIVE RESULTS:

A field experiment was established in autumn 2013 to compare stolon and rhizome development of four bermudagrass cultivars, including two seeded types ("LaPaloma" and "Yukon") and two sterile vegetative hybrids ("Patriot" and "Tifway"). The experimental design was a randomized complete block with three replications, for a total of 12 plots (2 x 2 m).

Fourteen stolons and fourteen rhizomes from each turf-type cultivar and the wild bermudagrass were collected randomly in the plots, as well as from wild bermudagrass plants growing near the plots, for a total of 70 stolons and 70 rhizomes. All stolons and rhizomes were cleaned as described in the protocol (step 2) before further measurement. Internode diameter and length were measured with a caliper and ruler, respectively, and the number of nodes was counted for each stolon or rhizome. The times necessary to clean and measure stolon and rhizome samples with the ruler and caliper were also recorded. Stolon and rhizome diameters were calculated as the means of all internode diameters measured. Total stolon and total rhizome lengths were calculated as the sum of all internode lengths. Furthermore, total scanned lengths and scanned diameters of each stolon and rhizome were measured using a digital image analysis system, as described in steps 3 and 5. The times necessary to measure stolon and rhizome traits by the digital analysis system were recorded. Each stolon and rhizome were then cut with scissors to

separate internodes from nodes, and the internodes were used to estimate the scanned internode diameter as described in steps 3 and 5. Pearson's correlation coefficients were calculated for stolons and rhizomes ($n = 70$ stolons, $n = 70$ rhizomes) between measured and scanned lengths, measured and scanned diameters, number of nodes and the absolute value of the difference between measured and scanned diameters, and measured diameters and scanned internode diameters. The lengths measured with the ruler were used to calibrate the lengths estimated through the digital image analysis system.

The regression analysis indicated a high correlation between stolon scanned length and measured length (**Figure 1a**), with a slope of 1.03 and intercept of -4.22, as well as between rhizome scanned length and measured length (**Figure 1b**), with a slope of 1.03 and intercept of 4.22. Cleaning by hand, 14 stolons and 14 rhizomes took an average time of 21 min and 24 s and 11 min and 12 s, respectively. The average time to measure length and diameter with a ruler and caliper was 14 min and 6 s for stolons and 13 min and 35 s for rhizomes. The scanning and software analysis of samples using WinRHIZO took an average of 11 min for stolons and 12 min and 4 s for rhizomes.

Measured and scanned diameters were also significantly correlated in both stolons and rhizomes. The relationships between measured and scanned diameter were close to 1:1, indicating a good fit of the data (**Figures 2a** and **2b**). However, the intercept indicated that digital image analysis system overestimated measured diameter, especially for lower values, and that higher values of rhizome diameter were underestimated. This overestimation may be due to stolon nodes that are scanned by the software, affecting the total projection surface that is used for calculating diameter (ratio between total projection surface and total length), and instead are excluded when measurements are made with the caliper. The correlation between the number of nodes and difference between diameter values obtained by both methods (measured and scanned) was significant only in stolons (**Figure 3a**); also, variations in the number of nodes explained only a small part of the variation of this difference ($R^2 = 14\%$). The significant correlation found between scanned internode diameter and measured diameter (slopes of 1.01 and 0.98 for stolons and rhizomes, respectively; intercepts of nearly zero) (**Figures 4a** and **4b**) demonstrates that internode diameter can be accurately estimated through the digital image analysis system as long as nodes are removed. Therefore, total stolon length and average diameter of samples composed by numerous stolons or rhizomes can be easily and accurately quantified using the digital image analysis system.

As part of an ongoing experiment, one turf sample (20 x 20 x 15 cm depth) was collected in each plot seasonally from autumn 2013 to summer 2015 and was handled as described in the protocol. The stolon and rhizome length per unit surface area (length density) and weight per unit surface area (weight density) of samples collected in July 2014 are presented in **Figure 5**. Differences in stolon length density were observed between the vegetatively propagated cultivars ("Patriot" and "Tifway") and seeded ones ("La Paloma" and "Yukon"). "Patriot" displayed the highest rhizome length density, followed by "Tifway" and the seeded cultivars. The stolon weight density was different for all cultivars, with "Patriot" showing the highest value followed by "Tifway", "La Paloma" and "Yukon". The vegetatively propagated cultivars also displayed higher rhizome

weight densities than the seeded cultivars. The development of stolon and rhizome length per unit surface area (length density) and weight per unit surface area (weight density) of cultivar Patriot throughout the study period are reported in **Figure 6**. Stolon length density displayed an increase from March 2014 to July 2014, and it did not vary from July 2014 to July 2015. Only a few rhizomes were found in samples collected in October 2013 and March 2014. Rhizome length density increased in July 2104, reaching its highest values, but decreased again in October 2014. Stolon weight density slightly increased from March to July 2014; however, a more rapid increase was observed from July to October 2014, with a subsequent decrease in March 2015. Rhizome weight density had a similar trend to rhizome length density, with its highest value in July 2014.

The software includes in the analysis all objects in the scanned image. An example of a digital image analysis layout from WinRHIZO software is presented (**Figure 7**), where lines of different color overlay objects (stolons) of varying diameter to calculate the total length per diameter class. We can observe that the analysis takes into account fragments of roots or leaves. As described in the step 3.9, it is possible to restrict the width and number of diameter classes that are analyzed. The histogram shows the distribution of lengths into selected diameter classes (**Figure 7**). This histogram can be used to assess the minimum diameter classes to be excluded. A visual observation of this graph in the upper part of the screen image highlights that the length has a normal distribution around an average mean diameter class, with the exception of the first two classes that show higher values than those fitting the normal distribution. Even if samples have been carefully cleaned, including these smaller classes, data analysis may affect the results, overestimating length density and underestimating average diameter. Our results show that the length of smaller classes (diameter < 0.2 mm) accounted for 13-32% of the total rhizome length values resulting from the software analysis (**Table 1**). Moreover, average diameter was underestimated from 2-17% (**Table 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Regression analysis of length values measured with the ruler against values estimated with the digital image analysis system of bermudagrass stolons²⁴ (a) and rhizomes (b). The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.* ²⁴.

Figure 2: Regression analysis of diameter values measured with the caliper against values estimated with the digital image analysis system of bermudagrass stolons²⁴ (a) and rhizomes (b). The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.* ²⁴.

Figure 3: Regression analysis of number of nodes of bermudagrass stolons²⁴ (a) and rhizomes (b) against absolute values of difference between diameter estimated with the digital image analysis system and measured with the caliper. The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.* ²⁴.

Figure 4: Regression analysis of diameter values measured with the caliper against values estimated with the digital image analysis system of bermudagrass stolons²⁴ (a) and rhizomes (b) for internodes only. The dashed line represents a 1:1 ratio. Panel A has been modified from Pornaro *et al.* ²⁴.

Figure 5: Example results of length and weight density of stolons and rhizomes from a field trial comparing four turfgrass bermudagrass cultivars (Patriot, Tifway, La Paloma, Yukon). Stolon length density (a), rhizome length density (b), stolon weight density (c), and rhizome weight density (d). Vertical bars represent standard errors of six replicates.

Figure 6: Example results of length and weight density of stolons and rhizomes from a field trial showing stolon and rhizome development of Patriot bermudagrass cultivar. Stolon length density (a), rhizome length density (b), stolon weight density (c), and rhizome weight density (d). Vertical bars represent standard errors of six replicates.

Figure 7: Example layout of digital image analysis from WinRHIZO software. The scanned image in the foreground and the bar charts in the upper part of the screen image show the length distribution in selected diameter classes. The colored lines indicate the image analysis, and each color corresponds to colors of diameter classes reported in the bar charts.

Table 1: Rhizome length density and rhizome average diameter with and without smaller diameter classes. Length density with and without including diameter classes smaller than 0.2 mm and their ratios (length of classes ≤ 0.2 mm/total length); and average diameter with and without including diameter classes smaller than 0.2 mm and their ratios (diameter including classes < 0.2 /diameter without including diameter classes < 0.2 mm).

DISCUSSION:

The protocol described here was developed and evaluated for the study of turfgrasses. However, it can be used over a range of stoloniferous or rhizomatous species with necessary adjustments according to their morphological characteristics, environmental conditions, and sample cleaning precision.

The average diameter estimated by means of this protocol cannot be compared to the internode diameter measured with a caliper. The digital image analysis includes nodes and internodes in the calculation of average diameter, that is the ratio between total projection surface and total length. As discussed by Pornaro *et al.*²⁴, average diameter obtained for bermudagrass stolons with WinRHIZO system overestimated average diameter values measured with the caliper at the internode. Stolon diameter is typically used to describe the diameter of stolon internodes and is a common parameter used for botanical description^{18,25}. For this reason, Pornaro *et al.*²⁴ pointed out that average diameter estimated through WinRHIZO system and the manually measured internode diameter describe two different morphology aspects.

The time required to perform this protocol remains a limiting factor for routine analysis. The most time-consuming phase is the cleaning of the samples (step 2.4). Based on our experience, cleaning one turf sample with a large amount of biomass (*i.e.*, 20 x 20 cm) requires approximately three people working for 2 to 4 hours. As described in the protocol, the cleaning process is necessary for both the digital analysis system and when using the caliper and ruler. When samples consist of a limited number of stolons/rhizomes, the time necessary to collect data with

the two methods is similar. However, as sample size is increased, the software-based method does not have a subsequent time increase, as the only limiting factor is the surface area of the scanner. On the contrary, the time necessary to measure organs with the ruler and caliper increases with the number of stolons or rhizomes composing the sample.

The study of stolon and rhizome traits in mature turfgrasses has always been based on the measurement of internode length and diameter and mass dry weight^{7,11,26,27}. Due to the large time required to process samples and the decrease in accuracy with the increase of sample size, manual measurements should be limited to a small number of stolons or rhizomes^{11,20,25}. As such, they may only be suitable for single-plant experiments. The advantage of an image analysis system over traditional methods is that it can measure the length of large stolon or rhizome samples and calculate both length density and specific weight (weight-length ratio).

This protocol allows for the measurement of stolon and rhizome length and calculation of length densities in samples with large biomass (for which stolon or rhizome weight is currently the only parameter used for morphology description). Stolon and/or rhizome length may be an important parameter in many studies that cannot be estimated with current techniques. Recent studies on different turf species⁶ have demonstrated that stolon weight and length densities are not always correlated, indicating that it may be desirable to measure multiple parameters to adequately assess the stolon and rhizome system. This method should be particularly suitable for cultivar or cultural management practices comparison.

Several steps within the protocol are critical for a successful estimation of length and average diameter of stolons and rhizomes. Because of high variability of plant morphology under different environment conditions, the number of samples (sample size) and ground area dimensions that should be sampled (sample dimension) should be carefully evaluated and be as representative as possible of the population in order to reduce data variability. Moreover, cleaning roots and leaves from the stolons before analysis is meticulous work requiring special attention to avoid overestimations. Lastly, before processing images, it is recommended to carefully select the width of diameter classes and minimum diameter using software options to exclude everything that is not a stolon or rhizome from the analysis. Each experiment requires the selection of a minimum diameter, as diameter varies with species and environmental conditions, including cultural practices.

DISCLOSURES:

The authors have nothing to disclose.

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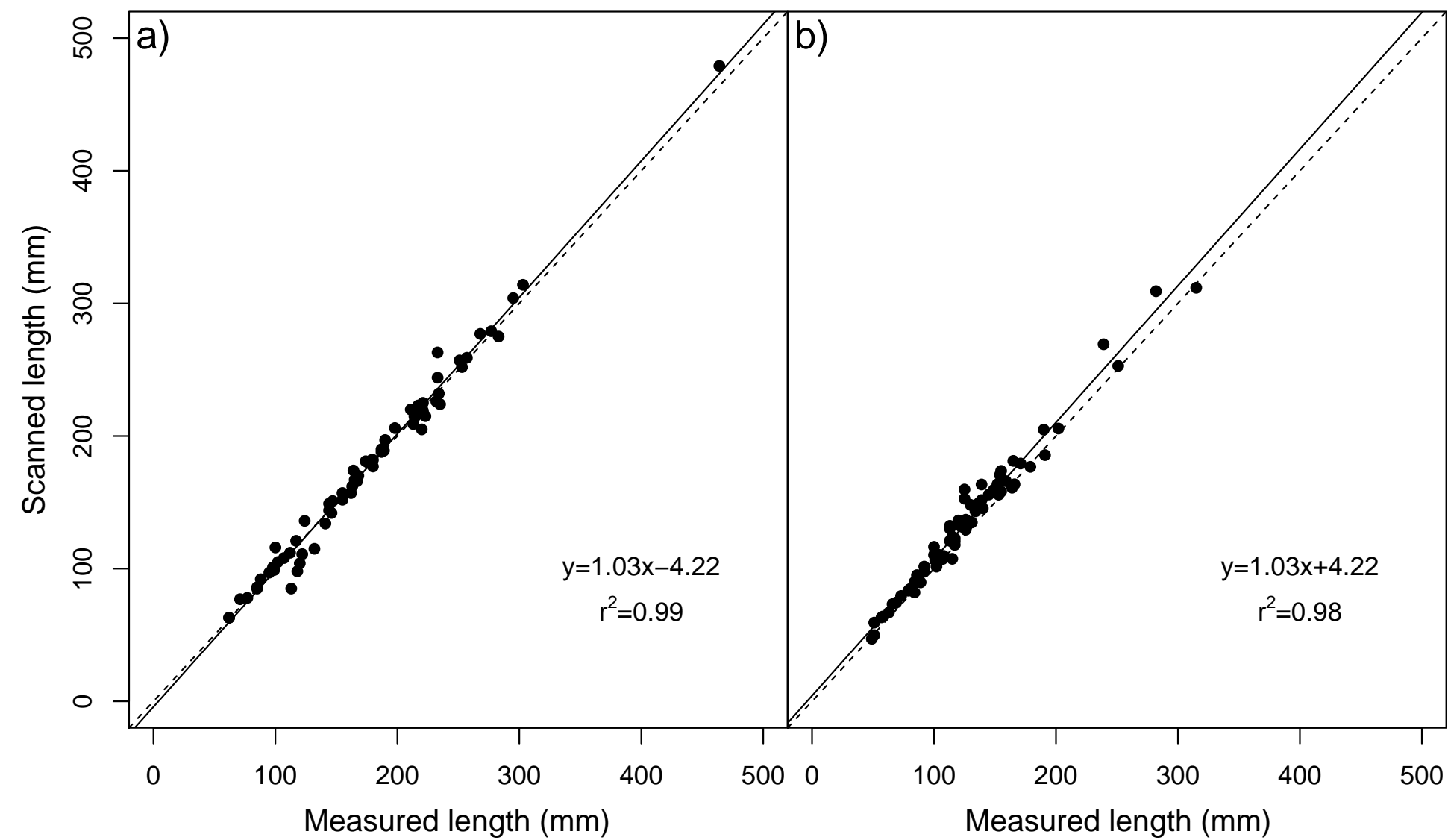
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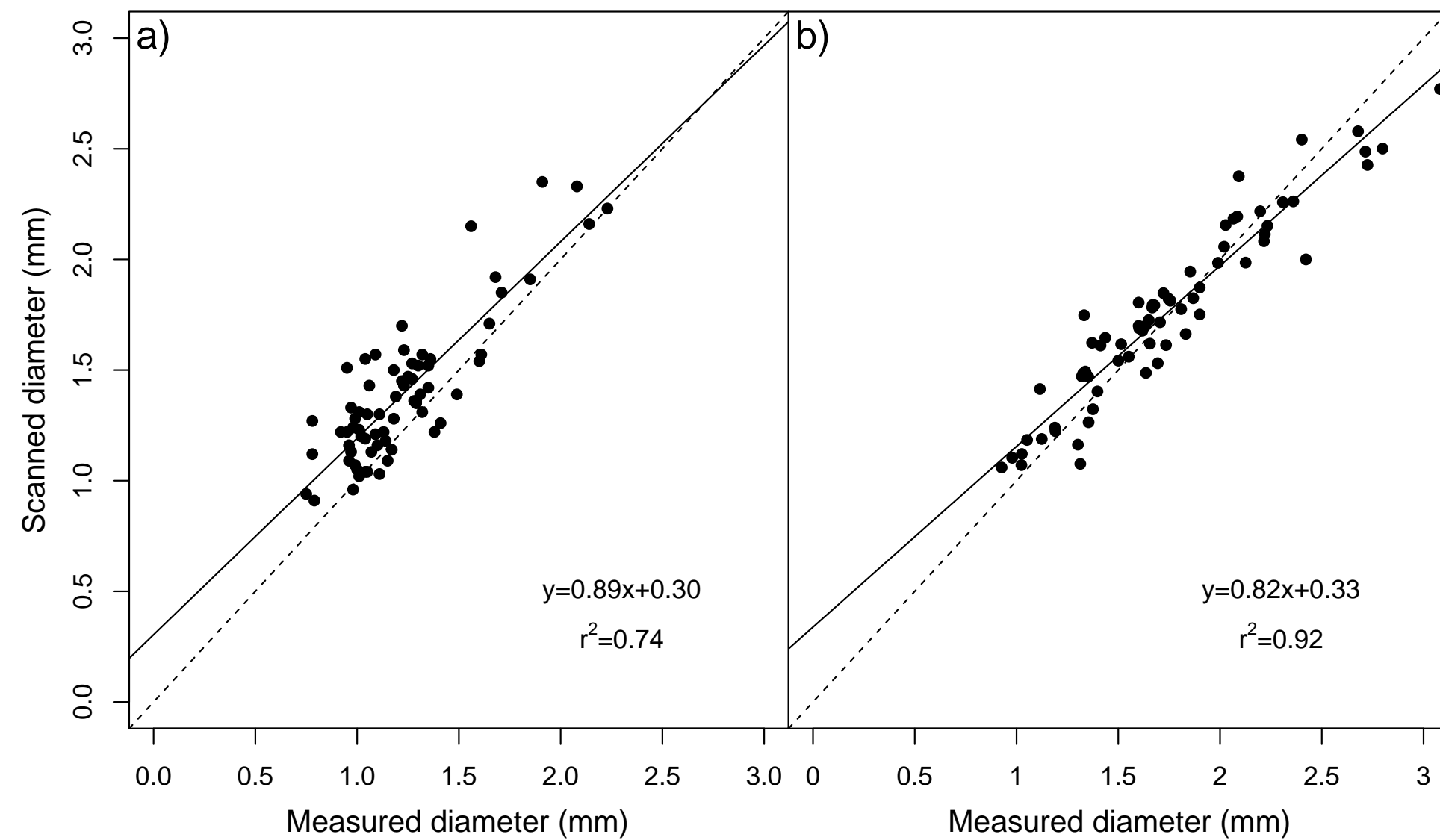
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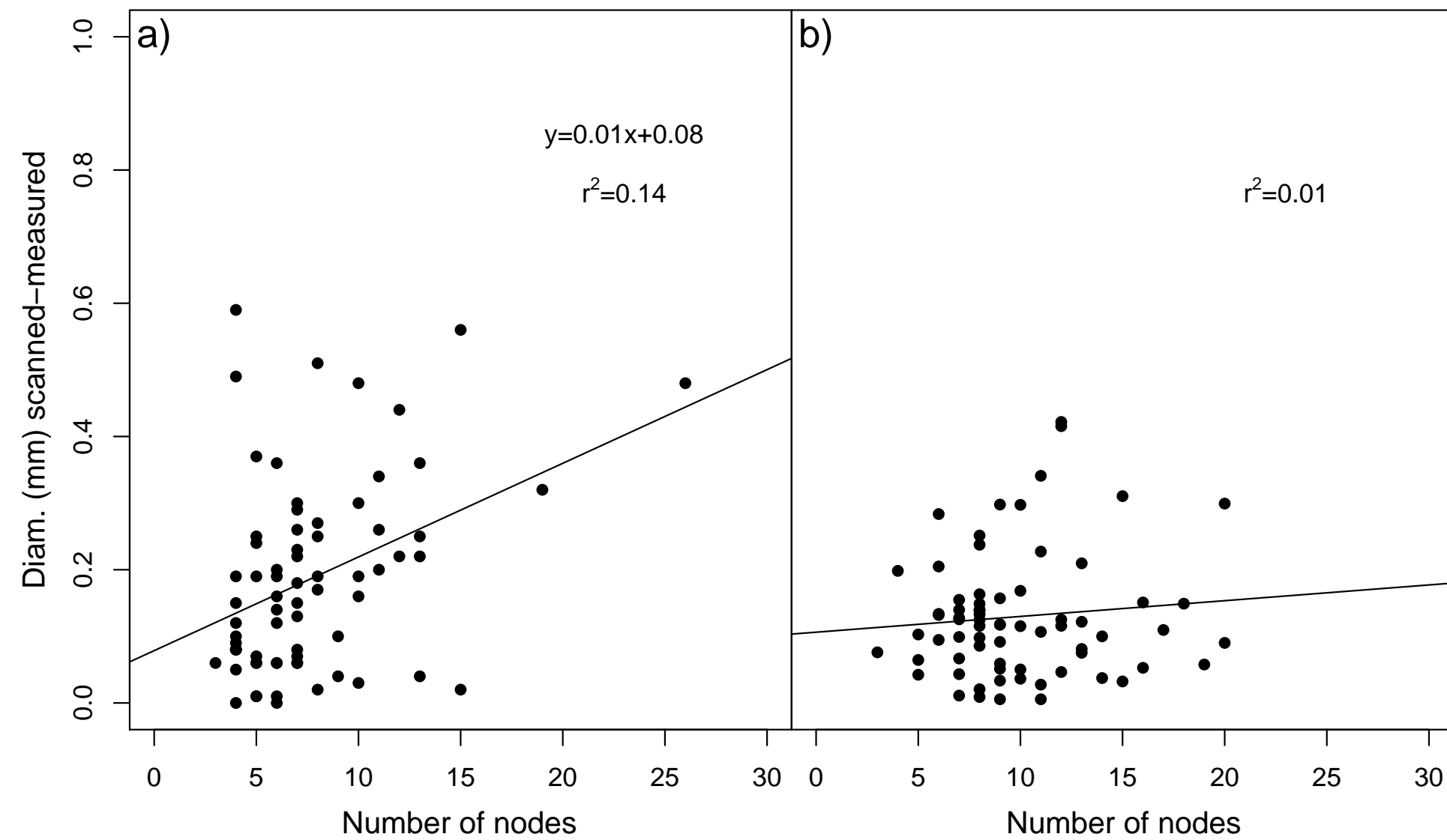
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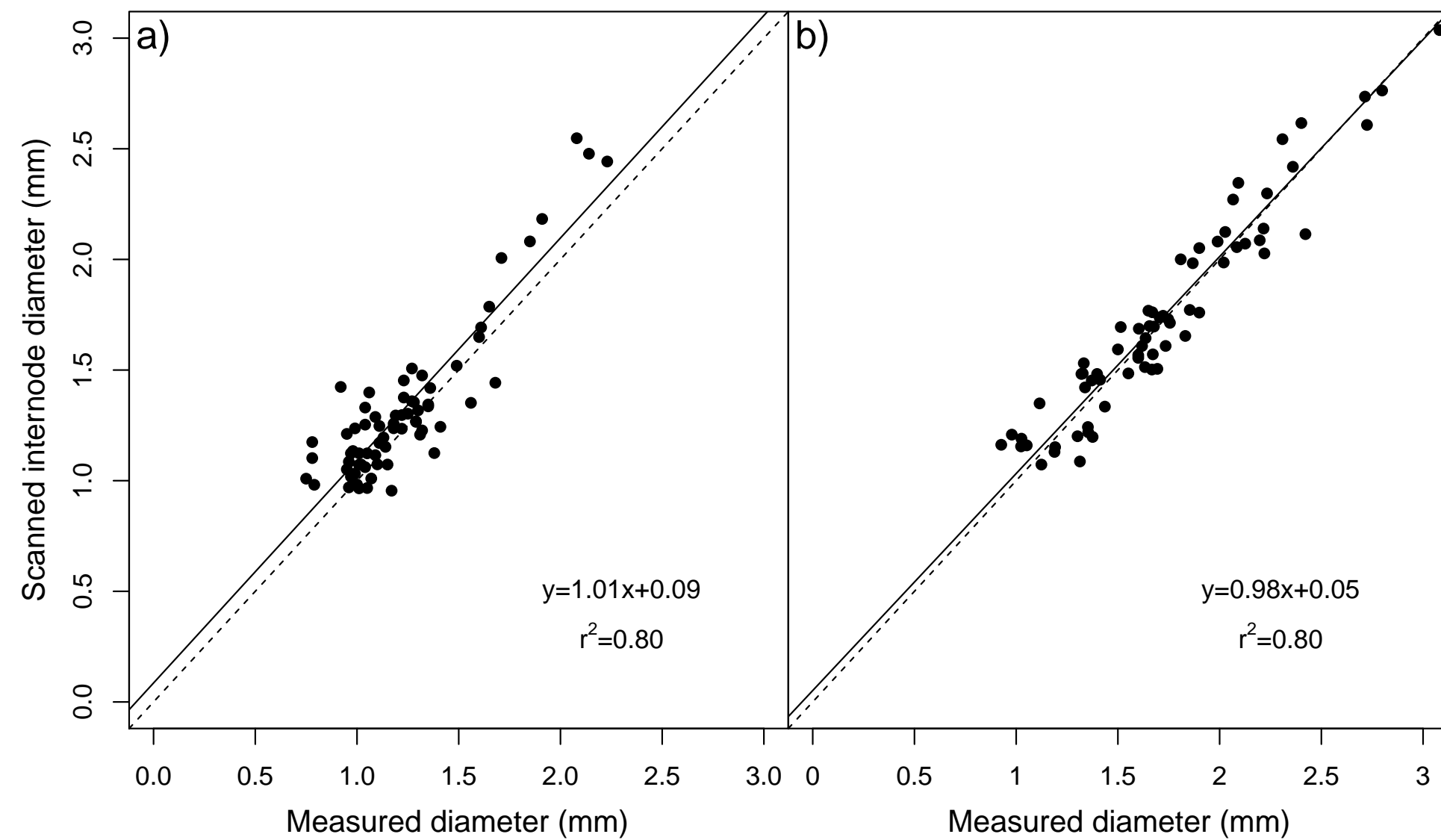
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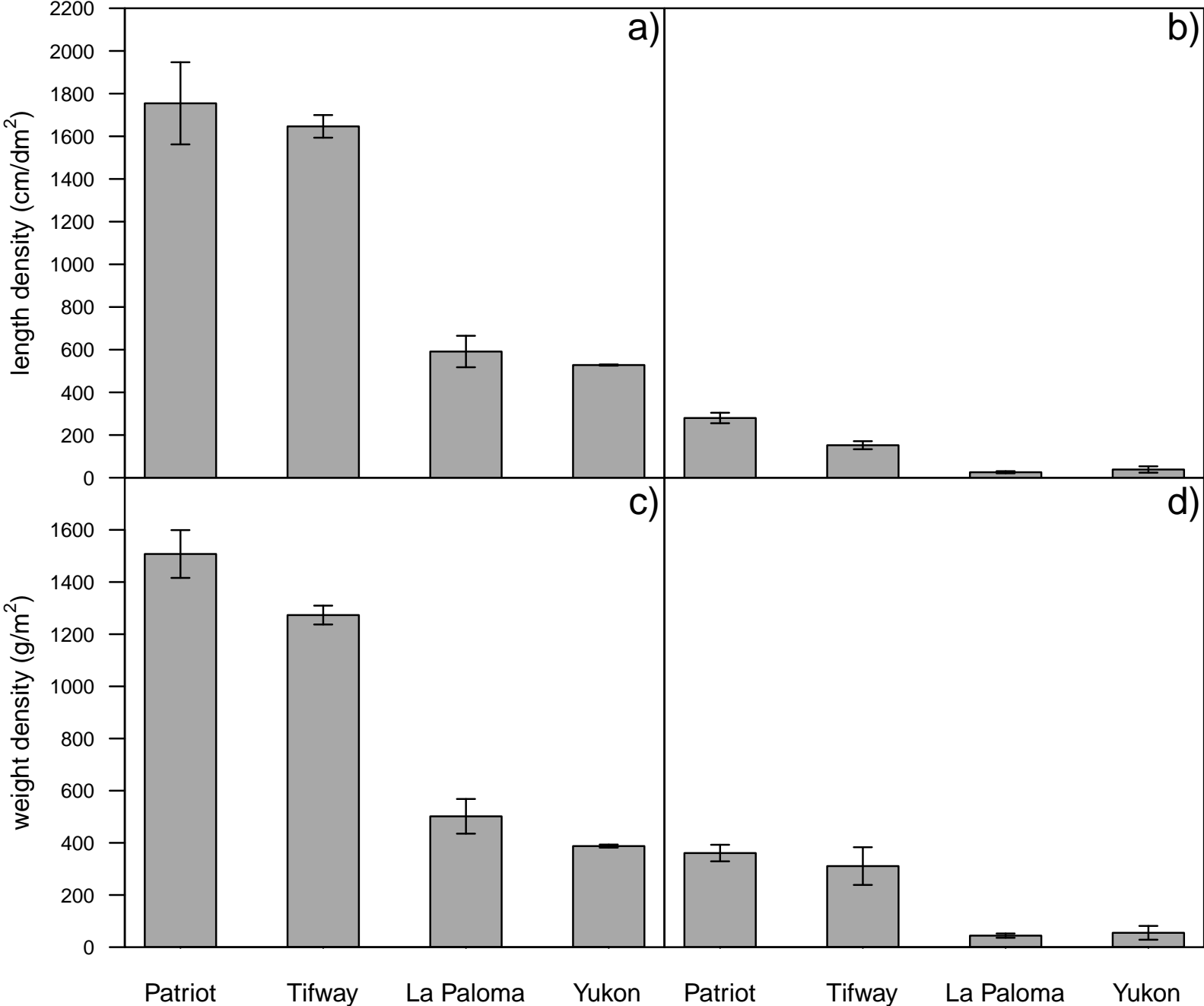
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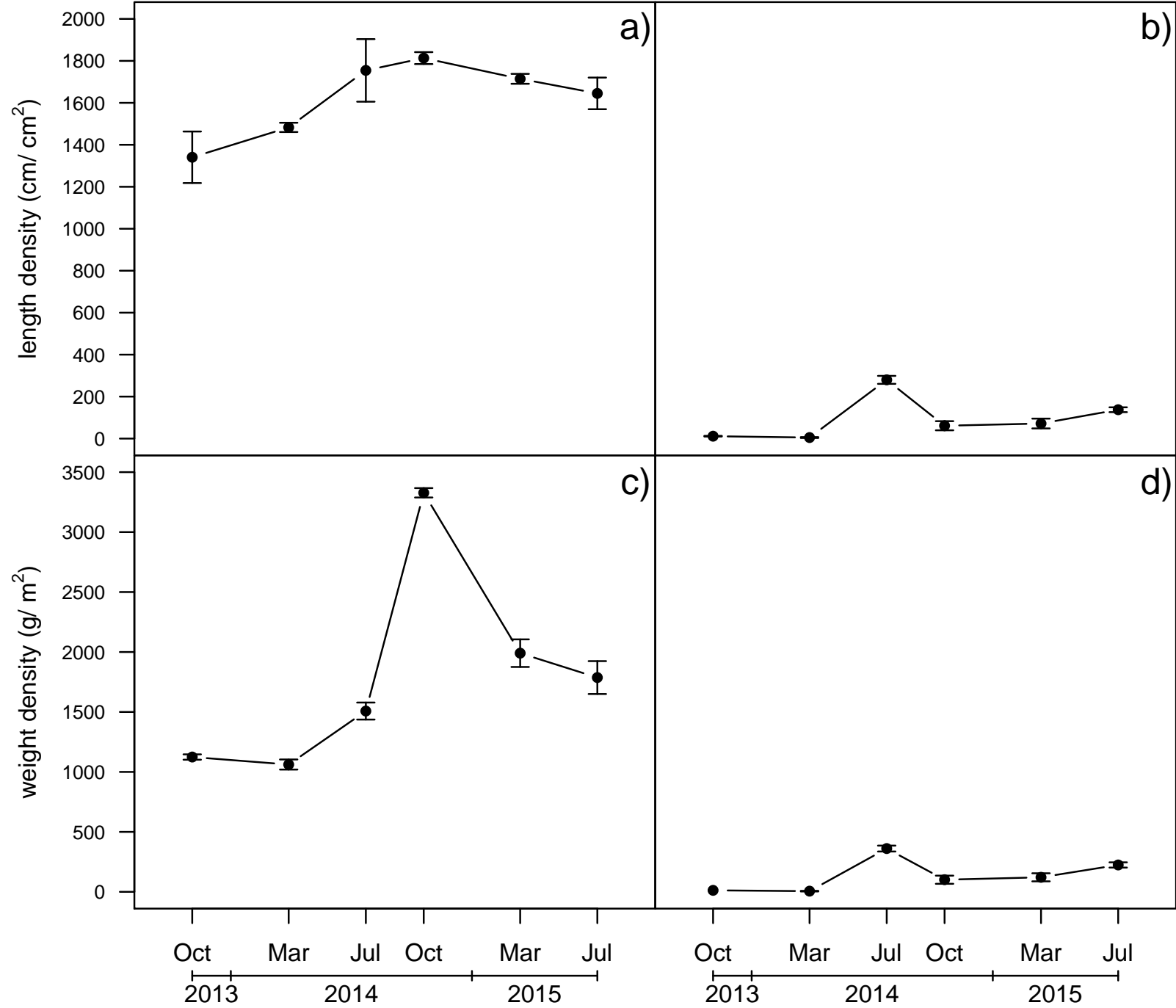














Cultivar	Block	Rhizome length (cm/dm ²)			Average diameter (mm)		
		< 0.2 mm	total	ratio ^a	< 0.2 mm	total	ratio ^b
Patriot	1	231	278	16.9	1.637846	1.5994	97.7
Patriot	2	304	349	12.8	1.620667	1.588371	98.0
Patriot	3	304	366	16.8	1.649918	1.621367	98.3
Tifway	1	184	231	20.6	2.149745	1.9951	92.8
Tifway	2	155	193	19.9	1.866253	1.76605	94.6
Tifway	3	119	150	20.9	1.877386	1.75865	93.7
La Paloma	1	17	23	24.4	2.139019	1.8904	88.4
La Paloma	2	26	38	31.6	2.101385	1.7455	83.1
La Paloma	3	34	47	27.5	2.033729	1.7354	85.3
Yukon	1	32	44	28.0	1.700155	1.4945	87.9
Yukon	2	17	25	33.2	1.68339	1.4284	84.9
Yukon	3	67	87	23.6	1.844721	1.6774	90.9

^a Length of classes ≤0.2 mm/total length

^b Total diameter/diameter of classes ≤0.2 mm

Name of Material/ Equipment		Company	Catalog Number	Comments/Description
laboratory tape	Any		NA	Tags may be used to label samples
plastic bags	Any		NA	Any plastic bag can be used to keep samples
paper bags	Any		NA	Any paper bag can be used to keep samples clean
paper towels	Any		NA	After samples have been washed with water
scissor	Any		NA	Any scissor with fine tips
aluminium box	Any		NA	Any aluminium box large enough to contain samples
trays	Any		NA	It is helpful to use plastic tray to hold samples
sieve with 0.5-1.5 mm openings	Any		NA	Any sieve
soil core sampler	Any		NA	We use core sampler for soil collection with
squared frame	Any		NA	To collect large samples we use squared frame
spade	Any		NA	We use spade to pull out samples delimited
precision electronic balance	Any		NA	Any precision electronic balance
laboratory oven	Any		NA	Any laboratory oven
freezer	Any		NA	Any freezer
WinRHIZO software	Regent Instruments Inc., Quebec		NA	Excluded the "basic" version
WinRHIZO scanner	Regent Instruments Inc., Quebec		NA	WinRHIZO system includes a scanner calibration
WinRHIZO scanner accessories	Regent Instruments Inc., Quebec		NA	WinRHIZO system includes accessories, as

es until they have been cleaned
d samples to avoid mold formation
er and before to clean them with scissors it is helpful to put them on a paper towel to absorb water

n the sample
oles during the cleaning process

h diameter of at least 8 cm
ame (10 x 10 cm, or 15 x 15 cm, or 20 x 20 cm)
d with squared frame

rated for the software
plastic tray and positioner, to be used with the scanner



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MEASURING STOLONS AND RHIZOMES THROUGH WINRHIZOTECHNOLOGY

Author(s):

PORHARO C., MACOLINO S., RICHARDSON M.D.

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Response to Reviewers comments of the paper entitled:
Measuring stolons and rhizomes of turfgrasses using a digital image analysis system

Dear Editor:

Thank you to you and to the anonymous reviewers for your time and efforts in reviewing our manuscript. Below please find our rebuttal statements (listed in blue) to each of the reviewers' comments and suggestion for changes.

Comments from Peer-Reviewers:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The text has been checked for spelling and grammar errors.

2. Please revise the following lines to avoid previously published text: 250-254, 262-269, 271-275, 282-284, 286-290, 292-294,

The text has been rewritten.

3. I would recommend not highlighting Step 5 for inclusion in the video as it consists mainly of calculations. Please note that the highlighting is only for the protocol section of the video. There will be an opportunity to discuss the results in the Representative Results section of the video.

The highlight of Step 5 has been removed.

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Reviewer #3:

Manuscript Summary:

The manuscript describe a technically sound piece of scientific research with data that supports the conclusions as well as the efficacy of the protocol. The topic of the present work is within the scope of the Journal. Please consider these minor suggestions that can improve the clarity of the present MS.

Minor Concerns:

-line 53: "on drought stress tolerance"...not only drought. I ll be more generic. please check here https://www.frontiersin.org/files/Articles/209303/fpls-07-01584-HTML/image_m/fpls-07-01584-t002.jpg

<https://doi.org/10.3389/fpls.2016.01584>

The term "drought" has been deleted to be more generic.

Field Code Changed

Field Code Changed

-line 54: "Stolons and rhizomes" I suggest to provide a botanical definition to help readers that are not acquainted with turfgrass science
Botanical definition has been added.

-line 150: "using a software application"? are you speaking about a thresholds setting in WinRHIZO, like in the point 3.6?
Threshold settings cannot exclude smaller organs. A correction of length and averaged diameter is needed after the software analysis. The process is described in the Protocol at Step 5.1. To clarify this point a reference to Step 5.1 has been added in line 154.

Reviewer #4:

Manuscript Summary:

For turfgrass, it is difficult to measure the length and diameter of each stolons or each rhizome using simple rulers and calipers. There are always some errors in traditional measurement methods. Compared with traditional methods of morphology, software-based image analysis system provides an alternative method which seems more efficient and accurate. This article explores the new protocol of morphological measurement which may provide new ideas for the morphological research of turfgrass and other crops. The results of the study indicated a high correlation between new protocol and traditional methods which are of high application value.

Major Concerns:

Cleaning is an important steps for a successful estimation of length and average diameter of stolons and rhizomes and it is meticulous work requiring special attention. The results show that it took 21 min 24 s for stolons cleaning and 11 min and 12 s for rhizomes cleaning. Measuring length and diameter with a ruler and caliper took an average of 14 min 06 s for stolons and 13 min 35 s for rhizomes. The scan and analysis of the sample using WinRHIZO took an average of 11 min 00 s for stolons and 12 min 04 s for rhizomes. Is software-based image analysis system more quick and convenient in the measurement of stolons and rhizomes in practical applications? It seems that the new method does not have much advantage because of cleaning time.

The cleaning is necessary for both measurements: software-based and ruler/caliper-based. The time necessary to have scanned and hand-measured data is almost the same when limited number of stolons/rhizomes is involved. Differences between the two methods concern measurements, the software-based method allows to analyse a limited or large number of organs taking the same time, the limiting factor is the scan surface size. On the contrary, the time necessary to measure organs by hand increases with the number of organs. The discussion has been improved about this issue in Lines 399-405.

Reviewer #5:

I have reviewed the revised manuscript entitled "Measuring stolons and rhizomes of turfgrasses using a digital image analysis system". This manuscript presents the WinRhizo software application for measuring some morphometric parameters of stolons and rhizomes of bermudagrass. Thus it is scope of JoVE journal. However, I do not recommend this manuscript for publication because of two reasons.

The first problem is autoplagerism. Authors republished significant part of data (Figure 1-4) from their article in Agronomy Journal (doi:10.2134/agronj2017.03.0187).

The second shortcoming is a lack of research problem. According to the producer, Regent Instruments Inc, the algorithms in WinRhizo allow to measure not only root parameters. WinRhizo is optimized for root measurements but it can be used to analyze other objects e.g. leaves. Thus, there is no novelty in WinRhizo application in stolon measurements.

Moreover, description and comparison of different cultivars of bermudagrass is also not a research problem. The differences in plant morphology between cultivars are usually very obvious. In this research not any explanation (or consequences) for these differences are presented.

[As suggested, this comment has not been answered.](#)

Reviewer #6:

Manuscript Summary:

An interesting manuscript that describes protocols for measuring stolon and rhizome length in Bermuda grasses using digital image software WinRHIZO. Correlations obtained for hand v digital analysis of stolon/rhizome length and stolon/rhizome diameter were >0.98 and 0.8 , respectively.

Major Concerns:

Rhizomes can easily penetrate down to 15 cms of the soil profile rather than 5 cm used in this study.

[In previous version of the manuscript we presented data from perennial ryegrass samples.](#)

[Vegetative organs in creeping perennial ryegrasses are stolons which grow at the soil surface or just below ground and samples were collected with a core sampler 8 cm diameter and 5 cm depth. Depth of samples presented in this paper was a misprint from the previous version. The depth has been corrected at line 108 and 304. The correct samples depth was 15 cm, based on our experience rhizomes of bermudagrass turf do not grow below this depth \(Rimi, F. 2012. Performance of warm season turfgrasses as affected by various management practices in a transition zone environment. PhD thesis. University of Padova\).](#)

Also, this manuscript is data poor, do the authors have additional data that can be used?

[The Result section has been improved \(Figure 6\).](#)

Did the correlations vary for different genotypes?

[As we described in the Results section, correlation was very good for length measures. We tested different cultivars and demonstrated that the precision of length measurements is not affected by the cultivar. Cultivars, species, genotype, could affect the correlation between measured and scanned diameter if they differ for morphological traits involving nodes. As reported in the text \(lines 386-389, “the average diameter estimated by means of this protocol cannot be compared to the internode diameter measured with a caliper. The digital image analysis includes nodes and internodes in the calculation of average diameter, that is the ratio between total projection surface and total length.” Furthermore, “Stolon diameter is typically used to describe the diameter of stolon internodes and is a common parameter used for botanical description. For this reason, Pornaro et al. pointed out that average diameter estimated through WinRHIZO system and the manually measured internode diameter describe two different morphology aspects.”](#)