Proximal sensing of Urochloa grasses increases selection accuracy

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Summary text for the online Table of Contents

Extensive areas in the Tropics are dedicated to livestock production. Grazing activities in these areas, however, are highly restricted by forage availability. By using sensors in place of conventional methods of forage evaluation, higher number of forages can be reliably evaluated, while incurring minimal additional cost. The use of digital cameras and hyperspectral sensors to evaluate forage characteristics and production were found effective and potentially useful for selecting outstanding hybrids.

Proximal sensing of *Urochloa* grasses increases selection accuracy

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Abstract

In the American Tropics, livestock production is highly restricted by forage availability. In addition, the breeding and development of new forage varieties with outstanding yield and high nutritional quality is often limited by a lack of resources and poor technology. Non-destructive high throughput phenotyping offers a rapid and economical means to evaluate large numbers of genotypes. In this study, visual assessments, digital color images, and spectral reflectance data were collected from 200 *Urochloa* hybrids in a field setting. Partial least squares regression (PLSR) was applied to relate visual assessments, digital image analysis and spectral data with shoot dry weight (DW), crude protein (CP) and chlorophyll content. Visual evaluations of biomass and greenness, digital color imaging, and hyperspectral canopy data were collected in 68, 40 and 80 minutes, respectively. Root mean squared errors of prediction for PLSR estimations of DW, CP, and chlorophyll were lower for digital image analysis followed by hyperspectral analysis and visual assessments. This study showed that digital color image and spectral analysis techniques have the potential to improve precision and reduce time for tropical forage grass phenotyping.

Keywords: High throughput phenotyping, *Urochloa*, tropical forage grasses, plant breeding.

Introduction

Livestock productivity depends on forage availability and quality. Grasses from the *Urochloa* (syn. *Brachiaria*) genus have been widely planted in the tropics as forage for grazing ruminant livestock and are considered the most important forages in the American Tropics (Miles et al. 2004). The International Center for Tropical Agriculture (CIAT) in Colombia conducts a *Urochloa* breeding program aimed at developing hybrids with outstanding performance on infertile, acidic soils with

43 superior forage productivity and nutritional quality. The hybrid development process is difficult
44 and time consuming. In a regular, three-year breeding cycle, over 7000 hybrids are produced by
45 open pollination, but fewer than 2% of these are retained for full evaluation. Approximately half
46 of the population is discarded based on their reproductive mode (sexual genotypes are discarded
47 and apomictic hybrids are kept); another major proportion is discarded based on visual evaluations;
48 and only a limited number of hybrids (approximately 100) are finally evaluated for different biotic
49 and abiotic stresses (Valheria Castiblanco, personal communication). The evaluation of genotypes
50 is restricted mainly by insufficient economic resources and technology for rapid screening.

51 Forage grasses exhibiting great biomass production and high nutritional quality are key
52 determinants of the productivity of grazing animals (Herrero et al., 2013). Therefore, evaluations
53 of shoot biomass production and quality parameters (i.e. crude protein) are among the most
54 important traits for improvement in any forage grasses breeding program. However, owing to the
55 destructive nature of these measurements and the insufficient economic resources, the evaluation
56 of these parameters is postponed to final stages of the breeding program characterized by a reduced
57 number of genotypes. Instead of analytical measurements of forage quality and destructive
58 biomass harvests, periodic visual evaluations of plant performance (i.e., plant biomass and
59 greenness) over time is traditionally used in *Urochloa* breeding programs to select superior plants
60 at initial stages of the breeding scheme (Miles et al. 2004; Miles 2007). These visual evaluations
61 are laborious and may not be sufficiently accurate especially in breeding populations characterized
62 by high genetic diversity and substantial genotype x environment interaction (Walter et al. 2012).

63 The use of new technologies for in-field non-destructive, high throughput phenotyping (HTP),
64 including digital image analysis and proximal hyperspectral sensing, offers the possibility to
65 precisely evaluate a larger number of genotypes than feasible in traditional ways, achieved at low

cost, and implemented in a short period of time (Montes et al. 2007; White et al. 2012; Andrade-Sanchez et al. 2014). Proximal hyperspectral sensing provides continuous information along the visual and near-infrared electromagnetic spectrum. This information often relates to plant traits and has successfully been studied in grasses to estimate quality parameters (Skidmore et al. 2010; Pullanagari et al. 2012; Thulin et al. 2012; Ferner et al. 2015; Safari et al. 2016), diversity (Lopatin et al. 2017) and nutrient content (Fava et al. 2009; Knox et al. 2012; Ramoelo et al. 2013; Adjorlolo et al. 2015; Foster et al. 2017). Likewise, plant image analysis for phenotyping purposes is based on image segmentation to separate the soil background and the plant for further quantification of regions of interest (Tucker 1979; Woebbecke et al. 1995; Camargo 2004; Hunt et al. 2005). Digital image analysis has also been used for quantifying vegetation indices related to plant growth, greenness and nutritional status (Meyer and Camargo 2008; Hunt et al. 2013). Very few reports of hyperspectral (Numata et al. 2008) or image analysis of *Urochloa* grasses exist in literature (Jimenez et al. 2017).

No studies combining hyperspectral information and image analyses and comparing them to conventional phenotyping methods is available. Moreover, hyperspectral data have not been used to evaluate target traits in *Urochloa* breeding programs. In this study, in-field visual evaluations, proximal hyperspectral data, and digital imaging were collected over canopies of *Urochloa* hybrids. Partial least squares regression was used to relate hyperspectral information to field measurements and machine learning (i.e. naive Bayes multiclass) was used to extract vegetation indices from overhead canopy images. The objectives of this study were to: 1) develop PLSR models for predicting CP, forage DW, and chlorophyll content; 2) extract plant traits from digital image analysis to relate with CP, forage DW, and chlorophyll; and 3) demonstrate the superiority of HTP techniques as compared to conventional visual evaluation of traits. Crude protein, forage

DW, and chlorophyll content were chosen as target traits in this study as they are key parameters determining both plant and cattle productivity. The development of HTP methodologies to evaluate tropical forages will increase the number of hybrids evaluated per selection cycle, thus permitting more intense selection and hence, genetic gain. The identification of new hybrids with outstanding performance (i.e. higher biomass, greener and high CP) will result in more productive pastures with concomitant increases in milk and meat production in livestock systems in tropical savannahs.

Materials and methods

Field experiment

Field data were obtained in August 2016 at the International Center for Tropical Agriculture (CIAT) in Cali, Colombia (Lat. 3° 29' N; Long. 76° 21' W; altitude 965 m). Four thousand *Urochloa* hybrids generated from crosses between the CIAT's *Urochloa* breeding program population SX12 and *U. decumbens* cv. Basilisk (CIAT 606) were initially planted in an andisol soil in an augmented block design and spaced at 1.5x1.5 m. These plants were visually evaluated four times (data not shown) for persistence, vigor and greenness after sequential cuttings every three months for one year. After that period, 200 hybrids were randomly selected for further visual and HTP analysis. These 200 hybrids, instead of the entire population, were selected for economic and practical reasons. Visual evaluations of biomass and greenness, imaging and spectra collection were performed after 3 months re-growth after cutting (see information below). Plant heights ranged from 20 to 50 cm and shoot architecture varied with both decumbent and erectus growth.

Visual evaluation

Plant biomass was assessed using a nine-point visual scale, where level '9' indicated high shoot biomass with many tillers and leaves while level '1' indicated stunted growth with fewer tillers

and leaves. Plant greenness was visually evaluated using a five-point visual scale, where level '5' represented intense dark green in all the leaves of the plant and level '1' indicated yellow-pale color in all leaves of the plant. This visual evaluation was conducted in 68 minutes one week before the HTP measurements (Table 1).

Imaging collection and analysis

Individual, digital color images for each of the 200 hybrids were taken at 1.2 m above the soil surface using a commercial digital 13-Megapixel camera (Coolpix P6000, Nikon, Japan) fixed to a buggy tractor. Digital images were saved in 4224 x 3168 pixel JPG format. The canopy cover (CC) and six vegetation indices including the normalized green red difference index (NGRDI), excess green index (ExG), excess red index (ExR), excess green minus excess red (ExGR), green ratio (GR) and green leaf index (GLI) were created using the formulae as indicated in Table 2. The canopy cover was extracted by dividing the total number of pixels representing the plant by the total number of pixels in each image. The vegetation indices were extracted using naive Bayes multiclass. Briefly, the distribution of colors in a set of digital color images (training set) was used to estimate the probability density function for each of the different region of interest (i.e. plant and background). Once the regions of interest were defined in the training set, the machine learning process was applied to all images to accurately classify and separate regions of interest. Therefore, every pixel in an image was classified into the previously defined plant and background classes. Every pixel characterizing the plant (but not the background) was then decomposed into red (R), green (G), and blue (B) channels. These channels were then normalized as follows:

$$r = \frac{R}{R+G+B}; g = \frac{G}{R+G+B}; b = \frac{B}{R+G+B}$$

Normalization makes the variations of light intensities uniform across the spectral distribution, thus, the individual color components (i.e. r,g,b) are independent from the overall brightness of the image (Cheng et al. 2011). Normalized channels were further used for the quantification of the vegetation indices (Table 2). Image analysis code was written in Java and run in ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Images were collected early in the morning to avoid beam solar radiation interferences. Digital images contained the whole plant in addition to the 23-cm diameter field-of-view (as indicated below for hyperspectral measurements, Supplementary Fig 1). The collection process took 40 minutes (Table 1).

Spectral collection and analysis

Hyperspectral field data collections were performed on clear days at full sun exposure around 11 am by positioning a hand-held field spectroradiometer (Fieldspec 2, Malvern Panalytical, Malvern, UK) directly above the plant canopy. The instrument was used with no foreoptics, which provided a 25-degree full conical angle field-of-view. To avoid soil background noise, the bare optical input was positioned at 50 cm from the top of the plant canopy to yield a 23-cm diameter field of view. The instrument collected information in 750 narrow wavebands from 325 to 1075 nm in 1 nm intervals. One or ten spectral scans were collected per plant and 50 plants were evaluated daily in about 20 minutes. Differences in the collection protocols were tested to evaluate the most effective way. Different spectra collection processes (1 or 10 scans) did not yield significant differences in the root mean squared error of prediction for the different traits evaluated (Supplementary Table 1). Radiometric collections over a 99% Spectralon panel (Labsphere, Inc., North Sutton, New Hampshire) were used to describe incoming solar irradiance throughout the data collection process. The radiometric collections over the calibration panel were made before starting and after every five canopy scans or when slight changes in solar irradiance due to cloud cover occurred.

The values of the Spectralon panel radiance were used to compute the canopy reflectance of the plants in each wavelength over the time of spectra collection. Subsequently, 401 bands from 500 to 900 nm were used for analysis. Based on visual inspection of reflectance spectra, these bands were typically less noisy, as compared to bands at the bounds of detector sensitivity. Spectral collection process was run in 80 minutes (Table 1).

Laboratory sample collections

Plants were immediately harvested after spectra collection. Aboveground tissue was removed by cutting the area defined by a 23-cm diameter plastic circle co-located with the spectral data collection area. Tissues were packed in plastic bags and stored on ice in a cooler in the field and then transported to the laboratory. The extraction of chlorophyll was performed by adding 100 mg of fresh tissue to 80% (v/v) cold methanol, and the mix was homogenized using a pestle in a mortar until the plant residue was clear and the solution was uniform. This solution was then filtered and absorbance was determined with a spectrophotometer (Synergy HT, Biotek, Winooski, USA). Total chlorophyll concentration was calculated according to Lichtenthaler and Welburn (1983). Dry weight (DW) was measured on an electronic balance (PB602S, Mettler Toledo, LLC, Columbus, OH, USA) after oven-drying the samples for three days at 60 °C. Nitrogen concentrations in the dry tissue were determined by using an automated nitrogen-carbon analyser (Sercon, Crewe, UK). *Urochloa* and common bean (*Phaseolus vulgaris*) leaves were used as reference tissues for confirmation of the reliability of the analyses. The crude protein content was calculated by multiplying nitrogen content with 6.25, as protein is assumed to contain 16% nitrogen on average.

Statistical analysis

Visual evaluations, digital image analysis, spectral reflectance, and plant trait data were incorporated into a partial least squares regression (PLSR) algorithm (Mevik and Wehrens 2007) within the R Project for Statistical Computing (http://www.r-project.org). Models were developed to predict each plant trait (i.e. CP, DW and chlorophyll) and to compare the precision for prediction of each of the different methods of phenotyping. Partial least squares regression was used in preference to conventional least squares analysis to reduce co-linearity effects. Thorp et al. (2011) provided the details on the PLSR methodology used in the present study. Briefly, if **Y** is an $n\times1$ vector of responses (i.e. CP, DW or chlorophyll content) and **X** is an n-observation by p-variable matrix of predictors (a set of visual evaluations, digital image analysis, or spectral reflectance data), PLSR aims to decompose **X** into a set of A orthogonal scores such that the covariance with corresponding **Y** scores is maximized. The X-weight and Y-loading vectors that result from the decomposition are used to estimate the vector of regression coefficients, β_{PLS}, such that

$$\mathbf{Y} = \mathbf{X}\,\boldsymbol{\beta}_{\mathbf{PLS}} + \boldsymbol{\varepsilon}$$

where $\boldsymbol{\varepsilon}$ is an $n\times1$ vector of error terms.

Leave-one-out cross validation was used to test model predictions for independent data. Results were reported for PLSR models with the number of factors that minimized the root mean squared error of cross validation. Pearson's correlation coefficients were calculated for the different traits extracted from digital color images taken from *Urochloa* hybrids.

Results

In this study, visual evaluations of biomass and greenness, digital color imaging and hyperspectral data were collected on 200 *Urochloa* hybrids in 68, 40 or 80 minutes, respectively (Table 1). High variability for the different characteristics of DW, CP and chlorophyll content evaluated on 200 *Urochloa* hybrids was found (Table 3).

Visual assessments

Partial least squares regressions for measured traits of DW, CP and chlorophyll based on visual evaluations of biomass and greenness performed with a root mean square error of prediction (RMSEP) of 8.47 g plant^{-1}, 1.76% and 0.60 mg g FW respectively (Fig 1).

Spectral data and digital image phenotyping

The PLSR models developed from the digital image analysis estimated DW, CP and chlorophyll with a RMSEP of 7.81 g plant^{-1}, 1.53% and 0.57 mg g FW, respectively (Fig 2). Differences on the correlation coefficients among traits extracted from image analysis indicated that including different indices into the model added independent information to build stronger PLSR models (Supplementary Fig 2). The contribution of each trait extracted from digital image analysis to the overall prediction of each destructively-measured trait is shown in Table 4. The GLI had the stronger positive influence on the PLSR model for predicting DW. The ExGR had the stronger positive influence on the PLSR model for predicting both CP and chlorophyll content.

The fitted PLSR models developed from 401 wavebands of canopy spectral reflectance estimated DW, CP and chlorophyll with a RMSEP of 7.90 g plant^{-1}, 1.63% and 0.55 mg g FW, respectively (Fig 3). The contribution of each spectral waveband to the overall prediction of each destructively-measured trait is shown in the Fig 4. In the PLSR model for DW, local extrema in regression coefficients were found at 701 and 674 nm, corresponding to red light near the inflection band and red light, respectively (Fig 4a). Strong positive contribution to DW estimation were with NIR (700-750), and a strong negative contribution with red light (674-640). In the PLSR models for CP and chlorophyll, regression coefficient plots exhibited strong positive contribution for traits estimation in the visible green light (Fig 4b and c). The PLSR models for CP contrasted wavebands in the visible spectrum with positive contribution from wavebands around 503 nm and negative

contributions from wavebands at 678 nm. Similarly, regression coefficients for total chlorophyll indicated strong positive contribution in the visible spectrum around 504 nm and negative contribution throughout the visible wavebands, especially at 625 and 643 nm (Fig 4c). This is sensible considering visible light absorption is increased with additional leaf chlorophyll.

Discussion

The results from this study demonstrate that the current visual assessment methodology at initial steps of the breeding cycle in the CIAT *Urochloa* breeding program can be improved using non-destructive HTP techniques. Color imaging, hyperspectral analysis, and PLSR models are more precise and faster than visual evaluations, thus increasing the number of plants evaluated in the tropical forage breeding program.

Visual evaluations of plant growth and greenness (characteristics associated with N content, and therefore CP and chlorophyll concentration in leaves) have traditionally been used to discard *Urochloa* hybrids at initial stages of plant phenotyping. The visual evaluation of an entire breeding population (i.e., 7,000 hybrids) is a slow, costly and tedious process, and is often biased by subjectivity and human fatigue, especially when phenotypic variation of such traits is high (Table 3). In this study, the estimation of DW, CP, and chlorophyll content was more precisely and consistently estimated by HTP techniques. Dry weight and CP predictions were more accurate using digital image analysis, followed by spectral analysis and visual evaluations. Chlorophyll content was better estimated by the analysis of 401 spectral wavebands, followed by color image analysis and finally visual evaluations (Fig 1, 2 and 3). The time required to run non-destructive HTP evaluations was considerably shorter by 28 minutes per 200 plants for color image analysis than visual evaluations, but longer by 12 minutes per 200 plants in hyperspectral than in visual evaluations (Table 1).

The moderate trends in the relationship between *Urochloa* canopy imaging and reflectance and measured DW, CP and chlorophyll may indicate that the method is not appropriate for very precise estimations of these traits. However, for breeding purposes where a large percentage of hybrids are discarded without detailed evaluation due to scarce resources, a difference in DW of 7.90 g plant^{-1} or a difference of 1.63% in the CP content of plants may be acceptable during initial stages of plant breeding. This moderate trend between *Urochloa* canopy analysis and measured traits in this study can be explained by dissimilarities in the canopy architecture of the *Urochloa* genotypes (Numata et al. 2008), as well as different growth patterns during recovery from cutting. The further evaluation of breeding populations with contrasting canopy architecture will improve the accuracy of the PLSR model to predict the targeted traits. Nonetheless, by combining both digital image and hyperspectral analysis techniques, higher precision accuracy for DW, CP and Chlorophyll content can be achieved.

The vegetation indices (see Table 2) extracted from color images of 200 *Urochloa* hybrids were originally developed to separate green plants from the background by extracting green and red colors from digital images. These indices have been related to different plant characteristics including biomass, chlorophyll content and nutritional status (Tucker 1979; Woebbecke et al. 1995; Camargo 2004; Hunt et al. 2005; Meyer and Camargo 2008; Hunt et al. 2013; Lee and Lee 2013; Wang et al. 2013). In this study, digital image analysis performed better than hyperspectral scanning analysis to estimate DW and CP (Fig 2 and 3). Nonetheless, the use of spectral analysis over grasses becomes more important when this technique is used to detect either nutritional or anti-nutritional compounds (i.e. metabolisable energy, digestibility, fiber) that are better estimated with the near-infrared regions of the electromagnetic spectra (Curran 1989; Pullanagari et al. 2012; Ferner et al. 2015). In this sense, the use of digital color image analysis and hyperspectral analysis

is complementary because by using both techniques a diverse set of plant traits can accurately be predicted and by adding extra factors to the prediction model, higher prediction accuracy can be achieved (cf. Numata et al. 2008). Future efforts will use data mining to fine-tune the spectral bands included in the PLSR model (Thorp et al. 2017), which can reduce model error and improve model fit statistics. Although testing multiple methods of analysis was not the intention of this study, future research could also test other techniques (e.g., artificial neural networks) for relating HTP measurements to plant traits.

The regression coefficients for the PLSR for DW and chlorophyll content obtained in this study highlight that the key wavelengths for the prediction of these traits occur in the green, red, red-edge and NIR regions of the electromagnetic spectrum (Fig 4). Previous hyperspectral studies have highlighted those regions as being highly representative for dry mass and chlorophyll content in plants (Lichtenthaler et al. 1996; Thenkabail et al. 2000; Mutanga and Skidmore 2004; Fava et al. 2009; Thorp et al. 2011; Adjorlolo et al. 2015; Dou et al, 2018). Although some similarities were found between wavebands among the different traits, the general regression coefficients differed among the traits, thus demonstrating that the reflectance data in a given waveband contributed differently toward the estimation of a given trait. Given the logistical burden to collect and analyze hyperspectral scans, the identification of informative key bands associated with each evaluated trait can improve the HTP process (Thorp et al. 2017). Results from this study will help guide selection of optimal bands in the construction of multispectral sensors tailored to predict specific traits of interest in tropical forage breeding programs.

The PLSR models for predicting DW, CP and chlorophyll content can be now used to evaluate the next generation of hybrids from the same *Urochloa* gene pool (i.e. *U. ruziziensis* – *U. brizantha* – *U. decumbens*). The accuracy of this prediction models relies on collection protocols similar to the

297 explained in the Materials and Methods section and evaluations on plants with comparable growth
298 characteristics as the hybrids evaluated here (i.e. about three months after regrowth). The
299 prediction accuracy will likely be reduced on larger plants with higher biomass (Hill 2004) and a
300 greater proportion of senescent leaves (Asner 1998). The development of more precise PLSR
301 models to predict variables of interest in a breeding program requires an ongoing effort. The
302 collection of ground data every year while making improvements to standardize collection
303 protocols and incorporate wider range of genotypes will result in more accurate and robust models.
304 Larger data sets will increase estimation precision.

305

306 **Conclusions**

307 In this study, 200 *Urochloa* hybrids were monitored in 40 and 80 minutes by digital imaging and
308 spectral analysis, respectively (Table 1). At this pace, more than 1000 *Urochloa* hybrids could be
309 evaluated in a period of less than 7 hours. This means that forage biomass and quality in a high
310 number of genotypes would be reliably evaluated with minimal increased acquisition costs relative
311 to destructive harvest. This demonstrates the superiority of HTP techniques as compared to
312 conventional visual evaluation of traits. The PLSR models for predicting CP, forage DW, and
313 chlorophyll content developed in this study supports the evaluation of higher numbers of genotypes
314 at initial stages of the breeding program. The greater numbers of plants evaluated reliably every
315 year in the *Urochloa* breeding program, the greater the genetic gain will be. Therefore, the use of
316 image analysis and hyperspectral monitoring over *Urochloa* hybrids canopies will benefit the on-
317 going breeding program. The application of this HTP method could be of great help in rural remote
318 areas lacking facilities to perform destructive harvest and plant chemical analysis. Research is
319 underway to improve the utility of proximal sensing by considering a greater range of canopy

architectural configurations and evaluating the potential to assess nutritional quality, including characteristics such as metabolisable energy, fiber, digestibility, lignin and cellulose fractions in *Urochloa* grasses.

Conflict of interest

The authors have no conflicts of interest to declare.

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482 **Table 1**. Phenotyping techniques used in the present study, the time of evaluation, its application,
483 advantages and disadvantages.
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Phenotyping technique	Time of evaluation*	Applications	Advantages	Disadvantages
Visual evaluation	68 min	Visual observations of different plant characteristics	Easy operation, low cost, evaluations can be performed under diverse conditions and environments	Evaluation of low number of genotypes, evaluation subjected to human bias and fatigue
Image analysis	40 min	Quantification of canopy cover and vegetation indices in the visible electromagnetic spectrum	Easy operation, low cost, greater number of plants evaluated, determination of several vegetation and water indices	Changes in ambient light conditions limit calculation of vegetation indices, data analysis is moderately complex
Hyperspectral analysis	80 min	Canopy reflectance information in the visible and near infra-red regions of the electromagnetic spectrum. Information can be used to predict biochemical composition of plants	Moderately easy operation, greater number of plants evaluated, determination of nutritional and biochemical composition of leaf/canopy	Low solar radiation or cloudy days limit analysis, sensor and white reference calibration is frequently needed, data analysis is complex

485 * The time of evaluation refers to 200 *Urochloa* plants evaluated under the conditions of the
486 present study.
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Table 2. Canopy cover and vegetation indices calculated from digital images of 200 *Urochloa* hybrids. Vegetation indices were extracted using a naive Bayes multiclass machine learning approach. Indices were then incorporated into a PLSR model to predict crude protein, dry weight biomass and chlorophyll content.

Plant traits	Name	Formula*	Reference
CC**	Canopy cover	Nc/Nt	-
NGRDI	Normalized green red difference index	(g-r)/(g+r)	Hunt et al., 2005
ExG	Excess green index	2g-r-b	Woebbecke et al., 1995
ExR	Excess red index	1.3r-g	Meyer et al., 1998
ExGR	Excess green minus excess red	ExG-ExR	Camargo 2004
GR	Green ratio	g/(r+g+b)	Tucker 1979
GLI	Green leaf index	(2g-r-b)/(2g+r+b)	Louhaichi et al. 2001

*r, g and b denote the normalized pixel values of each channel on the RGB colour mode.
** No normalization was performed for the canopy cover quantification. Nc= total number of pixels representing the canopy, Nt= total number of pixels in the picture.

504 **Table 3**. Plant traits measured in 200 *Urochloa* hybrids.

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Trait	Min	Max	Mean	CV (%)
Dry Weight (g plant^{-1})	6.74	64.1	30.22	34.81
Crude Protein (%)	6.76	21.58	11.23	19.68
Chlorophyll (mg g FW)	0.87	6.41	2.88	24.31

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521 **Table 4**. Regression coefficients of the fitted partial least square regression models of seven traits
522 extracted from digital image analysis. Positive and negative coefficients indicate positive and
523 negative influence on the prediction model, respectively.

Traits*	Dry weigth (g.plant^{-1})	Crude protein (% DW)	Chlorophyll (mg.g^{-1})
CC	3.760545	-0.23114345	-0.01673706
NGRDI	9.948634	0.08600517	-0.03532585
ExG	-14.3163	0.07760486	0.0730642
ExR	-32.126212	-0.39106455	-0.07758547
ExGR	3.724492	0.26592971	0.10326555
GR	-34.770799	-0.31158671	-0.03624834
GLI	80.87152	-0.31108316	-0.0357783

524 * CC= canopy cover, NGRDI= normalized green red difference index, ExG= excess green index,
525 ExR= excess red index, ExGR= excess green minus excess red, GR= green ratio and GLI= green
526 leaf index.
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Fig 1. Modeled versus measured dry weight, crude protein and chlorophyll content when fitting partial least square regression models to relate each biophysical characteristic to visual evaluations of biomass and greenness of 200 *Urochloa* hybrids.

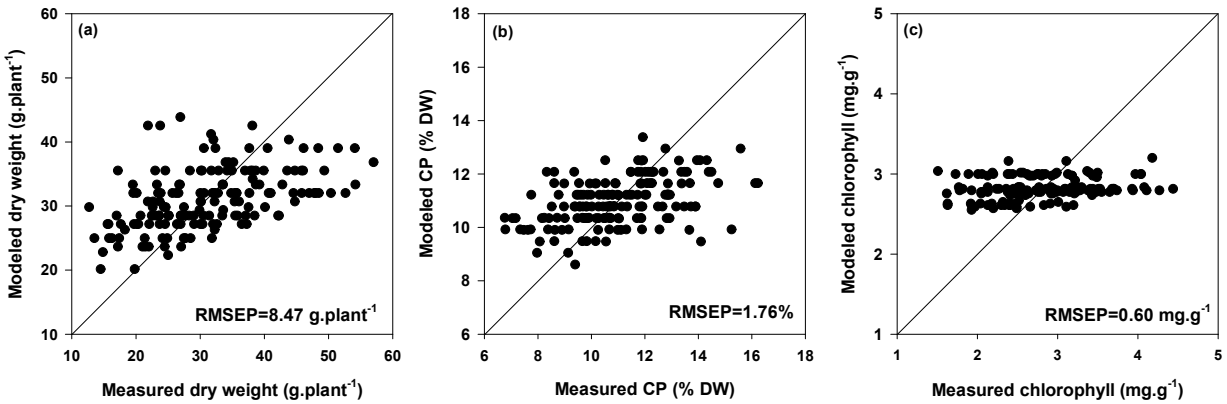
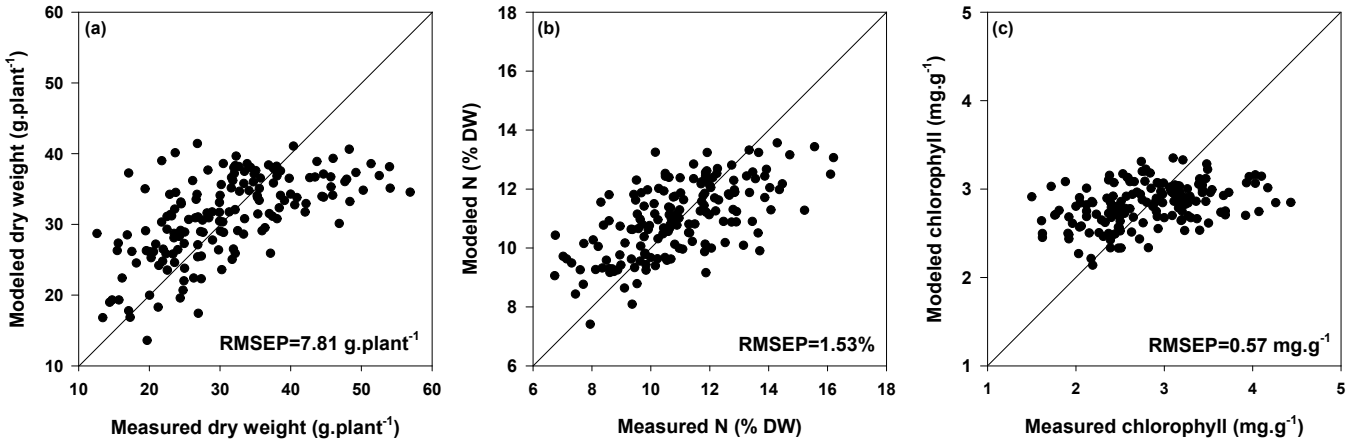


Fig 2. Modeled versus measured dry weight, crude protein and chlorophyll content when fitting partial least square regression models to relate each biophysical characteristic to digital image analysis of 200 *Urochloa* hybrids.



572 **Fig 3.** Modeled versus measured dry weight, crude protein and chlorophyll content when fitting
573 partial least square regression models to relate each biophysical characteristic to canopy spectral
574 reflectance of 200 *Urochloa* hybrids.

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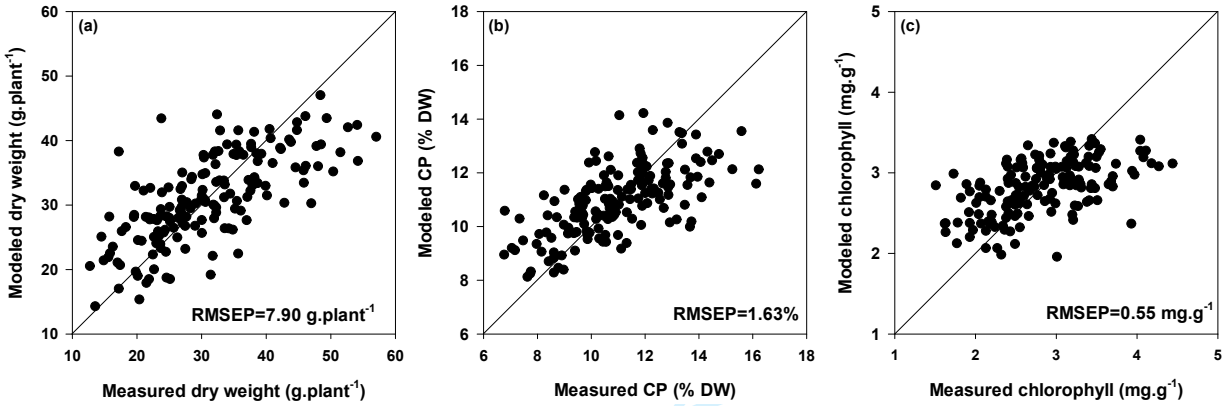

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587 **Fig 4.** Regression coefficients of the fitted partial least squares regression models for dry weight,
588 crude protein and chlorophyll content. The regression coefficients represents the contribution of
589 each spectral waveband to the overall prediction of each destructively-measured trait.

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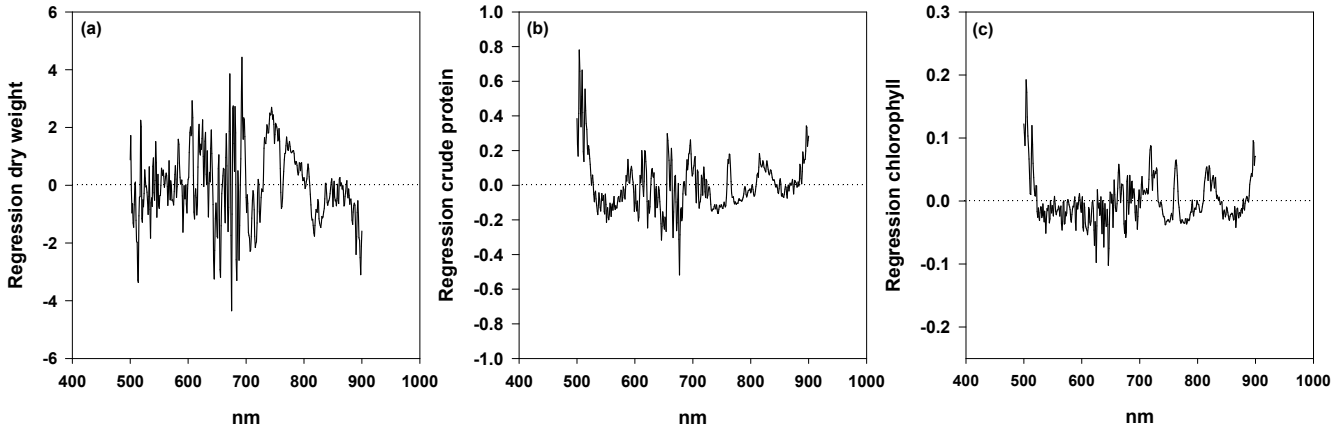

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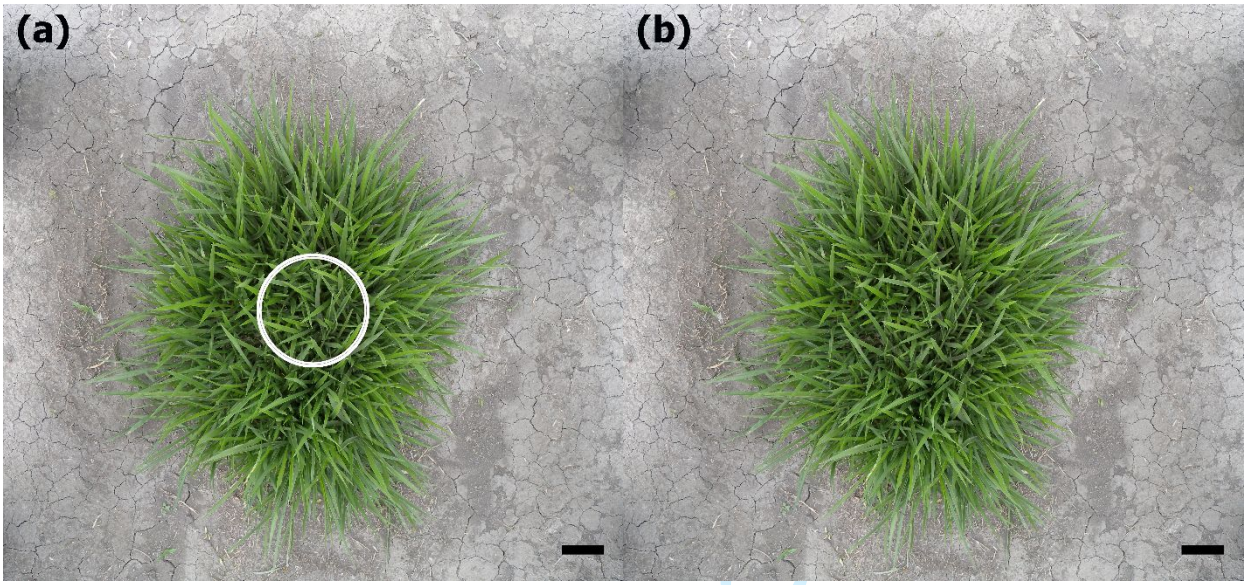
Supplementary information

Supplementary Table 1. Different protocols of spectral data collection and their respective root mean squared error of prediction (RMSEP) for crude protein, dry weight and chlorophyll content.

Collection	Trait	Factors¥	RMSEP
Day 1*	Dry weight (g plant^{-1})	4	9.23
	Crude protein (%)	11	1.29
	Chlorophyll (mg g FW)	4	0.49
Day 2*	Dry weight (g plant^{-1})	4	8.20
	Crude protein (%)	10	1.26
	Chlorophyll (mg g FW)	7	0.50
Day 3**	Dry weight (g plant^{-1})	4	7.63
	Crude protein (%)	11	2.07
	Chlorophyll (mg g FW)	5	0.54
Day 4**	Dry weight (g plant^{-1})	6	8.14
	Crude protein n (%)	2	1.21
	Chlorophyll (mg g FW)	3	0.58
All days	Dry weight (g plant^{-1})	6	7.90
	Crude protein (%)	5	1.63
	Chlorophyll (mg g FW)	5	0.55

Fifty plants were evaluated daily * One scan collected per plant. ** Ten scans collected per plant. ¥ Number of factors for which the root mean squared error of prediction was minimized in the model prediction.

626 **Supplementary Fig 1**. Schematic representation of the observation geometry of hyperspectral
627 analysis (a) and digital image analysis (b) techniques evaluated in 200 *Urochloa* hybrids. White
628 circle positioned at the center of the plant canopy in figure (a) represents the 23-cm field of view
629 of the spectroradiometer at a distance of 50mm from the plant canopy. For the digital image
630 analysis (figure b), the whole plant, and not the 23-cm section, was used for segmentation and
631 further analysis. Scale bar= 10 cm.



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642 **Supplementary Fig 2**. Binary relationships and Pearson's correlation coefficients between seven
643 plant traits extracted from digital images of 200 *Urochloa* hybrids. CC= canopy cover, NGRDI=
644 normalized green red difference index, ExG= excess green index, ExR= excess red index, ExGR=
645 excess green minus excess red, GR= green ration and GLI= green leaf index. Pearson's correlation
646 coefficients are indicated with their statistical significance as follows: *$P \leq 0.1$, **$P \leq 0.01$,
647 ***$P \leq 0.001$.
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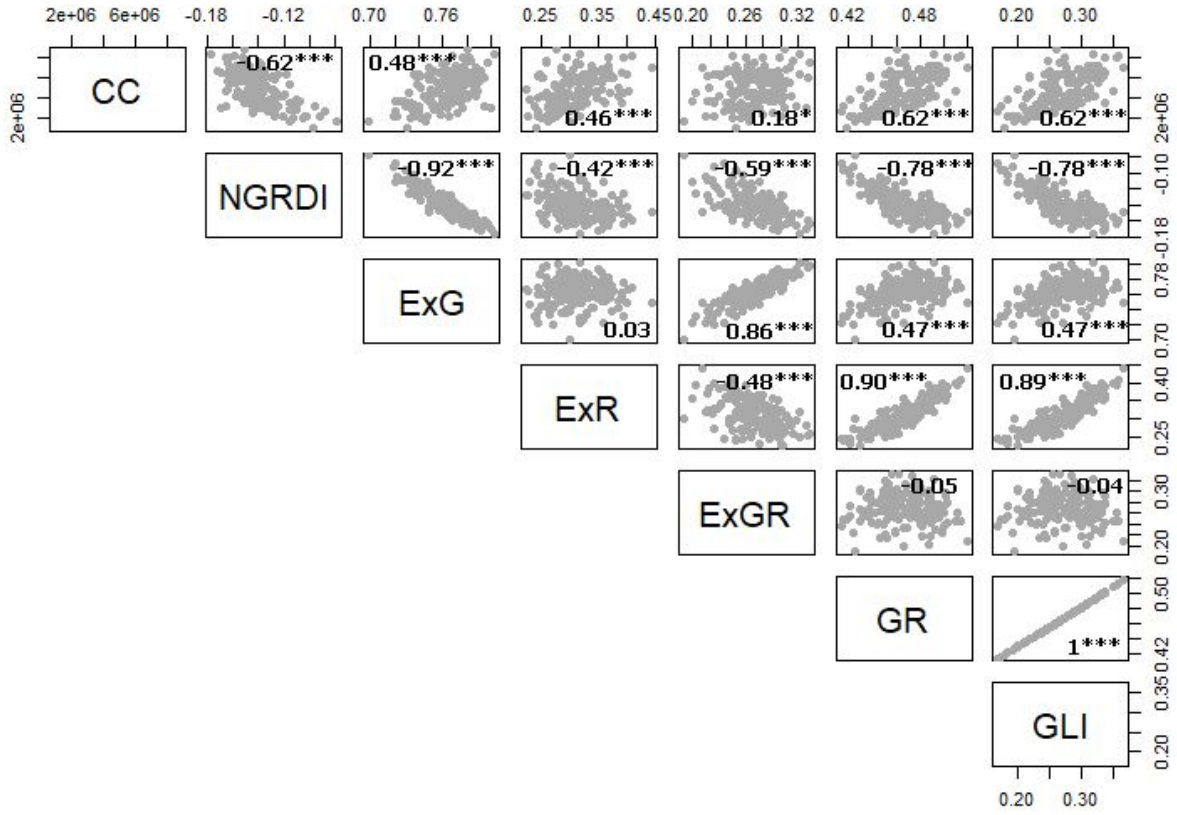

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Proximal sensing of *Urochloa* grasses increases selection accuracy

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Abstract

In the American Tropics, livestock production is highly restricted by forage availability. In addition, the breeding and development of new forage varieties with outstanding yield and high nutritional quality is often limited by a lack of resources and poor technology. Non-destructive high throughput phenotyping offers a rapid and economical means to evaluate large numbers of genotypes. In this study, visual assessments, digital color images, and spectral reflectance data were collected from 200 *Urochloa* hybrids in a field setting. Partial least squares regression (PLSR) was applied to relate visual assessments, ~~vegetation indices~~digital image analysis and spectral data with shoot dry weight~~, nitrogen (N) content~~ (DW), crude protein (CP) and chlorophyll content. Visual evaluations of biomass and greenness, digital color imaging, and hyperspectral canopy data were collected in 68, 40 and 80 minutes, respectively. Root mean squared errors of prediction for PLSR estimations of ~~dry weight, N~~DW, CP, and chlorophyll were lower for ~~vegetation indices~~ digital image analysis followed by hyperspectral analysis and visual assessments. This study showed that digital color image and spectral analysis techniques have the potential to improve precision and reduce time for tropical forage grass phenotyping.

Keywords: High throughput phenotyping, *Urochloa*, tropical forage grasses, plant breeding.

Introduction

Livestock productivity depends on forage availability and quality. Grasses from the *Urochloa* (syn. *Brachiaria*) genus have been widely planted in the tropics as forage for grazing ruminant livestock and are considered the most important forages in the American Tropics (Miles et al. 2004). The International Center for Tropical Agriculture (CIAT) in Colombia conducts a *Urochloa* breeding

program aimed at developing hybrids with outstanding performance on infertile, acidic soils with superior forage productivity and nutritional quality. The hybrid development process is difficult and time consuming. In a regular, three-year breeding cycle ~~(three years),~~ over 7000 hybrids are produced by open pollination, but fewer than 2% of these are retained for full evaluation. Approximately half of the population is discarded based on their reproductive mode (sexual ~~or~~genotypes are discarded and apomictic hybrids are kept); another major proportion is discarded based on visual evaluations; and only a limited number of hybrids (approximately 100) are finally evaluated for different biotic and abiotic stresses (Valheria Castiblanco, personal communication). The evaluation of genotypes is restricted mainly by insufficient economic resources and ~~lacking~~ technology for rapid screening.

~~Periodic~~Forage grasses exhibiting great biomass production and high nutritional quality are key determinants of the productivity of grazing animals (Herrero et al., 2013). Therefore, evaluations of shoot biomass production and quality parameters (i.e. crude protein) are among the most important traits for improvement in any forage grasses breeding program. However, owing to the destructive nature of these measurements and the insufficient economic resources, the evaluation of these parameters is postponed to final stages of the breeding program characterized by a reduced number of genotypes. Instead of analytical measurements of forage quality and destructive biomass harvests, periodic visual evaluations of plant performance (i.e., plant biomass and greenness) over time ~~has been~~is traditionally used in ~~the CIAT's~~ *Urochloa* breeding ~~program~~programs to select superior plants at initial stages of the breeding scheme (Miles et al. 2004; Miles 2007). These visual evaluations are laborious and may not be sufficiently accurate especially in breeding populations characterized by high genetic diversity and substantial genotype x environment interaction (Walter et al. 2012). ~~In this sense, the~~

The use of new technologies for in-field non-destructive, high throughput phenotyping (HTP), including digital image analysis and proximal hyperspectral sensing, ~~represents~~offers the possibility to precisely evaluate a larger number of genotypes than feasible in traditional ways, achieved at low cost, and implemented in a short period of time (Montes et al. 2007; White et al. 2012; Andrade-Sanchez et al. 2014).

Proximal hyperspectral sensing provides continuous information along the visual and near-infrared electromagnetic spectrum. This information often relates to plant traits and has successfully been studied in grasses to estimate quality parameters (Skidmore et al. 2010; Pullanagari et al. 2012; Thulin et al. 2012; Ferner et al. 2015; Safari et al. 2016), diversity (Lopatin et al. 2017) and nutrient content (Fava et al. 2009; Knox et al. 2012; Ramoelo et al. 2013; Adjorlolo et al. 2015; Foster et al. 2017). Likewise, plant image analysis for phenotyping purposes is based on image segmentation to separate the soil background ~~(i.e., soil)~~ and the plant for further quantification of regions of interest (Tucker 1979; Woebbecke et al. 1995; Camargo 2004; Hunt et al. 2005). Digital image analysis has also been used for quantifying vegetation indices related to plant growth, greenness and nutritional status (Meyer and Camargo 2008; Hunt et al. 2013). Very few reports of hyperspectral (Numata et al. 2008) or image analysis of *Urochloa* grasses exist in literature (Jimenez et al. 2017).

No studies combining hyperspectral information and image analyses and comparing them to conventional phenotyping methods is available. Moreover, hyperspectral data have not been used to evaluate target traits in *Urochloa* breeding programs. In this study, in-field visual evaluations, proximal hyperspectral data, and digital imaging were collected over canopies of *Urochloa* hybrids. Partial least squares regression ~~(PLSR)~~ was used to relate hyperspectral information to field measurements and machine learning (i.e. naive Bayes multiclass) was used to extract

vegetation indices from overhead canopy images. The objectives of this study were to: 1) develop PLSR models for predicting ~~N~~CP, forage ~~dry weight~~DW, and chlorophyll content; 2) ~~compute vegetation indices~~extract plant traits from digital image analysis to relate with ~~N~~CP, forage ~~dry weight~~DW, and chlorophyll; and 3) demonstrate the superiority of HTP techniques as compared to conventional visual evaluation of traits. ~~Hyperspectral data or image analysis have not been used to evaluate forage biomass, N or chlorophyll in *Urochloa* breeding programs. Moreover, no studies combining hyperspectral information and image analysis and comparing it to conventional phenotyping methods is available. Nitrogen, forage dry weight~~Crude protein, forage DW, and chlorophyll content were chosen as target traits in this study as they are key parameters determining both plant and cattle productivity. The development of HTP methodologies to evaluate tropical forages will increase the number of hybrids evaluated per selection cycle, thus permitting more intense selection and hence, genetic gain. The identification of new hybrids with outstanding performance (i.e. higher biomass, greener and high ~~N content~~CP) will result in more productive pastures with concomitant increases in milk and meat production in livestock systems in tropical savannahs.

Materials and methods

Field experiment

Field data were obtained in August 2016 at the International Center for Tropical Agriculture (CIAT) in Cali, Colombia (Lat. 3° 29' N; Long. 76° 21' W; altitude 965 m). Four thousand *Urochloa* hybrids generated from crosses between the CIAT's *Urochloa* breeding program population SX12 and *U. decumbens* cv. Basilisk (CIAT 606) were initially planted in an andisol soil in an augmented block design and spaced at 1.5x1.5 m. These plants were visually evaluated four times (data not shown) for persistence, vigor and greenness after sequential cuttings every

three months for one year. After that period, 200 hybrids were randomly selected for further visual and HTP analysis. These 200 hybrids ~~(and not~~, instead of the entire population~~)~~, were selected for economic and practical reasons. Visual evaluations of biomass and greenness, imaging and spectra collection were performed after 3 months ~~regrowth~~re-growth after cutting (see information below). Plant heights ranged from 20 to 50 cm and shoot architecture varied ~~from very prostrate to~~with both decumbent and erectus growth.

Visual evaluation

Plant biomass was assessed using a nine-point visual scale, where level '9' indicated high shoot biomass with many tillers and leaves while level '1' indicated stunted growth with fewer tillers and leaves. Plant greenness was visually evaluated using a five-point visual scale, where level '5' represented intense dark green in all the leaves of the plant and level '1' indicated yellow-pale color in all leaves of the plant. This visual evaluation was conducted in 68 minutes one week before the HTP measurements (Table 1).

Imaging collection and analysis

Individual, digital color images for each of the 200 hybrids were taken at 1.2 m above the soil surface using a commercial digital 13-Megapixel camera (Coolpix P6000, Nikon, Japan) fixed to a buggy tractor. Digital images were saved in 4224 x 3168 pixel JPG format ~~and vegetation indices were analyzed~~. The canopy cover (CC) and six vegetation indices including the normalized green red difference index (NGRDI), excess green index (ExG), excess red index (ExR), excess green minus excess red (ExGR), green ratio (GR) and green leaf index (GLI) were created using the formulae as indicated in Table 2. The canopy cover was extracted by dividing the total number of pixels representing the plant by the total number of pixels in each image. The vegetation indices were extracted using naive Bayes multiclass. Briefly, the distribution of colors in a set of digital color images (training set) was used to estimate the probability density function for each of the

different region of interest (i.e. plant and background). Once the regions of interest were defined~~,~~ in the training set, the machine learning process was applied to all images to accurately classify and separate regions of interest~~; therefore~~. Therefore, every ~~new~~ pixel in an image was classified into the previously defined plant and background classes. Every pixel characterizing the plant (but not the background) was then decomposed into red~~,~~ (R), green~~,~~ (G), and blue (~~RGB~~B) channels ~~for~~ . These channels were then normalized as follows:

$$r = \frac{R}{R+G+B}; g = \frac{G}{R+G+B}; b = \frac{B}{R+G+B}$$

Normalization makes the variations of light intensities uniform across the spectral distribution, thus, the individual color components (i.e. r,g,b) are independent from the overall brightness of the image (Cheng et al. 2011). Normalized channels were further used for the quantification of the vegetation indices~~. Seven vegetation indices including canopy cover, normalized red green difference index (Tucker 1979), excess green index (Woebbecke et al. 1995), excess red index (Meyer et al. 1998), excess green minus excess red (Camargo 2004), green ratio, and green leaf index (Louhaichi et al. 2001) were calculated.~~ (Table 2). Image analysis code was written in Java and run in ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Images were ~~collecting~~collected early in the morning to avoid beam solar radiation interferences. Digital images contained the whole plant in addition to the 23-cm diameter field-of-view (as indicated below for hyperspectral measurements, Supplementary Fig 1). The collection process took 40 minutes (Table 1).

Spectral collection and analysis

Hyperspectral field data collections were performed on clear days at full sun exposure around 11 am by positioning a hand-held field spectroradiometer (Fieldspec 2, Malvern Panalytical, Malvern, UK) directly above the plant canopy. The instrument was used with no foreoptics, which provided a 25-degree full conical angle field-of-view. To avoid soil background noise, the bare optical input was positioned at 50 cm from the top of the plant canopy to yield a 23-cm diameter field of view. The instrument collected information in 750 narrow wavebands from 325 to 1075 nm in 1 nm intervals. One or ten spectral scans were collected per plant and 50 plants were evaluated daily in about 20 minutes. Differences in the collection protocols were ~~deliberately done for comparison purposes.~~ tested to evaluate the most effective way. Different spectra collection ~~paradigms~~processes (1 or 10 scans) did not yield significant differences in the root mean squared error of prediction for the different traits evaluated (Supplementary ~~Fig~~Table 1). Radiometric collections over a 99% Spectralon panel (Labsphere, Inc., North Sutton, New Hampshire) were used to describe incoming solar irradiance throughout the data collection process. The radiometric collections over the calibration panel were made before starting and after every five canopy scans or when slight changes in solar irradiance due to cloud cover occurred. The values of the Spectralon panel radiance were used to compute the canopy reflectance of the plants in each wavelength over the time of spectra collection. Subsequently, 401 bands from 500 to 900 nm were used for analysis. Based on visual inspection of reflectance spectra, these bands were typically less noisy, as compared to bands at the bounds of detector sensitivity. Spectral collection process was run in 80 minutes (Table 1).

Laboratory sample collections

Plants were immediately harvested after spectra collection. Aboveground tissue was removed by cutting the area defined by a 23-cm diameter plastic circle co-located with the spectral data

collection area. Tissues were packed in plastic bags and stored on ice in a cooler in the field and then transported to the laboratory. The extraction of chlorophyll was performed by adding 100 mg of fresh tissue to 80% (v/v) cold methanol, and the mix was homogenized using a pestle in a mortar until the plant residue was clear and the solution was uniform. This solution was then filtered and absorbance was determined with a spectrophotometer (Synergy HT, Biotek, Winooski, USA). Total chlorophyll concentration was calculated according to Lichtenthaler and Welburn (1983). Dry weight (DW) was measured on an electronic balance (PB602S, Mettler Toledo, LLC, Columbus, OH, USA) after oven-drying the samples for three days at 60 °C. Nitrogen concentrations in the dry tissue were determined by using an automated nitrogen-carbon analyser (Sercon, Crewe, UK). *Urochloa* and common bean (*Phaseolus vulgaris*) leaves were used as reference tissues for confirmation of the reliability of the analyses. The crude protein content was calculated by multiplying nitrogen content with 6.25, as protein is assumed to contain 16% nitrogen on average.

Statistical analysis

Visual evaluations, ~~vegetation indices~~digital image analysis, spectral reflectance, and plant trait data were incorporated into a partial least squares regression (PLSR) algorithm (Mevik and Wehrens 2007) within the R Project for Statistical Computing (http://www.r-project.org~~)~~). Models were developed to ~~estimate~~predict each plant trait (i.e. CP, DW and chlorophyll) and to compare the precision for prediction of each of the different methods of phenotyping. Partial least squares regression was used in preference to conventional least squares analysis to reduce co-linearity effects. Thorp et al. (2011) provided the details on the PLSR methodology used in the present study. Briefly, if $\mathbf{Y}$ is an $n\times1$ vector of responses (i.e. ~~N, dry weight~~CP, DW or chlorophyll content)

and **X** is an *n*-observation by *p*-variable matrix of predictors (a set of visual evaluations, ~~vegetation indices~~digital image analysis, or spectral reflectance data), PLSR aims to decompose **X** into a set of *A* orthogonal scores such that the covariance with corresponding **Y** scores is maximized. The X-weight and Y-loading vectors that result from the decomposition are used to estimate the vector of regression coefficients, β_{PLS}, such that

$$\mathbf{Y} = \mathbf{X}\,\boldsymbol{\beta}_{\mathbf{PLS}} + \boldsymbol{\varepsilon}$$

where **ε** is an $n \times 1$ vector of error terms.

Leave-one-out cross validation was used to test model predictions for independent data. Results were reported for PLSR models with the number of factors that minimized the root mean squared error of cross validation. Pearson's correlation coefficients were calculated for the different traits extracted from digital color images taken from *Urochloa* hybrids.

Results

In this study, visual evaluations of biomass and greenness, digital color imaging and hyperspectral data were collected on 200 *Urochloa* hybrids in 68, 40 or 80 minutes, respectively (Table 1). High variability for the different characteristics of ~~dry weight, nitrogen~~DW, CP and chlorophyll content evaluated on 200 *Urochloa* hybrids was found (Table ~~2~~3).

Visual assessments

Partial least squares regressions for measured traits of DW, ~~N~~CP and chlorophyll ~~and~~based on visual evaluations of biomass and greenness performed with a root mean square error of prediction (RMSEP) of 8.47 g plant^{-1}, 1.76% and 0.60 mg g FW respectively (Fig 1).

Spectral data and digital image phenotyping

The PLSR models developed from ~~seven vegetation indices~~ the digital image analysis estimated DW, ~~N~~CP and chlorophyll with a RMSEP of 7.~~79~~81 g plant^{-1}, 1.53% and 0.57 mg g FW, respectively (Fig 2). Differences on the correlation coefficients among traits extracted from image analysis indicated that including different indices into the model added independent information to build stronger PLSR models (Supplementary Fig 2). The contribution of each trait extracted from digital image analysis to the overall prediction of each destructively-measured trait is shown in Table 4. The GLI had the stronger positive influence on the PLSR model for predicting DW. The ExGR had the stronger positive influence on the PLSR model for predicting both CP and chlorophyll content.

The fitted PLSR models developed from 401 wavebands of canopy spectral reflectance estimated DW, ~~N~~CP and chlorophyll with a RMSEP of 7.90 g plant^{-1}, 1.63% and 0.55 mg g FW, respectively (Fig 3).

The contribution of each spectral waveband to the overall prediction of each destructively-measured trait is shown in the Fig~~.~~ 4. In the PLSR model for DW, ~~three bands characterized the dry weight of *Urochloa*. Local~~local extrema in regression coefficients were found at ~~543, 668~~701 and ~~744~~674 nm, corresponding to ~~visible green light,~~ red light near the inflection band and ~~NIR radiation~~red light, respectively (Fig~~.~~ 4a). Strong positive contribution to ~~dry weight~~DW estimation were with ~~green light (543) and~~ NIR (~~744~~700-750), and a strong negative contribution with red light (~~668~~674-640). In the PLSR models for ~~N~~CP and chlorophyll, regression coefficient plots exhibited ~~a noisy pattern with less defined extrema.~~strong positive contribution for traits estimation in the visible green light (Fig 4b and c). The PLSR models for ~~N~~CP contrasted wavebands in the visible spectrum with positive contribution from wavebands around ~~513~~503 nm and negative contributions from wavebands at ~~676~~678 nm. ~~Wavebands at 600 nm and in the NIR contributed~~

~~less to the model for N (Fig. 4b). Regression~~Similarly, regression coefficients for total chlorophyll indicated strong positive contribution ~~from NIR wavelengths and strong~~ in the visible spectrum around 504 nm and negative contribution throughout the visible wavebands, especially ~~from 525 to~~at 625 ~~nm,~~ and ~~at the red edge at 705~~643 nm (Fig~~.~~ 4c). This is sensible considering visible light absorption is increased with additional leaf chlorophyll. ~~In the PLSR model for chlorophyll, local extrema in regression coefficients were found at 567, 674, 705 and 763 nm, which correspond to green light at the edge of yellow, red light, red light near the red inflection band and NIR radiation.~~

Discussion

The results from this study demonstrate that the current visual assessment methodology at initial steps of the breeding cycle in the CIAT *Urochloa* breeding program can be improved ~~by the use of~~using non-destructive ~~high throughput phenotyping~~HTP techniques. ~~The use of color~~Color imaging, hyperspectral analysis, and PLSR models ~~is~~are more precise and faster than visual evaluations, thus increasing the number of plants evaluated in the tropical forage breeding program.

Visual evaluations of plant growth and greenness (characteristics associated with N content, and therefore CP and chlorophyll concentration in leaves) have traditionally been used to discard *Urochloa* hybrids at initial stages of plant phenotyping. The visual evaluation of an entire breeding population (i.e., ~~4000~~7,000 hybrids) is a slow, costly and tedious process, and is often biased by subjectivity and human fatigue, especially when phenotypic variation of such traits is high (Table ~~2~~3). In this study, the estimation of DW, ~~N~~CP, and chlorophyll content was more precisely and consistently estimated by HTP techniques. Dry weight and ~~N~~CP predictions were more accurate using ~~vegetation indices~~digital image analysis, followed by spectral analysis and visual evaluations. Chlorophyll content was better estimated by the analysis of 401 spectral wavebands,

followed by color image analysis and finally visual evaluations (Fig 1, 2 and 3). ~~Likewise, the~~The time required to run non-destructive HTP evaluations was considerably shorter by 28 minutes per 200 plants for color image analysis than visual evaluations, but longer by 12 minutes per 200 plants in hyperspectral than in visual evaluations (Table 1).

The moderate trends in the relationship between *Urochloa* canopy imaging and reflectance and measured DW, ~~N~~CP and chlorophyll may indicate that the method is not appropriate for very precise estimations of these traits. However, for breeding purposes where a large percentage of hybrids are discarded without detailed evaluation due to scarce resources, a difference in DW of 7.90 g plant^{-1} or a difference of 1.63% in the ~~N~~CP content of plants may be acceptable during initial stages of plant breeding. This moderate trend between *Urochloa* canopy analysis and measured traits in this study can be explained by dissimilarities in the ~~*Urochloa* genotypes~~ canopy architecture of the *Urochloa* genotypes (Numata et al. 2008), as well as different growth patterns during recovery from cutting. The further evaluation of breeding populations with contrasting canopy architecture will improve the accuracy of the PLSR model to predict the targeted traits. Nonetheless, by combining both digital image and hyperspectral analysis techniques, higher precision accuracy for DW, CP and Chlorophyll content can be achieved.

The vegetation indices (see ~~materials and methods~~Table 2) extracted from color images of 200 *Urochloa* hybrids were originally developed to ~~extract~~separate green plants from the background by extracting green and red colors from ~~the image data to estimate~~digital images. These indices have been related to different plant characteristics including biomass, chlorophyll content and ~~the~~ nutritional status ~~of plants~~ (Tucker 1979; Woebbecke et al. 1995; Camargo 2004; Hunt et al. 2005; Meyer and Camargo 2008; Hunt et al. 2013; Lee and Lee 2013; Wang et al. 2013). ~~-~~In this study, ~~vegetation indices~~ digital image analysis performed better than hyperspectral scanning analysis to

estimate DW and ~~N~~NCP (Fig 2 and 3). Nonetheless, the use of spectral analysis over grasses becomes more important when this technique is used to detect either nutritional or anti-nutritional compounds (i.e. ~~protein~~metabolisable energy, digestibility, fiber) that are better estimated with the near-infrared regions of the electromagnetic spectra (Curran 1989; Pullanagari et al. 2012; Ferner et al. 2015). In this sense, the use of digital color image analysis and hyperspectral analysis is complementary because by using both techniques a diverse set of plant traits can accurately be predicted~~.~~ and by adding extra factors to the prediction model, higher prediction accuracy can be achieved (cf. Numata et al. 2008). Future efforts will use data mining to fine-tune the spectral bands included in the PLSR model (Thorp et al. 2017), which can reduce model error and improve model fit statistics. Although testing multiple methods of analysis was not the intention of this study, future research could also test other techniques (e.g., artificial neural networks) for relating HTP measurements to plant traits.

The regression coefficients for the PLSR for DW and chlorophyll content obtained in this study highlight that the key wavelengths for the prediction of these traits ~~were located~~occur in the green, red, red~~-~~-edge and NIR regions of the electromagnetic spectrum (Fig 4). Previous hyperspectral studies have highlighted those regions as being highly representative for dry mass and chlorophyll content in plants (Lichtenthaler et al. 1996; Thenkabail et al. 2000; Mutanga and Skidmore 2004; Fava et al. 2009; Thorp et al. 2011; Adjorlolo et al. 2015; Dou et al, 2018). Although some similarities were found between wavebands among the different traits, the general regression coefficients differed among the traits, thus demonstrating that the reflectance data in a given waveband contributed differently toward the estimation of a given trait. Given the logistical burden to collect and analyze hyperspectral scans, the identification of informative key bands associated with each evaluated trait can improve the HTP process (Thorp et al. 2017). Results from this study

will help guide selection of optimal bands in the construction of multispectral sensors tailored to predict specific traits of interest in tropical forage breeding programs.

The PLSR models for predicting DW, CP and chlorophyll content can be now used to evaluate the next generation of hybrids from the same *Urochloa* gene pool (i.e. *U. ruziziensis – U. brizantha – U. decumbens*). The accuracy of this prediction models relies on collection protocols similar to the explained in the Materials and Methods section and evaluations on plants with comparable growth characteristics as the hybrids evaluated here (i.e. about three months after regrowth). The prediction accuracy will likely be reduced on larger plants with higher biomass (Hill 2004) and a greater proportion of senescent leaves (Asner 1998). The development of more precise PLSR models to predict variables of interest in a breeding program requires an ongoing effort. The collection of ground data every year while making improvements to standardize collection protocols and incorporate wider range of genotypes will result in more accurate and robust models. Larger data sets will increase estimation precision.

Conclusions

In this study, 200 *Urochloa* hybrids were ~~successfully~~ monitored in 40 and 80 minutes by digital imaging and spectral analysis, respectively (Table 1). At this pace, more than 1000 *Urochloa* hybrids ~~can~~could be evaluated in a period of less than 7 hours. This means ~~more~~that forage biomass and quality in a high number of genotypes ~~could~~would be reliably evaluated with minimal increased acquisition costs ~~(compared~~relative to destructive harvest~~)~~. This demonstrates the superiority of HTP techniques as compared to conventional visual evaluation of traits. The PLSR models for predicting CP, forage DW, and chlorophyll content developed in this study supports

the evaluation of higher numbers of genotypes at initial stages of the breeding program. The greater ~~number~~numbers of plants evaluated reliably every year in the *Urochloa* breeding program, the greater the genetic gain will be. Therefore, the use of image analysis and hyperspectral monitoring over *Urochloa* hybrids canopies will benefit the on-going breeding program. ~~Likewise, the~~The application of this ~~methodology~~HTP method could be of great help in rural remote areas ~~without appropriate~~lacking facilities to perform destructive harvest and plant chemical analysis. ~~Additional studies on *Urochloa* plants with contrasting architectures need to be performed to optimize PLSR models. Moreover, more careful field measurements over plants with similar regrowth capacity are required~~Research is underway to improve the ~~prediction models. Furthermore,~~utility of proximal sensing by considering a greater range of canopy architectural configurations and evaluating the potential to assess nutritional quality ~~traits,~~ including ~~protein~~characteristics such as metabolisable energy, fiber, digestibility ~~and non-digestible fractions of the forage (~~, lignin and cellulose~~) must be evaluated through proximal hyperspectral sensing to improve phenotyping~~ fractions in *Urochloa* grasses.

Conflict of interest

The authors have no conflicts of interest to declare.

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Table 1. Phenotyping techniques used in the present study, the time of evaluation, its application, advantages and disadvantages.

Phenotyping technique	Time of evaluation*	Applications	Advantages	Disadvantages
Visual evaluation	68 min	Visual observations of different plant characteristics	Easy operation, low cost, evaluations can be performed under diverse conditions and environments	Evaluation of low number of genotypes, evaluation subjected to human bias and fatigue
Image analysis	40 min	Quantification of canopy cover and vegetation indices in the visible electromagnetic spectrum	Easy operation, low cost, greater number of plants evaluated, determination of several vegetation and water indices	Changes in ambient light conditions limit calculation of vegetation indices, data analysis is moderately complex
Hyperspectral analysis	80 min	Canopy reflectance information in the visible and near infra-red regions of the electromagnetic spectrum. Information can be used to predict biochemical ~~compositions~~composition of plants	Moderately easy operation, greater number of plants evaluated, determination of nutritional and biochemical composition of leaf/canopy	Low solar radiation or cloudy days limit analysis, sensor and white reference calibration is frequently needed, data analysis is complex

* The time of evaluation refers to 200 *Urochloa* plants evaluated under the conditions of the present study.

Table 2. Canopy cover and vegetation indices calculated from digital images of 200 *Urochloa* hybrids. Vegetation indices were extracted using a naive Bayes multiclass machine learning approach. Indices were then incorporated into a PLSR model to predict crude protein, dry weight biomass and chlorophyll content.

Plant traits	Name	Formula*	Reference
CC**	Canopy cover	Nc/Nt	-
NGRDI	Normalized green red difference index	(g-r)/(g+r)	Hunt et al., 2005
ExG	Excess green index	2g-r-b	Woebbecke et al., 1995
ExR	Excess red index	1.3r-g	Meyer et al., 1998
ExGR	Excess green minus excess red	ExG-ExR	Camargo 2004
GR	Green ratio	g/(r+g+b)	Tucker 1979
GLI	Green leaf index	(2g-r-b)/(2g+r+b)	Louhaichi et al. 2001

*r, g and b denote the normalized pixel values of each channel on the RGB colour mode.
** No normalization was performed for the canopy cover quantification. Nc= total number of pixels representing the canopy, Nt= total number of pixels in the picture.

Table 3. Plant traits measured in 200 *Urochloa* hybrids.

Trait	Min	Max	Mean	CV (%)
Dry Weight (g plant^{-1})	6.74	64.1	30.22	34.81
~~Nitrogen~~Crude Protein (%)	6.76	21.58	11.23	19.68
Chlorophyll (mg g FW)	0.87	6.41	2.88	24.31

Table 4. Regression coefficients of the fitted partial least square regression models of seven traits extracted from digital image analysis. Positive and negative coefficients indicate positive and negative influence on the prediction model, respectively.

Traits*	Dry weigth (g.plant^{-1})	Crude protein (% DW)	Chlorophyll (mg.g^{-1})
CC	3.760545	-0.23114345	-0.01673706
NGRDI	9.948634	0.08600517	-0.03532585
ExG	-14.3163	0.07760486	0.0730642
ExR	-32.126212	-0.39106455	-0.07758547
ExGR	3.724492	0.26592971	0.10326555
GR	-34.770799	-0.31158671	-0.03624834
GLI	80.87152	-0.31108316	-0.0357783

* CC= canopy cover, NGRDI= normalized green red difference index, ExG= excess green index, ExR= excess red index, ExGR= excess green minus excess red, GR= green ratio and GLI= green leaf index.

584 **Fig 1**. Modeled versus measured dry weight, ~~nitrogen~~crude protein and chlorophyll content when
585 fitting partial least square regression models to relate each biophysical characteristic to visual
586 evaluations of biomass and greenness of 200 *Urochloa* hybrids.

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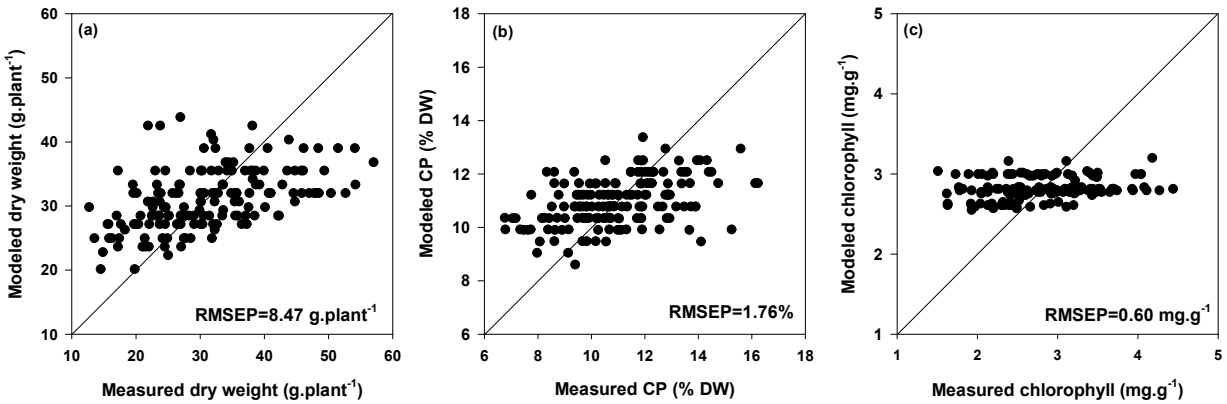

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Fig 2. Modeled versus measured dry weight, ~~nitrogen~~crude protein and chlorophyll content when fitting partial least square regression models to relate each biophysical characteristic to ~~vegetation indices~~digital image analysis of 200 *Urochloa* hybrids.

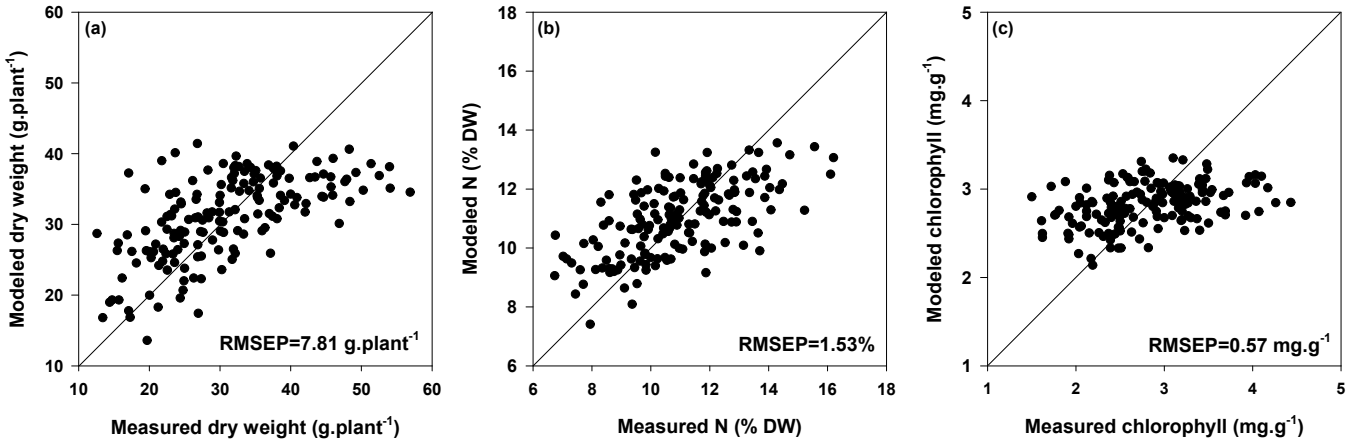


Fig 3. Modeled versus measured dry weight, ~~nitrogen~~crude protein and chlorophyll content when fitting partial least square regression models to relate each biophysical characteristic to canopy spectral reflectance of 200 *Urochloa* hybrids.

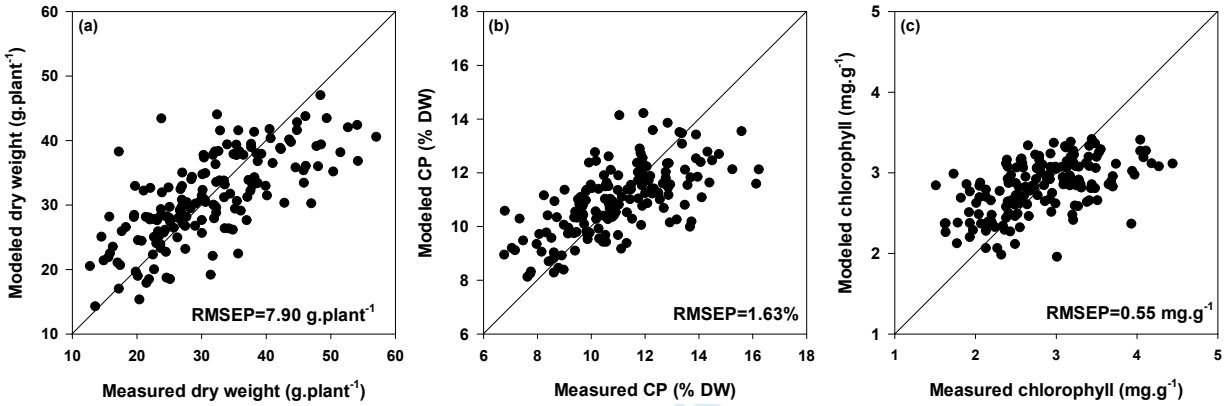
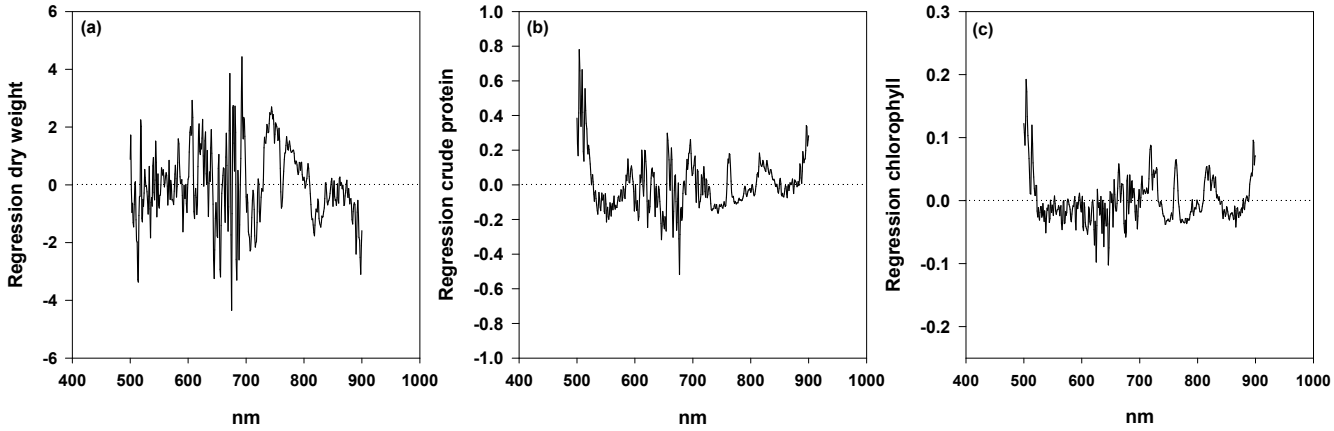


Fig 4. Regression coefficients of the fitted partial least squares regression models for dry weight, ~~nitrogen and chlorophyll content~~crude protein and chlorophyll content. The regression coefficients represents the contribution of each spectral waveband to the overall prediction of each destructively-measured trait.



Supplementary information

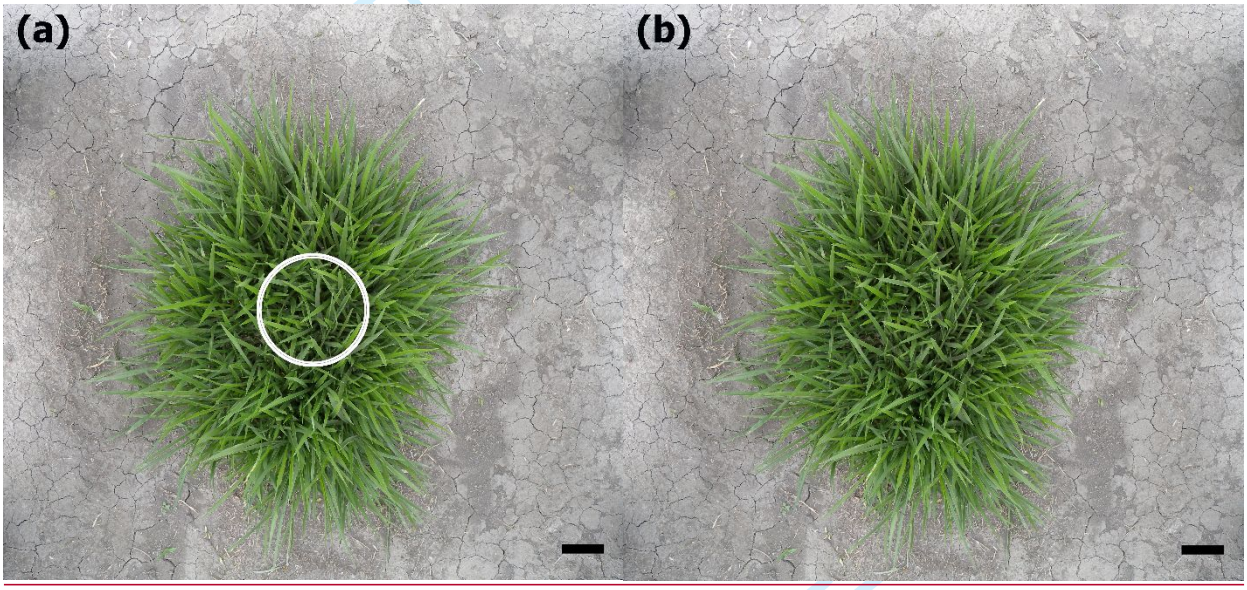
Supplementary Table 1. Different protocols of spectral data collection and their respective root mean squared error of prediction (RMSEP) for ~~nitrogen~~crude protein, dry weight and chlorophyll content.

Collection	Trait	Factors¥	RMSEP
Day 1*	Dry weight (g plant^{-1})	4	9.23
	~~Nitrogen~~Crude protein (%)	11	1.29
	Chlorophyll (mg g FW)	4	0.49
Day 2*	Dry weight (g plant^{-1})	4	8.20
	~~Nitrogen~~Crude protein (%)	10	1.26
	Chlorophyll (mg g FW)	7	0.50
Day 3**	Dry weight (g plant^{-1})	4	7.63
	~~Nitrogen~~Crude protein (%)	11	2.07
	Chlorophyll (mg g FW)	5	0.54
Day 4**	Dry weight (g plant^{-1})	6	8.14
	~~Nitrogen~~Crude protein n (%)	2	1.21
	Chlorophyll (mg g FW)	3	0.58
All days	Dry weight (g plant^{-1})	6	7.90
	~~Nitrogen~~Crude protein (%)	5	1.63
	Chlorophyll (mg g FW)	5	0.55

Fifty plants were evaluated daily * One scan collected per plant. ** Ten scans collected per plant. ¥ Number of factors for which the root mean squared error of ~~cross validation~~prediction was minimized in the model prediction.

671

672 **Supplementary Fig 1**. Schematic representation of the observation geometry of hyperspectral
673 analysis (a) and digital image analysis (b) techniques evaluated in 200 *Urochloa* hybrids. White
674 circle positioned at the center of the plant canopy in figure (a) represents the 23-cm field of view
675 of the spectroradiometer at a distance of 50mm from the plant canopy. For the digital image
676 analysis (figure b), the whole plant, and not the 23-cm section, was used for segmentation and
677 further analysis. Scale bar= 10 cm.



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Supplementary Fig 2. Binary relationships and Pearson's correlation coefficients between seven plant traits extracted from digital images of 200 *Urochloa* hybrids. CC= canopy cover, NGRDI= normalized green red difference index, ExG= excess green index, ExR= excess red index, ExGR= excess green minus excess red, GR= green ration and GLI= green leaf index. Pearson's correlation coefficients are indicated with their statistical significance as follows: *$P \leq 0.1$, **$P \leq 0.01$, ***$P \leq 0.001$.