

**Development of Advanced Phenomics Tools for
Molecular Breeding of Biomass Yield Improvement in
Perennial Ryegrass (*Lolium perenne*. L)**

Thesis submitted in total fulfilment of the requirements of the degree
of Doctor of Philosophy

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Bachelor of Plant Sciences

Master of Agriculture

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June 2020

School of Agriculture and Food

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Abstract

Increasing the biomass yield of perennial forage crops remains a crucial factor underpinning the profitability of grazing industries, and therefore is a priority for breeding programs. The rate of genetic gain for dry matter yield (DMY) in forage crops is likely to be increased with the development and application of genomic selection (GS) strategies. However, realising the full potential of GS will require an increase in the amount of phenotypic data and the rate at which it is collected. Phenotyping remains a critical bottleneck in the implementation of GS in forage species. Current assessment of DMY in forage crop breeding includes visual scores, sample clipping and mowing of plots, which are often costly and time-consuming. New ground and aerial-based platforms equipped with advanced sensors offer opportunities for fast, non-destructive and low-cost, high-throughput phenotyping (HTP) of plant growth, development and yield in a field environment.

This thesis aimed to develop, validate and deploy sensor-based non-destructive aerial, and ground-based HTP platforms to estimate DMY of perennial ryegrass plants in-field. Firstly, calibration and validation of aerial and ground based HTP platforms were developed to provide a non-destructive method to accurately estimate perennial ryegrass height and DMY. Secondly, the thesis compared a range of traits alone and in combination to demonstrate sensor based DMY estimation. Thirdly, this study demonstrated the application of combining normalised difference vegetative index (NDVI) from multispectral imaging and ultrasonic sonar estimates of plant height to estimate the seasonal distribution of DMY of 48,000 perennial ryegrass plants. Fourthly, the study discussed the application of developed platforms and data processing workflow which allows for an increase in the accuracy of genomic breeding values prediction in GS.

Calibration and validation results indicated that plant height measurements from ultrasonic sonar and light detection and ranging (LiDAR) sensors showed the potential to measure plant height with consistent repeatability under controlled conditions and in field trials. NDVI demonstrated the capability for non-destructive DMY estimation of individual ryegrass spaced planted and sward plots. However, DMY estimation from NDVI saturates as the biomass increases. The combination of plant height and NDVI was found to improve the DMY prediction accuracy by up to 10%. This was achieved by combining NDVI and plant

height with a simple multiplication combination (NDVI multiply by plant height), with the possibility to further improve the accuracy by combining the parameters in different models.

This thesis assesses further the feasibility of combining NDVI from multispectral imaging and ultrasonic sonar estimates of plant height to estimate DMV of single plants in a large perennial ryegrass breeding program. Results demonstrated that the best prediction models of DMV of spaced-planted perennial ryegrass plants come from the multiplicative combination of NDVI and plant height (NDVIsq_PH). The K-fold and random split cross-validation findings imply that the combination of NDVI and plant height improved prediction accuracy over the use of NDVI and plant height alone. This yielded an accuracy of the coefficient determination of DMV estimation of more than 0.63 and root mean square error (RMSE) for fresh herbage yield, and dry herbage yield was less than 33 gram per plant and 8 gram per plant, respectively across multiple growing seasons.

Therefore, to assess the application of combining NDVI and plant height for accurate DMV, a computational workflow was developed for image acquisition, data processing and analysis of spaced-planted perennial ryegrass plants. Fifty advanced breeding lines and commercial cultivars represented by a total of 48,000 individual plants were used to develop and validate the computation workflow for DMV prediction across three growing seasons. Combining NDVI and plant height of individual plants was a robust method to enable HTP of DMV estimation in a large population of ryegrass breeding. Similarly, the plot-level model indicated good to high-correlation between the predicted and measured DMV across three seasons with coefficient determination between 0.19- 0.81 and root RMSE values ranging from 0.09-0.21 kilogram per plot. The model was further validated using a combined regression of the three seasonal harvests. This study will have a significant contribution to the wide application of sensor technologies in forage research and plant breeding.

Keywords: High-throughput phenotyping; biomass yield; perennial ryegrass breeding; computational workflow; sensor technologies

Declaration

This is to certify that:

- (i) The thesis comprises only my original work towards the PhD except where indicated in the preface
- (ii) All photographs and illustrations are my original work except where indicated in the text
- (iii) Due acknowledgement has been made in the text to all other material used
- (iv) The thesis is fewer than 100,000 words in length, exclusive of tables, figures and bibliographies

Alem Gebremedhin Gebre

June 2020

List of Publications Included in the Thesis

List of Published Papers

Gebremedhin, A.; Badenhorst, P.E.; Wang, J.; Spangenberg, G.C.; Smith, K.F. (2019) Prospects for measurement of dry matter yield in forage breeding programs using sensor technologies. *Agronomy* **9**, 65.

Gebremedhin, A.; Badenhorst, P.; Wang, J.; Giri, K.; Spangenberg, G.; Smith, K. (2019) Development and validation of a model to combine NDVI and plant height for high-throughput phenotyping of herbage yield in a perennial ryegrass breeding program. *Remote Sensing* **11**, 2494.

Gebremedhin, A.; Badenhorst, P.; Wang, J.; Shi, F.; Breen, E.; Giri, K.; Spangenberg, G.; Smith, K. (2020) Development and validation of a phenotyping computational workflow to predict the biomass yield of a large perennial ryegrass breeding field trial. *Frontiers in Plant Science* **11**, 689

List of Posters Presented in International Conferences

Gebremedhin, A.; Badenhorst, P.; Wang, Noel Cogan, F.; Spangenberg, G.; Smith, K. (2019) Combining NDVI and plant height as a proxy to enable high-throughput phenotyping of herbage yield in perennial ryegrass. International Forage and Turf Breeding Conference. March 23-27, 2019, Orlando, Florida, The United States of America

Declaration for Thesis with Publications

Co-authors of the published papers (Chapters 2, 4 and 5) have been completed the co-author's authorisation form to be included in this thesis. The PhD candidate has contributed for more than 80% to the planning, execution and preparation of the published and unpublished chapters. All authors have certified the student's contribution for more than 50% of the planning, execution and preparation of the published papers. The candidate's advisory committee and principal supervisor have approved the inclusion of the published works in this thesis.



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Alem Gebremedhin Gebre	Enter signature here	28/01/2020

PRINCIPAL SUPERVISOR'S DECLARATION

Supervisor's name	Supervisor's signature	Date (dd/mm/yy)
Kevin Smith	Enter signature here	28/01/2020

B. PUBLICATION DETAILS (to be completed by the student)

Click on this box and on the “+” button in the bottom right corner to enter multiple publications.

Full title	Prospects for measurement of dry matter yield in forage breeding programs using sensor technologies		
Authors	Alem Gebremedhin, Pieter Badenhorst, Junping Wang, German Spangenberg and Kevin Smith		
Student's contribution (%)	80%	Volume/page numbers	9(2) 65
Journal or book name	Agronomy Journal		
Status	☐ Accepted and In-press ☒ Published ☐ In progress		Date accepted/ published 01/02/2019

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Authors	Alem Gebremedhin, Pieter Badenhorst, Junping Wang, Khageswor Giri, German Spangenberg and Kevin Smith		
Student's contribution (%)	80%	Volume/page numbers	11(2494)
Journal or book name	Remote Sensing Journal		
Status	☐ Accepted and In-press ☒ Published ☐ In progress		Date accepted/ published 25/10/2019



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Supervisor's name	Supervisor's signature	Date (dd/mm/yy)
Kevin Smith	<i>Kevin Smith</i>	23/06/2020

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Student's contribution (%)	70%	Volume/page numbers	11/ 689
Journal or book name	Frontiers in Plant Science		
Status	☐ Accepted and In-press ☒ Published ☐ In progress		Date accepted/ published 28/05/2020

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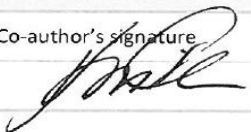
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Student's contribution (%)	80		
Journal or book name	Agronomy Journal		
Volume/page numbers	Volume 9 (6)/pages 19		
Status	☐ Accepted and In-press ☐ In progress	☒ Published	Date accepted/published 31/01/2019

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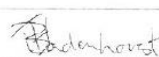
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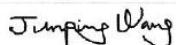
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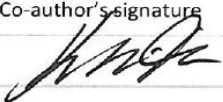
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Authors	Alem Gebremedhin, Pieter Badenhorst, Junping Wang, Khageswor Giri, German Spangenberg and Kevin Smith		
Student's contribution (%)	80		
Journal or book name	Remote Sensing		
Volume/page numbers	Volume 11(2494)/pages 19		
Status	☐ Accepted and In-press ☐ In progress	☒ Published	Date accepted/published 24/10/2019

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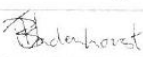
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Student's contribution (%) 80

Journal or book name Remote Sensing

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	☐ In progress		24/10/2019

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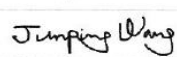
A. PUBLICATION DETAILS (to be completed by the student)

Full title	Development and validation of a model to combine NDVI and plant height for high-throughput phenotyping of herbage yield in a perennial ryegrass breeding program		
Authors	Alem Gebremedhin, Pieter Badenhorst, Junping Wang, Khageswor Giri, German Spangenberg and Kevin Smith		
Student's contribution (%)	80		
Journal or book name	Remote Sensing		
Volume/page numbers	Volume 11(2494)/pages 19		
Status	☐ Accepted and In-press ☐ In progress	☒ Published	Date accepted/published 24/10/2019

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Student's contribution (%)	70		
Journal or book name	Frontiers in Plant Science		
Volume/page numbers	Volume 11/pages 689.		
Status	☐ Accepted and In-press	☒ Published	Date accepted/published
	☐ In progress		28/05/2020

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German C. Spangenberg		22/6/2020



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Co-author's name	Co-author's signature	Date (dd/mm/yy)
Khageswor Giri		22/06/2020

Acknowledgements

This thesis has been realised with the support of the following people and organisations:

I would like to express my sincere gratitude to my main supervisor **Prof. Kevin Smith** for providing me with the opportunity to do my PhD under his supervision through The University of Melbourne and Agriculture Victoria Research-Hamilton Centre. **Prof. Smith** was extremely enthusiastic and supportive throughout the PhD process, not only academically but also emotionally. I am grateful for his consistent guidance, constructive criticisms and encouragement to maintain my academic passion and devotion. I would like to express my sincere gratitude to my co-supervisor **Dr. Pieter Badenhorst** for his unreserved technical and scientific support throughout the phenomics data analysis. **Dr Badenhorst** has always been open for constructive criticism and supported me to understand sensor technologies and application for plant phenotyping. I would also like to express my appreciation to my co-supervisor **Dr. Junping Wang**, for her consistent help and supervision. Our discussion was always fruitful, and your critical comments were always constructive and helpful. I would also like to express my appreciation to my co-supervisor **Prof. German Spangenberg**, for sharing his expertise and experiences.

My special acknowledgement also goes to the members of the Molecular Plant Breeding -Agriculture Victoria Research (AVR) staff at Hamilton and AgriBio for their precious support. All staff members including **Andrew Phelan, Carly Elliott, Darren Pickett, Jess Frankel, Peter Hardy, Greg Mason, Micaela Murray, Russel Elton** and **Darren Kean** introduced me to the perennial ryegrass breeding world, teach me how to deal with plant processing and data collection activities. **Andrew Phelan, Carly Elliott** played a great role, in doing all the flights and technical support. My acknowledgement also goes to **Dr. Fan Shi** and **Dr. Ed Breen** of the computational biology staff members, at AgriBio for their provision of the technical and professional assistance. I would like to appreciate another staff from AgriBio, Dr. Khageswor Giri, for his unreserved assistance in the statistical analysis process.

I would like to appreciate my fellow PhD students **Chinthaka Jayasinghe, Chaya Smith** and **Phat Nguyen** for all the discussion and debates throughout the PhD project. All of

you were a wonderful crew which leave me with amazing memories, and I look forward to continued engagements throughout our careers.

In three and a half years in Hamilton, I have met a lot of amazing people. **Steve Clark** (retired Research Scientist at AVR) was supportive from the start of my arrival to the end of my PhD study. **Steve** was an amazing person for providing me with accommodation at my arrival, and he was there in the highs and lows of my stay. He was also there for discussions, raising ideas and debates. Thank you so much for being a wonderful advisor, not only by providing a research assistantship but also by offering emotional support.

I would like to thank the DairyBio initiative program and The University of Melbourne, for financial support. The DairyBio initiative program supported me a lot through the DairyBio mentorship program where they set me up with a dairy farmer, and the experience was amazing.

To my family, thank you for instilling me a passion for education at a young age, and the derive to achieve the best. Particularly to my lovely mother **Kidan Gebremedhin** (Adeway), thank you for raising me with disciples. To my brother and mentor, you have always supported me through University life and my research. Thank you for your endless support throughout my studies. Finally, special thanks go to my beautiful wife, **Dr. Hiwet Aregawi**, for being a fantastic and supportive throughout the PhD journey. You have been an amazing wife and a mother of our son.

List of Abbreviations and Symbols

AVR	Agriculture Victoria Research
BPR	Plant Breeders' Rights
CASA	Australian Civil Aviation Safety Authority
cm	Centimetre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CSV file	comma-separated values file
Cultivar ID	Cultivar identification number
CV	Cross validation
DHY	Dry herbage yield
DMY	Dry matter yield
DN	Digital number
DSM	Digital surface model
FHY	Fresh herbage yield
FOV	Field of view
FVI	Forage value indices
g/plant	Grams per plant
G×E	Genotype × environment interaction
GB-HTP	Ground-based, high-throughput phenotyping
GCP	Ground control point
GE	Genome editing
GEBVs	Genomic estimation breeding values
GIS	Geographic information system
GM	Genetic modification
GPS	Geographic positioning system
GRNVI	Green–red, normalised difference vegetative index
GS	Genomic selection
GSD	Ground sample distances
GSS	Genomic sub-selection

GWS	Geodetic world system
HSV	Hue, Saturation, Value
HTP	High-throughput phenotyping
HY	Herbage yield
kg/plot	Kilograms per plot
kHz	kilohertz
LiDAR	Light detection and ranging
LP F ₂	F ₂ generations of different breeding lines and commercial cultivars of perennial ryegrass
m	Metre
m ²	Square metre
MAE	Mean absolute error
MAS	Marker assisted selection
MIAPPE	Minimum information about a plant phenotyping experiment
ML	Machine learning
MLR	Multiple linear regression
mm	Millimetre
MPE	Mean percentage error
ms ⁻¹	Metre per second
NDVI	Normalised difference vegetative index
NDVI*height	Combining NDVI and plant height
NDVIsq_PH	Combining of the squared NDVI and plant height
NIR	Reflectance at near-infrared waveband
nm	Nanometre
NSW DPI	New South Wales Department of Primary Industries
Plant ID	Plant identification number
PLSR	Partial least squares regression
ppm	parts per million
QGIS	Quantitative geographic information system
QTLs	Quantitative trait loci

r	Pearson correlation coefficient
R	Reflectance at red waveband
R ²	Coefficient of determination
RF	Random forest
RGB	Red-green-blue
RMSE	Root mean square error
RTK-GNSS	Real time kinematics-Global navigation satellite system
SI	Self-incompatibility
SONAR	Sound navigation and ranging
SVM	Support vector machine
TIFF file	Tagged image file format file
UAS	Unmanned aerial system
USH	Ultrasonic sonar height
UTC	Universal time coordinated
UTM	Universal Transverse Mercator
VDA	Victoria Department of Agriculture
VI	Vegetative indices
Vic	Victoria, Australia
WSC	Water soluble carbohydrate
%	Percent
°C	Degree centigrade
2D	Two dimensions
3D	Three dimensions
Note	The following names have the same meaning in this thesis: dry herbage yield and dry matter yield

Table of Contents

Abstract	i
Declaration	iii
List of Publications Included in the Thesis	iv
Declaration for Thesis with Publications	vi
Acknowledgements	xix
List of Abbreviations and Symbols	xxvi
Table of Contents.....	xxix
List of Figures	xxxii
List of Tables.....	xxxviii
Appendices	xxxix
Chapter 1. Introduction to Ryegrass Biomass Yield Improvement.....	1
1.1 Introduction	1
1.2 History of Perennial Ryegrass Breeding	3
<i>1.2.1 Objectives of ryegrass breeding.....</i>	<i>5</i>
1.3 Breeding Approaches.....	8
<i>1.3.1 Traditional Breeding</i>	<i>8</i>
<i>1.3.2 Marker Assisted Selection.....</i>	<i>8</i>
<i>1.3.3 Genomic Selection</i>	<i>9</i>
<i>1.3.4 Genetic Modification and Genome Editing</i>	<i>9</i>
1.4 Phenomic Assessments of Perennial Ryegrass.....	10
1.5 Thesis Back Ground and Significance	10
1.6 Research Questions	14
1.7 Research Aims	14
1.8 Outline of the Thesis	15
1.9 References	18

Chapter 2. Prospects for Measurement of Dry Matter Yield in Forage Breeding Programs Using Sensor Technologies.....	32
Chapter 3. Calibration of High-Throughput, Non-Destructive Phenotyping Technologies for Rapid Biomass and Plant Height Measurement of Perennial Ryegrass.....	52
3.1 Introduction.....	53
3.2 Materials and Methods.....	55
3.2.1 <i>Description of the PhenoRover Platform.....</i>	<i>55</i>
3.2.1.1 PhenoRover Platform Data Processing.....	58
3.2.1.2 General Steps for Geo-referencing Data.....	58
3.2.2 <i>Description of the Handheld GreenSeeker NDVI Data Collection.....</i>	<i>59</i>
3.2.3 <i>Description of the Aerial Platform</i>	<i>60</i>
3.2.3.1 Data Processing from UAS.....	61
3.2.4 <i>Data Collection for Sensors and Platforms Calibration.....</i>	<i>61</i>
3.2.4.1 Controlled Environment Experiments	62
3.2.4.2 Field Experiments.....	63
3.3. Results	64
3.3.1 <i>Plant Height Estimation in the Controlled Environment.....</i>	<i>64</i>
3.3.2 <i>In-field Plant Height and Herbage Yield Phenotyping</i>	<i>67</i>
3.3.2.1 Height and Herbage Yield Estimation for Individual Plant	67
3.3.2.2 Sward Plots Height and Herbage Yield Estimation	70
3.4 Discussion.....	73
3.5 Conclusions.....	76
3.6 References.....	77
Chapter 4. Development and Validation of a Model to Combine NDVI and Plant Height for High-Throughput Phenotyping of Herbage Yield in a Perennial Ryegrass Breeding Program.....	82

Chapter 5. Development and Validation of a Phenotyping Computational Workflow to Predict the Biomass Yield of a Large Perennial Ryegrass Breeding Field Trial.....	102
Chapter 6. General Discussion	119
6.1 Overview of Research	119
6.2 Calibration and Validation of Sensors on Phenotyping Platforms	121
6.3 Application of Developed High-Throughput Phenotyping Technologies for Dry Matter Yield Estimation	122
6.4 Challenges of Perennial Ryegrass High-Throughput Phenotyping	124
6.5 Modeling and Big Data Analysis	126
6.6 Incorporation of Sensor-Based Phenotypic Data into Genomic Selection in Breeding	126
6.7 Applications in Private Industry	128
6.8 General Conclusion and Future Outlook	128
6.9 References	129
7. Appendices	137

List of Figures

Chapter 1

Figure 1 Schematic representation of ryegrass growth and nutritive quality changes following defoliation (adapted from Donaghy 1998).....	3
--	---

Chapter 2

Figure 1 An aerial image of perennial ryegrass genomic subselection field trial which comprises individual plants at Hamilton Centre, Victoria, Australia.....	35
---	----

Figure 2 The ground-based phenotyping platform named as “PhenoRover” assembled in Hamilton Centre, AVR for field phenotyping of perennial ryegrass. (A) Semiautomated ground vehicle named PhenoRover; (B) list of deployed sensors on PhenoRover.....	39
---	----

Figure 3 The aerial-based unmanned aerial system (UAS) phenotyping platform in Hamilton Centre, AVR for field phenotyping of perennial ryegrass. (A) List of various semiautomated UASs; (B) list of sensors deployed on UASs.....	40
---	----

Figure 4 Workflow indicating image acquisition, processing and analysis	42
--	----

Chapter 3

Figure 1 The PhenoRover is a high-throughput phenotyping platform developed and deployed in Agriculture Victoria Research, Hamilton Centre, Australia. Annotations outline the major components of the system.	57
--	----

Figure 2 Scanned graph representative display of individual plants measured using LMS 400 sick LiDAR.....	59
--	----

Figure 3 An aerial platform (3DR Solo) equipped with a Parrot Sequoia multispectral camera on board for HTP of perennial ryegrass plants deployed in Agriculture Victoria Research, Hamilton Centre, Australia.	60
Figure 4 Picture of foam bricks plants used to validate ultrasonic sonar (a); and the correlation between height derived from ultrasonic sonar sensor and a ruler (b); and LiDAR and a ruler (c).....	66
Figure 5 Picture of artificial plants used to validate ultrasonic sonar (a) and the correlation between artificial plants height derived from ultrasonic sonar sensor and a ruler (b)....	67
Figure 6 Pictures of the perennial ryegrass sampled plants in pots (a) and the correlation between pot planted perennial ryegrass height derived from ultrasonic sonar sensor and a ruler (b) and LiDAR sensor and a ruler (c)	67
Figure 7 Pearson correlation coefficients between fresh herbage yield (FHY) and dry herbage yield (DHY) and plant height from the ruler, ultrasonic sonar, LiDAR of the PhenoRover system and NDVI from UAS measured at the time of three-leaf stage perennial ryegrass individual plants harvest.....	69
Figure 8 Pearson correlation coefficients between fresh and dry herbage yield and combining plant height from a ruler, ultrasonic sonar, and LiDAR of the ground-based platforms and NDVI from UAS measured at the time of harvest	70
Figure 9 Comparisons of herbage yield estimation methods; a) Relationship between plot fresh herbage yield and plot mean NDVI; b) Relationship between plot fresh weight and plot mean plant height; c) Relationship between plot fresh herbage yield and plot mean combination of NDVI and plant height (NDVI*plant height); d) Relationship between plot dry herbage yield and plot mean NDVI; e) Relationship between plot dry herbage yield and plot mean plant height, and f) Relationship between plot dry herbage yield and plot mean combination of NDVI and plant height (NDVI*plant height).....	72

Chapter 4

Figure 1 Study site: the perennial ryegrass experiment was conducted at the Hamilton Centre Research Station in Victoria State of Australia in 2017 and 2018. NDVI tiff file is used to display the field trial site sampled plants and non-sampled plants throughout the experiment time. 86

Figure 2 Aerial and ground-based platforms for high-throughput phenotyping (a) UASs platform and sensors; (b) PhenoRover system and sensors at the experimental site. 87

Figure 3 Workflow diagram of image processing and plant height extraction used in the GSS field trial. The steps in the left contain all procedures applied to extract single plants NDVI from the UAS images, whereas the steps in the right show the procedures followed to extract single plants plant height from the PhenoRover system. The box with the broken red line contains the methods and analysis procedures used to model the HY estimation. 89

Figure 4 Measured fresh herbage yield at four seasons of the year 2017 and 2018 (a, c) and dry herbage yield (b, d); A: autumn; W: winter; MS: mid-spring; LW: late winter; EW: early winter; ES: early spring; LS: late spring. 91

Figure 5 Scatterplots of plant height from ultrasonic sonar sensors vs. manually measured plant height using a standard ruler for the selected measurement date 92

Figure 6 Scatterplots of (a) NDVI extracted from UAS-based multispectral imaging vs. log-transformed dry herbage yield measurements of single plants in four seasons of the 2017 dataset; (b) plant height measured versus log-transformed dry herbage yield measurements of single plants in four seasons of the 2017 dataset; (c) NDVI extracted from UAS-based multispectral imaging vs. log-transformed dry herbage yield measurements of single plants in four seasons of the 2018 dataset; (d) plant height measured versus log-transformed dry herbage yield measurements of single plants in four seasons of the 2018 dataset..... 93

Figure 7 Scatterplots of (a) squared NDVI-value extracted from UAS-based multispectral imaging and plant height (NDVIsq_PH) vs. dry herbage yield measurements of single plants in four seasons of 2017; (b) NDVIsq_PH vs. dry herbage yield measurements of single plants in four seasons of 2018..... 94

Figure 8 Scatterplots of (a) combined regression of NDVIsq_PH vs. dry herbage yield measurements of single plants in four seasons of 2017; (b) combined regression of NDVIsq_PH vs. dry herbage yield measurements of single plants in four seasons of 2018 94

Figure 9 Scatterplots of observed and predicted dry herbage yield (grams/plant) in perennial ryegrass with linear regressions of various K-folds and random splits of cross-validation. The data points displayed in each plot represent the K-fold validation sets of (a) two-fold, (b) five-fold, (c) 10-fold, (d) 20- and (e) 60/40%, (f) 70/30% and (g) 80/20% random split from the 2017 harvest. The solid diagonals represent 1:1 line, and the dashed lines represent the fitted linear functions..... 96

Chapter 5

Figure 1 Workflow of analyzing geospatial images of spectral signals taken by REM sensor. Each circle represents a processing step and each rectangle represents an intermediate data type..... 106

Figure 2 Transformation of GeoTIFF images based on 4 corner GCPs. The green points are GCPs and the red polygon is the boundary for the perspective transformation. 107

Figure 3 Identification of plant blocks and plant rows. **(A)** Partial view of an NDVI UAV field image. The trace to right of this image represents the sum of the pixel values along each row of the image producing a vertical profile. **(B)** Overlay of identified horizontal dividing lines separating the four plant blocks. **(C)** The projection method applied to each plant block was used to identifying the plant rows in each plant block. Here the horizontal profile seen at the bottom of **(C)** constitutes the sum of the pixels along each pixel column within the given block. **(D)** Overlay showing the separation obtained between adjacent plant rows for a particular block..... 107

Figure 4 Profile processing. (A) Shows the projection profile from nine plant blocks. The blue overlay shows the result of performing a morphological opening using a line structuring element of length (plant row length)/2. (B) The opening was then processed using an erosion using a line structuring element of length (plant row length)/3. (C) Inverted regional minima. The eroded profile was processed to locate its regional minima. (D) Red vertical lines give the centers of each identified regional minima, while the blue vertical gives the boundary edge of each regional minima identified.....	108
Figure 5 Plant-row box growing results. (A) Initial estimate of plan-row boxes. (B) The same plant-row boxes after box growing.	108
Figure 6 Comparison of predicted DM of individual plants through automatically and manually derived polygons across growing seasons of winter2018 (A) , late spring2018_1 (B) , and late spring2018_2 (C)	110
Figure 7 Maps of perennial ryegrass NDVI, plant height (cm), predicted and measured DM (kg/plot) spatial variations at Winter2018 (A) , late Spring2018_1 (B) , and late Spring2018_2 (C) at harvest time.	110
Figure 8 Distribution of (A) NDVI, (B) plant height, and (C) predicted DM across three growing seasons of 48,000 individual perennial ryegrass genotypes.....	111
Figure 9 Variation of individual plants NDVI values among and within 50 perennial ryegrass cultivars/advanced breeding lines across three growing seasons.....	112
Figure 10 Variation of individual plants height among and within fifty perennial ryegrass cultivars/advanced breeding lines across three growing seasons.	113
Figure 11 Variation of individual plants biomass (predicted) among and within fifty perennial ryegrass cultivars/advanced breeding lines across three growing seasons...	114
Figure 12 Comparison of predicted and measured DM (kg/plot) based on spaced-planted trial.....	115

Figure 13 Comparison of predicted and measured DM (kg/plot) across three growing seasons combined 115

List of Tables

Chapter 1

Table 1 Number of ryegrass cultivars available in the Australian market (adapted from (Reed 2014) with modification)	5
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Chapter 4

Table 1 Overview of the data collected from individual plants, data collection date, sensors applied and equipment used for measurements	86
Table 2 Details of UAS data acquisition and processing quality.....	87
Table 3 Summary of cross-validation results for predicted fresh herbage yield	95
Table 4 Summary of cross-validation results for dry herbage yield.....	95

Appendices

Appendix I Poster presented at the International Forage and Turf Breeding Conference, Orlando, Florida, 2019	138
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Chapter 1. Introduction to Ryegrass Biomass Yield Improvement

1.1 Introduction

Cool-season grasses are the most important feed and turf crops in the temperate regions of the world. The main perennial grass species include perennial ryegrass (*Lolium perenne* L.), Phalaris (*Phalaris aquatica* L.), Italian ryegrass (*Lolium multiflorum* Lam.), tall fescue (*Festuca arundinacea* Schreb.), and cocksfoot (*Dactylis glomerata* L.) (Yamada *et al.* 2005; Talukder and Saha 2017) and these are widely regarded as the ideal forage species for animal feed in Australia (Reed 1996; Smith *et al.* 2001), Europe (Grogan and Gilliland 2011) and New Zealand (McDonagh *et al.* 2016). Perennial ryegrass is the dominant pasture and turf grass species in temperate regions and is mainly used for pasture-based animal feed (Hayes *et al.* 2013; Ludemann *et al.* 2015; Hendriks *et al.* 2017). There are around ten different species within the *Lolium* genus that are native to Europe, Asia and North Africa and several are grown extensively in Australia and New Zealand (Cunningham *et al.* 1994). Perennial ryegrass and Italian ryegrass are the most economically significant for pasture production (Wang *et al.* 2016a).

Perennial ryegrass occurs as diploid ($2n=14$) with an obligate outbreeding nature. However, tetraploid ($2n = 4x = 28$) cultivars of these species may be obtained by treating the species with colchicine leading to chromosome doubling (Ahloowalia 1967; Hume *et al.* 2010). The obligate outbreeding behaviour results in sizeable genetic diversity and has been due to the Z and S locus self-incompatibility (SI) mechanism (Thorogood *et al.* 2017). The Z and S gametophytic SI works together to determine the pollination of male and female gametophytes. During pollination, the pistil distinguishes the Z or S loci of its own pollen and prevents fertilisation (Shinozuka *et al.* 2009; Do Canto *et al.* 2018). This promotion of SI ensures heterozygosity and hybridisation within species by increasing variation. However, SI prevents the production of homozygous inbred lines, and the heterozygous nature of the parental plants means that they may carry deleterious recessive alleles that reach recurrent generation.

Perennial ryegrass naturally occurs in regions with contrasting climates from northern Europe and Asia with its extended cold season to southern Europe and north Africa with

a warmer, drier Mediterranean climate. The species is well suited to fertile soils, with average annual rainfall > 550 mm, but growth is restricted if the temperature exceeds 30°C. Seasonal production under rainfed conditions in temperate Australia is typically low in winter, high in spring and ceases as plants become dormant in summer. Growth resumes in autumn with the onset of seasonal rains. Ryegrass growth and morphology differs significantly due to genetic and environmental influences. Ryegrass generally has a relatively shallow root system with much of the root density concentrated in the topsoil (10-15cm). This results in poor persistence under drought and water-limited conditions (Nie *et al.* 2008; Cullen *et al.* 2014).

The life cycle of individual tillers determines the overall growth stage and nutritive value of the sward (Figure 1). Perennial ryegrass can persist for 30 or more years depending on management and climate (Grassland Society of Southern Australia Inc. 2008b). However, high herbage production can occur from year two onwards and typically lasts 5-10 years before it begins to decline. Maximum herbage accumulation and water-soluble carbohydrate (WSC) concentration occurs at the 2-3 leaves per tiller stage (Figure 1). This may also increase the herbage digestibility and animal intake (Miller *et al.* 2001). As a result, three management guidelines have been identified to optimise herbage production and nutritive quality parameters (Fulkerson and Donaghy 2001; Lee *et al.* 2010). These are i) graze between 2-3 leaf stage (2-3 leaves per tiller) (~3000 kg dry matter per hectare) when the net accumulation of herbage and WSC is at a high level; ii) leaving 4-6 cm (1,500–1,600 kg dry matter per hectare) post-cut residuals for regrowth, and iii) maintain a constant cover of green leave all year to maintain perenniality and plant survival.

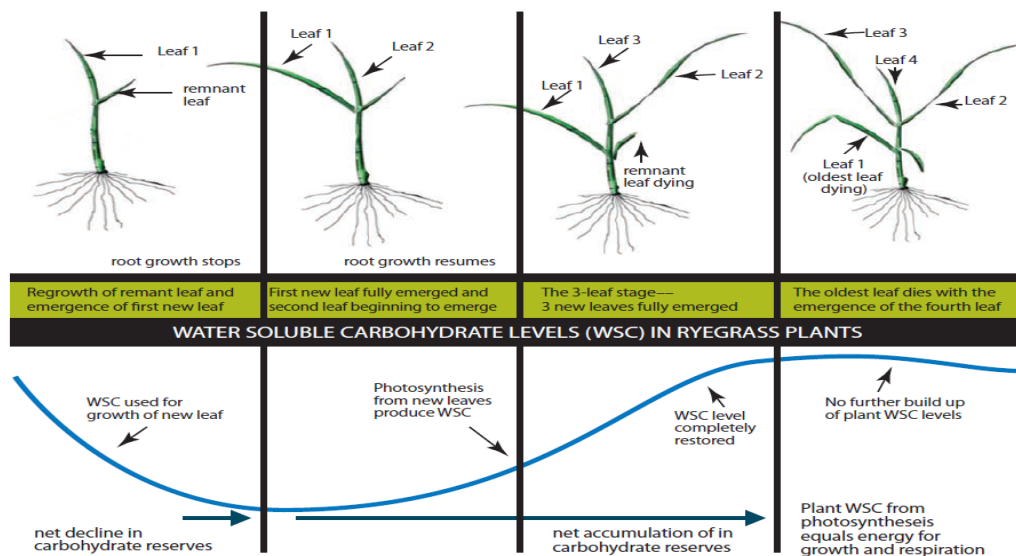


Figure 1 Schematic representative of ryegrass growth and nutritive quality changes following defoliation (adapted from Donaghy 1998)

1.2 History of Perennial Ryegrass Breeding

Perennial forage breeding commenced relatively in the recent compared to many cereal crops (Humphreys *et al.* 2006; Sampoux *et al.* 2011). Before the release of commercially bred cultivars, seeds produced by local farmers from acceptable strains were traded in European markets. These accessions were not evaluated for performance, stability, distinctiveness or uniformity. Introduction and evaluation of perennial grasses in Australia began in the 1850s, with deliberate introductions by early settlers (Cunningham *et al.* 1994). The Victorian Department of Agriculture began selection and evaluation in 1928 and the first perennial ryegrass cultivar called “Victorian” was released in 1936 (Reed 1996). Other government agriculture research organisations including the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Tasmanian Department of Primary Industries and Fisheries and New South Wales Department of Agriculture followed the Victoria Department of Agriculture in releasing new cultivars. From late 1980s to the early 1990s several cultivars of New Zealand and European origin were introduced and evaluated, but it was “Victorian” which dominated sown perennial ryegrass (80%) on farms in Australia at the time. This was due to the “Victorian” cultivar

being known for its persistence compared to the New Zealand and European cultivars introduced to south-eastern Australia at the time (Cunningham *et al.* 1994).

By the 1990s, Australia had established Plant Breeders Rights (PBR) legislation and this stimulated a greatly increased effort in cultivar registration (Reed 2014). PBR legislation changed the course of breeding in Australia by providing breeders with the incentive to pursue opportunities with commercial partners on recurrent selection, cultivar development, multiplication and distribution of seeds (Kingwell 2005). Private companies became involved in seed testing and certification services (e.g. Vic Seeds Pty Ltd). Today, the market drives the commercialisation of improved ryegrass cultivars. The number of cultivars in the Australian market increased steadily (Table 1) and in 2018 there were about 63 perennial and hybrid ryegrass cultivars and about 94 Italian and short-lived hybrid ryegrass cultivars in the Australian market (Australian Seed Federation list, 2018, <http://www.asf.asn.au/seeds/pasture-seed-database> /accessed on June 01, 2019). Private companies are selling cultivars in Australia from all over the world.

Table 1 Number of ryegrass cultivars available in the Australian market (adapted from (Reed 2014) with modification)

List of Agency and Year	Perennial and long-lived ryegrass	Italian and short-lived ryegrass
VDA recommended list 1978	4	3
VDA recommended list 1993	6	4
Vic. Pasture Varieties Committee approved list 1996	8	7
Pastures Australia, Pasture Picker website [2007–08]	47	28
NSW DPI, Pasture varieties book [2012–13]	69	67
Australian Seed Federation list 2018	63	94

VDA = Victoria Department of Agriculture NSW DPI = New South Wales Department of Primary Industries

1.2.1 Objectives of ryegrass breeding

Breeding for improved annual and seasonal herbage production, nutritive value, persistence (includes biotic and abiotic stress tolerance) and to a lesser extent, higher seed yields are the primary targets of forage breeders (Lee *et al.* 2012). However, the focus is mainly on the improvement of herbage production (Wilkins and Lovatt 2011; McDonagh *et al.* 2016). So far, an average genetic gain of 0.5% increase of ryegrass yield per year was achieved in the previous, even though this varies from continent to continent (Wang and Forster 2017). For instance, genetic gain per year in Australia and New Zealand was calculated to be 0.25 - 0.73% (Lee *et al.* 2012; Harmer *et al.* 2016), between 0.25 - 0.6% in Europe (Wilkins and Humphreys 2003) and between 0.0 - 0.1% in the USA (McDonagh *et al.* 2016). Similarly, there was a report of variation in average annual herbage yield increase of 0.52% for perennial ryegrass grown when managed for fodder

conservation and estimated 0.35% under simulated grazing management with cultivars of similar maturity and ploidy levels (McDonagh *et al.* 2016).

Generally, forage species have shown low genetic gain per year compared to cereal crops (1.3%) (Pratap *et al.* 2019). Different scenarios could be explained for lower genetic gain in forages (Woodfield 1999; Casler and Brummer 2008; McDonagh *et al.* 2016) including i) current selection methods may not be sufficient to detect small additive genetic variances within half-sib or full-sib families; ii) although results of heterosis breeding in ryegrass (Pembleton *et al.* 2015; Wang *et al.* 2016b) indicated high promise, it is not yet commercially exploited; iii) longer time to release new cultivars which may extend up to 15 years.

The improvement of herbage yield is further complicated as breeders often want to alter the distribution of yield across different seasons. Herbage yield improvements in key low production seasons have been achieved previously (Ward *et al.* 2013; O'Donovan *et al.* 2017). Breeders have applied various strategies to achieve better seasonal yield distribution: 1) by altering the flowering time - late heading dates may translate to increased herbage yield production as the vegetative growth stage is extended; 2) development of new cultivars with deep roots and stay-green growth habit that can tolerate water stress. These cultivars may not necessarily produce higher annual yield but instead shift the pattern of maximum production stage to balance with the low production times; 3) by incorporating endophyte into commercial cultivars. Endophytes are fungi that provide symbiotic benefit to the host plant (Karpyn Esqueda *et al.* 2017). The presence of asexual *Epichloë* endophytes associated with perennial ryegrass may increase plant growth, dry matter, seed production and resistance to biotic and abiotic stresses (Lowe *et al.* 2008). The fungi release chemicals (e.g. alkaloids) which assist the host plant to cope with pathogens, insect pests and abiotic stresses such as drought.

The genetic gain obtained through breeding and forage improvement can be translated easily into economic gain in terms of whole-farm herbage yield production (Ludemann *et al.* 2015). However, the genetic gain achieved through breeding may not be translated directly to animal weight gain. This may be related to the nutritive value of forage crops, which has a direct effect on animal performance (Ball *et al.* 2001). Breeding for high

nutritive value forages (e.g. digestibility) has generally received less attention (Smith *et al.* 1997), and a little effort has been made so far (McDonagh *et al.* 2016). In newly developed cultivars, quality parameters were often excluded from variety testing procedures except high sugar ryegrass cultivars (Smith *et al.* 2007). However, there is a limited success in the use of high sugar grasses in Australian perspective. This created knowledge gaps in understanding the complex traits of herbage nutritive value parameters, though it is known that improving the nutritive value of cultivars increases pasture intake by animals (Ludemann *et al.* 2013; Badenhorst *et al.* 2018). Perennial ryegrass nutritive value parameters included traits that contribute to the improvement of water-soluble carbohydrates concentration, crude protein, *in vivo* dry matter digestibility, ash content, acid detergent fibre, neutral detergent fibre, flowering time and ploidy level. Breeding for these parameters is expensive and it is difficult to prioritise nutritive value traits in terms of the ease and economic returns from their improvement through breeding (Smith *et al.* 1997; Smith *et al.* 2019).

Persistence is another important trait of perennial ryegrass as it affects the ability of a specific cultivar to remain productive over time. The persistence of a cultivar relates to the ability of a plant to withstand biotic and abiotic stresses over time. Abiotic stresses such as drought, water-logging, salinity and poor soil fertility are major causes of poor persistence under Australian conditions (Smith and Fennessy 2011). Biotic stresses include crown and stem rusts, barley yellow dwarf virus, insects and other invertebrates. Forage breeders mainly target genetic improvements of stresses (Bonos *et al.* 2006; Arojju *et al.* 2018; Knorst *et al.* 2018) through the implementation of symbiotic infection with fungal endophytes (Lee *et al.* 2012; Wiewióra *et al.* 2015). Thus, developing cultivars capable of resisting these stresses is a priority of breeding and will remain so for the foreseeable future (Smith and Fennessy 2011).

1.3 Breeding Approaches

1.3.1 Traditional Breeding

Breeders use various strategies to develop new ryegrass cultivars (Cunningham *et al.* 1994; Lee *et al.* 2012), including classical breeding like population improvement (recurrent selection), breeding synthetics (polycross) and hybrid breeding and molecular breeding strategies such as genomic selection, marker-assisted breeding and functional genomics. Classical breeding methods use recurrent selection to screen the best-performing genotypes among assessed population selected through repeated phenotyping and polycrossing of parent-progeny relationship. In this method, breeders start with a distinct number of genotypes and undertake multiple generations of selection. However, many genotypes may be required to maintain the superior genotypes considering the nature of self-incompatibility and resultant inbreeding depression (Lin *et al.* 2017). In the process, high biomass yielding genotypes and their interbreeding combinations to create a cultivar with a distinct improvement in the target trait may be achieved by repetitive phenotyping. The current method also exploits hybridisation breeding methods. The concept of hybridisation relates to a process of producing progeny from the crossing of two genetically different genotypes. This may create hybrid vigour (heterosis) where the progenies of two genetically divergent ryegrass parents perform better and produce more seed (Pembleton *et al.* 2015; Herridge *et al.* 2018).

1.3.2 Marker Assisted Selection

Advances in molecular biology over the last 20 years offer accurate manipulation of genetic factors for fast development of cultivars with increased yield, nutritive value and stress tolerance. This has encouraged scientists to apply various molecular tools for faster breeding of better cultivars (Spangenberg *et al.* 2001). These molecular breeding tools include marker-assisted selection (MAS) breeding, genome-wide association studies, genotyping by sequencing, genomic selection and genomic editing. The tools provided an opportunity for scientists to identify and incorporate genes for high-value traits in their

breeding program (Smith *et al.* 2007). MAS works by identifying molecular markers that have linkage disequilibrium with genes of targeted traits. Studies using MAS identified various quantitative trait loci (QTLs) associated with herbage yield, plant height, nutritive values, flowering time and disease resistance (Yamada *et al.* 2004; Cogan *et al.* 2005; Muylle *et al.* 2005; Pauly *et al.* 2012). The application of MAS assists breeders by accelerating the identification and selection of individual plants to reduce selection time. However, genomic selection (GS) may be more advantageous than MAS in accurately predicting agronomic traits (Hayes *et al.* 2013; Wang *et al.* 2014).

1.3.3 Genomic Selection

Despite important advancements in MAS application, the improvement of quantitative traits (such as yield) has been slow (Heffner *et al.* 2009). This was because such traits are affected by many genes of small effect (Fè *et al.* 2016) and these effects may be too small to detect. New genomic technologies like GS may overcome these problems. GS aims at applications of phenotypic and genotypic information to predict the performance of genotypes (Goddard and Hayes 2007; Pembleton *et al.* 2018). GS involves the collection of genotypic and phenotypic data from a training population that subsequently would be used to predict the breeding values of a validation population that only have genotypic information (Meuwissen *et al.* 2001). This method significantly increases the genetic prediction accuracies subject to sample size and small gene effects (Lin *et al.* 2017). Therefore, the training population should contain as much variation as possible to maximise the accuracy of predicted genomic estimation breeding values (GEBVs) of individual genotypes or families. Data on detailed measurements of plants that provide phenotypic information may play a crucial role in improving GEBVs and GS application in breeding programs.

1.3.4 Genetic Modification and Genome Editing

Genetic modification (GM) and genome editing (GE) serve as alternatives to traditional breeding methods to increase the pace of breeding (Williams *et al.* 2007; Fritsche *et al.*

2018). However, there is only a single, commercially available GM perennial forage species - Roundup Ready lucerne (*Medicago sativa*) despite extensive research on transgenes of perennial ryegrass and white clover (Badenhorst *et al.* 2016; Smith and Spangenberg 2016; Giraldo *et al.* 2019). Research into the development of GM ryegrass indicates the possibility of a significant increase in metabolisable energy (Badenhorst *et al.* 2018). The increase in metabolisable energy was achieved by up-regulation and the re-programming of fructan biosynthesis in ryegrass (Panter *et al.* 2017). However, a little work has been done so far to improve perennial ryegrass through targeted GE applications (Grogg *et al.* 2019).

1.4 Phenomic Assessments of Perennial Ryegrass

Phenomic assessments of major crops have evolved recently, with measurements moved from purely destructive, labour-intensive, time-consuming to rapid, non-destructive and quantitative sensing techniques (Furbank and Tester 2011; Araus and Cairns 2013; Reynolds *et al.* 2019). Sensor-based advanced phenotyping technologies have a great potential to evaluate large population non-destructively, and may robust the implementation of GS, GE, GM and MAS breeding approaches for fast identification of elite genotypes. The use of these technologies in forage breeding programs will enable the implementation of the selection of trait(s) non-destructively, rapidly and precisely. A detailed review of the potential application and limitation of sensor-based phenotyping technologies in forage breeding is published and presented as chapter 2 in this thesis (Gebremedhin *et al.* 2019b).

1.5 Thesis Back Ground and Significance

Investment in ryegrass improvement research in Australia is immense. Pioneering scientists are developing new breeding technologies to reduce the cost and numbers of years (typically from 10 to 15 years), required to develop new cultivars (Hayes *et al.* 2013; Lin *et al.* 2016). This may be achieved using the application of new genomic technologies, including GS. However, for the GS to be beneficial, it must overcome

practical challenges. The main challenge is the requirement for large and diverse training populations - sometimes impractical with small field trials. However, genetic markers used are expected to uncover nearly all genome information (Faville *et al.* 2018). Its implementation is by collecting genotypic and phenotypic data of the training population that subsequently would be used to predict the breeding values of the validation population that only have known genotypic information. However, various factors, including model performance, marker density, the similarity between training and breeding populations, population sizes of training and breeding populations and heritability of the target traits are barriers (Liu *et al.* 2018). So far, the assessment of phenotypes of these genotypes is often determined through manual measurements, visual scoring and destructive harvesting. These methods are labour intensive, costly, time-consuming and are often difficult to implement with large breeding programs.

High-throughput phenotyping (HTP) technologies have the potential to assess large breeding population non-destructively, rapidly and objectively. HTP involves the application of sensor-based assessments of plant morphological characters including herbage yield, plant height and leaf area index (Sha *et al.* 2018; Grüner *et al.* 2019; Pezzopane *et al.* 2019). Various sensor technologies including multispectral imaging, ultrasonic sonar, light detection and ranging (LiDAR), hyperspectral and visible imaging are used on mobile platforms to capture information rapidly.

HTP systems are being developed at the Agriculture Victoria Research Centre in Hamilton, that include aerial and ground-based in-field ryegrass phenotyping platforms equipped with an array of sensors for non-destructive, rapid and accurate phenotyping of ryegrass herbage yield, nutritive value and persistence traits, which are the core breeding traits of economic value in ryegrass improvement and new cultivar development (Lee *et al.* 2012). However, implementation of the HTP systems in identifying the desired traits and the best performing genotypes depends on the validity of the data collected from the sensors and platforms (Enciso *et al.* 2019).

Several aerial and ground-based HTP platforms have been validated to estimate plant height and biomass in the field for crops such as wheat, barley, sorghum, maize and rice (Montes *et al.* 2011; Bendig *et al.* 2013; Bendig *et al.* 2014; Madec *et al.* 2017; Wang *et al.* 2018). However, few papers have been reported related with the use of low to high

throughput platforms of sensor validation to describe ryegrass herbage yield, plant height and growth rate observations (Hunt *et al.* 2015; Lootens *et al.* 2016; Borra Serrano *et al.* 2017; Ghamkhar *et al.* 2019). The development of aerial and ground-based HTP platforms along with various data processing pipelines is essential to capture and process data of desired traits from hundreds of thousands of diverse space-planted genotypes (Gebremedhin *et al.* 2019). Calibration and validation of sensor technologies and HTP platforms will allow, therefore, the prediction of annual and seasonal herbage yield of large breeding populations at single plant, row and plot levels rapidly and objectively. This will replace current methods of visual scoring, sample clipping and mowing of plants or plots.

Aerial and ground-based HTP platforms make it possible to rapidly acquire various data from different sensors permitting repetitive cycles of measurements and enabling the user to collect an extensive amount of data. Images for canopy reflectance and above ground biophysical (e.g. plant height, leaf area index and canopy volume) data have been collected to monitor grassland growth and herbage yield (Jin *et al.* 2014; Zhang *et al.* 2016; Serrano *et al.* 2019). These experiments were used to extract vegetative indices (VI) (mainly from calculating reflectance at two or more different wavelengths, calculated VI from orthophotos), and plant height from point cloud data of crop surface models, LiDAR and ultrasonic sonar sensors. Normalised difference vegetative index (NDVI) is a widely used VI to estimate herbage yield and monitor growth rates of various crops including forages (Duan *et al.* 2017; Hogrefe *et al.* 2017; Hassan *et al.* 2018). NDVI characterises the spectral property of the upper canopy without penetrating the inner canopy spectral property. However, estimation of herbage yield may be affected by insensitivity of NDVI at high herbage yield conditions and this limits the differentiation of the rankings of well-performing genotypes from the poorly performed ones.

Plant height matrices, on the other hand, characterise a measurement of the shortest distance between the top and bottom of the canopy, which describes the entire vertical canopy structure (Holman *et al.* 2016). In previous studies, plant height measurements from ultrasonic sonar sensors were used to build an empirical relation between plant height and herbage yield (Pittman *et al.* 2015; Pittman and Butler 2019). Limitations with these matrices arise from the type of prediction in case it may not create a robust

estimation model when applied to an assessment of space-planted, individual ryegrass plants of great diversity both in tillers proliferation and growth characteristics.

In recent years, researchers have attempted to integrate aerial and ground-based spectral and plant height data to improve biomass estimation of wheat and barley crops (Bendig *et al.* 2015; Tilly *et al.* 2015; Lu *et al.* 2019). Ground-based platforms used to integrate NDVI of spectral data with plant height were also used to improve herbage yield estimation in forage crops (Pittman *et al.* 2015; Tilly *et al.* 2015; Schaefer and Lamb 2016). In these combinations, various regression models were applied to establish yield predictive models. Traditionally, scientists try to combine NDVI with plant height with simple integration (NDVI*plant height). However, there is no evidence that this is the best way of combining NDVI with plant height to get the maximum level of accuracy. Therefore, it is worth pursuing other potential options to integrate the two parameters. Identifying the best combination of NDVI and plant height is therefore essential.

Combining various spectral data and structural information may be considered to improve estimating herbage yield, but integration with models can improve applicability. Several studies applied different conventional regression and machine learning methods for herbage yield estimation. The selection of statistical techniques may take into account the uses of these methods separately or in combination to estimate herbage yield. (Viljanen *et al.* 2018) investigated herbage yield and height estimation of grass sward using a multispectral, photogrammetric camera. They evaluated the performance of multiple linear regression and random forest machine learning in which both methods provided equally accurate estimations of herbage yield from VIs and plant height. (Marabel and Alvarez-Taboada 2013) compared the performance of support vector machine (SVM) and partial least squares (PLS) regression to estimate grassland dry matter from spectrometer data with PLS performing better in predicting total dry matter.

Others have also attempted to develop accurate machine learning models to estimate biomass of wheat and maize crops in breeding programs (Han *et al.* 2019; Lu *et al.* 2019). In this thesis, regression models are being developed to estimate herbage yield of individual perennial ryegrass plants. This allows the estimation of herbage yield and plant

height of individual plants, rows and experimental plots of different breeding lines and varieties and their ranking according to their performance under field conditions.

Overall, this PhD thesis has developed a method to deploy drones and ground-based sensors to predict the biomass yield of grass plants in large breeding trials. Additionally, this thesis developed a computational data analysis pipeline to accurately measure the biomass yield of individual plants. The thesis findings allow the development of better pasture varieties for Australian dairy farmers.

1.6 Research Questions

The main questions of this thesis are as follows:

- Can sensor-based, non-destructive technologies on phenotyping platforms accurately estimate perennial ryegrass height and herbage yield?
- Can non-destructive, spectral (NDVI) and structural (plant height) information and the combination of NDVI and plant height accurately estimate herbage yield of individual plants?
- Can the predictive models developed through the combination of NDVI and plant height accurately predict seasonal herbage yield of individual perennial ryegrass plants?
- Can sensor-based non-destructive technologies on phenotyping platforms replace the current phenotyping methods with better accuracy, throughput and cost-effectiveness?

1.7 Research Aims

- To investigate the potential of using ground-based phenotyping platform for plant height and NDVI estimates
- To investigate the potential of using unmanned aerial system (UAS)-derived multispectral imaging for NDVI and herbage yield estimates

- To develop high-throughput methods that determine perennial ryegrass herbage yield using novel phenomics tools such as ultrasonic sensors, LiDAR, active NDVI sensors and multispectral cameras or combination of these
- To develop predictive models for herbage yield of individual perennial ryegrass plant by using data derived from non-destructive methods including plant height, NDVI and a fusion of plant height and NDVI in consecutive growing seasons and years
- To validate the application of developed predictive models, from sensor-based herbage yield estimation methods to evaluate automatic individual plants measurement
- To develop a method can be used to rapidly assess thousands of individual plants and develop prediction equations that can be used for phenotypic ranking of genotypes

1.8 Outline of the Thesis

This thesis consists of six chapters:

Chapter 1 discusses the background history of breeding, breeding objectives, the breeding approaches so far implemented for ryegrass herbage yield improvement and the challenges of delivering improved varieties and breeding lines sooner to the agricultural industry. The chapter also covers the significance of the thesis, with conceptualisation of the main questions raised, the objectives and some highlights and overviews of the thesis.

Chapter 2 is based on a published review article (Gebremedhin *et al.* 2019b, in the *Agronomy* journal) which begins by reviewing the limitations of current methods of herbage yield assessment. It describes new ground- and aerial-based platforms equipped with advanced sensor technologies offer opportunities for fast, nondestructive and low-cost HTP of herbage yield assessments. This section also reviews the importance of developing pipelines for “big data” acquisition, processing and analysis as well as combining these data with GS in order to achieve a higher rate of genetic gain in forages. The chapter also reviews the literature on perennial ryegrass HTP strategies, challenges and opportunities.

Chapter 3 evaluates the accuracy of the sensors used in measuring perennial ryegrass plant height and plant growth rate. Calibration and validation of these sensors on the ground and aerial-based platforms proved the potential to outperform conventional methods of phenotyping regarding throughput, cost, and accuracy. In this chapter, two sets of experiments are described which validated ground-based (PhenoRover system) and aerial-based platforms. The first experiment was conducted to calibrate and validate the use of ultrasonic sonar and LiDAR sensors on PhenoRover system to estimate individual perennial ryegrass plants. The results of this experiment showed that ultrasonic sonar and LiDAR sensors accurately estimated individual perennial ryegrass plant height. A second set of experiments, validated the PhenoRover (equipped with ultrasonic sonar LiDAR and GreenSeeker sensors) and the UAS (equipped with multispectral sensors) for estimating plant height, NDVI and fresh and dry herbage yield. Results indicated that sensors on PhenoRover system might be used to estimate plant height and herbage yield, whereas the highthroughput aerial-based NDVI can replace ground-based NDVI values obtained from a GreenSeeker.

Chapter 4 is based on published article (Gebremedhin *et al.* 2019a, Remote Sensing journal) which details the establishment of individual perennial ryegrass herbage yield estimation models by using data derived from non-destructive methods, including plant height, NDVI and a combination of plant height and NDVI in four different seasons over two consecutive years. This study investigated the use of aerial and ground-based HTP platforms equipped with multispectral imaging and ultrasonic sonar sensors to measure individual plants NDVI and plant height as a predictor of individual ryegrass plant herbage yield. The results show that NDVI and plant height alone can estimate herbage yield moderately. To improve the prediction ability of these parameters, the combination of the squared NDVI and plant height (NDVIsq_PH) was considered to be the best method to combine the parameters. This improved the prediction accuracy and validated the potential of non-destructive herbage yield estimation models. The method can be used to rapidly assess thousands of individual plants and the developed prediction equations can be used for the phenotypic ranking of genotypes.

Chapter 5 is based on a journal article submitted to the Frontiers in Plant Sciences journal. This chapter uses the models developed in Chapter 4 to estimate dry matter yield

of 48,000 individual perennial ryegrass plants in three different seasons of the year. The Chapter described and validated the model's ability to predict individual perennial ryegrass plant height and dry matter yield. These methods may be applied to the phenotypic ranking of cultivars and advanced breeding lines. Phenotypic selection for perennial ryegrass yield may be achieved for the rapid assessment of thousands of individual plants at low-cost.

Chapter 6 includes a general discussion, on the significance of using sensor-based high-throughput phenotyping technologies to predict perennial ryegrass plant height, herbage yield, growth rate and decision making in selecting genotypes for yield improvement, followed by conclusions and future perspectives.

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Chapter 2. Prospects for Measurement of Dry Matter Yield in Forage Breeding Programs Using Sensor Technologies

Chapter 2 is based on peer reviewed journal article that was published in the *Agronomy* journal in 2019

The details of the original article are as follows

Gebremedhin, A.; Badenhorst, PE.; Wang, J.; Spangenberg, GC.; Smith, KF
(2019) Prospects for measurement of dry matter yield in forage breeding programs using sensor technologies. *Agronomy*, **9**, 65.

Review

Prospects for Measurement of Dry Matter Yield in Forage Breeding Programs Using Sensor Technologies

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Received: 4 January 2019; Accepted: 31 January 2019; Published: 1 February 2019

Abstract: Increasing the yield of perennial forage crops remains a crucial factor underpinning the profitability of grazing industries, and therefore is a priority for breeding programs. Breeding for high dry matter yield (DMY) in forage crops is likely to be enhanced with the development of genomic selection (GS) strategies. However, realising the full potential of GS will require an increase in the amount of phenotypic data and the rate at which it is collected. Therefore, phenotyping remains a critical bottleneck in the implementation of GS in forage species. Assessments of DMY in forage crop breeding include visual scores, sample clipping and mowing of plots, which are often costly and time-consuming. New ground- and aerial-based platforms equipped with advanced sensors offer opportunities for fast, nondestructive and low-cost, high-throughput phenotyping (HTP) of plant growth, development and yield in a field environment. The workflow of image acquisition, processing and analysis are reviewed. The “big data” challenges, proposed storage and management techniques, development of advanced statistical tools and methods for incorporating the HTP into forage breeding systems are also reviewed. Initial results where these techniques have been applied to forages have been promising but further research and development is required to adapt them to forage breeding situations, particularly with respect to the management of large data sets and the integration of information from spaced plants to sward plots. However, realizing the potential of sensor technologies combined with GS leads to greater rates of genetic gain in forages.

Keywords: forage dry matter yield; high-throughput phenotyping; automation; imaging and image analysis

1. Introduction

Increasing the dry matter yield (DMY) of perennial forages remains a crucial factor underpinning the profitability of grazing industries [1], and is therefore a priority outcome for forage breeding programs. The rate of genetic gain in forage crops is lower (0.25–0.7% per year) than the main cereal crops (1.3% per year) [2–5]. DMY of perennial forages have high genotypic and phenotypic variability, with repeated phenotypic assessment required, a challenge which is exacerbated by the current shortcomings of phenotypic assessment methodology [6,7]. A typical

forage breeding program requires yield to be estimated multiple times throughout the year and on plants sown in various configurations, from individual plants and individually sown rows to densely sown plots [6,8]. Trait assessment and the separation of genotypic and environmental effects and genotype $\times$ environment interaction ($G \times E$), along with the associated challenges of data recording and analysis, remain rate-limiting in forage breeding programs.

These limitations restrict the number of seasons or environments that can be sampled with finite resources, thereby exacerbating this bottleneck to accelerating plant breeding [7,9]. Sample clipping and mowing are destructive and costly in addition to the time required for evaluating thousands of genotypes. In addition to determining forage yield through cutting and manual measurement, breeders have resorted to a visual estimation to allow higher throughput. Visual estimates on individually spaced plants or rows can be highly correlated to DMY but are still time-consuming and even with trained staff retain a certain degree of subjectivity [10,11]. The routine evaluation of a three-leaf stage single perennial ryegrass plant which includes measurement of plant height, leaf density score, cutting and bagging requires about 7 minutes for one person to perform. If the person works an 8-hour day, it would require 700 days to complete the evaluation of the 48,000-perennial-ryegrass-plant field trial located in Hamilton, Victoria, Australia (Figure 1). Application of the current phenotyping method is almost impossible to evaluate even more significant numbers like the 270,000 precisely planted ryegrass plant field trial at Hamilton [12].

Current phenotyping methods lack the resolution for the precise discrimination of genetic effects when the difference between genotypes may be small relative to those due to environmental effects. For example, measuring a particular trait of interest (e.g., DMY) is challenging because measurement is limited by the following factors: (1) the number of sampled plants from sections of experimental plots may not be the best representative, considering the genetic variability that can appear among genotypes; (2) mowing off all the plots is destructive and time-consuming; (3) samples harvesting and subsequent weighing to determine fresh weight and oven-drying to determine dry weight, as well as the logistics used to transport, may add more cost. Thus, development of robust and precise in-field forage phenotyping has the potential to replace destructive and costly phenotyping techniques as well as to provide options to utilise traits that were previously too complex to be assessed easily by breeders [4,13]. The estimation of forage yield is similar to the estimation of biomass, yield components and morphological functions of cereal crops [14,15]. Thus, developing robust and precise high-throughput phenotyping (HTP) methods that can increase the number of samples and record accurate data throughout the life cycle of the crop needs to be a priority for forage breeding.

On the other hand, the development of next-generation sequencing technologies for forage species [16–18] with associated reductions in the cost of genotyping has seen the development of genomic selection (GS) strategies for perennial forage species [8,19–21] with the potential to increase the rate of genetic gain and reduce the cost per unit of gain [21]. It is also evident that DMY improvement in ryegrass is not only achieved by genetic factors but also with improving management techniques to allow the expression of the plant's genetic potential [22]. However, realising the full potential of genomic selection will require an increase in amount and rate of collection of phenotypic data, and thus phenotyping remains a critical bottleneck and impediment to the implementation of GS in forage species like perennial ryegrass [21,23].

Limited attention to developing field-based, high-throughput phenotyping of forage crops has restrained the progress of ‘-omics’ technologies for forage improvement [24]. However, a review [25] indicated the potential, opportunities and challenges of sensor-based HTP tools in forage DMY, quality, drought and disease tolerance improvement. Other authors indicated the possibility of sensor-based HTP to determine perennial ryegrass DMY and plant base area of regrowth after cutting [26,27]. These methods have been validated in experiments with small populations but have not been optimised to screen larger field trials. Recently, various sensors, vehicle platforms, data loggers and real-time kinematic global navigation and satellite systems (RTK-GNSS), along with processing pipelines, have been used to capture phenotypic data in the field from single plants, rows and sward plots of 270,000 perennial ryegrass genotypes [12]. Most of these have been demonstrated in the

context of breeding programs, indicating the potential of utilising sensor-based technology for DMY phenotyping in forage improvement programs.

This review is structured in four sections. Firstly, we review the status of phenotyping forage DMY. Secondly, we discuss the advantages of advanced sensors deployed on the ground and aerial platforms for evaluating forage plant traits. The HTP platforms involve the deployment of proximal sensors with high-resolution imaging technologies accompanied by automation and precision to measure plant performance in large populations established as individual plants, rows, plots or swards. Thirdly, we review the “big data” challenges resulting from HTP, storage methods and management. Finally, we summarise the workflow of image processing and analysis as well as a method for modelling data postprocessing techniques.



Figure 1. An aerial image of perennial ryegrass genomic subsection field trial which comprises 48,000 individual plants at Hamilton Centre, Victoria, Australia.

2. Current Phenotyping Status of Forage Dry Matter Yield and Yield Components

Forage DMY is expressed as the amount of dried biomass obtained after clipping and removing the moisture available in the fresh forage biomass [28]. Methods of assessing DMY of forage can be grouped into direct and indirect measurement techniques. Direct methods that include sample clipping and mowing plots are widely used in breeding trials as a standard method to evaluate and phenotype individual plants, rows and swards plots in forage crops [10,11,29–31]. A range of indirect methods include rising plate meter, a ruler, a sward stick, visual score and others, which are useful to predict DMY, plant growth rate, plant height, number of tillers, leaf dimension and above-ground crown volume/density [6,32–34].

In the indirect methods of estimating DMY, calibration is made by developing empirical relationships between indirectly measured and actual sample values. Most indirect methods use inexpensive instruments and are nondestructive to predict DMY, and are thus applied widely to estimate DMY in various pasture simulation and grazing experiments [34–38]. Although these techniques are often easy to apply, estimations of DMY show inconsistent results with different species. For instance, plant density measured using a plate meter showed a low correlation ($r = 0.21$ to 0.41) with biomass across four different seasons of semiarid grassland [39], whereas a plate meter

measuring plant height showed high ($r = 0.79$ to 0.94) correlation with DMY for tall fescue pasture [40].

3. Sensors and Phenotyping Platforms

3.1. Sensors

The typical sensors used for ground-based, high-throughput phenotyping (GB-HTP) include passive sensors (e.g., red-green-blue, hyperspectral, fluorescence and thermal) [40–43] and active sensors (e.g., GreenSeeker, ultrasonic sonar and LiDAR laser scanner [44–46], among others) [4,47]. Active sensors possess a light- or sound-emitting unit whereas passive sensors use ambient sunlight as a light source. Active sensors, as a result, are independent of radiation differences so that they can operate in cloudy weather conditions. Active sensors often possess a narrow field of view (FOV), whereas passive sensors are less limited to FOV depending on the light source distance from target objects. Unlike active sensors, images obtained from passive sensors require radiometric calibration, adding more steps to the data-processing complexity.

3.1.1. Visible Digital Imaging

Visible digital imaging systems are mainly red-green-blue (RGB) sensors that use wavelengths ranging from 400 to 700 nm to capture two-dimensional (2D) images from the band ranges of red (630–690 nm), green (510–580 nm) and blue (450–495 nm) [40,48]. Images from RGB require adjustment and correction of image brightness, as well as 3D structure reconstruction, followed by geometric and radiometric calibrations to minimise image variability and to capture information from overlapping plant parts. There have been several reports of the estimation of DMY from RGB imaging systems: (1) RGB image analysis through commercial software to calculate the number of pixels and compare with the actual weight measured [26]. In this experiment, image analysis algorithms were developed in Math Lab to estimate DMY with significant correlations, $r = 0.74$ and $r = 0.93$, to visual scores and measured DMY, respectively. RGB images analysis methods were also used to estimate plant morphological parameters, like individual plants base area and tiller number, to understand the growth rate between two cuts of perennial ryegrass field trial [27]. (2) Images from RGB cameras can be used to calculate the vegetative index (VI) and correlate it with measured DMY. The VIs are the spectral transformation of two or more bands designed for a qualitative or quantitative measure of vegetation properties [49]. Limited work has been done so far in using RGB images for extracting VIs, but results showed a potential to estimate biomass [50]. (3) Generating high-resolution digital surface models from RGB images through height maps can also be used to estimate DMY from plant height correlations. The retrieval of plant height from RGB images is a relatively new method of automated height measurement [51]. Unmanned aerial system (UAS)-acquired overlapped images can be photo-merged and processed using the structure from motion algorithms to create a 3D point cloud using commercial software (i.e., PiX4D Mapper Pro, Lausanne, Switzerland, <http://pix4d.com>) [52]. The resulting point clouds are mostly saved as TIFF/CSV files to allow digital surface model (DSM) information to be available for export. A study describing the automation of perennial ryegrass height estimation using the DSM technique indicated a high correlation ($R^2 = 0.54$ – 0.63) between a ‘herbometre’ (a tool similar to rising plate meter [38]) and DSM, offering an alternative option to automating ryegrass plant height estimation [53].

3.1.2. Multispectral and Hyperspectral Imaging

Multispectral and hyperspectral imaging technologies are the most common HTP tools which may be deployed on the ground or aerial platforms. Multispectral imaging captures image data from a few specific narrow spectral bands (usually less than 10). Some multispectral imaging systems may be used to capture information at the RGB and near-infrared spectrum simultaneously. Hyperspectral imaging captures data from a broader range of spectral regions (400–2500 nm, usually more than 10 narrow bands). The use of continuous bands can be beneficial in using high-resolution images to describe the crop canopy in a more discrete and detailed manner.

The use of hyperspectral imaging systems to collect data across a broad range of wavelengths could have the advantage of refining old spectral indices and developing new VIs that may achieve higher correlations with morphological traits and DMY. Hyperspectral imaging systems operate to capture an image in the form of push-broom or snapshot sensor form. Hyperspectral push-broom systems work with a 2D detector array scanning the full spectrum simultaneously with spatial line scanning over time. Push-broom sensors collect spectral information on moving platforms in the form of scanned images or multiple points of the fibre optic spectrometer. The drawback of this sensor is that it requires moving along a platform (e.g., conveyor belt) where the movement of the platform may cause noise. Snapshot sensors are non-scanning sensors in which the entire data of an object can be captured in a single 3D data cube integration [54]. Snapshot sensors can avoid artefacts and noises since there is no requirement of movement while capturing the spectrum. Snapshot techniques, on the other hand, may not filter information, and thus image noise could be included. Generally, hyperspectral imaging systems are expensive and require technical knowledge to process the data. The use of continuous bands can be beneficial in using high-resolution images to describe the crop canopy in a more discrete and detailed manner. Therefore, application of machine learning and artificial intelligence to utilise all spectral information rather than simple VIs could have the advantage of refining new VIs that can achieve higher correlations with morphological traits.

Several VIs from multispectral and hyperspectral imaging systems have been calculated and utilised for nondestructive estimation of plant biomass [55–57]. For instance, the normalised difference vegetative index (NDVI) quantifies the vegetation density, greenness and health by calculating the difference in reflectance at the near-infrared and red region to the sum of reflectance at red and near-infrared regions [58]. The potential and limitations of NDVI for nondestructive forage DMY estimation are known [59–63]. However, at high canopy density, it may saturate and be less able to discriminate the difference in biomass yield [64–67]. This limitation can be alleviated by (1) applying narrowband vegetative indices like the red edge spectral reflectance, that could better estimate biomass than NDVI alone with less saturation at high biomass level [68]; (2) combining NDVI with other biophysical parameters (e.g., plant height) to overcome saturation and achieve accurate estimation [67,69–71]. The green-red, normalised difference vegetative index (GRNVI) has been shown to have a positive correlation with biomass estimation of corn, alfalfa and soybeans [72]. (3) There is an increased interest to use hyperspectral data to overcome the effect of saturation at higher DMY. Several narrow-band spectral signatures obtained from hyperspectral images may have a potential to indicate the subtle variations in reflected energy so that the canopy difference can be detected. This may lead to the introduction of new indices that may overcome issues associated with the saturation of NDVI in estimating DMY. The application of machine learning (ML) algorithms to refine and validate new VIs that can perform well across any growth stage may have great contribution in this regard [73].

3.1.3. Ultrasonic Sonar

Ultrasonic sonar, also called ultrasonic sonar height (USH), [74] measures the distance from a sensor to the plant canopy by detecting the time delay of sound echoes returning to the sensor from plant surfaces. Ultrasonic sonar enables significant improvement in sampling time and sample size compared to the manual measurement of canopy height. USH has been used for height measurement in cotton [75,76], blueberry [77,78], wheat [70,79], barley [38] and pasture grasses and legumes [35,80]. The use of ultrasonic sensors for plant height estimation may, however, be affected by the angle of divergence of the target plant (i.e., the field view becomes larger when the distance between the sensor and the targeted canopy increases, and the field view becomes smaller when the distance between the sensor the targeted canopy decreases). This results in a reduced accuracy of measurement. Similarly, the use of sonar estimates of height for biomass prediction may be affected by canopy structure [81] depending on the number and dimensions of a single leaf or a group of leaves that generate echoes [82].

3.1.4. Light Detection and Ranging (LiDAR)

Light Detection and Ranging (LiDAR) works based on laser light to measure the shortest distance to the target object from the LiDAR scanner (light source) by analysing the time of emission and the time of reflected light detection [83,84]. LiDAR technology provides an option to create and construct 3D point cloud data for filtering and segmenting canopy volume as well as for modelling plant architecture [85–87]. In recent years, LiDAR sensors have been utilised by researchers for the high-resolution and accurate high-throughput phenotyping of a range of forests and cereal crops. LiDAR can rapidly estimate canopy height, width, volume and other structural parameters [45,88,89]. The application of LiDAR covers a diverse range of crops from fruit trees and forests [90–94] to field crops and pasture grasses [95–98]. In perennial ryegrass, ground-based LiDAR was used to measure variation among 12 cultivars with high accuracy of fresh and dry biomass estimation ($R^2=0.76\text{--}0.78$) [99]. In this study, it is noted that only 12 cultivars in 30 rows were evaluated with a ground-based platform where simultaneous measurement of thousands of plots and cultivars will require the development of aerial LiDAR systems [100].

LiDAR was used in forage management systems with *Miscanthus giganteus* Keng under static and dynamic modes to measure plant height [101]. The authors indicated that accurate height measurement using LiDAR compared to manual measurements with an error of 4.2% for static and 3.8% for the dynamic mode. Measured plant height using LiDAR also correlates well with biomass for some forage crops (e.g., tall fescue), indicating the potential application of LiDAR to estimate biomass per unit area [69]. LiDAR measurements, combined with other sensors, for instance, plant height measured using LiDAR combined with NDVI (height $\times$ NDVI), may be used to improve biomass estimation [69].

LiDAR is an existing technology that can fulfil the current phenotyping demands of perennial ryegrass and other forage breeding programs, but its expense limits its application. Installations can cost up to several thousand dollars (though this is decreasing), depending on the complexity and performance parameters of the technology [102] as well as its technical demands [4,103]. Most LiDAR is incapable of giving measures of plant physiological attributes. Therefore, combining georeferenced LiDAR data with data from different sensor types that look on physiological properties may be required to evaluate the overall plant growth performance in the field. However, it is sometimes challenging to combine data that come from different sensors with various temporal and spatial ranges of resolution and units of measurement [104].

3.2. Phenotyping Platforms

3.2.1. Ground-Based Platforms

A platform is a facility or a physical carrier where proximal remote-sensing sensors are mounted for the acquisition of phenomic data (Figure 2). Platforms are important for increasing and accelerating the capture of phenotypic information so that genotypic correlations could be made under diverse environmental conditions. Over recent years, several ground-based, high-throughput phenotyping (GB-HTP) platforms (both automated and semiautomated) have been developed and deployed for phenotyping various crops, including maize, wheat, Bermuda grass and lucerne [68,103,105]. These platforms are equipped with sensors mounted on bicycles, robots and other vehicles (e.g., tractors and other all-terrain vehicles). These vehicles often deploy multiple sensors that enable data capture of plots, rows and individual plants [46,106,107]. Moreover, GB-HTPs typically have a higher payload that provides access to many sensors with relatively minimum additional data-postprocessing requirements [46,106,107]. However, GB-HTPs require a longer time for data capture than unmanned aerial systems. The vehicles can cause soil compaction through repeated traversing—a variation of environmental conditions (such as temperature, light, wind) from the start to the end of the data capture period, which makes thermal imaging systems unapplicable. There is also less precision in output data due to speed variations and an inability to collect data from waterlogged and rough surfaces.

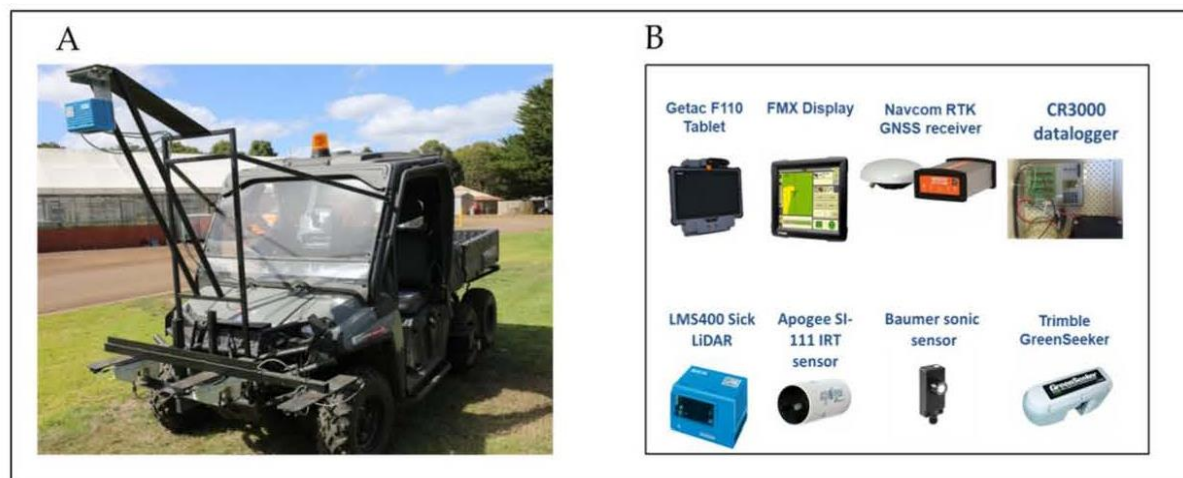


Figure 2. The ground-based phenotyping platform named as “Phenorover” assembled in Hamilton Centre, AVR for field phenotyping of perennial ryegrass. (A) Semiautomated ground vehicle named Phenorover; (B) list of deployed sensors on Phenorover.

3.2.2. Aerial Platforms

Aerial platforms may include various UAS types carrying different sensors, including digital cameras, multispectral imaging, hyperspectral imaging systems and LiDAR (Figure 3) (for detail on the integration of sensors to UASs see review in Tables 2 and 3 by [108] and [109], respectively). UASs have the potential to rapidly measure ground cover, plant height, biomass and leaf area index [110–115]. Another aerial platform that can perform phenotyping may include satellites, where they have the advantage of covering large areas at a time. However, satellites have mostly lower temporal and spatial resolution compared to UASs. It is also challenging to obtain satellite imagery at frequent time intervals without cloud interference. Furthermore, UASs have a lower cost of buying and deploying compared to the launching and buying of the high-resolution satellites [116]. UASs fly at a lower altitude than satellites (even < 100 m) allowing reasonable resolution and may cover large areas of measurement in limited timeframe compared to GB-HTP. UASs are affordable and available for repeated data collection without impacting the soil or plants [117,118].

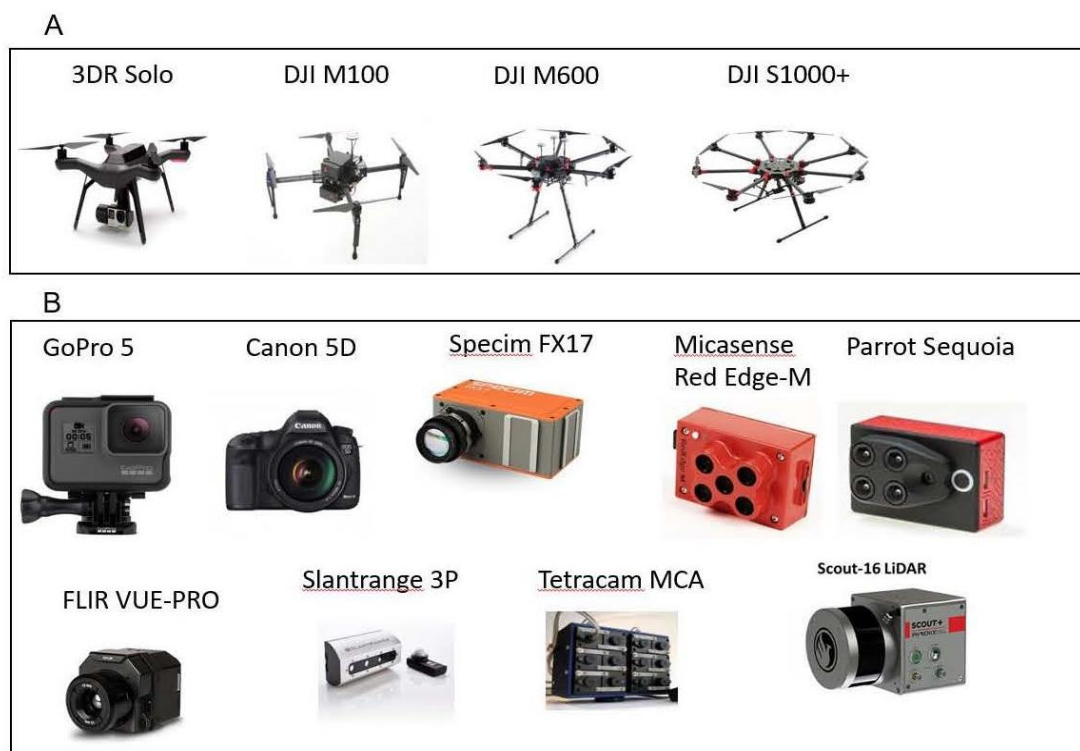


Figure 3. The aerial-based unmanned aerial system (UAS) phenotyping platform in Hamilton Centre, AVR for field phenotyping of perennial ryegrass. (A) List of various semiautomated UASs; (B) list of sensors deployed on UASs.

Limitations related to UAS phenotyping include operating safety regulations that limit payload and mode of operation [107]. Regarding payload and the flight specifications, some countries have restrictive flight operation rules, and this limits their application [116]. As a result, some researchers still prefer to invest in ground vehicles to increase the payload even though these are usually more expensive than UASs. However, recently UAS aviation regulation authorities have started to relax their rules. For instance, the Australian Civil Aviation Safety Authority (CASA) recently reduced licensing costs and introduced less strict legal and operational conditions for UASs [119].

In summary, the development of GB-HTP and aerial-based phenotyping platforms for forage breeding is promising and can provide reliable methods for nondestructive, cost- and time-efficient phenotyping methods for economically important traits. However, further research is required before these methods are routine. Calibration models need to be built that are robust across different environmental conditions and seasons to ensure applicability in multiharvest, multienvironment breeding programs. Moreover, sensors generate large amounts of data, and current processing methods often require manual interfacing and interaction from skilled operators [109,120]. Cost of data processing and analysis is another thing to be considered when developing phenotyping platforms and the automation of data extraction, analysis and interpretation is likely to aid the routine application of these methods in commercial programs. Image processing, data management techniques and statistical analysis and integration of sensors data will be covered in the following sections.

4. Image Processing

Sensor-based phenotyping platforms capture images with the interaction of electromagnetic radiation and the plant organ. The reflectance values vary with a wavelength of the radiation and chemical composition of the plants where many of the captured data require calibration, validation and standardising of the data-processing pipeline to produce quantitative values of phenotypes.

However, noise and variation in sensor position during the capturing of images can create difficulty in standardising of raw images. Thus, pre-data collection sensor calibration and preprocess images correction influence the final accuracy of quantitative phenotypic data output. However, the captured and preprocessed image data require technical image processing software and machine learning algorithm which is a challenging scenario to put the pipeline developed in place and extract biologically meaningful quantitative data easily [116]. Many general image-processing software and hardware solutions are available for phenotyping images from GB-HTP [121–124]. Most of the image-processing software requires processing workflow procedures, including image segmentation, classification, image calibration (geometric and radiometric) and extraction features in the form of geographic information system (GIS) deliverable data.

4.1. Geometric Calibrations

Geometric distortion effects from the original images from cameras are common in remotely sensed data due to the altitude differences between the camera and the target position on the ground. The traditional geometric calibration method is based on ground control points (GCP) on the experimental site. The GCPs are visible marks on the ground and are mostly set with high RTK global positioning systems [125,126]. Geometric calibration with the manual matching of GCPs can be time-consuming but correlates well with onboard RTK positioning [127]. There is also a possibility to perform geometric calibration without GCPs; this can be achieved by repeated observation and low-precision positioning and orientation systems data [108,127]. The limitation of this method is that the errors may be high, and precision can be reduced with small field trials or measurements on individual plants.

4.2. Radiometric Calibrations

Sensor-based captured images require calibration and correction to minimise the variation due to change in brightness, cloudiness or reflectance surface temperature during imaging time. This correction involves the matching of a calibration reflectance panel (e.g., MicaSense Inc., Seattle, WA, USA) that has different reflectance percentages to orthomosaic images [117]. Several approaches to the use of radiometric calibration panels have been used depending on the data acquisition and extraction methods used [117,126,128,129] for calibration of acquired images to the known standard reflectance value in contrast to the well adopted methodology used with satellite imagery [130].

4.3. Segmentation

Image processing starts with segmentation and classification of raw images into the background (soils, debris and so forth) and foreground (mainly plants and plant parts) ([131], Figure 4). The segmentation of images involves training and validating of the software in order to partition digital images so that it can decide on the grouping of pixels into plants and background based on geometry, texture, intensity and colour [131,132]. Alternatively, raw images can be transformed to another form of domain like hue, saturation, value (HSV), canny edge detection and Zhang-Suen thinning algorithm for segmenting plants from the soil, plant structure and plant geometry [133,134]. Image-processing software (e.g., Pix4D, eCognition Developer 9, Trimble, Munich, Germany) is used to create georeferenced orthomosaic photos and then erase the background through an object identification software application to remain with the plant features.

4.4. Feature Extraction

Quantitative extraction of target features from processed images, on the other hand, involves object identification, image colour combination, geometry and texture, as well as local and global 3D point cloud creation [135]. The features extracted are mainly used as a potential method to identify plant organs and characterise the organs to extract relevant features accordingly. Feature extraction results in creating a series of secondary traits like plant biophysical indices that can be obtained from the orthomosaic image as point files (shapefile and TIFF files) which are used for the prediction of

primary traits. Plant indices can be then calculated and processed for single plants, rows or plots based on user-defined georeferenced areas allowing for the quantitative measurements of various biophysical traits (Figure 4).

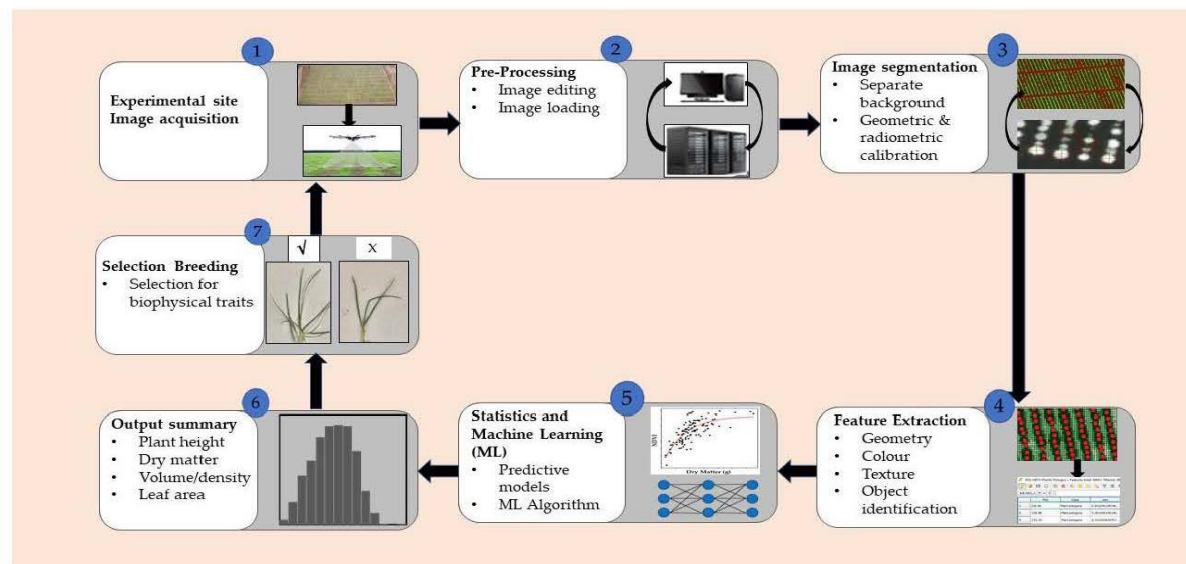


Figure 4. A diagram of workflow indicating image acquisition, processing and analysis. Image- and data-processing platform (in phenomics lab) requires data and image capture from an experimental field. Images and data obtained can be preprocessed to remove noise and artefacts and stored in a server for further processing. Images can be further identified, segmented and classified from the background and nontarget objects using the software. In the post-megadata processing, machine learning through predictive models would develop to the framework for data analysis and analysed data can be used to rank, visualise and screen at individual, row and sward plot levels.

5. “Big Data” Storage, Management and Challenges

High-throughput phenotyping produces large volumes of data (big data) through sensors-based imaging and scanning. Big data is defined as ‘big’ when it becomes hard to process the data with the existing powerful processing tools [136]. Most data that come from large field trials incorporate multisensor captured images and scanned data in 100 s and 1000 s of gigabytes. Therefore, it is sometimes challenging to remove noise, process and analyse big data at the same time. The effectiveness of data processing and analysis of big data can be improved by developing pipelines and scripts in combination with commercially available software packages to allow for automation of quantitative data extraction in a fast and shorter time frame. The main problem with big data processing comes from calibration effects (internal sensor calibration, deployment calibration and environmental noise calibration). One option we should take into consideration is being able to be selective regarding information to be computed from sensors and avoiding low efficiency caused by non-trait-related spectral ranges. The other option is moving away from these simple indices and getting the software to create predictive models using all available information captured.

To fulfil the demand for proper big data acquisition, storage and backup for reanalysis, global data management and support tools are critical [137]. For instance, global data management and support tools like the “Minimum Information About a Plant Phenotyping Experiment” (MIAPPE) can be developed to store, protect and allow the retrieval of data [138,139]. Data stored in global data management systems and support tools can also be freely downloaded by scientists for validation, further analysis, the design of new experiments and decision-making. To fulfil the standards of which and what phenotypic information to be in the global data management and support tool system, scientists, private funders and the public sector must agree on the standards and essential requirements to be included. Funding and institutionalisation of a global, sensor-based phenotypic

data management system will, therefore, help scientists to connect the genomic advancement achieved with the new phenotypic metadata to accelerate breeding [41,46,140].

6. Statistical Modeling

Application of HTP platforms to phenotype breeding trial at large scales presents challenges of data processing, data handling, data storing and application of suitable statistical methods and interpretation. Statistical and mathematical methods are required to analyse data from HTP platforms. The choice of statistical methods ranges from simple regression and partial least squares to advanced ML tools depending on the objective and hypothesis being tested. Several studies applied various regression models to calculate the relationship between measured biomass and spectral reflectance data [50,141,142]. These studies used regression models with limited features considered, where noise that comes from atmospheric effects, temporal effects and sensors limit the interpretation of the data. The models also tend to describe the relationship from a specific harvest rather than focus on developing predictive models. ML techniques evolved to deal with the noise from remote sensed data [143]. ML involves the development of quantitative predictive models, splitting of data sets into training, validation and testing subsets, as well as selecting appropriate mathematical models to apply; this data can be used for ranking, visualising and screening forage genotypes (Figure 4).

ML is a relatively new data analysis method in agriculture [144] with promising results and a vast potential. Limited studies have used various ML algorithms to estimate forage grass biomass from proximal remote sensing. For example, the performance of support vector machine (SVM) and partial least squares regression (PLSR) was evaluated for biomass estimation of grassland using data derived from a field spectrometer [145]. The authors suggested using PLSR as the most accurate model to estimate biomass. Another experiment compared the Multiple Linear Regression (MLR) and the Random Forest Estimation (RF) to estimate grass biomass from plant height models, RGB and VI features [146]. Both ML techniques provided accurate estimation results. ML has not been validated in data to determine the biomass of forage species in a breeding program.

However, several cereal crops breeding programs have used ML algorithms to predict plant growth, plant height, biomass and leaf counts [147–150]. The current applications of machine learning tools in plant science were reviewed [151] and indicated the significance of using SVM and artificial neural networks in identifying stress-tolerant genotypes. [126] Deep neural networks and ML have been used to accurately estimate vegetation indices for high-throughput phenotyping of wheat using aerial imaging [126]. Therefore, ML is now an exciting technique that can be widely applied in forage data analysis for integration, interpretation and further quantification of phenotypic traits at large population field trials, particularly those that may be required for genomic selection.

7. Concluding Remarks

Current forage DMY phenotyping involves manual measuring of samples and mowing of plots and visual scoring. They are either destructive, costly, time-consuming or subjective. Recent developments in advanced sensors technologies are changing the course of phenotyping towards a rapid, large-scale and more accurate direction. Advanced field phenomics involves deployment of proximal sensors and imaging technologies in the field accompanied by high-resolution, precision and large population scale of measurement at individual plants, rows and plots levels. Images captured from the proximal sensors produce multidimensional big data sets that require calibration, validation and analysis to translate them into biologically meaningful measurements. The advanced phenotyping technologies are now capable of assessing large populations in a robust and cost-efficient manner [152]. Sensor-based HTP of forage crops now attracts large investments from governments and nongovernment organisations with a long-term objective to increase the profitability of the pasture-based dairy and meat industry [12]. Therefore, applications of sensor-based forage phenotyping, managing of the “big data” that comes out from the sensors and analysing and standardising of the data require more significant focus from scientists in the area. Application of sensor-based phenotyping can reduce the cost and speed up data collection, phenotyping at a scale

we have never seen before and with high resolution. It will enable genomic selection and accelerate genetic gain for forage breeding.

Author Contributions: A.G. and K.F.S. conceived and designed the review; A.G. wrote the review manuscript; A.G., P.E.B., J.W., K.F.S., and G.C.S. reviewed and edited the review manuscript.

Funding: This research review was financially funded by the Victorian Department of Economic Development, Jobs, Transport and Resources and The University of Melbourne.

Acknowledgments: We acknowledge the staff of Hamilton Center (molecular plant breeding team) for sharing the knowledge and the information on phenomics resource on site. The authors thank Andrew Phelan and Chaya Smith for their valuable suggestions and comments on the overall idea of the review.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3. Calibration of High-Throughput, Non-Destructive Phenotyping Technologies for Rapid Biomass and Plant Height Measurement of Perennial Ryegrass

Chapter 3 is based journal article in preparation to be considered following the submission of the thesis.

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Calibration of high-throughput, non-destructive phenotyping technologies for rapid biomass and plant height measurement of perennial ryegrass. journal not yet decided

Chapter 3 Calibration of High-Throughput, Non-Destructive Phenotyping Technologies for Rapid Biomass and Plant Height Measurement of Perennial Ryegrass

3.1 Introduction

In the context of perennial ryegrass breeding, the early stages of selection mainly depend on multiple measurements of spaced-planted single plants (Wilkins 1991; Conaghan and Casler 2011). Most of the studies that have dealt with ryegrass genetic variation have focused on herbage yield and related traits such as plant height, plant volume, number of tillers, leaf area and leaf length assessments at harvest time and/or during the growth period (Smith *et al.* 2001; Khaembah *et al.* 2013; O’connor *et al.* 2015; Yates *et al.* 2019). Herbage yield and related traits are traditionally measured through laborious, destructive, or subjective methods, requiring substantial time and cost. Therefore, timely, efficient and accurate repeated measurements of these traits are difficult, especially with the requirements of large numbers of genotypes for selection (Gebremedhin *et al.* 2019b).

New sensors on aerial and ground-based platforms allow for rapid, non-destructive and high-throughput phenotyping (HTP) of large populations in the field (Haghighattalab *et al.* 2016; Singh *et al.* 2019). The use of these platforms for HTP of food crops has been widely implemented in recent years. Aerial-based platforms have been used to estimate many traits such as biomass of wheat, rice and maize (Cen *et al.* 2019; Han *et al.* 2019; Yue *et al.* 2019), or the plant height of cotton, wheat and barley (Brocks and Bareth 2018; Hassan *et al.* 2018; Feng *et al.* 2019) and the estimation of both biomass and plant height of ryegrass (Borra-Serrano *et al.* 2019). Similarly, ground-based platforms have been used to estimate the biomass, plant height and ground cover of wheat (Jimenez-Berni *et al.* 2018) and the plant height of sorghum (Thapa *et al.* 2018), wheat (Yuan *et al.* 2018). The validation of these technologies has recently commenced in ryegrass breeding (Gebremedhin *et al.* 2019a; Ghamkhar *et al.* 2019).

HTP proximal sensing methods have been well utilised within breeding programs for food crops, they have recently begun for application in improvement programs for livestock

forages. In recent years, some research has demonstrated the application of proximal sensors including red-green-blue (RGB) camera, light detection and ranging (LiDAR) and ultrasonic sensors to estimate the biomass, plant height, leaf expansion and growth traits of perennial ryegrass in breeding programs (Hunt *et al.* 2015; Lootens *et al.* 2016; Borra-Serrano *et al.* 2019; Gebremedhin *et al.* 2019a; Ghamkhar *et al.* 2019). For instance, a trolley-mounted RGB camera system for imaging of four month old spaced-planted ryegrass plants ($n = 50$) was developed to accurately estimate biomass ($r = 0.94$) of individual plants (Hunt *et al.* 2015). Similarly, manual imaging with RGB cameras on tripods was applied to phenotype lateral leaf expansion and growth ($r = 0.79-0.82$) of individual perennial ryegrass ($n = 501$) two weeks after cutting (Lootens *et al.* 2016). While these in-field measurements have some elements that may improve non-destructive phenotyping, they are limited by inefficient, low-throughput acquisition of multiple traits at multiple times. Recently, a new in-field phenotyping platform has demonstrated the capability of using LiDAR scanners to estimate biomass and growth rate of single row perennial ryegrass plants (Ghamkhar *et al.* 2019). However, this platform lacks Global Positioning System (GPS) capability and the application is unable to accurately identify and geo-reference each plant.

This chapter focuses on the deployment, calibration, and validation of ground and aerial-based platforms developed by the Agriculture Victoria Research (AVR), Hamilton Centre. The platforms include ultrasonic sonar, LiDAR, GreenSeeker, multispectral imaging, Real-Time Kinematics-Global Navigation Satellite System (RTK-GNSS) and data logger systems deployed on ground and aerial based vehicles. In this chapter calibration of sensors data were compared with manual measurements to developed a computational data analysis pipeline to accurately measure the biomass yield of individual plants. These workflows enabled the precise and rapid analysis of multiple types of data from various sensors.

The objective of this study was to develop, calibrate and validate the HTP methods for herbage yield and plant height estimation of perennial ryegrass using ultrasonic sonar and LiDAR systems on a ground-based vehicle and multispectral imaging systems on an unmanned aerial system (UAS). Sensor measurements were calibrated with manual measurements. To capture the genetic variations found in ryegrass, a comprehensive

range of controlled and field experiments were performed with the ground-based vehicle and the UAS platforms. Plant height from LiDAR and ultrasonic sonar systems were compared with height measurements. UAS derived normalised difference vegetative index (NDVI) based on image processing from multispectral imaging system was calibrated with ground-based NDVI measurements with handheld and vehicle mounted GreenSeeker unit systems and was then used to predict herbage yield alone or in combination with plant height.

3.2 Materials and Methods

All the field and controlled environment experiments used to evaluate the PhenoRover and UAS were conducted at Agriculture Victoria Research's Hamilton Centre (37.8211° S, 142.0653° E) located in South West Victoria, Australia. The average annual rainfall of the Hamilton Centre is approximately 680 mm with a mean daily temperature ranging between 11-26°C in summer and between 4-12°C in winter (McCaskill *et al.* 2016).

3.2.1 Description of the PhenoRover Platform

The PhenoRover platform is a Polaris Ranger 6x6 side by side vehicle system fitted with various sensors (Figure 1), and it was assembled at Hamilton in 2016. Two custom made steel mounts were attached to the PhenoRover system to provide mechanical support for the GreenSeeker, ultrasonic sonar and LiDAR sensors. The data acquisition system used was a CR3000 Campbell Scientific data logger, programmed to record plant height from the ultrasonic sensors and pre-processed NDVI data with latitude and longitude coordinates. Data from LiDAR was acquired using a custom-designed laptop with the manufacturer software installed. Various sensors were fitted to the PhenoRover to collect data from single plants, rows, and plots simultaneously. The PhenoRover platform comprises the following instruments.

- a) **Light detection and ranging (LiDAR):** The LiDAR model (Sick LMS400, SICK AG, Waldkirch, Germany) has a measuring range of 0.7 to 3.0 m with a laser scanning frequency of 230Hz to 500Hz. The sensor uses a visible red-light laser (650 nm) with

a measurement angle of 70°. The LiDAR scanner was mounted at 2.0 m above the surface at the front of PhenoRover. The system also has its uninterrupted power supply integrated on the back of the PhenoRover system.

- b) **Sound navigation and ranging (SONAR) sensor:** Six low-cost, ultrasonic sonar sensors (UNDK 30U6103/S14, Baumer, Switzerland) were used to measure the plant height of individual perennial ryegrass plants. The sensor uses an echolocation by emitting a cluster of waves from the sensor to the direction of the detected object and these waves bounce back off the object. The ultrasonic sonar sensors are mounted 0.6 m above the ground and arranged in three rows at 0.6 m apart. This was to allow measurements of plant height for 0.6 m spaced plants at the trial sites. The ultrasonic sonar sensors have a measuring range from 0.1 to 1.0 m with a sonic frequency of 240 kHz.
- c) **GreenSeeker:** Two GreenSeekers (GreenSeeker, Trimble, USA) that measure reflectance at the red (656 nm) and near-infrared bands (774 nm) (Tattaris *et al.* 2016). GreenSeekers were mounted at 0.6 m above-ground. On the PhenoRover front frame, the two GreenSeekers were pointing at nadir.
- d) **Power Unit:** It consists of a 12 v, 24 Amp-hour battery connected to the vehicle's main battery via a 20 Amp, 12 v DC to DC Matson Battery charger. This battery is connected to the CR3000, which allows for the distribution of the different power supplies (5V, 10V, and 12V) that are required for each sensor.
- e) **RTK-Global navigation satellite system (GNSS) antenna:** The RTK_GNSS antenna (Trimble FMX Integrated Display, Westminster, Colorado, USA) is mounted at the top part of the PhenoRover to provide universal time coordinated (UTC) and geographic position system (GPS), and it is displayed through a Trimble FMX monitor to generate geo-referenced data. A serial cable was used connect Trimble FMX monitor and the data data-logger for allowing data recording.
- f) **Data-logger:** The data acquisition system used in the PhenoRover system was CR3000 Campbell scientific (Campbell Scientific, Inc., Logan, Utah, USA) data

logger, programmed to record plant height from the ultrasonic sensors and the .csv files of pre-processed NDVI data with latitude and longitude coordinates.

- g) **Dell-computer:** Data from LiDAR was acquired using a custom-designed laptop with the manufacturer's software.

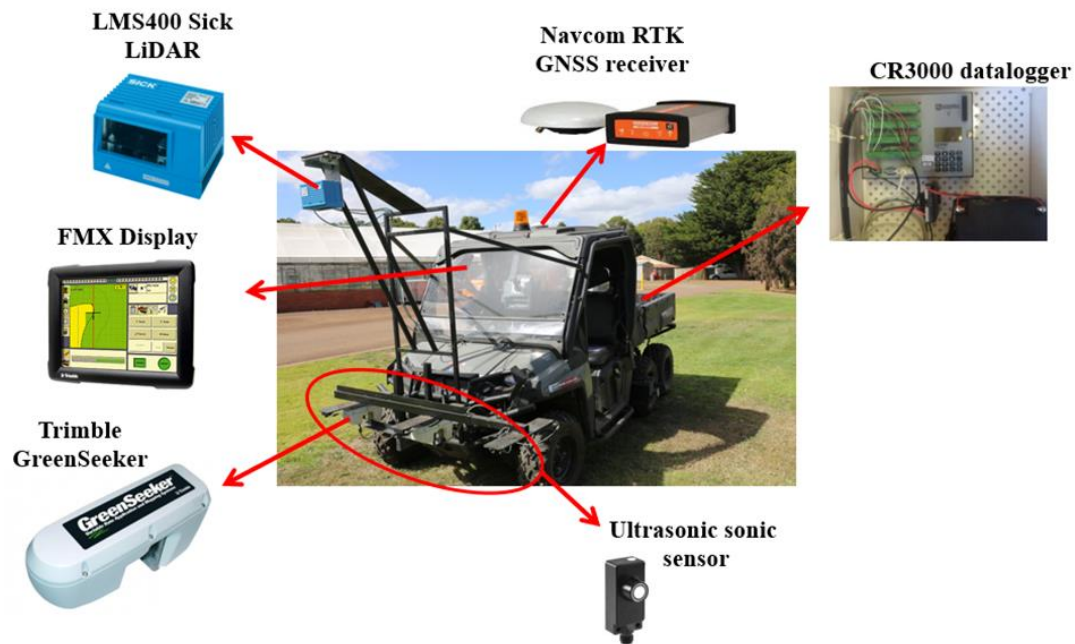


Figure 1 The PhenoRover is a high-throughput phenotyping platform developed and deployed in Agriculture Victoria Research, Hamilton Centre, Australia. Annotations outline the major components of the system.

For plant height measurements, two types of sensors - ultrasonic sonar and LiDAR - were used to collect data from single plants, rows, and plots. Six ultrasonic sonar sensors in three rows were installed onto a 1.45 m wide, custom-made steel bar, rigidly welded onto the PhenoRover. A single LiDAR sensor was installed onto a 2 m high custom-made steel bar rigidly welded to the PhenoRover. In addition, a GNSS antenna (AG-25) was connected on the roof of the PhenoRover and was connected to the RTK-GNSS receiver for generating georeferenced and position data. Ultrasonic sonar sensor measurements and the RTK-GNSS positioning were simultaneously recorded on a data logger system. LiDAR sensor measurements were recorded directly into a Dell computer.

3.2.1.1 PhenoRover Platform Data Processing

Phenotypic data were collected through the PhenoRover system, then transferred to a computer for further processing. The data collected from the sensors are exported and stored on a computer in the following formats. Ultrasonic sonar and GreenSeeker data were stored in the form of .dat and .shp files, respectively. The LiDAR data was stored in a .bin file format and consecutively transformed into a .txt file.

3.2.1.2 General Steps for Geo-referencing Data

Ultrasonic sonar and GreenSeeker sensors measurements were georeferenced in a similar approach to those described previously (Wang *et al.* 2016; Thompson *et al.* 2018; Gebremedhin *et al.* 2019a). The RTK-GNSS data was projected to the Universal Transverse Mercator (UTM) coordinate system. The field site is located in the UTM-World Geodetic System (GWS84) zone 54S. The Easting and northing offset of each sensor in relation to the RTK receiver is then used to calculate exact coordinates of each measurement for each sensor. Height data from the ultrasonic sensors were extracted from individual plant information using a quantitative GIS (QGIS Development Team, 2017, Raleigh, NC, USA) (<http://www.qgis.org>) based on each plant's boundaries. Geo-referenced boundary information is required to locate a measurement within a targeted individual plant. Therefore, rectangular area boundaries of plants' positions were established using the four corner coordinates in QGIS. Geo-referenced individual plant data were superimposed on pre-defined polygon shapefiles of the selected individual plants in QGIS. Polygons with ID numbers were manually drawn around the individual plants. The data processing and extraction were performed through the processing steps of the HTP geo-processor plug-in. The geo-processor under the HTP geo-processor plug-in completed the processing of plant height displaying the attribute table as a final output of individual plant heights along with the ID numbers of the plants.

For the LiDAR height data extraction, the height of individual plants was extracted manually from graphical readings of the SOPAS (SOPAS engineering tool 3.3.2, SICK, Inc., Minneapolis, MN, USA) software (Figure 2). Firstly, the distance between the sensor

and ground (cd) was determined, in which LiDAR height was fixed at 2 m from the ground. Graph of scanned individual plants shape was displayed on the software at the time of scanning. Reeding of the maximum height was recorded to calculate the maximum height of the scanned graph (ab). Finally, the height of the plant was determined as follows:

$$\text{Plant height} = cd-ab \dots\dots\dots 1$$

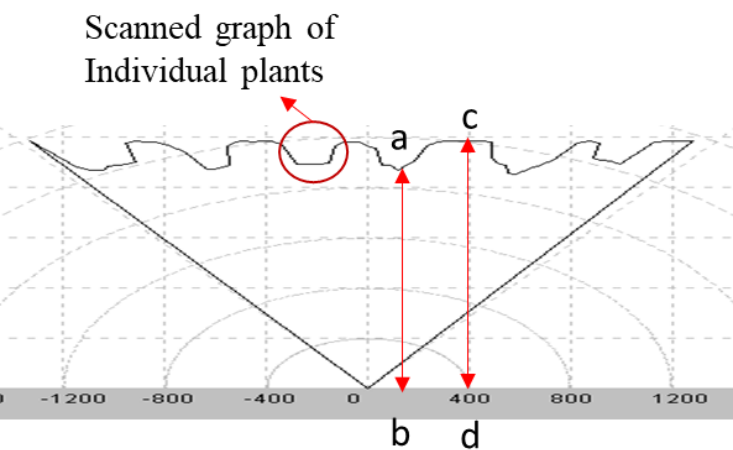


Figure 2 Scanned graph representative display of individual plants measured using LMS 400 sick LiDAR.

3.2.2 Description of the Handheld GreenSeeker NDVI Data Collection

Handheld GreenSeeker NDVI measurements were performed by walking at a similar speed to the PhenoRover. The handheld GreenSeeker was linked to display the plot numbers and timestamps of measurements. The measurements included the reflectance at the red and near-infrared as well as the calculated NDVI values. NDVI was calculated as follows;

$$NDVI = \frac{NIR - R}{NIR + R}$$

Where NIR stands for reflectance at near-infrared waveband and R stands for reflectance at red waveband. The information was saved in an iPad Pro-11 tablet computer (Apple, Cupertino, California, United States) as a .csv file.



Figure 3 An aerial platform (3DR Solo) equipped with a Parrot Sequoia multispectral camera on board for HTP of perennial ryegrass plants deployed in Agriculture Victoria Research, Hamilton Centre, Australia.

3.2.3 Description of the Aerial Platform

A low-cost UAS, 3DR Solo (3D Robotics, Berkeley, CA, USA) equipped with a Parrot Sequoia multispectral camera was used for HTP of the large perennial ryegrass breeding experiment at Hamilton, Victoria, Australia (Figure 3). Flights were conducted one day before the manual harvest. Images collected were set to have an 80% forward and 75% sideways overlap. The average ground sample distance was 1.99 cm/pixel for the 20 m altitude flight with a speed of 6 ms⁻¹. The flight paths were kept constant throughout all flight dates. Tower Beta software was used for accomplishment of flight missions.

3.2.3.1 Data Processing from UAS

Captured images were processed on Pix4DMapper Pro (Pix4D SA, Lausanne, Switzerland, <http://pix4d.com>) to generate an orthomosaic for each camera band. Nine ground control points (GCPs) were geolocated in the field. The GCP coordinates were captured with a Trimble RTK GPS (Navcom SF-3040, CA, USA), with a horizontal/vertical accuracy of 1 cm + 0.05ppm/2 cm + 1 ppm at each GCP points respectively. GCPs were made from checkered linoleum squares, fixed on the ground. Smaller GCPs were 35 x 35 cm in size, and larger GCP's were 50 x 50 cm. Maintenance of the GCPs was done regularly to keep them visible for the geo-referencing during data extraction. GCP positions were imported using the ray cloud editor, and manually tied to at least three images and automatically projected to the remaining images. Radiometric calibration of images was performed within the index calculator processing step of Pix4D using airinov calibration plate. Images of known reflectance values of the airinov plate were taken at the beginning of each flight. The image processing method elaborated by (Hassan *et al.* 2018) was followed. It included building point clouds, generate the digital surface model (DSM) as well as generating orthomosaics. Once index values were created as .shp files, the quality of the data was reviewed with root mean square error (RMSE < 2 cm) and the georeferenced orthomosaic .shp file was saved to the project file to finalise the processing. QGIS software was used for image segmentation. Polygon shapes that delineated to the measured plots and individual plants were generated with a specific plant ID defining the plant positioning boundaries. An index map was then overlaid with polygon shapes for each sampled plant in the coordinate reference system of the experimental area. Finally, the attribute table of the NDVI plot means was exported to an Excel spreadsheet file for further statistical analysis.

3.2.4 Data Collection for Sensors and Platforms Calibration

To evaluate and calibrate the sensors on PhenoRover and aerial platforms, we conducted experiments under controlled and field conditions.

3.2.4.1 Controlled Environment Experiments

For the controlled environment experiments, experiments were conducted under natural light and temperature conditions mainly on the PhenoRover system in a shade-house at the Hamilton Centre and details are presented as follows.

- To calibrate the accuracy of simultaneous height measurements using six ultrasonic sonar sensors and one LiDAR sensor (Figure 4a), foam bricks made from various sizes of polystyrene blocks were prepared. The bricks were prepared to various heights representing actual perennial ryegrass plant height ranges. Five bricks with heights ranging from 2 to 60 cm were used. Measurements were conducted with the stationary PhenoRover.
- The artificial plants experiment was conducted by considering their resemblance to actual ryegrass plants. Artificial plants were prepared from cable ties and foam bricks to evaluate the possibility of using ultrasonic sonar sensors to measure individual artificial plants (Figure 5a). Cable ties were arranged at a uniform height on foam bricks. The measured height of the artificial plants ranged from 10 to 35 cm. Artificial plant height measurements using six ultrasonic sonar sensors were calibrated at 10, 15, 20, 25, 30 and 35 cm height levels of the artificial plants. Two rounds of measurements were conducted with the stationary PhenoRover.
- To evaluate ultrasonic sonar's and LiDAR sensors' capability to measure individual ryegrass plants, individual perennial ryegrass plants were planted in one-litre pots and grown in a glasshouse at Hamilton Centre (Figure 6a). The plants were arranged perpendicular to the ultrasonic sonar and LiDAR sensors for measuring height with a stationary PhenoRover. Measurement of each plant was conducted using the six ultrasonic sonar sensors and the estimated plant height was averaged. Actual height was determined at three points within each pot from the soil surface to the tip of the grass leaves.

3.2.4.2 Field Experiments

Multiple field experiments were used to calibrate sensors and phenotyping platforms. Firstly, an established genomic sub-selection experiment was used to calibrate the possibility of estimating individual plant heights using LiDAR and ultrasonic sonar sensors. This was also used to extract NDVI and estimate herbage yield from individual plants. Secondly, an established field experiment was used to calibrate the possibility of estimating aboveground biomass in small-swards using NDVI or combination of NDVI and plant height measurements. The details of these experiments are as follows:

1) Genomic Sub-Selection Field Experiment

The Genomic sub-selection field experiment comprised of 48,000 spaced-planted perennial ryegrass plants, from 50 advanced breeding lines and cultivars, and was established in June 2016. It was a randomized completed block design with 10 replicates of the 50 lines and a total of 500 plots in a 50 (column) by 10 (row) layout. Each plot consisted of three rows of 32 individual plants each (i.e. 96 plants/plot). The spacing between row was 60 cm and the spacing between plant within a row was 25 cm. Hence, each plot was in an area of 8 m (length) $\times$ 1.8 m (width). Two sets of phenotypic data calibration experiments were conducted:

- a) For plant height calibration, single plant height data were collected using six ultrasonic sonars and one LiDAR on the PhenoRover system. Ultrasonic sonar sensors were arranged to measure three rows for every pass. Three breeding lines were used for this experiment. Plant height from a subset of 96 individual plants was measured by the PhenoRover system with a constant speed of $\sim 1.4 \text{ ms}^{-1}$ enabling each sensor to capture data at the three-leaf growth stage. Data from ultrasonic sonar sensor measurements and RTK-GNSS were recorded in the data logger. The data from LiDAR was stored in a laptop computer. For the manual measurements, a standard measuring ruler was used to measure the perennial ryegrass height of single plants from the ground level to the longest leaf of the canopy.

- b) For the above-ground herbage yield estimation, images were captured a day before harvest and processed following the image processing procedures (details in section 3.2.3) to extract NDVI data from single plants. All sampled plants were harvested individually and fresh biomass was immediately transferred to the laboratory for fresh herbage yield determination and then oven-dried at 60°C for 48 hours to determine dry herbage yield.

2) LP F₂ Field Evaluation Experiment

The LP F₂ field evaluation experiment comprised 72 plots of F₂ and F₁ generations of different breeding lines and commercial cultivars of perennial ryegrass. The experiment was established in June 2015 to compare the herbage production of F₁ and F₂ generations in the field. The size of each plot was 1.5 m x 1.5 m, with 100 seedlings planted in 10 x 10 grid. Plots were 1 m apart and the design of the experiment was split plot design with to replicate. The cutting regimes of the plots were aligned to the three-leaf growth stage.

For the phenotypic data collection, NDVI from handheld GreenSeeker, plant height measurement using a ruler and mowing off at 5 cm above ground level for all plots were performed. The data collected was used to calibrate the use of NDVI alone to estimate plots aboveground herbage yield or with combination with plant height (NDVI * plant height). Measurements of NDVI using a GreenSeeker sensor and plant height using a standard ruler were performed one day before the three-leaf stage mowing off. Mowed fresh herbage yield was immediately transferred to the laboratory for fresh herbage yield determination and then oven-dried at 60°C for 48 hours to determine dry herbage yield. Plant height measurements were performed at four different parts of each plot and the average height was used as the plot-level plant height.

3.3. Results

3.3.1 Plant Height Estimation in the Controlled Environment

Correlations between foam brick height measurements from six ultrasonic sonars and a LiDAR were compared with a ruler measurement (Figure 4). The R² values of the foam

bricks height measurements using the ultrasonic sonar and LiDAR versus a ruler was very high ($R^2 = 0.99$). Height estimation using ultrasonic sonar and LiDAR was comparable (RMSE < 0.4 cm). Ultrasonic sonar height measurements were comparable to ruler height measurements on the artificial plants ($R^2 = 0.99$, RMSE = 0.7 cm) (Figure 5). However, the reality of perennial ryegrass growth habit is more complex than that of the artificial plants in (Figure 6a). Thus, more complex artificial plant structures were constructed, and the ultrasonic sonar sensors could only record the maximum height neglecting the smaller artificial leaves (data not shown).

Furthermore, the ultrasonic sonar and LiDAR sensors of the PhenoRover system were calibrated with perennial ryegrass plants in pots (Figure 6). Results of plant height measurements of both ultrasonic sonar and LiDAR to ruler measurements indicated a high correlation ($R^2 = 0.86-0.93$). The estimation of plant height with LiDAR and ultrasonic sonar had low RMSE of 3.05 cm and 1.81 cm respectively.

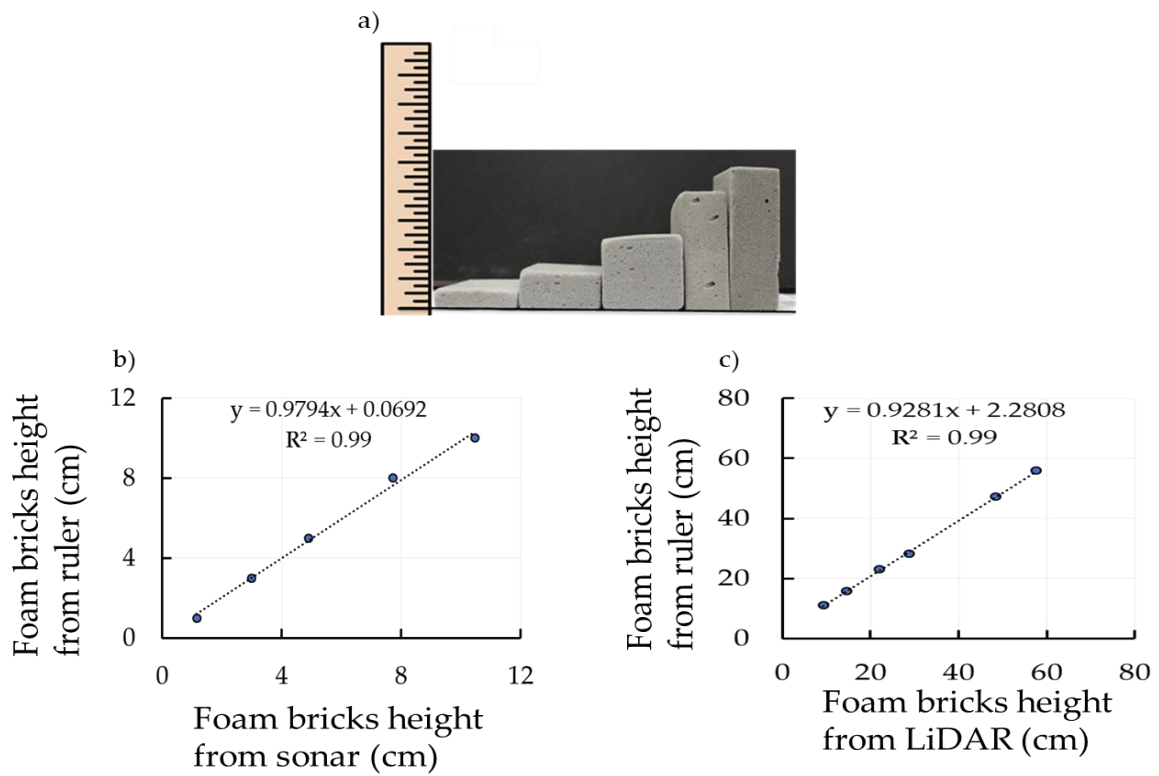


Figure 4 Picture of foam bricks plants used to calibrate ultrasonic sonar (a); and the correlation between height derived from ultrasonic sonar sensor and a ruler (b); and LiDAR and a ruler (c).

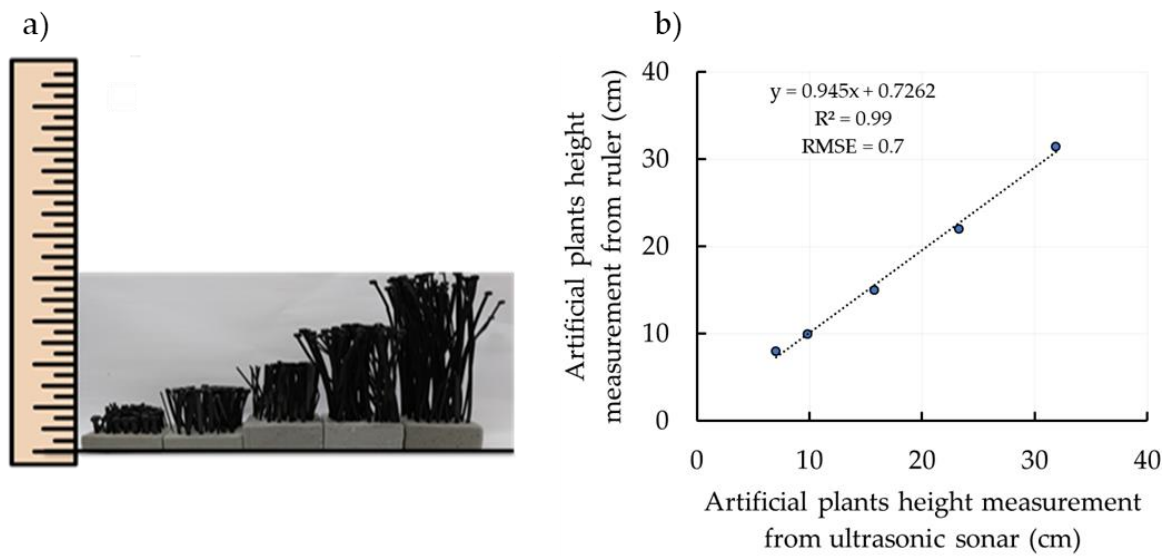


Figure 5 Picture of artificial plants used to calibrate ultrasonic sonar (a) and the correlation between artificial plants height derived from ultrasonic sonar sensor and a ruler (b)

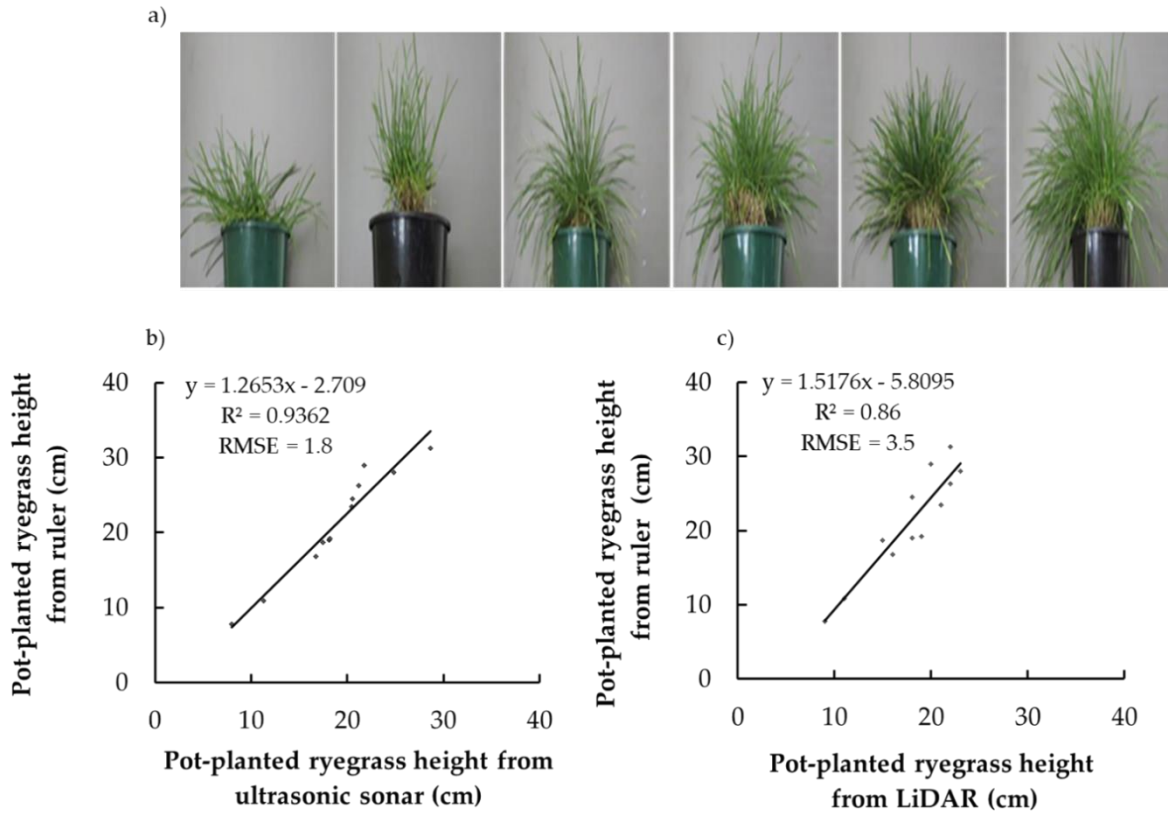


Figure 6 Pictures of the perennial ryegrass sampled plants in pots (a) and the correlation between pot planted perennial ryegrass height derived from ultrasonic sonar sensor and a ruler (b) and LiDAR sensor and a ruler (c)

3.3.2 In-field Plant Height and Herbage Yield Phenotyping

3.3.2.1 Height and Herbage Yield Estimation for Individual Plant

After confirming the ability of the UAS and PhenoRover system platforms to assess phenotypic traits at the controlled environment, the platforms were deployed in the field for individual perennial ryegrass plant phenotyping. Figure 7 shows the Pearson correlation coefficient (r) for the parameters of fresh herbage yield, dry herbage yield,

NDVI, plant height from ultrasonic sonars, a LiDAR and a ruler. Plant heights based on both ultrasonic sonar and LiDAR were significantly ($p < 0.001$) correlated ($r = 0.75-0.87$ respectively) with the manually measured plant heights. However, measurements using ultrasonic sonar versus a ruler showed slightly weaker correlation than with LiDAR ($r = 0.75$ vs $r = 0.87$). A significant correlation was also found between the plant height measurements from ultrasonic sonar and LiDAR sensors ($r = 0.75$).

The height measurements from the ultrasonic sonar and LiDAR, as well as NDVI from multispectral imaging, were used to estimate fresh/dry herbage yield. Fresh and dry herbage yield measurements showed a strong correlation with plant height from LiDAR and NDVI values ($r = 0.75-0.80$). A slightly lower correlation was recorded between plant height from ultrasonic sonar and measured fresh and dry herbage yield ($r = 0.67-0.68$). However, NDVI values were moderately correlated ($r = 0.44-0.56$) to plant height measurements from ultrasonic sonar, LiDAR, and a ruler.

Figure 8 shows the Pearson correlation coefficient between dry weight, a combination of measurements of NDVI and height from ultrasonic sonar (NDVI*Sonar), NDVI, and height from LiDAR (NDVI*LiDAR), NDVI and height from the ruler (NDVI*PH). Results indicated improvement in the correlation by combining NDVI and plant height to estimate fresh/dry herbage yield. Both NDVI*LiDAR and NDVI*PH values showed strong correlations ($r = 0.83-0.85$) with fresh and dry herbage yield ($p < 0.0001$). However, NDVI*Sonar produced slightly lower correlation ($r = 0.75$) with the fresh and dry herbage yield than the NDVI*LiDAR and NDVI*PH. The correlation between NDVI*LiDAR vs NDVI*PH showed higher correlation ($r = 0.90$) than NDVI*sonar vs NDVI*PH ($r = 0.82$) and NDVI*LiDAR vs NDVI*sonar ($r = 0.80$).

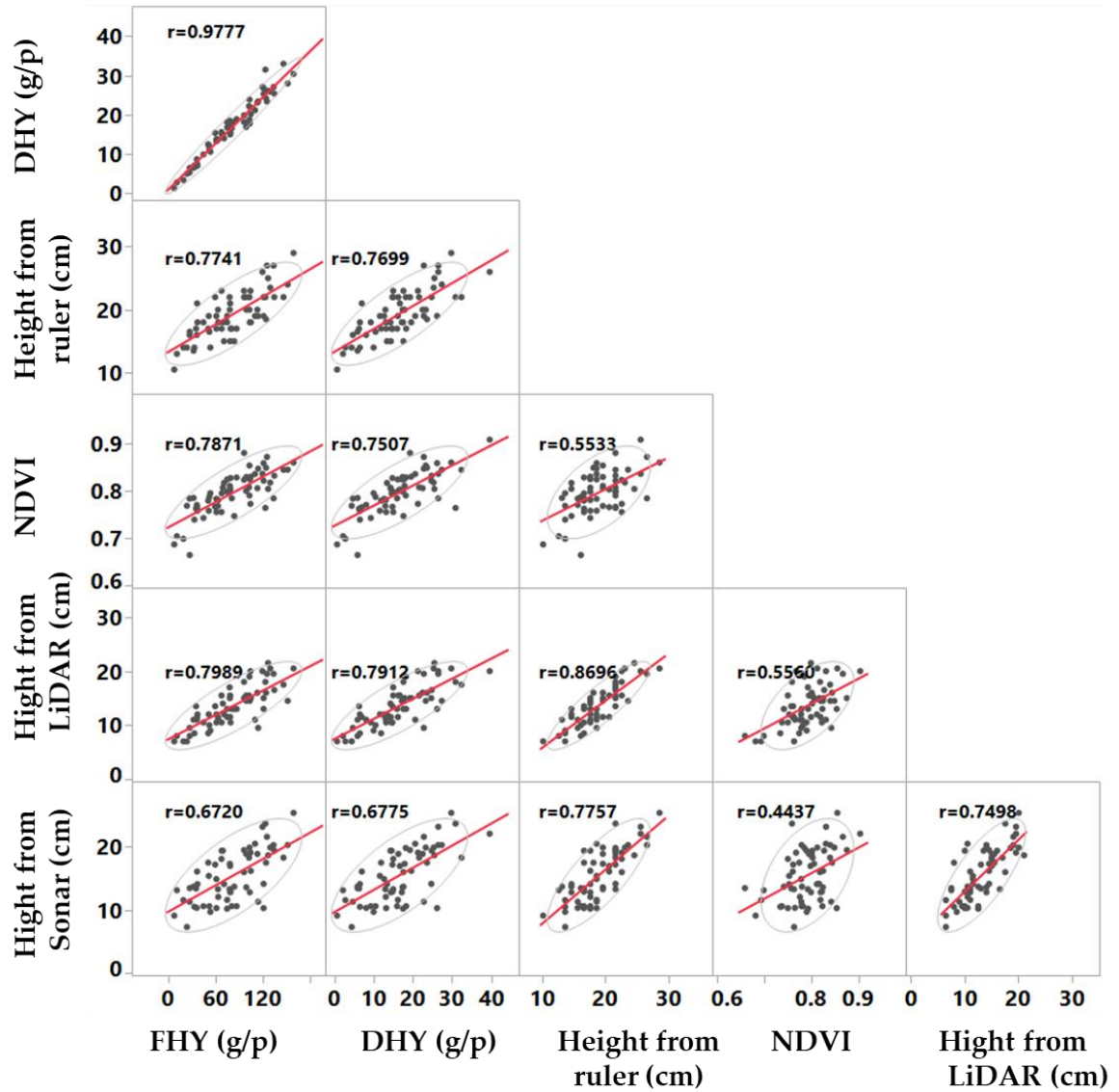


Figure 7 Pearson correlation coefficients between fresh herbage yield (FHY) and dry herbage yield (DHY) and plant height from the ruler, ultrasonic sonar, LiDAR of the PhenoRover system and NDVI from UAS measured at the time of three-leaf stage perennial ryegrass individual plants harvest. $P < 0.001$ for all correlations

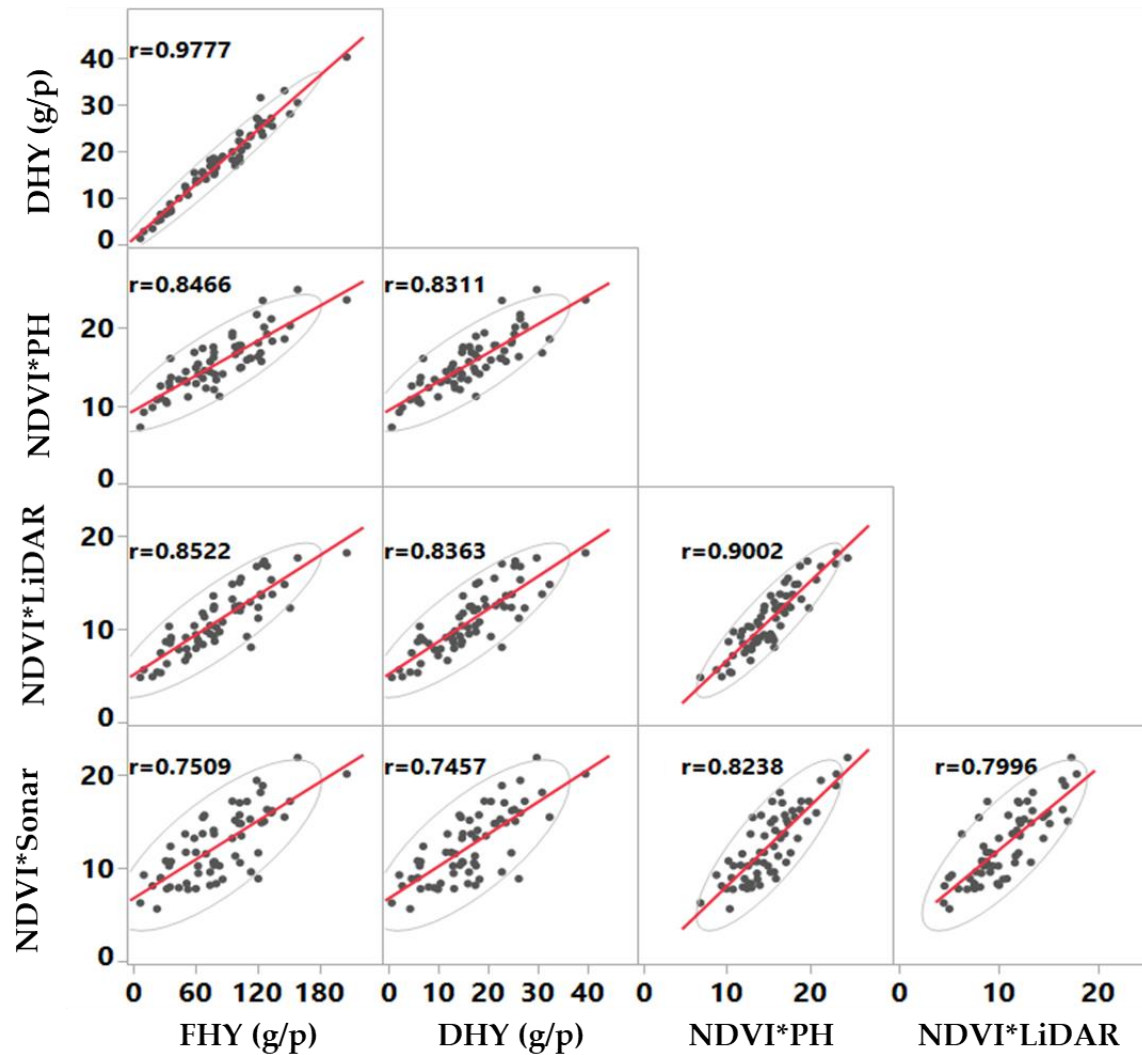


Figure 8 Pearson correlation coefficients between fresh and dry herbage yield and combining plant height from a ruler, ultrasonic sonar, and LiDAR of the ground-based platforms and NDVI from UAS measured at the time of harvest. $P < 0.0001$ for all correlations

3.3.2.2 Sward Plots Height and Herbage Yield Estimation

Data from the LP F₂ field trial experiment was used to evaluate and compare the NDVI and NDVI combined with plant height methodologies for estimating above-ground biomass. We evaluated the use of mean plot NDVI for fresh herbage yield (FHY) and dry herbage yield (DHY) estimation and we obtained strong correlations ($R^2 = 0.63$, RMSE

= 493 g) and ($R^2 = 0.57$, RMSE = 102.6 g) respectively (Figures 9 a & d). Similarly, plant height had high correlations with both FHY and DHY with R^2 values of 0.65 and 0.61 respectively (Figures 9 b & e). Incorporation of plant height improved the estimation accuracy of aboveground biomass with strong correlation of FHY ($R^2 = 0.73$, RMSE = 423.6 g) and DHY ($R^2 = 0.68$, RMSE = 90.5 g) (Figures 9 c & f). A combination of NDVI and plant height (NDVI*plant height) improved 7-10% prediction accuracy for both FHY and DHY. This also illustrates the robust estimation of FHY and DHY, overcoming the shortcomings of an NDVI- only approach.

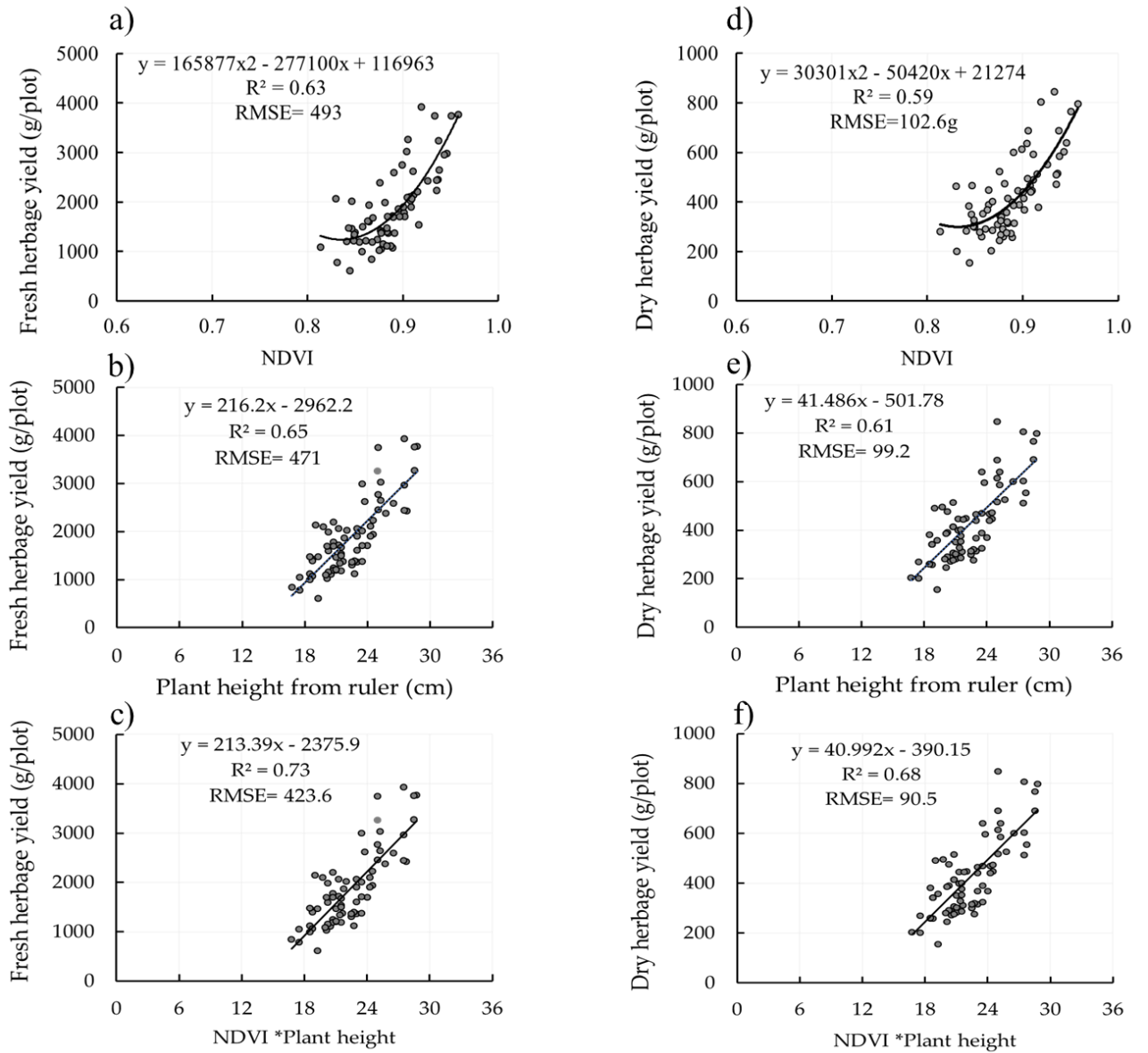


Figure 9 Comparisons of herbage yield estimation methods; a) Relationship between plot fresh herbage yield and plot mean NDVI; b) Relationship between plot fresh weight and plot mean plant height; c) Relationship between plot fresh herbage yield and plot mean combination of NDVI and plant height (NDVI*plant height); d) Relationship between plot dry herbage yield and plot mean NDVI; e) Relationship between plot dry herbage

yield and plot mean plant height, and f) Relationship between plot dry herbage yield and plot mean combination of NDVI and plant height (NDVI*plant height).

3.4 Discussion

Current forage yield phenotyping methods in breeding programs are mainly dependent on visual scoring, manual estimation, sample clipping, and mowing. However, these methods are highly time-consuming, costly and often subjective. Therefore, HTP platforms have been proposed to resolve the current assessment limitations (Walter *et al.* 2012; Gebremedhin *et al.* 2019b). In this chapter, the calibration and validation of the PhenoRover system and UAS phenotyping platforms to non-destructively phenotype perennial ryegrass height and herbage yield traits of extensive population breeding program are described. The deployments of the sensors on platforms provide several advantages for phenotyping herbage yield and related traits. These include: 1) the possibility of measuring traits continuously over the growth time of plants compared with the current approach that focuses on single time measuring; 2) measurements will be objective and more consistent; 3) measurements are faster, especially in experiments with large populations; 4) the collection of data for traits not amenable to current methods of phenotyping (e.g. pixel number, sward density, and volume of individual plants).

The PhenoRover system and UAS-based phenotyping system proved capable of measuring plant height and estimating herbage yield of perennial ryegrass. Results from the PhenoRover system with ultrasonic sonar and LiDAR sensors indicate the capability of replacing manual plant height measurements by automated real-time measurements. The ultrasonic sonar was successfully calibrated in the controlled environment ($R^2 > 0.93$, RMSE < 2 cm) with manual measurements of foam bricks, artificial plants, and pot-planted individual ryegrass plants. The LiDAR sensor was also successfully calibrated in the controlled environment ($R^2 > 0.86$, RMSE < 3.5 cm) with manual measurements of foam bricks and individual ryegrass plants in pots. Similar result was reported in a previous controlled environment study on maize plants (Reiser *et al.* 2018).

In the field experiments, the PhenoRover system with ultrasonic sonar and LiDAR sensors to estimate plant height indicated the possibility of automating plant height

measurements. Plant height measurements from ultrasonic sonar correlated well ($r = 0.78$) with manually measured plant height using a ruler. The estimation of plant height using ultrasonic sonar was comparable with previous experiments on Bermudagrass, alfalfa, wheat and barley (Pittman *et al.* 2015; Barmeier *et al.* 2016). Plant height measurements from LiDAR correlated well ($r = 0.87$) with manually determined plant heights. Plant height measurements LiDAR estimated individual plant height with high accuracy than ultrasonic sonar. However, the correlation coefficient of between manual measured height and LiDAR height was lower than other studies (Madec *et al.* 2017; Sun *et al.* 2017; Jimenez-Berni *et al.* 2018). Various reasons may explain this lower estimation: i) the limitations can be explained by the perenniality of ryegrass morphology at the time of measurement; ii) no action was taken for any noise removal technique, as occurred in other studies (Madec *et al.* 2017; Jimenez-Berni *et al.* 2018).

Multispectral imaging and GreenSeeker scanning were used to estimate the herbage yield of single plants and sward plots, respectively, with NDVI derived from UAS and ground-based proximal sensing. The results showed there were high correlations when NDVI derived from UAS was used to estimate single plants FHY and DHY with a correlation coefficient of ($r = 0.77$ or $R^2 = 0.59$). It was observed that when mean NDVI of sward plots was used with non-linear regression, a higher R^2 value, ranging 0.59-0.63, was found for fresh and dry herbage yield for perennial ryegrass swards. Similar results have been found for the biomass of maize ($R^2 = 0.52$) (Freeman *et al.* 2007) and rice ($R^2 = 0.69$) (Gnyp *et al.* 2014). Additionally, Pittman *et al.* (2015) observed R^2 values between 0.62-0.75 for NDVI when regressed with alfalfa, Bermuda grass and wheat dry herbage yield.

Plant height measured by ultrasonic sonar sensors predicts perennial ryegrass herbage yield moderately well ($r = 0.68$) at the three-leaf stage. Similar results have been reported (Fricke *et al.* 2011; Pittman *et al.* 2015; Moeckel *et al.* 2017). The estimation of herbage yield using the LiDAR sensor was better ($r = 0.8$) compared to that using ultrasonic sonar. However, the high cost and technical difficulties in extracting data of the LiDAR system is a major limiting factor for its application (Niu *et al.* 2019). The estimation of herbage yield using plant height also depends on the density of the plant structure (Fricke *et al.* 2011). In measurements through ultrasonic sensors, there is a high chance to reflect echo when there is a density canopy that could result in high correlation between plant height

and measured HY (Yuan *et al.* 2018). Similarly, there was a good correlation ($R^2 = 0.61$ - 0.65 or $r = 0.78$ - 0.81) between average sward plant height measured using a ruler and herbage yield. Similar results were found for tall fescue ($R^2 = 0.61$ or $r = 0.78$) (Schaefer and Lamb 2016) and alfalfa and Bermudagrass ($R^2 = 0.67$ or $r = 0.82$) (Pittman *et al.* 2015).

In previous work, the combination of spectral and structural information was reported to improve biomass estimation of cereal crops (Bendig *et al.* 2015; Tilly *et al.* 2015) and herbage yield estimation of forage crops (Safari *et al.* 2016; Schaefer and Lamb 2016). The results in this study confirmed that the addition of plant height into the herbage yield estimation model based on NDVI enhanced the model accuracy. When estimating HY based on the combination of NDVI and plant height, the estimation accuracy was improved by 7-10% for both fresh and dry herbage yield estimation of single plants and sward plots. However, combining NDVI and plant height may not always improve biomass predictions. For example, there was no improvement in the estimation performance when plant height derived from UAS-RGB imagery was added into the multivariable linear regression model based on vegetation indices (Niu *et al.* 2019). Therefore, further samples and combination methods for NDVI and plant height are required to see the practical significance.

While the results presented are encouraging, there are some limitation of this chapter that could be addressed going forward in the following chapters. This includes increasing the number of samples and replicating the calibration the sensors across seasons may prove the stability of the phenotypic system. The size of the samples used were small and experiments were performed with out further replication. Modeling of various forms of combining methods of NDVI and plant height will be much more powerful to check if the statistical difference among using NDVI, plant height and a combination of both to predict biomass of individual plants. Moreover, further samples that includes calibration and validation sets across seasons and years which are not included in this chapter must be taken in to consideration in further studies.

3.5 Conclusions

In this study, we calibrated and validated the ground-based PhenoRover system and Aerial-based UAS platforms for remote-sensing of plant height, NDVI and herbage yield. The PhenoRover system demonstrated a capacity for non-destructive NDVI and plant height measurements in the field using GreenSeeker, ultrasonic sonar and LiDAR. It also accommodated additional RTK-GNSS sensor to accurately geo-locate sensor data. The UAS demonstrated an ability to estimate NDVI values from images captured through multispectral cameras. In the future our UAS is expected to accommodate various sensors including RGB cameras, LiDAR, hyperspectral and thermal cameras to capture multiple information simultaneously.

The deployment of ground-based PhenoRover system and aerial-based UAS platforms were effective for perennial ryegrass plant height and herbage yield estimation. These platforms provide methods for accurate, non-destructive, time-effective estimation of herbage yield of single plants and sward plots in perennial ryegrass field experiment. The high-throughput nature of the platforms allows non-destructive monitoring and measuring of thousands of genotypes through the development of predictive models to estimate plant height and herbage yield.

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Chapter 4. Development and Validation of a Model to Combine NDVI and Plant Height for High-Throughput Phenotyping of Herbage Yield in a Perennial Ryegrass Breeding Program

Chapter 4 is based on peer reviewed journal article that was published in the Remote Sensing journal in 2019

The details of the original article are as follows

Gebremedhin, A.; Badenhorst, P.; Wang, J.; Giri, K.; Spangenberg, G.; Smith, K. (2019) Development and validation of a model to combine NDVI and plant height for high-throughput phenotyping of herbage yield in a perennial ryegrass breeding program. *Remote Sensing* **11**, 2494.

Article

Development and Validation of a Model to Combine NDVI and Plant Height for High-Throughput Phenotyping of Herbage Yield in a Perennial Ryegrass Breeding Program

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Received: 28 August 2019; Accepted: 21 October 2019; Published: 25 October 2019



Abstract: Sensor-based phenotyping technologies may offer a non-destructive, high-throughput and efficient assessment of herbage yield (HY) to replace current inefficient phenotyping methods. This paper assesses the feasibility of combining normalised difference vegetative index (NDVI) from multispectral imaging and ultrasonic sonar estimates of plant height to estimate HY of single plants in a large perennial ryegrass breeding program. For sensor calibration, fresh HY (FHY) and dry HY (DHY) were acquired destructively, and plant height was measured at four dates each in 2017 and 2018 from a selected subset of 480 plants. Global multiple linear regression models based on K-fold and random split cross-validation methods were used to evaluate the relationship between observed vs. predicted HY. The coefficient of determination (R^2) = 0.67–0.68 and a root mean square error (RMSE) between 5.43–7.60 g was obtained for the validation of predicted vs. observed DHY. The mean absolute error (MAE) and mean percentage error (MPE) ranged between 3.59–5.44 g and 22–28%, respectively. For the FHY, R^2 values ranged from 0.63 to 0.70, with an RMSE between 23.50 and 33 g, MAE between 15.11 and 24.34 g and MPE between ~22% and 31%. Combining NDVI and plant height is a robust method to enable high-throughput phenotyping of herbage yield in perennial ryegrass breeding programs.

Keywords: multispectral imaging; herbage yield; high-throughput phenotyping; NDVI; plant height; ultrasonic sonar

1. Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important temperate forage crops in Australia for dairy, meat and wool production and it makes a significant contribution to Australia's grazing industries valued at \$8 billion/annum [1]. Herbage yield (HY) improvement is one of the primary ryegrass breeding targets, yet it is not easy to improve because phenotyping herbage yield is laborious, costly and must be performed multiple times within a year and across multiple years [2–4]. The development of new cultivars may take up to 15 years, where repeated phenotyping of herbage yield and related traits is required at every harvest. However, performing measurements using

current methods on large numbers plants or plots is slow and expensive, making it difficult to include large numbers of individual plants or plots [5]. This makes it challenging to capture an accurate representation of the population and estimate the correct values for individual genotypes. Therefore, HY estimation requires rapid, non-destructive phenotyping methods that can also facilitate genomic tools (e.g., genomic selection, GS) to shorten the breeding time and accelerate genetic gain. However, the number of plants for GS is likely higher than the number of plants traditionally used by breeders to perform selection breeding (e.g., The DairyBio initiative has 48,000 individual plants for genomic sub-selection breeding) where phenotyping of individual plants require accurate evaluation [5]. In recent years, high-throughput phenotyping (HTP) technologies have brought new insights to evaluate phenotypic traits efficiently in large breeding programs [6–9].

Previous studies have used sensor-based data sources from aerial and ground-based platforms to estimate biophysical characteristics of various vegetations, including herbage yield of forage crops [5,10,11]. The aerial-based phenotyping platforms are suitable for lightweight red-green-blue (RGB), multispectral, and hyperspectral imaging systems and have used vegetative indices to build models for herbage yield [10,12] and biomass [13–15] estimation of pasture and cereal crops respectively. Normalised difference vegetative index (NDVI) is a widely used vegetative index for estimates of biomass [16–18] with limitations at high-biomass and density of crop cover [19]. Small, low-cost unmanned aerial systems (UAS) and satellite technologies (mainly equipped with multispectral sensors) are mainly deployed to execute the phenotyping task. The UAS system allows proximal sensing of plant phenotypes at a small experimental unit level with high spatial and spectral resolution [20–22].

Another alternative extensively used to estimate herbage yield is plant height [23–25]. Plant structural information metrics may be accessed from terrestrial and aerial sensors. The usefulness of this information depends on the spatial and temporal resolution of the data [13]. Different sensor types have been used to evaluate plant height, such as ultrasonic sonar and LiDAR on the ground and UAS [26–28] as well as structure from motion from digital cameras on UAS [13,29]. Among the mentioned sensors, ultrasonic sonar is inexpensive and user-friendly with broader application for sward pasture measurements [26,30,31]. However, the accuracy of plant height measurement from ultrasonic sonar may be limited by the distance between the sensor and target plant, plant growth habit and the size (density) of the leaves [26,30]. To overcome some of these limitations, fitting ultrasonic sonar to ground-based phenotyping platforms may provide accurate measurements. Indeed, the advantage of a ground-based platform is the suitability capturing data at high resolution with minimal errors [32].

Generally, the performance of vegetative indices from UAS can be limited by saturation at higher levels of crop canopy cover [19]. Combining vegetative indices from photogrammetric imagery and plant height information may improve herbage yield [5]. The combination of NDVI and plant height from terrestrial LiDAR may improve the herbage yield estimation by up to 20% [12]. A similar result has been observed in studies of herbage yield estimation of tall fescue (*Festuca arundinacea*), perennial ryegrass and phalaris (*Phalaris aquatica*) sward plots [33]. In these studies, forage crops were evaluated as swards or under conditions similar to those in commercial paddocks. However, assessment of ryegrass herbage yield at early stages of a breeding program requires an evaluation of large numbers of individual plants, grown under spaced conditions. Non-destructive estimations of plant height and NDVI may provide the structural and spectral information for forage breeders to select elite genotypes from large breeding populations to explore the full potential of GS.

Some biomass and herbage yield estimation methods use advanced multivariate regression techniques for the establishment of a direct relationship between proximally sensed and manually collected parameters. Previous studies have demonstrated the use of linear and non-linear regression models to predict plot biomass from sensor measured plant height in barley experimental plots, NDVI in cover crops and combined plant height, and NDVI in tall fescue [12], winter wheat and barley [15,34]. The performance of the models varies with the experiment and crop types used. However, most of the previous studies focus on plot-level field evaluations for grass and crop management, and no previous

studies have been performed that apply sensors in combination to determine the HY estimations of single plants. In this study, we develop various cross-validation models using NDVI from UAS-based multispectral imaging and plant height from the ground-based vehicle to predict fresh herbage yield (FHY) and dry herbage yield (DHY) in large perennial ryegrass breeding program.

The overall objective of the study was to establish herbage yield estimation models for individual perennial ryegrass plants by using data derived from non-destructive methods including plant height, NDVI and a fusion of plant height and NDVI for eight harvests during the 2017 and 2018 growing seasons. The model predictions were compared with manual measurements to validate them as predictors of HY for individual plants.

2. Materials and Methods

2.1. Experimental Setup

The experiment was carried out on a field at Agriculture Victoria Research's (AVR)-Hamilton Centre (37.8462°S, 142.07375°E, elevation 209 m) located in South West Victoria, Australia. The research site, approximately 0.78 ha, planted with 48,000 perennial ryegrass plants, was established in June 2016 as part of the DairyBio project. The field trial was established to perform genomic sub-selection (GSS, field hereafter) through applications of advanced phenomics and genomics tools. It contains 500 perennial ryegrass plots of fifty cultivars and advanced breeding lines planted as randomised complete block design. Each plot consists of 96 genotypes in a layout of three parallel rows, each with 32 spaced plants per row, 0.60 m spacing between rows, and 0.25 m spacing between individual plants within a row (Figure 1). The size of each plot is $\sim 14.4 \text{ m}^2$ (1.8 m $\times$ 8 m dimension) representing one cultivar/advanced breeding line per plot. Fertiliser application and weed management practices were performed regularly throughout the experiment period.

For this experiment, eight total harvests in the years 2017 and 2018 were performed. At each harvest, a subset of 475 (2017) and 426 (2018) plants from five breeding lines were harvested. Details of the manually collected data, data from the UAS and PhenoRover phenotyping platforms are listed in Table 1.

Two phenotyping systems, ground and UAS-based platforms were used for data collection. A ground-based phenotyping system was used to collect plant height measurements from single plants whereas the UAV used a multispectral imaging system to extract individual plant's NDVI values. The selected plants were assessed for height and cut for fresh and dry weight on the following day.

2.2. Data Collection Platforms

2.2.1. Unmanned Aerial Systems (UAS)

During 2017, aerial images were collected via a Parrot Sequoia (Parrot Drones S.A.S, Paris, France) multispectral camera aboard a 3DR Solo (3D Robotics, Berkeley, CA, USA) UAS at 20 m elevation (Figure 2a). Tower Beta software was used to conduct each flight. Airinov calibration plates (MicaSense Inc., Seattle, WA, USA) with known reflectance values were used to correct the reflectance values of the images.

During 2018, aerial images were collected via a RedEdge-M (RedEdge-M, MicaSense Inc., Seattle, WA, USA) multispectral camera aboard a DJI M100 (DJI Technology Co., Shenzhen, China) UAS to collect images at 20 m flight elevation. New UAS and camera system was used in this season due to the reason AVR decided to upgrade the carrying capacity of the UAS system at Hamilton Centre in the year 2018. Flights were conducted using the Pix4D capture software. Reflectance values from calibration tarps (Tetracam INC. Chatsworth, CA, USA) were used to correct the reflectance values of the images. The multispectral imagery was collected with 1.2 megapixels. The details of UAS data acquisition methods and processing accuracies are listed in Table 2.

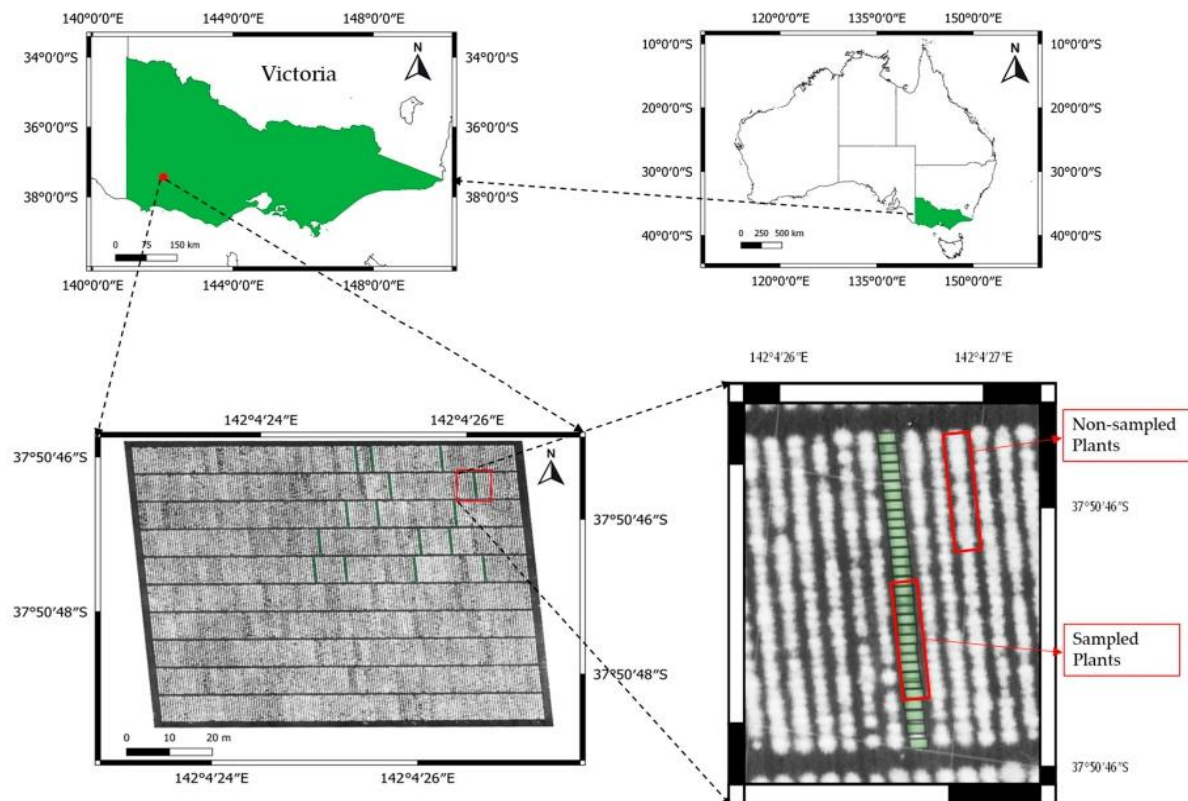


Figure 1. Study site: the perennial ryegrass experiment was conducted at the Hamilton Centre Research Station in Victoria State of Australia in 2017 and 2018. NDVI tiff file is used to display the field trial site sampled plants and non-sampled plants throughout the experiment time.

Table 1. Overview of the data collected from individual plants, data collection date, sensors applied and equipment used for measurements.

Date	Season of the Year	Number of Plants Sampled	Data Acquisition	Sensors/Equipment
9 May 2017	Autumn	475	UAS flight, manual height and fresh and dry HY	Parrot Sequoia, ruler and manual cut
4 July 2017	Winter			
11 September 2017	Early spring			
20 November 2017	Late spring			
19 June 2018	Winter	426	UAS flight, manual height, fresh and dry HY and ultrasonic sonar height	Parrot Sequoia, ruler, manual cut and ultrasonic sonar
20 August 2018	Late winter *			
23 October 2018	spring			
20 November 2018	Late spring			

* No ultrasonic sonar data.

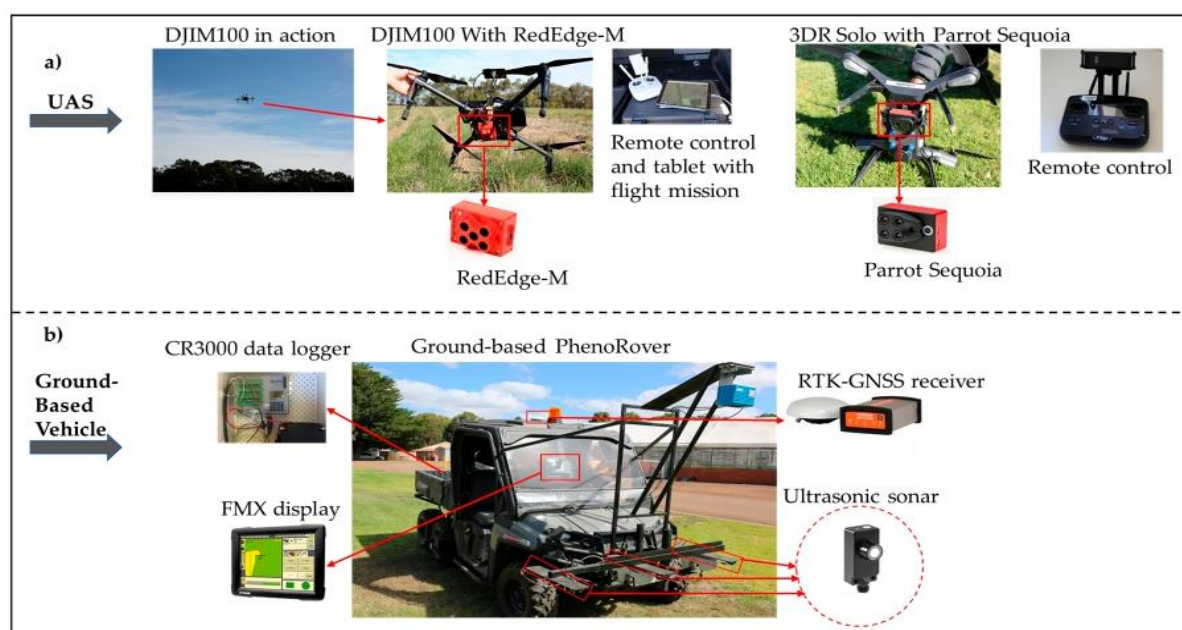


Figure 2. Aerial and ground-based platforms for high-throughput phenotyping (a) UASs platform and sensors; (b) PhenoRover system and sensors at the experimental site.

Table 2. Details of UAS data acquisition and processing quality.

Date	Season	Overlap (Forward/Side)	Flight (m/s)	Flight Time (Minutes)	Georeferencing RMSE (m)	GSD (m/pixel)
2017	Autumn	80%/75%	6	4	0.02	0.02
	Winter	80%/75%	6	4	0.01	0.02
	Early spring	80%/75%	6	4	0.01	0.02
	Late spring	80%/75%	6	4	0.01	0.022
2018	Early winter	75%/75%	6	4	0.01	0.02
	Late winter	75%/75%	6	4	0.03	0.02
	Early spring	75%/75%	6	4	0.04	0.02
	Late spring	75%/75%	6	4	0.03	0.02

2.2.2. Image Processing and NDVI Data Extraction

PiX4D Mapper Pro (version 4.3.31 Pix4D, Lausanne, Switzerland) (<https://pix4d.com>) was used to perform geolocation of captured images by generating orthomosaics. The initial main steps in this process included importing ground control points (GCPs), aligning and reoptimizing of images. The next step included building dense point clouds, building orthomosaic, performing radiometric calibration (2017 data) and calculating NDVI [21]. Nine GCPs were distributed across the field trial site for increasing accuracy and noise reduction (Figure 1). Accurate geographical locations of GCPs were obtained using a Trimble RTK GNSS receiver (Navcom SF-3040, Nav Com Technology Inc., Torrance, CA, USA), with a horizontal/vertical accuracy of 0.01 m + 0.5 ppm/0.02 m + 1 ppm respectively. The values of the geometric root mean square errors of the GCPs and ground sample distances (GSD) of the processed data are indicated in Table 2 were used to evaluate the accuracy of the orthomosaic images.

For the 2017 data, single plants NDVI value was calculated from the orthophotos using zonal statistics in the QGIS 2.18.2 (QGIS Development Team, 2017, Raleigh, NC, USA) (<https://www.qgis.org/en>) software environment. This was performed by manually projecting polygons on to each sampled single plant in the coordinate reference system of the trial.

For the 2018 data TIFF files of index values from individual bands were loaded to eCognition software (eCognition Developer 9, Trimble, Munich, Germany). In the eCognition workflow, calibration

of TIFF index values was performed based on the extrapolation of digital numbers from the calibration tarps of known reflectance. Next, the calculation of VI, manual segmentation, and classification of VIs were performed, followed by NDVI value extractions.

2.2.3. PhenoRover System Field Deployments

The PhenoRover system is a ground-based platform consisting of a Polaris Ranger 6 × 6 side-by-side vehicle system (Polaris Industries Inc., Medina, MN, USA) and a range of integrated sensors (Figure 2b). The PhenoRover phenotyping platform was designed for collecting pasture height data, and the development was completed at Hamilton in 2016. The PhenoRover was deployed at the beginning of 2018 and proceeded to collect data at each three-leaf stage harvest at an average speed of 1.4 ms^{−1}. Six ultrasonic sonar sensors (UNDK 30U6103/S14, Baumer group, Frauenfeld, Switzerland) were installed onto a sensor boom, which was 1.45 m wide custom-made steel rigidly welded to the PhenoRover front. The six ultrasonic sonar sensors in total collected height data in three rows and two columns of plants on each pass of the PhenoRover over the field trial. All ultrasonic sonar sensors were mounted at 0.6 m above the ground and arranged in three rows of 0.6 m apart in a nadir view. The ultrasonic sonars have a high repeatability accuracy of 0.0005 m. The ultrasonic sonar sensors have a measuring range from 0.1 to 1.0 m, with a sonic frequency of 240 KHz taking measurements at 10 Hz. This allows for height measurement to be captured every 0.05–0.10 m when driving at 1.4 ms^{−1}. In addition to the ultrasonic sensors, one Global Navigation Satellite System (GNSS) antennae (AG25, Trimble, Westminster, CO, USA) was installed at the rooftop of the PhenoRover system and connected to a real-time kinematics GNSS (RTK-GNSS) receiver (FMX Integrated Display, Trimble, Westminster, CA, USA) for generating georeferenced and geolocated sensor data. Moreover, to record the ultrasonic sonar measurements a Campbell Scientific CR3000 datalogger (Campbell Scientific, Inc., Logan, UT, USA) was fitted on the back of the PhenoRover system.

2.2.4. Plant Height Data Extraction from the Ultrasonic Sonar

Ultrasonic sonar sensors measurements were processed using the methodology of Thompson et al. [35] and Wang et al. [36] (Figure 3). Two main steps are followed for analysing the data: (1) georeferencing of sensor and RTK-GNSS data through projecting to the Universal Transverse Mercator (UTM) coordinate system. The UTM position of each sensor's measurement was calculated. The field site is in the UTM zone 54S; and (2) matching ultrasonic sensor data to individual plant information using QGIS to annotate sensor data to each plant's boundaries. Geo-referenced individual plants data were overlaid on pre-defined polygon shapefiles of the selected individual plants in QGIS. Polygons with ID numbers were manually drawn around the individual plants. The data processing and extraction were performed through the processing steps of the HTP geo-processor plug-in. This completes the processing of plant height, displaying an attribute table as a final output of individual plant height along with the ID numbers of the plants.

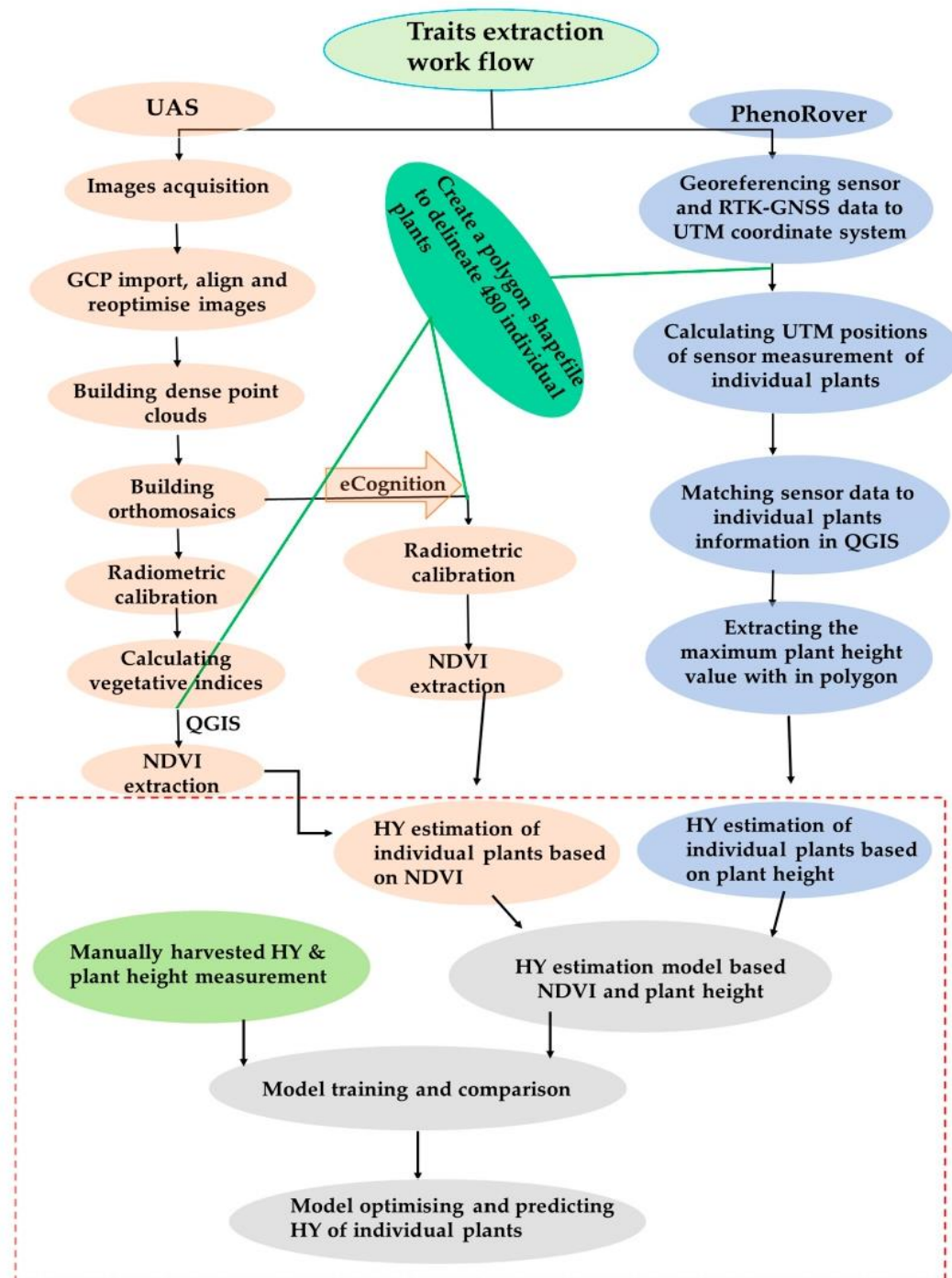


Figure 3. Workflow diagram of image processing and plant height extraction used in the GSS field trial. The steps in the left contain all procedures applied to extract single plants NDVI from the UAS images, whereas the steps in the right show the procedures followed to extract single plants plant height from the PhenoRover system. The box with the broken red line contains the methods and analysis procedures used to model the HY estimation.

2.2.5. HY Data Collection

Individual plants' HY data from manual harvests were collected in the years 2017 and 2018 under four harvesting dates of different seasons of each year (Table 1). The timing was determined

by the growth stage of the single plants, in which the 2–3 leaf stage was considered as a standard simulated grazing stage [37]. Thirty-two plants from the third row of the sampled plot were selected for plant height and herbage yield measurement. The destructive measurements of FHY and DHY of single plants were obtained by cutting plants at the 0.05 m above-ground level. FHY was measured from individual plants, and all samples were subsequently dried in an oven for 48 h at 60 °C for the determination of DHY. Additionally, individual plant height was measured using a standard ruler from the base of each plant to the highest upright standing leaf.

2.3. Estimation Models of HY through Sensors Data Combination

Data from the ground and aerial-based platforms were combined to obtain the most suitable model for individual plants' herbage yield estimation. Firstly, UAS-based NDVI data extracted from each plant through the process in Figure 3 were modelled to estimate HY at every seasonal harvest. Secondly, manually measured plant height and PhenoRover-based plant height data extracted from individual plants through the process in Figure 3 were compared. Thirdly, the manually measured and PhenoRover-based plant height data were modelled to estimate HY. To improve the estimation of accuracy of FHY and DHY, combining the spectral and structural information may be necessary [12,15,31]. In this study, different multiplicative combination methods of NDVI and plant height were assessed, including NDVI × plant height, NDVI × plant height × plant height and, NDVIsq × plant height (NDVIsq_PH). NDVIsq_PH showed the best combination to estimate FHY and DHY with higher accuracy.

2.4. Statistical Analysis

The relationship of herbage yields with plant height and NDVI were analysed using general linear regression models (Figure 3). The measurement taken at the individual plant level was the unit of analyses. Residuals versus fitted values plots were examined to determine the need for data transformation to ensure the normality of residuals with constant variance. When deemed necessary, data were logarithmically transformed before the final analyses. All analyses were performed in R version 3.5.2 (R Core Team 2018, Vienna, Austria).

On some data collection dates (all of 2017 and one day in 2018), plant heights from ultrasonic sonar was not available due to technical issues with the GPS of the PhenoRover system. For these dates, manually measured plant height with a standard ruler was used. For the testing, the estimation of plant height using ultrasonic sonar, a linear regression relationship with the manually measured plant height was performed on three collection dates of 2018.

A parsimonious model for FHY/DHY was developed by using variables such as NDVI values, manually measured plant height (PH)/ plant height from ultrasonic sonar sensors, the product of NDVI square and plant height (NDVIsq_PH) and seasons. The parsimonious model selected was:

$$FHY/DHY \sim intercept + NDVIsq_PH + Season + NDVIsq_PH.Season \quad (1)$$

The predictive accuracy of the model was determined by splitting data into calibrations/training and validation/test sets, which is often referred to as cross-validation. Cross-validation can be carried out in many ways, but we focused on two methods (1) k-fold cross-validation and (2) random split of the dataset. In K-fold cross-validation, we randomly split the data into k-folds (k = 2, 5, 10 and 20) and K-1 folds (sets) of data used as calibration/training set and model parameters were estimated using this data. Using these model parameters, we predicted the FHY/DHY for the remaining one-fold (set) of the data. The cross-validation process is then repeated k times, with each of the k-fold (sets) used exactly once as the validation/test data.

In random split approach, we randomly split data into 60%/40%, 70%/30% and 80%/20%, where 60%, 70% and 80% of the data were used as calibration/training and 40%, 30% and 20% of the data were used

as validation/test set, respectively. Each of the random split strategies was carried out 10,000 times, and results averaged.

The model accuracy was measured using coefficient of determination (R^2), root mean squared error (RMSE), mean absolute error (MAE), and mean percentage error (MPE) [21]. The R^2 is the correlation coefficient between measured and predicted herbage yield. RMSE is the square root of the mean squared difference between observed herbage yield and predicted herbage yield. MAE is the mean of the absolute difference between observed herbage yield and predicted herbage yield and mean percentage error is the mean of the error percentage ($(\text{observed herbage yield} - \text{predicted herbage yield}) / \text{observed herbage yield} \times 100$).

3. Results

3.1. Seasonal Herbage Yield Variation

Figure 4 shows a box mean of measured FHY and DHY of individual plants across four seasons of 2017 and 2018. The FHY value per plant (82.48–127.18 g) varied across seasons in 2017 and (32.80–96.50 g) in 2018. Similarly, the DHY value per plant varies (20.04–36.24 g) in 2017 and (9–22.29 g) in 2018. Moreover, measured seasonal FHY and DHY of the years 2017 and 2018 indicated a wide variability of biomass values (~1.41–428 g) for each measurement season for the individual plants. This suggests that herbage yield had enough variation to use to correlate the NDVI and plant height from ultrasonic sonar sensors.

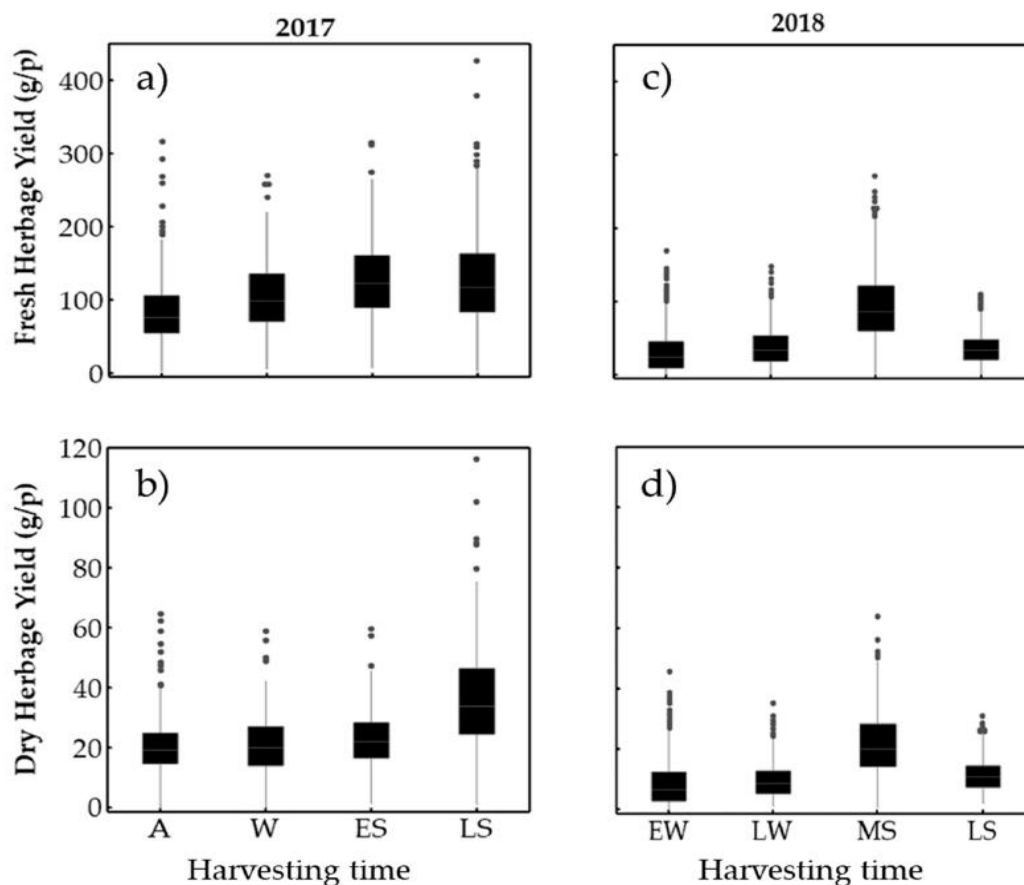


Figure 4. Measured fresh herbage yield at four seasons of the year 2017 and 2018 (a,c) and dry herbage yield (b,d); A: autumn; W: winter; MS: mid-spring; LW: late winter; EW: early winter; ES: early spring; LS: late spring.

3.2. Correlation between Manual and Sonar Plant Height

A strong correlation ($R^2 = 0.76$) was observed between manual and ultrasonic sonar plant height (Figure 5). Plant height measurement using ultrasonic sonar slightly overestimated height, mainly when plants are small, which may result from the uneven surface of the measured field trial. The slope of the regression line was close to one, and the RMSE was 2.98 cm, and this implies that the use of ultrasonic sonar is useful to estimate plant heights of space-planted perennial ryegrass single plants.

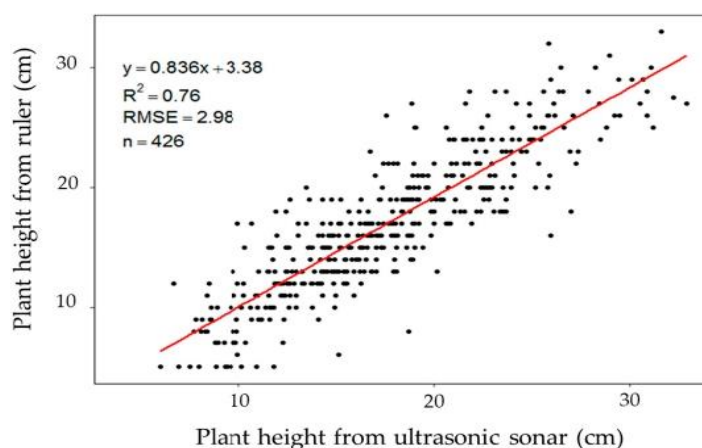


Figure 5. Scatterplots of plant height from ultrasonic sonar sensors vs. manually measured plant height using a standard ruler for the selected measurement date of 19 June 2018.

3.3. Individual Plant Herbage Yield Model Evaluation

The R^2 for linear regression models of log-transformed DHY estimation from NDVI ranges between 0.35 and 0.73 (Figure 6a,c). The variation in accuracy of seasonal HY estimation indicates that NDVI as an estimator can be limited by saturation at high seasonal HY accumulation. A linear relation between DHY and plant height showed variable R^2 , which was between 0.15 and 0.65 (Figure 6b,d). Lower estimation accuracy of the mid and late-spring of 2018 ($R^2 = 0.12$ – 0.15).

Figure 7a,b showed seasonal linear regression models of DHY estimation from NDVI_{sq}_PH that improved the R^2 averagely with up to 10% in 2017 and 24% in 2018 compared to plant height and NDVI alone. Looking at the combined seasonal estimation models, yearly estimation values of DHY from NDVI_{sq}_PH were consistent with the individual season models ($R^2 = 0.67$) (Figure 8a,b), indicating the possibility of applying the global model of HY estimation instead of specific (seasonal) models.

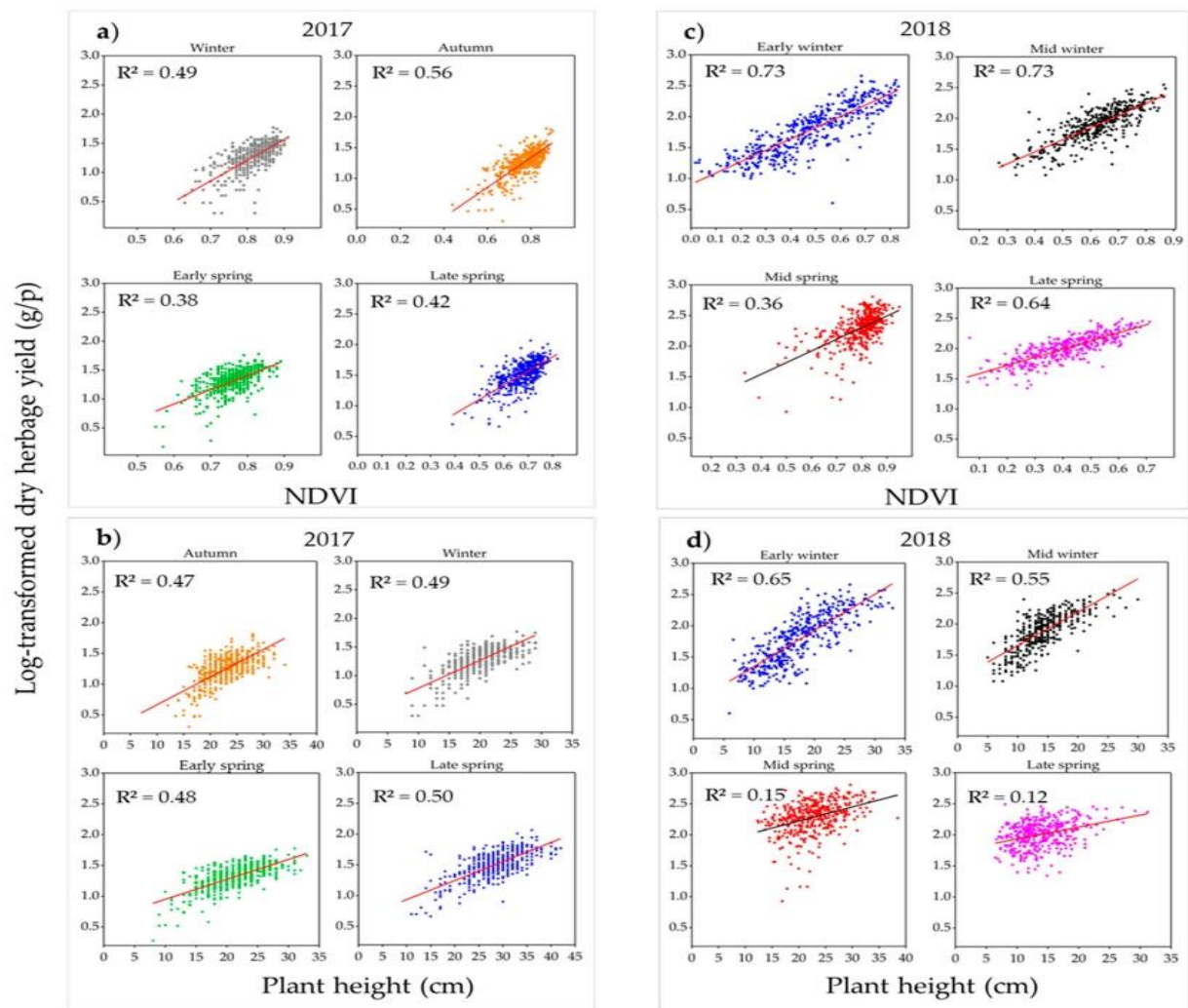


Figure 6. Scatterplots of (a) NDVI extracted from UAS-based multispectral imaging vs. log-transformed dry herbage yield measurements of single plants in four seasons of the 2017 dataset; (b) plant height measured versus log-transformed dry herbage yield measurements of single plants in four seasons of the 2017 dataset; (c) NDVI extracted from UAS-based multispectral imaging vs. log-transformed dry herbage yield measurements of single plants in four seasons of the 2018 dataset; (d) plant height measured versus log-transformed dry herbage yield measurements of single plants in four seasons of the 2018 dataset. p -value < 0.001 for all regression equations.

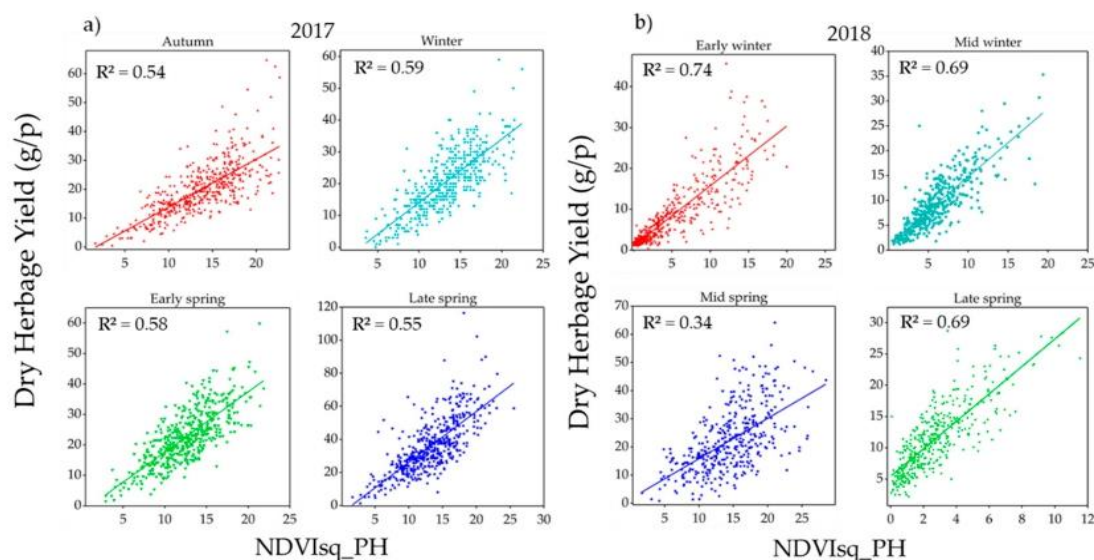


Figure 7. Scatterplots of (a) squared NDVI-value extracted from UAS-based multispectral imaging and plant height (NDVisq_PH) vs. dry herbage yield measurements of single plants in four seasons of 2017; (b) NDVisq_PH vs. dry herbage yield measurements of single plants in four seasons of 2018. p -value < 0.001 for all regression equations.

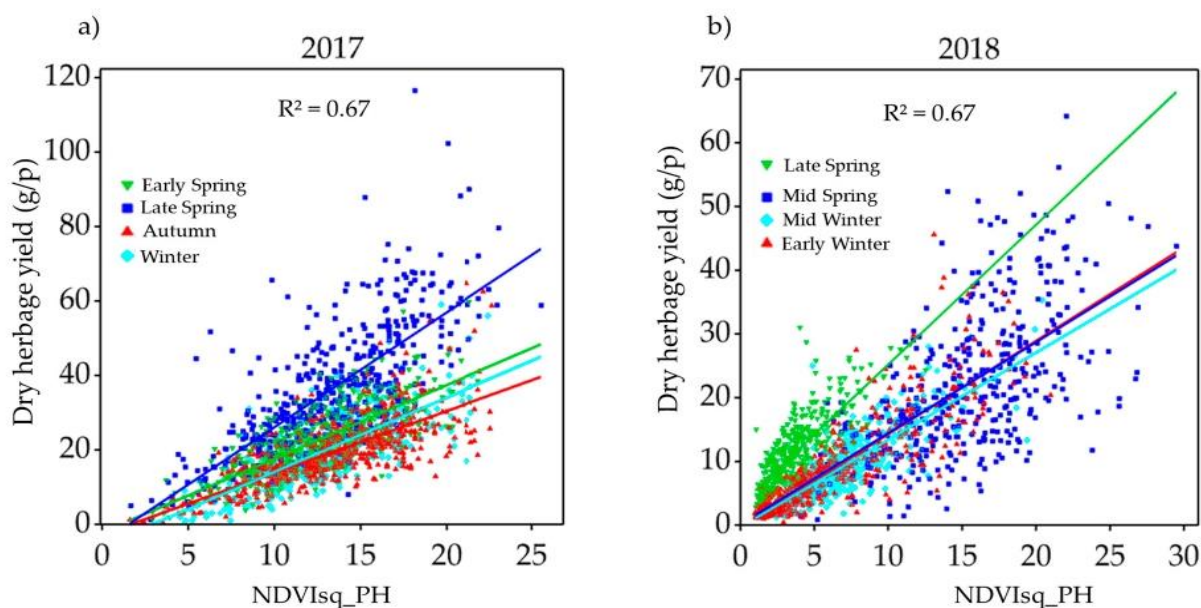


Figure 8. Scatterplots of (a) combined regression of NDVisq_PH vs. dry herbage yield measurements of single plants in four seasons of 2017; (b) combined regression of NDVisq_PH vs. dry herbage yield measurements of single plants in four seasons of 2018. p -value < 0.001 for all regression equations.

3.4. Herbage Yield Prediction

Tables 3 and 4 show the details of cross-validations (CVs) of multiple linear regression models to estimate FHY and DHY yield, respectively. Looking at Table 3, K-fold CV at K = 2, 5, 10 and 20 indicated a similar level of estimation accuracies across the 2017 and 2018 datasets. The R^2 values from the K-fold CV of the 2017 dataset remain similar to that of 2018 (0.63–0.64 vs. 0.69–0.70). However, the RMSE and MAE values from the K-fold CV of the 2017 dataset were higher than those of 2018. The MPE% of 2018 K-fold CV was higher than that of 2017. Similarly, random splits of the yearly

dataset into 10,000 simulated repetitions of 60%/40%, 70%/30%, and 80%/20% indicated similar R^2 values (0.64–0.69) like the K-fold CV in both experimental years. The RMSE and MAE values of the 10,000 random splits were similar to the K-fold within the year but significantly higher in 2017 than 2018.

Table 3. Summary of cross-validation results for predicted fresh herbage yield (g/p). CV method: cross-validation method; R^2 : coefficient of determination; RMSE: root mean square error; MAE: mean absolute error; MPE: mean percentage error.

CV Method	Partition	2017				2018			
		R^2	RMSE (g)	MAE	MPE%	R^2	RMSE (g)	MAE	MPE%
2-folds	2	0.63	32.81	24.31	22	0.69	23.80	15.21	30
5-folds	5	0.64	32.66	24.30	22	0.69	23.67	15.11	30.63
10-folds	10	0.64	32.60	24.29	22	0.69	23.71	15.16	30
20-folds	20	0.64	32.59	24.30	22	0.70	23.50	15.15	30
Random split *	60%/40%	0.64	32.77	24.34	22	0.69	23.96	15.50	30.70
Random split *	70%/30%	0.64	32.77	24.33	22	0.69	23.92	15.49	30.68
Random split *	80%/20%	0.64	32.71	24.31	22	0.69	23.91	15.49	30.66

* = Random split results based on 10,000 simulations.

In the DHY estimation, K-fold and random split CV showed relatively similar accuracy for the R^2 values (0.66–0.67) of the 2017 and 2018. The RMSE, MAE, and MPE values from the K-fold and random split CV were in the range of 7.53–7.60 g, ~5.44 g and ~22%, respectively, for the 2017 dataset (Table 4). This indicated K-fold CV (at different values of K) and the 10,000 simulated random splits of 60%/40%, 70%/30% and 80%/20% showed no sign of variation in these values irrespective of the sample size. Similarly, RMSE, MAE, and MPE values of the 2018 dataset were 5.44–5.52 g, 3.59–3.67 g and ~28%, respectively. The RMSE and MAE of the CV values were generally smaller in 2018 than the 2017 dataset, whereas the MPE was reverse.

The scatterplot in Figure 9 shows an example of the graphic representation of observed vs. the predicted DHY. The DHY validation shows the data points generally closer and slightly deviated from the 1:1 line, indicating DHY prediction methods may be used to accurately estimate HY of individual ryegrass plants.

Table 4. Summary of cross-validation results for dry herbage yield (g/p). CV method: cross-validation method; RMSE: root mean square error; R^2 : coefficient of determination; MAE: mean absolute error; MPE: mean percentage error.

CV Method	Partition	2017				2018			
		R^2	RMSE (g)	MAE	MPE%	R^2	RMSE (g)	MAE	MPE%
2-folds	2	0.66	7.57	5.43	22	0.67	5.50	3.60	27.76
5-folds	5	0.67	7.56	5.44	22	0.67	5.46	3.60	27.62
10-folds	10	0.67	7.57	5.43	22	0.67	5.50	3.59	28
20-folds	20	0.67	7.53	5.43	22	0.68	5.43	3.59	27.65
Random split *	60%/40%	0.67	7.59	5.44	22	0.67	5.52	3.67	28.34
Random split *	70%/30%	0.67	7.60	5.44	22	0.67	5.52	3.66	28.31
Random split *	80%/20%	0.67	7.58	5.44	22	0.67	5.51	3.66	28.29

* = Random split results based on 10,000 simulations.

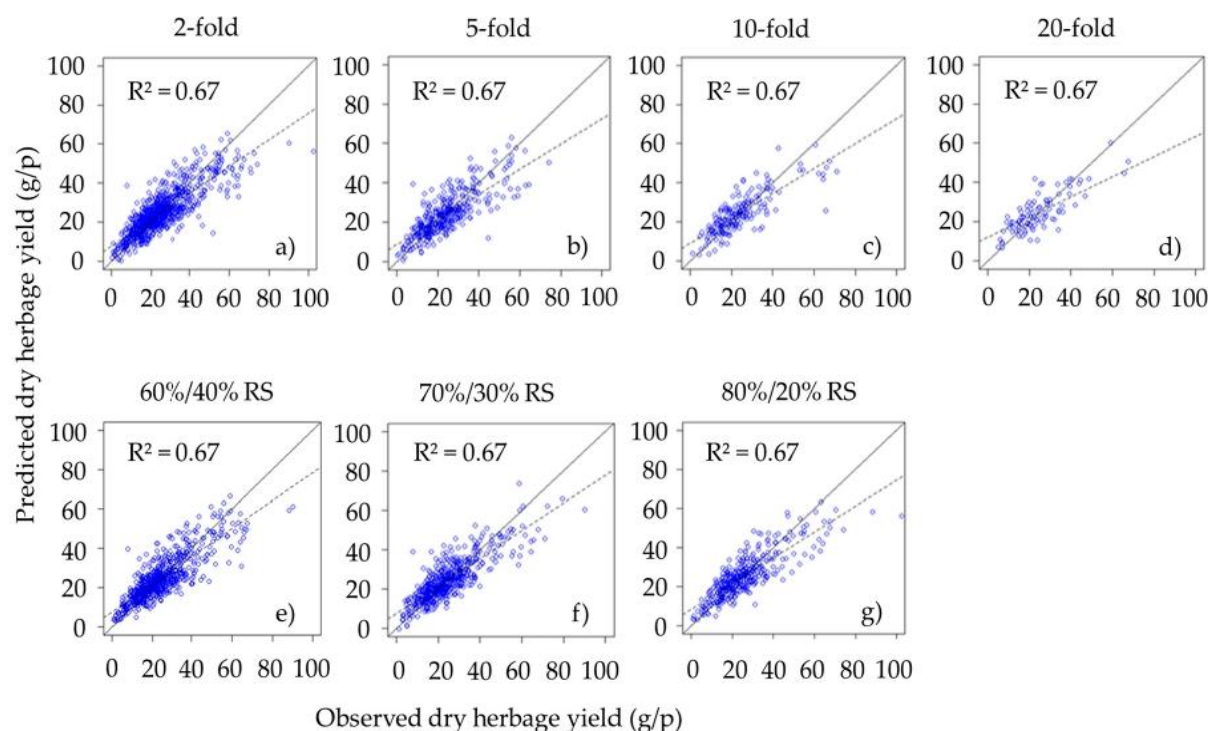


Figure 9. Scatterplots of observed and predicted dry herbage yield (grams/plant) in perennial ryegrass with linear regressions of various K-folds and random splits of cross-validation. The data points displayed in each plot represent the K-fold validation sets of (a) two-fold, (b) five-fold, (c) 10-fold, (d) 20-fold and (e) 60/40%, (f) 70/30% and (g) 80/20% random split from the 2017 harvest. The solid diagonals represent 1:1 line, and the dashed lines represent the fitted linear functions. p -value < 0.0001 for all regression functions.

4. Discussion

Current, HY phenotyping is time-consuming and relies mainly on visual scoring. HTP platforms that would improve the speed and accuracy of HY phenotyping have, so far, only been applied at the plot level [20,29], and no one has conducted phenotyping at the individual plant level except on trees [38–40]. Various sensor-based assessments of phenotyping at the individual plant level is required to implement molecular breeding techniques, like GS. However, the implementation of GS implies cross-validation on the evaluation of the genetic and phenotypic breeding values of individuals for accurate selection of superior genotypes [41,42]. GS, in perennial ryegrass, has currently followed the trend of evaluating the HY of sward plots of cultivars and breeding lines [43,44]. The phenotypic selection at individual plants level is required to explore the full potential of GS programs. Sensor-based phenotyping technologies offer a non-destructive, high-throughput and efficient assessment of HY replacing current inefficient phenotyping methods [5]. In this study, we explained the potentials of sensor-based HTP technologies to estimate FHY and DHY through the development and cross-validation of various prediction models.

The seasonal HY distribution in the 2017 and 2018 showed that HY from the 2017 harvest dates was higher compared to 2018 in all seasons. The notable decline in dry matter yield as the year progressed may result from the drought stress and repetitive cutting [45].

Plant height can be obtained using manual and ultrasonic sonar-based measurements [26–28,46]. Automation of plant height measurements using sensors offers an excellent advantage for assessing large breeding populations rapidly and objectively [5]. A high correlation was observed between plant height from a standard ruler and an ultrasonic sonar sensor. Similar results of R^2 (0.56–0.7) from UAS-derived grassland height and RMSE of 3.50 cm from LiDAR-derived wheat height were reported. However, other reports achieved higher R^2 (0.90–0.98) applying ground-based LiDAR on wheat and

cotton, aerial based-RGB ($R^2 = 0.87\text{--}0.98$) on barley [23,24] and ground-based sonar sensors ($R^2 = 0.86$) on cotton [47]. Most of these studies conducted at the plot level were of the canopy structure of plants growing as a sward plot and may be different from space-planted single plants.

The correlation between log-transformed seasonal DHY vs. NDVI of individual ryegrass plants was moderate, with R^2 ranging between 0.36 and 0.73 across seasons. The significant variations of R^2 (up to 37%) of estimation accuracies occur among the seasons; mainly, it could result from the effect of saturation at the late growth stage [19]. In previous sward studies, similar R^2 values (0.38–0.56) of NDVI vs. DHY were reported in alfalfa, Bermuda grass and tall fescue [12,28,48]. However, R^2 values in these studies were slightly lower compared to studies on grain crops, including wheat, barley, and maize [49–51]. This could be related to the vigour of canopy growth habit difference between forage and grain crops, where NDVI could be influenced [52].

Plant height is another widely used structural and physical parameter used to estimate the biomass of various crops [20,23,53,54]. Previous studies on grasslands indicated moderate to high correlation ($R^2 = 0.59\text{--}0.81$) on predicting dry herbage yield from plant height measurements [45]. In the current study, the R^2 of plant height vs. log-transformed DHY was more variable ($R^2 = 0.12\text{--}0.65$) than NDVI across seasons. Moreover, correlations were slightly lower compared to previous results in the literature [13,55]. Specifically, lower accuracies were obtained from the spring 2018 experiments due to early flower initiation from drought stresses. In this study, we believe the surface of these small plants may have reflected weak sound echoes, which made it difficult for the ultrasonic sonar to detect accurate distance from the top canopy of the plant. Furthermore, when the plant canopy is a mixture of leaves and sparse reproductive tillers there is a greater variability in height and the surface that interacts with the sensor. Similar results of low accuracies were reported to detect thin spikes of wheat at the time of maturity compared to clustered young leaves [26].

The NDVI values explain the spectral properties of the plant canopy, whereas plant height is related to the vertical structure and growth rate of a plant [53,56]. Combining the physical and spectral parameters was successful in grasping spectral and structural information and improved the regression estimation of aboveground biomass [20]. The results show combining NDVI and plant height (NDVIsq_PH) data exhibited up to 10–24% improvement in prediction accuracy of DHY than using either NDVI or plant height alone. Schaefer and Lamb [12] investigated the combination of NDVI and LiDAR plant height to improve the estimation accuracy of tall fescue HY by up to 20%. However, this study made on single measurement experiment without looking at combined information of changes in plant performance for multiple growing seasons.

The validation of the model performance of observed vs. predicted values of FHY and DHY showed similar accuracies across all the K-fold ($k = 2, 5, 10$, and 20) and random split (60/40%, 70/30% and 80/20%) with $R^2 = 0.63\text{--}0.70$ across the two seasons dataset. We excluded FHY results in some of our results since data points deviated similarly from the 1:1 line, indicating that either FHY or DHY prediction methods will be enough to estimate HY of individual ryegrass plants. However, FHY results were included in the cross-validation results to match up with some of the results of the whole GSS result with only FHY datasets. Our cross-validation results were consistent with the results of [15], who applied random split CV to estimate fresh and dry biomass of barley from the fusion of NDVI and plant height. The authors obtained ($R^2 = 0.67\text{--}0.79$) from linear regression. The R^2 , RMSE, MAE and MPE of yearly regression models performed similarly irrespective of the CV type used, suggesting that models developed are stable and reliable across all K-fold and 10,000 repetitive random splits. However, regression models from previous studies showed variability in model performance at different random split [15,23,24]. The reason for this was, a small number of samples ($n = 48\text{--}194$) were used in these studies, and experiments were performed with not more than three harvests compared to the current study ($n = 1626\text{--}1900$) with a total of eight seasonal harvests in two years. Several other studies indicated combined VIs (other than NDVI) and plant height performed better for biomass estimation than VIs alone [13,15,20,57].

In the current study, year-for-year RMSE, MAE and MPE values of the CV showed variations in values. For instance, the RMSE and MAE values of the 2017 dataset were more significantly higher than the 2018 dataset, suggesting the plants from the 2017 harvest dates were larger with larger FHY and DHY values. It was impossible to compare the RMSE and MAE values from our results with the literature as there is no work done on single plants in the previous research where works focused on measurements of kg per plot or in a given area (square metre/hectare). Contrary to the RMSE and MAE, the MPE values from the 2017 dataset were slightly smaller compared to the 2018 dataset.

We found a high correlation by combining NDVI and plant height to predict DHY of individual ryegrass plants at the 2–3 leaf growth stage. This was proved by cross-validation using multiple linear regression model, and we still need to apply more non-linear regression methods that include machine learning techniques with more datasets from larger study samples may improve the prediction models further and be able to rank genotypes non-destructively and objectively.

5. Conclusions

This study is the first to integrate various sensors on aerial and ground-based phenotyping platforms that implement combining spectral and structural information to estimate HY of space-planted perennial ryegrass in the context of the large-scale breeding program. The results of this study demonstrate that the best prediction of DHY of space-planted perennial ryegrass single plants come from the multiplicative combination of NDVI and Plant height (NDVIsq_PH). The K-fold and random split CV findings imply that the combination of NDVI and plant height improved prediction accuracy over the use of NDVI and plant height alone. This yielded an accuracy of the coefficient determinations of HY estimation more than 0.63 and RMSE for FHY, and DHY was less than 33 g/plant and 8 g/plant, respectively. The application of various K-fold and random split methods do not change the prediction accuracy. We propose this non-destructive and rapid HY phenotyping technique to replace the current slow and time-consuming HY yield phenotyping methods. Therefore, this method can be assessed for effective estimation of thousands of individual plants and develop prediction equations that can be used for phenotypic ranking of genotypes for HY.

Author Contributions: P.B., J.W., K.S. and G.S. conceived the experiment. A.G. performed the experiment. A.G. and P.B. organised and processed the PhenoRover system and UAS data. K.G. and A.G. developed the R-programmed script and finalised the data analysis. A.G. wrote the paper. P.B., J.W. and K.S. contributed to the overall experimental design and supervision. All authors assisted in editing and improving the paper.

Funding: This research was funded by Agriculture Victoria and Dairy Australia through the DairyBio initiative and The University of Melbourne.

Acknowledgments: We acknowledge the plant molecular breeding staff of Hamilton Centre (Agriculture Victoria Research, AVR) for managing the field experiments. The authors would like to thank Andrew Phelan and Carly Elliott for doing all the flights and some of the PiX4D data processing. The authors would also like to thank Daren Pickett, Russel Elton, Daren Kean, Greg Mason, Chinthaka Jayasinghe and Chaya Smith for their contribution to manual harvesting of samples. We would also like to acknowledge Senani Karunaratne of AVR (Ellinbank Centre) for his valuable comments on the data analysis section.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 5. Development and Validation of a Phenotyping Computational Workflow to Predict the Biomass Yield of a Large Perennial Ryegrass Breeding Field Trial

Chapter 5 is based on published paper in Frontiers in Plant Science in May 2020

The details of the original article are as follows:

Gebremedhin, A.; Badenhorst, P.; Wang, J.; Shi, F; Breen, E; Giri, K.; Spangenberg, G.; Smith, K. (2020) Development and validation of a phenotyping computational workflow to predict the biomass yield of a large perennial ryegrass breeding field trial. *Frontiers in Plant Science* **11**, 689.



Development and Validation of a Phenotyping Computational Workflow to Predict the Biomass Yield of a Large Perennial Ryegrass Breeding Field Trial

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Edited by:

Søren K. Rasmussen,
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Reviewed by:

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Specialty section:

This article was submitted to
Plant Breeding,
a section of the journal
Frontiers in Plant Science

Received: 28 January 2020

Accepted: 30 April 2020

Published: 28 May 2020

Citation:

Gebremedhin A, Badenhorst P, Wang J, Shi F, Breen E, Giri K, Spangenberg GC and Smith K (2020) Development and Validation of a Phenotyping Computational Workflow to Predict the Biomass Yield of a Large Perennial Ryegrass Breeding Field Trial. *Front. Plant Sci.* 11:689. doi: 10.3389/fpls.2020.00689

Increasing dry matter yield (DMY) is the most important objective in perennial ryegrass breeding programs. Current yield assessment methods like cutting are time-consuming and destructive, non-destructive measures such as scoring yield on single plants by visual inspection may be subjective. These assessments involve multiple measurements and selection procedures across seasons and years to evaluate biomass yield repeatedly. This contributes to the slow process of new cultivar development and commercialisation. This study developed and validated a computational phenotyping workflow for image acquisition, processing and analysis of spaced planted ryegrass and investigated sensor-based DMY yield estimation of individual plants through normalized difference vegetative index (NDVI) and ultrasonic plant height data extraction. The DMY of 48,000 individual plants representing 50 advanced breeding lines and commercial cultivars was accurately estimated at multiple harvests across the growing season. NDVI, plant height and predicted DMY obtained from aerial and ground-based sensors illustrated the variation within and between cultivars across different seasons. Combining NDVI and plant height of individual plants was a robust method to enable high-throughput phenotyping of biomass yield in ryegrass breeding. Similarly, the plot-level model indicated good to high-coefficients of determination (R^2) between the predicted and measured DMY across three seasons with R^2 between 0.19 and 0.81 and root mean square errors (RMSE) values ranging from 0.09 to 0.21 kg/plot. The model was further validated using a combined regression of the three seasons harvests. This study further sets a foundation for the application of sensor technologies combined with genomic studies that lead to greater rates of genetic gain in perennial ryegrass biomass yield.

Keywords: high-throughput phenotyping, biomass, perennial ryegrass, NDVI, plant height, computational workflow

INTRODUCTION

Increasing biomass yield is the most important trait to improve during the breeding of perennial ryegrass (Smith et al., 2001; McDonagh et al., 2016; Herridge et al., 2018; Ghamkhar et al., 2019). However, biomass yield is a complex trait, which varies with the number and density of tillers, regrowth after defoliation and growth habit; across a range of seasons, environments, and plant age (Yates et al., 2019). Furthermore, the early stages of perennial ryegrass breeding programs depend on the assessment of populations based on large numbers of genotypes planted as spaced plants or small sward plots in the field (Lootens et al., 2016; Ghamkhar et al., 2019). These assessments involve multiple measurement and selection procedures across seasons and years to repeatedly evaluate biomass yield (Leddin et al., 2018). Biomass yield assessment methods are time-consuming, mainly relying on visual scores or destructive harvesting, drying and weighing of samples. This contributes to the slow process of new cultivar development and commercialization. The application of sensor-based high-throughput phenotyping (HTP) technologies on aerial and ground-based mobile platforms have the potential to offer a non-destructive, rapid and efficient method to assess biomass yield in the field (Inostroza et al., 2016; Gebremedhin et al., 2019b; Ghamkhar et al., 2019).

The possibility for the application of HTP for automated biomass assessments on an objective and accurate basis has been demonstrated for forage grasses (Walter et al., 2012; Barrett et al., 2015). In other species, various remote sensing methods have been calibrated and validated to conduct measurements at a single plant, plot or experimental units, paddock and landscape scales (Rahman et al., 2014; Hunt et al., 2015; Inostroza et al., 2016; Punalekar et al., 2018). Satellite-based remote sensing methods have been used to assess grassland biomass at a landscape scale (Barrachina et al., 2015). However, low temporal and spatial resolution limits the applicability of satellite remote sensing for breeding (Tattaris et al., 2016). For instance, satellite imaging tools have spatial resolution ranging from 1.24 to 260 m (Chawade et al., 2019), which limits their application on small experimental plots. Proximal sensing platforms have the advantage of capturing phenotypic data from thousands of genotypes at high spatial and temporal resolution (Haghighattalab et al., 2016). The platforms equipped with various sensors may function to capture information of multiple traits from field plots and individual plants.

A variety of aerial and ground-based HTP platforms have been developed for non-destructive biomass estimation of wheat (Yue et al., 2017; Jimenez-Berni et al., 2018), barley (Bendig et al., 2014; Brocks and Bareth, 2018), triticale (Bussemeyer et al., 2013), and rice (Devia et al., 2019). Specifically, small grassland experiments (10 sampled plots of 1 m² each), demonstrated non-destructive biomass estimations of timothy and ryegrass from plant height (Rueda-Ayala et al., 2019). Other experiments of biomass estimation on ryegrass (Borra-Serrano et al., 2019) and a mixture of timothy and meadow fescue (Viljanen et al., 2018) were demonstrated using aerial platforms. In these studies, vegetative indices, plant height and photogrammetric features were validated, and the models from these surrogates were

used to develop biomass estimation models under plots and small paddock level ($n \leq 96$ sampled plots) experiments. Thus, the general application of aerial and ground-based phenotyping methods in these experiments showed there is a potential to increase throughput and hence to improve the precision to individual plants level biomass yield estimation. Therefore, development and validation of a phenotyping computational workflow of data acquisition, processing and analysis could be applied to implement automated biomass yield estimation in large-scale ryegrass breeding program. We have recently shown that normalized difference vegetative index (NDVI) and plant height correlated with perennial ryegrass biomass yield in four seasons (Gebremedhin et al., 2019a). The results showed combining NDVI and plant height to be a robust method to enable high-throughput phenotyping of biomass yield in a ryegrass breeding program.

The objective of the study was to validate a computational phenotyping workflow for image acquisition, processing and analysis of spaced-planted perennial ryegrass to estimate the biomass yield of 48,000 individual plants through NDVI and plant height data extraction. The workflow resulted in the accurate, non-destructive biomass yield estimation of spaced planted perennial ryegrass across growing seasons in a field environment. The outcome of the study contributes to the application of sensor technologies combined with genomic selection for greater rates of genetic gain in forages.

MATERIALS AND METHODS

Field Experiment and Plant Material

Advanced perennial ryegrass breeding lines and commercial cultivars were established in a field experiment in June 2016 at Agriculture Victoria Research, Hamilton Centre, Victoria, Australia (37.8464°S, 142.0737°E) (Supplementary Figure 1), as part of a project to implement genomic selection (GS) in perennial ryegrass. Fifty advanced perennial ryegrass breeding lines and commercial cultivars were planted in a randomized complete block design with ten replications of each. Each line or commercial cultivar was coded 1–50 to conceal their identities as requested by the owners of the breeding lines and cultivars. Each replication was considered as a plot and contained three rows of 32 spaced plants each (i.e., 96 plants/plot). The experimental unit was, therefore, a plot of 8 × 1.8 m. The spacing between plants was 25 cm and between rows was 60 cm. The GS experiment contained a total of 48,000 individual plants in 10 blocks. The total area of the field experiment was 8,100 m².

Phenotypic Data Collection

For the non-destructive biomass estimation, ground and aerial-based proximally sensed data were collected a day before each harvest. The harvests were allocated to seasons according to the protocol of Leddin et al. (2018). The field experiment was harvested three times in 2018, one in June 2018 called winter 2018, and two in the late spring season of October 2018 and November 2018 called late-spring2018_1 and late-spring2018_2, respectively. No harvests were conducted in autumn or summer

due to slow growth and drought. In these three harvests, two sets of data collection scenarios were conducted at the same time.

Firstly, 480 individual plants from three breeding lines were selected and manually harvested at 5 cm above ground level in each of the seasonal harvests. The manually harvested measurements were used to develop and validate dry matter yield (DMY) estimation model from the combinations of NDVI and plant height values from sensors (Gebremedhin et al., 2019a). The models developed from these individual plants were used to estimate biomass of the 48,000 plants for the winter2018, spring2018_1, and late-spring2018_2 seasonal harvests.

Secondly, mechanical harvesting of the 500 plots of the GS field was conducted using a Gianni Ferrari combine harvester (Gianni Ferrari s.r.l., Reggio, Italy) to validate the plot level correlation between estimated and measured DMY. In the late-spring2018_1 and late-spring2018_2 measured DMY data wasn't collected from 100 plots since these plots were allocated for flowering score experiment. Plant height data from a few plots of the late-spring2018_2 was not received due to technical issues of the Phenover driving at that specific date.

Aerial Images Acquisition and Processing

Multispectral images acquisition was conducted using a RedEdge-M sensor (RedEdge, MicaSense Inc., Seattle, WA, United States) attached to DJI M100 quadcopter (DJI Technology Co., Shenzhen, China). The RedEdge-M capture images simultaneously at five bands including blue (465–485 nm), green (550–570 nm), red (663–673 nm), red edge (712–722 nm), and near-infrared (820–860 nm). It also has GPS and sensor and incident light sensors. The flight mission was planned by Pix4D Capture software.

Aerial images were collected using the unmanned aerial system (UAS) on a weekly basis over the GS trial site, and three sets of data from 2018 (winter2018, late-spring2018_1, and late-spring2018_2) was used for this analysis. Imaging dates were synchronized with each harvest. Flight operations were conducted under bright, sunny weather conditions to minimize noise from environmental variation. The UAS flight altitude was set at 20 m above ground level, and the flight speed was 6 m/s with 75% side and forward overlap of images. At this flight altitude and speed, the spatial resolution of the images was 2 cm/pixel. The same flight path was followed on each date. Multispectral ground calibration targets (Tetracam Inc., Chatsworth, CA, United States) with known reflectance values (6, 11, 22, and 33%) was used for radiometric calibration by developing regression equation between digital numbers (DN) vs. the reflectance percentage.

Computational Workflow for Data Extraction

Before the first flight, nine ground control points (GCPs), (50 × 50 cm checkered linoleum flooring) were distributed across the field experiment to improve the georeferencing accuracy. The GCPs were used for manual assignment of absolute geographic coordinates to the ortho-mosaic images. For image processing,

Pix4Dmapper Pro (version 4.3.31 Pix4D, Lausanne, Switzerland, <https://pix4d.com>) was used to process raw images into ortho-mosaics. The workflow of analyzing geospatial images of spectral signals (Figure 1) is described below.

Ortho-Mosaic Processing, Calibration, and NDVI Extraction

Ortho-mosaic processing have five main steps; including image alignment, image geo-referencing, building dense point clouds, ortho-mosaics, and vegetative indices map, were followed for image processing. For detailed processing steps, please refer to following previous studies (Khan et al., 2018; Dobbels and Lorenz, 2019). Ortho-mosaic images were generated and stored as geo-referenced TIFF files. Assessment accuracy of processed ortho-mosaic images was conducted by assessing the quality report from the Pix4D processing. After the ortho-mosaic processing was completed, reflectance calibration and NDVI extraction were conducted using eCognition. For radiometric calibrations, linear correlations were developed between ortho-mosaic images of DN and their known reflectance values for each band. The equation developed was used to convert DN to corrected reflectance values of the ortho-mosaic TIFF file. The corrected ortho-mosaic TIFF file was used to develop the NDVI extraction workflow in eCognition. NDVI was calculated from the red and near-infrared reflectance values (Khan et al., 2018).

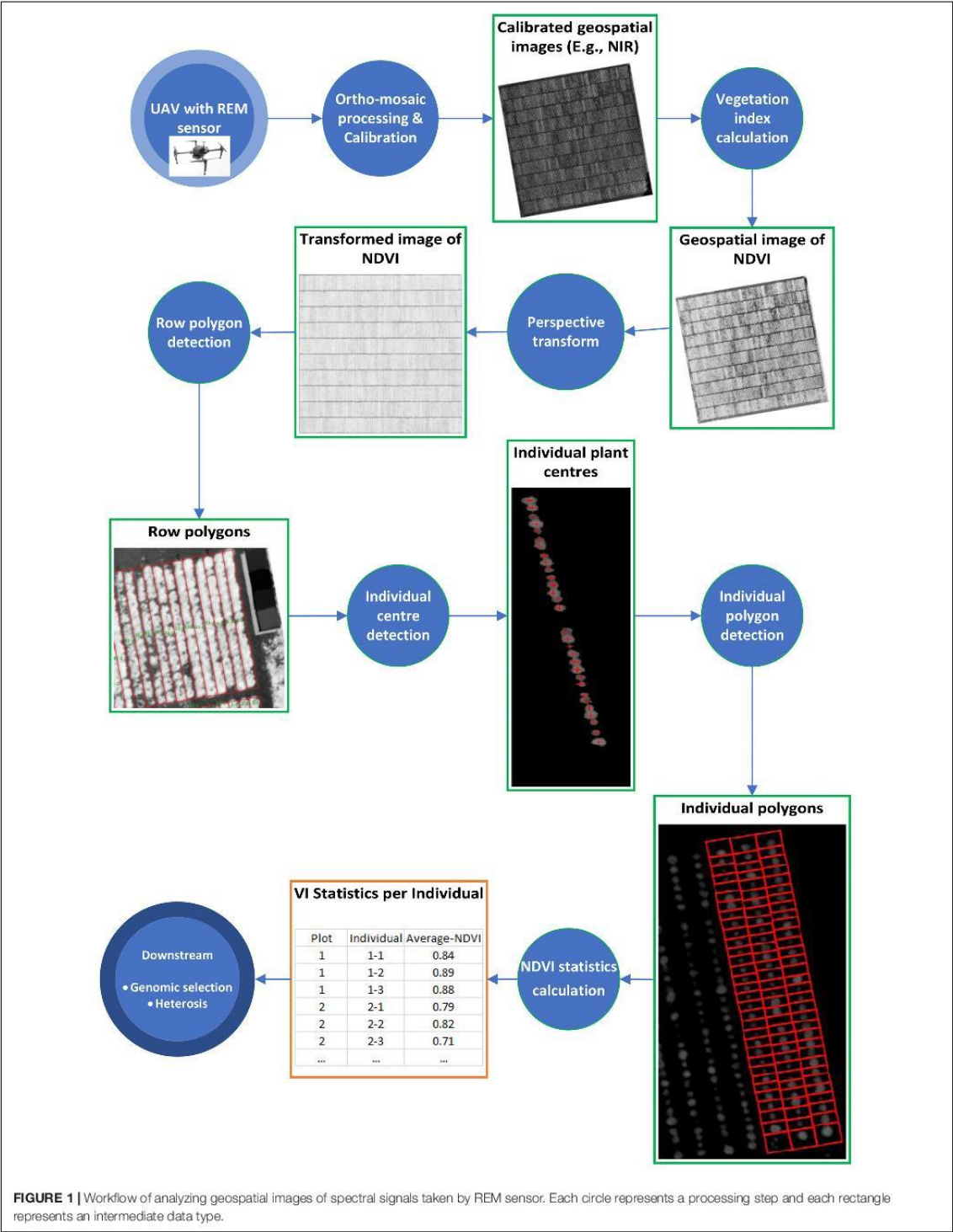
We generated a polygon as the boundary of each row of 32 plants as well as a polygon around the boundary of each individual plant with an identification number (plant ID) from the calibrated NDVI images above. The process comprises of four components:

Perspective Transform

Four corner GCPs in the field were used to form a polygon bounding the area of interest. The area contained in this polygon for each NDVI image was subsequently cropped and rotated into a rectangular shape using a perspective transformation (Figure 2), where the plant rows and blocks were essentially orthogonal to the image axes. Identification of plant row polygons.

A set of 1,500 polygons, each of which contains a single row of plants, was generated based on each transformed NDVI image (time point). The best polygon for each plant row, which we refer to as a consensus polygon, was subsequently chosen and applied to all downstream analysis later.

Plant rows (Figure 3D) for each transformed NDVI image were identified using projection methods, whereby the sum of the image pixels along the image columns and image rows was used to produce vertical and horizontal projection profiles. These profiles were then processed (Figure 4) to identify the centers of each trough (low-intensity regions) separating either the plant blocks (Figure 3B) or the plant rows (Figure 3D). Briefly: first, the plant blocks were identified by taking the projection of the image pixels values along the x-axis as shown in Figure 3A where the horizontal projection is seen in the profile to the right of the sub-image. The actual processing steps used to identify the troughs between the plant-blocks are laid out in Figure 4, from which it is seen that only one external parameter is required;



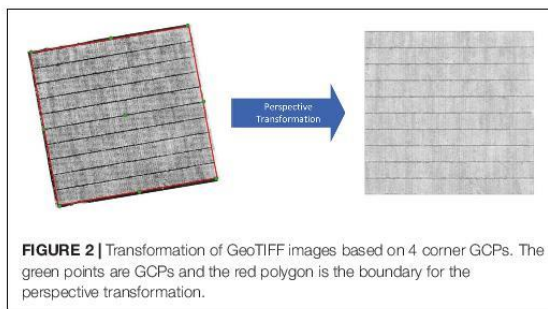


FIGURE 2 | Transformation of GeoTIFF images based on 4 corner GCPs. The green points are GCPs and the red polygon is the boundary for the perspective transformation.

that is the expected length of the trial plant rows. Next, once each plant block was isolated (Figure 3C) the individual plant rows were then identified using the same process outlined in Figure 4 in each plant block, but where the projection was along the y-axis, and the plant-row length parameter was replaced with the expected plant width/diameter.

The edges associated with each plant-block and plant-row (Figure 4D, blue vertical lines) were then used to construct an initial estimate of each plant-row polygon/box (Figure 5A). As can be seen in Figure 5A, the initial estimates of the plant-row boxes underestimated the extent of each box in the vertical direction. The amount of underestimation in this case was deliberate and was controlled by the size of the erosion in the profile processing (Figure 4B). This was done to compensate for the lack of alignment in the actual start and end vertical position of each planted plot-row in each block (Figure 5A). However, this meant the trial plants between two vertically aligned plant-boxes were not assigned to a plant-row box. Therefore, to assign each of these plants to a plant-row-box a box growing procedure was developed as shown in Figure 5B.

In the box growing procedure, each initial plant-row box (Figure 5A) is linked with the boxes directly above and or below it. Each initial plant-row box is linked to at most two other boxes. The region between two linked boxes can be defined by a rectangle, with the top edge of the link delineated by the bottom edge of the top box, and the bottom edge of the link delineated by the top edge of the lower box. The sides of the rectangular linking region are defined by the minimum and maximum pixel x-positions taken from the intersection of the x-positions defined by the top and bottom boxes. Therefore, the width of a linked region is defined by the maximum x-position minus the minimum x-position plus 1.

Next, for each of the linking regions, a projection profile was constructed from the row-wise sums of the pixels in each of the rows in the linking region. Let such a profile be represented as $f(x)$, where $x \in \{1, \dots, n\}$ and n is the number of image pixel-rows between the linked boxes. $f(1)$ represents the sum of pixels along the top edge of the link and $f(n)$ represents the sum of the pixels along the bottom edge of the link. Let t represent a positive threshold value. The threshold t is set to the average background (non-plant) pixel intensity and is scaled during box growing to the number of pixels between the upper and lower edges of the growing boxes. This threshold can be determined from the image

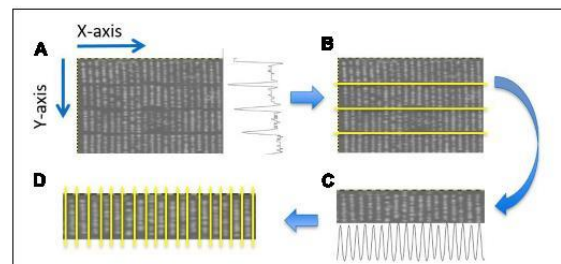


FIGURE 3 | Identification of plant blocks and plant rows. (A) Partial view of an NDVI UAV field image. The trace to right of this image represents the sum of the pixel values along each row of the image producing a vertical profile. (B) Overlay of identified horizontal dividing lines separating the four plant blocks. (C) The projection method applied to each plant block was used to identifying the plant rows in each plant block. Here the horizontal profile seen at the bottom of (C) constitutes the sum of the pixels along each pixel column within the given block. (D) Overlay showing the separation obtained between adjacent plant rows for a particular block.

or can be supplied by the user. The details of the box growing procedure are outlined in the following pseudo-code:

```

Let  $j = 1$ ;  $k = n$ ;  $a = 0$ ;  $s = \sum_{i=1}^n f(i)$ ;  $t = \text{specified threshold}$ 
width = number of pixels in a link row
while( $j < k$ ) {
  if ( $f(j) > f(k)$ )
     $a += f(j)$ ;
     $j++$ ;
  else if ( $f(j) == f(k)$ )
     $a += f(j) + f(k)$ ;
     $j++$ ;  $k--$ ;
  else
     $a += f(k)$ ;
     $k--$ ;
   $t1 = t * \text{width} * (n-j+1)$ ;
  if ( $s-a \leq t1$ )
    break;
}
Add to bottom edge of top box  $j-1$  rows;
Add to top edge of bottom box  $n-k$  rows;

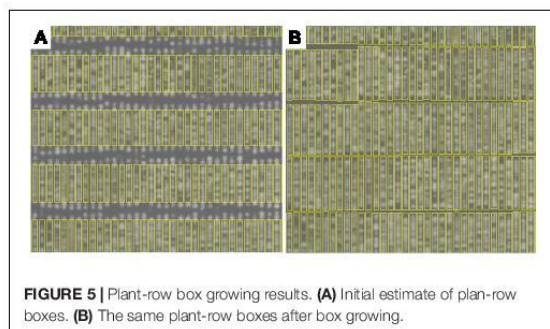
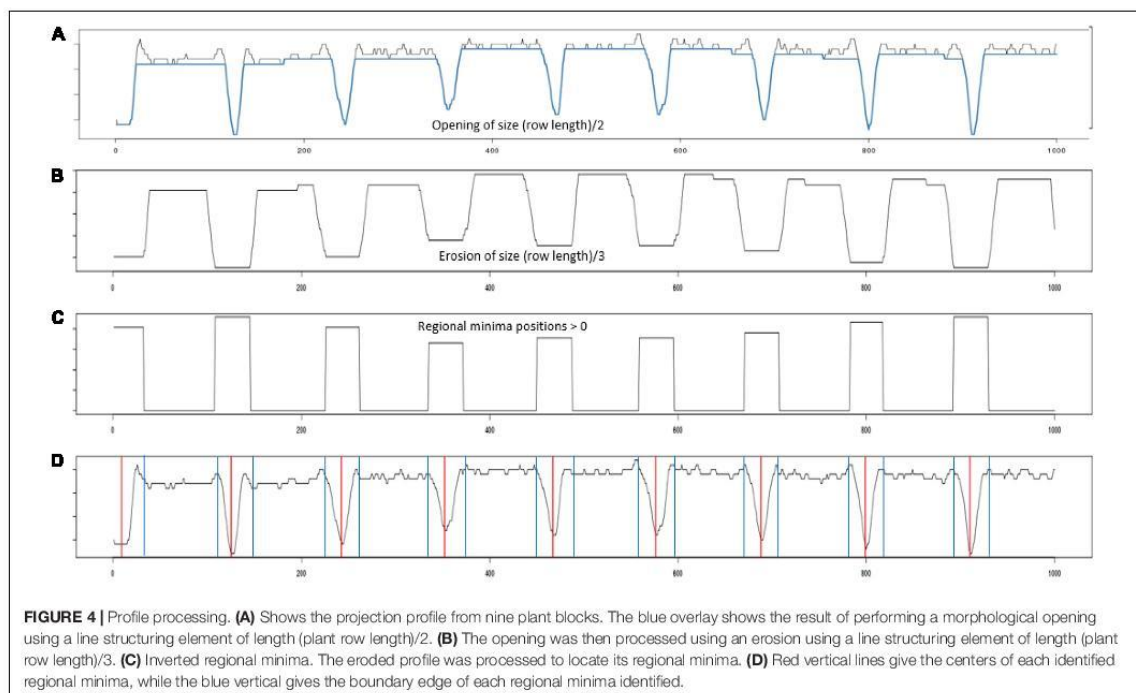
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Choosing Consensus of Plant Row Polygons

As the plant row polygons were detected for each image separately, we chose a best set of polygons that could be applied to all downstream analysis. In order to choose the most stable set of polygons, the variance of the area of the 1,500 polygons for each transformed NDVI image (time point) was calculated and compared across all time points. The plant row polygons identified from the image captured at 31/03/2017 had the least variance in polygon area and were chosen as the consensus.

Individual Center Detection

To identify single plants within the GS field trial, we applied the following 2-step process.



Firstly, a center point of each individual plant was identified. The NDVI ortho-mosaic TIFF image (captured date: 31/03/2017), from which the consensus of plant row polygons was developed, and subsequently processed in Pix4D was imported into eCognition (eCognition developer 9.2, Trimble, Munich, Germany). The template editor algorithm in eCognition was used to allow for the identification of individual plants. A test template was created by selecting 35 samples (Width: 20; Height 20; Context: 4) which were representative of various sizes of perennial ryegrass plants. The correlation between the generated test template and selected samples was 0.774. A small-region of the NDVI layer was randomly selected to validate the generated template and predict individual plants

within the area selected. The identified plants within template were then manually verified by the user as being correct, false, or unsure predictions to allow eCognition to refine the prediction model. After the template validation, test parameters values were obtained [ground truth tolerance (pixels): 3 and threshold: 0.6]. The template applied to the test region correctly identified 281 plants, missed 18 plants and incorrectly identified 2 samples as plants. The generated template was then updated based on the test area and the updated template, which had a sample correlation (0.917) to the samples within the selected area. This template matching algorithm was then applied to the trial area. All points predicted outside of the plant-row box was discarded. Manual visual quality control was performed within each plant-row box and further points added or removed to ensure that it was correct representative of the plants within the image were included.

Individual Polygon Detection

Once all plant-row-boxes were constructed, and all plant centers identified, a simple procedure was used to assign each plant-center to its enclosing plant-row-box. Once all plant-centers were assigned to a plant-row box, individual plant-boxes needed to be constructed around each plant center. This was done by (1) using the midpoint between adjacent plants-centers to create a horizontal line connecting the left and right edge within each plant-row-box and (2) the enclosing boxed region containing a plant-center was then identified and used to determine the coordinates for each plant-box. Consequently, each plant-row

polygons was split into 32 individual plant polygons (bottom right **Figure 1**). Each individual plant polygon was also annotated by an identification number (plant ID) and was used to calculate the NDVI statistics for each plant in eCognition.

Ground-Based Plant Height Data Acquisition and Processing

In the ground-based platform, “PhenoRover,” a Polaris ranger side-by-side vehicle was used for ryegrass morphometric data collection. Details of the PhenoRover platform and data processing method was discussed in our previous paper (Gebremedhin et al., 2019a). In brief, the vehicle has six ultrasonic sonars for plant height measurement attached to a welded steel bar at 0.6 m above-ground. For accurate geolocating of each sensor’s data, a single Real-Time-Kinematic Global Navigation Satellite System (RTK-GNSS) unit was placed on the top of the vehicle. Sensors data was stored on a data logger (CR3000, Campbell Scientific, Inc., Logan, UT, United States). For extracting data, we first projected the latitude and longitude coordinates to Universal Transverse Mercator (UTM) coordinate system and calculated the sensor position followed by matching sensor data to individual plants in the quantitative GIS software interface. The same polygons used to extract NDVI were superimposed on top of each geo-referenced data in the quantitative GIS software interface to extract plant height.

Destructive Biomass Measurements

The aboveground biomass was harvested manually at 5 cm height. Destructive biomass harvest of 480 space-plants was collected following the same procedure described previously (Gebremedhin et al., 2019a). In addition, mechanical cutting of all the GS field experiment plots was conducted to measure total fresh biomass weight (kg/plot) at the row or plot level. In this case, biomass was measured at each seasonal harvest from all 500 plots. This data was used to validate the computational phenotyping workflow with manually derived polygons as well as to compare manually collected biomass with predicted biomass using the workflow.

Data Analysis

The variability distribution of phenotyping data was statistically analyzed using R version 3.6.1, R Development Core Team, Vienna, Austria. Non-destructive biomass estimation of individual plants was described using the combination of NDVI and plant height. Spatial distribution variance maps of NDVI, plant height, predicted and measured DMV in three seasons were compared. Histogram of distribution graphs was prepared in Tableau Software (Tableau2019.4, Seattle, Washington, United States). The predicted DMV of spaced-planted individuals was therefore estimated using the NDVI and height combination (NDVIsq_PH) using the following general and seasonal formulas derived from Gebremedhin et al. (2019a):

Universal Equation:

$$\text{Biomass} = \text{intercept} + \text{NDVIsq_PH} + \text{Season} + \text{NDVIsq_PH} \times \text{Season} \quad (1)$$

Seasonal Equations:

$$\text{winter2018} = -4.62 + \text{NDVIsq_PH} \quad (2)$$

$$\text{late} - \text{spring2018_1} = -4.62 + (5.088 \times \text{NDVIsq_PH}) + 5.76 + (1.49 \times \text{NDVIsq_PH}) \quad (3)$$

$$\text{late} - \text{spring2018_2} = -4.62 + (5.088 \times \text{NDVIsq_PH}) + 16.86 + (3.68 \times \text{NDVIsq_PH}) \quad (4)$$

The statistical regression between the predicted and measured DMV during each harvest was compared at single plant and plot level. Similarly, a model considering all three harvests of biomass estimation was also developed. Coefficient of determination (R^2) and root mean square error (RMSE) were used to evaluate and quantify the accuracies of regression models of phenotyping variables. Normality of the data was verified by checking the residual vs. fitted values of plots to ensure the normality of residuals with constant variance.

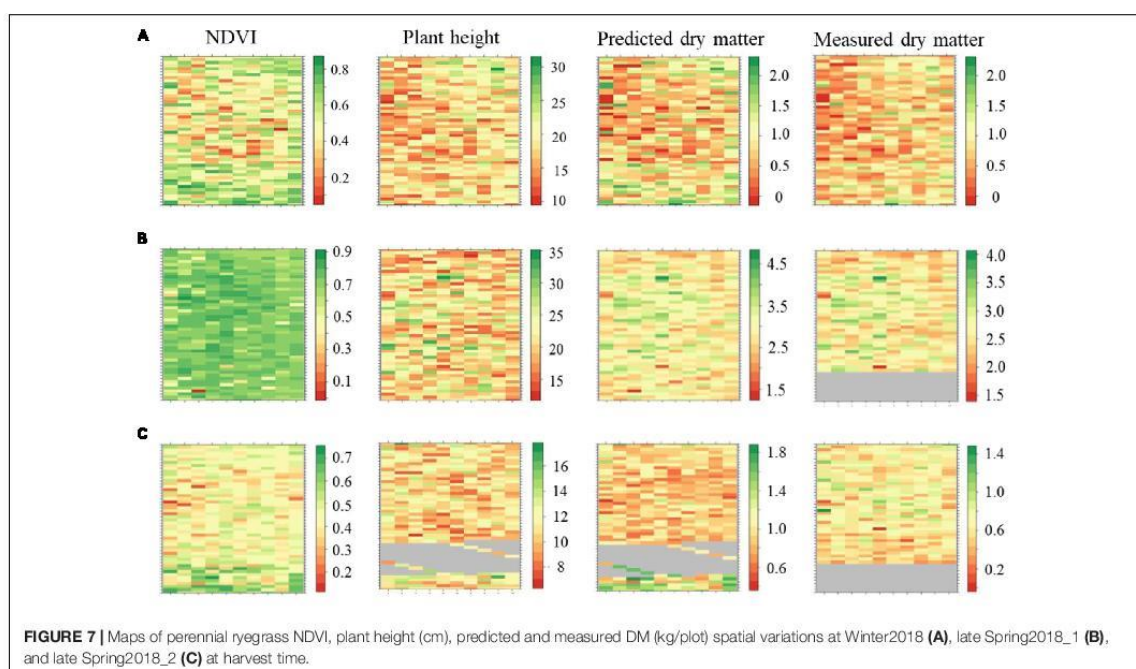
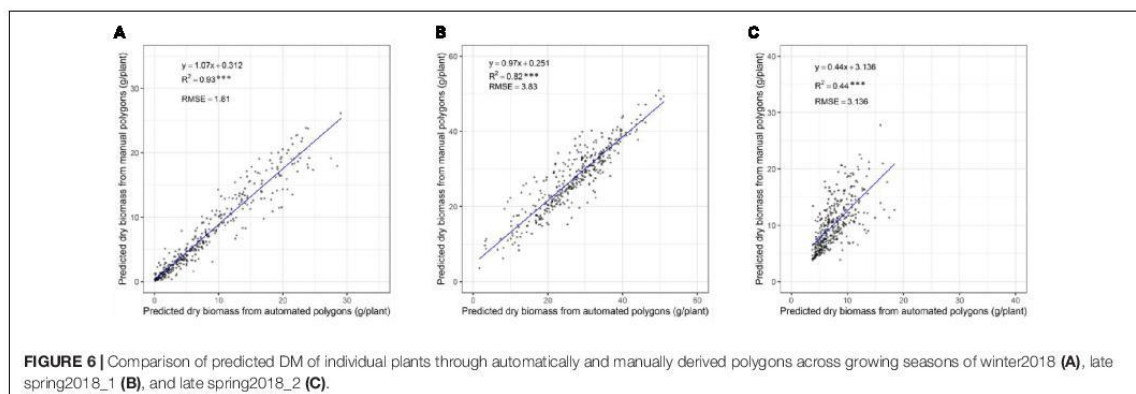
RESULTS

Comparison of Automated and Manually Derived Delineated Polygons

In our previous validation experiment, we developed a model to predict the DMV of individually drawn polygon by combining NDVI and plant height (Gebremedhin et al., 2019a). Polygons used to extract data were manually, but it is difficult to apply this into 48,000 plants. As a result, a computational phenotyping workflow was applied to extract NDVI and plant height information. To evaluate the robustness of the automatically derived polygons, the predicted DMV of individual plants from automated and manually derived polygons was compared across three different seasons (**Figures 6A–C**). The results show a strong coefficient of determination between predicted DMV from automated and manual polygons with R^2 values ranging 0.82–0.93 for the winter2018 and late-spring2018_1 season and RMSE values ranging from 1.81 to 3.83 g/plant. For the late-spring2018_2, harvest a moderate coefficient of determination ($R^2 = 0.44$) of DMV prediction was obtained. These results indicate that automatically built polygons can replace manually derived polygons for plant height and NDVI accurate extraction, and biomass prediction for all 48,000 plants in our field experiment.

Seasonal Spatial Variation of Biomass and Other Traits

The plant height, NDVI and predicted DMV variables were retrieved using a computational data processing workflow developed in this experiment (**Figures 7A–C**). NDVI showed seasonal variability with increased values observed in late-spring2018_1 compared to winter2018 and late-spring2018_2. Most of the plots in the late-spring2018_1 indicated NDVI was at saturation point at the time of harvest. Spatial variations of NDVI values were observed in the middle, top and lower part of the field experiment in all seasons. For instance, the center

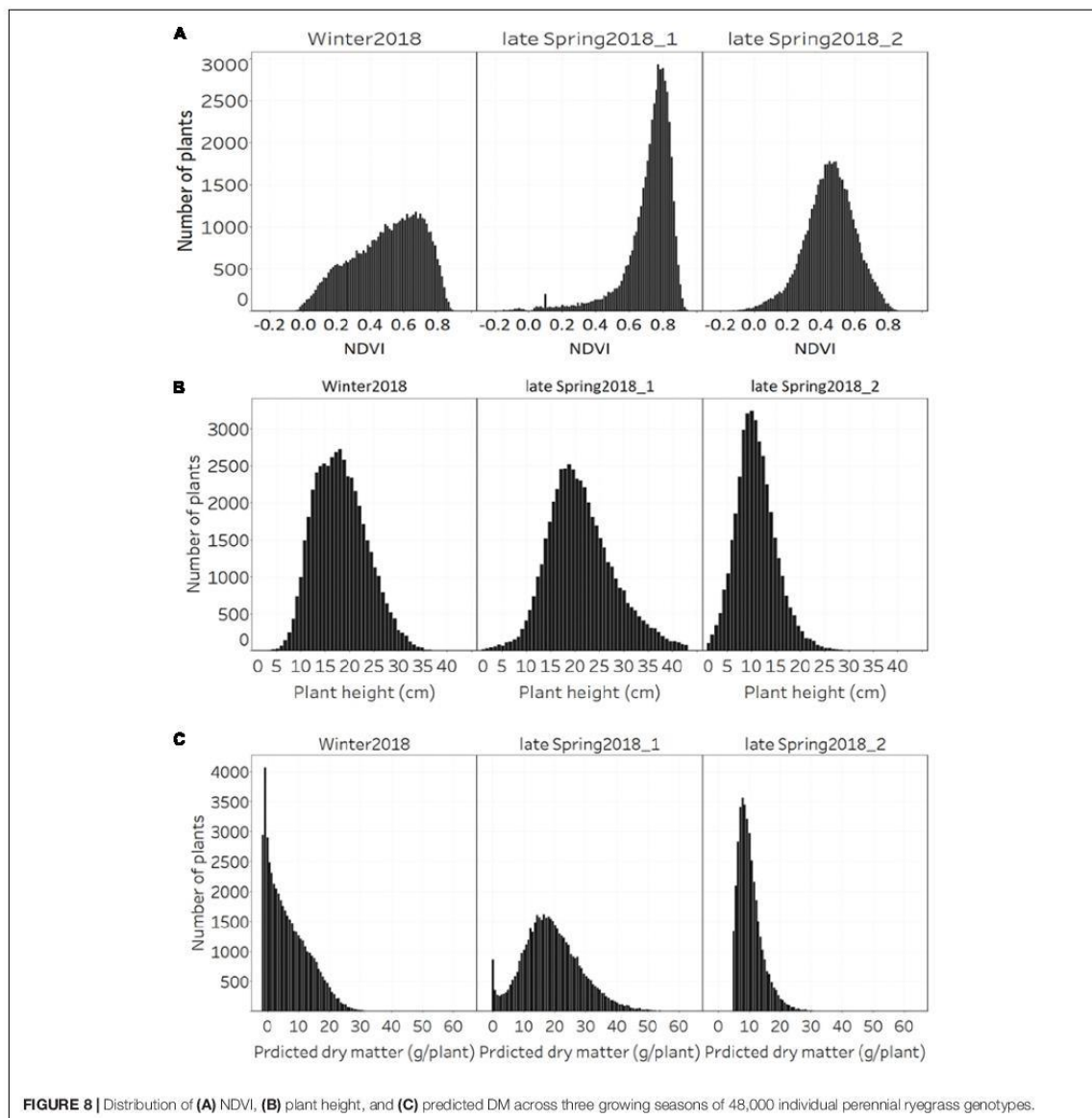


part of the GS trial site showed explicitly lower NDVI values in winter2018 compared to an upper and lower location within parts of the field experiment. Seasonal plant height and predicted biomass variation occurred in the winter2018 followed by peak accumulation in the late-spring2018_1 then decreased into late-spring2018_2.

Distribution of NDVI, Plant Height, and Predicted Biomass Across Growing Seasons

The distribution of NDVI, plant height and predicted biomass across three growing seasons, of the entire population of

48,000 individual perennial ryegrass genotypes was estimated (Figures 8A–C). Figure 8A shows the NDVI distribution variation of individual plants across seasons. The winter2018 season had a wider spread of NDVI values at the time of harvest for most genotypes, with some already reaching saturated NDVI values. In the late-spring2018_1, the narrow spread of NDVI values distribution was skewed toward the right indicating that most of the individual plants reached the NDVI saturation point at the time of harvest. In the late-spring2018_2 harvest, relatively normalized distribution of NDVI values was observed signifying some individual plants were harvested earlier than the 2–3 leaf stage.



The plant height of spaced-plants showed a normal distribution across all growing seasons (Figure 8B). Taller plants were observed at the late-spring2018_1 harvest followed by the winter2018 and late-spring2018_2 harvests, respectively. Figure 8C shows the predicted DMY distribution variation of individual plants across seasons. The winter2018 and late-spring2018_2 seasons histogram distributions were similar with left-skewed distributions of narrow-spread values at the time of harvest for most genotypes. The predicted DMY distribution of the late-spring2018_1 harvest was observed

to be normally distributed, contrary to the winter2018 and late-spring2018_2 harvests.

Comparison of Seasonal Distribution of NDVI, Plant Height, and Predicted Biomass of Cultivars

Figure 9 displays the NDVI histogram distribution variance of 50 cultivars/breeding lines for each of the three harvests. Cultivars showed large relative differences in growth and development

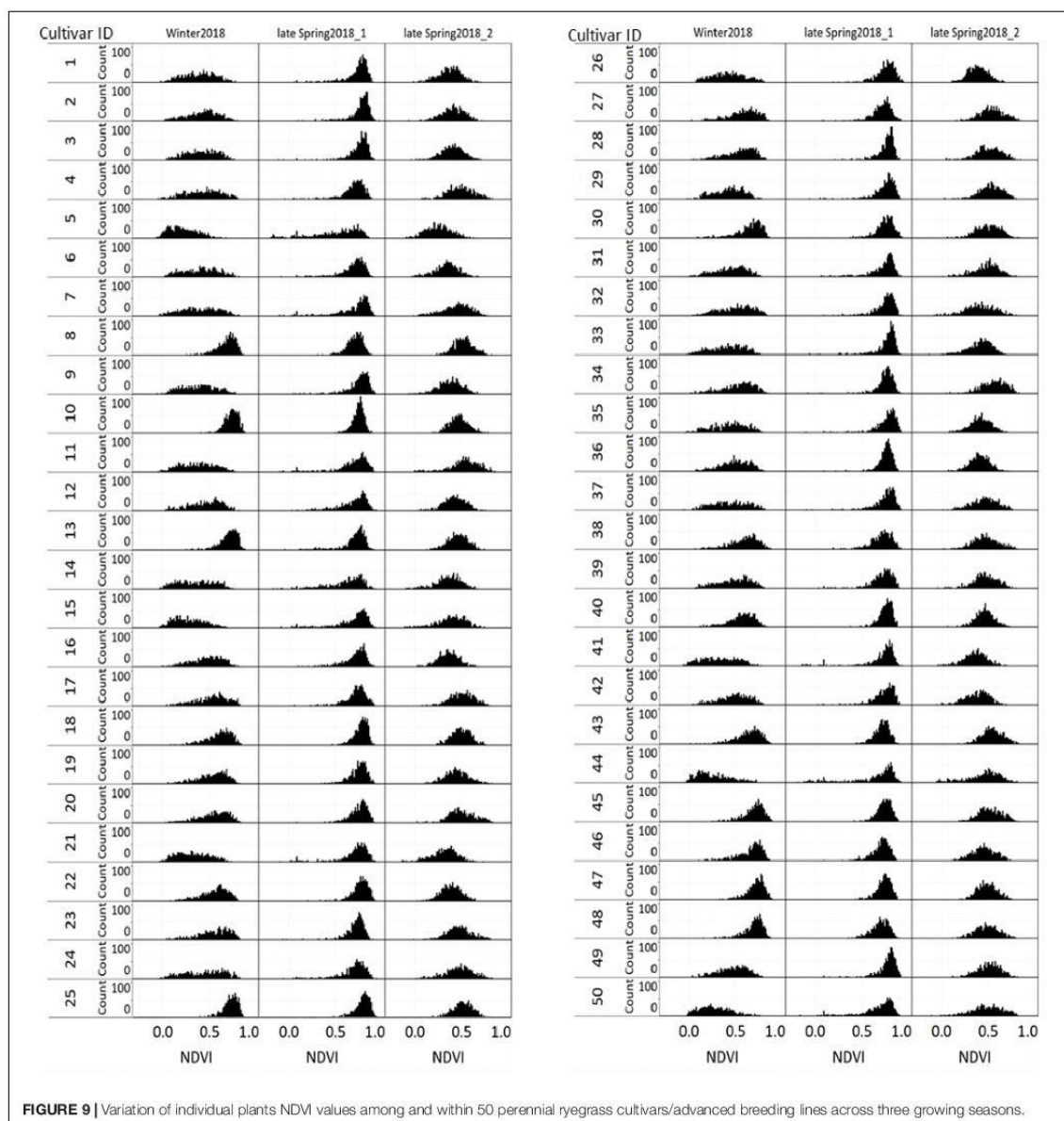


FIGURE 9 | Variation of individual plants NDVI values among and within 50 perennial ryegrass cultivars/advanced breeding lines across three growing seasons.

between and within a harvest. For example, cultivars designated with a Cultivar ID number 8, 10, 13, 25, 45, 46, 47, and 48 reached saturation and the overdue harvest was observed in winter2018.

Figure 10 displays the plant height variation distribution of 50 cultivars/breeding lines across three seasons. Similar to the NDVI histogram distribution, the plant height variation of each cultivar was obvious at the winter2018 harvest. For instance, cultivars designated with Cultivar ID number 8, 10, 13, 25, 45, 46, 47, and 48 showed higher height values than their counterparts.

Similarly, plants with Cultivar ID 5, 9, 12, 14, 16, 21, 33, 35, 36, and 41 showed a wider distribution at the late-spring2018_1 harvest. The plant height distribution for the late-spring2018_2 harvest did not show any major histogram structural differences among cultivars.

Figure 11 displays the variation of individual plant biomass (predicted) among and within 50 perennial ryegrass cultivars/advanced breeding lines across three growing seasons. Similar to the plant height and NDVI, cultivars designated with a

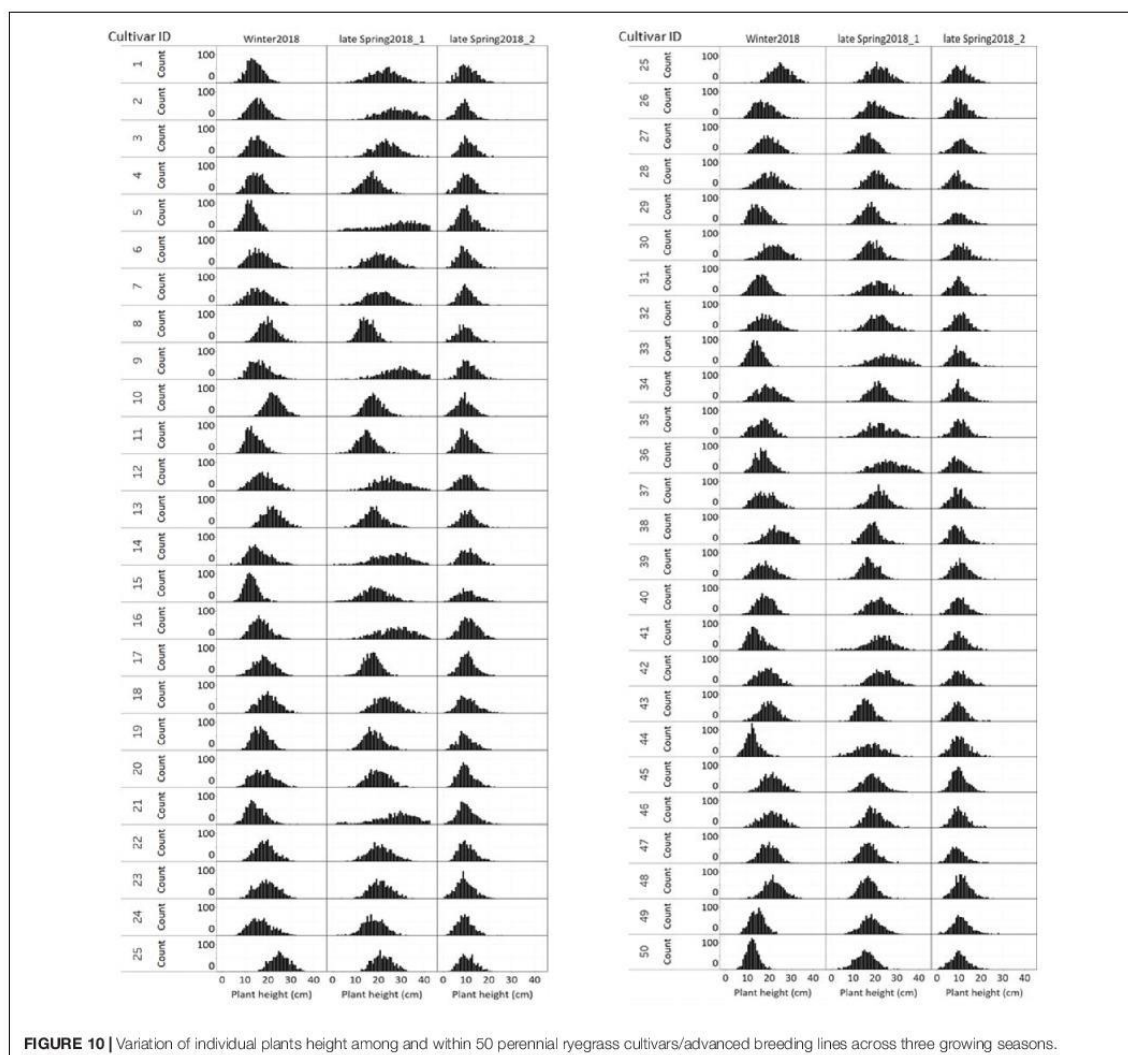


FIGURE 10 | Variation of individual plants height among and within 50 perennial ryegrass cultivars/advanced breeding lines across three growing seasons.

Cultivar ID number 8, 10, 13, 25, 45, 46, 47, and 48 showed higher height values. However, the histogram of predicted DMY for each cultivar at the late-spring2018_1 harvest showed more variation compared to the winter2018 and late-spring2018_2 harvests. Moreover, varietal differences in DMY distribution of some cultivars showed a clear difference at the winter2018 harvest.

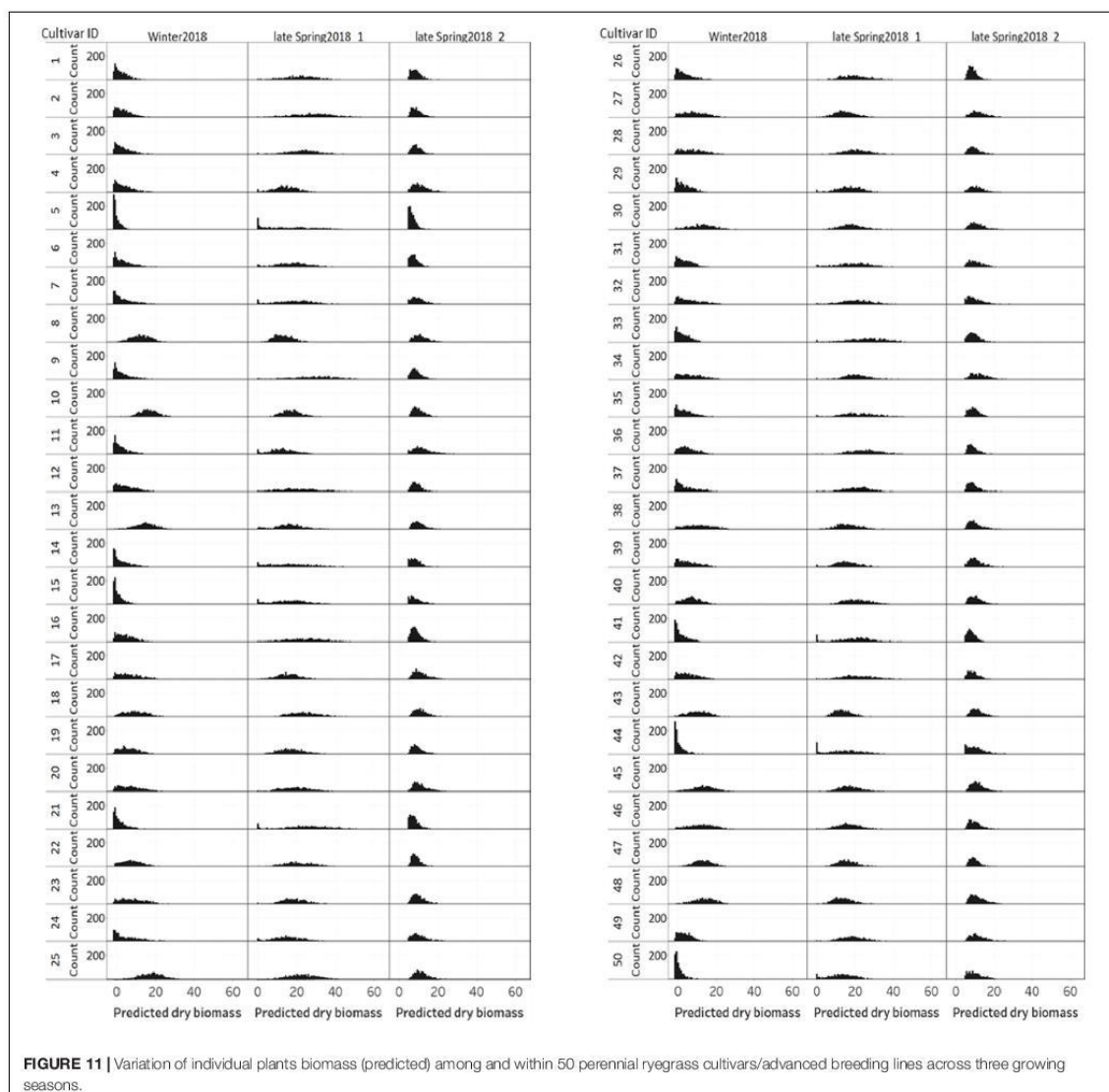
Plot Level Seasonal Biomass Estimation Model

The automated biomass prediction of spaced individual plants was translated to plot-level estimates. The sum of predicted biomass from spaced planted 96 individual plants within the plot was calculated to determine predicted biomass per plot and compared with the mechanically harvested and measured

biomass data. Significantly high coefficients of determinations were observed between measured and predicted DMY ($R^2 = 0.19$ – 0.81) at all three harvests (**Figures 12A–C**). Subsequently, RMSE values ranged from 0.09 to 0.21 kg/plot was observed at these harvests. When combining all harvests into a single regression of estimated and measured biomass (**Figure 13**), the best coefficients of determination had a slope, R^2 and RMSE values of 0.93, 0.94, and 0.21 kg/plot, respectively.

DISCUSSION

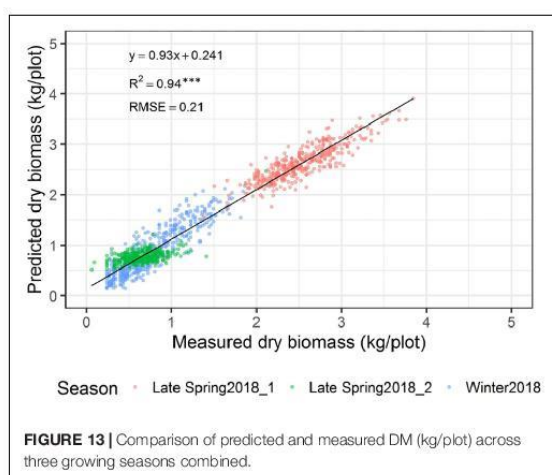
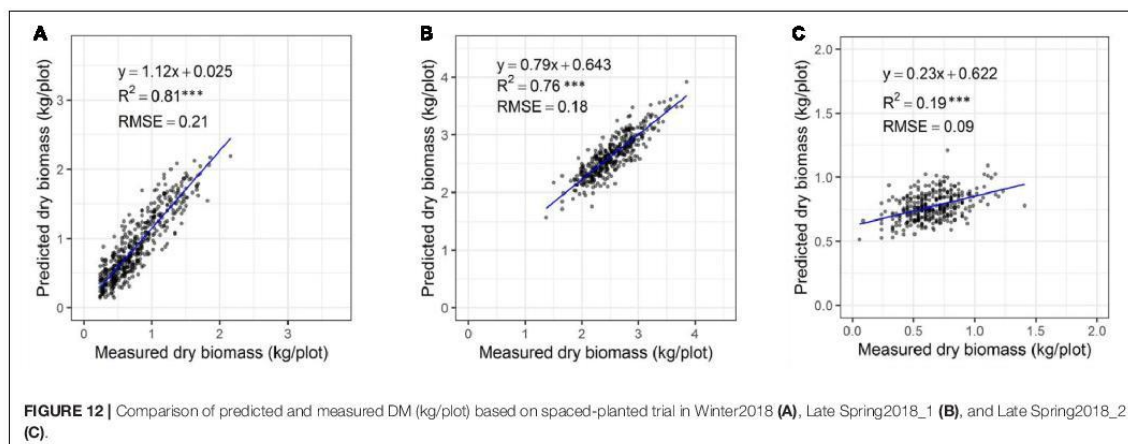
In commercial ryegrass breeding, experiments with thousands of individual space-plants may be used for evaluation and



selection (Hayes et al., 2013; Lin et al., 2016). Phenotyping of these genotypes requires repeated evaluation across growing seasons and years. This is essential to select, potentially high yielding genotypes to take into future stages of a breeding program. However, phenotyping of large populations, by the available traditional methods of visual scores, manual cutting, bagging and drying is difficult to implement in terms of time and cost. This study developed and validated ground-and aerial-based platforms equipped with advanced sensors for high-throughput phenotyping of ryegrass biomass yield. This resulted in the development of a computational workflow for image acquisition, processing, and analysis to

predict biomass yield based on vegetative indices and plant height from 48,000 ryegrass plants. With this method, it was possible to assess traits including predicted biomass of 48,000 individual plants in 6 h from data acquisition to data extraction. This method may provide a robust method for ranking of genotypes and make faster progress in breeding programs.

Manually drawing thousands of polygons has been indicated difficult and considered as time-consuming and automation of polygons for 38,000 plots was expected to increase the throughput and accuracy of traits extraction (Shi et al., 2016). A coefficient of determination between



the predicted DMY from automated and manually derived polygons in the late-spring2018_2 was moderate ($R^2 = 0.44$). This could be due to smaller plant height architecture at that particular time, which coincided with early drought onset in the spring season of 2018. The canopy of these smaller plants may have reflected weak sound echoes from the surface of each plant, which may result in a greater variability in height values that resulted lower R^2 .

Biomass yield distribution of perennial ryegrass cultivars may vary across growing seasons and years (Brock et al., 1996; Chapman et al., 2015). These variations mainly occur due to genetic merit of productivity and seasonal growth rate differences of cultivars during the vegetative growth stage. The ability to measure how much differences exist mainly depends on destructive harvest or visual score, which is restricted by low-throughput (Walter et al., 2012; Ghamkhar et al., 2019). Sensor-based non-destructive biomass

yield predictive methods have the potential to replace this with applicability in large scale screening of breeding lines across seasons and years. Generally, individual plants NDVI, plant height and predicted biomass estimations showed variations across seasons. The distribution of NDVI values was wider in the winter2018 than late spring2018_1 and late spring2018_2. This indicates the various genotypes responded differently to cold season conditions (Förster et al., 2018). However, plant height and DMY predictions in the late spring2018_1 indicated higher values in which ryegrass appeared to have rapid growth and faster accumulation of dry matter in the spring season (Wang et al., 2019).

In this study, NDVI, plant height and predicted biomass estimations from the UAS and PhenoRover showed obvious temporal and spatial variabilities that mainly arises from genetic and environmental interactions. In the winter2018 harvest, NDVI histograms of some cultivars (Cultivar ID 8, 10, 13, 25, 45, 46, 47, and 48) indicated they were at NDVI saturation point before the harvest. This was further confirmed by the estimates of the plant height and DMY of these cultivars. These methods of accurate non-destructive estimates may provide a better perspective to facilitate breeding selection and ranking of ryegrass genotypes. The application of this non-destructive biomass prediction will enable the development of seasonal forage value indices (FVI) which combine biomass yield and economic values to select best performing commercial cultivars (Leddin et al., 2018; Giri et al., 2019). At the late-spring2018_1 harvest, plant height increased, and NDVI values saturated for all cultivars. The increase in plant height was from the fast growth rate in the spring season and NDVI value peaked and reached saturation before harvest. This result agrees with previous studies in wheat (Li et al., 2019) and buffelgrass (Mutanga and Skidmore, 2004) where NDVI tended to saturate at high canopy density. However, in the late-spring2018_2 lower NDVI, plant height and predicted biomass yields were observed for some plants. This indicates there was an early-onset of a late spring

drought stress, that can restrict leaf growth (Cyriac et al., 2018), and plants with a thin canopy density are expected to reflect limited echoes and NDVI values (Condorelli et al., 2018; Yuan et al., 2018).

Experimental plots in this study were represented by three sets of rows containing 32 spaced-planted individuals each (total 96 plants/plot), in which individual plants level biomass prediction has reflected an accurate representation of plots biomass prediction. The results showed a significantly higher coefficients of determination between the predicted and measured DMV across three seasons with $R^2 = 0.19$, 0.75, and 0.81 at the winter2018, late-spring2018_1 and late-spring2018_2 harvests, respectively, and RMSE values ranging from 0.09 to 0.21 kg/plot. Subsequently, the overall regression of the three harvests indicated significantly high coefficients of determination ($R^2 = 0.94$) and RMSE 0.21 kg/plant. Similar coefficients of determination trends ($R^2 = 0.76$ – 0.89) were observed in previously reported pasture studies when various sensors were used to estimate biomass yield of tall fescue and ryegrass (Safari et al., 2016; Ghamkhar et al., 2019).

In this study, we validated a computational workflow for image acquisition, processing, and analysis to predict the biomass yield based on vegetative indices and plant height measurements of 48,000 ryegrass plants. Previous studies indicated the use of NDVI for ranking cultivars of ryegrass (Wang et al., 2019), field pea, canola, and spring wheat grain yield (Brian McConkey et al., 2004) and lint yield in cotton (Hugie et al., 2018). Considering the plant height and NDVI as a surrogate to predict DMV of individual and plot-level plants, there is a great potential to apply our workflow to be used for ranking of genotypes and cultivars across growing seasons and years. Our method also has the potential to further pave a foundation for the application of sensor technologies combined with GS that leads to faster rates of genetic gain in forages. To further improve the stability and accuracy of the automated workflow, other traits including vegetative indices other than NDVI, volume and leaf area features may be included by incorporating measurements through sensors including LiDAR and various camera systems (Ghamkhar et al., 2019; Yates et al., 2019).

CONCLUSION

The computational phenotyping workflow developed in this study indicated that automatically built polygons could replace manually derived polygons for plant height and NDVI accurate extraction from 48,000 plants. The NDVI, plant height, predicted and measured DMV showed accurate seasonal and spatial variations, which may contribute to identifying differences among genotypes of varying genetic background. Thus, the proposed computational phenotyping workflow proved to enable non-destructive and objective prediction of individual plants NDVI, plant height and DMV

in three different seasons. Subsequently, the observed plot level linear relationship between predicted and measured DMV indicated capability of the developed computational workflow to estimate plot-level DMV from the individual plants' prediction. Therefore, this study demonstrated the potential of the validated computational workflow to conduct seasonal and annual ranking of breeding lines and commercial cultivars that may be used for the application of genomic selection that paves to greater rates of genetic gain in forages.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KS, PB, JW, and GS conceived the research. KS, PB, and JW designed and participated in charge of overall supervision of the study. KS and AG drafted the manuscript. AG wrote the manuscript with input from all authors. AG and KG analyzed the data. PB and AG analyzed images and plant height data. EB, PB, and FS developed the automated polygons used to segment images and plant height data from the spaced plant trial.

FUNDING

The authors acknowledge financial support from Agriculture Victoria, Dairy Australia, and the Gardiner Foundation.

ACKNOWLEDGMENTS

We would like to thank the plant molecular breeding staff of Hamilton Centre (Agriculture Victoria Research) for managing the field experiments, Andrew Phelan and Carly Elliott for doing all the flights and some of the PiX4D data processing. We would also like to thank Daren Pickett, Russel Elton, Daren Kean, Chinthaka Jayasinghe, Chaya Smith, and Phat Nguyen for their contribution to manual and mechanical harvesting of samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.00689/full#supplementary-material>

FIGURE S1 | Experimental field site: A genomic sub-selection (GSS) experiment on perennial ryegrass at Hamilton Centre Research Station in Victoria State of Australia was conducted in three growing seasons in 2018. RGB ortho-mosaic image is used to display the GSS field experiment site, ground control points, and delineated polygons.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6. General Discussion

6.1 Overview of Research

Perennial ryegrass is the primary source of feed in the dairy industry in temperate Australia (Ludemann *et al.* 2013). Developing cultivars with improved dry matter yield (DMY) is the primary objective for ryegrass breeders. In recent years, advances in genomic selection breeding methodologies have been implemented to accelerate genetic gain through increasing the accuracy of genomic breeding values in perennial ryegrass (Lin *et al.* 2016; Lin *et al.* 2017). However, the advancement of high-throughput phenotyping (HTP) platforms has been relatively slow in forage breeding programs (Lootens *et al.* 2016; Gebremedhin *et al.* 2019b).

Field phenotyping is the basic component of crop improvement as it is important to explore a crop's genetic merits and the environmental influence on effects of morphological characters (Rebetzke *et al.* 2019). Therefore, phenotyping forage grasses with highly diverse growth habits under field conditions may be critical for the characterisation and non-destructive capturing of phenotypic information such as DMY from thousands of genotypes rapidly and objectively. Linking these phenotypic data with genomic information from candidate genes, quantitative trait loci or mapping the whole genome may increase the rate of genetic gain through molecular breeding.

Recently, various field-based HTP platforms have been applied for non-destructive estimation of forage agronomic traits (Chapter 2). Various sensors have been deployed on platforms including handheld, small carts and trolleys, ground vehicles and unmanned aerial systems (UAS). These deployments were used mainly to phenotype traits of spaced planted and sward plot of breeding field trials. For instance, RGB (red-green-blue) imaging cameras were deployed on a tripod (Lootens *et al.* 2016) or custom-made trolley (Hunt *et al.* 2015) to estimate dry matter yield (DMY) from individual plants. The mobile platform with a LiDAR scanner described by (Ghamkhar *et al.* 2019) was successfully deployed to develop DMY prediction in single-row plots. However, the phenotyping platforms (Hunt *et al.* 2015; Lootens *et al.* 2016) were used to phenotype between 500-1100 individual ryegrass genotypes, but both have low-throughput that could limit the application on large field trials. The platforms mentioned above

were also designed without a global positioning system (GPS) which restricts the capability for extracting georeferenced data from target plants. UAS equipped with RGB and multispectral imaging systems were used to estimate DMY in plots of perennial ryegrass (Borra-Serrano *et al.* 2019) and white clover (Inostroza *et al.* 2016). These experiments were conducted on small plots, and these haven't been used to estimate individual plant DMY.

This thesis obligates ground and aerial-based platforms under development performed at Victoria, Hamilton. The platforms were fitted with ultrasonic sonar, light detection and ranging (LiDAR), GreenSeeker, multispectral imaging, real-time kinematic-global navigation satellite system (RTK-GNSS) and data logger system. The deployment of the array of these sensors on platforms enabled the establishment of a method that allows calibration and validation of the sensors to capture phenotypic information from individual plants, rows and sward plots (Chapter 3). Additionally, accurate geo-spatial location of single plants (error ≤ 4 cm) was achieved with accurately defined georeferencing, allowing for the quantitative measurements of spectral and plant height traits. In an automated manner, computational workflow for data acquisition, processing, and analysis enabled to predict the DMY based on normalised difference vegetative index (NDVI) and plant height measurements of 48,000 ryegrass plants was developed (Chapter 5). The phenotypic computational workflow could be used as a baseline workflow for developing biomass prediction model from our field trials that consist of a global perennial ryegrass reference population of 270,000 individual plants from 1300 varieties/breeding lines (Chapter 2). The implementation of the computational workflow may further contribute towards the application of sensor technologies combined with genomic selection for greater rates of genetic gain in forages, as part of the large forage breeding program at Agriculture Victoria Research and more broadly.

This thesis aims to develop, validate and deploy of sensor-based, non-destructive aerial and ground-based HTP platforms to estimate biomass of perennial ryegrass plants in-field. Firstly, calibration and validation of aerial and ground-based HTP platform provided a non-destructive approach for perennial ryegrass height and DMY phenotyping. Secondly, the thesis demonstrated how sensor-based phenotyping methods could improve perennial ryegrass research and support more effective and efficient forage breeding programs. Thirdly, this study demonstrated the application of combining NDVI from multispectral imaging and ultrasonic sonar estimates of plant height to estimate the seasonal distribution of biomass of 48,000 single plants for genomic selection in perennial ryegrass. Fourthly, the study discussed the application of developed platforms and data processing workflow which could allow to an increase the

accuracy of predicting genomic breeding values in genomic selection. In this thesis, four research questions have been developed (Chapter 1 section 1.6). Results provided a proof of concept of the application of sensor-based DMV estimation techniques, mainly answering the questions raised. The summary discussion of the thesis is provided as follows:

An optimal ground and unmanned aerial system (UAS)-based HTP platform were developed for phenotyping large breeding populations of perennial ryegrass based on the results of this thesis. The following on-board sensors were deployed: a high-resolution multispectral camera for canopy spectral information measurements at different perennial ryegrass growth stages and ultrasonic sonar and LiDAR sensors for plant height and canopy structure measurements. With the rapid improvement of platforms, sensor technologies and processing software, the next generations of platforms may provide semi- or fully data acquisition, processing and analysis with minimal human involvement.

6.2 Calibration and Validation of Sensors on Phenotyping Platforms

In 2016, a ground-based vehicle “PhenoRover system”, incorporating six ultrasonic sonar sensors, a LiDAR system, an RTK-GPS unit and data loggers, was constructed to collect morphological and spectral information from individual plants, plots and paddocks. All sensors were attached to the vehicle using a custom-made rigid steel frame to ensure the stability of the sensors on the platforms (Chapter 3). The sensor mounting height was kept constant to maintain consistency throughout the data collection period.

The aerial platform used in this study integrated a Parrot Sequoia multispectral camera system on a 3DR-Solo UAS, which can capture data in four narrow bands of green (550nm band centre), red (660nm band centre), RedEdge (735nm band centre) and near-infrared (790nm band centre) simultaneously. Constant data acquisition protocols were followed during flight and image acquisition. For instance, flight paths and mission plans were kept constant since mission planning is necessary for accurate data acquisition (Pepe *et al.* 2018).

At the of beginning of the PhD study, the ultrasonic sonars and LiDAR sensors as well as the RTK-GNSS navigation system had been fitted to the “PhenoRover system”, for plant height calibration and validations studies (Chapter 3). Results of Chapter 3 indicated that plant height measurements from ultrasonic sonar and LiDAR sensors showed the potential to replace a ruler with consistent repeatability for the controlled condition and field trials. Further plant height

measurements from ultrasonic sonar and LiDAR sensors accurately estimated DMY of individual perennial ryegrass plants. Results from Chapter 3 were supported by previous studies (Hazard *et al.* 2006; Borra-Serrano *et al.* 2019), where plant height data was used either directly as a primary trait or to develop linear regression for DMY estimation. NDVI from multispectral imaging and GreenSeeker demonstrated the capability for non-destructive moderate DMY estimation of individual ryegrass spaced planted and sward plots, respectively. However, Estimation accuracy using NDVI occurred to decrease due to the obvious saturation as the biomass increases (Gu *et al.* 2013; Schaefer and Lamb 2016). Therefore, the combination of plant height and NDVI was found to improve the DMY prediction accuracy by up to 10%. This was achieved by combining NDVI and plant height with a simple multiplication combination (NDVI*plant height), with the possibility to further improve the accuracy by application of different combinations for the parameters.

Calibration and validation of the aerial and ground-based HTP platforms proposed in this Chapter were successfully implemented. The calibration (e.g., geometric and radiometric images calibration), processing and analysis of sensors data, and combining data from different sensors to improve prediction accuracy were effectively executed for accurate prediction of plant height and DMY of ryegrass plants.

6.3 Application of Developed High-Throughput Phenotyping Technologies for Dry Matter Yield Estimation

Vegetative index and plant height information from the HTP platform can be combined to develop biomass estimation models in various crops (Tilly *et al.* 2015; Schaefer and Lamb 2016; Lu *et al.* 2019; Niu *et al.* 2019). Chapter four of this thesis explains the development of biomass estimation models by combining NDVI and plant height from multispectral imaging and ultrasonic sonar respectively (Gebremedhin *et al.* 2019a). The main finding of this Chapter was the integration of spectral and structural data from individual ryegrass plants to estimate DMY at the 3-leaf growth stage. Plant height and NDVI were used to estimate DMY separately and estimation models showed moderate accuracies. Therefore, various combinations of these parameters were tested, and higher accuracies were achieved by squaring the NDVI and multiplying the product with plant height values (NDVIsq_PH). Cross-validation models developed through this combination exhibited up to 24% in prediction accuracy of individual

plant's biomass. Various cross-validation models have been evaluated to predict non-destructive DMY estimation (Bendig *et al.* 2014; Brocks and Bareth 2018; Chen *et al.* 2018), where the stability of models was not always constant due to various reasons. Unlike these results, our evaluation model was accurate and stable. Thus, the proposed method can be assessed for applicability to accurately estimate thousands of diverse individual plants in GS spaced-plant experiments (Hayes *et al.* 2013; Lin *et al.* 2016).

Each sensor produces data which requires different systems of extraction and subsequent processing. Data extraction from ultrasonic sonars on the PhenoRover system was relatively simple and developing the processing workflow was successfully accomplished on quantitative GIS (Chapter 4). However, data from multispectral imaging sensors require long post-acquisition and image processing workflows that could incorporate analysis stitching of images based on geometric locations and radiometric calibrations. Various software programs including PiX4D, eCognition and quantitative GIS with various processing steps were applied to extract vegetative indices from these images. These sensors have medium-range complexity in developing processing pipelines. For instance, LiDAR and hyperspectral imaging could generate huge data files, and this results in high processing complexity in terms of extracting and cleaning of data.

Data extraction methods indicated in Chapter 4 did not take into consideration the automation of delineated polygons on plant boundaries. The polygons used for data extraction (e.g., NDVI and plant height) were drawn manually, and preparation of these polygons was slow and time-consuming. Application of this method to the large population field trial may not be feasible and automation and identifying the polygons for delineation of plants boundary is necessary (Shi *et al.* 2016).

Development of HTP phenotyping tools has been suggested as a methodology to increase genetic gain and reduce breeding cycle in forage crops (Walter *et al.* 2012; Barrett *et al.* 2015). Phenotypic information from these tools has the potential to enhance genomic selection and physiological screening and to reveal quantitative trait loci associated with multiple traits of various crops (Pauli *et al.* 2016; Halperin *et al.* 2017; Zhang *et al.* 2017; Sun *et al.* 2019). In the last decade HTP data acquisition and processing have been rapidly customised through deployments of commercial HTP platforms, various software and computer vision techniques (Bolger *et al.* 2019; Shakoar *et al.* 2019). Breeding for high DMY in forage crops is likely to be benefited from the development of HTP which could highly increase the amount of phenotypic data and the rate at which it is collected (Ghamkhar *et al.* 2019; Wang *et al.* 2019).

The fifth Chapter addressed the development and validation of a computational phenotyping workflow to predict the biomass of 48,000 individual plants in a perennial ryegrass breeding field trial. Developing an efficient workflow for non-destructive estimation of traits of thousands of plants is necessary to accelerate the selection of genotypes based on the accurate ranking of commercial cultivars and breeding lines (Chawade *et al.* 2019; Zhao *et al.* 2019) that could lead to a shorter cycle of breeding and greater rates of genetic gain in forages. At the start of my PhD, there was no working protocol in place to process the data from 48,000 individual plants planted in the GSS field trial. This required a great effort from the project team across molecular breeding and computational biology to develop a computational workflow for plant height and images acquisition, processing and analysis. Interesting results indicating the applicability of this workflow to the estimation of plant biomass through normalised difference vegetative index (NDVI) and plant height data extraction. The NDVI, plant height and dry matter distribution of individual plants may contribute to developing a robust method for deciding the time of harvest for each cultivar in different growing seasons. However, the growth rate of cultivars varies so the ranking of growth rate and biomass accumulation also vary accordingly.

Moderate to high correlation accuracy was achieved for plot-level biomass estimation across three seasons. Similar results have been reported in plot-level non-destructive biomass estimations of ryegrass (Borra-Serrano *et al.* 2019) and other crops (Brocks and Bareth 2018; Han *et al.* 2019; Yue *et al.* 2019). The model was further confirmed to be reliable by evaluating the combined regression of the three seasonal harvests. Similarly, the plot-level model indicated good to high-correlation between predicted and measured DM across three seasons with R^2 between 0.19- 0.81 and root mean square errors (RMSE) values ranging from 0.09- 0.21 kg/plot. The model was further validated using a combined regression of the three seasonal harvests. This study further paves a foundation for the application of sensor technologies combined with genomic studies that would lead to greater rates of genetic gain in ryegrass DMY.

6.4 Challenges of Perennial Ryegrass High-Throughput Phenotyping

Various factors affect the development of high-throughput phenotyping in perennial ryegrass including the heterogeneity of genotypes that arise from variations due to genetic effects. High-heterogeneity in perennial ryegrass is both an opportunity and a challenge. The presence of

heterogeneity provides an opportunity to broaden selection intensity by minimising the chance of inbreeding depression in the breeding cycle. Understanding the effect of structural variation on spectral characteristics are important for DMY estimations (Cen *et al.* 2019; ten Harkel *et al.* 2020). Capturing the complex canopy structural information from individual plants may be challenging considering the diversity of growth habit for each genotype in the field.

Developing phenotyping platforms has entered a new era of automated measurement of biomass using multiple sensors. The design of phenotyping platforms needs to consider an application to phenotype multiple traits at a time. Relatively little research has been conducted on the use of HTP for traits other than DMY (Jayasinghe *et al.* 2019; Smith *et al.* 2019). Quality and persistence are affected by multiple traits and breeders will need to have clear criteria on which traits are most important to be assessed through HTP in breeding programs. Identifying nutritive quality and persistent traits is difficult since various traits could be considered as representative phenotypic traits, and there is no criterion in place which trait to be prioritised (Smith *et al.* 1997; Smith and Fennessy 2011).

At the start of this PhD, no standard workflow was in place to estimate the biomass of individual plants while identifying and standardising background soil. The background of images vary greatly with sensor types and weather condition at the time of data acquisition. In particular, the radiometric calibration and standardisation of images from aerial platforms were successful (Chapter 3). Similarly, geometric calibrations of images from aerial platforms as well as plant height from ground vehicles were standardised with a maximum error of <4 cm (Chapter 4; Gebremedhin *et al.* 2019a). This creates a great opportunity for industries and breeding companies who can assess DMY non-destructively with maximum accuracy. However, the processing techniques and developed protocols for extracting data and analysis of the so-called “big data” require computational and advanced statistical knowledge. An easily accessible domain that works for primary users (plant scientists) has not been developed and this challenge requires development of a coherent workflow for extracting and analysing trait information.

Sensor technology has advanced rapidly in the last decade and a wide choice is now available. The cost of these sensors has also decreased greatly in the last few years (Reynolds *et al.* 2018). This provides opportunities for researchers to make more extensive use of sensor technologies in breeding for biomass improvement research (Furbank *et al.* 2019; Gebremedhin *et al.* 2019b). However, forage breeding research is often underfunded (Stewart and Hayes 2011) and this may leave researchers struggling to use these new technologies.

6.5 Modeling and Big Data Analysis

Developing adaptable, reliable and less expensive data modelling and analysis is important to deal with the big data sets from HTP platforms. Standardising and developing sophisticated models and big data-driven selection strategies will benefit plant breeding by changing the way breeders select traits (Morota *et al.* 2019; Shakoor *et al.* 2019). These may be different from the primary traits (e.g. plant height and biomass) used to evaluate genotypes in breeding programs.

In this thesis, computational phenotyping workflow was proposed for DMY estimation from the NDVI and plant height data, as a potential replacement for destructive harvesting and visual scores of single plants. Workflows to capture big data sets and computational processing have been developed with assistance from Agriculture Victoria Research staff from AgriBio, Melbourne and the Hamilton Centre. This provided a unique opportunity to analyse DMY data from 48,000 spaced-plants in three seasonal harvests. A future challenge is methodology to incorporate analysed phenotypic data into genomic selection models. Details of using these data to improve genetic predictions will be discussed in the next section.

6.6 Incorporation of Sensor-Based Phenotypic Data into Genomic Selection in Breeding

The success of achieving high genomic estimates of breeding values in GS depends on the size of the population used as training population, the relationship between the training and validation populations and the use of multi-location and multi-seasonal traits measurement data. As a result, GS requires an adequate range of phenotypic information from various genotypes grown in variable field condition. The rapid development of field HTP platforms provided an opportunity for accurate genetic predictive models in GS (Sun *et al.* 2019). Field HTP platforms have great potential to assess phenotypic traits non-destructively by significantly increasing the size of the population and the spatial and temporal scale of measurements. In previous studies, data from field HTP platforms were incorporated either as secondary traits (e.g., vegetative indices) (Rutkoski *et al.* 2016) or directly as non-destructively measured primary traits (e.g., plant height) (Watanabe *et al.* 2017).

Incorporation of the traits as univariate and multivariate in genomic prediction model may enhance the accuracy of genomic prediction. Incorporating NDVI and canopy temperature

from field HTP platforms, for example, improved the accuracy of a genomic prediction model for grain yield by up to 70% (Rutkoski *et al.* 2016). Similarly, incorporation of green normalised difference vegetative and canopy temperature in the early cycle of GS predictive models improved accuracy by up to 145% (Sun *et al.* 2019). These experiments were conducted mainly on the genetic prediction of grain yield of food crops.

The integration of genomic and field HTP phenotypic information may be useful to accelerate the rate of genetic gain and shorten the cycle of ryegrass breeding. There has been limited research done to date that integrates trait data from field HTP platforms to improve the accuracy of predictive genetic models for biomass estimation. Traits like NDVI and plant height have a high correlation with biomass estimation, and there is potential to incorporate these into the predictive model in GS to significantly improve the selection accuracy of superior genotypes. NDVI and plant height may be used in combined form or as a univariate. Access to these variables has become easier in our breeding program in terms of the temporal and spatial phenotypic data that can be captured (Chapter 5). This helps to develop accurate predictive models across seasons and years that uncover the complexity of DMV improvement. Most importantly, the application of phenotypic information from our HTP platforms could be flexible in utilising phenotyping data ranging from individual plants to rows and different sizes of plots. This provides an opportunity to obtain phenotypic information from genetically variable families. Finally, the field HTP platform and computational workflow developed in this thesis are expected to be useful to explore more traits (e.g. number of pixels, leaf area, plant volume) and these can further contribute to the development of robust genetic predictive model of biomass for GS in ryegrass breeding programs. Furthermore, these methods developed in this thesis may be used in the future as baseline for advancing other application methods including automated scoring of large breeding nurseries, parental selection and to enable automatic evaluation of population/family/cultivar performances as evidenced in other crops (Xu *et al.* 2020).

The workflow developed in this thesis presented some limitations with lower accuracies in some of the harvesting seasons. Specifically, lower accuracies were obtained from the Late-spring 2018_2 experiments (chapters 4 and 5) due to early flower initiation from drought stresses. In this study, we believe the surface of these small plants may have reflected weak sound echoes, which made it difficult for the ultrasonic sonar to detect accurate distance from the top canopy of the plant. Furthermore, when the plant canopy is a mixture of leaves and sparse reproductive tillers there is a greater variability in height and the surface that interacts

with the sensor. Further re-evaluation considering seasonal variation accuracies across years combined with non-learner models (e. g., machine learning) may be required to further improve and recalibrate the algorithm developed.

6.7 Applications in Private Industry

Private industries have leading roles in the improvement and implementation of current ryegrass breeding programs (Stewart and Hayes 2011). The implementation of breeding programs by these companies is mainly done on small sward plot trials, which depends on performing a phenotypic evaluation of cultivars or breeding lines with limited resources. Private breeding companies are predominantly expected to benefit from the improvements and cost reductions of field HTP platforms (Araus *et al.* 2018). However, further development of the phenotyping platforms, data processing, storage and analysis capabilities would be difficult to achieve by the forage breeding companies since they tend to be smaller than those developing major food crops (Harmer *et al.* 2016). Private forage breeding companies may not directly benefit from our large-scale phenotyping platform due to its cost. However, the developed dashboard of our platforms will provide a foundation for developing low cost- HTP platforms that can perform phenotyping and other activities including precision agriculture and this would be within the reach of small private forage breeding companies.

6.8 General Conclusion and Future Outlook

Realising the full potential of genomic selection in forage breeding requires an increase in the amount of phenotypic data and the rate at which it is collected. To shorten the breeding cycle of perennial ryegrass, this thesis suggests that HTP methods have the potential to change the way we phenotype forage crops currently in breeding programs. Advanced sensor based HTP technologies developed in this thesis indicated the potential to automate phenotyping of traits so that non-destructive assessments of thousands of genotypes in large breeding trials could be implemented. In addition, combining data from different sensors enabled to improve biomass prediction models by up to 24% compared to the estimation by individual sensors. With this analysis of a series of validation models proved the stability of the models developed (chapter

4), predictive models could be re-evaluated and calibrated to phenotype traits of various perennial and annual forage crops across seasons and years.

Moreover, the introduction of additional sensors to be able to detect more traits and incorporate into the model effectively may provide an option to acquire several traits at once saving time. For instance, in addition to the parameters (plant height and NDVI) used to develop our model, the inclusion of other parameters may further improve the estimation model. Parameters including vegetative indices other than NDVI may present additional information about the canopy biochemical and spectral characteristics. Utilising these additional vegetative indices separately or in combination with plant height metrics may further improve the prediction accuracy of DMY estimations of single plants. Furthermore, integrating other sensors into both aerial and ground platforms (e.g. Red-Green-Blue cameras, thermal cameras, hyperspectral cameras and LiDAR) may allow the incorporation of different stress-related traits and the three-dimensional structural architecture of the plant (Lu *et al.* 2019; Jin *et al.* 2020; ten Harkel *et al.* 2020). These sensors may provide an expansion to the number of phenotypic features like pixel number, leaf area measurement, volume and canopy density and these may increase the accuracy of DMY and plant height estimation.

Furthermore, with the use of machine learning and modern statistical tools, it will be important to explore the application of non-linear regression algorithms for better use of the big data sets obtained from sensor technologies. The applications of these machine learning algorithms may support to exploit the significance of multi-temporal and spatial data from various sensors. The main challenge, however, remains in the data processing. Data processing capabilities from sensors require people with technical knowledge that extends beyond biology. A modern computer algorithm with advanced statistical knowledge that could summarise these sophisticated data systems will need to be readily accessible by plant breeders.

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7. Appendices

Combining NDVI and Plant Height as a Proxy to Enable High-Throughput Phenotyping of Herbage Yield in Perennial Ryegrass Breeding

AGRICULTURE VICTORIA

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INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is one of the most widely used forage species in temperate regions. Methods for assessment of herbage yield (HY) include visual scores, sample clipping and mowing. These methods are often costly and time-consuming. The use of multispectral imaging and light detection and ranging (LiDAR) technologies on aerial and ground platforms offer a non-destructive, high-throughput and efficient method of phenotyping to accelerate screening of HY in the field. A sensor-based phenotyping system was developed that combines normalised difference vegetative index (NDVI) and plant height measurements to estimate the fresh herbage yield (FHY) and dry herbage yield (DHY) of single plants rapidly and objectively.

OBJECTIVES

This study aims to evaluate different sensors to develop phenotyping tools for fresh and dry herbage yield estimation.

METHODS AND RESULTS

1) Data collection

A subset of 475 plants from five breeding lines was used to measure NDVI, FHY & DHY in this experiment (Fig. 1a). Out of the 475 plants, 84 were used for plant height measurement using standard ruler, LiDAR and sonar sensors. A 3DR Solo Quadcopter with a Parrot sequoia-multispectral imaging system was used to capture images, and NDVI from single plants was extracted using PIX4D and QGIS software.

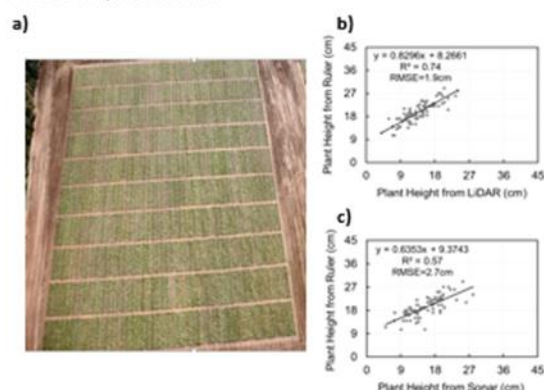


Figure 1. a) An aerial image of a perennial ryegrass field trial, which comprises 48,000 individual plants at Agriculture Victoria, Hamilton Centre, Victoria, Australia. b) regression of LiDAR and manual measured plant height. c) regression of Sonar and manual measured plant height.

2) Estimation of individual plants height using sensors

Plant height measured by sonar and LiDAR sensors on single plants was highly correlated to the manual measurements using a ruler (Fig. 1bc).

However, measurements made with the LiDAR sensors were more accurate in predicting plant height compared to sonar.

3) Estimation of fresh and dry herbage yield using sensors

DHY prediction from NDVI and plant height (Fig. 2ab) indicated that plant height measurements predicted DHY slightly better than NDVI. Combining NDVI and height indicated 6-18% more accurate in prediction of FHY & DHY than using either NDVI or height alone (Fig. 3ab).

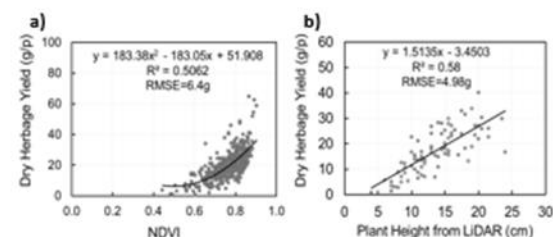


Figure 2. Scatterplots of measured dry herbage yield of single plants and calculated NDVI values (a) and measured dry herbage yield of single plants and plant height estimation using LiDAR (b).

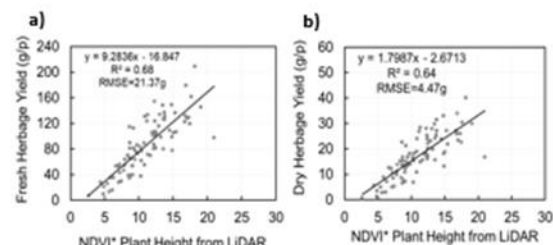


Figure 3. Scatterplots of measured fresh herbage yield (a) and measured dry herbage yield (b) of single plants vs. the multiplicative combined value (NDVI*LiDAR) of NDVI from multispectral images and plant height from LiDAR sensor.

EXPECTED OUTCOMES

- Development of an aerial and ground-based, high-throughput phenotyping system to predict herbage yield at single plant, row and plot levels for perennial ryegrass breeding programs.
- Development of seasonal yield prediction equations that can be used for phenotypic ranking of genotypes.

ACKNOWLEDGEMENTS

This work is supported by Agriculture Victoria, Dairy Australia, Royal Barenbrug Group, Barenbrug Australia and The University of Melbourne.

