Project title:	*PhD studentship - Manipulating growth rates at the plant scale to reduce in field leafy salad and vegetable crop variability*
Project number:	CP 177
Project leader:	Dr Jim Monaghan
Report:	Annual report, December 2019
Previous report:	None
Key staff:	William Johnson
Location of project:	Harper Adams University
Industry Representative:	Rob Parker, G's Fresh; Harry Wilder, Barfoots of Botley
Date project commenced:	24 September 2018

DISCLAIMER

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Jim Monaghan

Reader – Fresh Produce

Harper Adams University

Signature .... Date .. 19/12/2019.....................

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GROWER SUMMARY

Headline

This preliminary study has shown that 40-50% of the variation in transplant growth for iceberg lettuce and Tenderstem™ can be explained by cotyledon size variation.

Background

Variability in the growth of leafy vegetable crops such as lettuces or brassicas leads to variation in weight and maturity at harvest. This causes a significant issue for growers as they work to meet customer specifications. While breeders have made significant advances in reducing the genetic components of variation, the lack of uniformity in the growing environment both during transplant raising and field growth significantly affects relative intra-plant growth rates. The cause of the variability in the transplant stage is not fully understood, but a number of factors are likely to be involved and it is known that optimised lighting and watering during transplant production could have significant benefits in reducing the variation at the start of the crop cycle.

N.B. This is a summary of a one-year study as a component of a wider PhD studentship. The studentship ended after one year and the work should be considered as preliminary. A following PhD studentship (2020 -2024) is studying the control of variation of transplanted lettuce and tenderstem in the field.

Summary

1. Cotyledon size is related to transplant fresh weight

This preliminary study has shown that 40-50% of the variation in transplant growth can be explained by cotyledon size variation. This also highlights that other factors account for a significant proportion of variation in transplant size.

2. Cotyledon size variation can be imaged and modelled at a tray-scale.

Cotyledon size can be imaged and modelled at a tray scale. This work has developed a preliminary methodology that could be automated to image cotyledons and extract data from multiple tray images. This could be refined to produce information on variation between and within trays during propagation.

3. Location within tray affects the accuracy of the cotyledon to fresh weight model

Spatial analysis of the fit of the general growth relationship between cotyledon size and subsequent transplant fresh weight showed that the relationship did not fit as well in the edges of the trays, most markedly for lettuce where plants were in general smaller than the model predicted.

Financial Benefits

Not available at this stage of the study

Action Points

Minimise obvious sources of variation in the propagation of transplants. Take particular care with the edges of the trays where blocks/modules may be more prone to drying out and being shaded.

SCIENCE SECTION

Introduction

Variability in the growth of leafy vegetable crops such as lettuces or brassicas leads to variation in weight and maturity (Kerbirou et al., 2013). This causes a significant issue for growers as they must harvest heads of a uniform size and weight to meet customer specifications. While breeders have made significant advances in reducing the genetic components of variation, the lack of uniformity in the growing environment both during transplant raising and field growth significantly affects relative intra-plant growth rates. The cause of the variability in the transplant stage is not fully understood, but a number of factors are likely to be involved and it is known that optimised lighting and watering during transplant production could have significant benefits in reducing the variation at the start of the crop cycle.

The aim of these studies was to quantify the natural levels of variation within iceberg lettuce (*Lactuca sativa*) and Tenderstem™ (*Brassica oleracea italica* X *brassica oleracea alboglabra*) transplant seedling growth. Cotyledon size was modelled against fresh weight at transplant stage.

Hypotheses:

1. Cotyledon size is related to transplant fresh weight
2. Cotyledon size variation can be imaged and modelled at a tray-scale.
3. Location within tray affects the accuracy of the cotyledon to fresh weight model

N.B. This is a summary of a one-year study as a component of a wider PhD studentship. The studentship ended after one year and the work should be considered as preliminary. A following PhD studentship (2020 -2024) is studying the control of variation of transplanted lettuce and tenderstem in the field.

Materials and methods

This experimental work was carried out at Harper Adams University Crop and Environment Research Centre glasshouses, with seed and plant material supplied from G's Fresh (lettuce) and Sakata Seed (Tender stem).

This programme of work assessed cotyledon size in relation to fresh weight for transplant stage seedlings. Repetitions of the experiment were staggered throughout the year, and the data has been normalised to compare between these repetitions. Experimental work was carried out to establish the levels of variation present at cotyledon stage, and whether that effects transplant stage fresh weight. This required the development of an image analysis protocol within ImageJ as the methodology used in preliminary work carried out at G's Fresh was insufficient.

By studying large numbers of transplants/modules the aim of the work was to model in a comparable way, with cotyledon size as a continual variable rather than emergence day as a factor, the fresh weight of lettuce and Tenderstem transplant seedlings.

Computer Vision and Image Analysis of Plant Material

Image analysis of plant material varies between specific target plant, goal of analysis and data required, however they all follow a basic work flow (Ibaraki and Gupta, 2015):

- Acquisition of an image of the target object
- Pre-processing of the image for facilitating further processing
- Selection of pixels of interest
- Extraction of characteristic features

An initial approach to collecting image data required the user to manually select and define the areas of interest. One of the major limitations of the system was the time this manual selection took. Although this approach is effective at selecting and measuring individual plant sizes, it proved inefficient and labour intensive when processing large number of images, with each tray photographed having 176 plants. Therefore, further development of this system into a methodology which could process images with greater efficiency was prioritised to complete this work.

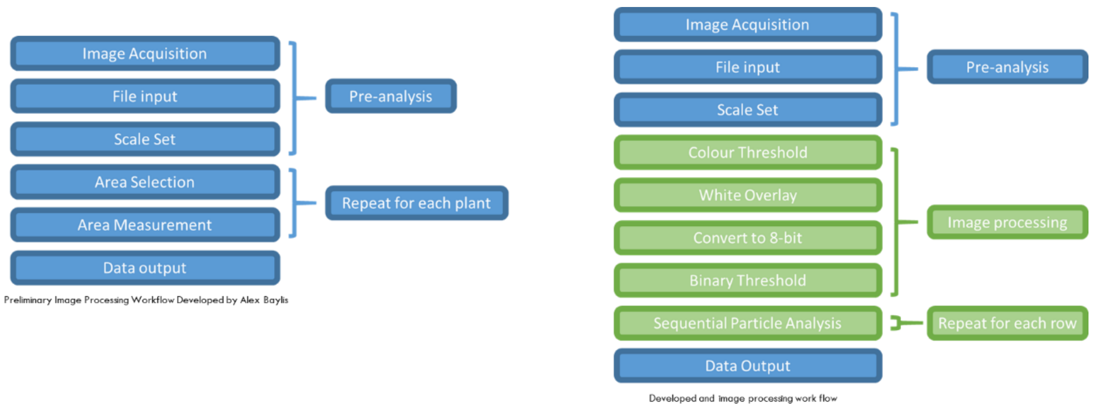

Figure 1. Development of imaging methodology; the green boxes highlight the additional units required to automate the image capture and analysis.

Elements of the system which were selected for improvement were the area selection and the area measurement. In the initial system, this required the manual selection and measurement of each individual plant. Selection of the areas needed through different image segmentation methods was investigated for this work, and notable progress was made using K-means and K-nearest-neighbour clustering for running full automated segmentation using MATLAB™. A simpler and more labour intensive, but more practical approach, using a series of thresholding techniques was used to select the areas for measurement.

Selection of Areas through Thresholding

Digital colour image files are described by three values, for example R:G:B. Image thresholding is the selection of pixels within an image based on their values. Digital colour images files are described by three values and there are number of file types which can be used to describe images in different ways. This colour thresholding in ImageJ allows for the selection of pixels which describe leaf material. Thresholding the image in different colour spaces can also give different levels of accuracy, and noise within the segmented area and the optimum colour space needs to be identified, with subsequent trialling to establish the thresholding in each colour space (García-Mateos et al., 2015; Hernández-Hernández et al., 2017).

L*a*b* was the optimum colour space to work in for this experimental system, both from its application in the literature and in the ease in application to this work. L represents the lightness of the pixel, which when set to include all values (0-255) removes the issue of inconsonant lighting within an image. The a* values represent a spectrum from green to red. When segmenting the image in this space it therefore is simple to select pixels within the full range of green (0-125) to select the green material of the cotyledon. b* value represents a spectrum from blue to yellow (García-Mateos et al., 2015). With this set to include all values, there is selection of a high number of pixels which do not represent cotyledons, however selecting the yellow range and excluding the blue (125–255) removes this interference. When running a colour threshold in ImageJ, the system automatically over lays the image with block colour to display the pixels being selected. By setting this overlay to white and then converting the image into an 8-bit greyscale file, the original image and the white overlay are converted into a single file, with a single value (0-255) describing each pixel.

It is then possible to select just the white pixels generated by the overlay, using a binary threshold (value 255 – 255). This converts the image into a binary image, in which pixels either have a value of 0 (black), or 255 (0).

Measurement of Areas through Particle analysis

Thresholding as above allowed the selection of the cotyledons in an image in a replicable and defined manner. The next step to developing this methodology was to develop an efficient manner of extracting the areas of these defined regions. With a binary image, a particle analysis can be run in ImageJ. This assesses every pixel within the image, starting in the top left-hand corner and moving along each row in turn, and measuring the area of each cluster of black pixels with a value of 0. This then outputs the data and overlays the image with assigned numberings. However, the sequence the particle analysis measures the cotyledons will not correspond to the row and column due to the method of searching the image for particles.

A number of solutions to this problem were trialled. Segmenting the image into separate files, each with one column of plants, and then converting these files into an image stack, a single file containing multiple images in a sequence, and the running the particle analysis, successfully extracted the data in the correct order. However, the processing of the images in to these stacks manually proved time consuming. Therefore, a simpler and more time efficient method was implemented. Manually selecting specific columns within the image, and then running the particle analysis on each column separately, proved an efficient and reliable method for extracting the data.

The Developed System

The system as developed in this work can handle the extraction of 176 cotyledon surface area data points in approximately 20 minutes. A protocol for this methodology was produced and implemented by a crops department intern to extract data. The method for analysing the size of a high number of cotyledons from a single image is widely applicable within this project, and for other researchers both at Harper Adams University and at other institutions.

Experimental Procedures

Experiments were set up at HAU (Table 1), the time between planting and harvesting was dependant on the development of the plant and therefore differed between experiments. These disparities were compensated for when normalising and analysing the data.

Table 1. Experiments involved in the cotyledon modelling study

Rep	Crop	Location
2.0.1	Lettuce	CERC Glasshouse
2.0.2	Tenderstem	CERC Glasshouse
2.1.1	Lettuce	Harper Adams University main campus glasshouse
2.1.2	Tenderstem	Harper Adams University main campus glasshouse

Seed Tray Preparation and Seedling Cultivation

Each repetition of the experiments used six trays of peat blocks sourced from G's Fresh, with 176 individual peat blocks per tray, giving a maximum of 1056 plants per experiment. The trays are 664 mm by 441 mm, with 11x16 blocks of 35x35x40 mm size. Pre-planted trays were provided by G's Fresh for experiment 2.0.1, with the remaining experiments being planted by hand at HAU. Iceberg lettuce *(Lactuca sativa)* of variety Gondar was used for 2.0.1 and 2.1.1, and Tenderstem (*Brassica oleracea italic X Brassica oleracea alboglabra*) sprouting broccoli Inspiration F1 was used for planting 2.0.2 and 2.1.2. The environmental conditions; temperature and light levels, were recorded.

Assessment of Transplant Stage Maturity

The trays were assessed when ~70% of the plants were passed the third true leaf stage, for both lettuce and Tenderstem. The fresh weight data was normalised around the mean of each tray to give relative fresh weight for comparison between experiments, taking into account some of the effects of any disparities between crop maturity assessments.

Assessment of Seedling Fresh Weight

Seedlings were harvested by cutting the stem of the plant at soil surface level, and then weighed to two decimal places on a scale. Fresh weight data was recorded for specific individual plants.

Data Handling

Zero values and outliers were removed manually in Excel. The cotyledon surface area readings and fresh weight values were then normalised around the mean for each tray [relative n =n(/(sum(x^1:x^{nth})/n^{th})], to give relative cotyledon and relative fresh weight values. This allowed for comparison between experiments, and to give preliminary results. Script is currently being developed in R™ to re-handle to data in a more uniform and repeatable method.

Results

Experiment 2 - Results

Zero values were removed from cotyledon surface area and fresh weight. These values were then normalised see described in the materials and methods.

Modelling the un-normalised Data

Prior to normalising the data as described in the materials and methods, linear regression on the data sets screened to remove zero values was carried out in Genstat. The significance of both data sets was $p < 0.001$, with variation in lettuce cotyledon area accounting for 30.5% of the variation in transplant size (Fig 1), with a standard error of 1.1, and variation in Tenderstem cotyledon area accounting for 12.7% of the variation in transplant size (Fig 2), with a standard error of 0.5.

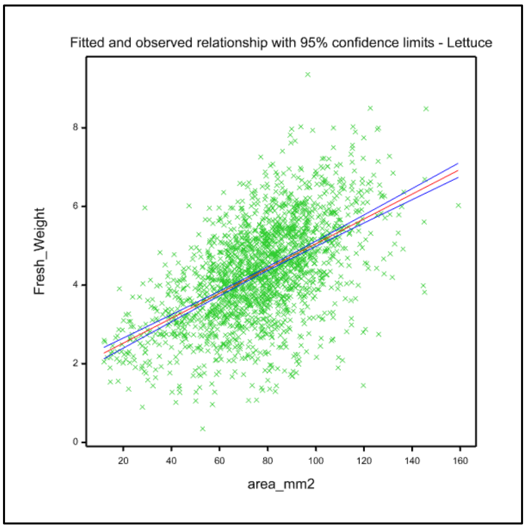

Figure 2. Linear Regression - Lettuce

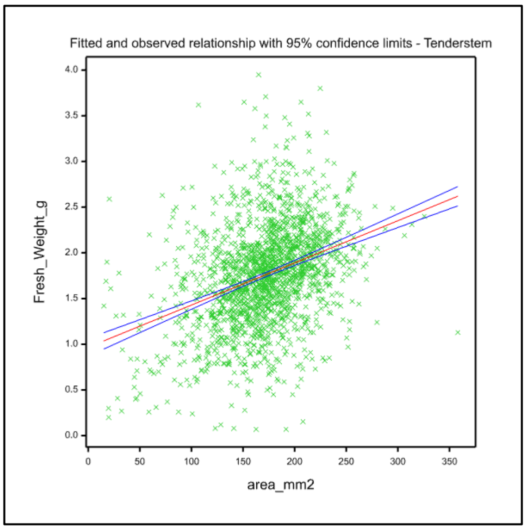

Figure 3. Linear Regression - Tenderstem

Linear Regression of Normalised Data

The data was normalised around the mean of each tray, and linear regression on the data was carried out, in Genstat. The significance of both data sets is $p < 0.001$, with variation in lettuce cotyledon area now accounting for 53.0% of the variation in transplant size (Fig 4), with a standard error of 0.2, and variation in Tenderstem cotyledon area accounting for 39.3% of the variation in transplant size (Fig 5), with a standard error of 0.2.

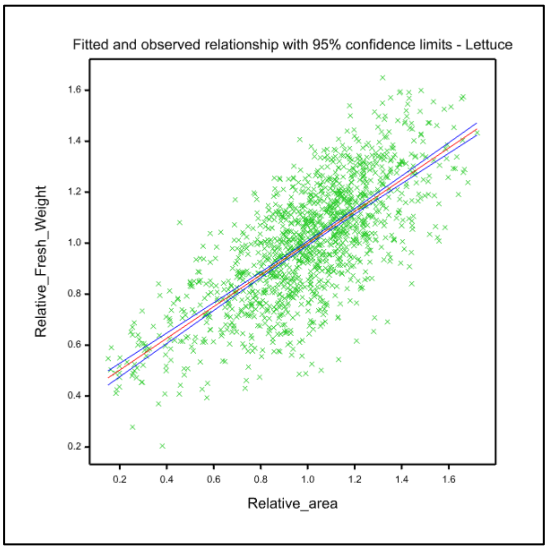

Figure 4. Linear Regression of Normalised Data - Lettuce

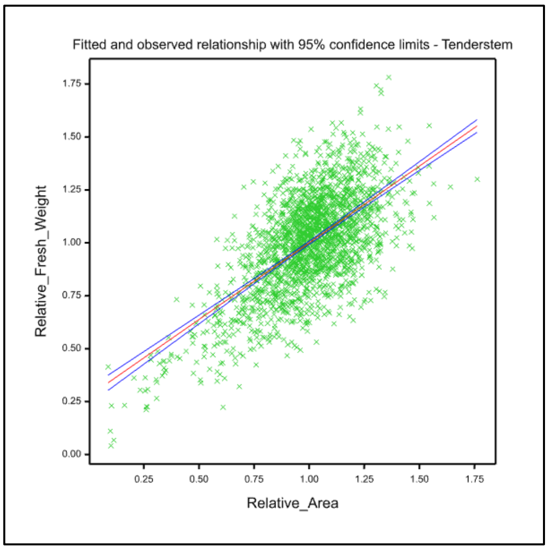

Figure 5. Linear Regression of Normalised Data - Tenderstem

Relative Fresh Weight Spatial Variation in Lettuce

A two-way ANOVA was performed on normalised lettuce data with column and row as factors against variable relative fresh weight. Column, but not row has a statistically significant effect on the relative fresh weight of lettuce transplants with p values of <0.001 and 0.168 respectively. This generates predictions from a regression model, which can then be plotted in Excel with conditional formatting to visualise the patterns of relative fresh weight variation (Fig 6). A one-way ANOVA on column and relative fresh weight was then carried out to generate Tukey's confidence intervals in R™ with the displayed violin plot and bar graph (Fig 7).

1.0461	0.9821	1.1277	1.1793	1.0962	1.0262	1.0974	0.9776	1.0787	1.0824	1.1133	0.9616	0.8636	0.8424
1.0845	1.121	1.0694	1.1054	1.0607	1.025	1.0975	1.1022	1.0395	0.965	0.9825	1.1051	0.8426	0.8636
1.021	1.1321	0.9198	0.9186	0.9788	1.0325	1.0264	0.8693	0.8849	1.0831	1.0499	0.9024	0.9728	0.9068
1.0402	0.973	1.0109	1.0619	0.9336	1.0019	1.1184	0.9898	0.9506	1.103	1.0723	0.9876	0.8095	0.8956
1.1467	1.022	1.061	0.9733	1.131	1.0996	1.0506	0.8221	1.0722	0.9632	0.9494	0.9554	0.9284	0.7828
0.8328	1.0235	1.1317	1.0208	0.9881	0.9811	1.1004	1.0397	0.9102	0.8707	0.9019	0.9874	1.0516	0.8529
1.0585	1.0178	0.9699	0.9325	0.9966	0.9649	0.9957	0.9215	0.9677	0.935	1.0142	0.9414	0.9751	0.8203
0.9617	1.0436	1.0296	1.0123	0.9985	1.1412	1.0197	1.0274	1.0674	1.0144	0.906	0.914	0.9804	0.931
1.056	1.1032	1.0414	0.9606	1.1435	1.0724	0.9528	1.0173	1.0278	1.0307	1.0349	1.0361	0.9169	0.7931

Figure 6. Spatial Model of Lettuce Relative Fresh Weight

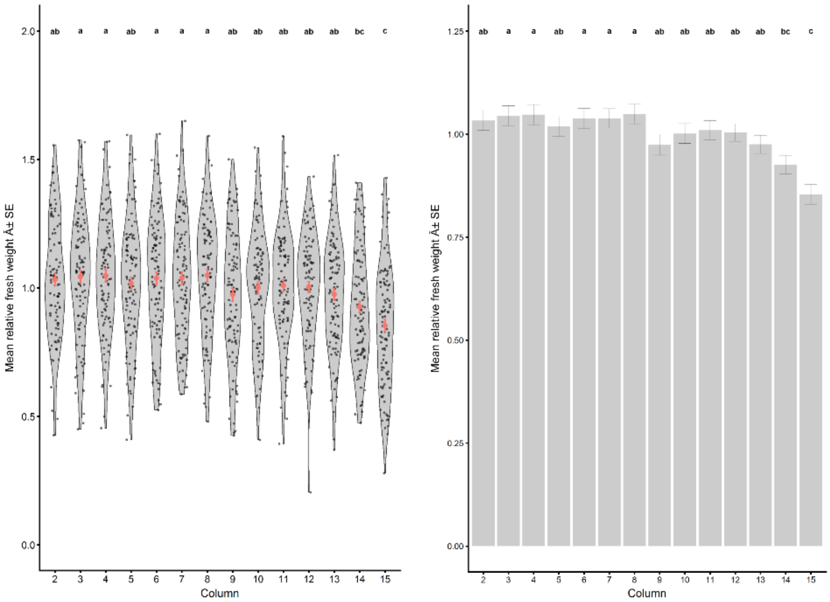

Figure 7. Violin Plot and Bar graph of Lettuce column Spatial Variation

Fresh Weight Spatial Variation in Tenderstem

A two-way ANOVA was performed on normalised tenderstem data with column and row as factors against variable relative fresh weight. Column and row had statistically significant effect on the relative fresh weight of tenderstem transplants with p values of <0.001. This generates predictions from a regression model, which can then be plotted in excel with conditional formatting to visualise the patterns of relative fresh weight variation (Fig 8). One-way ANOVAs on column, and row, a relative fresh weight were then carried out in R™ to generate Tukey's confidence intervals. These were then plotted in row and column in simple bar graphs and violin plots demonstrating the distribution of values in row and column (Fig 9).

0.832	0.935	1.018	0.976	0.994	1.084	1.081	1.125	1.081	1.002	1.062	1.01	0.941	0.837
0.798	0.925	1.027	0.933	0.995	0.922	1.052	1.009	1.011	1.013	0.977	0.949	0.892	0.856
0.809	0.855	1.014	1.023	1.031	1.019	1.115	1.087	1.064	1.041	0.978	0.99	0.944	0.891
0.917	0.907	0.939	1.081	1.043	1.155	0.975	0.979	1.078	1.079	1.048	0.979	0.997	0.844
0.945	0.972	1.02	1.147	1.09	1.034	1.046	1.109	1.165	1.109	1.02	1.132	0.972	0.99
0.858	0.935	1.041	1.043	0.95	1.154	1.082	1.082	1.093	1.077	1.035	0.96	0.927	1.059
0.892	0.908	0.998	1.025	1.175	1.085	1.203	1.015	1.078	1.049	1.114	0.948	0.94	1.069
0.867	0.893	1.007	1.061	1.053	1.036	1.141	1.055	1.041	1.094	1.092	0.93	0.916	0.781
0.799	0.886	0.971	0.955	1.067	1.109	1.157	0.968	1.014	1.148	0.995	0.975	0.833	0.924

Figure 8. Spatial Model of Tenderstem Relative Fresh Weight

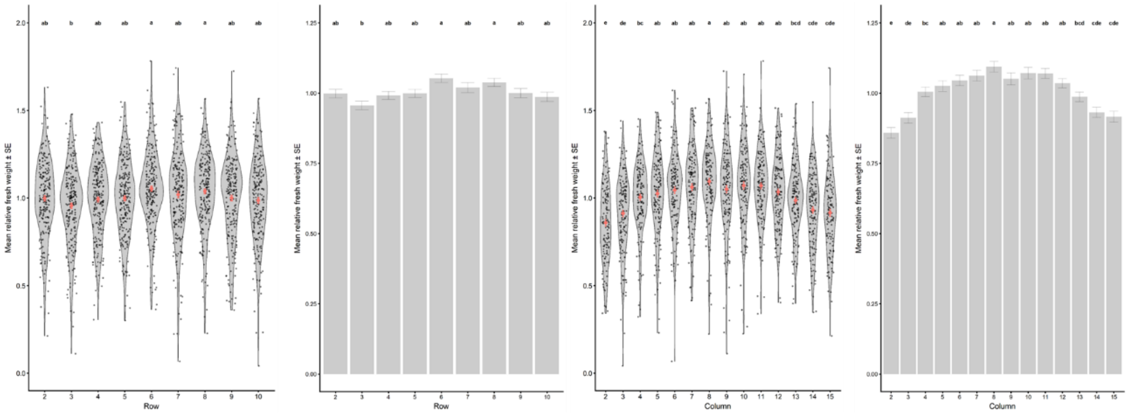

Figure 9. Violin Plots and Bar Charts – Tenderstem Spatial Variation of Row and Column

Assessing and mapping the spatial impact on the model

To assess whether spatial location influenced the accuracy of the linear model, the residuals were calculated, squared and then square rooted to transform the values into positive values. These were then assessed against row and column in a two-way ANOVA and predicted values were generated by the analysis. These were plotted in Excel and conditional formatting was used to visualise locations within the trays where variation from the model was occurring for lettuce (Fig 10) and Tenderstem (Fig 11), separately. Column but not row significantly affected the accuracy of the model for both lettuce and Tenderstem. The Tukey's test on both crops shows a significant deviation from the model in column 15.

0.1276	0.099	0.1065	0.1225	0.1289	0.1389	0.1252	0.1282	0.0915	0.0671	0.1045	0.0907	0.1634	0.2376
0.1388	0.1633	0.1238	0.1155	0.1276	0.1075	0.116	0.1424	0.1066	0.1167	0.1008	0.0976	0.1738	0.1863
0.1183	0.1317	0.1363	0.1137	0.1257	0.1101	0.1388	0.0754	0.1836	0.1284	0.115	0.1276	0.124	0.1851
0.091	0.074	0.1699	0.1419	0.1039	0.1508	0.1541	0.0929	0.1123	0.113	0.1097	0.1216	0.2193	0.2137
0.1923	0.1734	0.1635	0.0971	0.1715	0.1092	0.1499	0.0913	0.1126	0.1094	0.0649	0.1286	0.1826	0.2118
0.0956	0.0706	0.0784	0.1324	0.1117	0.1202	0.1143	0.1146	0.1095	0.1449	0.0764	0.1586	0.1553	0.2165
0.0913	0.1058	0.1159	0.1119	0.0784	0.128	0.0953	0.1187	0.0831	0.1247	0.1096	0.1087	0.1201	0.2008
0.1327	0.1487	0.1342	0.1524	0.134	0.1367	0.1698	0.1282	0.1534	0.1382	0.1557	0.1135	0.1386	0.1912
0.0919	0.1333	0.1015	0.1416	0.1483	0.1324	0.1158	0.1647	0.1129	0.1129	0.0782	0.073	0.1676	0.2509

Tukey's 95% confidence intervals Column

	Mean	
12	0.1013	a
13	0.1139	a
11	0.1156	ab
10	0.1157	ab
9	0.1167	ab
2	0.1217	ab
3	0.1217	ab
4	0.1251	ab
7	0.1252	ab
5	0.1255	ab
6	0.1259	ab
8	0.1318	ab
14	0.1603	b
15	0.2098	c

Figure 10. Lettuce residual spatial model

0.165	0.1237	0.1722	0.1589	0.1783	0.1428	0.1707	0.1539	0.1479	0.0934	0.1315	0.1329	0.1635	0.2116
0.1998	0.1133	0.1268	0.1542	0.1041	0.115	0.1171	0.1444	0.1309	0.127	0.1474	0.1348	0.1885	0.1942
0.1648	0.1285	0.1122	0.1206	0.1509	0.1397	0.127	0.119	0.1066	0.0817	0.1257	0.1256	0.1482	0.2395
0.1503	0.1162	0.0869	0.1778	0.1023	0.1675	0.1281	0.1592	0.1397	0.1338	0.116	0.1078	0.1406	0.2429
0.1218	0.1278	0.1305	0.2043	0.1998	0.1524	0.1391	0.1612	0.1434	0.1251	0.1545	0.1167	0.1447	0.162
0.1565	0.134	0.1558	0.1292	0.1532	0.1708	0.1879	0.1489	0.1888	0.1271	0.1466	0.1038	0.1152	0.2164
0.2096	0.1628	0.1345	0.0907	0.1787	0.1401	0.1904	0.1557	0.0858	0.1174	0.1058	0.1411	0.1383	0.169
0.1307	0.1653	0.1198	0.1288	0.1078	0.1126	0.137	0.1446	0.1612	0.0951	0.1321	0.1432	0.1968	0.2258
0.1618	0.1529	0.1293	0.1504	0.1585	0.1773	0.1742	0.1205	0.1155	0.1215	0.1264	0.1389	0.2086	0.1941

Tukey's 95% confidence intervals Column

	Mean	
11	0.1138	a
13	0.1266	ab
4	0.1300	ab
12	0.1318	ab
10	0.1358	ab
3	0.1362	ab
9	0.1450	ab
7	0.1452	ab
5	0.1454	ab
6	0.1476	ab
8	0.1510	ab
14	0.1595	b
2	0.1615	bc
15	0.2042	c

Figure 11. Tenderstem residual spatial model

Discussion

The aim of this study was to assess the contribution of cotyledon size variation to transplant seedling fresh weight variation. As part of this work package, an image analysis protocol for extracting a high number of target values from a single image was developed in ImageJ. This protocol allowed work to be carried out assessing the size of cotyledons without destructively harvesting them, a useful tool for modelling plant development. Within the current literature, there is very little work on cotyledons size, and subsequent growth, with some research into herbivory within chalk land grass species (Hanley & May, 2006). Cotyledons have been shown to contribute to the development of the seedlings in three phases. Firstly, the cotyledon acts as a respiratory reserve as the plant develops. Secondly the cotyledons fully expand and photosynthetically contribute to the seedling development, and thirdly the cotyledon material is translocated within the plant (Zhang & Zhou, 2008). This mechanism is associated with the assumption that larger cotyledons result in larger developed plants, this work supports this assumption and the simple modelling showed that 39.3% and 53.0% of variation in relative fresh weight in tenderstem and lettuce respectively was accounted for by cotyledon size. This suggests that approximately 50% of variation in transplant size is related to factors other than size of cotyledon.

It should be noted that both the lettuce and Tenderstem seedlings were raised in lettuce block trays, which include a raised edge with handles at the ends of the trays. The same trays were used for each crop to allow direct comparison at this stage of the study (brassicas are commercially propagated in handle-less module trays).

Clear spatial effects on the accuracy of the models was observed i.e. the relationship between cotyledon size and transplant fresh weight was less reliable towards the edges of the trays. The Tukey's test for column spatial variation in relative fresh weight of lettuce showed an interesting trend. The outermost column 15, was significantly smaller than would have been predicted from the cotyledon size from all the columns other than column 14, marking a distinct edge effect even within the screened data set. This finding in the lettuce seedlings is supported by recent work at HAU (CP 121) where significant spatial variation was observed in lettuce transplant size during propagation where although plant size varied across the trays (0.4 – 1.8 g fresh weight/plant after 22 d), smaller plants were more common at the ends of the trays. These size differences have been shown to lead to significant growth differences following planting.

When looking at the similar analysis for Tenderstem, both row and column had significant effect on relative fresh weight. Within the column analysis there are more groupings and overlaps than in the lettuce data. This may be explained by the more upright growth habit of tenderstem seedlings, avoiding the shading effect of the tray handles.

The cause of the variability in the transplant stage is not fully understood, but a number of factors are likely to be involved including optimised lighting and watering during transplant production.

Conclusions

1. *Cotyledon size is related to transplant fresh weight*

This preliminary study has shown that 40-50% of the variation in transplant growth can be explained by cotyledon size variation. This also highlights that other factors account for a significant proportion of variation in transplant size.

2. *Cotyledon size variation can be imaged and modelled at a tray-scale.*

Cotyledon size can be imaged and modelled at a tray scale. This work has developed a preliminary methodology that could be automated to image cotyledons and extract data from multiple tray images. This could be refined to produce information on variation between and within trays during propagation.

3. *Location within tray affects the accuracy of the cotyledon to fresh weight model*

Spatial analysis of the fit of the general growth relationship between cotyledon size and subsequent transplant fresh weight showed that the relationship did not fit as well in the edges of the trays, most markedly in the lettuce where plants were in general smaller than the model predicted.

Knowledge and Technology Transfer

Will Johnson presented an overview of this work at a G's Growers meeting and the AHDB Annual PhD conference in 2019.

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