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Title	Photoperiod and circadian regulation of early development in Lolium perenne (L.) varieties
Authors	Richards, Michael
Publication date	2022-09-28
Original Citation	Richards, M. 2022. Photoperiod and circadian regulation of early development in Lolium perenne (L.) varieties. MRes Thesis, University College Cork.
Type of publication	Masters thesis (Research)
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Photoperiod and circadian regulation of early development in *Lolium perenne* (L.) varieties.

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September 2022

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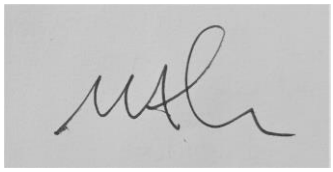
Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.



M.A.Richards

Abstract

This research describes a protocol for the rapid assessment of early growth photoperiodic performance of the agronomically important perennial ryegrass *Lolium perenne,* within long-day (LD) and short-day (SD) photoperiods and provides a preliminary assessment of candidate primer sequences for genes involved in ryegrass circadian regulation. The *in-vitro* screening process allowed us to compare the effects of LD and SD photoperiods upon both germination rates and root and shoot development. We were able to show a correlation between the performance *in vitro* with traits from the field based agricultural Pasture Profit Index (PPI), which would allow for a faster screening process for plant breeders. Working *in-vitro* and being able to control environmental parameters such as temperature would speed the process of screening ryegrass genotypes for useful traits within Irish agriculture and facilitate the exploration of circadian rhythm in ryegrass fitness and its adaptability to climate change.

Acknowledgements

This work was brough to an abrupt halt by the closure of the school due to COVID-19. It could not have been finished without the on-line support and encouragement of Dr Rossana Henriques, for which I am extremely grateful.

I would also like to thank all the academics, technicians, and staff at UCC for their support and encouragement. And my children, for always asking questions.

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1. Introduction.

1.1 Breeding and agriculture

The grassland biome is estimated to cover 25-37% of the Earth's land mass and the life cycle of grasses (Poaceae) exhibit both annual and perennial growth habits, with perennial grasses reproducing either sexually by seed or through asexual vegetative cloning (tillering) which allows a grass sward to develop when grazed. (Blackmore et al., 2015, Crowley and Wingler, 2019, Hopkins et al., 2007). Whilst the International Plant Names Index (www.IPNI.org) lists 80 species of *Lolium*, many are synonyms and less than ten species are recognised; *Lolium perenne* L.(*2n=2x=14*) is grown throughout Northern Europe and the temperate regions as an important C3 photosynthetic forage crop with high digestibility and ability to withstand frequent grazing (Armstrong, 1917, Van Huylenbroeck et al., 1999). Commercial breeding of diploid perennial ryegrass began in the 1930's at the Welsh Plant Breeding Station followed by colchicine induced tetraploids in The Netherlands, and whilst teraploids can provide higher establishment, disease resistance and tillering rates, they are less persistent in the field than diploids (Connolly and Park, 2001). Both diploids and tetraploids have adapted to a wide range of habitats and provide an array of genotypic and phenotypic variation which may be accessed for traditional breeding programmes through poly-crossing or hybridisation (Harper et al., 2019, Velmurugan et al., 2016). However, selection difficulties arise due to perennial ryegrass being an outcrossing, wind pollinated species, which displays a two locus self-incompatibility system designated S and Z which limits hybrid vigour (Thorogood et al., 2002). Leverage is being gained through genetic approaches such as Marker Assisted Selection (MAS) in Quantitative Trait Loci (QTL) and *L. perenne* was the first grass within the *Lolium spp.* to be sequenced, which helped identify genomic regions associated with agriculturally important traits (Byrne et al., 2015, Connolly and Park, 2001, Sartie et al, 2011).

1.2 The Pasture Profit Index

Within Ireland, perennial ryegrass accounts for approximately 95% of grass seed sales and is the most important forage species within the country; growth is photoperiod dependent, with substantial increases in biomass in the late vegetative and mid flowering phases and when soil temperatures reach 5°C at 10cm depth (Phelan et al, 2013, Fig.1).

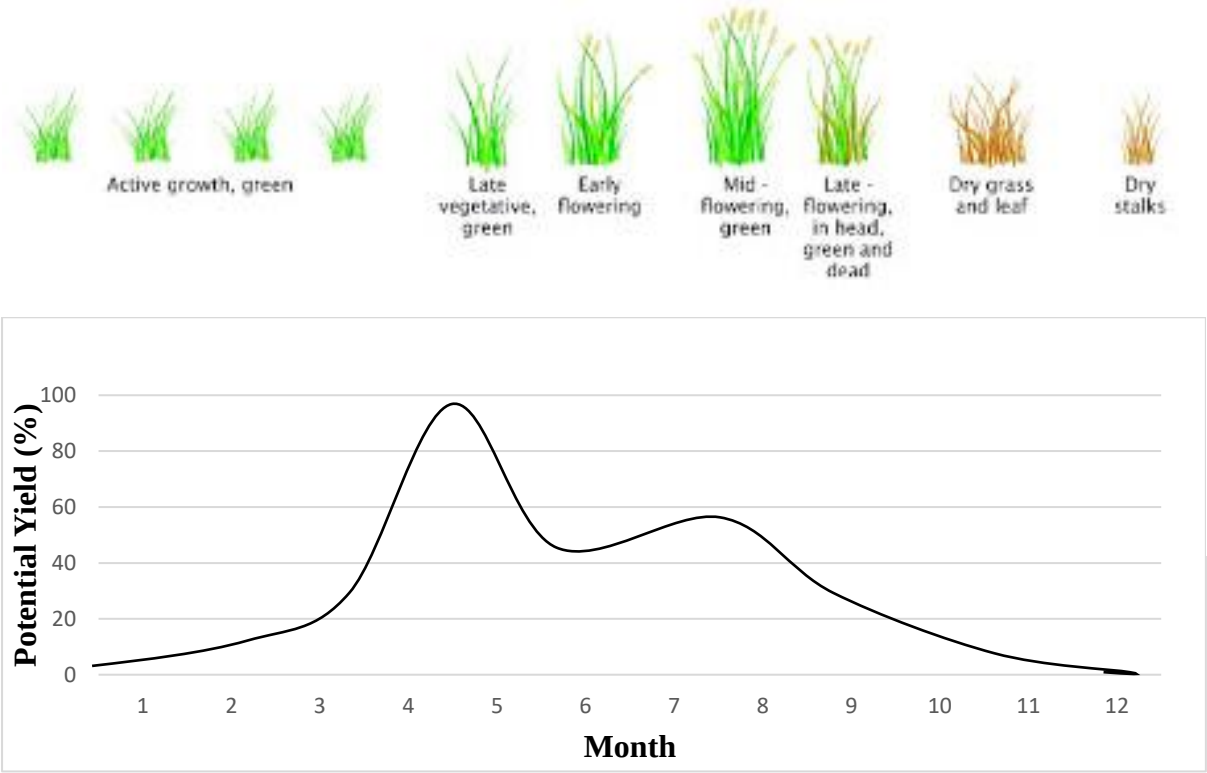


Fig.1 Seasonal growth of perennial ryegrass in Ireland, showing the potential yield (%) over the 12month period showing the peaks of growth occurring between March-June and August-September (modified from Phelan et al., 2013).

Breeding is undertaken by the state's agricultural agency Teagasc, and private companies such as Goldcrop; assessing traits of importance collected in the field allows for evaluation between varieties, such as total seasonal and annual yield, persistency, sward density, ploidy, flowering time (heading date), and annual dry matter per hectare (DMHa) content for silage. This

generates the Pasture Profit Index (PPI), and these indices provide information for farmers to selecting varieties deemed most appropriate for their agronomic needs (Teagasc, 2020). The LD results from *in-vitro* grown plants in this research correlates with summer growth from the PPI, and SD to early spring or late autumn in the field. Research from Teagasc is also being placed upon efficient fertiliser inputs and extending the growing seasons of these varieties to enhance the sustainability and therefore the security and profitability of Irish agriculture (Burns et al, 2015, Conaghan et al., 2008).

Recommended Intermediate & Late Perennial Ryegrass Varieties 2020

			Pasture Profit Index Values € / Ha / Year							Spring	Summer	Autumn	Total Yield	Mean DMD	1st Cut Silage	2nd Cut Silage	Ground Cover
			Total	Sub-Indices						(t DM/ha)	(t DM/ha)	(t DM/ha)	(t DM/ha)	(g/kg)	(t DM/ha)	(t DM/ha)	Score
Variety Name	Ploidy	Heading Date	PPI	Spring Growth	Summer Growth	Autumn Growth	Quality	Silage	Persist.	*1.10**	*7.03**	*2.36**	*10.49**	*840.7**	*4.65**	*4.05**	*6.1**
Aberclyde	T	25-May	**205**	51	42	34	41	38	0	1.31	7.20	2.22	10.73	852.1	5.25	3.73	5.4
Abergain	T	4-Jun	**192**	21	38	42	52	39	0	1.13	7.10	2.29	10.52	854.3	5.05	4.06	5.6
Abermagic	D	28-May	**187**	33	45	70	17	22	0	1.20	7.29	2.55	11.04	845.1	4.87	3.73	6.3
Nashota	T	3-Jun	**186**	43	33	34	32	44	0	1.27	6.98	2.22	10.47	846.8	4.99	4.31	6.2
Aberchoice	D	11-Jun	**176**	19	45	51	43	18	0	1.12	7.29	2.37	10.78	849.4	4.37	4.24	6.0
Aberwolf	D	30-May	**169**	56	34	37	10	33	0	1.34	6.98	2.25	10.57	841.7	5.04	3.85	7.0
Astonconqueror	D	27-May	**169**	76	28	38	-7	34	0	1.47	6.84	2.26	10.56	837.6	5.49	3.29	6.5

Table 1. Sample of the Pasture Profit Index table produced by the Irish state agrigultural agency Teagasc. Issued yearly, this shows the Variety name, Ploidy, and Heading Date. The PPI rank, Sping, Summer, Autumn Growth, Quality, Silage, and Persistency are allocated a value (€/Ha/Year). Spring, Summer, Autumn, Total Yield, 1st and 2nd cut Silage are in tons of dry matter per hectare (t/DM/Ha), and a score is given for Ground Cover (See also Appendix A, Table 17)

1.3 Photoperiodism

Lolium perenne is believed to have originated in the Mediterranean, with migratory routes and settlements in Africa, the Near East and Europe (Blackmore et al., 2015, McGrath, 2008). Its ability to adapt to more temperate regions was due to the natural selection of daylength neutral genotypes that flower early in the summer and can therefore take advantage of the subsequent long days (Cresswell et al., 2001).

As 90% of Ireland's farmland is grassland (Connolly and Park, 2001), swards of perennial ryegrass are a distinctive feature of the Irish landscape, with productivity increasing in the spring and early summer, before declining from late summer onwards as temperature and solar radiation decreases (Fig.1. Hurtado-Uria et al, 2013). Heading date (defined as the point when 50% of tillers show ear emergence) can vary throughout the summer and affects herbage quality, an important seasonal trait as a forage crop (Wang and Forster, 2017).

Classifying photoperiodic response is usually based upon flowering, which is initiated by the plant's perception of increasing day length and rising temperatures (Garner and Allard, 1920). An induction of a short-day photoperiod (SD) and lower temperature (vernalisation) is required. Plants which initiate reproductive strategies during a longer photoperiod, such as ryegrass, are long-day photoperiod (LD) plants, and the underlying genetic mechanism for this response is regulated by different pathways, one of which is the circadian clock (Harper et al., 2019, Higgins et al., 2010).

1.4 Circadian rhythm and the molecular clock

The Earth's 24-hour rotation produces an oscillating light and dark cycle (the photoperiod), as well as variations in temperature; these exogenous signals lead to a biological cycle referred to as circadian rhythm (Gehan et al., 2015, Harmer 2009, Millar 2016). As perennial ryegrass

expanded its geographical distribution across latitudinal clines, the response to LD and SD photoperiods and decreasing temperatures was a key adaptive trait in regulating flowering time and hence maximising reproductive fitness (Blackmore et al., 2015, Millar, 2016). The mechanism underpinning molecular responses to the photoperiod is referred to as the circadian clock, a network of genes which function as the plant's endogenous timekeeper. Advances made in the plant model species *Arabidopsis thaliana*, and through mathematical modelling, has shown how this is set in motion by the photoperiod which resets the daily oscillation in the central complex. Downstream of this oscillator complex are the outputs, such as flowering, hormone signalling, growth, metabolism, and biotic and abiotic stress responses (Fig.2. Belbin et al., 2019, Henriques et al., 2018, Inoue et al., 2018, Nohales and Kay, 2016).

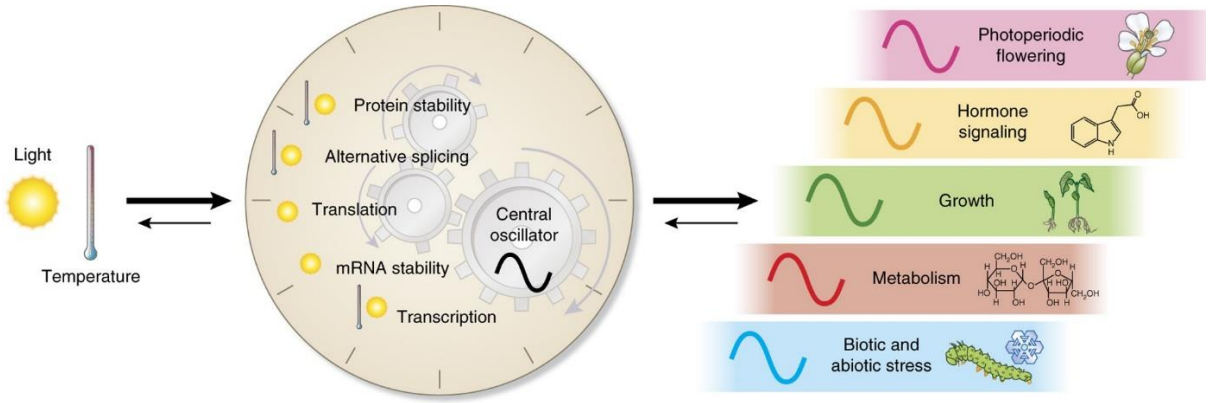


Fig.2. Basic view of the molecular mechanisms at the core of the oscillator, showing input/output pathway signals, and potential responses. (modified from Nohales and Kay, 2016).

The oscillator, input and output pathways are the core clock components and earlier research showing temperature and photoperiod sensitivity reciprocally regulating the transcription of morning and evening expressed genes, has now been expanded to include repression feedback loops forming a clock repressilator (Fig.3. Nohales and Kay, 2016). The primary components are *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY)*, which both accumulate by dawn. These two genes are gradually repressed during the

day by the *PSEUDO RESPONSE REGULATOR (PRR)* family (*PRR9, PRR7, PRR5),* and *TIME OF CAB EXPRESSION (TOC 1),* the latter the first identified evening clock gene shown to be repressed and possibly, together with *CCA1*, the earliest evolutionary circadian clock components (Henriques et al., 2018, Nohales and Kay, 2016). The daytime gene family are likewise inhibited by the evening phased gene complex *(EC)* comprising *EARLY FLOWERING (ELF 3, ELF 4),* and the transcription factor *LUX*. The loop is completed full circle by *CCA1* and *LHY* repressing the evening complex at dawn and this cycle maintains the circadian rhythm (Oakenfull and Davis, 2017, Davis and Ronald, 2017).

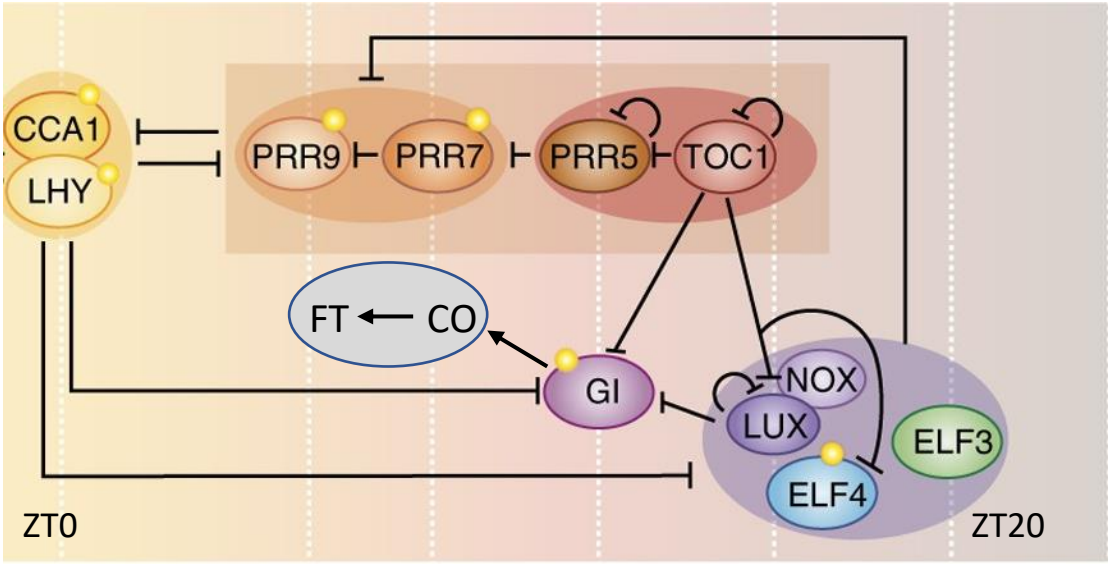


Fig.3 Simplified schematic showing the sequential expression of the core clock components over a 24hr diurnal cycle, with onset of light referred to as ZT0 (*Zeitgeber Time),* when *CCA1* and *LHY* repress the expression of the *PRR*-encoding genes, then *TOC1*, *GIGANTEA (GI*) and finally the EC members *LUX*, *ELF3* and *ELF4*. Sequentially expressed are *PRR9*, *PRR7*, *PRR5* and *TOC1*, which represses the expression of *CCA1* and *LHY*. In the evening, *TOC1* represses all previously expressed components, in addition to *GI*, *LUX* and *ELF4*. Subsequently, the EC

maintains the repression of *GI* and represses *PRR9* and *PRR7*. In the flowering pathway, *GI* initiates *FLOWERING LOCUS T (FT)* through the activation of *CONSTANS (CO*), the latter accumulating through response to lengthening photoperiod in LDs (modified from Nohales and Kay, 2016).

1.5 Circadian regulation within other plant species.

Whilst most knowledge on circadian clock regulation lies within the dicot model species *Arabidopsis*, monocots evolutionarily diverged ~127 to 191 million years ago (Bell et al., 2010). However, genetic conservation with the grasses allows for the identification of potential circadian clock components from both *Oryza sativa* (Rice, SD) and *Brachypodium distachyon* (LD), which provide orthologue sequences that can be accessed through comparative genomic databases (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_monocots/, https://www.arabidopsis.org/,). For example, *GIGANTEA (GI)* is a large, relatively unexplored protein involved in a wide range of physiological processes (Mishra and Panigrahi, 2015). Within *Brachypodium* it is known to be a positive regulator connecting the clock with the flowering time master regulator *CONSTANS (CO)* which activates transcription of *FLOWERING LOCUS T (FT)* in the leaf phloem cells (Johannson and Staiger, 2015, Woods et al., 2014). Also interacting with *GI* is *ZEITLUPE (ZTL)*, a subunit of a ubiquitin ligase complex which is stabilised by blue light during the day (Johannson and Staiger, 2015). Red and far-red light signals for flowering are interpreted by phytochromes *phyA, phyB, phyC*, but to date only *phyC* has being shown to be an essential component of flowering in monocots (Woods et al., 2014). These endogenous timekeeping light signals converge with the clock cycle through the interaction of *TOC1* and *PHYTOCHROME INTERACTING FACTORS (PIFs)* and research in *Arabidopsis* shows that *PIF3* interacts with *TOC1* to regulate hypocotyl growth and appears to be a key feature in the evolution of LD flowering responses in temperate grasses (Soy et al.,

2016, Woods et al., 2014). Whilst the vernalisation pathway is a critical component of flowering response, this does not lie under circadian control (Paina et al., 2016, Wang and Forster, 2017).

In summary, these endogenous timekeeping components allows those plants with the most efficient photoperiodic circadian rhythm to gain a competitive advantage and increase fitness through explicit control of flowering time development. Understanding the diurnal gene expression of the clock genes *CO*, *FT*, and *GI* has the potential for us to enhance plant fitness (Gehan et al., 2015, Wang and Foster, 2017).

1.6 Gene expression

For effective RT-PCR analysis, reference (or housekeeping) genes are required for normalisation against the gene of interest (GOI). For model plant species, commonly used reference genes include *beta-Actin (ACTB), Tubulin (TUB), glyceraldeyde-3-phosphate dehydrogenase (GAPDH), Ubiquitin (UBQ), Ribosomal 18sRNA, TATA-Box Binding Protein (TBP), Elongation-Factor (EF-1α)* and *Translation Elongation Factor (TEF)* (Huang et al., 2014, Lee et al., 2010). These can be used to help establish the functional validation of clock genes for *L. perenne*, which is challenging with a non-model monocot such as perennial ryegrass (Harper et al.,2019). Verification of the amplification success of such primers can be undertaken with regular Polymer Chain Reaction (PCR) before progressing to RT-PCR. To track circadian gene expression, a time course is initiated for the collection of plant material at specific moments through a 24hour period, starting at ZT0 to ZT24. 'ZT' refers to Zeitgeber ('time giver'), the stimulus which generates circadian rhythm, in this case first light (Fig.16, Millar, 2016).

1.7 Importance of this research

In traditional ryegrass breeding without genetic screening, field-based performance is assessed through morphological characteristics rated by the International Union for Protection of Varieties (UPOV) (McGrath, 2008). Whilst this provides an assessment of phenotypic variation, field trials require a long growth period. The aim of this *in-vitro* research is to provide an evaluation of early growth performance, such as germination rate, and from just 5 to 7 days after germination gain some indication of the potential mature growth performance in the field. Growing *in-vitro* within temperature-controlled growth chambers provides us with the ability to mimic the LD and SD photoperiods of winter and summer and then collect plant material over a diurnal cycle and analyse gene expression. Improving traits for climate change could include adaptation to an extended growing season due to climate change leading to an increaed biomass, and to study potential stresses such as warmer winter temperatures and delayed cold hardening (Hopkins and Del Prado, 2007). Unlike selective breeding processes for the cereals, which has been ongoing since the advent of farming, commercial perennial ryegrass breeding has only been undertaken since the 1930's. Whilst Aberystwyth University alone holds ~6000 accessions of *Lolium* Spp., the estimate on annual genetic gain for biomass production over this past century is estimated to be as low as 0.25-0.6% (Harper et al., 2019). Understanding the responses to photoperiod *in-vitro* could lead to improving variety selection for the field, to deal with changing climate change scenarios.

Tracking the expression of circadian clock genes through time facilitates understanding of the relationship between genotype and phenotype and the potential of exploiting this knowledge, and here we show how we set up such an experiment. These *in-vitro* conditions provide an ability to track circadian gene expression and lead to a better understanding of genetic mechanisms behind QTL agronomic traits such as heading date, biomass, forage quality, cold tolerance, and biotic/abiotic stress.

1.8 Research objectives

- To set up a reproducible *in-vitro* protocol to characterise photoperiodic growth regulation of perennial ryegrass in the early developmental stage under simulated LD and SD photoperiods and,
- To characterise the different varieties and assess if there is a correlation between genotype and photoperiod and,
- To screen for varietal performance in relation to the Pasture Profit Index and establish if this is a method which we can adapt for field trials at later dates.
- To assess candidate housekeeping genes and test the primer efficiency of novel circadian clock genes within perennial ryegrass, and to determine their oscillation under different photoperiod conditions to further understand circadian gene expression within *L. perenne.*

2. Materials and Methods

2.1 Plant Materials

For this study, seventeen commercial varieties of *Lolium perenne* were supplied by Irish breeding programmes from Teagasc and Goldcrop, having different performance characteristics in the PPI (Appendix A, Table 17). Whilst ryegrass tetraploids have increased grazing efficiency, for this research only diploids were selected due to their lesser complexity for primer design and sequencing.

2.2 Plant Growth Chambers

1. Light and temperature-controlled growth chambers programmable for SD [8hr light/16hr dark] and LD [16hr light/8hr dark], were set to a baseline temperature of 22°C. Seeds were sterilised in either Solution A (70% ETOH) or Solution B (60% bleach, 40% Triton X-100 (0.05 stock)), with germination synchronised by stratifying for 7 days at 4°C darkness, before being placed with scutellums and embryos in alignment into MS Medium basal salt mixture, pH to 5.7 within 6cm square petri dishes. Germination rates were recorded after 72 hours.

2.3 RNA Extraction, Quantification and PCR

1. RNA was obtained by grinding frozen plant material (~10 seedlings providing ~0.5mL of ground material) using the QIAGEN RNeasy® plant mini kit and the BIOLINE Isolate II RNA plant kit, a two-step on column purification process followed by a DNAse digestion to provide RNA in solution, with quantification through Nanodrop spectrophotometry. Regular PCR cycle was 95°C/5mins, 95°C/1min, 58°C/45secs, 72°C/3mins, 72°C/5mins for 30 cycles, and visualised on Tris-Acetate-EDTA (TAE) RNA gel,

2.4 cDNA Synthesis

cDNA synthesis followed the Affinity Script Q-PCR agilent Kit protocol with 2X MasterMix, Oligo dT primer and Affinity Script RT/RNase block (see Table 1). RNA gel contained 1% agarose in TEA buffer, followed by RT-PCR (Table 2)

2.5 Statistical analysis

1. One-way and two-way ANOVA performed using SPSS statistical software.
2. Tukey Post Hoc test was performed to assess statistical difference.

Table 2. cDNA synthesis Affinity Script Q-PCR Agilent Kit Mix for Oligo dT primer

Reagents	1 x
RNAse free dH_2O	(Based upon Nanodrop Results)
2X MasterMix	10µL
Oligo dT primer	3µL
RT/RNAse block	1µL
RNA	1µg in vol up to 6µL

Table 3. RT-PCR mix (in final volume of 10µL)

Mix	1 x
Fwd Primer	0.2µL
Rev Primer	0.2µL
$StdH_2O$	3.6µL
SYBR	5µL
cDNA (1:20)	1µL

3. Results

3.1. Section 1. Establishment of a reproducible protocol for assessing early developmental responses in perennial ryegrass

3.1.1 Methodology

The aim of this project was to develop an efficient protocol for analysing the early developmental growth of perennial ryegrass under different photoperiods, and the possibility of correlating these findings with the field grown Pasture Profit Index (Appendix A, Table 17). This strategy could help create a much faster screening process for potentially superior ryegrass varieties than having to engage in field trials. Research started by testing 4 varieties of diploid *L. perenne* (Table 4), two of which are represented as being high performers in the PPI, and two as low performers, to provide some initial comparisons.

Table 4: Abbreviations used for the four diploid varieties assessed for photoperiodic regulation together with their PPI rank, 2020. Seed stock for this first assay was provided by Teagasc.

Section 1 LD and SD	Variety	Varietal Code	PPI Rank 2020
	Abergreen	AG	192 (High)
	Abermagic	AM	187 (High)
	Clanrye	C	68 (Low)‡
	Kerry	K	73 (Low)

‡ PPI data from 2019

Ryegrass varieties AG, AM, C and K (twenty seeds per plate) were grown under long day (LD) and short day (SD) photoperiods in controlled growth chamber conditions (Materials and Methods: Section 2.2). With this experimental set up, this generated 5 biological replicates for LD (Experiments 1,2,3,5 and 6) and 4 biological replicates for SD (Experiments 1,4,6 and 7). Each plate was photographed at midday (±1hr) between days 3 - 7 between mid March and early June (Fig.4). These photographs were then analysed in ImageJ software, with root and shoot length recorded for data analysis. In this initial set up both the first leaf, primary, and seminal roots were measured. Germination rates were also analysed (Table 5) to track both root and shoot developmental responses, and fresh and dry weights were collected (Table 7). These results are presented below.

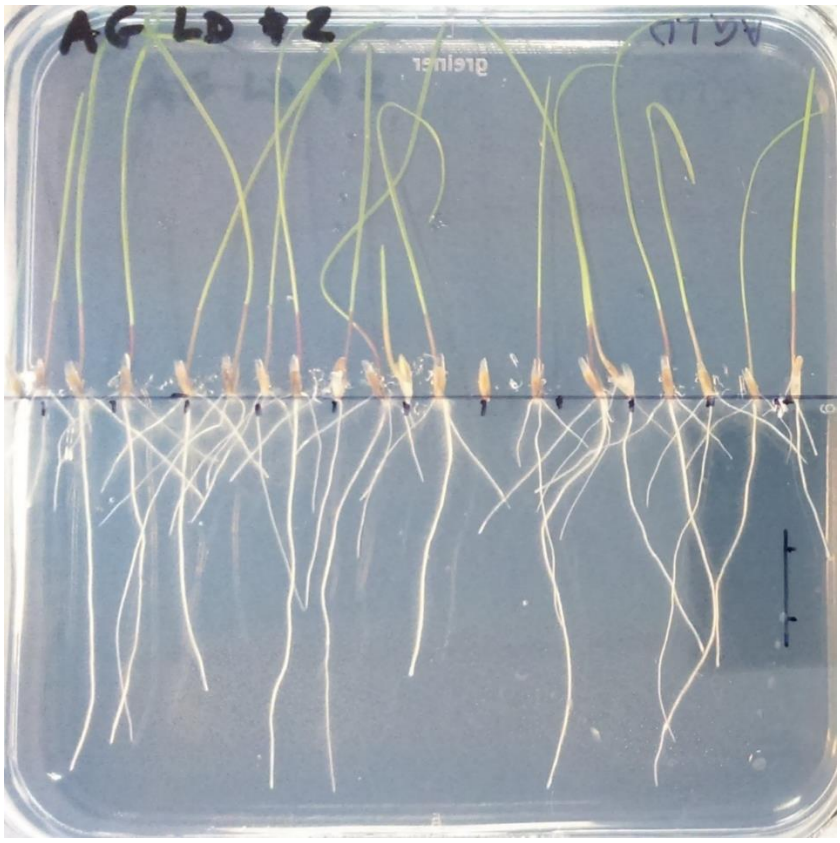


Fig.4. Typical plate showing *in-vitro* growth. AG roots and shoots at day 5 in LD. Black ‡ line on lower right of plate represents 1cm. Black dots on the centre line are from marking

germination rates. This plate shows the shoots of AG beginning to touch the upper edge of the petri dish, measuring after this date prone to error. Day 5 was thus considered to be the optimum timepoint for statistical analysis and provided enough material from all varieties for RNA extraction.

3.1.2 Germination rates

Twenty seeds of each variety from 6 biological replicates in both LD and SD (except for AG in LD, which only had 5 replicates due to contamination) were assessed 72 hours after exposure to light using a stereomicroscope and radicle protrusion recorded. Table 5 shows the average % germination rate and standard deviation. AG in both LD and SD photoperiods showed the highest germination rates (LD = 92%, SD = 89.2%) and this result correlates with its high ranked position in the Pasture Profit Index. This was followed by K both in SD (72.5%), and LD (67.5%). Kerry is a low ranked performer in the PPI table (PPI=73), but in this assay had a higher germination rate than C in both LD (60%) and SD (55%). AM was the second highest performer from the PPI table (PPI=187), yet in all biological replicates assessed was consistently poor, suggestive of unreliable seed stock.

Table 5: Percentage germination rates of AG, AM, C and K seeds after 72h in the light in LD and SD. Values are an average of each % germination under LD or SD photoperiod from 6 biological replicates (5 for AG in LD).

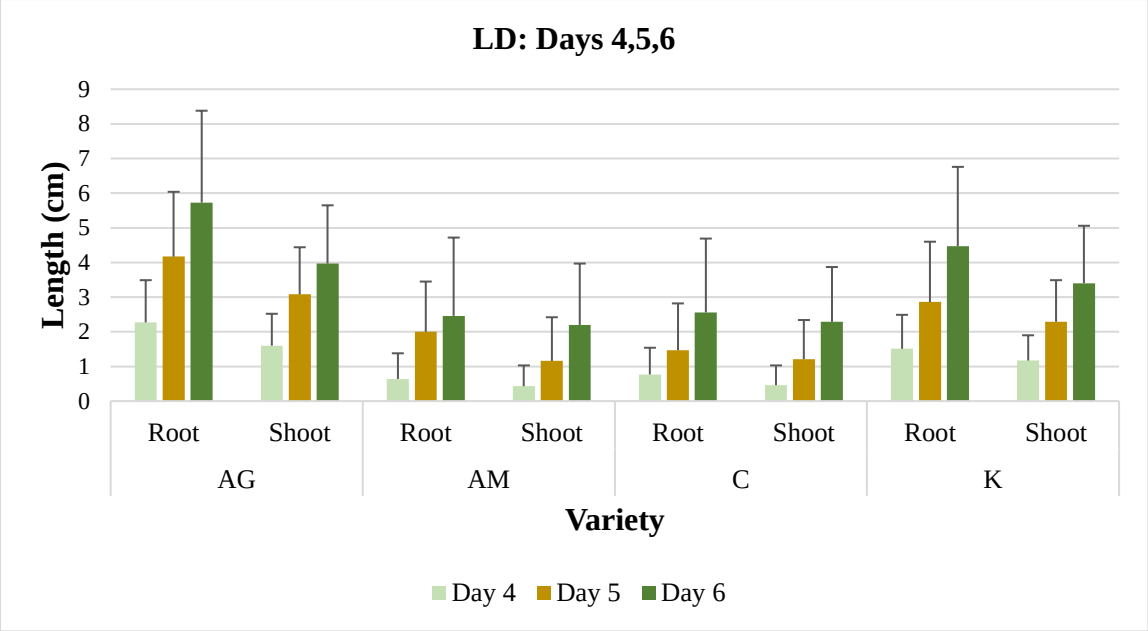
Photoperiod	Variety	% Germination72hrs after light	Stdev
LD	AG	92	±6.9
	AM	45	±17.8
	C	60	±23.1
	K	67.5	±27.2
SD	AG	89.2	±18.3
	AM	49.2	±30.7
	C	55	±22.7
	K	72.5	±26.6

3.1.3 Root and shoot analysis

Root and shoot lengths were recorded from day 3 - 7 with photographs taken at a similar time each day (±1h) and measurements recorded in ImageJ sofware. Days 4, 5 and 6 were chosen as the most representative for root and shoot length, as beyond this date the normal distribution of AG and K were skewed, probably due to the size constraint of the petri dish (Fig 4). Whilst a degree of contamination was considered acceptable for assessing germination rates, only non-contaminated plates were used for root and shoot measurements. A minimum number of sixty seeds were used per variety, with averages calculated from three biological replicates showing

normally distributed data, (experiments 2,3 and 6 in LDs, and experiments 4,6, and 7 in SDs). As expected, there was an overall increase in biomass over this 3-day period, except for AM which did not seem to develop properly, due to either unreliable seed stock or contamination, and was removed from subsequent analysis. Fig.5 shows the average results for root and shoot length in LDs or SDs. Similar results were recorded from field -grown plants in the PPI, in that the PPI top performer, AG, developed longer roots and shoots in both photoperiods tested, followed by K, C and AM. root, and shoot ratios were also calculated for these experiments (Table 6). Results showed statistically significant differences in varietal root length under LDs except for K, which has a similar performance both in LDs and SDs.

A remarkably high difference in shoot length under both photoperiods was not observed, although root lengths appeared to show more similarity in both photoperiods than the shoots. Whilst AG correlated with the PPI as being the top performer, K also showed high performance in both photoperiods.



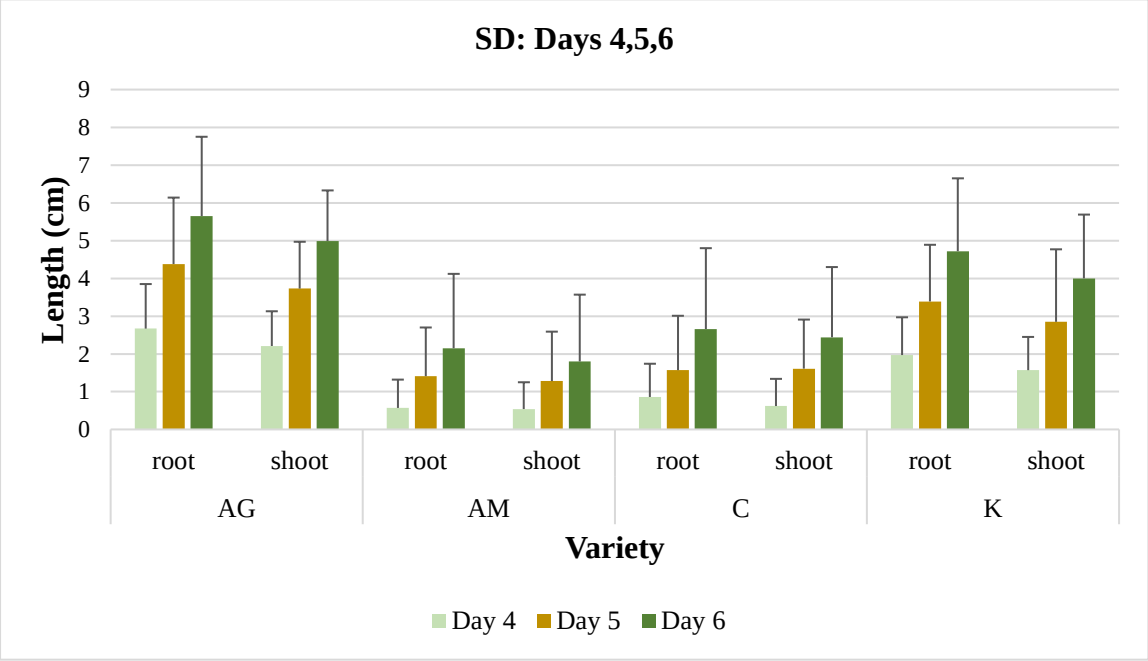


Fig 5. Root and shoot length of varieties AG, AM, C and K between days 4-6 in LDs and SDs. Upper panel: LD photoperiod. Lower panel: SD photoperiod. Results presented are an average of three biological replicates. Error bars represent standard deviation.

Table 6. Root and shoot ratios for AG, AM, C and K at days 4-6 in LD and SD. 3 biological replicates per photoperiod.

LD	Day 4	Day 5	Day 6	SD	Day 4	Day 5	Day 6
AG	1.42	1.35	1.44	AG	1.17	1.2	1.13
AM	1.49	1.72	1.19	AM	1.1	1.05	1.94
C	1.67	1.21	1.18	C	0.97	1.39	1.09
K	1.29	1.25	1.31	K	1.19	1.25	1.18

Root development was greater than shoot development throughout LD for all the varieties, except for C in SD at day 4, when shoot growth was slightly increased. The root and shoot ratio for K was most the most consistent between the photoperiods.

To further understand the relationship between LDs and SDs, fresh and dry weights (g) at day 7 from experiments 5, 6 (LD) and 4, 6 and 7 (SD) were recorded. Roots and shoots were separated and individually weighed to assess biomass partitioning as an indicator of the plants resource allocation under different photoperiods, and an average and standard deviation calculated (except for SD Dry) (Table 7). A minimum of 15 seedlings from 2 biological replicates per variety were measured (except AG where we could only recover data from one biological sample), and results are presented as an average weight per plant by dividing the total weight by the number of seedlings. The results showed that the biomass of shoots was greater in SD than LD, which correlated with our results from Fig.5.

Table.7. Fresh and dry weights (g) of varieties AG, C and K at day 7 after exposure to light in LD and SD. Obtained from 2 biological samples: LD experiments 5 and 6; SD experiments 4, 6 and 7.

Variety	Organ	LD Fresh	LD Fresh Stdv	LD Dry	LD Dry Stdv	SD Fresh	SD Fresh Stdv	SD Dry	SD Dry Stdv‡
AG	Root	0.072	±0.017	0.0233#	±0.09	0.0553	±0.075	0.0547	±n/a
	Shoot	0.120	±0.068	0.0082#	±0.052	0.1809	±0.004	0.1586	±n/a
C	Root	0.053	±0.001	0.0079	±0.054	0.0573	±0.002	0.0314	±n/a
	Shoot	0.184	±0.020	0.0102	±0.071	0.1127	±0.003	0.0989	±n/a
K	Root	0.076	±0.003	0.0043	±0.072	0.0794	±0.002	0.024	±n/a
	Shoot	0.130	±0.067	0.0093	±0.07	0.2045	±0.003	0.1146	±n/a

Only one biological sample

‡ No stdev calculated as only a total weight was recorded for root and shoot

3.1.4 Statistical analysis: one-way ANOVA LD and SD

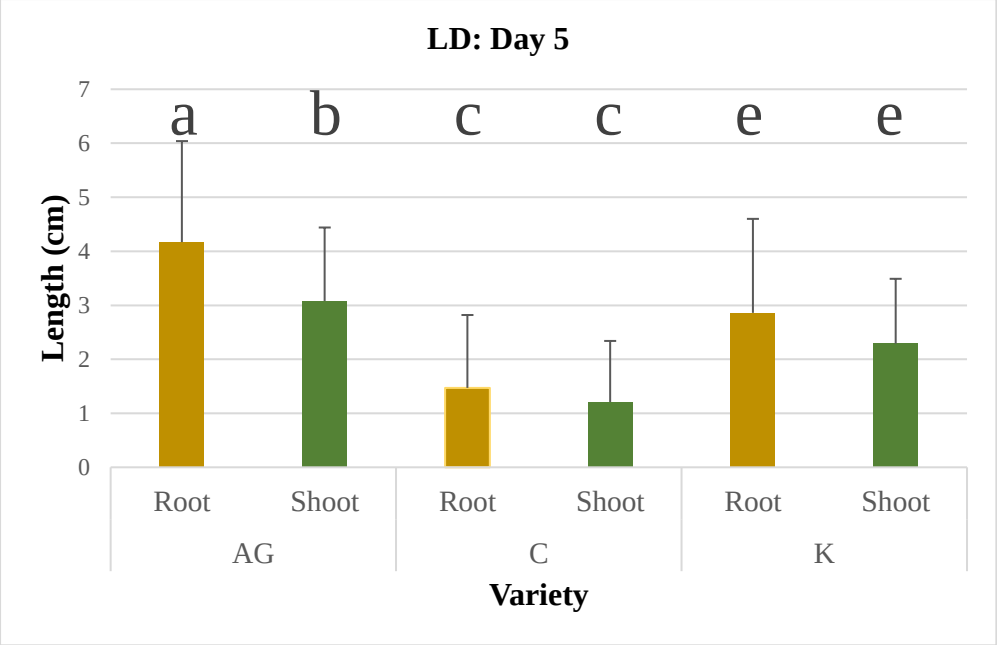
Differences between root and shoot lengths *within* each variety, showed that in SD at Day 5, there was no statistical difference between root and shoot lengths of the varieties. In LD, there was no statistical difference between C root and shoot length, but there was a statistical difference between root and shoot lengths in varieties AG (p-value 0.01) and K (p-value 0.00) (Fig.6). When we compared root and shoot lengths *between* the varieties, a statistically

significant difference was observed (p value 0.00) between the three varieties in both LDs and SDs and the Tukey post hoc test was used to identify where this statistically significant difference lay.

From the Tukey test results from LD roots a statistically significant difference was observed between AG and C, and AG and K root lengths (Fig.5). In LD shoots, it was observed that as in roots, AG and C displayed the most significant difference, although AG and K shoot length was still statistically significant. This showed that under LDs, all three varieties had statistically significant differences in both root and shoot lengths.

SD roots showed a statistical significance between AG, C and K root lengths (all p-values 0.00). In SD shoots, again AG and C displayed the most statistically significant difference with slightly less difference between AG and K. These results showed that under SDs, all three varieties share the same statistical differences as with LDs. Of note is that AG roots are similar within both LD and SD photoperiods at >4cm, and less dependent on photoperiod than K, which increased growth in the SDs. C maintains similar root lengths of ±1.5cm regardless of photoperiod, and whilst shoot length increases under SDs it is considerably shorter against AG and K. Statistically significant differences occurred between the root and shoot lengths of all the varieties in LD (p-values 0.00), but was less significant in SD.

Within LD, AG and K roots showed similar responses to this photoperiod (p-value 0.02), yet within shoots the similarity lay between C and K (p value = 0.017). To extend analysis a two-way ANOVA was undertaken.



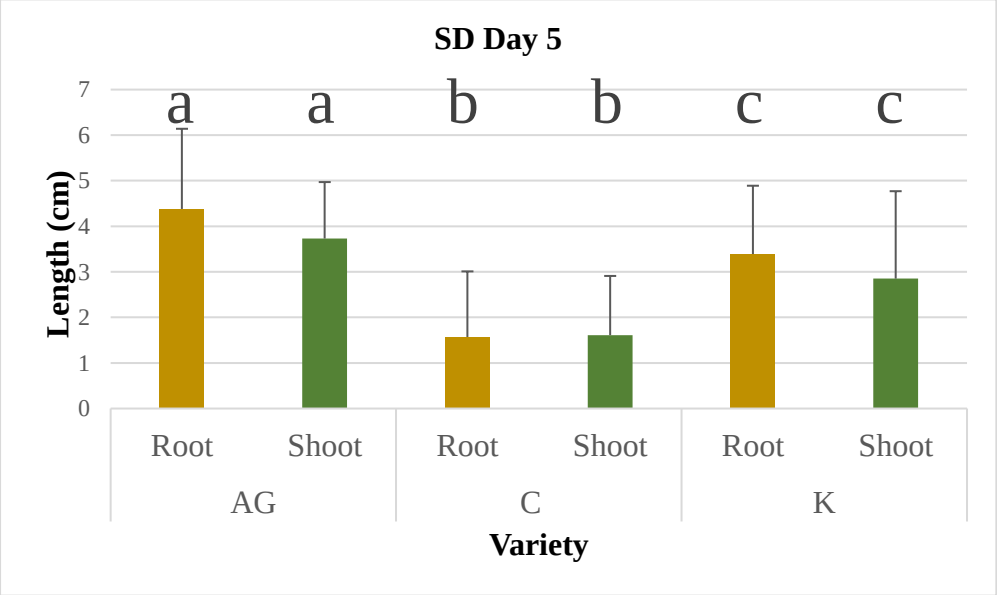


Fig.6. One-way ANOVA, showing the statistical significance between root and shoot length of varieties AG, C and K in LD and SD. Upper panel: root and shoot lengths at day 5 in LD. Lower panel: root and shoot lengths at day 5 in SD. Error bars represent standard deviation. Statistical significance was determined by one-way ANOVA followed by Tukey test. Statistically significant differences are shown by different letters.

3.1.5 Statistical analysis: two-way ANOVA LD and SD

A two-way ANOVA was performed to check for statistical significance (p-value <0.05) between the three varieties AG, C and K, and the two photoperiods LD and SD, against the dependent variable of root and shoot length (Appendix B). In LD, there was a statistically significant difference between the three varieties and the photoperiod (p-value 0.001). Within SD, there was slightly less statistical difference between the varieties and photoperiod (p-value 0.003) and that AG appears to be less dependent on photoperiod for root and shoot length.

3.1.6 Response to increased light intensity in SD

Finally, an experiment was undertaken to assess ryegrass performance in SDs when exposed to increased light intensity (Fig.7 below). For this experiment, fourteen plates (with twenty seeds per plate) of AG were exposed to an increased light intensity of 168µmol·m^{-2}·s^{-1} (increased from 100 µmol·m^{-2}·s^{-1}). This allowed for correlation between root and shoot lengths at day 5 with the average of three biological replicates from the previous SD analysis.

Results showed that shoots grew considerably shorter at the increased light level, and that root length increased slightly. Table 8 below shows the root and shoot ratio of AG and an average of the fresh weights (g). This showed that the ratio between root and shoot at increased light levels was 4.25, compared to 1.2, ~4x higher than under normal light intensity. However, the fresh weight of both roots and shoots remained constant between the two light levels, which indicated the same biomass.

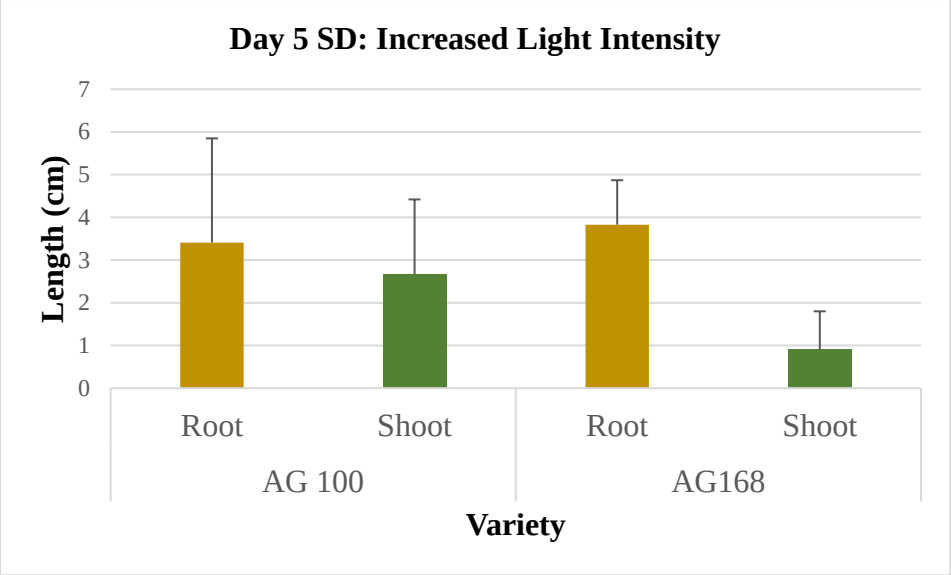


Fig.7. Root and shoot length of AG at day 5 in SD in both normal and increased light intensity. Bars on the left (AG100) are the average of three plates from Experiments 4,5 and 6 (three biological replicates) grown under 100 µmol·m^{-2}·s^{-1}. Those on the right (AG168) are the average of fourteen plates from Experiment 8 grown under 168µmol·m^{-2}·s^{-1}.

Table 8. Root and shoot ratio of AG at day 5 in SD, in both normal and increased light intensity, with average of fresh weights (g) of three biological replicates from normal light intensity of 100 µmol·m^{-2}·s^{-1} (AG100), and fourteen plates (280 seedlings) from increased light intensity of 168µmol·m^{-2}·s^{-1} (AG168).

AG SD	AG100	AG168
Root/Shoot Ratio	1.2	4.25
Roots fresh weight (g)	0.0553	0.0555
Shoots fresh weight (g)	0.1809	0.1835

3.2 Section 2. Analysis of photoperiodic regulation of perennial ryegrass early development

3.2.1 Methodology

Making use of a small group of varieties (n=4) allowed an efficient methodology to generate reliable results from this *in-vitro* protocol. Optimising seed sterilisation techniques and ascertaining the most suitable time points for recording root and shoot lengths for statistical analysis in Section 1, allowed for the assessment of another group of varieties (n=14) (Table 9), to determine how photoperiod would impact on perennial ryegrass early-stage development from different seed stock.

For the discussion, the varieties were divided into three groups based upon their PPI ranking of high, medium, or low performance. This group also included three non-PPI varieties; Esquire (a dwarf amenity variety), and Talbot and Vigour, two older varieties which were established prior to the PPI and in use within Europe since 1968.

Twenty seeds per variety were grown under LD photoperiod in controlled growth chambers (Materials and Methods: Section 2.2). With this new set of varieties, three experiments generated two biological replicates in LD. Data from another study from this laboratory (Dalton, 2020) utilising SD was also available but limited to six varieties due to contamination. Varieties were again photographed between days 3 - 7 at the same time each day (±1hr) with measurements undertaken in ImageJ, but for this group, only the primary root was measured. There was considerable variation in growth between high, middle, and low performers (Fig.8). Germination rates, and root and shoot developmental responses in LD were also analysed and the results presented below.

Table 9. Abbreviations used for the fourteen diploid varieties assessed for photoperiodic regulation together with their PPI rank in 2020.

Section 2 LD only	Variety	Varietal Code	PPI (2020)
	Astonconqueror	ACON	165 (High)
	Astonking	AKIN	116 (Medium)
	Drumbo	D	117 (Medium)
	Esquire[1]	E	N/A
	Glenroyal	GR	96 (Low)
	Gusto	GU	161 (High)
	Kerry[2]	K	98 (Low)
	Majestic	MJ	66 (Low)
	Moira	MO	187 (High)
	Nifty	N	193 (High)
	Oakpark	OAK	118 (Medium)
	Rosetta	R	156 (High)
	Talbot[3]	T	N/A
	Vigour[4]	V	N/A

1 Amenity dwarf variety.

[2] Kerry in this section is different seed stock from Section 1.

[3] and [4] are older pre-PPI varieties

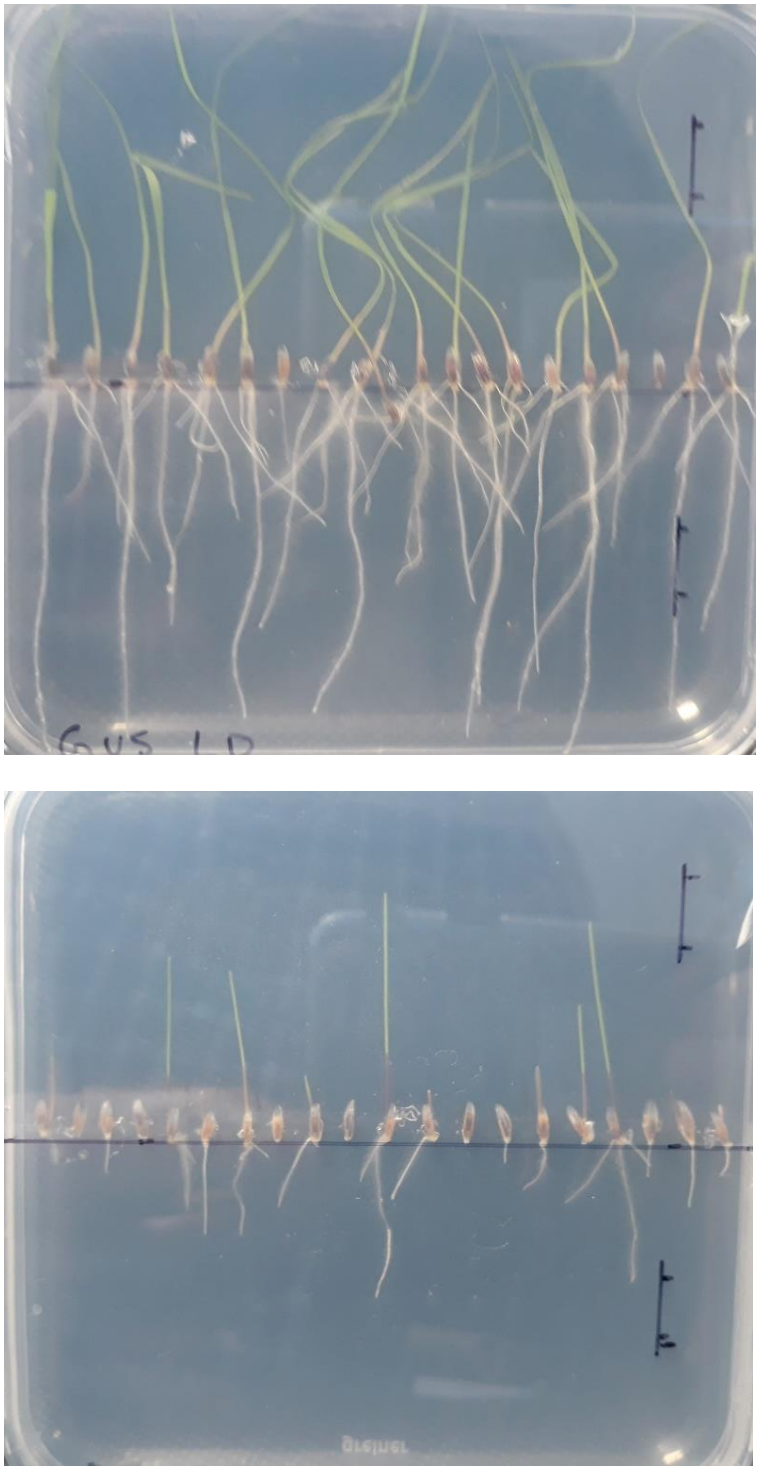


Fig. 8. GU and D seedlings at day 5 in LD. Upper figure: GU, a high-ranking variety in the PPI, showing extensive root and shoot establishment. Lower figure: D, a low-ranking variety from the PPI. Black ‡ lines at right side of image represents 1cm. Unlike Section 1, day 5 was sub optimal as varieties such as D were still germinating whilst GU and R were too large to be measured accurately, which could be overcome by utilising larger (~12cm) plates.

Table 10. Germination rates for the fourteen varieties. Values are average of % germination determined after 72 hours exposure to light under LD and SD. 60 seeds per variety in 3 biological replicates for LD. 40 seeds in 2 replicates for SD.

LD	Variety	% Germination 72 hours after light	Stdev	SD	Variety	% Germination 72 hours after light	Stdev
	ACON	93	±0.47		ACON	97.5	±0.50
	AKIN	80	±2.45		AKIN	85	±2.00
	D	6	±0.94		D	0‡	±0.00
	E	73	±3.40		E	70	±2.00
	GR	35‡	±0.50		GR	15‡	±3.00
	GU	90	±0.00		GU	92.5	±0.50
	K	93	±0.94		K	87.5	±2.50
	MJ	71	±2.87		MJ	77.5	±0.50
	MO	86	±1.25		MO	77.5	±2.50
	N	62‡	±3.50		N	32.5‡	±6.50
	OAK	90	±0.82		OAK	95	±0.00
	R	86	±0.94		R	92.5	±1.50
	T	77.5‡	±1.50		T	32.5‡	±6.50
	V	67.5‡	±1.50		V	N/A	±N/A

‡ Missing sample due to contamination

3.2.2 Germination Rates

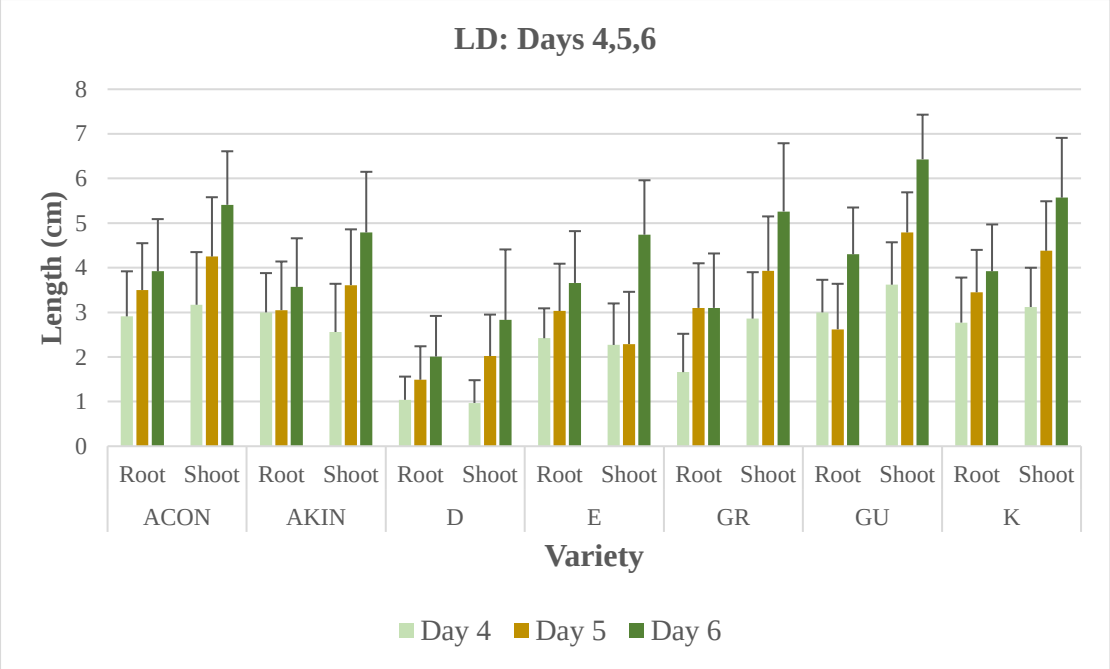
Twenty seeds of each variety from three biological replicates in LD and two biological replicates in SD (except for D, GR, N, T and V) were assessed 72 hours after exposure to light and radicle protrusion recorded (Table 10).

In both LDs and SDs, ACON, GU and OAK had the highest germination rates. K, despite being a low performer in the PPI, maintained a high germination rate in LD and SD as in Section 1. N and MJ had low rates of germination, the latter being a better performer in SD. The lowest germination rates were D and GR, the latter having contamination which may have affected the result. Overall, germination rates were higher in SD than LD, with a very pronounced difference with the non-PPI variety T.

3.2.3 Root and shoot analysis LD

As in Section 1, root and shoot length was recorded from days 3 - 7 with photographs taken at midday each day (±1hr) in late November and early December, and these measurements recorded in ImageJ. Contamination was a major factor influencing results in this section, as varieties of the new seed stock needed a harsher sterilisation protocol which was not developed until after experiment 2. This resulted in having to combine data from three experiments in SD, and after discarding results from contaminated plates, only one set of data was available for the varieties ACON, AKIN, E, GU, K, MJ, MO, and R in SD.

For LD, contamination was less severe, and two biological replicates were available for the fourteen varieties to track their growth over days 4-6 (Fig. 9 below). A large range of root and shoot lengths was observed. Days 4-6 were still selected from LD as being the most representative to graph the daily increase in root and shoot lengths, as this also provided correlation with the results from Section 1,



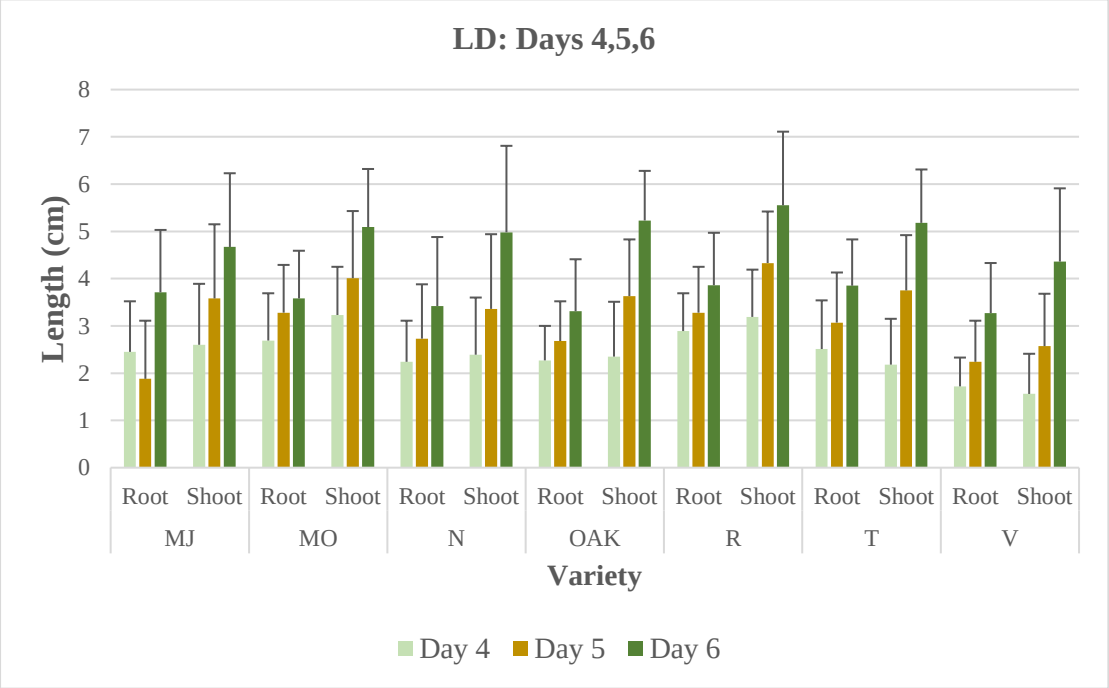


Fig 9. Showing the root and shoot lengths of the fourteen varieties between days 4-6 in LD. Upper panel: Varieties ACON -K. Lower panel: Varieties MJ-V. A minimum of forty seeds from two biological replicates for varieties D, E, GR, N, OAK, T and V in LD.

Unlike Section 1, shoots grew longer than roots. Only the primary root was measured.

In LD roots, the only variety which grew >3cm at day 4 was GU, followed by ACON and R. As in section 1, K was a comparatively high performer in LD. At day 6, the only varieties which >4cm were GU, with ACON, K, MJ, MO, and R. The lowest performance in LD roots were D, GR, N, OAK, and V.

In LD shoots, the top performer >6cm was again GU, followed by >5cm ACON, GR, K, MO, N, OAK, R and T, the latter being an older variety with no PPI rating. All other varieties were >3cm, except for D. The dwarf amenity variety E in LD was also a significant performer in both root and shoot length, and was also the only variety together with GR, GU, and MJ to not gain length every day.

Root and shoot ratios were calculated to show the relationship between the two organs (Table 11). In this analysis, unlike the four varieties in Section 1, the 14 varieties in Section 2 displayed greater daily shoot length (especially at day 6), when compared to roots, except for AKIN, D (day 4), E (day 4 and 5), GU (day 5), T (day 4) and V (day 4 and 5).

Fresh and dry weights are not included in this analysis due to lack of biological replicates.

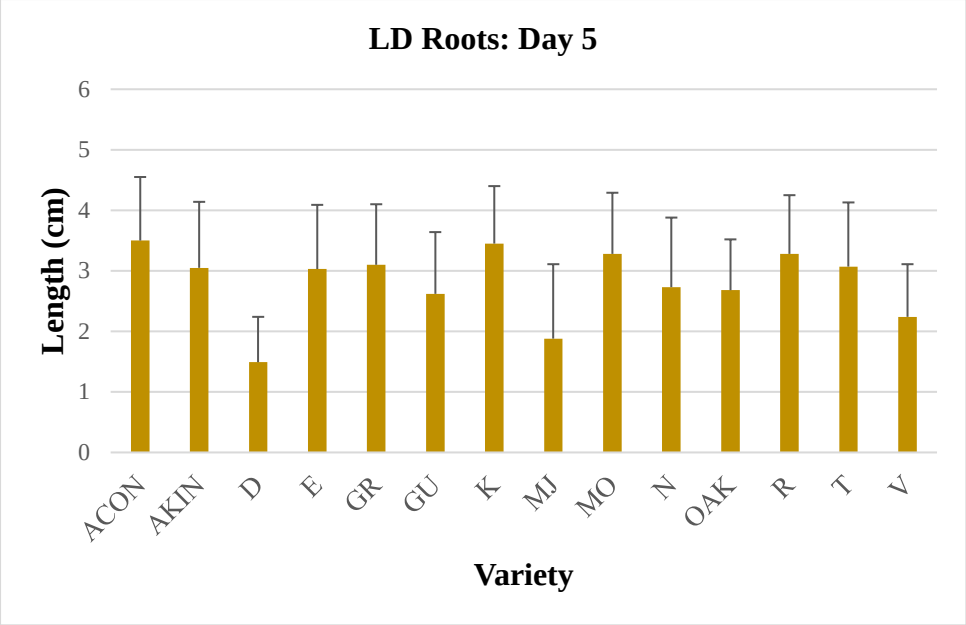
Day 5 was again selected as the optimum time point to generate a one-way ANOVA, but this was restricted to varieties D, E, GR, N, T and V in SD due to contamination.

Table 11. Root and shoot ratio between days 4-6 of fourteen varieties in LD

Variety	Day 4	Day 5	Day 6
ACON	0.91	0.82	0.72
AKIN	1.17	0.84	0.75
D	1.07	0.74	0.71
E	1.06	1.32	0.77
GR	0.58	0.78	0.59
GU	0.82	1.82	0.69
K	0.89	0.78	0.70
MJ	0.94	0.52	0.79
MO	0.83	0.81	0.70
N	0.93	0.81	0.69
OAK	0.96	0.74	0.63
R	0.90	0.76	0.69
T	1.15	0.82	0.74
V	1.10	0.87	0.75

3.2.4 Statistical Analysis: one-way ANOVA LD

One-way ANOVA was performed and showed statistical significance (p-value 0.00) between all the varieties for roots and shoots. A Tukey test was performed to identify where the differences occurred between the varieties (Appendix B) and Levene's tests showed homogeneity of variance.



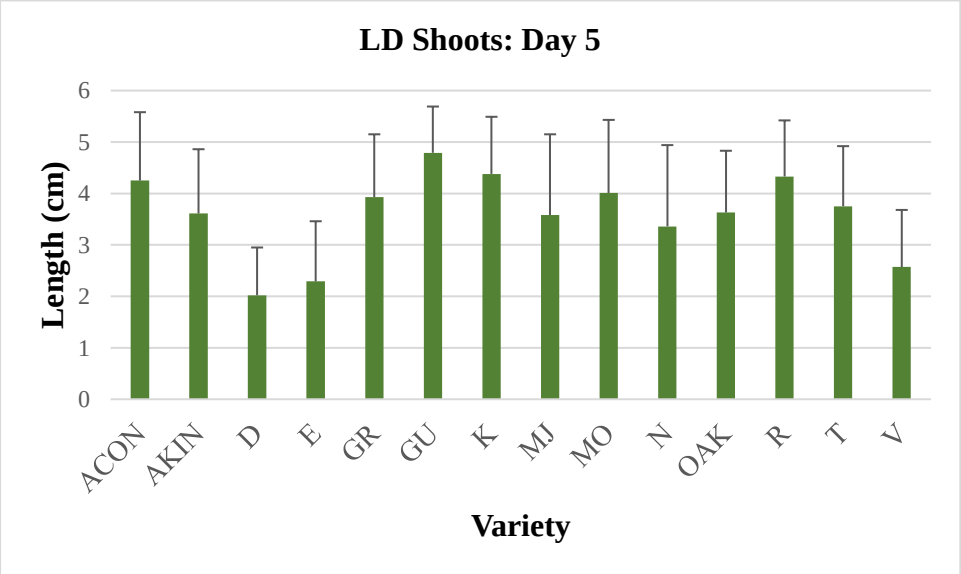


Figure 10. Showing the root and shoot length of the fourteen varieties at day 5 in LD. Upper panel: LD Roots. Lower panel: LD Shoots. Error bars represents standard deviation. Three experiments provided two biological replicates for the one-way ANOVA analysis and the Tukey test. For this figure, statistical differences with compact lettering were not undertaken due to space restriction but p-values of each variety are shown in Table 12.

LD root top performance were ACON and K with root length >3.5cm. AKIN, GR, MO, R, GR, and the two non-PPI varieties E and T. LD had root length ~3cm. GU, N and OAK, followed

by MJ and V had root length >2cm, the latter a non-PPI variety, and the lowest performer was D ~1.5cm

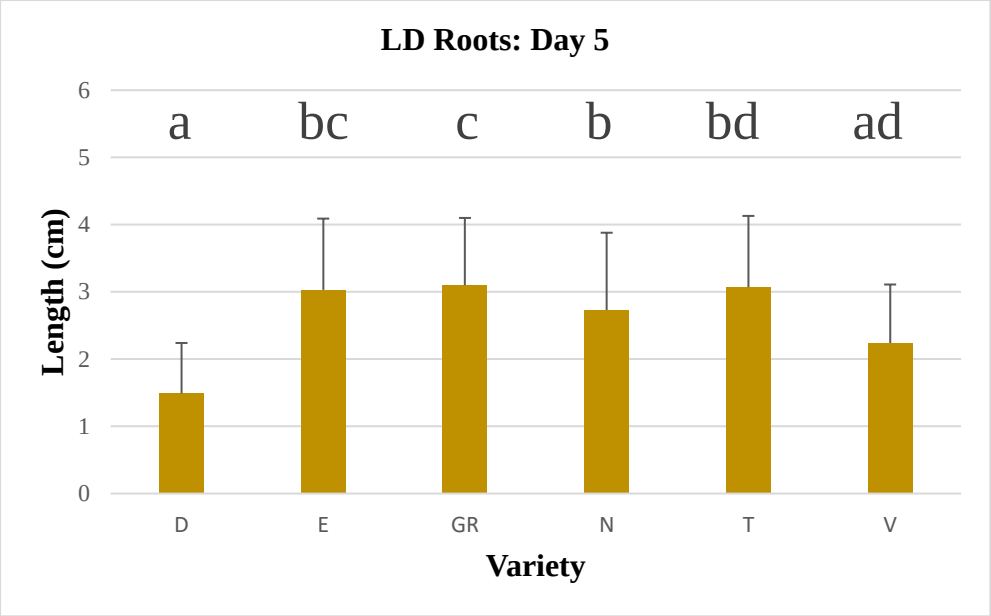
LD shoot top performer was GU with shoot length~5cm. ACON, K and R had shoot length >4cm. AKIN, GR, MJ, N, OAK and T had shoot length >3.5cm. The lowest performer was again D <2cm, followed by E and V, the latter two again being non-PPI varieties. See also Figs.11 and 12 for statistical differences between varieties D, E, GR, N, T and V in LDs and SDs.

There was statistical significance between roots and shoots. For LD, there was statistical significances (p value 0.00) between D and varieties E, GU, K, MJ, MO, N, OAK, R, T and V. *In-vitro* performance matched the PPI, except for N, which did not achieve a high performance in the experiments to match its PPI ranking.

3.2.5 Statistical Analysis: one-way ANOVA LD and SD

Due to contamination, only six varieties from SD could be selected for analysis to compare with LD (Fig.11). These were D, E, GR, N, T and V, with 2 biological replicates from experiments 1 and 2.

Root and shoot lengths in SD were considerably shorter than in LD, which was to be expected in a shorter photoperiod, but which was the opposite from results in Section 1 (Discussion, Section 4.4.1).



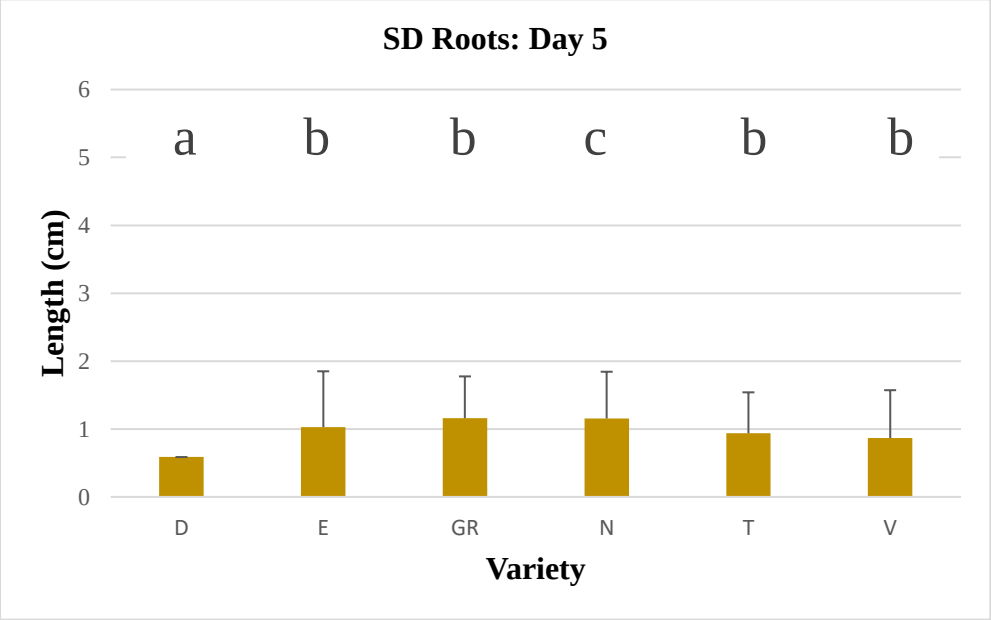
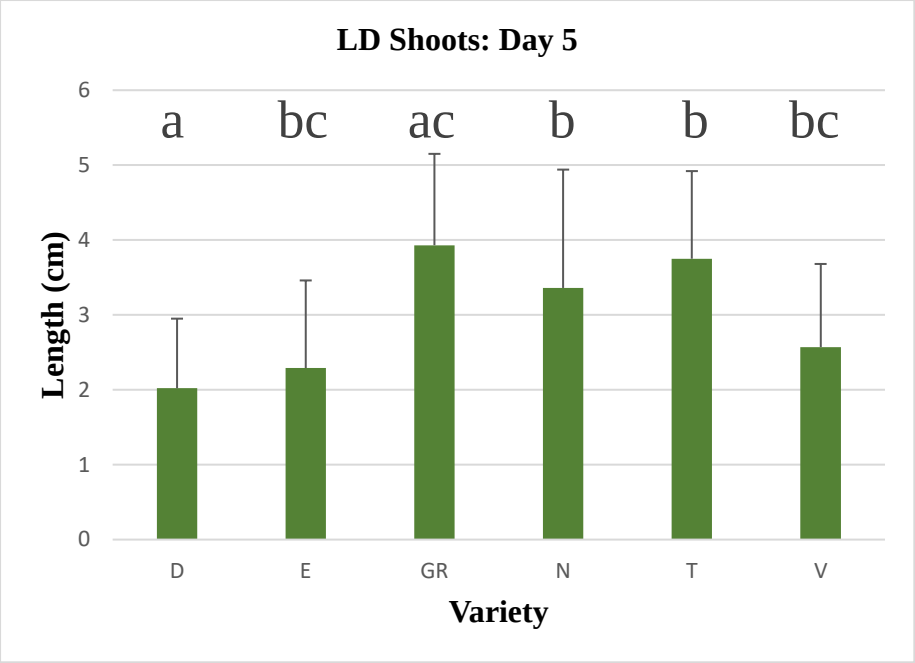


Fig.11. One-way ANOVA for roots, showing the statistical significance between varieties D, E, GR, N, T and V in LD and SD at day 5. Upper panel: LD roots. Lower panel: SD roots. Error bars represent standard deviation. Statistical significance is represented with different letters. For statistically significant one-way ANOVA p-values in LD, refer to Table 11, and to Table 12 for SD.



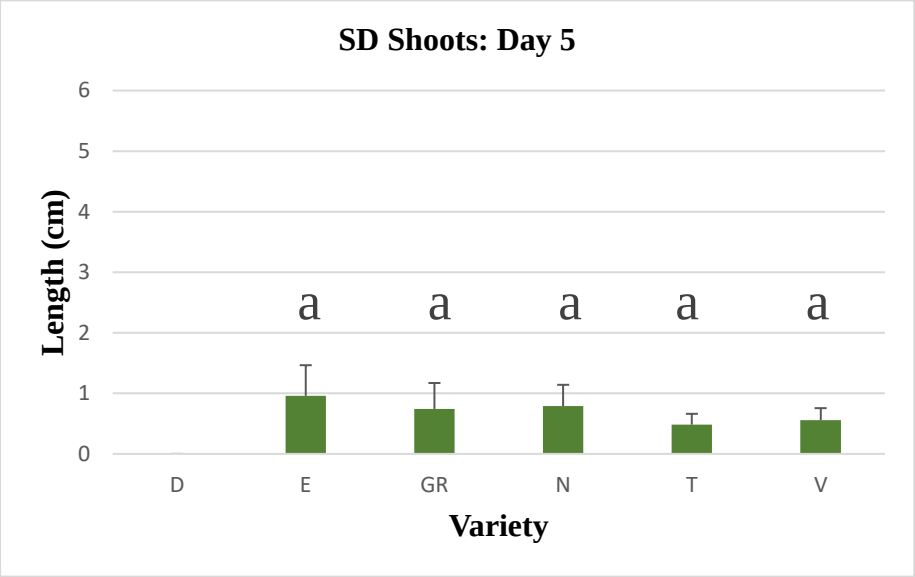


Fig.12. One-way ANOVA for shoots, showing the statistical significance between varieties D, E, GR, N, T and V in LD and SD at day 5. Upper panel: LD shoots. Lower panel: SD shoots. Error bars represent standard deviation. There was no statistical significance between the SD shoots. For statistically significant one-way ANOVA p-values in SD, refer to Table 12.

In LD roots, E, GR and T ~3cm showed a greater response to the extended photoperiod. D was the poorest performer, but had no statistical difference from V. There was also no statistical difference between E, N, and T, and no statistical difference between E and GR.

In LD Shoots, the top performer was GR and N, followed by E, T and V. There was no statistical difference between E, GR, T and V. The lowest performer was again D, and together with N were statistically different from the rest of the group.

In SD roots, all varieties except D grew ~1cm, and the top performers were E, GR, and N. There was no statistical difference between E, GR, T and V.

In SD shoots, the lowest performance was again D, which had no growth at all by day 5 and was removed from statistical analysis. T had lower performance than in LD. The highest performing varieties were E, GR, and N. There was no statistical difference between the varieties.

In conclusion, in LD, shoots were more advantaged by the extended photoperiod than roots.

In SD, shoots were affected by the shorter photoperiod than roots, with D failing to germinate by day 5.

Table 12. Summary table for use with Figs 11 and 12, being the results of the SD root one-way ANOVA showing those varieties with statistically significant relationships (there was no statistical significance in shoots).

SD ROOTS		
Variety	Comparison	P value
D	E, N, T, V	0.000
	GR	0.018
E	GR	0.006
GR	T	0.002
T	V	0.034

To avoid bias in the results, contaminated plates were removed from statistical analysis and only six varieties of the fourteen were assessed.

3.2.6 Root and shoot analysis LD and SD

Root and shoot ratios were calculated for comparison between LDs and SDs. Due to contamination, only the root and shoot ratio for day 5 could be calculated as a comparison with LD with the six varieties D, E, GR, N, T and V (Table 13). This showed that the ratio increased for varieties GR, N, T and V under SDs, except for E, and D, the latter with no shoot growth in SD.

Table 13. Root and shoot ratio of varieties D, E, GR, N, T and V at day 5 in LD and SD

Variety	LD	SD
D	0.74	0.00
E	1.32	1.07
GR	0.78	1.56
N	0.81	1.47
T	0.82	1.96
V	0.87	1.55

3.2.7 Summary of results

Section 1 showed a correlation between the *in-vitro* protocol of four varieties with the field-based PPI, except for K (which was also a high performer in Section 2, despite coming from different seed stock). It was also observed that in terms of photoperiod dependency, SD compared with LD in both root and shoot length and weights, which was not to be expected. C was shown to be less dependent upon photoperiod than AG or K, but statistically significantly different, being much shorter in both LDs and SDs. Experiment 8 showed increasing light levels from 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to 168$\mu mol \cdot m^{-2} \cdot s^{-1}$ increases root length, reduces shoot length by ~25%, but does not affect biomass.

Section 2 also showed correlation between the *in-vitro* protocol with the 11 varieties chosen from the PPI except for K as in Section 1. It was observed that seedlings growing under LDs developed longer roots and shoots and displayed an increase in biomass when compared with SD-grown seedlings, as to be expected from established observations.

This research also revealed that with the non-PPI varieties, E developed long roots and shoots *in-vitro* despite being described as having a dwarf phenotype in the field. Variety T displayed longer root and shoot development when grown under LD, but significantly underperformed in SD. especially roots. This research would allow rapid elucidation of photoperiodic regulation of perennial ryegrass early development for transfer to the field. Molecular analysis was undertaken to gain insight into the genetic mechanisms behind photoperiod regulation.

3.3 Section 3. Molecular Analysis

3.3.1 Existing ryegrass database sequences

For gene analysis, a search was undertaken for ryegrass sequences that had already been published and validated, relating to those genes known to be involved in either circadian or photoperiod regulation. Orthologues from *Oryza sativa (Os)*, *Arabidopsis thaliana (At)*, and the grass functional model species *Brachypodium distachyon (Bradi)* were BLAST searched using the NCBI and PLAZA gene bank databases to check for sequences showing high homology with ryegrass. This facilitated the generation of primer sequences for potential photoperiodic and circadian genes, as well as reference genes for correlation of gene expression when undertaking RT-PCR analysis (Table 15). The aim of this research was to establish a reliable protocol for analysis of gene expression in perennial ryegrass varieties grown under different photoperiods. These results could then be correlated with the analysis of plant growth responses such as root/shoot length and begin to provide some novel insights into circadian regulation.

3.3.2 Preliminary time course and RNA extraction

To combine previous phenotypic analysis with gene expression, an experiment was designed to collect *in vitro*-grown seedlings at day 5 at time points ZT3 and ZT9 (ZT = *Zeitgeber*, a 'time-giving' exogenous signal, here refering to light). The varieties tested were AG, AM, C and K. RNA for further analysis, was ascertained by a regular agarose gel (Fig.13).

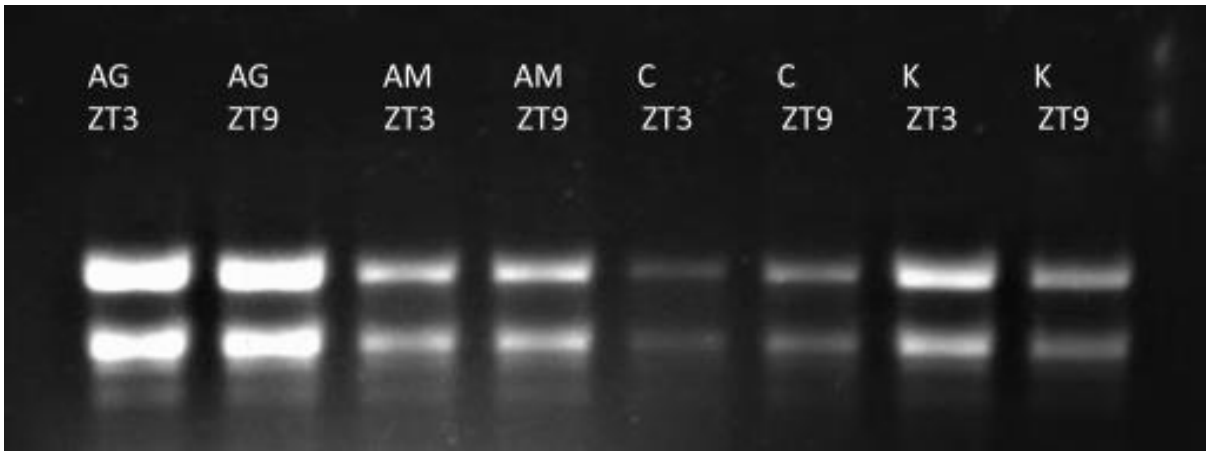


Fig.13. RNA profile of varieties AG, AM, C and K in LD. The RNA of AG and K was extracted using the Qiagen extraction kit, the RNA of AM and C was extracted using the Bioline kit (see Materials and Methods, Section 2.3).

Results of the RNA extraction showed strong expression of the four varieties collected at the two different time points. Despite good expression of AM, this was excluded from further study due to the previous inconsistencies noted regarding germination. The quality of the RNA was also assessed through spectrophotometry (Table 14). As real time PCR analysis requires cDNA synthesis from a preliminary 1µg of total RNA, we could confirm that this extraction method provided an adequate RNA concentration for further experiments. It was observed that the Qiagen Plant RNeasy kit provided higher yields than the Bioline.

Table 14: RNA concentration of varieties AG, AM, C and K in LD at time points ZT3 and ZT9, determined by Nanodrop analysis using Qiagen and Bioline RNA extraction kits.

Cultivar	ZT	ng/µL	RNA extraction kit
AG	ZT3	652.7	Qiagen
AG	ZT9	567.3	Qiagen
AM	ZT3	291.1	Bioline
AM	ZT9	312.3	Bioline
C	ZT3	110.5	Bioline
C	ZT9	180.4	Bioline
K	ZT3	332.6	Qiagen
K	ZT9	402	Qiagen

3.3.3 Time course cDNA synthesis and regular PCR

After confirming RNA expression levels, cDNA synthesis protocols were tested to ensure gene expression by RT-PCR would be succesful. In this preliminary experiment varieties AG, C and K were tested, with samples collected at ZT3 and ZT9, then placed into the -80°C storage (Fig. 13. Materials and Methods 2.4).

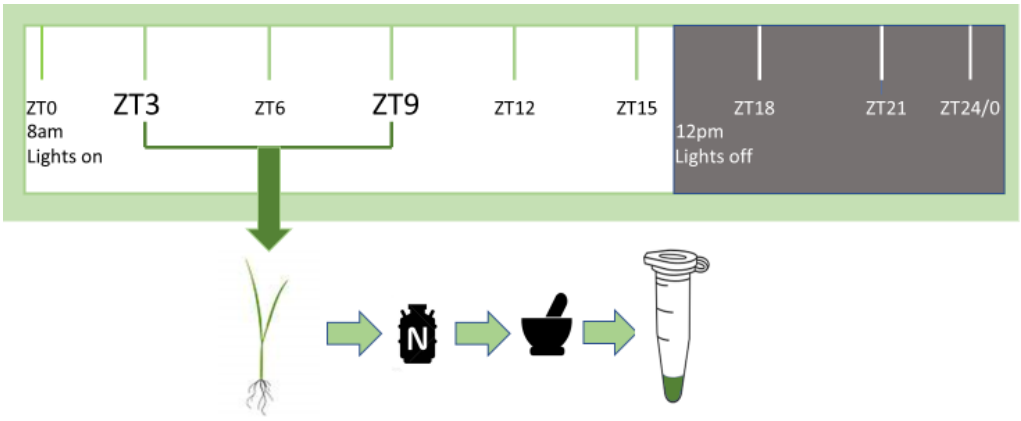


Fig14. Schematic showing time course and collection points ZT3 and ZT9 of AG, C and K. ~10 seedlings provided ~0.5mL of ground material after being immersed in liquid nitrogen.

cDNA synthesis was undertaken, and by combining the four varieties, an initial semi-quantitative RT-PCR assay was performed to test 11 primer combinations (amplifying between 150-200 bp sequences from the candidate genes) (Table 15).

A fragment of the expected size was detected in most of the candidate genes. In *LpFT3q1* and *LpFT3q2* the fragment size was higher than expected (of 170bp and 214bp respectively) This result could be due to unspecific binding or genomic DNA contamination, although this RNA extraction protocol included a DNAse digestion step. Only *LpCOq2* amplified a fragment of the expected size at ZT9, probably correlated with the specific pattern on *LpCO* accumulation (Martin et al., 2004). Previous results for *LpGIq1* and *LpGIq2* primers were confirmed in a previous assay as being good quality ~200bp primers suitable for qPCR (O'Sullivan, 2019).

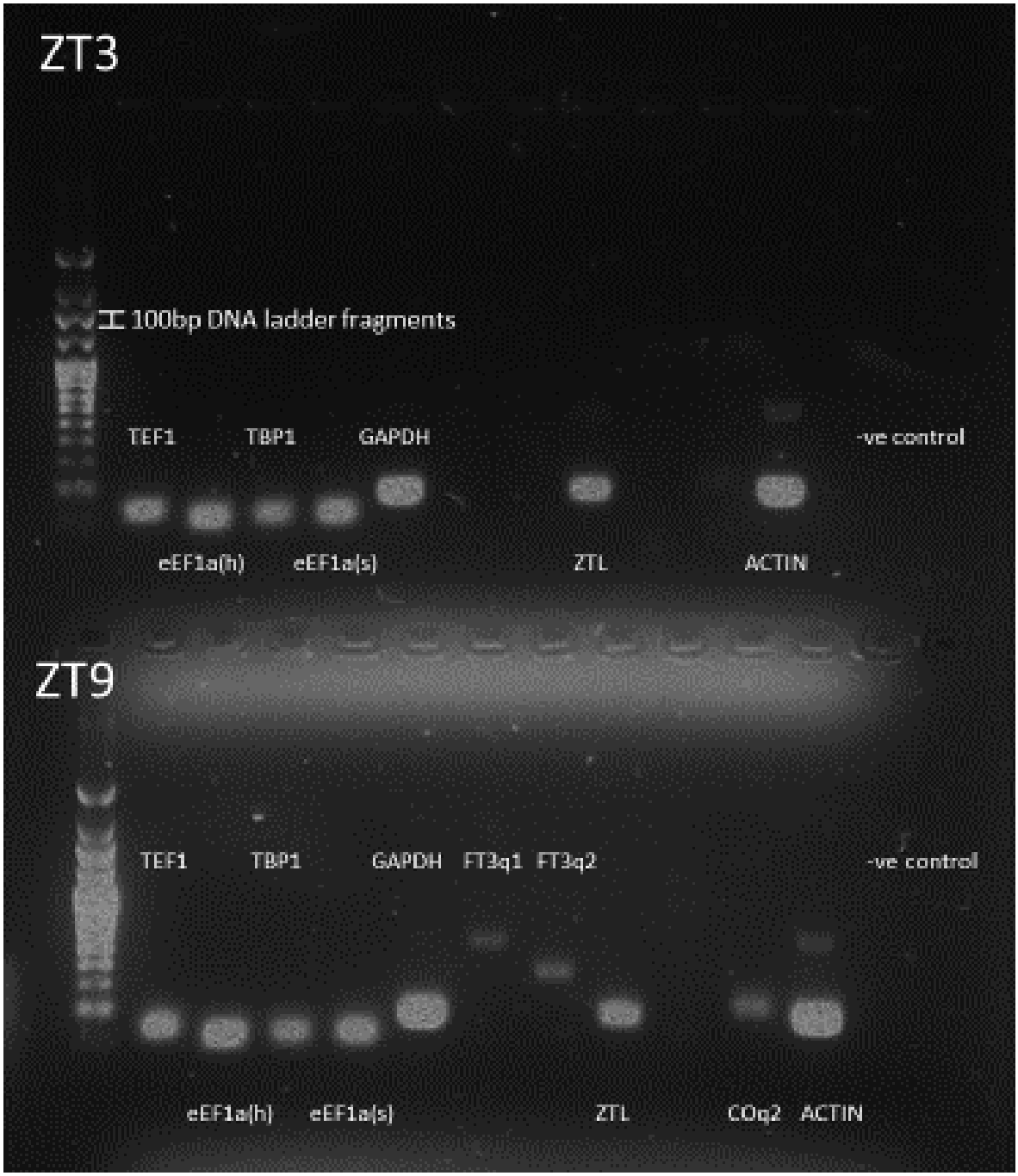


Fig.15. Semi-quantitative RT-PCR to test candidate primer pairs for qPCR analysis. cDNA used was combined from AG, C and K varieties at ZT3 and ZT9 time points. All primers were designed for *Lolium perenne* (Lp). Left to right: *LpTEF1, LpeEF1a(h), LpTBP1, LpeEF1a(s), LpGAPDHq1, LpFT3q1, LpFT3q2, LpZTLq1, LpCOq2* and *LpACTINq1*. DNA ladder on the left shows 100bp fragments. All primers except for *LpFT3q1* and *LpFT3q2* show 180-220bp fragments.

Table 15: Candidate genes and primer sequences selected and tested for efficiency with *L.perenne*. LpCOq2F/R, LpGIq1F/R, LpGIq2F/R and LpACTINq1F/R results obtained from earlier research within this laboratory.

Gene	Primer Sequence	Reference
LpTEFq1F	5'-CGT GTG ATC GAG AGG TTT GA-3'	Huang et al., 2014
LpTEFq1R	5'-CGA ATT TCC AGA GGG CAA TA-3'	,,
LpeEF1a(h)q1F	5'-ATG TCT GTT GAG CAG CCT TC-3'	Lee et al., 2010
LpeEF1a(h)q1R	5'-GCG GAG TAT ATA AAG GGG TAG C-3'	,,
LpTBP1q1F	5'-TGC TTA GTT CCC CTA AGA TAG TGA-3'	Lee at al., 2010 Huang et al., 2014
LpTBP1q1R	5'-CTG AGA CCA AAC ACG ATT TCA-3'	,,
LpeEF1a(s)q1F	5'-CCG TTT TGT CGA GTT TGG T-3'	Lee et al., 2010
LpeEF1a(s)q1R	5'-AGC AAC TGT AAC CGA ACA TAG C-3'	,,
LpGAPDHq1F	5'-CTT GCT CCT CTT GCT AAG GTT A-3'	Martin et al., 2004
LpGAPDHq1R	5'-CTT GGC AGC TCC AGT GCT-3'	,,
LpFT3q1F	5'-TCC GCA CCA CTA ACC TCA GG-3'	Skot et al., 2011
LpFT3q1R	5'-AGG TTG GGA TCG CTT GGA CTT-3'	,,
LpFT3q2F	5'-AAG TCC AAG CGA TCC CAA CCT-3'	Skot et al., 2011
LpFT3q2R	5'-GTC CCT GGT ATT GAA GTT CTG GC-3'	,,
LpZTLq1F	5'-AGT TCC AGG GTG ATC TGC TG-3'	Huang et al., 2014
LpZTLq1R	5'-TCA CAA CTT CCT TTG CCA CA-3'	,,

LpCOq1F	5’-AGA GCA GGC TGC ATC GAT GA-3’	Martin et al., 2004	
LpCOq1R	5’-TCC ATG GAC AGA GAG GCC AA-3’	,,	
LpCOq2F	5’-GGA ATA TGC AGA GAA GGA AGG GAG C-3’	This study	
LpCOq2R	5’-GCA GAT CTA CCA CCG TAT TGT CTG GT-3’	,,	
LpGIq1F	5’ -TCC ACC CCT AGA TGG GCT GTT 3’	O’Sullivan McCormack	2019 2019
LpGIq1R	5’ -TGA AAC AAG CGA GCG TAT GGT TCA AGA 3’	,,	
LpGIq2F	5’ -TGG GCT CCA GAT GCA CTT GAT 3’	,,	
LpGIq2R	5’- ACC AGC TCT CAT AGA CAT TGC AGT TC 3’	,,	
LpACTINq1F	5’- GTT GGA CTC TGG TGA TGG TGT GA 3’	,,	
LpACTINq1R	5’ – GTC TCT GAC AAT TTC CCG CTC AGC 3’	,,	

3.3.4 RT-PCR

For RT-PCR analysis, fluorescence is to be above an arbitrary threshold to determine the Ct value, and SYBR Green was chosen as the fluorescent reporter. Standard curves can be generated from a dilution series (1:4, 1:16, 1:64) constructed from reference samples of the primers to be tested, and results normalised using a housekeeping gene. Here, all the cDNAs of the four varieties AG, AM, C and K were combined, and a series of dilutions at 1:4 to calculate primer efficiency by q RT-PCR.

Table 16 shows the results for three primer pairs tested: *GIq1, GIq2* and *ACTINq1* (efficiency is shown as %). Results show that *GIq2* could be used further to determine the transcript levels of *GIGANTEA*. However, a suitable housekeeping candidate gene would need to be optimised.

Table 16. Results for the three primer pairs tested: *GIq1, GIq2* and *ACTINq1* (efficiency is shown as %)

Primer	Efficiency (%)
GIq1	73.246107
GIq2	95.2979454
ACTINq1	48.7352107

3.3.5 Future research

Plant material was collected at six time points from varieties AG, K, R and V, from both LD and SD photoperiods, over a 24hour period time course and placed into -80°C (Fig.16).

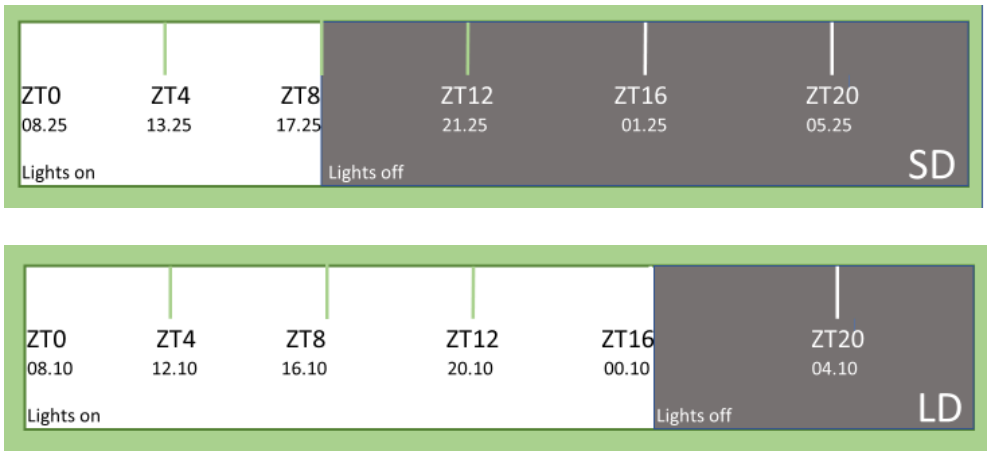


Fig.16. Time course showing both ZT and actual time collection points of varieties AG, K, R and V, from LD and SD photoperiods.

Research was to be finalised by utilising the primers established in Table 15 to track circadian gene expression through LD and SD, to gain an understanding of how the four varieties of ryegrass are responding to photoperiod. This work was brough to a halt by the closure of the school due to COVID-19.

4. Discussion

4.1 Experimental design

Performance was observed of *in-vitro* of ryegrass varieties AG, AM, C and K, followed by ACON, AKIN, D, E, GR, GU, K, MJ, MO, N, OAK, R, T and V (Results, Section 1 and 2) There is no established methodology for assessing *in vitro* early growth of perennial ryegrass, but by selecting day 5 there was sufficient length and biomass for statistical analysis, and to provide enough material for RNA extraction (Barrero et al., 2012).

4.2 Germination rates

Seed dormancy is controlled by both the genotype and the environmental cues it receives such as humidity, the quality of light (red and far-red) and nutrient availability; hormonal regulation can then fine tune seed germination to these environmental conditions (Carrera-Castano et al, 2020). To synchronise germination, a 7-day stratification protocol from *Brachypodium distachyon* was utilised which proved to be highly efficient, as this species has similar seed morphology to ryegrass and is a recognised model species in the developmental biology of temperate grasses (Betekhtin et al., 2020, Vogel and Hill, 2008).

Germination encompasses a range of physiological events, from imbibition to radicle protrusion, and seeds displaying characteristics such as high germination percentages and

cotyledon emergence are important as these will correlate with yield and actual field performance (Finch-Savage and Bassel, 2016). Whilst for commercial applications, there are emerging technologies such as phenotyping platforms for generating automated seed imaging (Colmer et al., 2020). in this research a stereomicroscope was utilised to assess radicle protrusion, which is an accurate proxy for frequency and speed of germination (Finch-Savage and Bassel, 2016).

Whilst the *in-vitro* germination results from Section 1 surprisingly showed that germination rates in SD's correlated with LD's, there is some research showing that photoperiod does not affect germination rates of grasses (Lin et al., 2018, Martins et al., 2017). The use of growth chambers programmed to 22°C was not a limiting factor with germination as moisture levels were adequate (see discussion on temperature in Section 4.4.1 below). In Section 1, Experiments 2 and 3, the palea and lemma were removed, which significantly improved germination rates (Vogel and Hill, 2008, but this procedure does not mimic the seeds' natural environment and was subsequently removed.

4.3 Seed sterilisation and contamination.

Like many cool season grasses, *L. perenne* is often affected with endophytic fungi (such as *Neotyphodium lolii*), and saprophytes associated with harvesting seed in wet weather or if lodging has occurred (Bajaj et al., 2006, Wiewiora, 2014). Whilst in the field, the relationship between ryegrass and endophytes has been shown to be mutualistic, the absence of fungal growth is an important determinant in the success or failure of *in-vitro* plant growth (Clay et al., 1987). A wide range of liquid phase sterilising agents have been previously employed *in-vitro*, such as sulphuric acid, mercuric chloride, sodium hypochlorite and ethanol in dilutions of up to 90% (Josekutty et al., 2011). In this research, some varieties were more prone to

contamination, which could be due to the presence of different fungal and bacterial species (Wiewiora, 2014), and a more stringent sterilisation procedure using 70% ethanol was employed which did not harm the integrity of the seeds metabolism, nor affect germination rates (Materials and Methods, Section 2.1).

4.4 Early growth and plant development

4.4.1 Section 1

In the field, the root structure of ryegrass is fibrous and grows adventitiously from underground nodes, its development influenced by dynamic factors such as water and nutrient availability, as well as soil structure. (Humphreys et al., 2018). Such constraints are removed when growing *in-vitro* as the plant has sufficient nutrient requirements and, unlike in the field, the roots are also exposed to light. Phototropic responses in roots have been recorded since the 19th century, and work in *Arabidopsis* has shown that light exposure to the roots can change the entire plant physiology with multiple effects (Yokawa et al., 2014). As roots *in vitro* were exposed to light due to clear growing media, this may have affected phenotypical development (and may not provide field relevant growing conditions for ryegrass) The plants response to the deviating microclimatic conditions within the growth chambers may affect comparison with field grown plants (Shukla et al., 2020). Circadian gene expression maintains different rhythms within specific plant organs (Greenwood et al., 2019, Ma et al., 2019), but this research did not differentiate between root and shoot material when harvesting for gene expression analysis. This analysis could be done in future studies.

In Section 1, both the primary and seminal root structures were measured. For Section 2, the primary root was measured. Within monocots, both these root types do not persist and are replaced by crown and brace roots which facilitates the rapid formation of a grass sward (Del

Bianco and Kepinski, 2018), but these measurements provided a good proxy for establishing the length and rate of growth for the varieties under analysis (Results, Tables 6 and 11).

The plants response to exogenous stimuli, may have resulted in the different root/shoot lengths between the 2 assays, which were undertaken at different times of the year. In section 1, measurements were taken from mid march to mid June, correlating with peak growth for ryegrass in the field environment. Assays in Section 2 were undertaken in late November to early December, correlating with its dormancy period. For example, the results from Assay 1 for Kerry at Day 5 in LD, show a decreased root and shoot length in comparison to the results for Assay 2 despite the same *in-vitro* environmental conditions, which could be developed in future research.

Section 1 showed that varieties AG, C and K in SDs had longer roots and shoots and increased fresh and dry weights when compared with LD's. This contradicted the literature both from *in-vitro* and field studies, which have shown that under LDs, additional photosynthate generated by more hours of light exposure are invested in more growth, increasing Dry Matter (DM) weights in the summer (LD) than in the spring and autumn (SD) (Hurtado-Uria et al., 2013). Whilst there is some literature on ryegrass suggesting that growth in LDs and SDs may be comparable, or even longer under SD's (Adams and Langton, 2005), research here suggests that there may have been a problem with the seed stock or analysis. The role of temperature could also be considered; in the field, shoot length increases rapidly >5°C after cold hardening and vernalisation, but when temperatures reach >20°C the difference between LD and SD is less pronounced (Hay, 1990). This research was undertaken with the growth chamber at 22°C, and it has been shown that monocots adjust their growth more to temperature than light (Henriques et al., 2018).

Evolutionarily, the circadian clock-controlled response to SD's has been shown to become enhanced as a competitive advantage for some varieties of ryegrass as a ruderal strategy (Wingler and Crowley, 2020), and at higher latitudes, plants are exposed to increased red to far-red ratios at the beginning and end of the day for up to 4 hours. The light spectrum within the growth chambers was consistent under LD and SD and did not correlate with natural photoperiodic exposure (Foster et al., 2018).

Analysis of photoperiodic responses in roots and shoots was expanded by increasing the number of varieties.

4.4.2 Section 2.

Section 2 showed that all fourteen varieties had longer root and shoots and increased fresh and dry weights in LD's which was to be expected from previous literature (Hurtado-Uria et al., 2013). Ryegrass is classified as a LD plant in terms of flowering time regulation, and photoperiod is an important regulatory component of this plant growth response (Wang and Forster, 2017). This research showed different varietal responses to LD's and SD's, with AG displaying less dependency on photoperiod whilst also a high ranked performer in the PPI; varieties which can efficiently adjust their photoperiodic response to both LD's and SD's enhances their adaptability to local climates.

It is important to note that ryegrass is categorised as either an early, intermediate, or late flowering variety based upon the date of ear emergence. As growth is limited by winter temperatures, varieties that provide early spring growth are economically important (Fig.1, Foster et al., 2018, Hurtado-Uria et al., 2013) and intermediate varieties such as AG which head later in May display a more versatile genotype that can increase biomass under SD's. Late

varieties such as K are displaying a phenotype which require LD, and this is more pronounced with varieties such as D, which did not germinate in SD due to its LD entrainment.

4.5 Gene Expression, photoperiod, and flowering

4.5.1 Circadian clock genes.

CONSTANS (CO), *GIGANTEA (GI)*, and *FLOWERING LOCUS T (FT3)* were confirmed as candidate primers. Research has already established that *CO* mediates day length responses between the core clock components and the floral integrators (Andres and Coupland, 2012), and its expression is promoted by the upstream circadian clock component *GI*, a conserved molecular scaffold which probably acts as an inductor protein (Gagic, 2007, Mishra and Panigrahi, 2015). *GI* primer efficiency was tested, and *LPGIq2* shown to be >90% (Table 16); several other candidate primers could also be tested by semi-quantitative RT-PCR and detect the transcripts of *CO*, even if we could not proceed with our molecular analysis by qPCR.

There are significant molecular differences between plants becoming entrained to either LD or SD photoperiods, and *LpCO* transcripts were found at the time point ZT9 in LD (Song et al., 2015). *CO* expression is stable in light and accumulates in the afternoon and evening in LD's (Matsubura, 2014). Previous findings showed that this flowering regulator can still be detected at the early growth stages of perennial ryegrass (Martin et al., 2004. O'Sullivan, 2019), and this high level of expression with light is necessary for floral induction (Jackson, 2009). Previous research has only been undertaken with soil grown plants either in the field, glass house or growth chamber, requiring substantially longer growth periods than this rapid screening protocol facilitates (Gagic, 2007).

Flowering response genes such as *FT* have been largely studied in rice, a SD plant, but it has also been shown within *Arabidopsis* that *FT3* is known to be expressed in SD, which was confirmed in results displaying weak expression of *LpFT3* in LD (Fjellheim et al., 2014).

4.5.2 Reference genes.

Previous research on housekeeping genes for *L. perenne* is not as established as for other grass species such as rice, but existing ryegrass literature suggested *EF1*, *GAPDH*, *TBP*, *TEF*, *ACTIN*, and *ZTL* as suitable candidates for reference genes (see P.17, Huang et al., 2014, Lee et al., 2010, Martin et al., 2008). *ZTL* also functions in the circadian complex as a blue light photoreceptor known to interact with *GI* and remove the expression of *CO* (Bendix et al., 2015, Jackson, 2009). Ideally, stable expression should occur across all varieties, and this research data uniquely showed all these regulators expressed as early as 5 days in perennial ryegrass seedlings (Fig.15). Primers *LpGADPH* and *LpeEF1a(h)* were strongly expressed which correlates with previous research suggesting these are very stable candidates to use for further research (Martin et al., 2008). It has also been shown that photoperiod affects phase shift changes in *LpCO, LpGI* and the transcription levels of *LpFT3*, and results here correlated with previous work showing that primers *LpTBP1* and *LpeEF1a(s)* were stably expressed with a similar profile (Gagic, 2007).

Understanding how to modulate the numerous genes involved in the flowering of ryegrass will be a key component in gaining adaptive strategies to deal with climate change (Velmurugan et al., 2016). Over 200 genes have been established to be involved in flowering time regulation within *Arabidopsis* alone (Peng et al., 2016) and the photoperiodic pathway within *L. perenne* will require more exploration by utilising the advances being made in cost efficient sequencing

technologies to provide linkage mapping and association studies, but function will still need to be categorised from field and *in-vitro* studies such as this one (Harper et al., 2019, Paina et al., 2016, Wang and Foster, 2017).

4.6 Conclusion

Ryegrass genotypes which respond to increased agricultural inputs such as irrigation and fertilisers have been the focus of most crop breeding strategies this last century, but it is becoming more important to develop new varieties of *Lolium perenne* which can accept reduced agricultural inputs, yet still provide the necessary increases in yield to meet population demand. Being able to quantify the photoperiodic response of ryegrass would allow for the selection of varieties that could be optimised for increasing valuable traits such as early spring growth, and to adapt to changing regional rainfall patterns (Hurtado-Uria et al., 2013, Wingler and Hennessy). This research has contributed to establishing a reliable protocol that can be repeated, and with a small amount of laboratory time, used to quickly assess ryegrass varieties that will take to the field far quicker than current methodologies.

This experimental design illustrates the potential for assessing ryegrass cultivars at the early growth phase by undertaking comparative experiments in LD and SD photoperiods. Even though the data from Section 1 showed some varieties performing better in SD than LD, it was still possible to assess varietal performance in relation to their response to both photoperiod and to the Pasture Profit Index with accuracy (Appendix A, Table 17). Future work *in-vitro* would allow full control of environmental parameters such as light, temperature and nutrients, and to explore plant stressors such as drought. Research on temperature was not undertaken, but as cold winter temperatures facilitate flowering, such research could assist in developing

genotypes more sensitive to flowering time regulation that could exploit anticipated warmer winter temperatures. The circadian clock complex is involved in critical responses to photoperiod, and further research into the circadian rhythm of *L. perenne* may facilitate the shortening or lengthening of the duration of growth to adapt to regional conditions and lead to more sustainable agricultural systems (Byrne et al, 2015).

5. References

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6. Appendices

6.1 Appendix A

Table 17. Showing the 2020 Pasture Profit Index (PPI) ranking of all the varieties tested from Teagasc and Goldcrop, showing the cost benefit value (€) of each variety.

Variety	Code	Spring Growth	Autumn Growth	Total Yield (Rank)	Quality (Rank)
Abergreen	AG	58	62	173	4
Abermagic	AM	44	70	161	19
Clanrye	C	27	15	73	-30
Kerry	K	42	35	108	-23
Astonconqueror	ACON	79	34	142	-4
Astonking	AKIN	64	22	114	-15
Drumbo	D	29	34	90	24
Esquire	E	n/a	n/a	n/a	n/a
Glenroyal	GR	25	40	96	-11
Gusto	GU	67	55	153	15
Kerry‡	K	42	35	108	-23
Majestic	MJ	35	36	97	-38
Moira	MO	118	49	188	-30
Nifty	N	80	57	187	-23
Oakpark	OAK	34	33	97	-8
Rosetta	R	85	40	150	-16
Talbot‡	T	n/a	n/a	n/a	n/a
Vigour‡	V	n/a	n/a	n/a	n/a

‡ non-PPI varieties

Table 18. Summary for use with Figs.10,11 and 12) root and shoot one-way ANOVA LD

LD SHOOTS Variety	Comparison	P value	LD ROOTS Variety	Comparison	P value
ACON	D, GR, N, V	0.000	ACON	D, GR, N, V	0.000
	MJ	0.002		MJ	0.001
AKIN	D	0.000	AKIN	D, GR, V	0.000
	GR	0.001		N	0.001
	V	0.009			
D	E, GU, K, MJ, MO, N, OAK, R, T, V	0.000	D	E, GU, K, MJ, MO, N, OAK, R, T	0.000
	V	0.024		V	0.001
E	D	0.000			
	GR	0.007	E	E, GR	0.006
	V	0.045			
GR	GU, K, MO, OAK, R	0.000	GR	GU, K, MO, OAK, R	
	T	0.009		T	0.002
GU	N, V	0.000	GU	N, V	0.000
K	N, V	0.000	K	MJ	0.037
	MJ	0.001		N, V	0.000
MJ	OAK	0.001	MJ	OAK	0.049
	R	0.002		R	0.026
	MO	0.008			
MO	N	0.003	MO	N	0.001
	V	0.000		V	0.000
N	OAK, R	0.000	N	OAK, R	0.000
OAK	V	0.000	OAK	V	0.000
R	V		R	V	0.000
			T	V	0.034

6.2 Appendix B

Table 19. One-way ANOVA for root and shoot of varieties AG, AM, C and K in LD

ROOTS LD. Dependent variable: Length (cm)

(I) Variety	(J) Variety	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
AG	C	2.42600*	.38798	.000	1.4923	3.3597
	K	1.05600*	.38798	.023	.1223	1.9897
C	AG	-2.42600*	.38798	.000	-3.3597	-1.4923
	K	-1.37000*	.38798	.002	-2.3037	-.4363
K	AG	-1.05600*	.38798	.023	-1.9897	-.1223
	C	1.37000*	.38798	.002	.4363	2.3037

SHOOTS LD. Dependent variable: Length (cm)

AG	C	1.63550*	.26484	.000	.9982	2.2728
	K	.88200*	.26484	.004	.2447	1.5193
C	AG	-1.63550*	.26484	.000	-2.2728	-.9982
	K	-.75350*	.26484	.017	-1.3908	-.1162
K	AG	-.88200*	.26484	.004	-1.5193	-.2447
	C	.75350*	.26484	.017	.1162	1.3908

Table 20. One-way ANOVA for root and shoot of varieties AG, AM, C and K in SD

ROOTS SD. Dependent variable: Length (cm)

AG	C	3.09300*	.27608	.000	2.4286	3.7574
	K	1.40050*	.27608	.000	.7361	2.0649
C	AG	-3.09300*	.27608	.000	-3.7574	-2.4286
	K	-1.69250*	.27608	.000	-2.3569	-1.0281
K	AG	-1.40050*	.27608	.000	-2.0649	-.7361
	C	1.69250*	.27608	.000	1.0281	2.3569

SHOOTS SD. Dependent variable: Length (cm)

AG	C	2.39050*	.23175	.000	1.8328	2.9482
	K	.82700*	.23175	.002	.2693	1.3847
C	AG	-2.39050*	.23175	.000	-2.9482	-1.8328
	K	-1.56350*	.23175	.000	-2.1212	-1.0058
K	AG	-.82700*	.23175	.002	-1.3847	-.2693
	C	1.56350*	.23175	.000	1.0058	2.1212

Table 21. Two-way ANOVA for root and shoot of varieties AG, AM, C and K in LD and SD

Roots. Dependent variable: Length (cm)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	244.389[a]	5	48.878	35.370	.000
Intercept	931.817	1	931.817	674.297	.000
Variety	193.836	2	96.918	70.133	.000
Photoperiod	28.429	1	28.429	20.572	.000
Variety * Photoperiod	22.125	2	11.062	8.005	.001
Error	157.538	114	1.382		
Total	1333.744	120			
Corrected Total	401.927	119			

a. R Squared = .608 (Adjusted R Squared = .591)

Shoots. Dependent variable: Length (cm)

Corrected Model	127.816[a]	5	25.563	34.981	.000
Intercept	585.769	1	585.769	801.570	.000
Variety	95.089	2	47.545	65.060	.000
Photoperiod	24.024	1	24.024	32.875	.000
Variety * Photoperiod	8.702	2	4.351	5.954	.003
Error	83.309	114	.731		
Total	796.894	120			
Corrected Total	211.124	119			

a. R Squared = .605 (Adjusted R Squared = .588)

Table 22. One-way ANOVA for root of the fourteen varieties in LD.

Root. Dependent variable: Length

(I) Variety	(J) Variety	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
ACON	AKIN	.50340	.29917	.920	-.5093	1.5161
	D	3.24813*	.29917	.000	2.2354	4.2609
	E	.94545	.29917	.095	-.0673	1.9582
	GR	2.13835*	.29917	.000	1.1256	3.1511
	GU	.37200	.29917	.994	-.6407	1.3847
	K	.33607	.29917	.998	-.6767	1.3488
	MJ	1.37825*	.29917	.001	.3655	2.3910
	MO	.51032	.29917	.912	-.5024	1.5231
	N	1.84865*	.29917	.000	.8359	2.8614
	OAK	.36377	.29917	.995	-.6490	1.3765
	R	.30367	.29917	.999	-.7091	1.3164
	T	.86235	.29917	.192	-.1504	1.8751
	V	1.91295*	.29917	.000	.9002	2.9257
AKIN	ACON	-.50340	.29917	.920	-1.5161	.5093
	D	2.74473*	.29917	.000	1.7320	3.7575
	E	.44205	.29917	.971	-.5707	1.4548
	GR	1.63495*	.29917	.000	.6222	2.6477
	GU	-.13140	.29917	1.000	-1.1441	.8813
	K	-.16732	.29917	1.000	-1.1801	.8454
	MJ	.87485	.29917	.174	-.1379	1.8876
	MO	.00693	.29917	1.000	-1.0058	1.0197
	N	1.34525*	.29917	.001	.3325	2.3580
	OAK	-.13962	.29917	1.000	-1.1524	.8731
	R	-.19972	.29917	1.000	-1.2125	.8130
	T	.35895	.29917	.995	-.6538	1.3717
	V	1.40955*	.29917	.000	.3968	2.4223
D	ACON	-3.24812*	.29917	.000	-4.2609	-2.2354
	AKIN	-2.74473*	.29917	.000	-3.7575	-1.7320
	E	-2.30268*	.29917	.000	-3.3154	-1.2899
	GR	-1.10978*	.29917	.018	-2.1225	-.0970
	GU	-2.87612*	.29917	.000	-3.8889	-1.8634
	K	-2.91205*	.29917	.000	-3.9248	-1.8993
	MJ	-1.86987*	.29917	.000	-2.8826	-.8571
	MO	-2.73780*	.29917	.000	-3.7505	-1.7251

	N	-1.39947*	.29917	.000	-2.4122	-.3867
	OAK	-2.88435*	.29917	.000	-3.8971	-1.8716
	R	-2.94445*	.29917	.000	-3.9572	-1.9317
	T	-2.38578*	.29917	.000	-3.3985	-1.3730
	V	-1.33518*	.29917	.001	-2.3479	-.3224
E	ACON	-.94545	.29917	.095	-1.9582	.0673
	AKIN	-.44205	.29917	.971	-1.4548	.5707
	D	2.30268*	.29917	.000	1.2899	3.3154
	GR	1.19290*	.29917	.006	.1802	2.2056
	GU	-.57345	.29917	.816	-1.5862	.4393
	K	-.60937	.29917	.743	-1.6221	.4034
	MJ	.43280	.29917	.975	-.5799	1.4455
	MO	-.43513	.29917	.974	-1.4479	.5776
	N	.90320	.29917	.138	-.1095	1.9159
	OAK	-.58168	.29917	.800	-1.5944	.4311
	R	-.64177	.29917	.669	-1.6545	.3710
	T	-.08310	.29917	1.000	-1.0958	.9296
	V	.96750	.29917	.078	-.0452	1.9802
GR	ACON	-2.13835*	.29917	.000	-3.1511	-1.1256
	AKIN	-1.63495*	.29917	.000	-2.6477	-.6222
	D	1.10978*	.29917	.018	.0970	2.1225
	E	-1.19290*	.29917	.006	-2.2056	-.1802
	GU	-1.76635*	.29917	.000	-2.7791	-.7536
	K	-1.80227*	.29917	.000	-2.8150	-.7895
	MJ	-.76010	.29917	.384	-1.7728	.2526
	MO	-1.62802*	.29917	.000	-2.6408	-.6153
	N	-.28970	.29917	.999	-1.3024	.7230
	OAK	-1.77457*	.29917	.000	-2.7873	-.7618
	R	-1.83467*	.29917	.000	-2.8474	-.8219
	T	-1.27600*	.29917	.002	-2.2887	-.2633
	V	-.22540	.29917	1.000	-1.2381	.7873
GU	ACON	-.37200	.29917	.994	-1.3847	.6407
	AKIN	.13140	.29917	1.000	-.8813	1.1441
	D	2.87612*	.29917	.000	1.8634	3.8889
	E	.57345	.29917	.816	-.4393	1.5862
	GR	1.76635*	.29917	.000	.7536	2.7791
	K	-.03593	.29917	1.000	-1.0487	.9768
	MJ	1.00625	.29917	.053	-.0065	2.0190
	MO	.13832	.29917	1.000	-.8744	1.1511
	N	1.47665*	.29917	.000	.4639	2.4894

	OAK	-.00823	.29917	1.000	-1.0210	1.0045
	R	-.06833	.29917	1.000	-1.0811	.9444
	T	.49035	.29917	.934	-.5224	1.5031
	V	1.54095*	.29917	.000	.5282	2.5537
K	ACON	-.33607	.29917	.998	-1.3488	.6767
	AKIN	.16732	.29917	1.000	-.8454	1.1801
	D	2.91205*	.29917	.000	1.8993	3.9248
	E	.60938	.29917	.743	-.4034	1.6221
	GR	1.80227*	.29917	.000	.7895	2.8150
	GU	.03593	.29917	1.000	-.9768	1.0487
	MJ	1.04218*	.29917	.037	.0294	2.0549
	MO	.17425	.29917	1.000	-.8385	1.1870
	N	1.51258*	.29917	.000	.4998	2.5253
	OAK	.02770	.29917	1.000	-.9850	1.0404
	R	-.03240	.29917	1.000	-1.0451	.9803
	T	.52628	.29917	.892	-.4865	1.5390
	V	1.57687*	.29917	.000	.5641	2.5896
MJ	ACON	-1.37825*	.29917	.001	-2.3910	-.3655
	AKIN	-.87485	.29917	.174	-1.8876	.1379
	D	1.86988*	.29917	.000	.8571	2.8826
	E	-.43280	.29917	.975	-1.4455	.5799
	GR	.76010	.29917	.384	-.2526	1.7728
	GU	-1.00625	.29917	.053	-2.0190	.0065
	K	-1.04218*	.29917	.037	-2.0549	-.0294
	MO	-.86793	.29917	.184	-1.8807	.1448
	N	.47040	.29917	.952	-.5423	1.4831
	OAK	-1.01448*	.29917	.049	-2.0272	-.0017
	R	-1.07458*	.29917	.026	-2.0873	-.0618
	T	-.51590	.29917	.906	-1.5286	.4968
	V	.53470	.29917	.880	-.4780	1.5474
MO	ACON	-.51032	.29917	.912	-1.5231	.5024
	AKIN	-.00693	.29917	1.000	-1.0197	1.0058
	D	2.73780*	.29917	.000	1.7251	3.7505
	E	.43513	.29917	.974	-.5776	1.4479
	GR	1.62803*	.29917	.000	.6153	2.6408
	GU	-.13832	.29917	1.000	-1.1511	.8744
	K	-.17425	.29917	1.000	-1.1870	.8385
	MJ	.86793	.29917	.184	-.1448	1.8807
	N	1.33833*	.29917	.001	.3256	2.3511
	OAK	-.14655	.29917	1.000	-1.1593	.8662

	R	-.20665	.29917	1.000	-1.2194	.8061
	T	.35203	.29917	.996	-.6607	1.3648
	V	1.40263*	.29917	.000	.3899	2.4154
N	ACON	-1.84865*	.29917	.000	-2.8614	-.8359
	AKIN	-1.34525*	.29917	.001	-2.3580	-.3325
	D	1.39947*	.29917	.000	.3867	2.4122
	E	-.90320	.29917	.138	-1.9159	.1095
	GR	.28970	.29917	.999	-.7230	1.3024
	GU	-1.47665*	.29917	.000	-2.4894	-.4639
	K	-1.51258*	.29917	.000	-2.5253	-.4998
	MJ	-.47040	.29917	.952	-1.4831	.5423
	MO	-1.33833*	.29917	.001	-2.3511	-.3256
	OAK	-1.48488*	.29917	.000	-2.4976	-.4721
	R	-1.54498*	.29917	.000	-2.5577	-.5322
	T	-.98630	.29917	.065	-1.9990	.0264
	V	.06430	.29917	1.000	-.9484	1.0770
OAK	ACON	-.36377	.29917	.995	-1.3765	.6490
	AKIN	.13962	.29917	1.000	-.8731	1.1524
	D	2.88435*	.29917	.000	1.8716	3.8971
	E	.58168	.29917	.800	-.4311	1.5944
	GR	1.77458*	.29917	.000	.7618	2.7873
	GU	.00823	.29917	1.000	-1.0045	1.0210
	K	-.02770	.29917	1.000	-1.0404	.9850
	MJ	1.01448*	.29917	.049	.0017	2.0272
	MO	.14655	.29917	1.000	-.8662	1.1593
	N	1.48488*	.29917	.000	.4721	2.4976
	R	-.06010	.29917	1.000	-1.0728	.9526
	T	.49858	.29917	.926	-.5142	1.5113
	V	1.54918*	.29917	.000	.5364	2.5619
R	ACON	-.30367	.29917	.999	-1.3164	.7091
	AKIN	.19972	.29917	1.000	-.8130	1.2125
	D	2.94445*	.29917	.000	1.9317	3.9572
	E	.64178	.29917	.669	-.3710	1.6545
	GR	1.83467*	.29917	.000	.8219	2.8474
	GU	.06833	.29917	1.000	-.9444	1.0811
	K	.03240	.29917	1.000	-.9803	1.0451
	MJ	1.07458*	.29917	.026	.0618	2.0873
	MO	.20665	.29917	1.000	-.8061	1.2194
	N	1.54498*	.29917	.000	.5322	2.5577
	OAK	.06010	.29917	1.000	-.9526	1.0728

	T	.55868	.29917	.842	-.4541	1.5714
	V	1.60927*	.29917	.000	.5965	2.6220
T	ACON	-.86235	.29917	.192	-1.8751	.1504
	AKIN	-.35895	.29917	.995	-1.3717	.6538
	D	2.38578*	.29917	.000	1.3730	3.3985
	E	.08310	.29917	1.000	-.9296	1.0958
	GR	1.27600*	.29917	.002	.2633	2.2887
	GU	-.49035	.29917	.934	-1.5031	.5224
	K	-.52628	.29917	.892	-1.5390	.4865
	MJ	.51590	.29917	.906	-.4968	1.5286
	MO	-.35203	.29917	.996	-1.3648	.6607
	N	.98630	.29917	.065	-.0264	1.9990
	OAK	-.49858	.29917	.926	-1.5113	.5142
	R	-.55867	.29917	.842	-1.5714	.4541
	V	1.05060*	.29917	.034	.0379	2.0633
V	ACON	-1.91295*	.29917	.000	-2.9257	-.9002
	AKIN	-1.40955*	.29917	.000	-2.4223	-.3968
	D	1.33518*	.29917	.001	.3224	2.3479
	E	-.96750	.29917	.078	-1.9802	.0452
	GR	.22540	.29917	1.000	-.7873	1.2381
	GU	-1.54095*	.29917	.000	-2.5537	-.5282
	K	-1.57687*	.29917	.000	-2.5896	-.5641
	MJ	-.53470	.29917	.880	-1.5474	.4780
	MO	-1.40262*	.29917	.000	-2.4154	-.3899
	N	-.06430	.29917	1.000	-1.0770	.9484
	OAK	-1.54917*	.29917	.000	-2.5619	-.5364
	R	-1.60927*	.29917	.000	-2.6220	-.5965
	T	-1.05060*	.29917	.034	-2.0633	-.0379

*. The mean difference is significant at the 0.05 level.

Table 23. One-way ANOVA for shoots of the fourteen varieties in LD

Shoot. Dependent variable: Length

(I) Variety	(J) Variety	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
ACON	AKIN	.71418	.38095	.838	-.5754	2.0038
	D	3.57397*	.38095	.000	2.2844	4.8636
	E	.89287	.38095	.524	-.3967	2.1825
	GR	2.41012*	.38095	.000	1.1205	3.6997
	GU	-.13650	.38095	1.000	-1.4261	1.1531
	K	-.04537	.38095	1.000	-1.3350	1.2442
	MJ	1.65485*	.38095	.002	.3653	2.9444
	MO	.15890	.38095	1.000	-1.1307	1.4485
	N	1.76455*	.38095	.000	.4750	3.0541
	OAK	-.02508	.38095	1.000	-1.3147	1.2645
	R	.00177	.38095	1.000	-1.2878	1.2914
	T	.92507	.38095	.463	-.3645	2.2147
	V	2.19635*	.38095	.000	.9068	3.4859
AKIN	ACON	-.71417	.38095	.838	-2.0038	.5754
	D	2.85980*	.38095	.000	1.5702	4.1494
	E	.17870	.38095	1.000	-1.1109	1.4683
	GR	1.69595*	.38095	.001	.4064	2.9855
	GU	-.85067	.38095	.606	-2.1403	.4389
	K	-.75955	.38095	.770	-2.0491	.5300
	MJ	.94067	.38095	.434	-.3489	2.2303
	MO	-.55528	.38095	.974	-1.8449	.7343
	N	1.05037	.38095	.253	-.2392	2.3400
	OAK	-.73925	.38095	.802	-2.0288	.5503
	R	-.71240	.38095	.841	-2.0020	.5772
	T	.21090	.38095	1.000	-1.0787	1.5005
	V	1.48217*	.38095	.009	.1926	2.7718
D	ACON	-3.57397*	.38095	.000	-4.8636	-2.2844
	AKIN	-2.85980*	.38095	.000	-4.1494	-1.5702
	E	-2.68110*	.38095	.000	-3.9707	-1.3915
	GR	-1.16385	.38095	.126	-2.4534	.1257
	GU	-3.71047*	.38095	.000	-5.0001	-2.4209
	K	-3.61935*	.38095	.000	-4.9089	-2.3298
	MJ	-1.91913*	.38095	.000	-3.2087	-.6295
	MO	-3.41508*	.38095	.000	-4.7047	-2.1255
	N	-1.80943*	.38095	.000	-3.0990	-.5198

	OAK	-3.59905*	.38095	.000	-4.8886	-2.3095
	R	-3.57220*	.38095	.000	-4.8618	-2.2826
	T	-2.64890*	.38095	.000	-3.9385	-1.3593
	V	-1.37762*	.38095	.024	-2.6672	-.0880
E	ACON	-.89287	.38095	.524	-2.1825	.3967
	AKIN	-.17870	.38095	1.000	-1.4683	1.1109
	D	2.68110*	.38095	.000	1.3915	3.9707
	GR	1.51725*	.38095	.007	.2277	2.8068
	GU	-1.02937	.38095	.284	-2.3190	.2602
	K	-.93825	.38095	.438	-2.2278	.3513
	MJ	.76197	.38095	.766	-.5276	2.0516
	MO	-.73398	.38095	.810	-2.0236	.5556
	N	.87167	.38095	.565	-.4179	2.1613
	OAK	-.91795	.38095	.476	-2.2075	.3716
	R	-.89110	.38095	.528	-2.1807	.3985
	T	.03220	.38095	1.000	-1.2574	1.3218
	V	1.30347*	.38095	.045	.0139	2.5931
GR	ACON	-2.41012*	.38095	.000	-3.6997	-1.1205
	AKIN	-1.69595*	.38095	.001	-2.9855	-.4064
	D	1.16385	.38095	.126	-.1257	2.4534
	E	-1.51725*	.38095	.007	-2.8068	-.2277
	GU	-2.54662*	.38095	.000	-3.8362	-1.2570
	K	-2.45550*	.38095	.000	-3.7451	-1.1659
	MJ	-.75528	.38095	.777	-2.0449	.5343
	MO	-2.25123*	.38095	.000	-3.5408	-.9616
	N	-.64558	.38095	.916	-1.9352	.6440
	OAK	-2.43520*	.38095	.000	-3.7248	-1.1456
	R	-2.40835*	.38095	.000	-3.6979	-1.1188
	T	-1.48505*	.38095	.009	-2.7746	-.1955
	V	-.21378	.38095	1.000	-1.5034	1.0758
GU	ACON	.13650	.38095	1.000	-1.1531	1.4261
	AKIN	.85067	.38095	.606	-.4389	2.1403
	D	3.71047*	.38095	.000	2.4209	5.0001
	E	1.02937	.38095	.284	-.2602	2.3190
	GR	2.54662*	.38095	.000	1.2570	3.8362
	K	.09112	.38095	1.000	-1.1985	1.3807
	MJ	1.79135*	.38095	.000	.5018	3.0809
	MO	.29540	.38095	1.000	-.9942	1.5850
	N	1.90105*	.38095	.000	.6115	3.1906
	OAK	.11142	.38095	1.000	-1.1782	1.4010

	R	.13827	.38095	1.000	-1.1513	1.4279
	T	1.06157	.38095	.238	-.2280	2.3512
	V	2.33285*	.38095	.000	1.0433	3.6224
K	ACON	.04537	.38095	1.000	-1.2442	1.3350
	AKIN	.75955	.38095	.770	-.5300	2.0491
	D	3.61935*	.38095	.000	2.3298	4.9089
	E	.93825	.38095	.438	-.3513	2.2278
	GR	2.45550*	.38095	.000	1.1659	3.7451
	GU	-.09112	.38095	1.000	-1.3807	1.1985
	MJ	1.70022*	.38095	.001	.4106	2.9898
	MO	.20427	.38095	1.000	-1.0853	1.4939
	N	1.80992*	.38095	.000	.5203	3.0995
	OAK	.02030	.38095	1.000	-1.2693	1.3099
	R	.04715	.38095	1.000	-1.2424	1.3367
	T	.97045	.38095	.380	-.3191	2.2600
	V	2.24172*	.38095	.000	.9521	3.5313
MJ	ACON	-1.65485*	.38095	.002	-2.9444	-.3653
	AKIN	-.94067	.38095	.434	-2.2303	.3489
	D	1.91913*	.38095	.000	.6295	3.2087
	E	-.76197	.38095	.766	-2.0516	.5276
	GR	.75528	.38095	.777	-.5343	2.0449
	GU	-1.79135*	.38095	.000	-3.0809	-.5018
	K	-1.70022*	.38095	.001	-2.9898	-.4106
	MO	-1.49595*	.38095	.008	-2.7855	-.2064
	N	.10970	.38095	1.000	-1.1799	1.3993
	OAK	-1.67992*	.38095	.001	-2.9695	-.3903
	R	-1.65308*	.38095	.002	-2.9427	-.3635
	T	-.72977	.38095	.816	-2.0194	.5598
	V	.54150	.38095	.979	-.7481	1.8311
MO	ACON	-.15890	.38095	1.000	-1.4485	1.1307
	AKIN	.55528	.38095	.974	-.7343	1.8449
	D	3.41508*	.38095	.000	2.1255	4.7047
	E	.73398	.38095	.810	-.5556	2.0236
	GR	2.25123*	.38095	.000	.9616	3.5408
	GU	-.29540	.38095	1.000	-1.5850	.9942
	K	-.20427	.38095	1.000	-1.4939	1.0853
	MJ	1.49595*	.38095	.008	.2064	2.7855
	N	1.60565*	.38095	.003	.3161	2.8952
	OAK	-.18397	.38095	1.000	-1.4736	1.1056
	R	-.15713	.38095	1.000	-1.4467	1.1325

	T	.76618	.38095	.759	-.5234	2.0558
	V	2.03745*	.38095	.000	.7479	3.3270
N	ACON	-1.76455*	.38095	.000	-3.0541	-.4750
	AKIN	-1.05037	.38095	.253	-2.3400	.2392
	D	1.80943*	.38095	.000	.5198	3.0990
	E	-.87167	.38095	.565	-2.1613	.4179
	GR	.64558	.38095	.916	-.6440	1.9352
	GU	-1.90105*	.38095	.000	-3.1906	-.6115
	K	-1.80992*	.38095	.000	-3.0995	-.5203
	MJ	-.10970	.38095	1.000	-1.3993	1.1799
	MO	-1.60565*	.38095	.003	-2.8952	-.3161
	OAK	-1.78962*	.38095	.000	-3.0792	-.5000
	R	-1.76278*	.38095	.000	-3.0524	-.4732
	T	-.83947	.38095	.627	-2.1291	.4501
	V	.43180	.38095	.997	-.8578	1.7214
OAK	ACON	.02508	.38095	1.000	-1.2645	1.3147
	AKIN	.73925	.38095	.802	-.5503	2.0288
	D	3.59905*	.38095	.000	2.3095	4.8886
	E	.91795	.38095	.476	-.3716	2.2075
	GR	2.43520*	.38095	.000	1.1456	3.7248
	GU	-.11142	.38095	1.000	-1.4010	1.1782
	K	-.02030	.38095	1.000	-1.3099	1.2693
	MJ	1.67992*	.38095	.001	.3903	2.9695
	MO	.18397	.38095	1.000	-1.1056	1.4736
	N	1.78962*	.38095	.000	.5000	3.0792
	R	.02685	.38095	1.000	-1.2627	1.3164
	T	.95015	.38095	.416	-.3394	2.2397
	V	2.22142*	.38095	.000	.9318	3.5110
R	ACON	-.00177	.38095	1.000	-1.2914	1.2878
	AKIN	.71240	.38095	.841	-.5772	2.0020
	D	3.57220*	.38095	.000	2.2826	4.8618
	E	.89110	.38095	.528	-.3985	2.1807
	GR	2.40835*	.38095	.000	1.1188	3.6979
	GU	-.13827	.38095	1.000	-1.4279	1.1513
	K	-.04715	.38095	1.000	-1.3367	1.2424
	MJ	1.65308*	.38095	.002	.3635	2.9427
	MO	.15713	.38095	1.000	-1.1325	1.4467
	N	1.76278*	.38095	.000	.4732	3.0524
	OAK	-.02685	.38095	1.000	-1.3164	1.2627
	T	.92330	.38095	.466	-.3663	2.2129
	V	2.19458*	.38095	.000	.9050	3.4842

T	ACON	-.92507	.38095	.463	-2.2147	.3645
	AKIN	-.21090	.38095	1.000	-1.5005	1.0787
	D	2.64890*	.38095	.000	1.3593	3.9385
	E	-.03220	.38095	1.000	-1.3218	1.2574
	GR	1.48505*	.38095	.009	.1955	2.7746
	GU	-1.06157	.38095	.238	-2.3512	.2280
	K	-.97045	.38095	.380	-2.2600	.3191
	MJ	.72978	.38095	.816	-.5598	2.0194
	MO	-.76617	.38095	.759	-2.0558	.5234
	N	.83947	.38095	.627	-.4501	2.1291
	OAK	-.95015	.38095	.416	-2.2397	.3394
	R	-.92330	.38095	.466	-2.2129	.3663
	V	1.27128	.38095	.058	-.0183	2.5609
V	ACON	-2.19635*	.38095	.000	-3.4859	-.9068
	AKIN	-1.48217*	.38095	.009	-2.7718	-.1926
	D	1.37763*	.38095	.024	.0880	2.6672
	E	-1.30347*	.38095	.045	-2.5931	-.0139
	GR	.21378	.38095	1.000	-1.0758	1.5034
	GU	-2.33285*	.38095	.000	-3.6224	-1.0433
	K	-2.24172*	.38095	.000	-3.5313	-.9521
	MJ	-.54150	.38095	.979	-1.8311	.7481
	MO	-2.03745*	.38095	.000	-3.3270	-.7479
	N	-.43180	.38095	.997	-1.7214	.8578
	OAK	-2.22142*	.38095	.000	-3.5110	-.9318
	R	-2.19458*	.38095	.000	-3.4842	-.9050
	T	-1.27128	.38095	.058	-2.5609	.0183

*. The mean difference is significant at the 0.05 level.