A peer-reviewed version of this preprint was published in PeerJ on 11 September 2018.

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Förster L, Grant J, Michel T, Ng C, Barth S. 2018. Growth under cold conditions in a wide perennial ryegrass panel is under tight physiological control. PeerJ 6:e5520 https://doi.org/10.7717/peerj.5520

Growth under cold conditions in perennial ryegrass is under tight genetic control

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Background. Perennial ryegrass is a cool-season grass species from the family Poaceae and is widely cultivated in temperate regions because it exhibits rapid growth and establishment, and possesses high forage quality. The extension of the growing season in Ireland in spring and autumn is a breeding target to make farming more profitable since a grass-fed diet based on grazing is the cheapest way of nutrition for ruminants. **Methods.** Fifty-seven perennial ryegrass accessions were screened for their ability to grow under typical Irish spring conditions as taken from long term temperature records in controlled climate chambers. They were grown in low temperature (8°C/2°C day/night) and control conditions (15°C/8°C day/night) in three consecutive independent experiments. Fresh weight, height, chlorophyll content and electrolyte leakage were measured, and these parameters were used to rank plant performance under low temperature growth conditions. **Results.** The results showed that height, yield and electrolyte leakage are excellent measures for the impact of cold stress tolerance. Little variation in growth was seen under cold stress, but a wide variety of responses were observed under control conditions. **Discussion.** Our results suggest that cold stress is under tight genetic control. Interestingly, the various genotypes responded differentially to more amenable control conditions, indicating that a quick response to more amenable growth conditions is a better target for breeding programmes.

Growth under cold conditions in perennial ryegrass is under tight genetic control

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Abstract

Background. Perennial ryegrass is a cool-season grass species from the family Poaceae and is widely cultivated in temperate regions because it exhibits rapid growth and establishment, and possesses high forage quality. The extension of the growing season in Ireland in spring and autumn is a breeding target to make farming more profitable since a grass-fed diet based on grazing is the cheapest way of nutrition for ruminants. **Methods.** Fifty-seven perennial ryegrass accessions were screened for their ability to grow under typical Irish spring conditions as taken from long term temperature records in controlled climate chambers. They were grown in low temperature (8°C/2°C day/night) and control conditions (15°C/8°C day/night) in three consecutive independent experiments. Fresh weight, height, chlorophyll content and electrolyte leakage were measured, and these parameters were used to rank plant performance under low temperature growth conditions. **Results.** The results showed that height, yield and electrolyte leakage are excellent measures for the impact of cold stress tolerance. Little variation in growth was seen under cold stress, but a wide variety of responses were observed under control conditions. **Discussion.** Our results suggest that cold stress is under tight genetic control. Interestingly, the various genotypes responded differentially to more amenable control conditions, indicating that a quick response to more amenable growth conditions is a better target for breeding programmes.

Keywords

Cold stress, acclimation to cold stress, perennial ryegrass, genetic control

Introduction

Perennial ryegrass (*Lolium perenne* L.), a cool-season grass species from the family Poaceae with a self-incompatible and outcrossing nature (Cornish et al., 1980), is one of the most important perennial grasses worldwide (Wilkins, 1991). Perennial ryegrass is widely cultivated in temperate regions because it exhibits rapid growth and establishment, and it also possesses high forage quality (Casler & Duncan, 2003). Temperature has a major impact on the growth of temperate forage grasses, and soil temperature was identified as the main determinant of growth in the South of Ireland. Developmental factors also determine the growth of grass, and productivity is highest in late spring and early summer, declining later in the summer (Hurtado-Uria et al., 2013). Often there is a distinct peak of leaf extension in spring. These seasonal effects are demonstrated by transfer from cold into warm conditions in perennial and Italian ryegrass (Peacock, 1975; Parsons & Robson, 1980; Davies et al., 1989). Low rates of growth were observed at temperatures down to 0°C, whereas leaf elongation of perennial ryegrass increases strongly at temperatures above 5°C (Peacock, 1975; Brereton et al., 1985). However, there is little known to-date about genotypic differences in growth responses under cool conditions. Wilson (1975) demonstrated selection for genotypes within a variety of perennial ryegrass for low and high respiring genotypes at 8° C and 25° C, and Brereton and McGilloway (1999) studied varietal differences amongst eight varieties under natural cool Irish Spring and winter conditions. Cold or chilling stress results from temperatures cool enough (0 to 15°C) to induce injury without forming ice crystals in plant tissues whereas freezing stress induces injury at temperatures below 0°C. Plants from temperate climatic regions are considered to be chilling tolerant to variable degrees (Sanghera et al., 2011). Cold acclimatisation involves the remodelling of cells and tissues, and the reprogramming of metabolism and gene expression (Thomashow, 1999). Low temperature stress also inhibits various metabolic reactions resulting in altered phenotypic characteristics (Chinnusamy et al., 2007).

Limited solar radiation and short day length may limit photosynthesis during the winter months in temperate grasslands, resulting in source limitations. Once light conditions become more favourable, growth can be restricted by processes that inhibit cell division and expansion at low temperatures. Growth under these conditions becomes sink-limited (Wingler, 2015). Hormone signalling pathways play an important role in this regulation, in particular gibberellic acid (GA) signalling. GA also determines the growth of grass species, e.g. leaf extension (Stapleton &

Jones, 1987), and in the induction of the fructan-degrading enzyme, fructan exohydrolase to promote growth after defoliation (Morvan et al., 1997). Brassinosteroids may also contribute to growth by stimulating cell division and expansion (Fridman & Savaldi-Goldstein, 2013), and regulating GA production in *Arabidopsis* (Unterholzner et al., 2015). Additionally, brassinosteroid signalling has also been shown to be important in determining growth in grass species (Thole et al., 2012). Secondary signals such as abscisic acid (ABA) and reactive oxygen species (ROS) can also regulate responses to cold via calcium-based signalling systems. It has been observed that ROS accumulation in cells following exposure to various abiotic stresses appear to have a strong influence on cold regulation of gene expression (Lee et al., 2002).

The primary reactions of photosynthesis are temperature-independent and catalysed by photosystem I (PSI) and photosystem II (PS II) to trap light energy and transform it into redox potential energy through a combination of photophysical and photochemical processes, leading to charge separation. Temperature-dependent biochemical reactions convert this redox potential energy to stable reducing power in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH), the establishment of a *trans*-thylakoid ΔpH by the oxygen-evolving complex, and the plastoquinone pool of the intersystem electron transport to synthesise adenosine triphosphate (ATP) by chemiosmosis (Ensminger et al., 2006). Low temperatures can inhibit electron transport by increasing membrane viscosity through alterations in the biophysical properties of thylakoid lipids and decreasing the rates of the enzymatic reactions involved in light absorption, energy transfer and transformation (Huner et al., 1998).

Koç et al. (2010) observed a decline of chlorophyll content induced by low temperature in two pepper cultivars under cold stress conditions, likely due to oxidative stress. This was confirmed by Rinalducci et al. (2011) who observed a reduction in of the chlorophyll content over time in spring wheat at low temperatures (Rinalducci et al., 2011).

The objectives of this work were to investigate the extent to which natural variation towards cold tolerance in perennial ryegrass can be found, and how growth characteristics vary in genotypes under cold and ambient control conditions.

Material and Methods

Plant Materials

Fifty-seven accessions of perennial ryegrass (*Lolium perenne*) were used and they include commercial accessions, breeder's seeds, and ecotypes from a range of geographical locations and breeding programmes (Supplemental Materials Table 1). The criteria used for selecting the accessions for experiments were adaptation to (1) temperate climates and (2) high land climates, and (3) a range of materials from world-wide breeding materials from different climatic zones.

Soil Preparation

Soil used in all experiments was first sterilised and then mixed with 400g of slow release nitrogen (38% N:38% ureic nitrogen), 400g of nitrogen (27% N), 450g of muriate of potash (50% K) and 400g of superphosphate for 125 kg of soil. The soil mix (Westland Horticulture) consisted of 80% peat, 10% soil, 5% perlite, 5% grit, and was sterilised with steam for 48 hr before use.

Plant Growth

Plants were sown in the glasshouse in a mini lawn, to simulate a meadow in a pot, at ambient conditions into fertilised soil and watered every two days and cut fortnightly. After 4 to 5 weeks, they were cut to 4 cm in height and transferred into climate controlled plant growth chambers (Microclima 1750, Snijders, NL), one pot of each accession into each chamber, namely one control chamber and one with cold settings. The experiment was repeated three times, Experiments 1, and 3 in 3 litre pots and Experiment 2 in 1 litre pots. Humidity settings and light intensity settings were 70% humidity and photosynthetic photon flux density (PPFD) of 320 $\mu mol\ m^{-2}\ s^{-1}$, 12h/12h light and dark in both chambers across all three experiments. The temperatures in the control chamber were 15°C during the day and 8°C at night. Temperature settings in the cold treatment chamber were 8°C during the day and 2°C at night. The cold temperatures were chosen to represent a very cool Irish spring day while the control conditions are typical of a typical Irish spring day (see Supplemental Materials Table 2). Plants were kept in the controlled environments for 73 days. The controlled environment chambers were monitored with a temperature and humidity sensor per chamber (Lascar EasyLog EL-USB-2) positioned between the plants. The experiments took place from November 2015 to August 2016 (Experiment 1: November 2015 to January 2016, Experiment 2: February 2016 to April 2016;

Experiment 3: May 2016 to August 2016).

Parameters Measured

Plant height was measured weekly over a period of 73 days. Height was determined from three points within the pot from the soil surface to the tip of the grass leaves. Chlorophyll content was measured using a SPAD chlorophyll meter (Spectrum Technologies Inc., USA) from five leaves within each pot by measurement of the optical density difference at two wavelengths, 650nm and 940nm. Electrolyte leakage was determined at days 40 and 70. Two leaves (5 cm long) were cut and then transferred to 25 ml centrifuge tubes containing distilled water and kept for 24 hr in the dark at room temperature. Conductivity of the solution was measured in µS before autoclaving at 121°C for 20 min. The conductivity was measured again once the temperature was at ambient room temperature and taken as absolute conductivity to calculate electrolyte leakage using the equation shown below.

$$electrolyte\ leakage = \frac{conductivity\ after\ 24h}{conductivity\ after\ autaclaving} * 100$$

Fresh weights of the plants were determined at day 73 where all plants were cut to 4 cm in height before drying for three days at 70° to determine dry weight. Growth rate was determined by comparing the fresh weight of the plants under cold stress to those under control conditions.

Statistical Analysis

Statistical analyses were conducted using SAS 9.4. A log transformation was applied to the raw data as most of the phenotypic data were not normally distributed (all experimental raw data are accessible as supplemental materials raw data table). To determine if there were significant differences between cold and control treatments, a t-test at the 95% confidence level was used.

A general linear model analysis followed by a Tukey post-hoc test was applied in order to find groups with significant differences. The Glimmix procedure in SAS 9.4 was used, including interaction between treatment and experiment number in the model. The Tukey post-hoc test divides the accessions into groups where least squares means with the same letter are not

significantly different for $\alpha=0.05$. Pearson correlations were calculated for the traits measured at the end of the experiments 1, 2 and 3.

Repeated measures analysis was used to examine changes over time for the height and the chlorophyll content values for all 3 experiments.

Growth curves were calculated with the Glimmix procedure by fitting the linear trend ($y=a+bx$) and also the quadratic model ($y=a+bx+cx^2$) to test for evidence of curvature. Plots of raw data showed that the two treatments had a consistent pattern across days. Individual accessions appeared to show more variability in curve shape but in terms of the treatment groups this appeared to be negligible and simpler curves were fitted to describe the overall behaviour. A simple quadratic function was found to give a good fit to the data and clearly showed differences between treatment groups. The model fitted accounted for treatment, experiment and the response over time. The accessions do not appear explicitly in this model but they are included as subjects in a random coefficients analysis. The analysis fits an overall curve for the factors in the model and the subjects (each accession for each treatment for each experiment) are modelled as having the same form but randomly different from each other. All the subjects were combined to produce the overall curve and the deviations of the individual subject curve from the overall curve gives the measure of variance/error. Fitted coefficients were estimated and compared and residual checks were made.

Results and Discussion

The experimental conditions were chosen to represent typical late winter and early spring temperatures in Ireland. As seen in the climate data of Ireland (Supplemental Data Table 2), the average daily maximum temperatures in January and February are around 8°C, rising to nearly 15°C in May. The average daily minimum for these months also corresponds with the chosen night settings. The cold stress was severe enough to effect significant differences in the measured plant traits between cold and control conditions (Table 1). Peacock (1975) demonstrated that temperature in the region of the stem apex exerted the greatest influence on the rate of extension of perennial ryegrass leaves. We thus deemed the chosen temperature regime as suitable to test the influence of cool temperatures on varietal spring growth. Additionally, the duration of day

and night are similar in spring in Ireland, with the equinox on the 20th of March, hence plants were experimental grown under 12 hr day and 12 hr darkness.

The fresh weight of the *L. perenne* accessions was lower under cold conditions (median 10.92 g) compared to plants grown under control conditions (median 37.00 g), and the interquartile range of the fresh weight was higher in the control than in the cold treatment (25.66 g compared to 16.62 g) (Table 1, Figure 2). No significant differences were observed among the accessions tested under cold stress conditions. Wilson (1975) did not find in a diverging selection of two cultivar S23 groups any differences in winter growth. However, significant differences were observed between the different accessions under control conditions ($p > 0.05$), and the responses can be categorized into five groups according to a Tukey ranking test (Supplemental Materials Figure 1). Wilson (1975) reported on a good broad sense heritability for growth in low and high respiring S23 cultivar genotype groups under amenable 25°C day time temperatures. Substantial differences in the fresh weight between the experiments were found. This suggests a notable sensitivity of perennial ryegrass to small differences.

Plant heights measured on day 67 were significantly different between the accessions grown under cold (13.67 cm) and control (28.00 cm) conditions. The plants grown under control conditions also exhibited a higher interquartile range of 8.75 cm compared to 4.54 cm under cold conditions (Table 1 and Figure 2). There were two significantly different groups for plant height under cold stress and three significantly different groups under control conditions (Supplemental Materials Figure 2).

Electrolyte leakage from leaves at day 40 (9.52) and day 70 (11.58) were higher for plants grown under cold conditions compared to plants grown under control conditions at day 40 (8.18) and day 70 (7.68) (Table 1, Figure 2). There were two Tukey test ranking groups for electrolyte leakage which overlap for the two treatments (Supplemental Materials Figure 3). Higher electrolyte leakage value at day 70 compared to day 40 in the cold stressed leaves suggests an increase of cell membrane damage or loss of membrane integrity under cold stress conditions. In general, higher electrolyte leakage in the cold stressed leaves provides an indication of membrane damage occurring even at temperatures above 0°C.

The chlorophyll content at day 63 exhibited a greater variability for plants grown under cold conditions with an interquartile range of 11.32, compared to 5.92 for plants grown under control conditions (Table 1). However differences were not significantly different under cold and control conditions (Supplemental Materials Figure 4). For 23 out of 57 accessions, chlorophyll content was higher in the cold stress conditions. In aging leaves, sugar accumulation can down-regulate photosynthetic gene expression and accelerate leaf senescence (reduction in green colour), but this response is abolished in cold acclimation resulting in a longer photosynthetic lifespan (summarized by Wingler 2015). Photosynthesis could be further investigated in relation to stomatal conductance and chlorophyll conductance measurements. Combining these measurements enables the collection of information on photosynthetic performance of the accessions under cold stress conditions.

No correlations were found for chlorophyll content, height, and fresh weight under control conditions. Additionally, there were no correlations between chlorophyll content and electrolyte leakage (Table 2). We observed significant correlations between plant height and fresh weight under control conditions. Negative correlations were observed between electrolyte leakage and chlorophyll content under control conditions. We observed positive correlations between fresh weight, plant height, chlorophyll content, and electrolyte leakage under cold conditions. Plant height under cold conditions was positively correlated with chlorophyll content and electrolyte leakage (Table 2). There was a positive correlation between fresh weight and height with the electrolyte leakage although higher electrolyte leakage values indicate higher membrane damage. This aspect of the results could be further investigated using a lipidomics approach to assess changes in composition of cell membranes. The fresh weight is considered as the most important trait in this study because yield is in general the most important trait for farmers. The height is highly correlated with the fresh weight with a correlation coefficient of 0.85 under control conditions, and a similar correlation coefficient of 0.88 under cold stress conditions. Additionally, height measurement data from earlier time points during the growth period can be used as a good proxy for the fresh weight at the end of the growth period.

The analysis fits an overall curve for the factors in the model and subjects (each accession for each treatment for each experiment) are seen as having the same form in experiments 2 and 3, but are randomly different from each other. Experiment 1 differed from experiments 2 and 3 in shape (Figure 3). Individual accessions appeared to show more variability in curve form but in terms of the treatment groups this variation appeared to be negligible and simpler curves were fitted to describe the overall behaviour (Supplemental Materials Figure 5). A simple quadratic function was found to give a good fit to the data and clearly showed differences between treatment groups. The model fitted accounted for treatment, experiment and the response over time. The analysis fits an overall curve for the factors in the model and accounts for subjects (each accession for each treatment for each experiment). Experiment as a factor was found to interact with treatment and with the fitted coefficients for the time response and there were also interactions between treatment and the fitted coefficients, resulting in significantly different curve shapes across the experiments (Figure 3). The coefficients, linear and quadratic, for each treatment are all significant which means that there is a linear trend and curvature for both treatments. When comparing coefficients for treatments a stronger trend (greater magnitude and more positive) for the control and stronger curvature (greater magnitude and more negative) for the control were found.

The growth differences between cold treatment and an ambient control at 15 degree C in experiments 2 and 3 were comparable to differences in growth observed at similar temperatures by Peacock (1975). The growth response in experiment 1 in the control treatment was much lower and had a different shape. This could be due to being carried out in a different season compared to experiments 2 and 3 which might have impacted on the run performance of the control chamber. The experiments were run sufficiently long (73 days) to see long term effects of cold temperatures. To mimic field conditions, the grass were sown in mini lawns to simulate competition of the single plants for nutrients, light and water as they would experience in the field. Additionally, the mini lawns were grown in the glasshouse for up to five weeks, and cut twice prior to the start of the treatments.

Conclusions

We conclude that growth under cold conditions as in our experiment is under tight genetic control which did not allow in the 57 accessions from a wide variety of origins any variation in growth response. However looking at more amenable temperatures as experienced during a warm spring period, large differences in growth amongst the 57 accessions can be found. This should enable the selection of varieties which are amenable to sort pulses of enhanced temperatures and sun conditions. Support for this hypothesis can be found in a pilot study on winter growth in eight accessions of perennial ryegrass which found improved growth in early heading varieties over the winter (Brereton & McGilloway, 1999). Furthermore detailed physiological studies on stomatal functioning should be carried out to support the selection of varieties that can better respond to enhanced temperatures and light conditions. Also, in further experimentation on the effect of extended cold stress on perennial ryegrass, root growth measurements should be included, and changes in the root/shoot ratio, as well as root architecture traits be investigated.

Acknowledgements

The authors acknowledge the contributions of intern students to physiological measurements: Annabelle Gilgen, Tarah Kearney, Dominik Petrovic, Shirley Bernard and Emma Power. We are indebted to Teagasc and Goldcrop (Ireland), Agroscope (Switzerland), IBERS (UK) and Barenbrug (The Netherlands) for supplying us with seeds. We acknowledge careful recommendations of Prof. Marcel Jansen (UCC) and Dr. Wieland Fricke (UCD) in improving the drafting of this manuscript.

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Table 1(on next page)

Medianand interquartile range across the traits fresh weight (g), height (in cm),electrolyte leakage, and chlorophyll content.

Median and interquartile range across the traits fresh weight (g), height (in cm), electrolyte leakage, and chlorophyll content.

Table 1 Median and interquartile range across the traits fresh weight (g), height (in cm), electrolyte leakage, and chlorophyll content.

Trait measured	Cold stress		Control	
	Median	Interquartile Range	Median	Interquartile Range
Fresh weight	10.92	16.62	37.00	25.66
Height (day 39)	11.00	4.75	21.00	13.67
Height day (day)	13.67	4.54	28.00	8.75
Electrolyte leakage day 40	9.52	5.52	8.18	3.66
Electrolyte leakage day 70	11.58	3.35	7.68	4.20
Chlorophyll content (day 39)	23.94	8.28	26.64	9.68
Chlorophyll content (day 63)	31.98	11.32	32.82	5.92

Table 2(on next page)

Pearson correlation coefficients for measured traits, fresh weight, height (at day 67), chlorophyll content (at day 70), and electrolyte leakage (at day 70) (significance is indicated below the coefficients).

Pearson correlation coefficients for measured traits, fresh weight, height (at day 67), chlorophyll content (at day 70), and electrolyte leakage (at day 70) (significance is indicated below the coefficients). Correlations for cold treatment are in bold; correlations for control treatment are not in bold.

Table 2. Pearson correlation coefficients for measured traits, fresh weight, height (at day 67), chlorophyll content (at day 70), and electrolyte leakage (at day 70) (significance is indicated below the coefficients). Correlations for cold treatment are in bold; correlations for control treatment are not in bold.

	Fresh weight	Height day	Chlorophyll content	Electrolyte leakage
Fresh weight		0.85 ***	0.14	0.09 ***
Height	**0.88** ***		0.14	0.22 **
Chlorophyll content	**0.58** ***	**0.54** ***		-0.47 ***
Electrolyte leakage	**0.24** ***	**0.25** ***	**0.03**	

Figure 1(on next page)

Fresh weight of the57 accessions of perennial ryegrass under cold treatment and control conditionsat day 73.

Fresh weight of the 57 accessions of perennial ryegrass under cold treatment and control conditions at day 73. The results are from three separate experiments (experiment 1, 2 and 3).

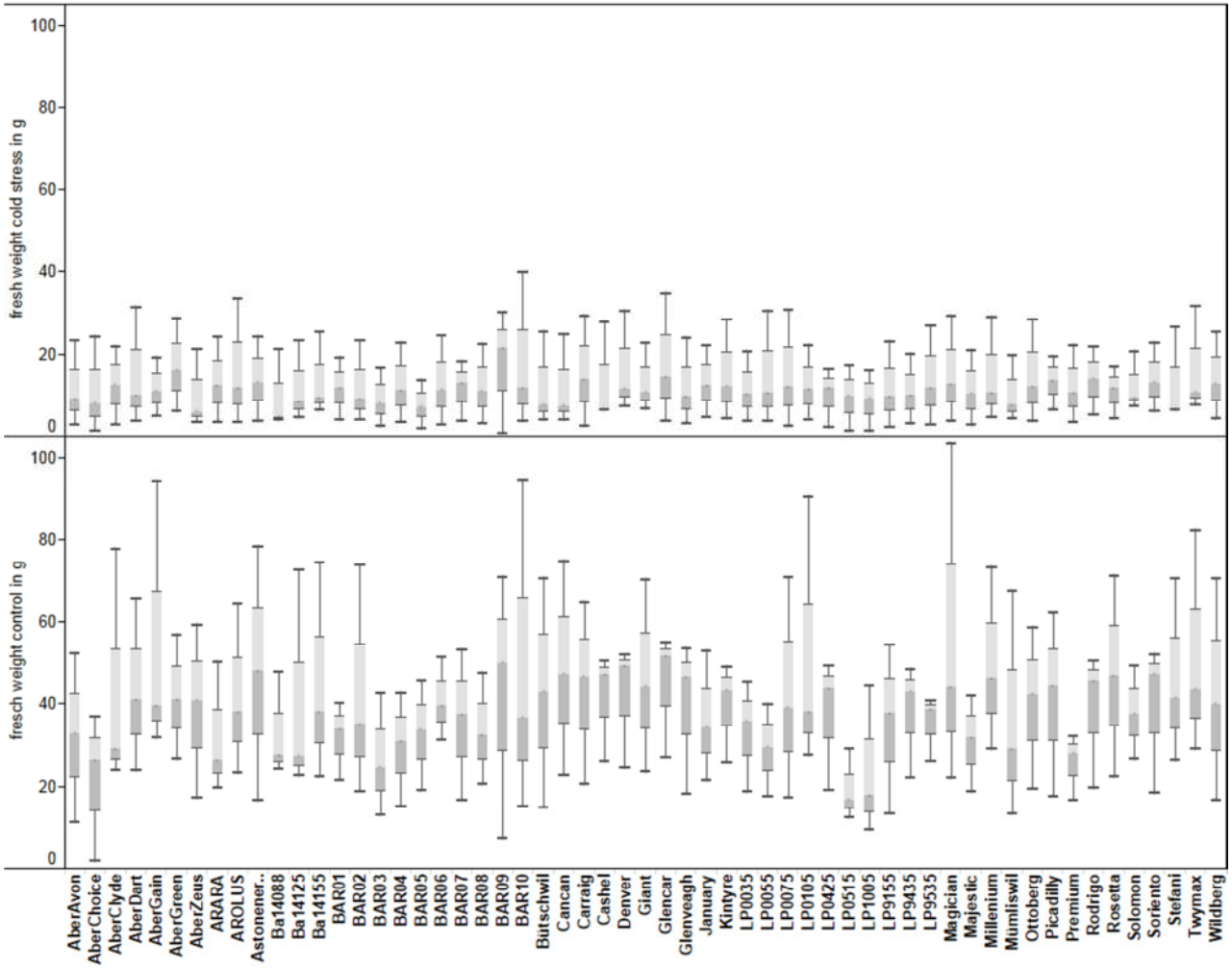

Figure 1 Fresh weight of the 57 accessions of perennial ryegrass under cold treatment and control conditions at day 73. The results are from three separate experiments (experiment 1, 2 and 3).

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Figure 2(on next page)

Box and whisker plot for plant height day 67, fresh weight at day 73, and electrolyte leakage day 40 and day 70under cold and control treatment.

Box and whisker plot for plant height at day 67 and fresh weight at day 73, and electrolyte leakage day 40 and day 70 under cold and control treatment. The results are derived from three experiments.

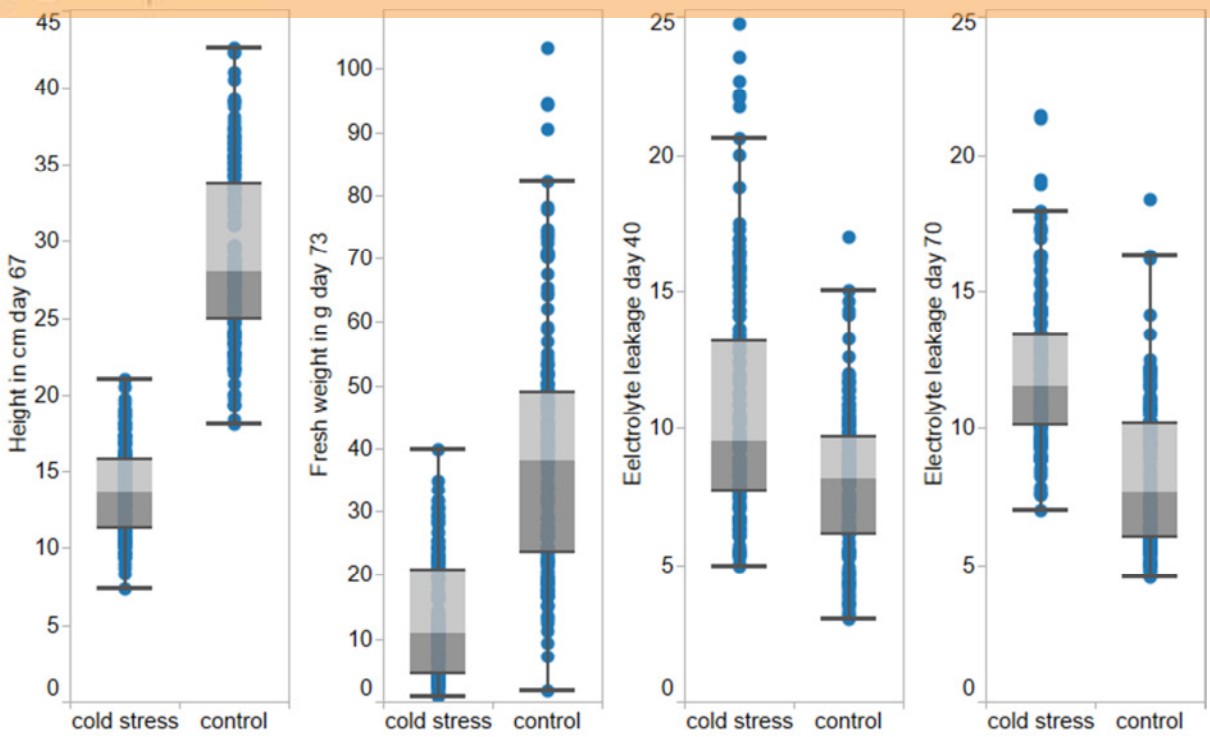

Figure 2 Box and whisker plot for plant height at day 67 and fresh weight at day 73, and electrolyte leakage day 40 and day 70 under cold and control treatment. The results are derived from three experiments.

Figure 3(on next page)

Response over time comparisons for plantheight (cm) at days 0, 35 and 65.

Response over time comparisons for plant height (cm) at days 0, 35 and 65 (The accessions are included as subjects in the analysis).

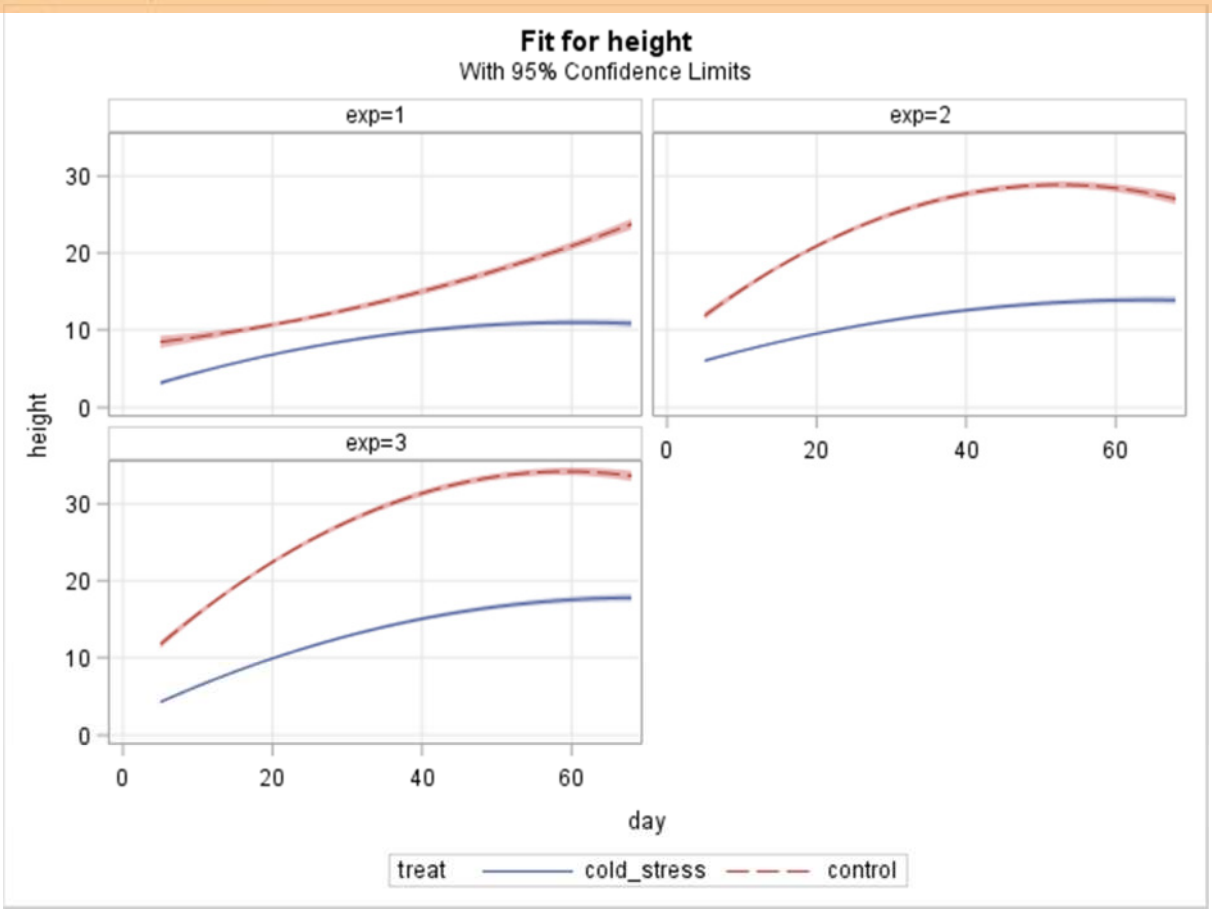

Figure 3: Response over time comparisons for plant height (cm) at days 0, 35 and 65 (The accessions are included as subjects in the analysis).