THE EFFECT OF NITRATE AND GIBBERELLIN TREATMENT ON CARBON STORAGE AND NITROGEN UPTAKE EFFICIENCY FOLLOWING DEFOLIATION OF *LOLIUM PERENNE*

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Abstract

Agriculture accounts for almost 50 % of New Zealand's greenhouse gas emissions. The high level of nitrogenous fertilisers used to stimulate plant growth causes emissions of NO_2 and leaching of N into waterways. In order to address these issues and improve N uptake efficiency in *Lolium perenne* L., a widely used forage grass, the effect of the day (post-defoliation) of N application, in conjunction with the addition of GA_3, on plant growth was observed. Parameters measured included N uptake and fructan levels which are the main storage carbohydrate of grasses. Results indicate that the application of fertiliser between day 6 and 11 post-defoliation may be beneficial in increasing N uptake and reducing leaching of N as carbohydrate levels have been restored in leaves, leaving the plant less C starved and more able to take up N. The application of GA_3 was not found to have an effect on N uptake. The effect of N form and concentration on root system architecture *in vitro*, indicates that arginine, an organic form of nitrogen, may be beneficial in improving N uptake and growth of plants if used as a fertiliser, compared to nitrate, a commonly used inorganic form of fertiliser.

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1 Introduction

1.1 Background

The New Zealand Government has set a goal of doubling the value of its agricultural production by 2025 from $32 billion in 2014 to $64 billion (Ministry for Primary Industries, 2014). Dairy conversion is a major driver of this growth projection as New Zealand exports 90% of the milk produced each year, which represents around 33% of the global dairy export market (Ministry for Primary Industries, 2015). To reach these goals there needs to be new approaches focusing on plant growth efficiency instead of merely increasing inputs of water and fertiliser. As agricultural productivity grows, so is it necessary to adapt agricultural practices to mitigate detrimental effects that such intensive systems can have on the environment.

Dairy farms are a particularly high intensive system, with nitrogen (N) inputs onto perennial rye grass (*L. perenne*) pasture around 20 kg N ha^{-1} 21 d^{-1} rotation and is now regulated under a cap in many regions.

In the dairy system, there is a typical rotation of 21 days where grazing occurs at day zero and fertiliser is applied two to six days later under irrigation, then the field is left resting for the remaining period (Miller *et al*., 2001). Grazing necessarily defoliates the grass, removing photosynthetic capacity. During the regrowth period, photosynthetic capacity increases, facilitating the transition of leaves from net carbon (C) sink to source. Concomitantly, the uptake rate of N, which is needed for growth, also needs to increase during this period. Both regrowth after grazing and N uptake simultaneously demand C from carbohydrate reserves.

It is therefore likely that such simultaneous resource allocation to these two processes creates a limitation of regrowth.

1.2 *Lolium perenne* and New Zealand Agriculture

Perennial ryegrass (*L. perenne)* is a dominant forage crop in the dairy farm, as well as other pastoral systems. It is the most widely sown perennial forage grass in many temperate regions, due to in part to its tolerance to a range of environmental conditions and grazing as well as high digestibility (Wilkins, 1991; Wilkins & Humpreys, 2003; Lestienne *et al*., 2006). In order to increase plant growth, nitrogenous fertilisers are applied (Robertson & Vitousek, 2009). Urea and nitrate are commonly used nitrogenous fertiliser. Over the past 50 years, the use of nitrogenous fertilisers has increased as the world's population grows, and diets improve. The application of N to soils, whilst improving yields, can have detrimental effects to the environment as detailed in the next section.

Of concern, is the fact the agriculture is responsible for almost 50 % of New Zealand's greenhouse gas emissions, and whilst New Zealand accounts for less than 1 % of total global emissions, on a per capita basis New Zealand is ranked 12th in the world (Ministry for the Environment, 2007). Steps to reduce emissions as a result of agricultural practices have been taken, such as instigating the Emissions Trading Scheme which aims to reduce greenhouse emissions (Ministry for the Environment, 2017).

1.3 Nitrogen

Nitrogenous fertilisers are applied at an annual rate globally of approximately 85-90 metric tonnes annually (Good *et al*., 2004). The production of these fertilisers also depends upon the Haber-Bosh process, accounting for 3-5% of the world's natural gas usage.

The high usage of nitrogen (N) in fertilisers causes it to leach from the soils, leading to eutrophication, resulting in the death of aquatic life. Leaching of N to waterways also cause disease, resulting in the need for chlorination of drinking water. There are also concerns about the conversion of fertiliser on the ground to nitrous oxide, which is a greenhouse gas with almost 300 times more global warming potential than carbon dioxide. Nitrous oxide may be regarded as being as important as ozone-depleting anthropogenic emissions (Ravishankara *et al*., 2009; Davidson & Kanter, 2014). It is estimated that the 66% of anthropogenic emissions are in the form of nitrous oxide derived from agricultural inputs (Davidson & Kanter, 2014). These emissions are a result of the nitrification and denitrification of fertilisers applied as well as further downstream effects such as nitrate leaching, and the detrimental effects are increased as nitrogenous fertilisers are applied in excess (Grant *et al*., 2000).

If the usage of nitrates as fertilisers on farms can remain static or be reduced, the detrimental effects of high nitrate use in fertilisers could be mitigated. One of the primary issues regarding the leaching of nitrogen and the subsequent production of greenhouse gases is the lack of synchronisation in the time that N is applied and the amount the plant can effectively uptake (Masclaux-Daubresse *et al*., 2010; Hodge *et al*., 2000; Brakin *et al*., 2015).

Depending on the form, concentration, and the N status of the plant, soil N can either stimulate or inhibit root growth (Walch-Liu & Forde, 2008). This is achieved through root to shoot signalling (Walch-Liu & Forde, 2008; Zhang *et al*., 2007). When nitrogen is available in excess it has the effect of inhibiting root growth (Ruffel *et al*., 2011), demonstrating that root development is plastic in response to nitrogen availability allowing a plant to adapt to different environments (Hodge, 2009).

There are also well-known interactions between nitrate and hormones (Ruffel *et al*., 2011; Sakakibara *et al*., 2006; Kiba *et al*., 2010). Krouk *et al.* proposed a model outlining the interactions of nitrogen with hormones, but this model neglected to consider the role carbon metabolism, which, it is hypothesised, plays a pivotal role in nitrogen uptake and metabolism (Krouk *et al*., 2010). Guo *et al*. have developed this model further by proposing that cytokinin regulates nitrate uptake in a C dependent manner (Guo *et al*., 2017).

Nitrogen in the soil is available in organic and inorganic forms. Inselsbacher & Näsholm (2012) used a micro dialysis technique to sample organic and inorganic nitrogen concentrations in soils in a more minimally-invasive manner than previously used. This has resulted in a change in the understanding of the relative abundances of these nitrogen groups found in the soil, showing that organic nitrogen flux in the soil has potentially a greater impact on nitrogen availability than previously thought (Inselbacher & Näsholm, 2012; Brakin *et al*., 2015; Streeter *et al*., 2000). It is possible for nitrogen to be taken up by the plant in both organic and inorganic forms (Jämtgård *et al*., 2010; Näsholm *et al*., 2009). As well as being essential for growth of plants, nitrogen is also essential for growth of microorganisms in the soil, some of which form beneficial relationships with the roots of the

plant enabling uptake of nutrients whilst acquiring products such as carbohydrates in return.

Three physical processes precede the uptake of nitrogen by the root: mass flow, diffusion and interception (Barber, 1995). Assimilation of nitrate is followed by reduction to nitrite, then to ammonium, before assimilation to amino acids which are used to build proteins responsible for many roles including essential catalysis (Masclaux-Daubresse *et al*., 2010). The reduction of nitrate to nitrite is catalysed by nitrate reductase, at which point it is further reduced to ammonium by nitrate reductase (Meyer & Stitt, 2001). This is an energy, and carbon expensive reaction, requiring NADH or ferredoxin as reducing power (Masclaux-Daubresse *et al*., 2010). In addition, carbon skeletons are essential to form amino acids that are the basis for the subsequent synthesis of secondary metabolites (Masclaux-Daubresse *et al*., 2006; Lam *et al*., 2003).

Of all the N forms available to plants, nitrate dominates agricultural systems and has thus received the greatest level of attention. There are two systems for nitrate and urea uptake: high affinity (HATS) and low affinity (LATS) nitrate transport systems, which are composed of constitutive and nitrate-inducible components (Miller *et al*., 2007). LATS has a functionally dominant role under high nitrate conditions, whereas HATS is of greater import in low nitrate conditions. Two gene families of nitrate transporters are defined according to this functional definition: NRT1 and NRT2 (Forde, 2000; Williams & Miller, 2001). It is generally assumed that the NRT1 gene family mediates the LATS, whilst NRT2 gene family mediates HATS, with both family members including members regulated at the transcriptomal level (Wang *et al*., 1998; Liu *et al*., 1999). Amino acid uptake is facilitated by the amino acid

permease (AAP) gene family: AAP5 mediates uptake of L-arginine and L-lysine – both cationic amino acids (Svennerstam *et al*., 2008).

1.4 Water Soluble Carbohydrates

Grasses have evolved in response to grazing pressure by storing high levels of carbohydrates in their stubble and roots (Lestienne *et al*., 2006). Carbohydrate synthesis occurs as a result of photosynthesis. Carbohydrates are an energy source and the backbone for all structures in the plant, being converted into molecules such as amino acids, glycans and, cellulose. Carbohydrate storage in ryegrass has been postulated as an important factor determining nitrate uptake rates after defoliation (Louahlia *et al*., 2008). In order the improve nitrogen uptake efficiency, the interaction between carbohydrate reserves and nitrogen uptake has been investigated to improve nitrogen uptake efficiency, as it has been shown that when carbohydrates are not the limiting factor, nitrogen is and vice versa (Morvan-Bertrand *et al*., 1999; Guo *et al*., 2017; Roche *et al*., 2017).

The predominant carbon storage molecule in *L. perenne* is fructan. Fructans are products of polymerisation of fructose based on sucrose which are mainly stored in the vacuole of cells in the base of stubble and roots (Pollock & Cairns, 1991; Carins & Gallagher, 2004). Accumulation of sucrose leads to fructan synthesis by fructose transferases (Cairns *et al*.,

2000; Ritsema & Smeekens, 2003). The initial step involves the addition of fructose, feeding further polymerisation (French, 1989).

In the stubble of *L. perenne*, fructans preferentially accumulate in the elongating zone (Schnyder & Nelson, 1987; Schnyder *et al*., 1988). This allows for there to be rapid re-foliation following defoliation, which can be further sustained by carbohydrate sourced from the sheath and the roots (Schnyder & de Visser, 1999). Concentrations of fructans decrease after cell elongation ceases and during active deposition of the cell wall (Allard & Nelson, 1992). Fructan degradation is important in mobilising stored carbohydrate and is mediated by fructan exohydrolases (Chalmers *et al*., 2005).

Morvan-Bertand *et al.* found that fructan levels in sheath and elongating leaf bases strongly influenced shoot yield during the first two days after defoliation, indicating, that over this time period little photosynthesis is occurring, and regrowth is supported by fructans which are degraded by fructan exohydrolase (Morvan-Bertrand *et al*., 1999). After this time point, the initial carbohydrate levels did not continue to correlate with leaf dry matter accumulation, indicating that photosynthesis was now responsible for further accumulation of leaf dry matter (Morvan-Bertrand *et al*., 1999). This represents a transition in the leaf from a carbon sink to a carbon source. The initial regrowth was supported by hydrolysis of fructans in the elongating leaf bases, followed an hour and a half later by fructan mobilised from the leaf sheaths (Morvan-Bertrand *et al*., 2001).

1.5 The Relationship Between Carbon and Nitrogen

Both carbon and nitrogen are essential elements to plant growth, being the basis around which energy and proteins are formed. Defoliation as a result of grazing leads to loss of both carbon stores and photosynthetic potential. This leads to a carbon demand, which is then increased by the application of fertiliser, and subsequent N uptake. Nitrogen uptake decreases WSC content of plants through down regulating fructan synthesis and demanding carbohydrates for uptake and assimilation (Rasmussen *et al*., 2008; Morcuende *et al*., 2004). In the dairy system, there is competition of resources between growing new photosynthetic material that has been lost due to defoliation and the uptake of nitrogen (Louahlia *et al*., 2008). Remobilisation of carbohydrates following defoliation for the synthesis of amino acids and regrowth post defoliation relies on cross talk between N and C signals (Roche *et al*., 2017). Cytokinin has been implicated in the regulation N and C cross talk (Roche *et al*., 2016; Roche *et al*., 2017; Guo *et al*., 2017). When the carbohydrate limitation has been relieved, nitrogen availability is a limiting factor to plant growth (Morvan-Bertrand *et al*., 1999). It has been proposed that the application of fertiliser should be deferred until such time as plants have reinstated their C resources post defoliation (Roche *et al*., 2017). This enables the plants to recover their photosynthetic pathways more efficiently and may improve the quantity of regrowth produced.

1.6 Gibberellins

Gibberellins (GA) are plant hormones associated with a number of plant processes, but of most interest in this research, GA has been documented as having a significant role in shoot elongation (Cosgrove & Sovonick-Dunford, 1989). GA enhances shoot elongation through relaxing the cell wall, though it has also been shown to occur as a result of cell division in some species (Cosgrove & Sovonick-Dunford, 1989; Sachs, 1965). This occurs as a result of a inhibition of the growth repressor DELLA (Hedden & Sponsel, 2015) High concentrations of GA have an inhibitory effect on root growth, whilst lower concentrations lead to the cell elongation of roots (Stowe & Yamaki, 1957; Tanimoto, 2012).

In previous research, the application of gibberellin has been shown to increase leaf elongation, stimulating regrowth post defoliation (Cai *et al*., 2016; Percival, 1980; Zaman *et al*., 2014; Zaman *et al*., 2016). In addition, the application of GA in some cases has been shown to increase dry mass yields when combined with nitrogenous fertilisers (Zaman *et al*., 2016; Zaman *et al*., 2014; Ghani *et al*., 2014). Application of GA have been shown to reduce fructan exohydrolase activity, with no apparent co-regulation with other plant hormones, nor change in fructan or sucrose levels of the plant (Gasperl *et al*., 2016). The use of GA in agricultural systems has been suggested to aid in the reduction of N-leaching from soils and NO_2 emissions through elevated plant growth behaviours (Ghani *et al*., 2014; Whitehead & Edwards, 2015). However, the long-term effects of GA application on pasture growth have yet to be investigated. There is, however, anecdotal evidence that the application of GA can cause a decrease in pasture yields the following season after it was applied (Paula Jameson, personal communication, Boom *et al*., 2015; Matthew *et al*., 2010). GA has been proposed as a tool to increase the growing season for New Zealand pasture. It has been reported that

the effects of GA are seasonally dependent, with the suggestion that the application of gibberellin in increasing pasture yield is most effective in the winter (Parsons *et al*., 2013).

1.7 Root System Architecture

Understanding root system architecture is important for understanding how plants acquire nutrients from the soil (Ho & McCannon, 2004). It is fair to say that the workings of roots are poorly understood in comparison to their aboveground counterparts. Classically, roots with a diameter of less than 2 mm have been classified as fine roots which are responsible for the acquisition of nutrients (McCormack *et al*., 2015). However, this may not be an accurate representation of all the roots making up this group, it has been proposed they could be split further into two distinct groups: absorptive and transport fine roots (McCormack *et al*., 2015).

Roots respond to the presence of nitrate in the soil – presence increases primary root growth, and lateral root development, as well as root branching when combined with L-glutamate (Walch-Liu & Forde, 2008). In low N conditions roots typically forage for nutrients and this is characterised by an increase in lateral root length and number, whereas in N rich conditions the opposite strategy is taken (Ruffel *et al*., 2011).

Nitrogen uptake and assimilation is a carbon demanding process, and the uptake of nutrients stimulates growth which in turn stimulates nutrient uptake and assimilation. The

use of amino acids as an N source is of interest because an amino acid has four nitrogen (N) atoms present, making it a nitrogen rich compound compared to more traditionally used fertilisers such as nitrate or urea which are nitrogen poor in comparison. Arginine is one such amino acid, and has the potential to be taken up as a whole molecule and assimilated with less energy expenditure than standard fertilisers (Miller & Cramer, 2005). It has been demonstrated that uptake of intact amino acids by plants is possible and can be directly assimilated by plants (Näsholm *et al*., 2009; Forsum *et al*., 2008; Weigelt *et al*., 2005; Streeter *et al*., 2000). Application of arginine instead of nitrate could allow the plant to continue to take up assimilate and convert nitrogen at a lower carbon cost; thereby increasing growth in plants.

1.8 Aims and Objectives

This thesis is concerned with investigating the application of nitrogen (N) and gibberellic acid (GA_3) on the growth habits of perennial ryegrass. This research was carried out with the view of increasing productivity in the dairy system and mitigating the detrimental effects of intensive farming on the environment. There were two main objectives of this research, which were investigated using two different systems: deep pots and vertical plates.

The first objective was concerned with determining the best time post-defoliation to apply N in terms of plant regrowth and biomass accumulation. This was done in deep pots to imitate field conditions, and N was added over a time series following defoliation. ^{15}N uptake was measured to determine the time post-defoliation that plants were no longer

limited in their ability to take up N by the demands of regrowth. A sequential hypothesis to this was that the exogenous application of the plant hormone GA_3 would cause a shift in the pathway by which plants store carbohydrates, leading to increased growth, and nitrogen uptake efficiency. This work was repeated over two seasons: summer and autumn. The rationale behind this was that previous studies had indicated that the application of GA_3 may have different effects in different seasons (Parsons *et al*., 2013).

The second objective of this research was to examine the effect of organic and inorganic forms of nitrogen at different concentrations on root system architecture (RSA). For these experiments, *L. perenne* was grown in quasi-sterile conditions using transparent media and plates to facilitate the visualisation of the roots as they developed. Concurrently, the effect of GA_3 was investigated on RSA to determine if the application of GA_3 led to a decrease in below ground growth. This was a preliminary investigation into the impact of the use of different fertilisers on RSA, which is essential in the acquisition of nutrients and water for the plant.

2 Materials and Methods

2.1 Deep Pot Experimental Design

The deep pot experiment was designed to determine the best day, post defoliation, to apply nitrogen, and whether the addition of GA_3 influenced grass growth and carbohydrate (fructan) accumulation patterns in perennial grass. This experiment was also designed to simulate field conditions as closely as possible whilst being able to control and hence minimise the variation that would occur under field conditions. Fertiliser free substrates were used in order to control the N content of the substrate the plants were grown in. This experiment was carried out in autumn (set up: February 2016, experimental rotation: 17th June – 23rd July 2016) and summer (set up: September 2016, experimental rotation: 9th January– 9th February 2017) - to compare growth over the two seasons.

Pots were sown with *L. perenne* c.v. nui at a rate of 40.6 kg/ha in a grid pattern with rows spaced 40 mm apart and seeds spaced 10 mm apart from each other in the row (see Figure 2-1 and Figure 2-2). To simulate the action of grazing by cows, leaves were defoliated using scissors to 40 mm above soil level. Instead of using a 21-day rotation cycle which is customary in the dairy industry a 28-day cycle was used here to determine if it may be beneficial to extend the rotation length.

2.1.1 Design of autumn experiment

Culvert pipes 280 mm in diameter and one metre deep were used in this experiment. This depth was chosen as it had been shown that roots can grow this deep, with the majority of

root mass in the top 0 – 200 mm below the soil surface (Bolinder *et al*., 2002; Crush *et al*., 2005). The pipes were filled with a N-free potting mix. Seeds were sown in pots in the pattern shown in Figure 2-1, they were allowed to establish for 11 weeks before the first defoliation. Two preliminary rotations of 28 days in length each were conducted. Plants were defoliated to 40 mm above soil level on every 28th day. On the third experimental rotation, plants were defoliated as previously with the exception of intact plants.

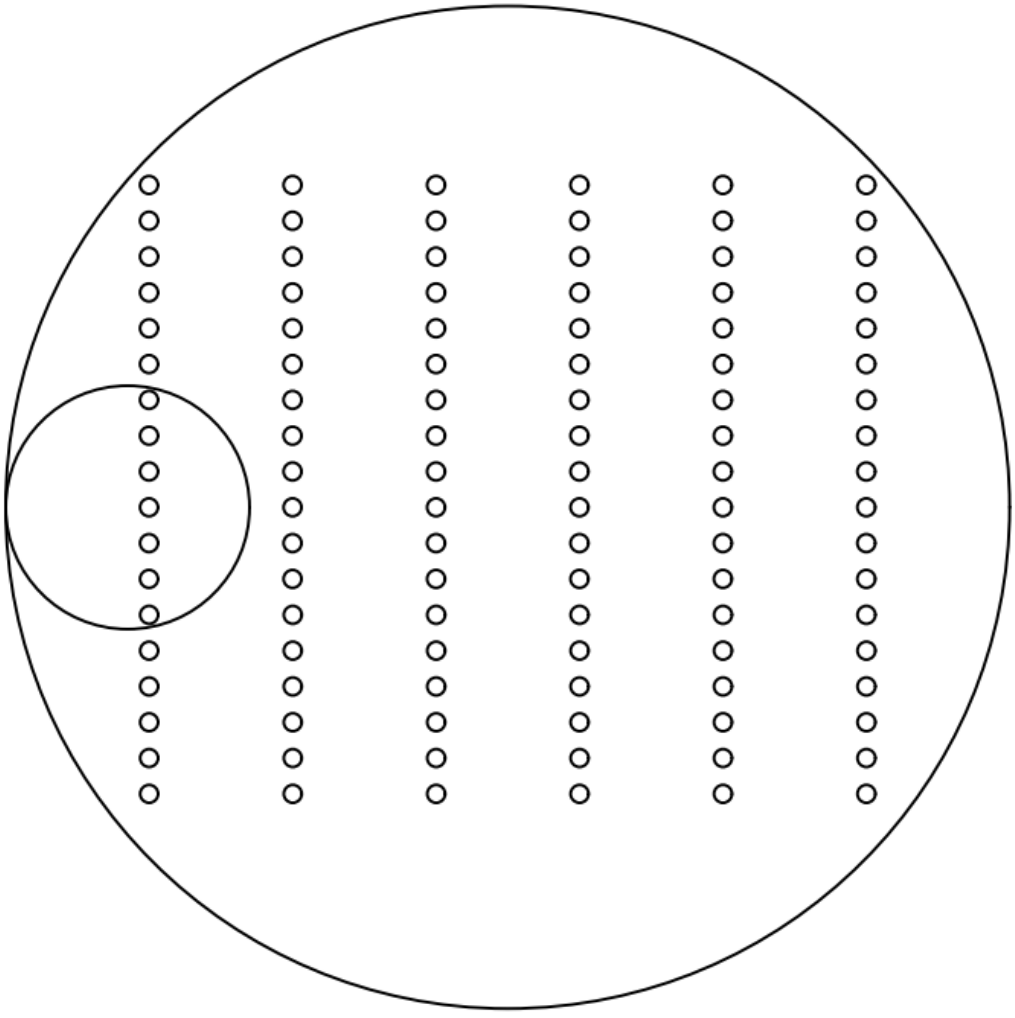

Figure 2-1: Pot layout of autumn experiment. *L. perenne* seeds were sown in a grid pattern 200 x 180 mm. Rows were spaced 40 mm apart whilst seeds were spaced 10 mm apart along the row as indicated by small dots. The small circle indicates the core that was taken for ^{15}N sampling during the 28-day rotation.

On days 0, 2, 4, 7, 11, 21, and 28 post defoliation different treatments were applied. These were carried out between 10.30 and 11.30 am each day.

GA_3, or the control solution to the whole area of the pot until run off. Solutions were prepared immediately prior to application.

- GA_3 solution: 24 µl GA_3 (30 ppm in 80% ethanol), 8 µl silwet, 80 ml mili-q water.
- Control solution: 24µl 80% ethanol, 8 µl silwet, 80 ml mili-q water.

A pipe with an inner diameter of 68 mm was inserted into the pot to a depth of 100 mm. This subsection of the pot was treated with urea at a rate of 20 kg/ha enriched with 10% ^{15}N ($K^{15}N0_3$ ≥98% purity) and incubated for 24 hours for ^{15}N analysis to determine N uptake efficiency. ^{15}N samples were destructively harvested after 24 hours by removal of cores. The cores were washed to remove all dirt in water, and rinsed in 0.1 mM $CaSO_4$ to remove any residual ^{15}N from the exterior of plants. Plants were cut at the root crown to separate roots from the plant, and 40 mm above the root crown to yield stubble and leaf blade material. Roots were thoroughly washed to remove all bark.

The remainder of the pot was left undisturbed for the remaining time until harvest which took place from day 30-34 post defoliation. All remaining material in the pot was harvested to the depth of 200 mm (Bolinder *et al*., 2002; Crush *et al*., 2005). All root material was removed from the soil and washed. Material was divided into roots (all below ground growth), stubble (growth from the root crown to 40 mm above) and leaf blades (all remaining material more than 40 mm above the root crown).

2.1.2 Design of summer experiment

This experiment followed the same experimental design as the previous experiment with the exception that a range of improvements were applied as follows.

Pots were cut to half the size (0.5 m tall, 280 mm diameter), to reduce the amount of work involved with harvesting them. Pots were filled to 400 mm with the same nitrogen free potting mix. The top 100 mm was filled with a mix of 25% plasterers sand and 75% soil. This was because it was decided to harvest only the top 100 mm of roots from the pots after visual confirmation that roots were most prolific in the region 0-100 mm below ground in the previous autumn experiment. Yates Lawn Master Revival Lawn Food was applied at a rate of 10 kg N per ha – half that used in the experimental rotation – two weeks after germination, and two weeks after the first and second defoliation event in order to keep the plants healthy and assist in growth. Additionally, the replicates were spread across the span of six days to improve the efficiency of final harvest and reducing the effects of time.

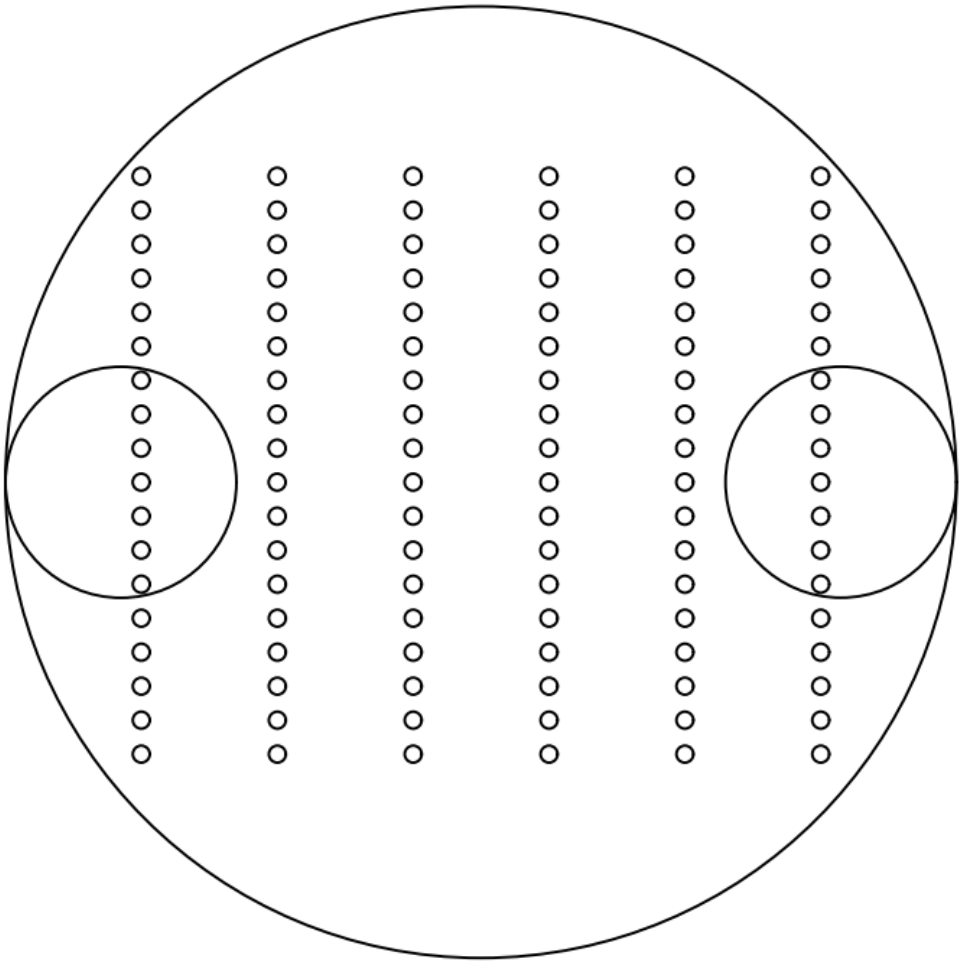

Figure 2-2: Pot layout of summer experiment. *L. perenne* seeds were sown in a grid pattern 200 x 180 mm. Rows were spaced 40 mm apart whilst seeds were spaced 10 mm apart along the row as indicated by small dots. The small circles indicate the cores that were taken for ^{15}N sampling during the 28-day rotation. In the event that only 1 core was taken instead of two, the core was positioned to the left as seen in Figure 2-1 above.

On the third experimental rotation plants were defoliated to 40 mm at 7 am. Treatments of GA_3 ^{15}N and N were applied to D0 and intact plants at 9 am. On further days: 2, 4, 6, 11, 21, 28, treatments were applied between 9 and 10 am. All time of defoliation, application and harvesting were at an earlier time of the day relative to the autumn rotation to take into account the difference in the time sunrise occurred.

GA_3, or the control solution to the whole area of the pot until run off. Solutions were prepared immediately prior to application.

- GA_3 solution: 24 µl GA_3 (30 ppm in 80% ethanol), 8 µl silwet, 80 ml mili-q water.

- Control solution: 24µl 80% ethanol, 8 µl silwet, 80 ml mili-q water.

A pipe with an inner diameter of 68 mm was inserted into the pot to a depth of 100 mm. This subsection of the pot was treated with urea at a rate of 20 kg/ha enriched with 10% ^{15}N ($K^{15}N0_3$ ≥98 % purity) and incubated for 24 hours for ^{15}N analysis to determine N uptake efficiency. ^{15}N samples were destructively harvested after 24 hours by removal of cores. They were washed to remove all dirt in water, and rinsed in 0.1 mM $CaSO_4$ to remove any residual ^{15}N from the exterior of plants. Plants were cut at the root crown to separate roots from the plant, and 40 mm above the root crown to yield stubble and leaf blade material. Roots were thoroughly washed to remove all bark.

The remainder of the pot was treated with a urea solution at a rate of 20 kg N per ha and left undisturbed for the remainder of the growing period. Harvesting of this material took place on day 29 and 30 post defoliation over six separate days. Shoot removal occurred between seven and nine am whilst sheath removal occurred between eight and eleven am. Shoots were determined as the material 40 mm above the soil, sheaths were determined as the material from the soil level to 40 mm above the soil level. Shoots and sheaths were immediately frozen in liquid N before being placed in -20 °C freezer prior to being dried to determine dry mass. Roots were harvested from 9 am. For two replicates, the top 100 mm of roots only were harvested. For two further replicates, roots from the pot were harvested. For the remaining replicate, the top 100 mm and the remainder of roots from 100 – 500 mm below soil level were harvested and processed separately. After removal from pots, roots were kept on ice until it was possible to wash and remove all dirt from the samples.

2.1.3 Morphological measurements

Tiller number, leaf number and leaf length were measured on day zero and day 28 for all pots. Percentage canopy cover was measured every two to three days by taking photos from directly above. The number of green pixels in each image was counted and percentage green pixels calculated.

2.1.4 ^{15}N analysis of summer deep pots

Analysis of ^{15}N content of plants was only carried out on plants from the summer experiment after dry weight measurements from the autumn rotation indicated very little growth over the period. After being collected from the field, samples were freeze dried and ground using a ball mill to <200 µm. These samples were then sent to the Waikato Stable Isotope Unit where total N was determined using isotope ratio mass spectrometry. ^{15}N content was determined by continuous-flow mass spectrometry after samples were combusted and the gases separated in an automated Europa Scientific 20/20 isotope analyser.

Percentage N derived from fertiliser (NDFF) was calculated:

$$NDFF = \left(\frac{\%\ ^{15}N\ excess\ in\ sample}{\%\ ^{15}N\ excess\ in\ fertiliser}\right) \times 100$$

Using NDFF % N applied that was present in the plant was calculated:

$$\%N\ uptake = \left(\frac{\%\ NDFF\ \times mass\ harvested}{rate\ of\ N\ application}\right) \times 100$$

These calculations were carried out as described by the International Atomic Energy Agency (International Atomic Energy Agency, 2001).

2.1.5 Fructan analysis of summer deep pots

Subsamples of samples taken and ground for ^{15}N analysis were taken for fructan analysis. This was only carried out on plants from the summer experiment due to little growth occurring over the autumn experiment. Fructan levels were measured in the stubble of plants (plant material from the root crown to 40 mm above) over the four week (28 day) growing period. Fructan concentration of plant material was determines based on Association of Official Analytical Chemists (AOAC) method 999.03 and the Megazyme International fructan-assay kit (Megazyme, 2016). Previously it had been demonstrated that it was possible to miniaturise the kit by a factor of 27 (Revanna *et al*., 2010), however despite numerous attempts this was not able to be replicates, smaller factors of 20 and 10 to miniaturise the assay kit with no success. Thus, the kit was used at the level recommended by the manufacturers.

Perennial ryegrass stubble samples were dispersed in water and filtered before the analysis. To remove sucrose, starch and reducing sugar samples were treated with sucrase plus β-amylase, pullulanase and maltase and incubated, then further treated with the addition of alkaline borohydride and dilute acetic acid. Fructans were hydrolysed with the addition of *exo*- and *endo*-inulinase and incubated. PAHBAH was added to colour reducing sugars in a final incubation at 100 °C for 6 minutes. Solutions were promptly cooled to room temperature and read against reagent blank at 410 nm. Samples of fructan flour and sucrose of known fructan content were analysed with each set of analyses along with reagent blanks and d-fructose standard samples.

2.2 Vertical Plates

To examine the root system architecture (RSA) of plants, *L. perenne* was grown on clear vertical plates filled with transparent N-free Hoagland's media. This allowed for manipulation of N content and form provided in the media. Several experiments were performed to determine the effect of form, concentration on the RSA. In addition, the effect of GA_3 applied to leaf blades on the RSA was investigated. Analysis of root growth was able to take place without disturbing the plants by removing them from their growth media, through the use of transparent plates and media.

2.2.1 Seed sterilisation methodology

Growth of *L. perenne* on N-free Hoagland media plates had previously been unsuccessful due to high rates of infection. A system for sterilising *L. perenne* seeds was developed so that the rate of infection decreased. The effect of sterilisation on growth rate and vigour of the seedlings was investigated.

Personal communication with another lab group researcher indicated that the removal of the hull from the seed may lead to lower infection rates, as they had experienced similar problems with the growth of rice. Thus, experiments involved the removal of the hull from the seed. Three different methods were tried along with two controls methods where the seed was left intact without removal of the hull (Table 2-1). For each treatment, seeds were plated immediately following sterilisation. They were then incubated in the dark at room temperature before transferring onto plates with different treatments and being put into a growth room on day three when observations were carried out on day ten.

	Method 1	Method 2	Method 3	Method 4	Method 5
Intact Seeds	5 minutes in 75 % ethanol Wash with milli-q water 5 minutes in 75 % ethanol Wash with milli-q water 15 minutes in 0.5 % SDS Wash with mili-q water	5 minutes in 75 % ethanol Wash with milli-q water 5 minutes in 75 % ethanol Wash with milli-q water 15 minutes in 0.5 % SDS Wash with mili-q water		5 minutes in 75 % ethanol Wash with milli-q water 5 minutes in 75 % ethanol Wash with milli-q water 15 minutes in 0.5 % SDS Wash with mili-q water	5 minutes in 75 % ethanol Wash with milli-q water 5 minutes in 75 % ethanol Wash with milli-q water 15 minutes in 0.5 % SDS Wash with mili-q water
					30 seconds in 75 % ethanol Wash with milli-q water 5 minutes in 0.5 % SDS Wash with milli-q water
Removal of Hull	Removal of hull with forceps	Removal of hull with forceps	Removal of hull with forceps		
De-hulled Seeds	30 seconds in 75 % ethanol Wash with milli-q water 5 minutes in 0.5 % SDS Wash with milli-q water	30 seconds in 75 % ethanol Wash with milli-q water 5 minutes in 0.5 % SDS Wash with milli-q water perform this step twice	30 seconds in 75 % ethanol Wash with milli-q water 5 minutes in 0.5 % SDS Wash with milli-q water		

Table 2-1: Table demonstrating different methods used for seed sterilisation

2.2.2 Plate and solution preparation

Seedlings were grown in a quasi sterile system on plates of two sizes (300 x 300 mm and 100 x 100 mm) were used to grow seedlings in. These plates had 2 holes cut in the top of the plate in order for plates to be placed vertically, allowing roots to grow down the plate in a natural response to gravity, and ensuring leaf material did not come into contact with the growth medium. Plates were filled with transparent N-free Hoagland's media that was supplemented with N in differing concentrations (5 and 0.05 mM) and in two different forms organic (arginine) and inorganic (nitrate). GA_3 solutions were applied by submerging leaves in GA_3 solution for 10 seconds. This was to reduce the risk of cross aerosolised GA_3 solution spreading across treatments. Run off of solution from leaves into the plate was reduced through the use of paper towels. GA_3 solutions were prepared along a gradient from 0.01 ppm to 30 ppm with two controls as follows:

- Control 1: water
- Control 2: water + 0.01% silwet
- 0.01 ppm: water + 0.01% silwet + GA_3
- 0.04 ppm: water + 0.01% silwet + GA_3
- 0.12 ppm: water + 0.01% silwet + GA_3
- 0.36 ppm: water + 0.01% silwet + GA_3
- 1.1 ppm: water + 0.01% silwet + GA_3
- 3.3 ppm: water + 0.01% silwet + GA_3
- 10 ppm: water + 0.01% silwet + GA_3
- 30 ppm: water + 0.01% silwet + GA_3

2.2.3 Plant growth

Sterilised seeds were germinated for five days in the dark before being transferred onto plates filled with transparent media. These plates were wrapped in tin foil to exclude light from the roots, as exposing roots to light causes changes not only in the RSA but whole plant morphology as discussed above. Plant roots were monitored by scanning them on a flat-bed scanner. These images were then analysed in RootNav to examine the root system architecture.

2.2.4 Root system architecture analysis

RootNav was used to analyse the RSA (Pound *et al*., 2013). Root Nav uses an algorithm after start and end point of roots have been identified to determine the most likely path the root has grown along. To speed the image processing time in RootNav code was written in MatLab to clean up the images, removing imperfections such as condensation on the plate, bubbles in the media or bacterial growth on plates.

3 Statistical Analysis

3.1 Deep Pots

A two-factor ANOVA was used to analyse data (for ANOVA tables, see appendices, section 7.1.2). Error bars are the standard error of the mean. Floating error bars demonstrate least significant differences from the mean of all points of the same GA_3 treatment (either $+GA_3$ or $-GA_3$).

Effects from blocks were investigated – there is an effect of different rows in the deep pots. The difference between blocks is likely the result of a temperature gradient, running North to South (see section 7.1.1). Pots were arranged as demonstrated below in Figure 3-1. The black colour of the pots may have affected temperature inside the pots, causing this variability. Previously, vertical gradients in temperature have been shown to impact plant growth in terms of biomass production and concentrations of N and C (Füllner *et al*., 2012).

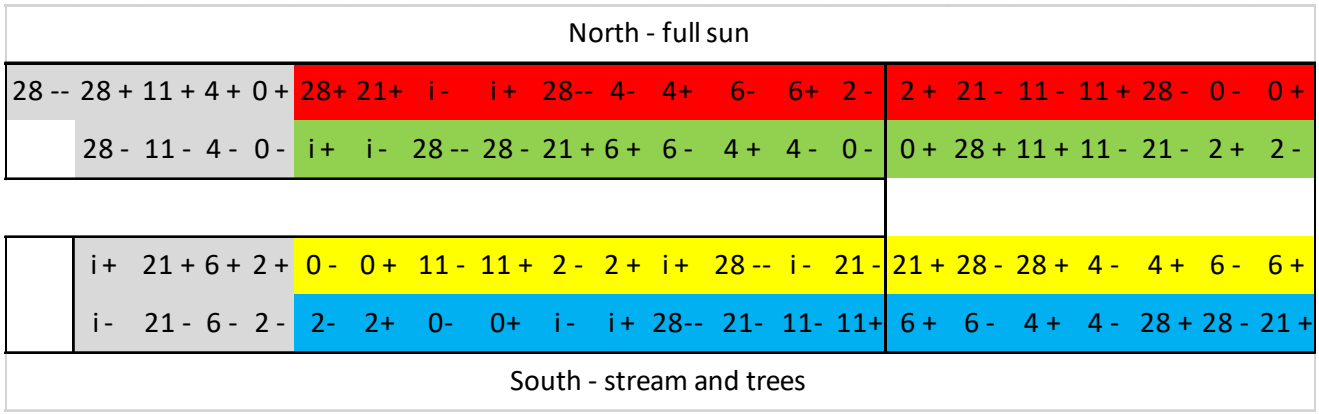

Figure 3-1: Set up of pots in summer experiment. Different colours indicate different replicates. Green and yellow pots were time replicate number 1, red and blue pots time replicate number 2 and grey pots time replicate number 3. Lines indicate the lay-out of 3 irrigation systems. All pots were defoliated on D0 to 40 mm above soil level with the exception of intact "i" pots. Each pot was assigned a number as determined as the day post defoliation they were treated. There were 3 GA_3 treatments: nil: "-"; plus: "+". Additionally, there was a control treatment "28- -" which was left untreated for the duration of the experiment.

3.2 Vertical Plates

Two-factor ANOVAs were used to investigate treatment effects. These are displayed in graphs below. Error bars are the standard error of the mean whilst floating bars indicate the least squared difference from the mean for significant points.

4 Results

4.1 Autumn Deep Pots

Shoot length, tiller number, leaf number and fresh and dry weights were measured in the autumn rotation. None of the morphological measurements showed any significant differences, therefore, the data is not shown.

4.1.1 Dry mass measurements

Figure 4-1 displays the dry mass of plants from the end of the autumn rotation. As can be seen from the figure, little growth occurred over the experimental rotation possibly due to low light intensity, cool temperatures. Any significant data points are most likely a result of natural variation within the biological material and sampling systems.

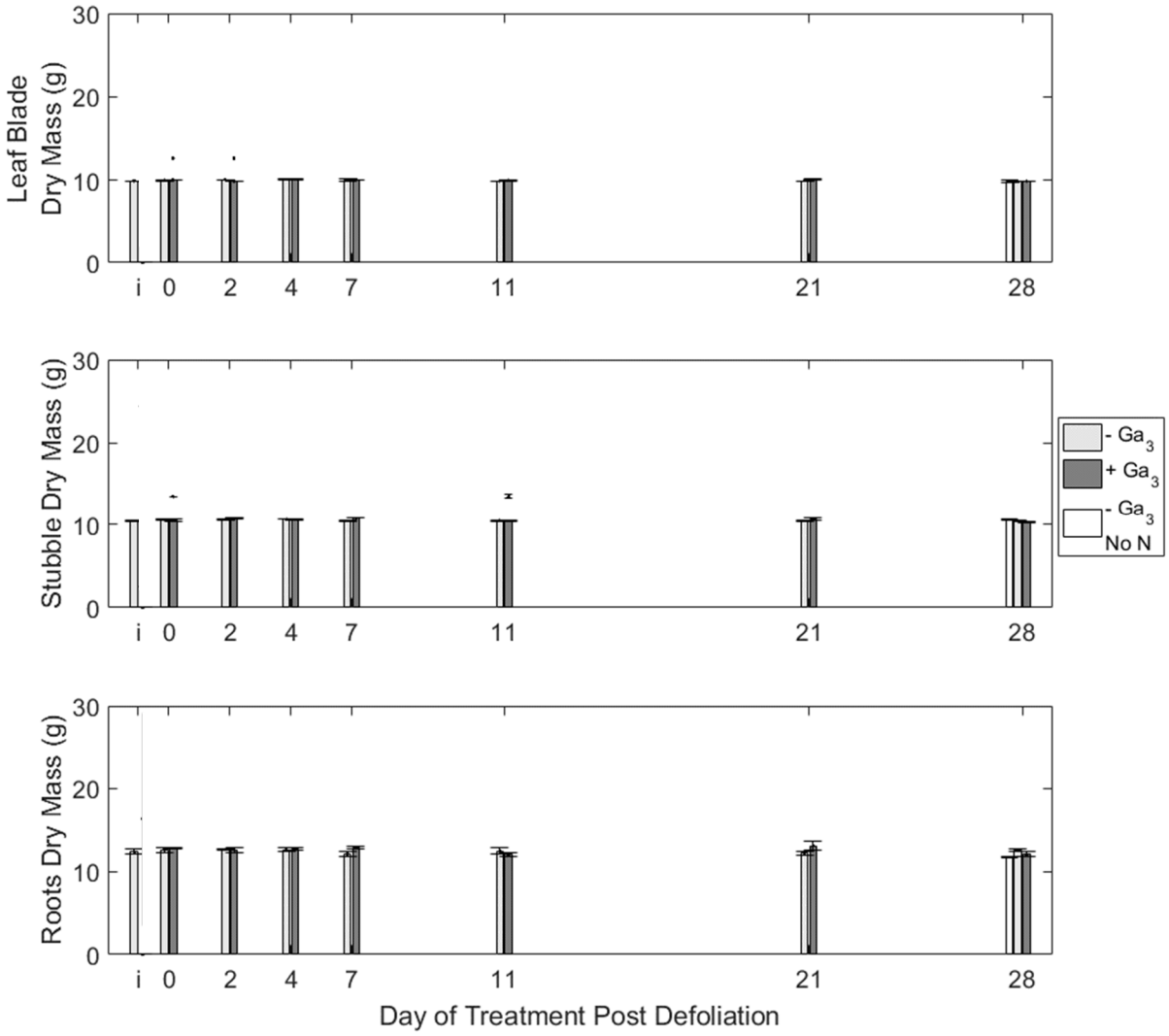

Figure 4-1: Dry mass of plants harvested at the end of the autumn 28-day rotation with roots to a depth of 200 mm. Plants were defoliated on D0 to 4 cm above soil level. Treatments were applied at days post defoliation indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied.

4.2 Summer Deep Pots

ANOVA determinations from the summer deep pot trial revealed that the main treatment effect was that of day of treatment post defoliation. However, no significant differences between treatments with or without GA_3 were observed. The graphs below highlight some of these differences.

4.2.1 Growth over three rotations

Figure 4.2 demonstrates changes in leaf length over the first two preliminary rotations and the final experimental rotation in the control plants that did not have fertiliser or GA_3 applied to them. Rotations were 28 days in length, on day zero all plants were defoliated to 40 mm above soil level. Variability in the slope of the growth lines can be explained by the bias seen between the blocks of pots as mentioned above (Figure 7-1, Figure 7-2, Section 7.1.1).

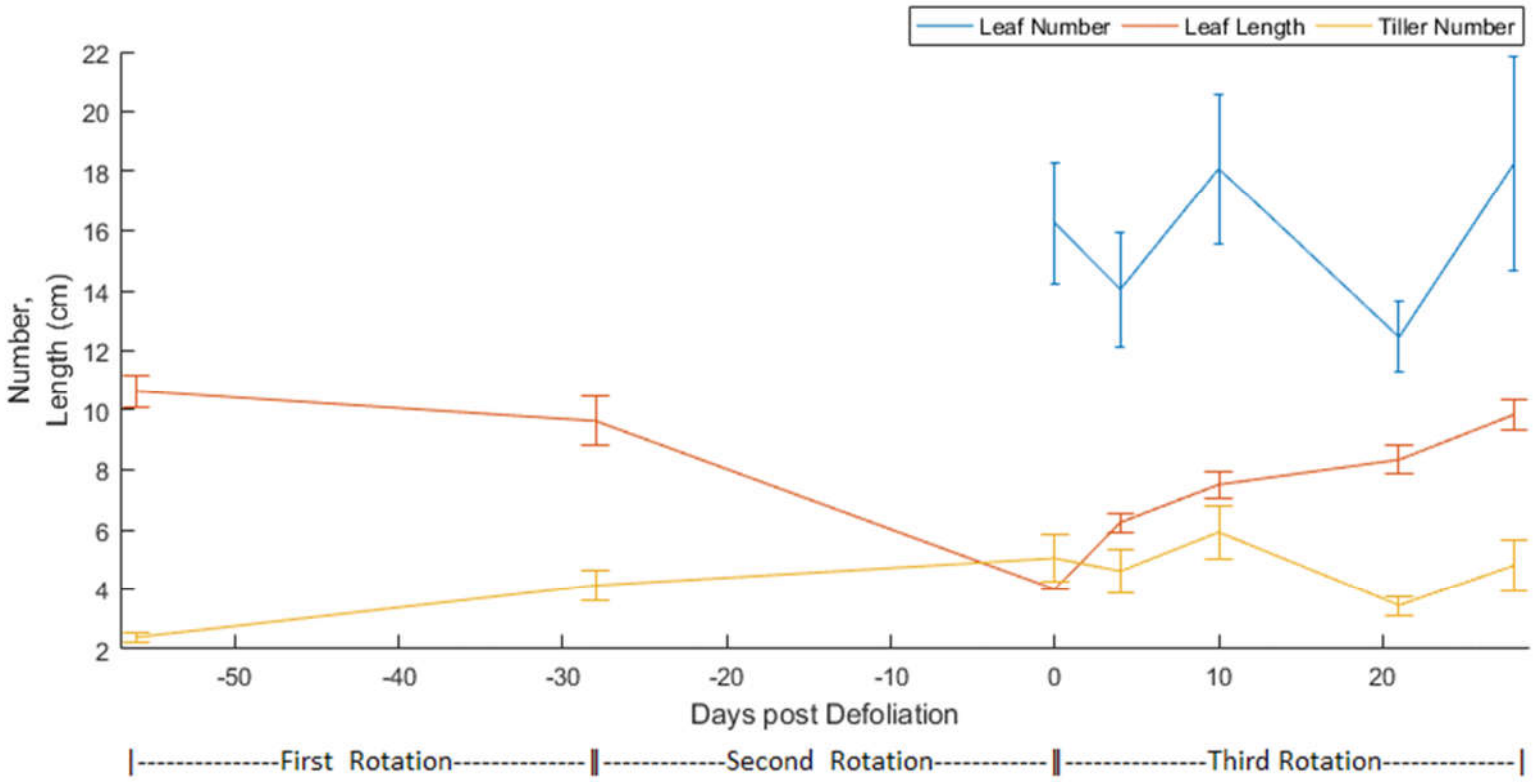

Figure 4-2:Leaf length, leaf number and tiller number measured over the 3 rotations of the experiment in control plants – plants left untreated by N or GA_3. Plants were measured at D0 of each rotation prior to being cut to 40 mm above ground level for Rotation 1 and Rotation 2. Plants were measured on D-56, D-28, D0, D4, D10, D21, D28. N=8 for all points except D4, D10, D21 where N=4

4.2.2 Dry mass measurements

The following figures illustrate the effect of time post defoliation of application of fertiliser, with and without GA_3 over a growth period of 28 days on dry weight accumulation over the 28-day period.

Dry weight of sample plants was measured on days that treatments were applied, as well as at the end of the rotation. For those plants treated on day 21 post defoliation there was a significant decrease in all plant tissues irrespective of whether GA_3 was applied (Figure 4-3). For those plants treated on day 0, there is significantly lower mass in the leaf blade for both treatments and the roots without GA_3 applied (Figure 4-3), with a concurrent increase in stubble mass for both treatments and roots with GA_3 applied. However, the effects of different treatments over time do not show the same significant points (Figure 4-4). Figure 4-4 shows a relative decrease in shoot mass in plants treated on days 0 and 28 post defoliation with GA_3, as well as decreases in root mass in plants treated on days 0 and 6 post defoliation. Overall, at the end of the harvest there is an apparent trend that application of GA_3 can lead to a relative decrease in below ground mass compared to those plants not exposed to GA_3.

Figure 4-5 shows the dry weight of roots harvested at the end of the third experimental rotation. Pots were divided into two groups, for some the whole pot was harvested, whilst for others only the top 100 mm of soil was collected to harvest roots from (the rational for this was discussed in the section 2.1.2). The mass of roots harvested from the top 100 mm was consistently in the region of half of the weight of roots harvested from the whole pot.

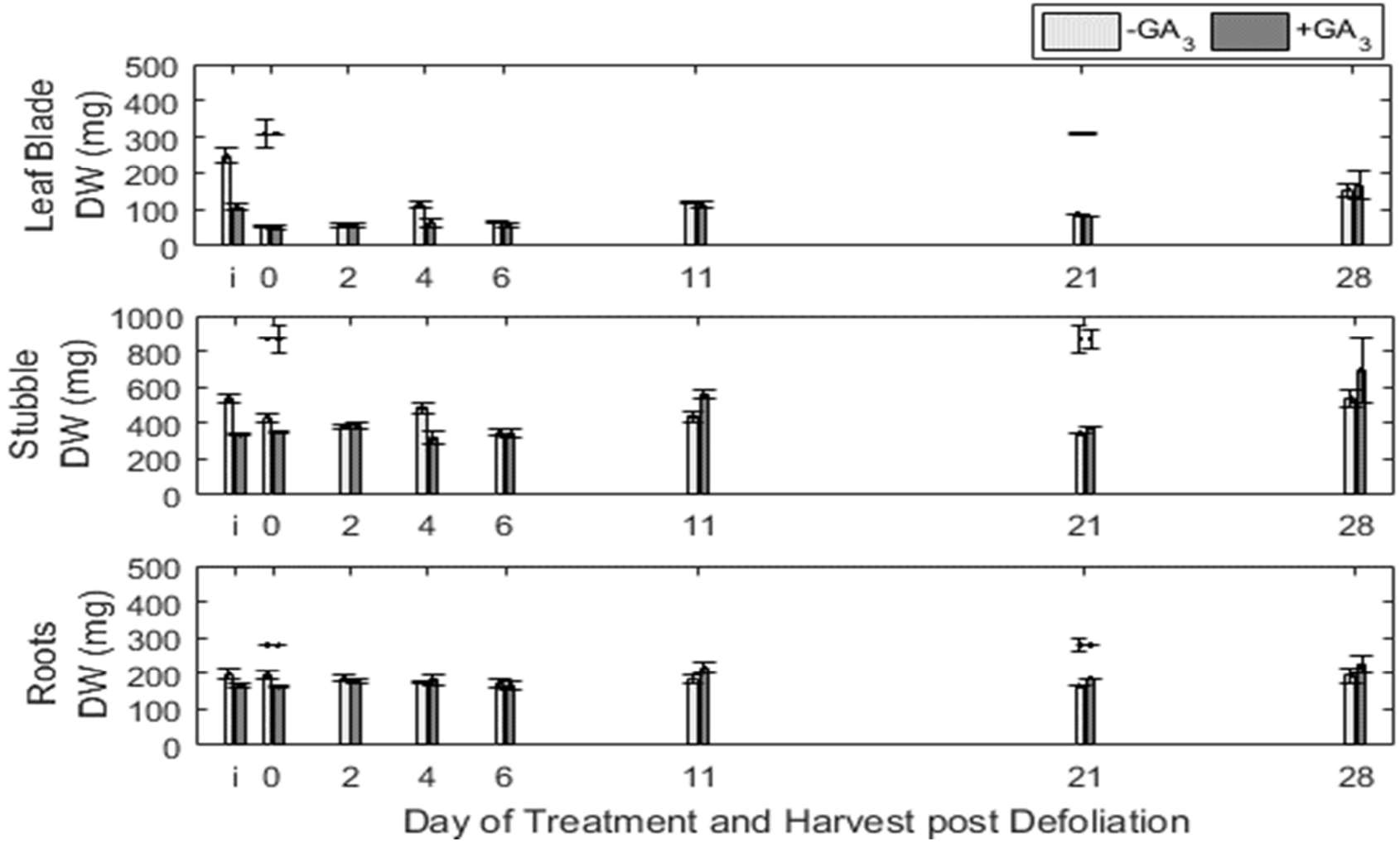

Figure 4-3: Dry weight of samples harvested from pots for ^{15}N analysis during the 28-day experimental rotation for different plant tissues: Tissue Blade (plant material above 40 mm from the root crown); Stubble (root crown - 40 mm above); Roots (all below ground growth). All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

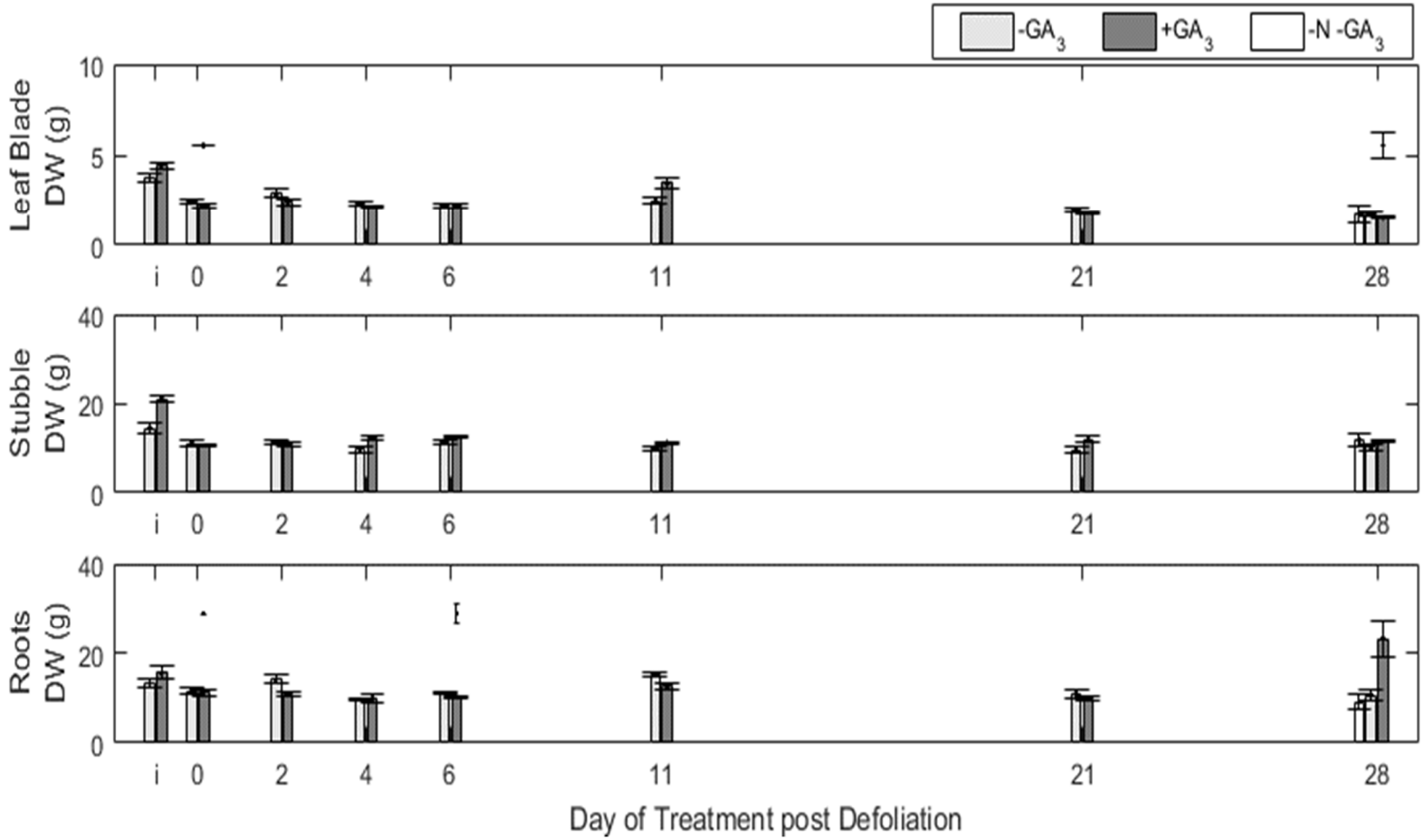

Figure 4-4: Dry weight of different plant tissues at the end of the rotation period: Tissue Blade (plant material above 40 mm from the root crown); Stubble (root crown - 40 mm above); Roots (all below ground growth). All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

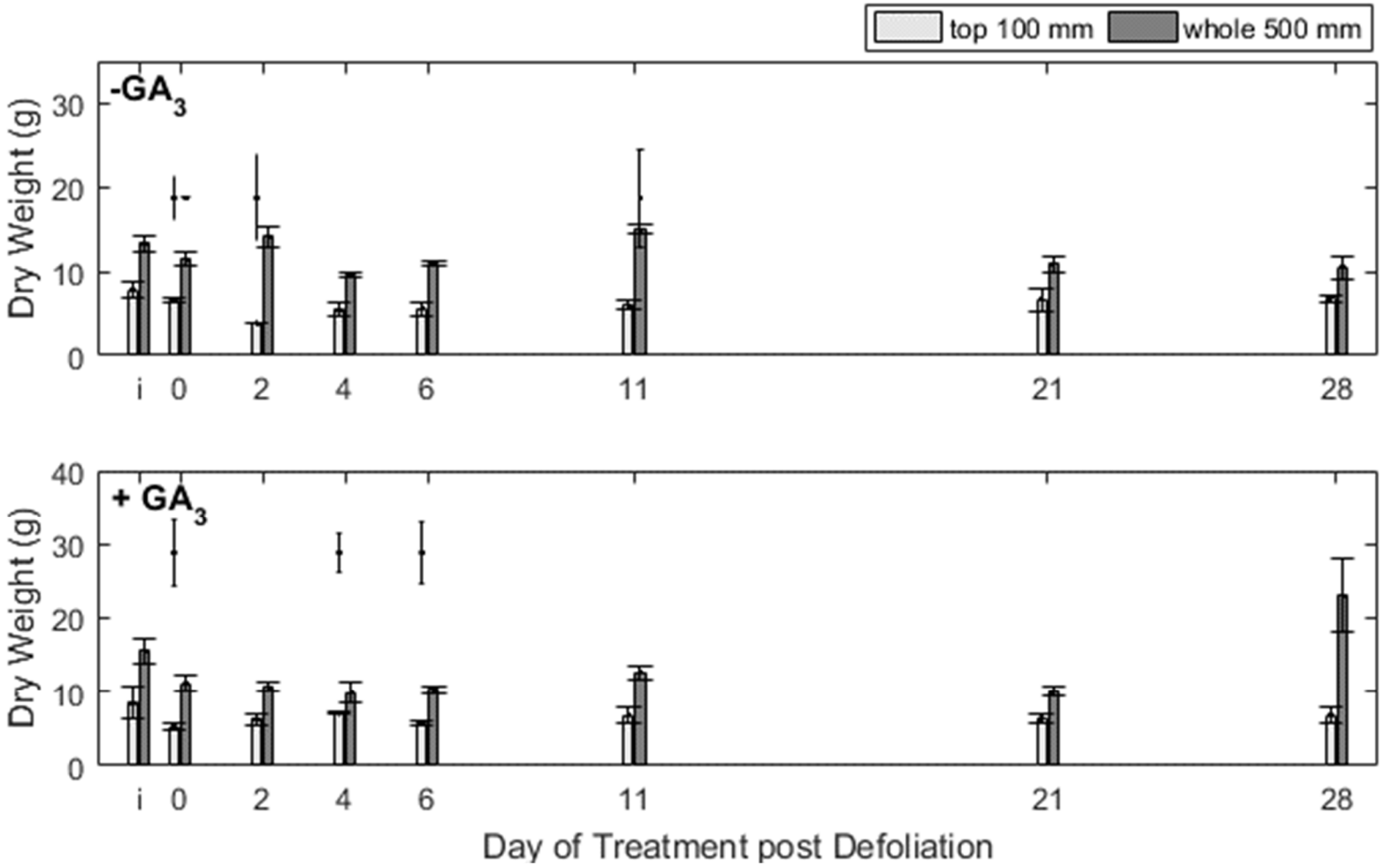

Figure 4-5: Dry weight of roots harvested at the end of the rotation period. Pots were divided into two groups where the whole pot (500 mm) was harvested, and the top 100 mm of soil was harvested. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

4.2.3 Nitrogen uptake

These figures demonstrate nitrogen uptake efficiency in plants over time, and the effect of co-incidental GA_3 treatment. ^{15}N labelled fertiliser was applied at different time points over the 28-day growth period (section 2.1.2), samples were then prepared and analysed as described in Section 2.1.4.

Nitrogen uptake rate was lowest in the leaf blades between in plants treated at days 0 and 4 post defoliation across all treatments but most significantly in plants to which GA_3 had been applied, with a marked increase in uptake occurring at day 21 +GA_3 (Figure 4-6). There was a significant decrease in N uptake rate in the stubble on plants treated at days 6 and 11 post defoliation without GA_3 treatment. At day 21 post defoliation both treatments showed a significant increase in uptake rate. In the roots there was no significant difference in N uptake rate over the different treatments.

Percentage nitrogen derived from fertiliser was highest in the roots and lowest in the shoots (Figure 4-7) and is the same whether the plants were treated with GA_3 or not, the only significant differences being that of day of application.

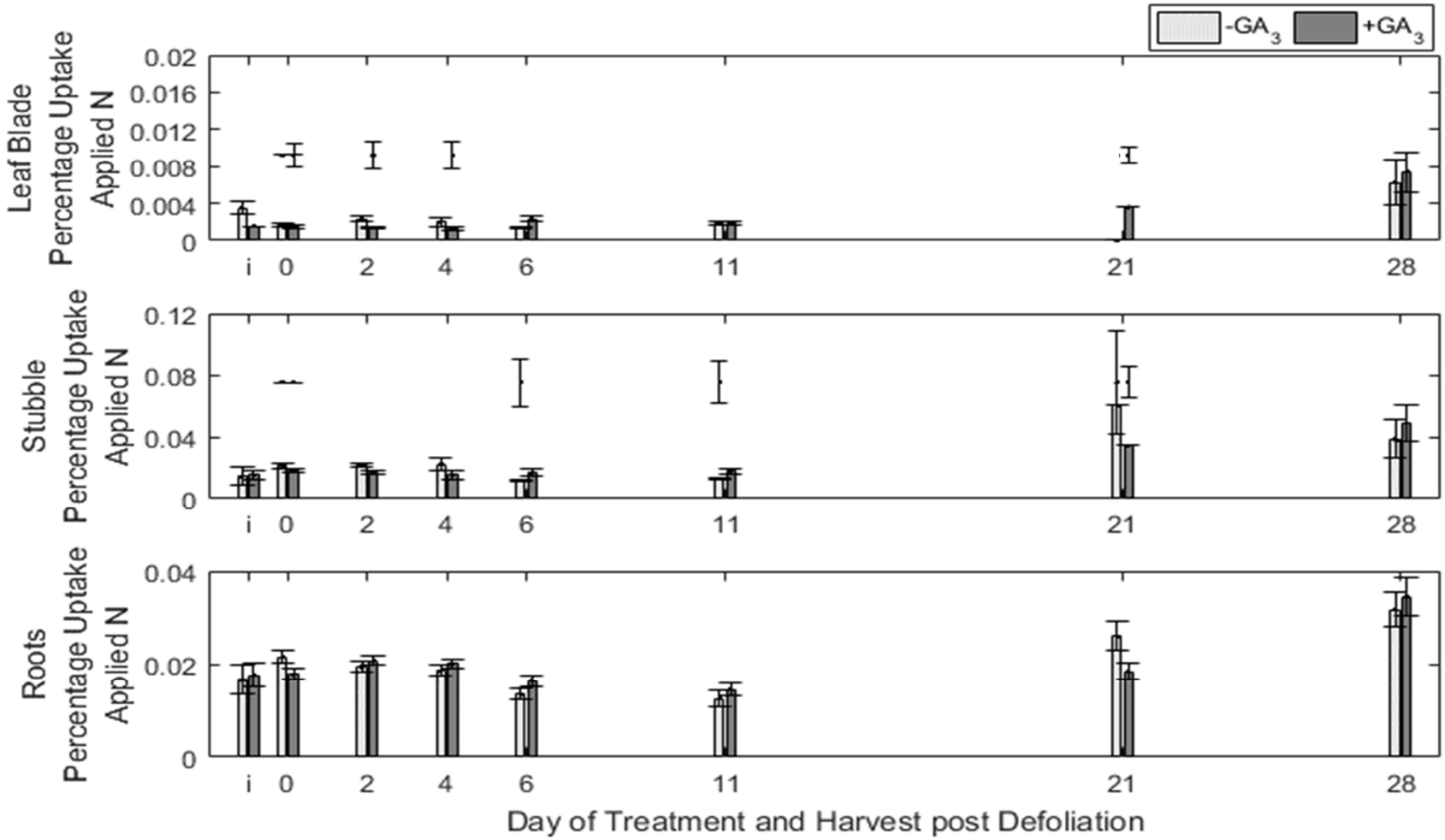

Figure 4-6: Percentage uptake of applied nitrogen 24 hours after N (with or without GA_3) treatment in different plant tissues, i.e. the uptake rate of applied N in plant tissues. Tissue Blade (plant material above 40 mm from the root crown); Stubble (root crown - 40 mm above); Roots (all below ground growth). All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

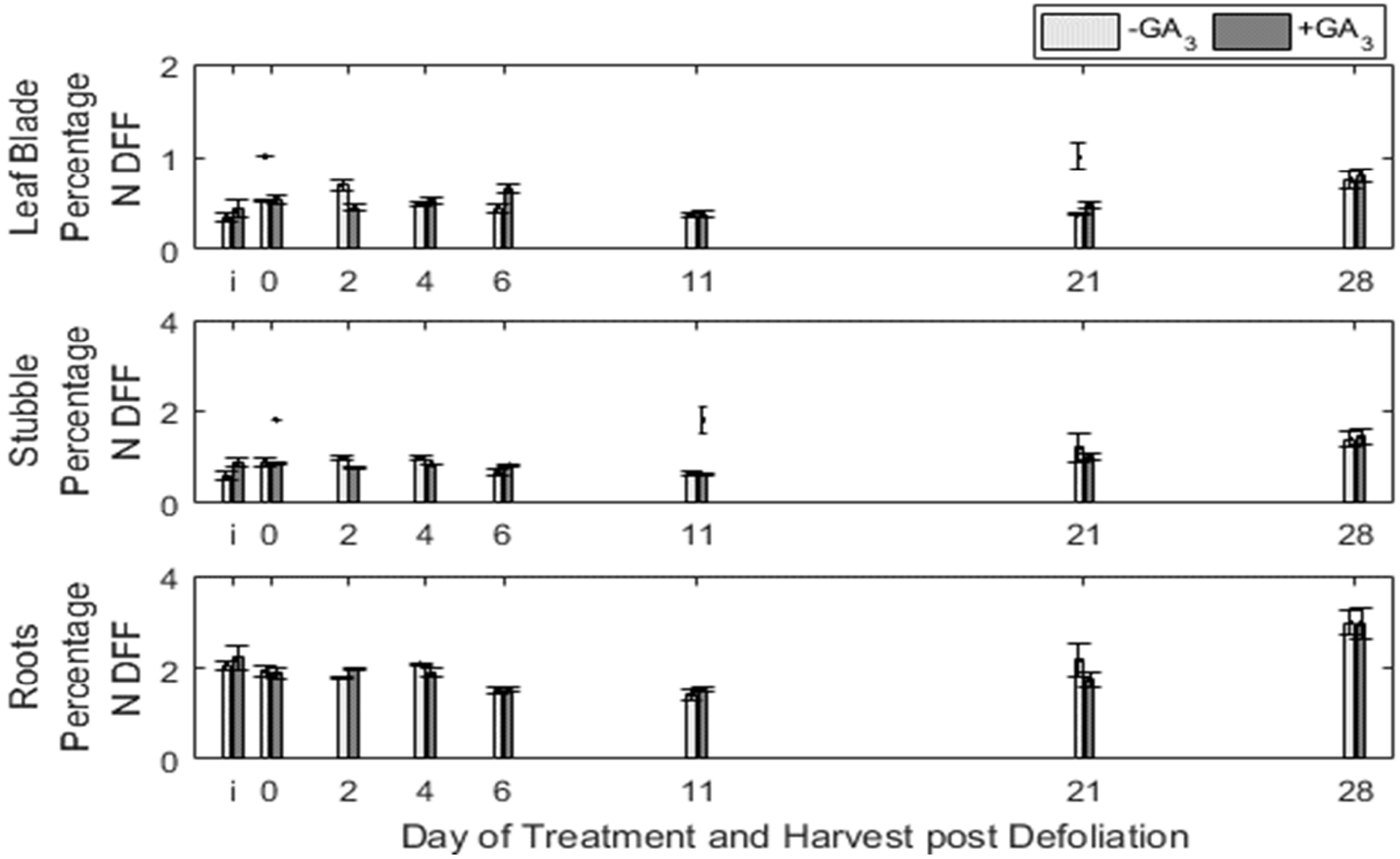

Figure 4-7: Nitrogen derived from fertiliser, i.e. the percentage of total N in plant tissues that was derived from fertiliser. Tissue Blade (plant material above 40 mm from the root crown); Stubble (root crown - 40 mm above); Roots (all below ground growth). All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

4.2.4 Carbohydrate storage patterns

Fructan is the main storage carbohydrate in *L. perenne*, and is primarily found in the stubble and roots of plants. The trend of fructan accumulation over time shown in plants not treated with GA_3 is illustrated in Figure 4-8. Fructans were observed to be mobilised from the stubble to support shoot growth, leading to an initial decrease in fructan content (Figure 4-8). The decrease starts to be reversed between day 4 and 6. Figure 4-8 shows that on day 6, with application of GA_3, there was a significant decrease in fructan content. This data also shows little change in fructan content from day 11 to day 28 when GA_3 was not applied.

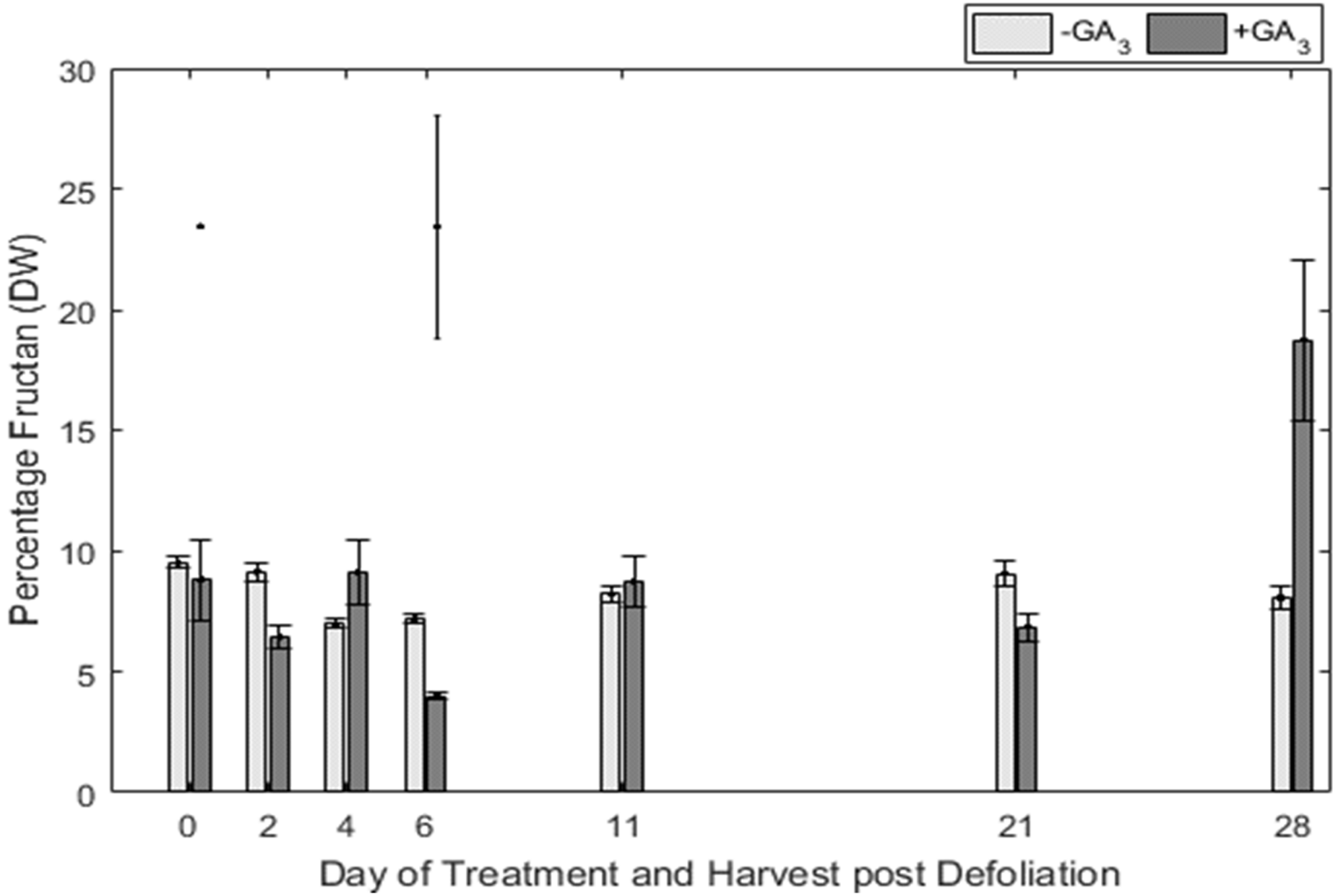

Figure 4-8: Fructan accumulation in the stubble following defoliation. All plants were cut on day zero, and fertiliser was applied on days indicated above. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

4.2.5 Morphological data

There was no significant difference between leaf number or tiller number over the 28-day rotation between any treatments (see section 7.1.2.5). However, there was an effect of day of treatment post defoliation on shoot length (Figure 4-9). Leaf length of plants left intact at the beginning of rotation 3 were longer than all other plants, a difference that persisted until day 10 post defoliation. Although the ANOVA indicated that there was no effect of GA_3 treatment, there was a difference by day four post defoliation between those plants treated with GA_3 and nitrogen on day two post defoliation compared to all other plants treated with GA_3 and nitrogen.

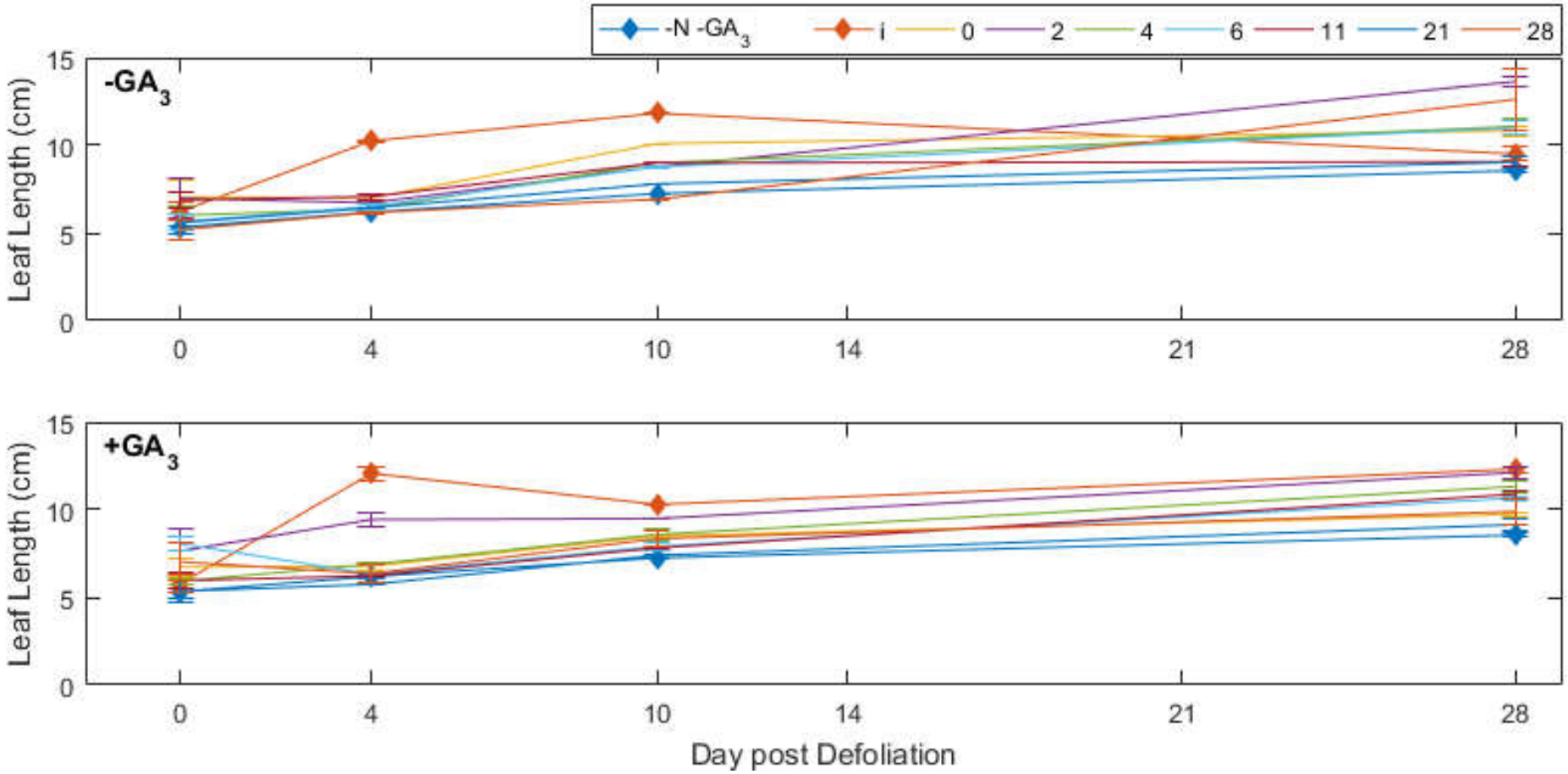

Figure 4-9: Leaf length over the 28 day rotation starting from day 0 post defoliation. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars indicate standard error of the mean.

4.2.5.1 Percentage canopy cover

Determination of canopy cover by analysis of percentage green pixels did not yield useful results. It would be expected that there would be more difference in the percentage green pixels from day 0 to day 28 (Figure 4-10). However, in the experiment this was not observed. This may be in part due to a lack of control of light levels and colour. As a result, this method did not detect the visual differences noted as occurring over time, as evidence by the little change between day 0 and day 28 post defoliation, despite leaf blade growth occurring (Figure 4-9).

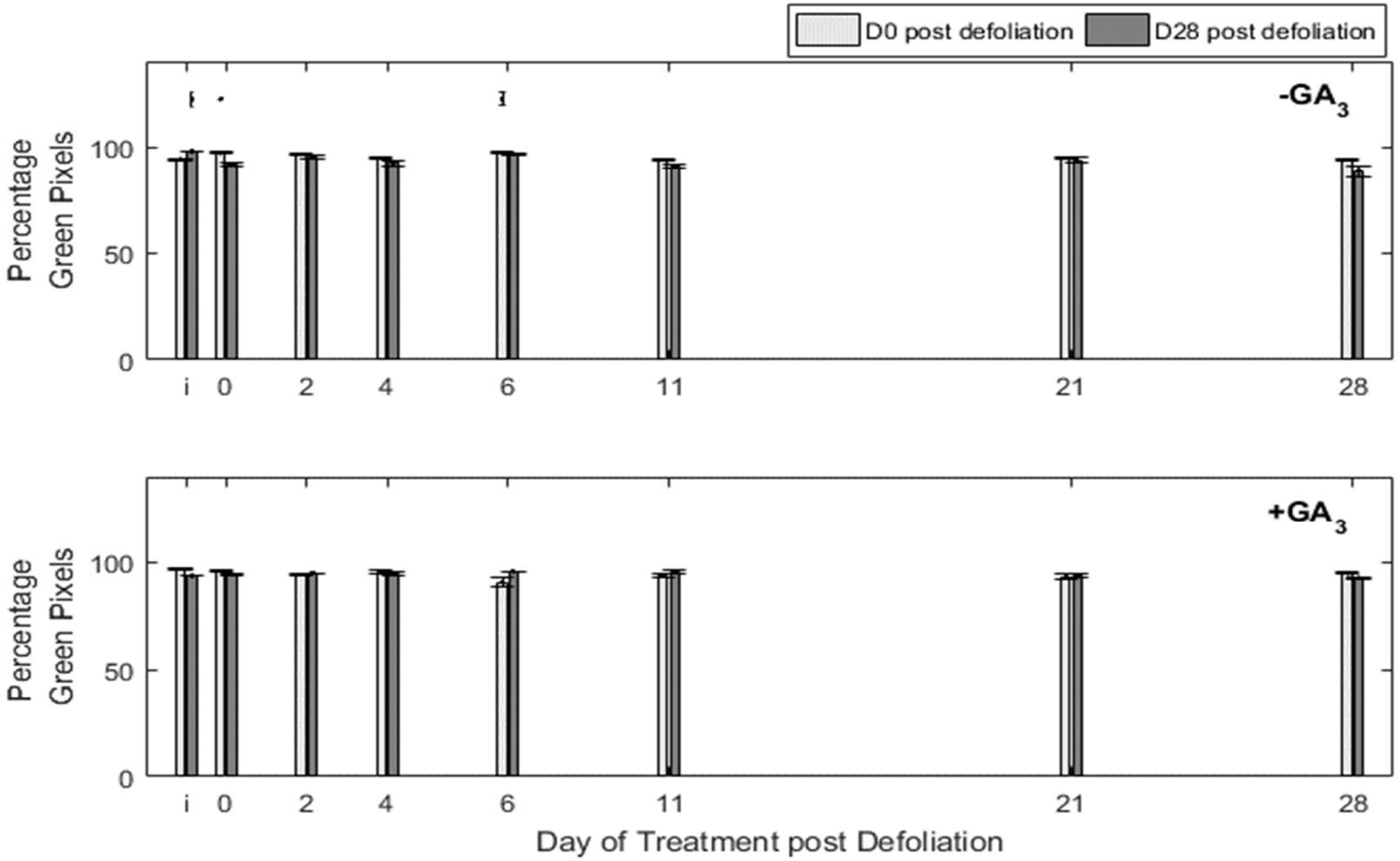

Figure 4-10: Percentage of green pixels in each pot on day 0 and day 28. Panel 1 shows pots without GA_3 treatment, panel 2 those pots with GA_3 treatment. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

4.3 Vertical Plate Plant Growth

4.3.1 Seed sterilisation

Table 4-1 demonstrates that hull removal decreased infection rate and increased germination rate. Method 1 (Table 2-1) seems to be the method that maintains the best balance between germination rate and infection rate and so this method was used for sterilisation of seeds. It would be useful if the time could be shortened for this process (which was the aim of method 5). It is possible that soaking for 5 minutes prior to hull removal in ethanol instead of milli-q water would result in a lower infection rate. The differences between methods 1 and 2, which included a 2nd wash after hull removal seemed to retard the germination rate and vigour of the seedlings without a decreased rate of infection.

Method	Day	% Germinated	% Infected
1	3	57.73	15.45
1	6	78.10	25.42
2	3	46.41	22.05
2	6	50.26	33.59
3	3	0	100
3	6	38.46	100
4	3	6.67	6.67
4	6	6.67	13.33
5	3	40	13.33
5	6	66.67	40

Table 4-1 percentage of germinated and infected *L. perenne* seedlings using different methods of sterilisation as detailed in Table 2-1

4.3.2 Root system architecture (RSA)

No significant difference was observed in the RSA of plants when exposed to a gradient of GA_3 treatments, so data are not shown.

The number of primary and lateral roots (Figure 4-11) was not significantly different between treatments, although it is demonstrated that at higher concentrations, the number of lateral roots is decreased. The total length of roots and the length of lateral roots (Figure 4-12) was significantly less for those plants treated with 5 mM arginine compared to other treatments.

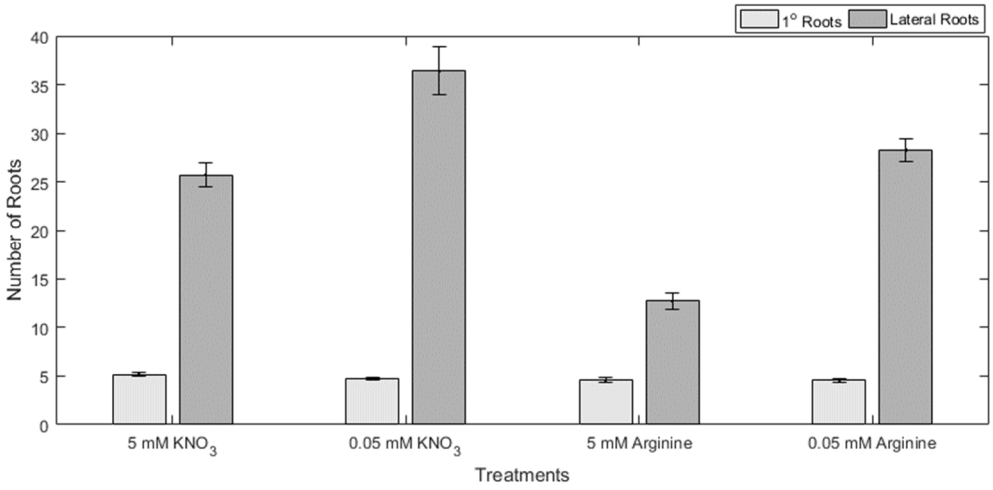

Figure 4-11: Total number of primary and lateral roots as influenced by N provided in different forms (potassium nitrate and arginine) and concentrations (5 and 0.05 mM) of *L. perenne* seedlings grown on Hoagland's media 10 days after transfer

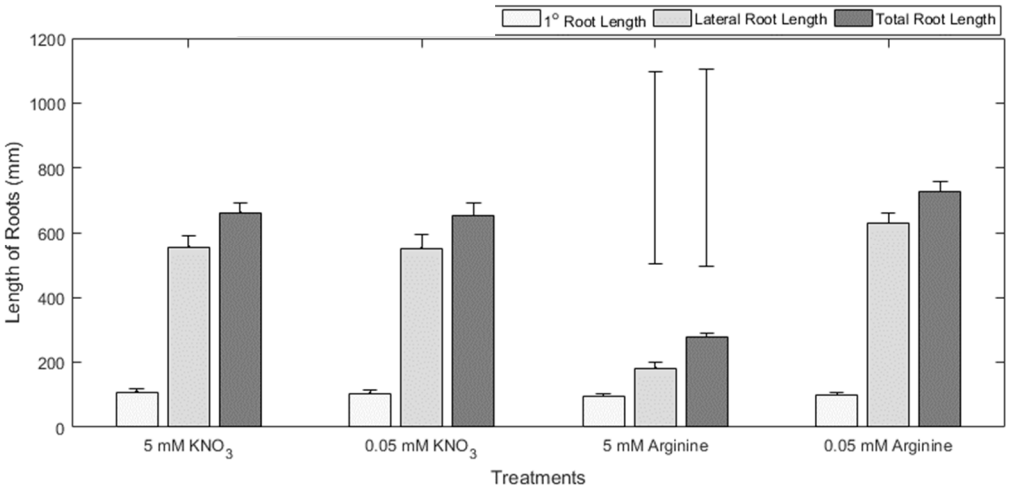

Figure 4-12: Total length of plant, total primary root length and total lateral root length as influenced by N provided in different forms (potassium nitrate and arginine) and concentrations (5 and 0.05 mM) of *L. perenne* seedlings grown on Hoagland's media 10 days after transfer

5 Discussion

The experiments were successful in generating results which confirmed that most of the functional roots are those in the top 100 mm (Figure 4-4), with approximately 50 % of root dry mass accounted for in this volume (Bolinder *et al*., 2002; Crush *et al*., 2005). It is also important to note that the trends in root mass are not the same when considering the top 20 % of roots as with 100 % of roots (as illustrated by the differences observed in Figure 4-5). This further indicates partitioning of roots by function as suggested by McCormack *et al.* (2015)

The experiment also illustrated that there was an increase in dry mass yield from day 21 to day 28 post defoliation (Figure 4-3). This increase coincided with a marked increase in rate of N uptake on day 21 post defoliation, would indicate that pasture growth would benefit from being extended by seven days, with fertiliser addition occurring on day 21 post defoliation. However, there was no significant difference between these two time points in terms of fructan content of the stubble (Figure 4-8), indicating that little benefit may be gained. This will be further explored in future work examining the fructan content of other plant tissues and tissues from the end of the experimental period.

Cross-talk between C and N metabolism has previously been demonstrated (Roche *et al*., 2017) and used to explain plant growth in response to fertiliser treatment. This was also observed in the current research which illustrated an antagonistic relationship shown between fructan content and NDFF in stubble tissues (Figure 4-7, Figure 4-8, Figure 7-35). Figure 4-4 shows that at the end of the harvest period those plants treated at day 6 and day

11 have statistically significant increases in dry mass. Additionally, at these time points the antagonism between C and N content in stubble reverses (Figure 4-7, Figure 4-8, Figure 7-35), indicating that plants are no longer carbon starved as a result of defoliation, and are able to uptake N. Consequently, these data would indicate that the best time to apply fertiliser would be between day six and eleven post defoliation.

Overall there was no statistically significant effect observed form the addition of GA_3, either in terms of dry mass or fructan accumulation. However, there are trends in the data showing a decrease in mass of below ground growth when GA_3 is applied (Figure 4-4). This observation may explain anecdotal evidence of poor pasture growth in the season following the application of GA_3 (Paula Jameson, personal communication; Boom *et al*., 2015). Trends of reduced mass in below ground growth with concurrent increases in mass of above ground growth would confirm the hypothesis that application of GA_3 causes an immediate shift of fructan away from storage locations in the stubble and roots and into the leaves where it can be mobilised for shoot elongation. Fructan analysis of leaf blades and roots will be conducted in the future, as well as analysis of fructan accumulation in all plant tissues at the end of the experimental period.

Data in Figure 4-8 concerning treatments where GA_3 was applied to plants does not show a consistent decline in the fructan in the stubble tissue. The error statistic for day 4 post defoliation is relatively large, so a repetition of this analysis is required to verify the results. This would also seem to be the case for day 28 post defoliation. Assuming these points to be outliers, a trend can be observed of an initial depletion of fructans at a faster and more sustained rate in plants treated with GA_3 than that of plants not treated with GA_3. This would imply that the application of GA_3 is driving cell elongation in the shoots resulting in an

increase in fructan accumulation in the shoots where it can be utilised more quickly through GA_3 acting as a regulator of fructan exohydrolase (Cai *et al*., 2016; Morvan *et al*., 1997). Although this would seem to be contradictory to the work by Gasperl *et al*. (2016) who showed application of GA_3 causing upregulation of FEH and thus increasing the pool of free fructan being mobilised and supporting shoot growth would agree with previous findings that application of GA_3 improves pasture growth (Zaman *et al*., 2016; Zaman *et al*., 2014; Ghani *et al*., 2014).

Whilst the application of GA_3 may aid in the reduction of NO_2 emissions and N leaching, measurement of such effects was beyond the scope of this experiment (Whitehead & Edwards, 2015). In agreement with previous research by Zaman *et al.* (2014) the current study demonstrated that there was no evidence to suggest that the application of GA_3 increased the uptake efficiency of N (Figure 4-6, Figure 4-7) (Zaman *et al*., 2014). In this set of experiments, most grass growth occurred in summer and not autumn as is shown by comparing Figure 4-1 and Figure 4-4. However, there was no evidence to suggest that the application of GA_3 over the autumn period increased plant growth by rescuing plants from a strategy of limited growth as previously proposed (Zaman *et al*., 2014; Parsons *et al*., 2013).

Total and lateral root length was shown to be affected by different forms and concentrations of nitrogen (Figure 4-12). Abundance of N negated the need for roots to extensively forage for N, but could negatively impact the plant in the long run by not having

an extensive root system to support itself in times of stress such as drought or poor nutrient availability. The significant differences noted in plants treated with a high concentration of arginine indicates that foraging for nitrogen was no longer necessary and so energy was diverted into other above ground growth (Miller & Cramer, 2005; Hodge, 2009; Ruffel *et al*., 2011).

No significant effect of GA_3 application was noted on the roots or overall morphology of the plants *in vitro.* This could have been due to the short amount of time plants were exposed to treatments. If this experiment was to be repeated with a longer exposure to treatments, significant differences may be observed.

Despite the benefits of this work being carried out on vertical plates with Hoagland's media as the growth substrate to allow imaging of roots, drawbacks include that the media does not imitate heterogeneity of nutrient dispersion in soil, nor the consistency of soil itself (Clark *et al*., 1999). It would be interesting to observe if differences in root growth persisted in plants grown in soil and fertilised with N in different forms and concentrations.

5.1 Conclusions and implications

Although there are no strong significant differences noted in N uptake post defoliation, the data suggest that it may be possible to increase N uptake (and subsequently reduce N leaching) by extension of the grazing rotation from 21 to 28 days, with application of fertiliser on day 21. However, more work is needed to confirm if this would make a significant difference in increasing yields and reducing excess application of N. This should maintain productivity for the farmer of that pasture. However, this would require extra land for grazing to be able to extend the grazing period, and/or supplementary feed to be provided to cows.

The relationship demonstrated between N and C content of stubble tissues throughout the experiment, and dry mass yield at the end of the experiment more strongly indicate that growth could be maximised by the application of fertiliser treatments between day six and eleven days post defoliation. However, further research is needed to determine if the results found in this study are applicable to the field in a 21-day grazing rotation. In addition, this finding has more potential to be applicable to the dairy system, by not requiring additional pasture or supplementary feed. This work has taken this area of research closer to a true field environment. Ongoing experiments are needed to further investigate the data and the trends presented here, which may be masked due to the high variability within the results.

Application of GA_3 seems to neither hinder nor assist in the growth of *L. perenne*, but there is evidence presented to suggest that the application to GA_3 could have long term negative

effects on the pasture which may not be apparent in one grazing cycle, as a result of an apparent shift in fructan accumulation away from plant tissues where it is typically stored and an overall trend of decreased below ground dry mass. It is important that this work be repeated and that the impact of application of GA_3 over multiple rotations on all plant tissues is characterised.

The use of arginine has potential to aid in increasing productivity of the agricultural sector whilst reducing detrimental effects to the environment resulting from agricultural activity through conservation of plant energy for above ground growth. This research demonstrates that the application of arginine at high concentrations can significantly decrease the number of lateral roots of the plant, compared to other treatments, presumably because the plant no longer needs to forage extensively for nutrients.

Furthering this research by carrying out experiments on a medium such as a sterile sand or soil substrate instead of Hoagland's media for a longer duration would allow for better characterisation of whole plant growth and partitioning of resources for above and below ground growth. This would demonstrate if the use of amino acids in fertilisers could aid in the increase of productivity, or the reduction of fertiliser leaching from fields. This research would have larger ramifications for the agricultural community if carried out on soil and if there were investigations into the effect on the mycorrhizal community over time.

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7 Appendices

7.1.1 Bias between blocks in summer treatment

Because of high variability observed in data, additional effects were investigated. Bias was found based on position of pots by comparing the mass of each set of treatments (assuming that they should be the same) and comparing the mass to the position. The close the data points to zero the less bias as a result of positioning. The gradient of performance runs from North – South, but not East to West (Figure 3-1) indicating that there may be a possible confounding factor of a temperature or shading gradient.

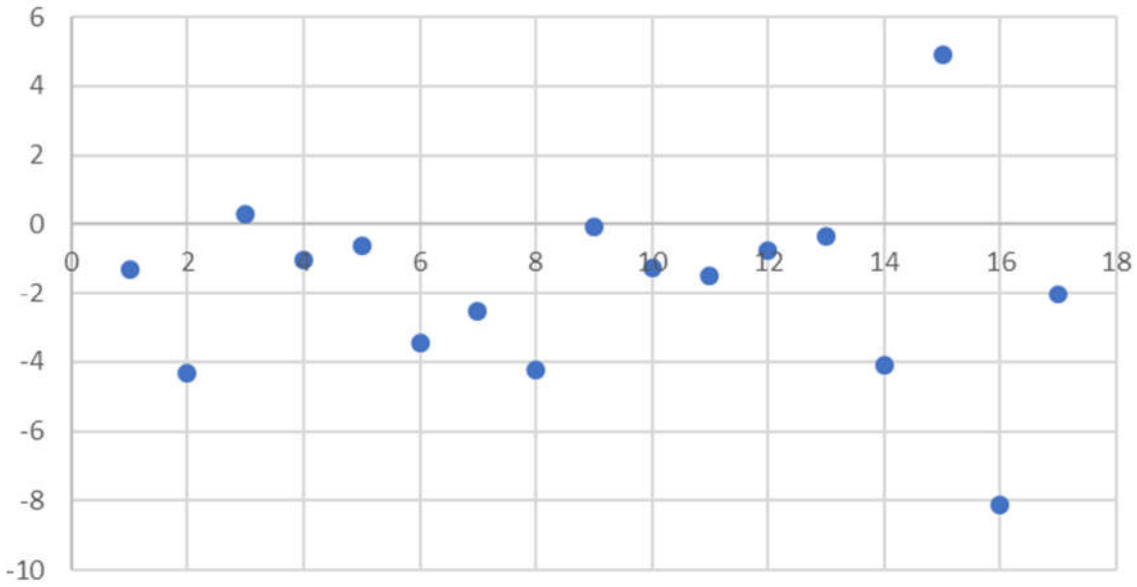

Figure 7-1: Position bias in the N - S direction.

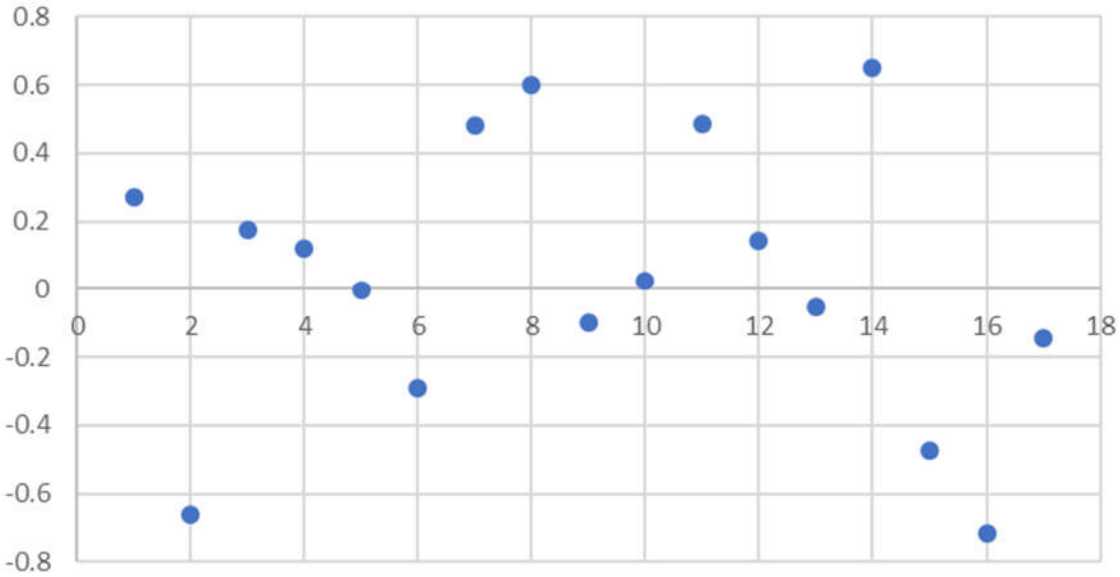

Figure 7-2: Position bias in the E - W direction

7.1.2 ANOVA tables

7.1.2.1 Autumn deep pot dry weights

These ANOVA tables show that no significant difference was found in dry mass measurements for plants treated with GA_3 and N at different times post defoliation during the Autumn rotation.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.22695	6	0.03783	1.5	0.2077
Treatment	0.00559	1	0.00559	0.22	0.6404
Day*Treatment	0.05521	6	0.0092	0.37	0.8954
Error	0.83036	33	0.02516		
Total	1.14349	46			

Figure 7-3: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in leaf blades.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.47443	6	0.07907	0.75	0.6106
Treatment	0.03009	1	0.03009	0.29	0.5958
Day*Treatment	0.23974	6	0.03996	0.38	0.8857
Error	3.45967	33	0.10484		
Total	4.21036	46			

Figure 7-4: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in stubble..

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	1.5211	6	0.25352	0.18	0.9798
Treatment	0.2316	1	0.23156	0.17	0.686
Day*Treatment	3.1215	6	0.52025	0.37	0.8904
Error	45.9435	33	1.39223		
Total	50.5896	46			

Figure 7-5: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in roots.

7.1.2.2 Summer deep pot dry weights

These ANOVA tables in Figure 7-6 - Figure 7-8 show a significant effect of day of treatment and harvest post defoliation across all tissue types. These data relate to Figure 4-3.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	65917.6	6	10986.3	8.85	0
Treatment	659.1	1	659.1	0.53	0.4707
Day*Treatment	2849.1	6	474.8	0.38	0.8856
Error	47185	38	1241.7		
Total	117202.9	51			

Figure 7-6: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on dry mass during the 28 day harvest on the day of treatment in leaf blades.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	367599.2	6	61266.5	2.99	0.0172
Treatment	764.5	1	764.5	0.04	0.8479
Day*Treatment	122329.5	6	20388.2	0.99	0.4428
Error	778751.3	38	20493.5		
Total	1259331.3	51			

Figure 7-7: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on dry mass during the 28 day harvest on the day of treatment in stubble.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.00241	6	0.0004	3.85	0.0028
Treatment	0	1	0	0	0.9481
Day*Treatment	0.00024	6	0.00004	0.39	0.8837
Error	0.00573	55	0.0001		
Total	0.00839	68			

Figure 7-8: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on dry mass during the 28 day harvest on the day of treatment in roots.

These ANOVA tables in Figure 7-9 - Figure 7-11 show a significant effect of day of treatment and harvest post defoliation in leaf blades only. These data relate to Figure 4-4.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	11.4836	6	1.91393	2.45	0.0362
Treatment	0.0305	1	0.03048	0.04	0.8442
Day*Treatment	3.5223	6	0.58704	0.75	0.6112
Error	42.1992	54	0.78147		
Total	57.4305	67			

Figure 7-9: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in leaf blades.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	8.432	6	1.4053	0.18	0.9816
Treatment	20.673	1	20.6727	2.62	0.1112
Day*Treatment	21.088	6	3.5146	0.45	0.8448
Error	425.831	54	7.8858		
Total	476.798	67			

Figure 7-10: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in stubble..

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	211.99	6	35.3314	1.63	0.1763
Treatment	5.13	1	5.1293	0.24	0.6306
Day*Treatment	259.73	6	43.2881	2	0.1001
Error	607.44	28	21.6942		
Total	1089.19	41			

Figure 7-11: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on dry mass at the end of the 28 day harvest in roots.

7.1.2.3 Nitrogen uptake

These ANOVA tables Figure 7-12 - Figure 7-14 show a significant effect of day of treatment across all tissue types. These data relate to Figure 4-6.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.00015	5	3.01376e-05	5.36	0.0008
Treatment	0	1	2.12245e-08	0	0.9513
Day*Treatment	0.00001	5	1.50229e-06	0.27	0.9282
Error	0.00022	39	5.62018e-06		
Total	0.00039	50			

Figure 7-12: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % uptake applied N in leaf blades.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.00385	5	0.00077	5.15	0.0011
Treatment	0.00001	1	0.00001	0.08	0.7761
Day*Treatment	0.00039	5	0.00008	0.52	0.7609
Error	0.00553	37	0.00015		
Total	0.00984	48			

Figure 7-13: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % uptake applied N in stubble.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.00241	6	0.0004	3.85	0.0028
Treatment	0	1	0	0	0.9481
Day*Treatment	0.00024	6	0.00004	0.39	0.8837
Error	0.00573	55	0.0001		
Total	0.00839	68			

Figure 7-14: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % uptake applied N in roots.

These ANOVA tables Figure 7-15 - Figure 7-17 show a significant effect of day of treatment across all tissue types. These data relate to Figure 4-7

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	0.97942	6	0.16324	3.2	0.0095
Treatment	0.01322	1	0.01322	0.26	0.6129
Day*Treatment	0.26316	6	0.04386	0.86	0.5308
Error	2.65357	52	0.05103		
Total	3.90479	65			

Figure 7-15: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % N derived from fertiliser in leaf blades.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	3.5074	6	0.58457	2.81	0.0189
Treatment	0.052	1	0.05199	0.25	0.6191
Day*Treatment	0.2363	6	0.03938	0.19	0.9785
Error	11.0194	53	0.20791		
Total	14.824	66			

Figure 7-16: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % N derived from fertiliser in stubble.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	15.0131	6	2.50218	3.3	0.0075
Treatment	0.0557	1	0.05567	0.07	0.7873
Day*Treatment	0.6687	6	0.11145	0.15	0.9889
Error	42.4322	56	0.75772		
Total	58.1697	69			

Figure 7-17: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" (-GA_3, +GA_3) on % N derived from fertiliser in roots.

7.1.2.4 Stubble carbohydrate content

Figure 7-18 demonstrates a significant effect of day of treatment, and a significant additive effect of day of treatment combined with treatment with or without GA_3. These data relate to Figure 4-8

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	276.6	6	46.0995	3.12	0.0105
Treatment	5.5	1	5.5026	0.37	0.544
Day*Treatment	299.34	6	49.8905	3.38	0.0066
Error	811.71	55	14.7584		
Total	1341.9	68			

Figure 7-18: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on fructan content of stubble tissues.

7.1.2.5 Morphological data

No significant differences were noted in the ANOVA as a result of day of treatment post defoliation or type of treatment. These data relate to Figure 4-10.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Day	195.66	7	27.9515	0.87	0.5374
Treatment	20.56	1	20.5593	0.64	0.4275
Day*Treatment	264.93	7	37.847	1.17	0.3303
Error	2063.53	64	32.2427		
Total	2544.68	79			

Figure 7-19: ANOVA table showing the effects of "Day" (day of treatment) and "Treatment" ($-GA_3$, $+GA_3$) on canopy cover on day 28

7.1.2.6 Root system architecture

Figure 7-20 and Figure 7-21 ANOVA tables demonstrate no significant effect of form or concentration of N applied on the number of primary or lateral roots. These data relate to Figure 4-11.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Concentration	0.3214	1	0.32143	0.13	0.7179
Form	0.8929	1	0.89286	0.37	0.548
Conc*Form	0.3214	1	0.32143	0.13	0.7179
Error	57.7143	24	2.40476		
Total	59.25	27			

Figure 7-20: ANOVA table show the effects of "Concentration" (5 or 0.05 mM) and "Form" (arginine or nitrate) of N on the number of primary roots after 10 days.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Concentration	1.4	1	1.44	0	0.9879
Form	638.9	1	638.89	0.1	0.75
Conc*Form	107.8	1	107.81	0.02	0.8958
Error	147543.7	24	6147.65		
Total	148291.8	27			

Figure 7-21: ANOVA table show the effects of "Concentration" (5 or 0.05 mM) and "Form" (arginine or nitrate) of N on the number of lateral roots after 10 days.

Figure 7-22 - Figure 7-24 relate to Figure 4-12. These figures show no effect of N form or concentration on total length of primary roots. They do show a significant effect of concentration of N on the total length of all roots and total length of lateral roots. Additionally demonstrated is an effect of form of N on total root length and an additive effect of form and concentration on total length of lateral roots.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Conccentration	1.4	1	1.44	0	0.9879
Form	638.9	1	638.89	0.1	0.75
Conc*Form	107.8	1	107.81	0.02	0.8958
Error	147543.7	24	6147.65		
Total	148291.8	27			

Figure 7-22: ANOVA table show the effects of “Concentration” (5 or 0.05 mM) and “Form” (arginine or nitrate) of N on the total length of primary roots after 10 days.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Concentration	343848.2	1	343848.2	4.73	0.0397
Form	148928.3	1	148928.3	2.05	0.1652
Conc*Form	357889.9	1	357889.9	4.92	0.0362
Error	1744730.6	24	72697.1		
Total	2595397	27			

Figure 7-23: ANOVA table show the effects of “Concentration” (5 or 0.05 mM) and “Form” (arginine or nitrate) of N on the total length of lateral roots after 10 days.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Concentration	1209.14	1	1209.14	8.52	0.0075
Form	782.29	1	782.29	5.51	0.0275
Conc*Form	41.29	1	41.29	0.29	0.5947
Error	3408	24	142		
Total	5440.71	27			

Figure 7-24: ANOVA table show the effects of “Concentration” (5 or 0.05 mM) and “Form” (arginine or nitrate) of N on the total length of primary and lateral roots after 10 days.

7.1.3 Morphological Measurements over Experimental Rotation

The following figures in this section demonstrate measurements of morphological features of plants from the summer experimental deep pots. The only data that has any significant differences are those of leaf length over the 28 day period (Figure 7-25 and Figure 7-26) the data provided here to demonstrates the variability of the data-set and to give an idea of trends observed over time.

The significant points data from Figure 7-25 and Figure 7-26 have previously been shown in Figure 4-9.

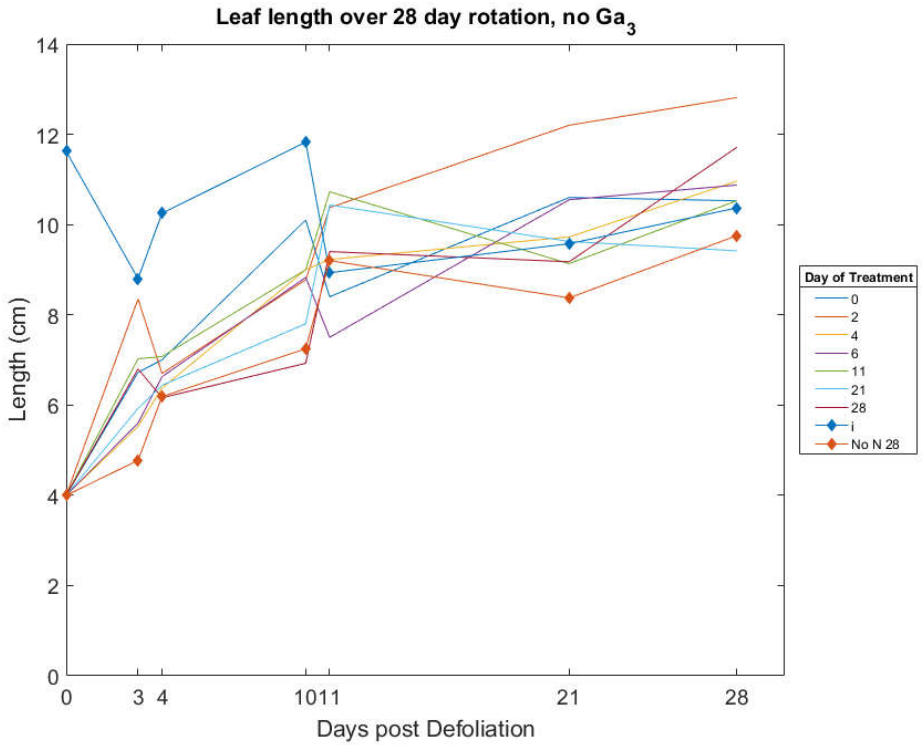

Figure 7-25: Leaf Length measured from ground level to tips of blade over 28 days following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga_3 was applied. Graph displays all treatments with no Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied.

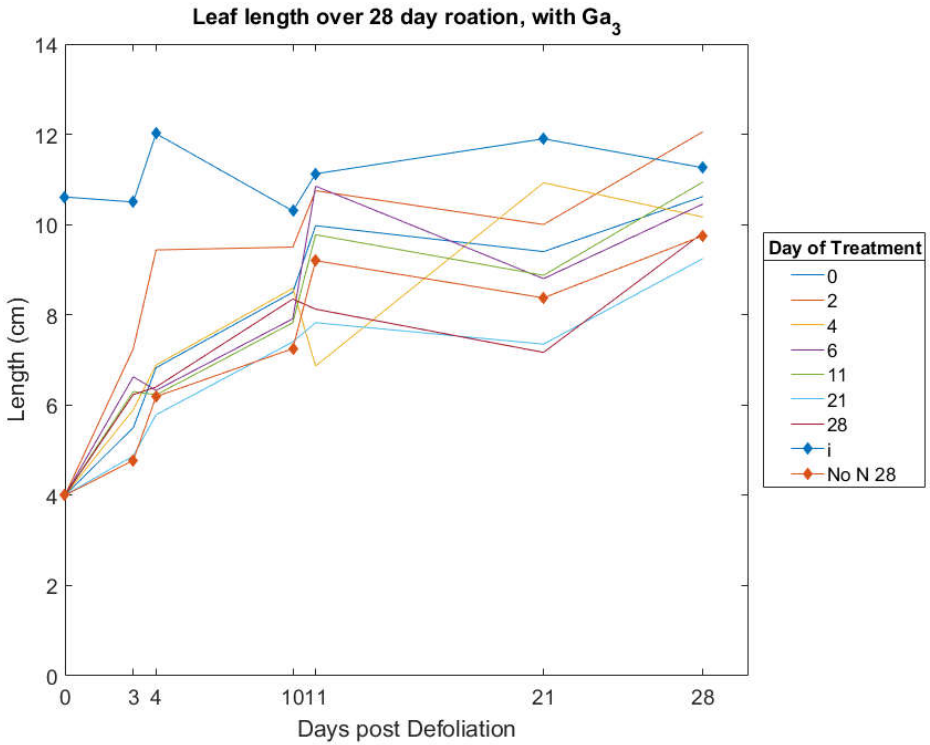

Figure 7-26: Leaf Length measured from ground level to tips of blade over 28 days following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga3 was applied. Graph displays all treatments with Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied.

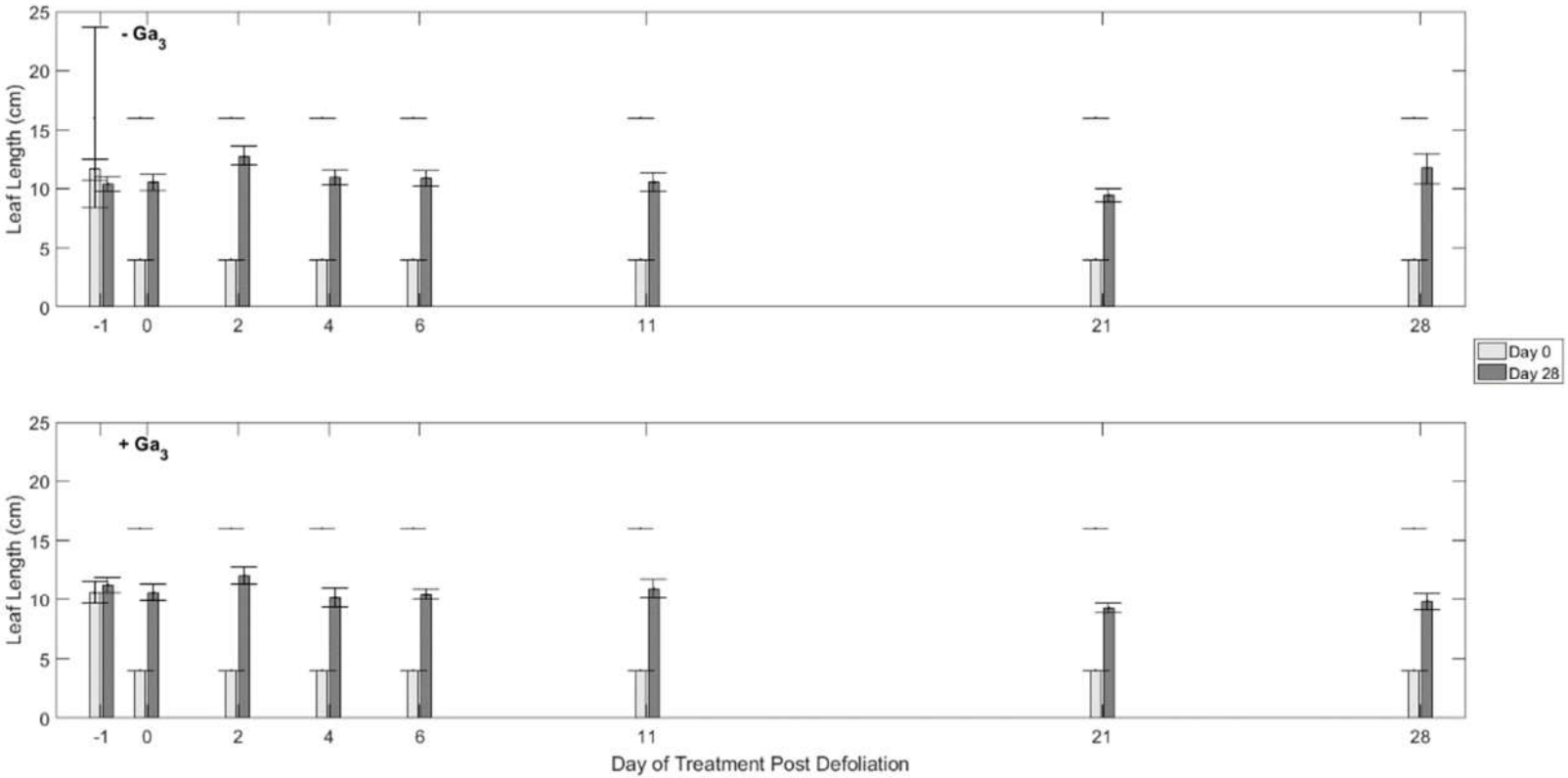

Figure 7-27: Graph comparing leaf length on day zero and day 28. Panel 1 shows treatments without GA_3 application, panel 2 shows treatments with GA_3 application. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

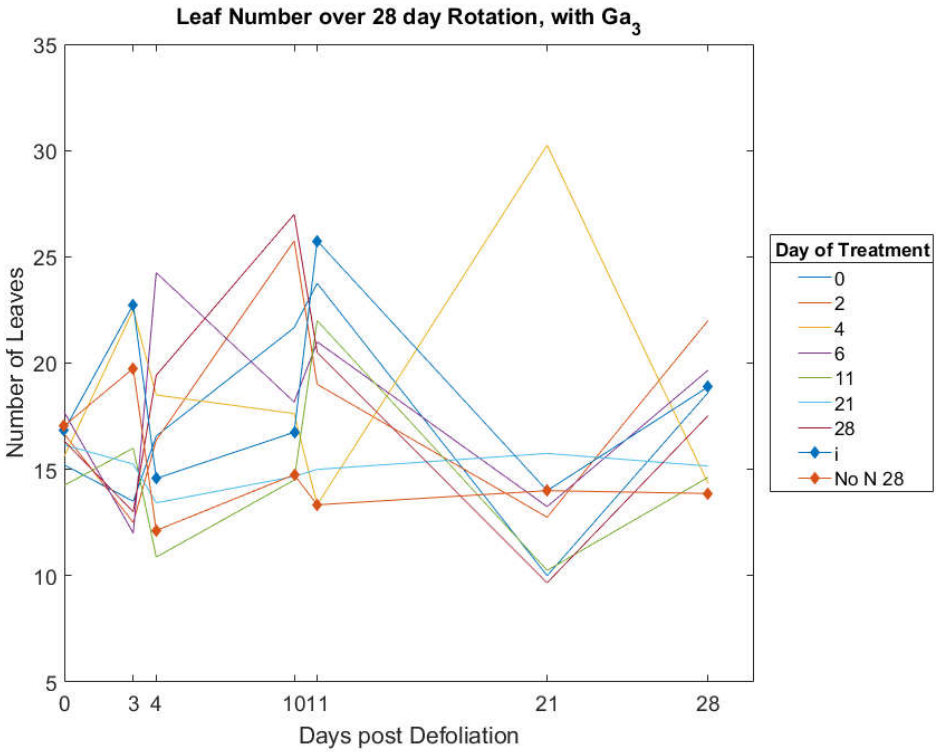

Figure 7-28: Leaf number of plants following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga_3 was applied. Graph displays all treatments with no Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

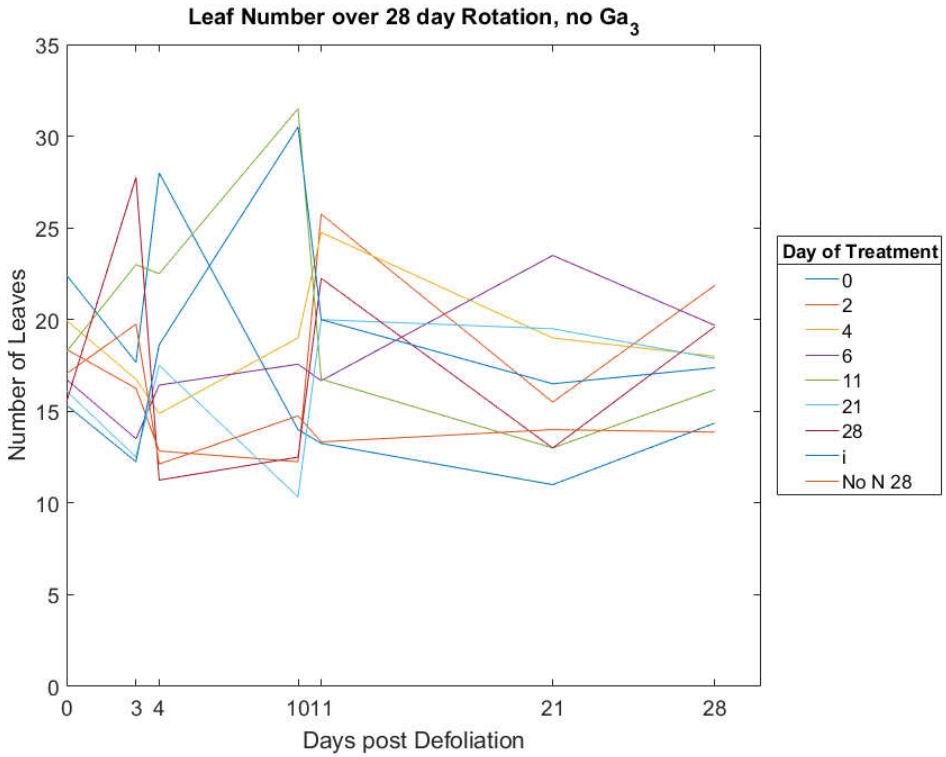

Figure 7-29: Leaf number of plants following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga3 was applied. Graph displays all treatments with Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

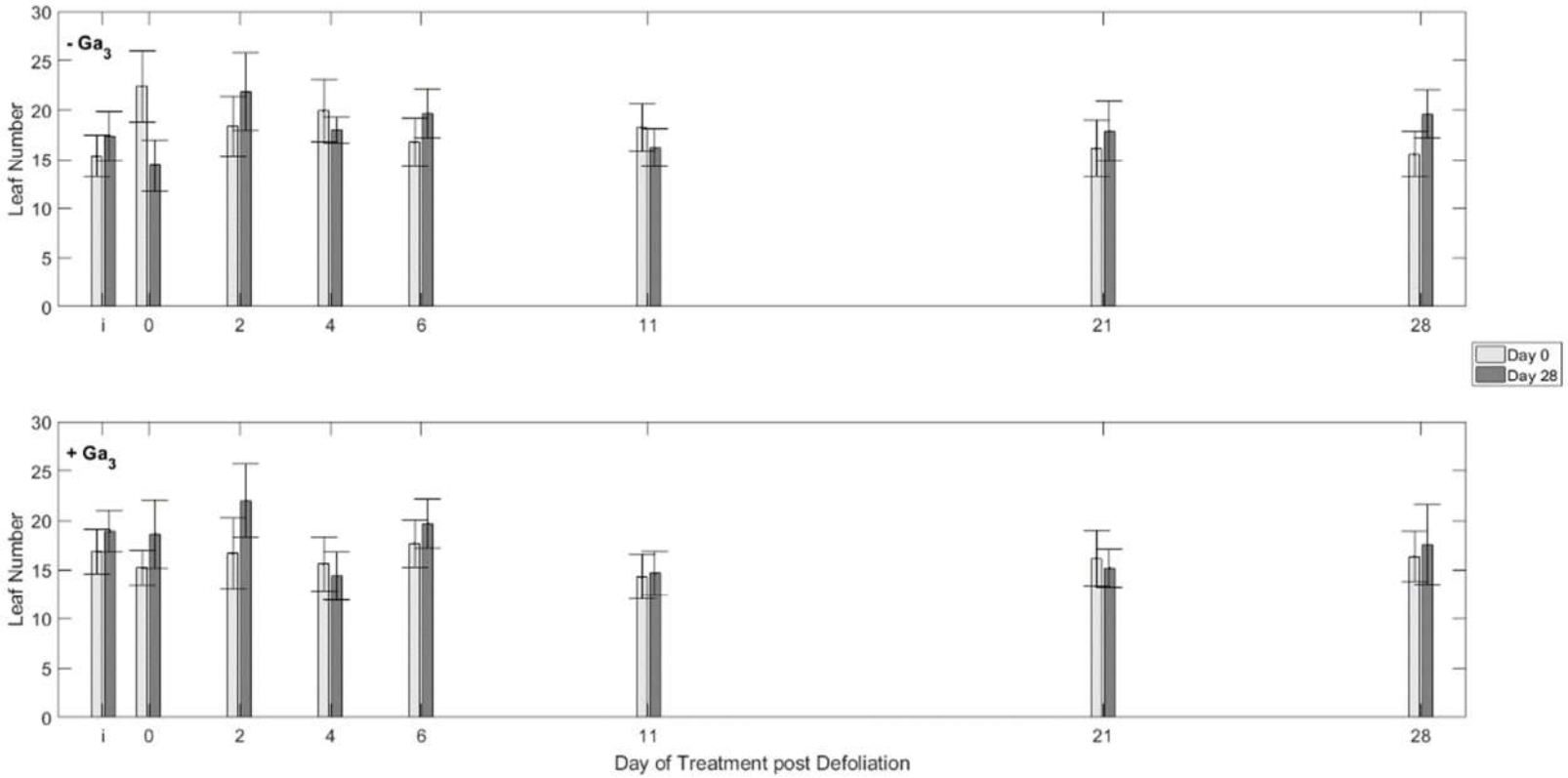

Figure 7-30: Graph comparing leaf number on day zero and day 28. Panel 1 shows treatments without GA_3 application, panel 2 shows treatments with GA_3 application. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

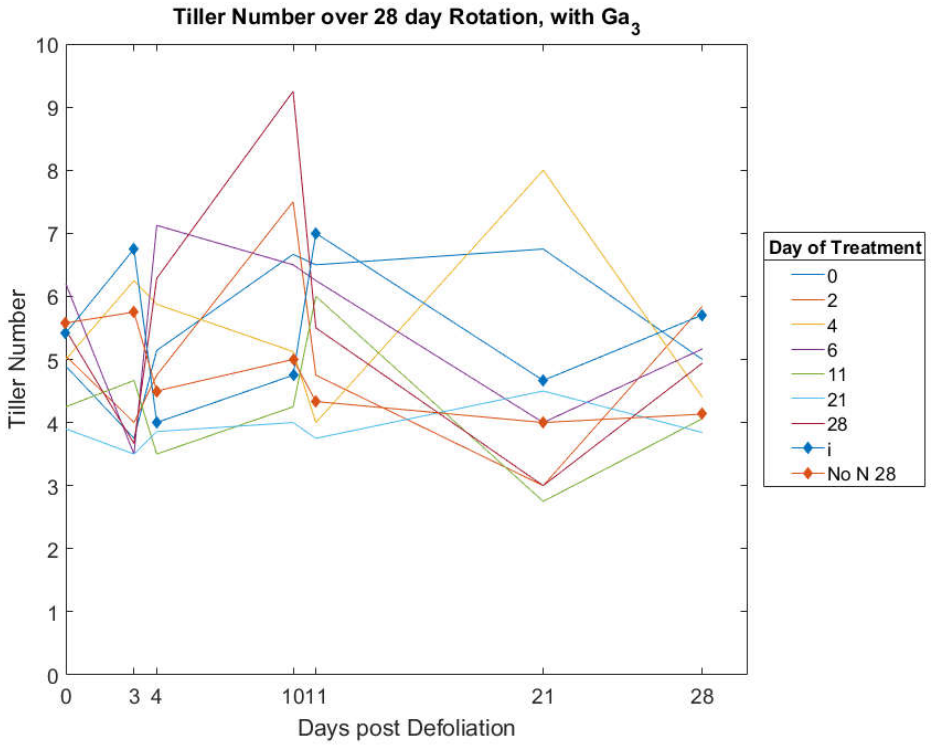

Figure 7-31: Tiller number of plants following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga_3 was applied. Graph displays all treatments with no Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

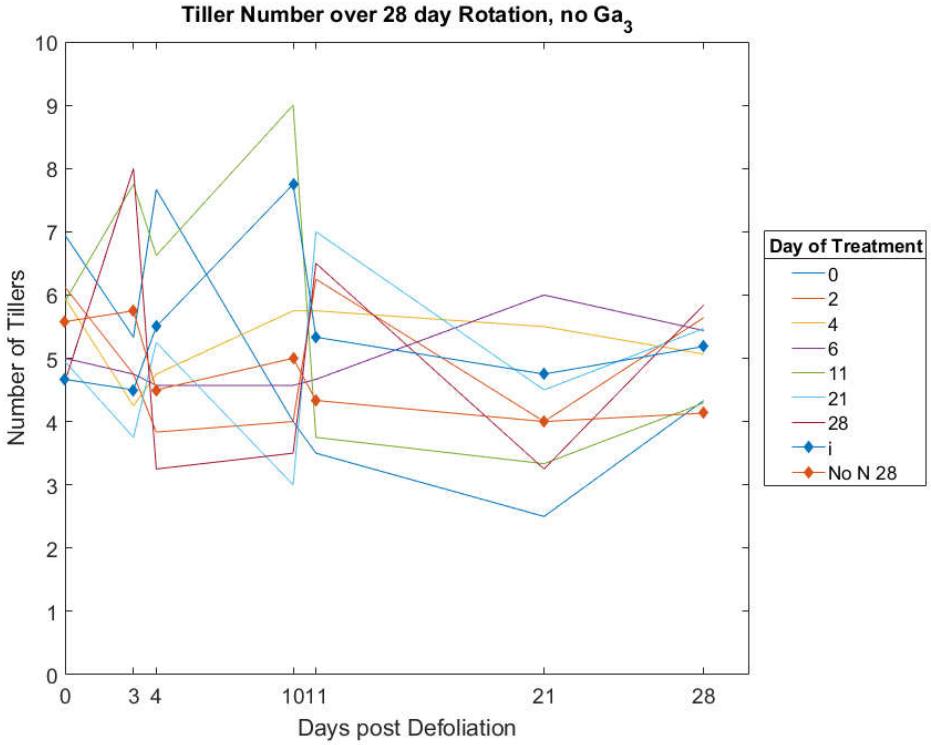

Figure 7-32: Leaf number of plants following defoliation on day zero (with the exception of intact (i) plants). An additional third treatment was carried out on day 28 as a control: no nitrogen or Ga3 was applied. Graph displays all treatments with Ga_3 applied. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

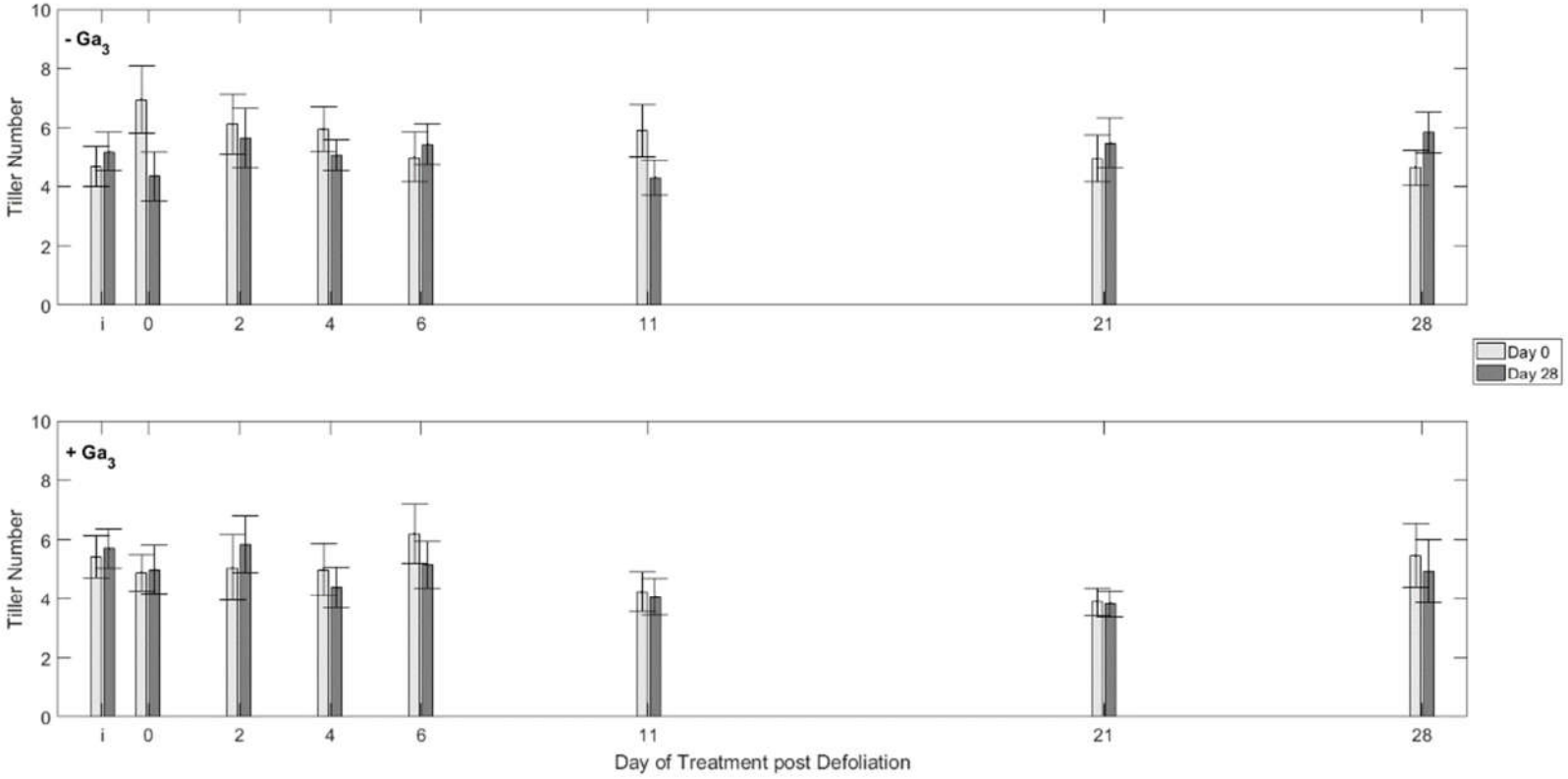

Figure 7-33: Graph comparing tiller number on day zero and day 28. Panel 1 shows treatments without GA_3 application, panel 2 shows treatments with GA_3 application. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

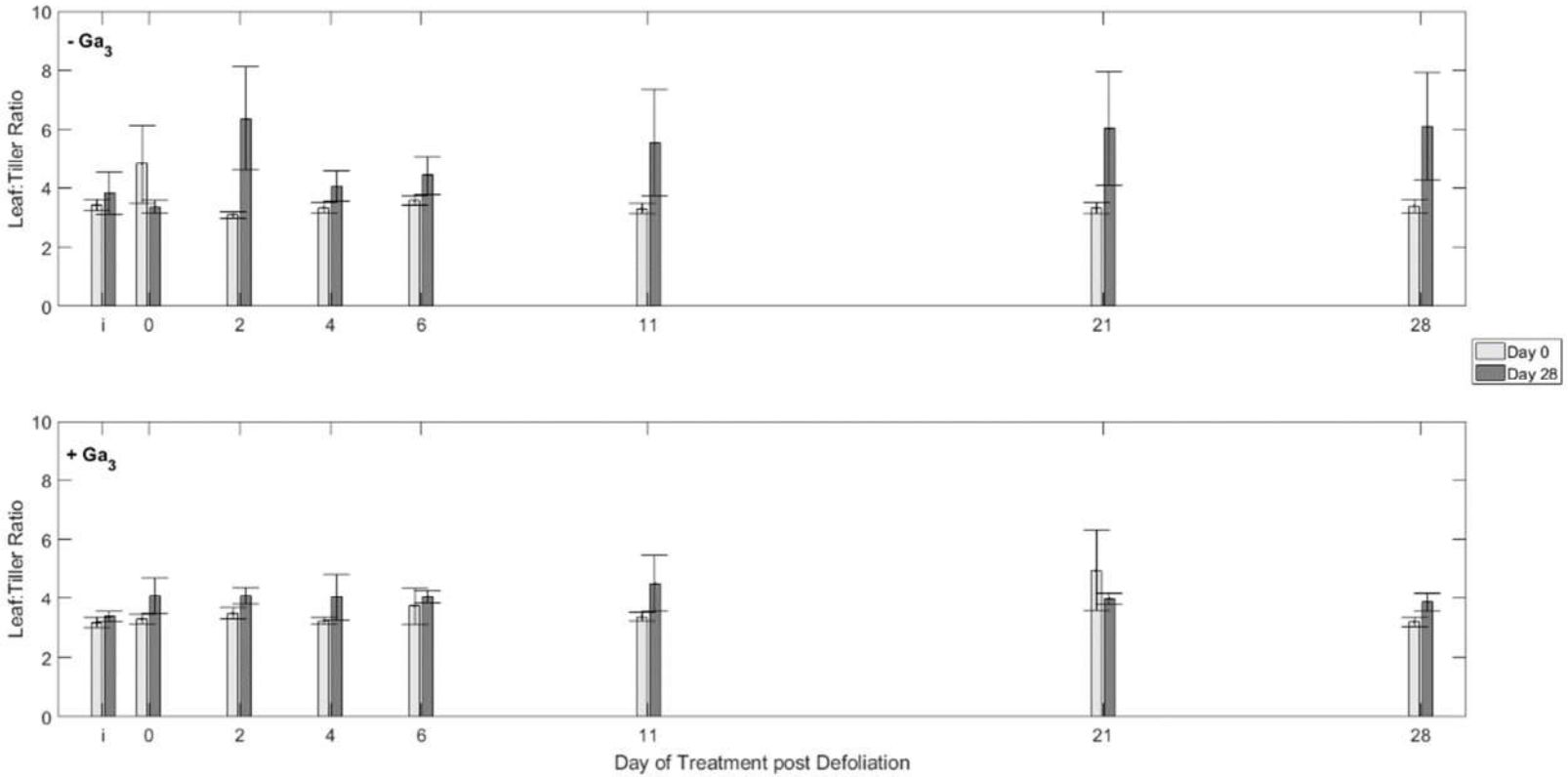

Figure 7-34: Graph comparing leaf to tiller ratio on day zero and day 28. Panel 1 shows treatments without GA_3 application, panel 2 shows treatments with GA_3 application. All plants were cut on day zero, and fertiliser was applied on days indicated above. For treatment "i" plants were left uncut and treatment applied at day 0. An additional third treatment was carried out on day 28 as a control: no nitrogen or GA_3 was applied. Error bars are the standard error of the mean, floating bars indicate LSD from mean for significant points.

7.1.4 Directly comparing nitrogen and fructan content of plants

Figure 7-35 shows in direct contrast the data presented in Figure 4-7 and Figure 4-8. There is a clear trend of NDFF decreasing whilst percentage fructan content increases and *vise versa*. This is discussed more above in the discussion and conclusion.

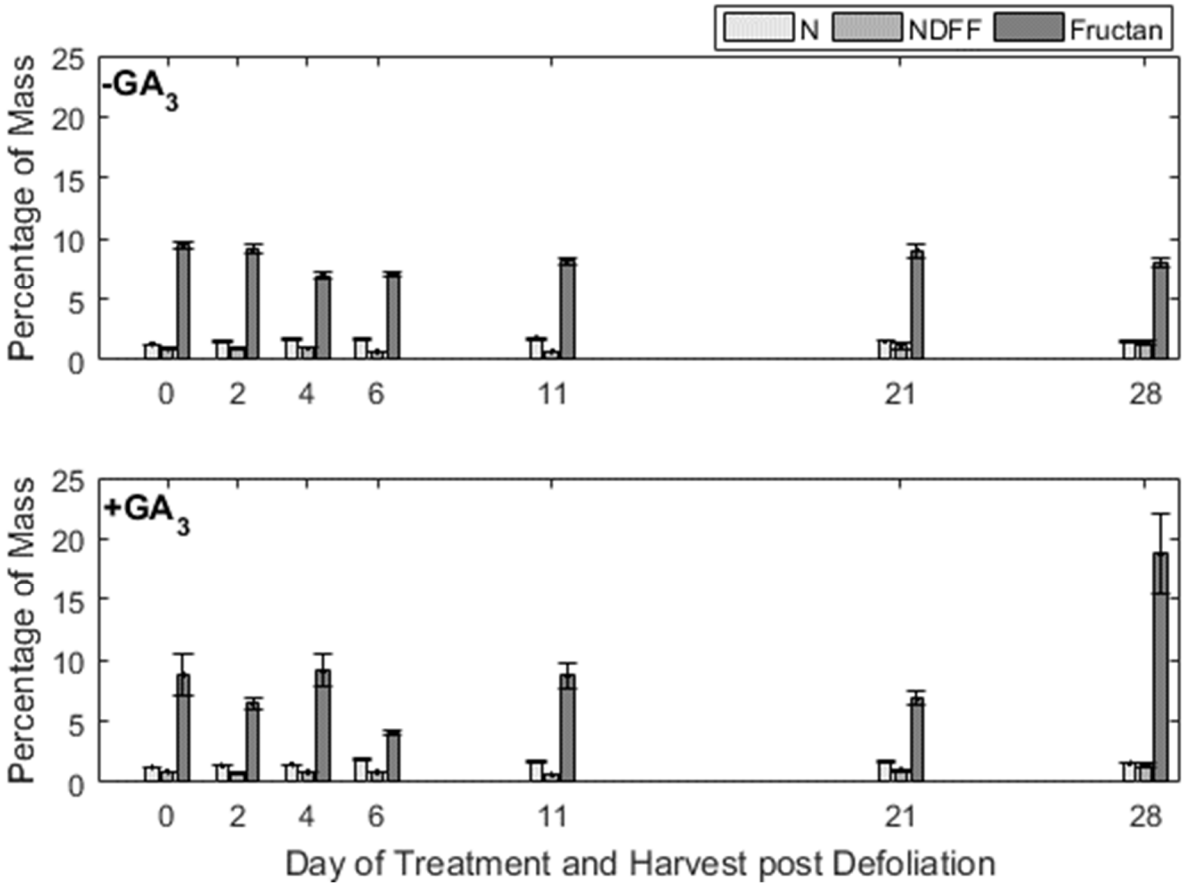

Figure 7-35: "N" percentage total N, "NDFF" percentage of N derived from fertiliser, "Fructan" percentage fructan in stubble of plants. Error bars indicate standard error of the mean. Floating LSD bars are emitted for clarity, see figures in results to see LSD bars (Figure 4-7, Figure 4-8)