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The impact of carbon dot nanomaterials on two cultivars of common wheat

Ellen Martin

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master's by Research in the Faculty of Life Sciences.

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Abstract

We are, without a doubt, facing some of the biggest agricultural challenges to date, with projected increases in crop demands and climate change severely threatening crop production. Furthermore, current projected increases in crop yields are not high enough to meet demand in the coming decades. Historically, agricultural intensification has increased crop yields, yet current agricultural practices are unsustainable and contribute to environmental destruction. Therefore, there is a pressing need for new technologies to aid in sustainably intensifying agriculture. Carbon dot (CD) nanomaterials have been presented as sustainable biostimulants, able to enhance nutrient uptake and use efficiency in plants; herein, their effects on two cultivars of common wheat, *Triticum aestivum* cv. Apogee and Paragon, are investigated. Firstly, confocal microscopy confirmed successful uptake of CDs from soil. Then, CDs were revealed to significantly increase photosynthetic activity in a number of ways; the chlorophyll contents of flag leaves was increased and the expression of chlorophyll a-b binding proteins was upregulated, increasing light-harvesting capacity; oxygen-evolving enhancer proteins were upregulated, and the rate of electron transport was significantly increased; the rate of non-photochemical quenching (NPQ) was decreased and NPQ-related genes were down-regulated; and photoprotective mechanisms were up-regulated, including increased flavanol production, ROS scavenging, and modulated photosystem stoichiometry, shown by the up-regulation of photosystem I genes and down-regulation of photosystem II genes. Importantly, dual application of CDs and NPK fertilisers enhanced crop yields and ear development, more so than CD or fertiliser treatment alone. Overall, CDs are a promising biostimulant candidate for use in agriculture and enhance NPK fertiliser use. Further work is needed to quantify the optimum concentrations of CDs and NPK fertilisers, and with optimum concentrations likely to be cultivar-dependent, further work is needed to assess how best to tailor application to a wider range of wheat cultivars.

Dedication and Acknowledgments

I would like to thank my supervisor Dr. Heather Whitney for the opportunity, support, and endless patience throughout my time as a student, and I would like to thank my lab group for making my time so enjoyable. In particular, I would like to thank Katie Higginbottom and Florence MacDuff for answering countless questions and giving help and advice whenever I asked, and for the years of friendship and support. I would like to thank my partner Sam Windsor, my housemate Vesper Cain, and my sister Hannah Martin for keeping me sane over the last few years and encouraging me to keep going even when the rest of the world came to a stop. Lastly, I would like to thank Lucia Primavesi for providing endless advice and training whenever I asked, and Helen Martin, who never failed to notice when my hair was a new colour and whose compliments made my day.

I couldn't have completed my degree without the help of many people at the University. In no particular order, I would like to thank: the Galan Research Group at the University of Bristol for kindly synthesising and providing carbon dots; Tom Pitman and Alanna Kelly for their assistance in sowing and maintaining the plants in the University of Bristol GroDome; Hilary McCarthy for providing help and advice on using the chlorophyll fluorometer; the Wolfson Bioimaging Facility for their training, support, and access to the facility equipment (BBSRC grant BB/F011709/1); Cara Doyle for assistance with experimental design, confocal training, and general advice; Ashutosh Sharma for kindly permitting me to use the DUALEX and providing training and advice; the five undergraduate project students – Jenna Ventress, Todd Buesnel, Emma Narbett, Patrick Bernon, and Beth Howley – whom I supervised while running my pilot project and who assisted with collecting the plant height, leaf temperature, ear development, and biomass measurements in the autumn-sown wheat; Gilda Varliero for processing the extracted RNA, running the transcriptomics analysis, and providing advice throughout the planning process; the BCAI for funding the MinION sequencing work; and everyone else in Lab 246, 324, and 321 who I worked alongside and trained with during my time at the University, I will miss it very much.

Covid-19 Statement

During my first term, I completed a pilot study on which I based the experimental design of my project. The idea of the pilot study was to collect a brief set of preliminary data and work out logistics. Then, alongside my reading, I would use this experience to plan where to take my project. It was successful and I was able to plan my experiment going forward, and I decided I would focus on the impact of CDs on photosynthesis. Furthermore, funding was secured for MinION sequencing and so it was decided that my project would consist of two parts: a) an expanded repeat of my pilot study to investigate physiological impacts more thoroughly, and b) a parallel transcriptomics study to investigate genetic impacts; both parts would focus on photosynthesis. Together, these two datasets would allow for a thorough look into how the nanomaterials were impacting the plants.

I spent the first months of 2020 preparing to start my experiment, which included growing up approximately 400 wheat plants, ensuring the stock of nanomaterials was replenished, and training on/booking all relevant equipment and resources. With all going to plan, I was due to being collecting data in the last week of March 2020. However, on the 16th of March the UK lockdown was announced, and a week later the lab was shut down until further notice.

Due to the closure of the lab, I lost all of my plants, any access to facilities, and was unable to collect my data. By the time that the lab re-opened, I had a severely reduced amount of time in which to complete my experiment. Furthermore, it was announced that the transcriptomics work would not restart until the following December, which was too late for me. Therefore, it was decided that it would be best to complete a condensed version of my original experimental design, focusing on photosynthesis but without the genomic component, and with a second type of CD treatment to try and collect more data. When the lab re-opened, there were very strict rules about who was allowed in the building and at what time. We had to book time in the lab, and we had to split time equally between everyone in the lab group. Therefore, as well running a condensed experiment, I had much less time to take the measurements. With only a 'skeleton crew' of staff on site and now having to work alone, it was also not possible to obtain training on equipment I had planned on using.

Despite these challenges, I was able to collect a second dataset. With the exception of the photosynthesis experiments, my second dataset mirrored the pilot study as I was unable to expand it due to the constraints from Covid. Therefore, rather than viewing the first dataset as a pilot study, I decided to view my two datasets as repeats from different seasons and investigate the effect of CDs under different photoperiods, which would allow me to analyse my data from a different perspective and adapt to the challenges. Furthermore, my supervisor announced that two new master's students would be joining the lab and that, if I was still interested, I could harvest the plant samples for transcriptomics but that the new students would analyse the data instead. Knowing that it would mean the transcriptomics work would still be carried out, which to me was the most important part, I agreed, and the final data chapter of this thesis presents that work.

In spite of the pandemic, I feel I managed to collect a good dataset. However, the factors leading up to data collection should be considered when reading this body of work, as it represents the best effort I could give in an extremely troubling time.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: Ellen Martin

Date: 31/07/2022

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Abbreviations

CO ₂	Carbon dioxide
CD	Carbon dot
DEGs	Differentially expressed genes
ETR	Electron transport rate
GCD	Glucose-functionalised carbon dot
GDP	Gross domestic product
GHG	Greenhouse gas
LDR	Light-dependent reaction
LHC	Light-harvesting complex
LHCI	Light-harvesting complexes of PSI
LHCII	Light-harvesting complexes of PSII
LIR	Light-independent reaction
NBI	Nitrogen balance index
NPQ	Non-photochemical quenching
NPK	Nitrogen, phosphorous, and potassium
O ₂	Oxygen
PAM	Pulse modulated amplitude
PCD	PEG-functionalised carbon dot
PEG	Polyethylene glycol
PSI	Photosystem I
PSII	Photosystem II
RC	Reaction centre
RCI	Reaction centre of PSI
RCII	Reaction centre of PSII

ROS	Reactive oxygen species
UV	Ultraviolet
Y(II)	Effective quantum yield of PSII
Y(NO)	Quantum yield of non-regulated energy dissipation
Y(NPQ)	Quantum yield of regulated energy dissipation

1. Introduction

1.1. The challenges facing agriculture today

We are, without a doubt, facing some of the biggest agricultural challenges to date. By 2050, global population is projected to reach 9.7 billion and, consequently, the demand for crops will double (FAO, 2009b; Ray et al., 2013; Tilman et al., 2011). Despite efforts to meet projected demands, current crop yields are not sufficient to feed the population in coming decades (Ray et al., 2013). Four crops provide two-thirds of calories worldwide – maize, rice, wheat, and soybean (FAO, 2013). However, despite how heavily these crops are relied upon, current rates of crop yield increases are much slower than in previous decades; for example, between 1960 and 2000, the growth in global cereal yields decreased by 1.7% (FAO, 2009a, 2009b; Tian et al., 2021; Tilman et al., 2011). Recent projections paint a bleak picture, with yields unlikely to continue increasing past 2050 (Schaubberger et al., 2018; Tian et al., 2021). Therefore, it is vital that efforts are made to increase crop yields (OECD/FAO, 2020).

Increasing demand for crops is not the only problem agriculture is facing, however, with climate change presenting an increasingly severe threat to crop yields (Challinor et al., 2014). Extreme weather events will become commonplace, with droughts, floods, and extreme temperatures occurring much more regularly than before (Bailey-Serres et al., 2019; Deryng et al., 2014; Wing, De Cian, & Mistry, 2021). Pests and pathogens will emerge in an increasingly unpredictable manner (Llewellyn, 2018). Freshwater shortages and shrinking groundwater levels will worsen, and with agriculture responsible for 70% of global freshwater usage, this will present a severe threat to crop yields (FAO, 2009b; OECD/FAO, 2020). Climate change will, without a doubt, severely reduce crop yields if changes are not made. Alarmingly, for every 1°C increase in global temperature, it is projected that the yields of the top four crops will decrease by up to 7.4% (Zhao et al., 2017). While agriculture faces enormous challenges at the hands of climate change, it is a major contributor to climate change itself. Past increases in yields have been achieved by unsustainable agricultural intensification at the expense of the environment, and today agriculture is responsible for 11% of global greenhouse gas (GHG) emissions. Of these GHG emissions, 13% derive from the use of synthetic fertilisers, yet crop yields are dependent on them. Similarly, agriculture is dependent on biodiversity for the production of crops, yet biodiversity is severely threatened by agricultural intensification (FAO, 2009b). With agricultural GHG emissions projected to increase by 6% by 2029, agriculture must radically change if it is to mitigate and adapt to climate change (OECD/FAO, 2020).

1.2. Historic solutions to agricultural problems

Despite the challenges facing agriculture today, it is not the first time that crop yields have threatened food security. In the 20th Century, there were valid concerns that crop yields were not increasing fast enough in order to meet demand. Against the odds, crop yields were massively increased during a period now known as the 'Green Revolution', with overall food production doubling between 1960 and 2000 (Khush, 2001). To achieve this, high-yielding crop varieties were developed through the introduction of dwarfing genes and the intensified use of agricultural inputs such as water, pesticides, and fertilisers (Berry et al., 2015; Hedden, 2003; Reynolds et al., 2009). Today, crop breeding continues to offer hope for agriculture, whereby traits such as high yields, pest and pathogen resistance, climate resilience, and more efficient use of water and fertilisers can be introduced into crops (Chen et al., 2021; Gao, 2021; Llewellyn, 2018; Tian et al., 2021). However, to meet projected crop demands, the rate of genetic improvement in crops must double by 2050 (Voss-Fels, Stahl, & Hickey, 2019). Current rates of genetic improvement are too slow, and the theoretical maximum yields of new crop varieties are not realised in the field (Fischer & Edmeades, 2010; Hall & Richards, 2013; Xu et al., 2017). While novel breeding technologies like CRISPR/Cas9 offer new ways to precisely introduce new traits at an accelerated pace, crop breeding is unlikely to be the sole solution (Li & Yan, 2020).

Like crop breeding, the intensified use of synthetic nitrogen, phosphorous, and potassium (NPK) fertilisers strongly benefitted crop yields in the Green Revolution (David Tilman, 1998). However, NPK fertilisers have their own problems. Their production produces large amounts of GHGs and excess amounts in soils leads to polluting run-off into water systems (Cowie, 2004; Li, Wiedmann, & Hadjikakou, 2019). Furthermore, the use of phosphorous within NPK fertilisers is a problem in itself, with phosphorous being an unsustainable resource that is being depleted much faster than it can be restored (Jupp et al., 2021; Li et al., 2019). Therefore, moving towards more sustainable fertiliser use will be essential for improving crop yields and mitigating climate change.

Current crop yields are reliant on NPK fertilisers and intensifying agriculture without increasing fertiliser inputs may have once seemed impossible. However, novel technologies offer potential solutions. One example is biostimulants. Defined simply as substances or microorganisms that enhance plant growth, biostimulants are a novel technology which show great promise for agricultural applications (Calvo, Nelson, & Kloepper, 2014). Rather than continuing to increase NPK fertiliser inputs, complementing the application of NPK fertilisers with biostimulants can cause an enhancement effect, increasing yields without increasing the volumes of NPK fertilisers required

(Cai et al., 2019; Caradonia et al., 2019; La Torre, Battaglia, & Caradonia, 2016; Lal, 2006; Schutz et al., 2017; Van't Padje, Werner, & Kiers, 2021). Similarly, crop management strategies can be used to optimise fertiliser use, with integrated crop management strategies having been shown to reduce nitrogen fertiliser inputs in rice by 10% (Chen et al., 2021). If NPK fertiliser application was bolstered by the application of biostimulants, and if crop management strategies were implemented, agricultural GHG emissions would decrease without decreasing yields (Maraseni et al., 2021).

1.3. Carbon dots – a solution?

In the search for new technologies for the sustainable intensification of agriculture, nanomaterials have garnered significant research interest. Defined simply by their nanoscale size, nanomaterials encompass a huge array of different materials with many unique properties. In agriculture, nanomaterials have been developed as nanofertilisers, nanopesticides, seed nanoprimer, and nanosensors, all with the common aim of increasing yields and reducing the reliance on unsustainable inputs and practices, such as the increased use of NPK fertilisers (Ashraf et al., 2021; do Espirito Santo Pereira et al., 2021; Dwivedi et al., 2016). In 2004, Xu et al. purified single-walled carbon nanotubes and accidentally discovered what are now known as carbon dot (CD) nanomaterials (Xu et al., 2004). With a quasi-spherical structure and a size of <10nm, CDs have garnered interest in many scientific fields due to their strong fluorescence, relative low toxicity and biocompatibility, water solubility, and cheap cost of production (Bhattacharya et al., 2016; Qu et al., 2012; Song et al., 2013; Zheng et al., 2015). CDs have been produced from a wide variety of materials, from candle soot and sugar through to plant matter and bacteria, and can be synthesised in a matter of minutes (Hill et al., 2016; Liu, Ye, & Mao, 2007; Qin et al., 2019; Zhou et al., 2012; Zhu et al., 2009). In recent years, attention has turned to the sustainable production of CDs from waste materials, with CDs having been successfully produced from single-use plastic and sewage sludge (Chaudhary et al., 2021; Hu & Gao, 2020). Furthermore, CDs can undergo surface functionalisation to drastically change their properties, opening up avenues for a huge array of potential uses (Havrdova et al., 2016; Swift et al., 2018). Similarly, the feedstock material used to produce CDs can lead to unique properties, from tissue-specific binding to antimicrobial activity (Mintz et al., 2019; Qin et al., 2019). Therefore, CDs present a quick, cheap, and extremely useful nanomaterial.

CDs have already shown great promise in a number of biological fields. Due to their small size and strong fluorescence, CDs easily cross cell membranes and are ideal candidates for bioimaging and biosensing (Ji et al., 2020; Su et al., 2020; Zhu et al., 2009). CDs also show great promise in biomedicine as candidates for drug delivery, due to their ability to conjugate drugs to their surface for site-specific release (Lin, Bao, & Wu, 2019; Wang et al., 2013). One innovative use of CDs in

biomedicine is in the treatment of brain-related diseases. CDs derived from tryptophan can cross the blood-brain barrier, offering potential in imaging and treating brain-related diseases such as brain tumours and Alzheimer's (Chung et al., 2020; Mintz et al., 2019). More recently, CDs have become promising agricultural candidates, showing a wide range of biostimulating activities (Mukherjee et al., 2016). CDs improve crop physiology, increasing the rates of water and nutrient uptake, seed germination, stem and root elongation, photosynthesis, and grain production (Li et al., 2019; Saxena, Maity, & Sarkar, 2014; Swift et al., 2021; Swift, et al., 2019; Tripathi & Sarkar, 2014; Wang et al., 2018b). Similarly, CDs significantly increase the nitrogen-fixing activity of *Azobacter chroococcum* by 158%, which increases soil fertility and reduces nitrogen fertiliser requirements (Aasfar et al., 2021; Wang et al., 2018a).

1.4. The present study: aims and objectives

There is an urgent need to find new technologies to sustainably increase crop yields. Wheat is a vital crop, being the most important source of vegetable protein worldwide (OECD/FAO, 2020). Finding novel ways to increase wheat yields will therefore be vital for food security. Herein, this body of work aimed to investigate the impacts of CDs on two cultivars of common wheat, *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon, with comparison to and in conjunction with synthetic NPK fertilisers, to assess the suitability of CDs as a novel agricultural biostimulant.

To assess the biostimulant suitability of CDs, a number of research objectives were followed:

1. Assess the ability of wheat plants to uptake CDs from soil
2. Investigate the impacts of CDs on plant height and nitrogen status
3. Investigate the impacts of CDs on photosynthesis and stomatal conductance
4. Assess the impacts of CDs on ear development and biomass
5. Briefly examine the impacts of CDs on photosynthesis genes
6. Examine the impacts of CDs on wheat plants as a whole and bring together a 'big picture' view on their suitability as a biostimulant in an agricultural context

2. Materials and Methods

2.1. Experimental design

2.1.1. Datasets presented

As outlined in the Covid statement, two datasets are presented in this body of work:

1. A dataset where two cultivars of common wheat, *Triticum aestivum* cv. Apogee and Paragon, were sown in September and harvested in December
2. A dataset where the same wheat cultivars were sown in June and harvested in September.

For brevity, these datasets will be referred to as the ‘autumn-sown’ and ‘spring-sown’ wheat respectively.

2.1.2. Wheat cultivars used

Two cultivars of common wheat were grown, *T. aestivum* cv. Apogee and *T. aestivum* cv. Paragon. These two cultivars have different properties and were selected for this reason. *T. aestivum* cv. Apogee is a full-dwarf cultivar, meaning it is extremely short yet relatively very high yielding. It is a fast-growing, photoperiod-insensitive cultivar, growing optimally in longer photoperiods; when grown under optimum conditions, ears emerge after just 23 days (Koerner, 1997). On the other hand, *T. aestivum* cv. Paragon is a non-dwarf, photoperiod-sensitive cultivar. While it grows fastest in longer photoperiods, like *T. aestivum* cv. Apogee, it has the highest yields when grown more slowly in shorter photoperiods (Shaw et al., 2012). Together, these two species effectively represent opposite ends of the spectrum for *Triticum aestivum*, providing a wide-field look at the impacts of CDs on common wheat.

2.1.3. Plant growth

Across both datasets and across both wheat cultivars, plants were grown identically. Plants were grown in the University of Bristol GroDome in a glasshouse where the environment was constantly controlled. The ambient temperature was maintained at 22°C and a 16-hour photoperiod was maintained by supplementary lighting (Attis7 LED Growth Lights, 80-120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Seeds were sown in Levington F2 compost with added exemptor granules (0.4g/L) and were kept under lids for one week after sowing to maintain a high humidity to facilitate germination. Plants were grown in trays of ten, with three trays per experimental treatment. Individual outer trays were used for all plant trays, and these were watered individually to prevent cross-contamination between experimental treatments. Watering was carried out three times a week on Mondays, Wednesdays,

and Fridays, initially with 500ml per tray, increasing to 1L per tray as the plants grew. Water was applied using a bottom-up method by pouring into the base of the outer tray to ensure all plants were watered equally. The trays were randomly arranged in a Latin square and re-arranged weekly to prevent confounding factors.

2.1.4. Experimental treatment

Experimental treatment began when the wheat had developed to Zadoks stage 3, which was approximately three weeks post-germination (Zadoks, Chang, & Konzak, 1974). This was done to allow for comparison of results to previous works of others in the lab group (Swift et al., 2021).

The Zadoks decimal code refers to the growth stages of cereals. It can be summarised as:

0. Germination
1. Seeding growth
2. Tillering
3. Stem elongation
4. Booting
5. Awn emergence
6. Flowering
7. Milk development
8. Dough development
9. Ripening

While each stage is split further into sub-stages, these were not used in the present study and so are not presented here (Zadoks, Chang, & Konzak, 1974).

Once plants had reached Zadoks stage 3, experimental treatments were applied weekly until harvest. In *T. aestivum* cv. Apogee, plants were harvested at 5 weeks post-germination, after 3 weeks of treatment, in the autumn-sown wheat, and at 10 weeks post-germination, after 7 weeks of treatment, in the spring-sown wheat.

In *T. aestivum* cv. Paragon, plants were harvested at 8 weeks post-germination, after 5 weeks of treatment, in the autumn-sown wheat, and at 10 weeks post-germination, after 7 weeks of treatment, in the spring-sown wheat.

As stated in Section 2.1.3., the plants were watered three times a week. For application of the experimental treatments, the treatments were mixed into the watering regime applied on Wednesdays. To do so, 1L solutions of experimental treatment were made up in glass bottles, and

these were then applied to the plants following the same bottom-up watering method as outlined in Section 2.1.3. The aim of mixing the experimental treatment into the standard watering regime was to prevent over-watering and maximise equal treatment uptake across all plants in each tray. The experimental treatments used are summarised in Table 2.1, below.

Table 2.1 – A summary of the experimental treatments used to treat two cultivars of common wheat, T. aestivum cv. Apogee and T. aestivum cv. Paragon. Treatments were applied weekly from three-weeks post germination through to harvest. Harvest occurred at 5 weeks and 8 weeks post-germination in the autumn-sown T. aestivum cv. Apogee and T. aestivum cv. Paragon respectively, and occurred at 10 weeks post-germination in both the spring-sown T. aestivum cv. Apogee and T. aestivum cv. Paragon.

Autumn-Sown Wheat		Spring-Sown Wheat	
Treatment Name	Concentration (per tray of 10 plants, applied weekly)	Treatment Name	Concentration (per tray of 10 plants, applied weekly)
Water	1L water	Water	1L water
Fertiliser	40mg ASDA Grow Your Own Soluble Plant Food added to 1L water	Fertiliser	40mg ASDA Grow Your Own Soluble Plant Food added to 1L water
Glucose-functionalised carbon dots	33mg of glucose-functionalised carbon dots added to 1L water	Glucose-functionalised carbon dots	33mg of glucose-functionalised carbon dots added to 1L water
Glucose-functionalised carbon dots & fertiliser	40mg ASDA Grow Your Own Soluble Plant Food and 33mg of glucose-functionalised carbon dots added to 1L water	Polyethylene glycol-functionalised carbon dots	33mg of polyethylene glycol-functionalised carbon dots added to 1L water
		Glucose-functionalised carbon dots & fertiliser	40mg ASDA Grow Your Own Soluble Plant Food and 33mg of glucose-functionalised carbon dots added to 1L water
		Polyethylene glycol-functionalised carbon dots & fertiliser	40mg ASDA Grow Your Own Soluble Plant Food and 33mg of polyethylene glycol-functionalised carbon dots added to 1L water

2.1.5 Carbon dot synthesis

All carbon dots (CDs) were synthesised by the Galan Research Group at the University of Bristol following a protocol developed by Hill et al. (2016). The CDs produced are made up of a crystalline core with an amorphous surface, and these are referred to as ‘core CDs’. These core CDs feature aromatic regions and can undergo surface functionalisation (Hill et al., 2016). Core CDs were then functionalised with glucose and polyethylene glycol (PEG) following the work of Swift *et al.* (2018) and Doyle et al. (2019). Glucose-functionalised CDs and PEG-functionalised CDs were selected as they have been shown to be more biocompatible than core CDs (Havrdova et al., 2016; Swift et al., 2018; Swift et al., 2021). The glucose-functionalised CDs were used in the autumn-sown wheat, and

both glucose-functionalised CDs and PEG-functionalised CDs were used in the spring-sown wheat, as outlined in the Covid Statement. Lastly, at very high concentrations ($<100 \text{ mg L}^{-1}$) CDs have been shown to inhibit plant growth rather than promote it (Chen et al., 2016; Chen et al., 2018). Therefore, a lower dose was chosen in lieu of previous works of others in the lab group, as presented in Table 2.1 (Swift et al., 2021).

2.1.6. Light levels and time of year

Although the plants were grown identically in both sets of wheat, there was one key difference between the two: the time of year grown, as described in Section 2.1.1. As seen in Table 2.2 below, although the ambient temperature within the glasshouse remained stable due to the constantly-controlled environment, the average day length differed greatly by almost 5 hours on average.

Table 2.2 – Temperature and daylength data within the glasshouse as the wheat was grown.

Temperature data was collected automatically by the Building Management System (BMS) which remotely monitors and controls the glasshouse temperature, and the daylength data was researched online (Time and Date, 2021).

	September Wheat				June Wheat				
	September	October	November	December	June	July	August	September	
Maximum Temperature (°C)	26.40	24.70	22.50	22.60	23.30	23.20	26.70	23.00	
Minimum Temperature (°C)	21.50	21.40	21.20	21.50	21.60	21.50	21.60	21.50	
Average Temperature (°C)	22.10	22.00	21.90	21.90	22.10	22.10	22.10	22.10	
Total average temperature:				21.98 °C	Total average temperature:				22.1 °C
Maximum Day Length (hrs/mins/secs)	13:34:57	11:38:32	09:40:58	08:12:20	16:21:13	16:32:11	15:22:34	13:32:03	
Minimum Day Length (hrs/mins/secs)	11:42:26	09:44:33	08:14:25	08:10:19	16:33:10	15:25:42	13:35:53	11:39:28	
Average Day Length (hrs/mins/secs)	12:38:41	10:41:32	08:57:41	08:11:19	16:27:11	15:58:56	14:29:13	12:35:45	
Total average day length (hrs/mins/secs):				10:07:19	Total average day length (hrs/mins/secs):				14:52:47

Although supplementary lighting was used to maintain a 16-hour photoperiod ($80\text{-}120 \mu\text{mol m}^{-2} \text{s}^{-1}$), natural sunlight varies greatly and provides significantly more light, ranging anywhere from $700\text{-}1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Dorm Grow, 2021; LEDTonic, 2019). From the daylength data presented in Table 2.3, it is evident that the two sets of wheat were exposed to natural light for different lengths of time. On average, the autumn-sown wheat was exposed to 10 hours of natural light per day whereas the spring-sown wheat was exposed to 15 hours of natural light per day. Unlike *T. aestivum* cv. Apogee wheat, which is a photoperiod-insensitive cultivar, *T. aestivum* cv. Paragon wheat is highly photoperiod-sensitive (Koerner, 1997; Shaw, Turner, & Laurie, 2012). Therefore, this difference in

exposure to natural light may have been a confounding factor and will be considered when discussing results in subsequent chapters.

2.3.6. Statistical analysis

Shapiro-Wilk tests were used to determine the distribution of data, in order to choose an appropriate statistical test. Where the distribution of the data departed significantly from normality ($p < 0.05$), non-parametric statistics were chosen. Here, Kruskal-Wallis tests were used to test for significant differences between groups. Where a significant difference was found ($p < 0.05$), Mann-Whitney U tests were used for pairwise comparisons, with Bonferroni correction applied to account for multiple comparisons. Where data were normally distributed, One-Way ANOVA tests were used to test for significant differences between groups, with Tukey post-hoc tests selected for pairwise comparisons. All data was statistically analysed using SPSS v.26.0 (IBM, 2021) and figures were produced using Microsoft Excel v.2206 (Microsoft, 2022).

To assess the suitability of CDs as a biostimulant, a number of analyses were made:

1. All treatments were analysed relative to the water treatment group, to assess the impacts of each treatment against what was effectively a 'no treatment' control
2. Glucose CD and PEG CD treatments were analysed relative to the fertiliser treatment, to compare the biostimulant activity of CDs against a standard NPK fertiliser
3. Glucose CDs & fertiliser, and PEG CDs & fertiliser treatments were analysed relative to the fertiliser treatment, glucose CDs were analysed relative to the glucose CDs & fertiliser treatments, and PEG CDs were analysed relative to the PEG CD & fertiliser treatments, with the aim of testing whether CDs act synergistically with fertiliser

3. The uptake of carbon dots from soil

3.1. Introduction

3.1.1. Carbon dot fluorescence and application

Carbon dots (CDs) are highly fluorescent nanomaterials by nature, able to absorb light in the UV range (Hill et al., 2016; Xu et al., 2004). Therefore, CDs can be imaged using fluorescent confocal microscopy (Lemenager et al., 2014).

To apply CDs to plants, a number of methods can be used. Application of CDs by watering onto soil has proven to be a successful method. In-depth analysis of CD uptake from the soil by roots has revealed that CDs are able to easily cross the biological barriers of roots and are transported throughout the plant within the vascular system (Tripathi & Sarkar, 2014). Another method, whereby CDs are applied by foliar spray, has also shown to be successful and has been used for both gene editing and siRNA delivery (Doyle et al., 2019; Schwartz et al., 2020).

3.1.2. Carbon dot application and aims

Due to the ease of application and scale of the experiment, a water-on method was chosen in the present study. Before beginning to test the suitability of CDs as a biostimulant, it was essential to verify if there had been successful uptake of CDs by wheat plants following water-on application. To do this, confocal microscopy was used to image CDs in mature leaf tips. This chapter will aim to present the successful uptake of CDs in two cultivars of *T. aestivum* via a watering on method, as verified by confocal microscopy.

3.2. Methods

3.2.1. Plant growth and sample harvesting

Two cultivars of common wheat, *T. aestivum* cv. Apogee and *T. aestivum* cv. Paragon, were grown and treated as stipulated in the 'Materials and Methods' chapter. To harvest samples for confocal microscopy, the tips of mature flag leaves were harvested after three and five weeks of treatment in the spring-sown and autumn-sown wheat respectively. The leaf tips were then mounted onto glass slides for use on the confocal microscope, and were used immediately after harvesting.

3.2.2. Confocal microscopy

The samples were imaged using confocal microscopy. A Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope was used. The settings used were as follows:

1. Chlorophyll – 514nm excitation, 644-713nm emission, 20% power
2. CDs – 405nm excitation, 415-470nm emission, 15% power

Using these settings, the samples were imaged using a 63X oil immersion objective lens. Then, the captured images were analysed to look for the presence of CDs, which is seen as a characteristic blue fluorescence.

3.3. Results

Using the images captured by confocal microscopy, it was confirmed that CDs were present in the CD-treated plants, visible as a characteristic blue fluorescence as presented in Figure 3.1 below. It was also confirmed that there were no CDs present in the non-CD treated plants, as there was no blue fluorescence to be seen. This meant that there had been no contamination between treatment groups.

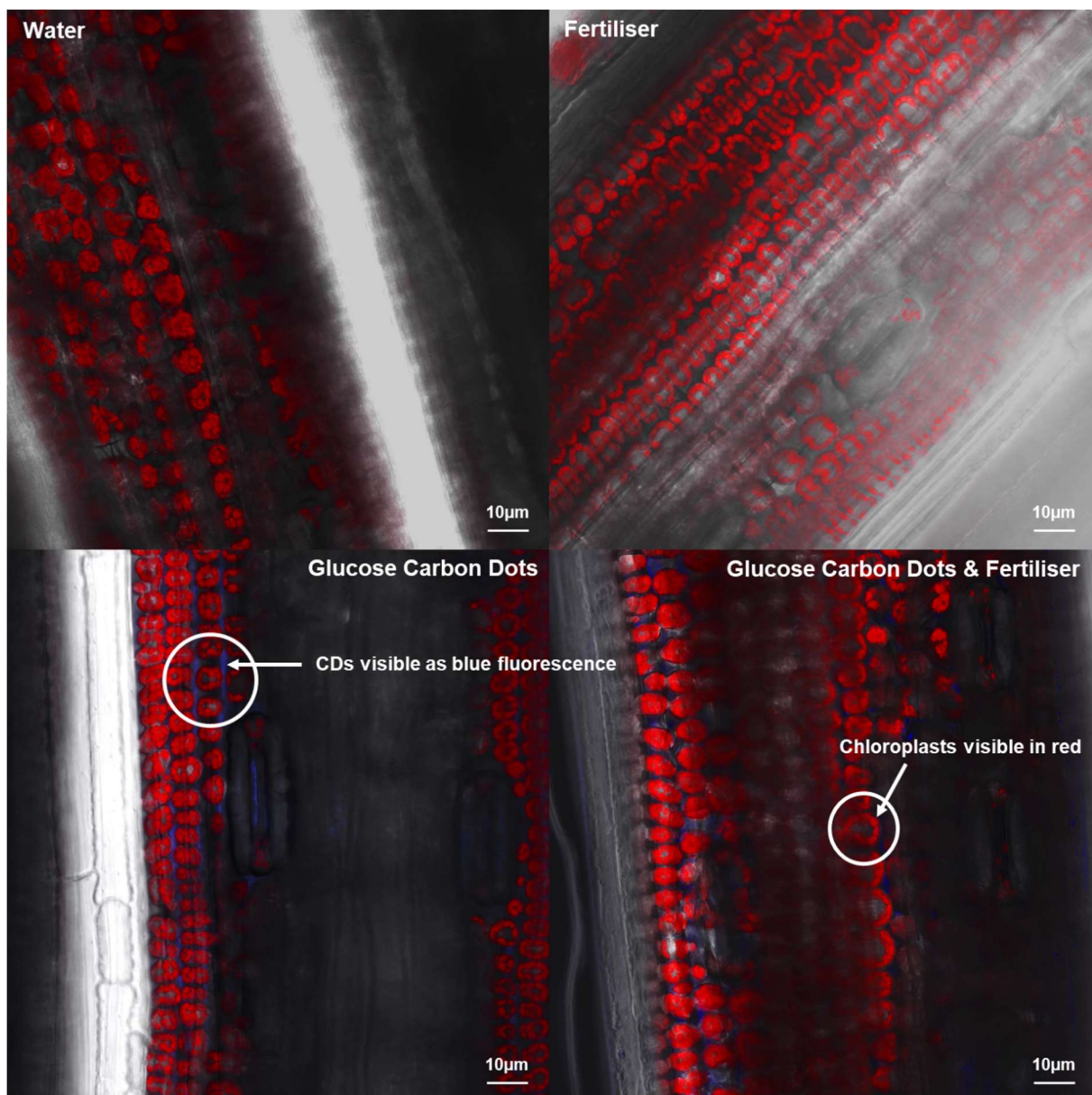


Figure 3.1 – Confocal microscopy images of autumn-sown *Triticum aestivum* cv. Apogee. Mature flag leaves were harvested 5 weeks post-germination and imaged using a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope. Carbon dots (CDs) were visible in CD-treated plants as a characteristic blue fluorescence, as labelled on the images. Images were taken at 63x magnification with chlorophyll fluorescence depicted in red (excitation 514nm, emission 644-713nm) and CD fluorescence depicted in blue (excitation 405nm, emission 415-470nm). Scale bars are shown on the bottom right of each image.

3.4. Carbon dot uptake summary

Confocal microscopy images confirmed that there had been successful uptake of CDs from soil in wheat plants, and that the CDs had been transported up to the leaf tips.

4. The impacts of carbon dots on plant height and nitrogen status

4.1. Introduction

4.1.1. The historic importance of plant height in wheat

Plant height is a major factor in determining wheat yield. In the past, wheat yields have showed a positive relationship with plant height, with taller plants producing larger, more numerous grains (Law, Snape, & Worland, 1978). However, taller plants are more susceptible to lodging (A. Navabi, 2006). Lodging occurs when a plant stem is unable to support the weight of the plant, and as a result the plant breaks and bends over. In wheat, lodging is a significant problem, reducing yields by up to 80%. Many factors contribute to the likelihood of lodging, including fertilisers. Throughout the 20th Century, the use of fertilisers was essential to increase yields. High fertiliser inputs massively increased crop yields by facilitating the development of larger, more numerous ears. However, they also increased height, and the combination of taller crops developing heavier ears meant the chance of lodging drastically increased as plants were unable to support the increased ear weights (Berry et al., 2004).

The introduction of dwarfing genes during the Green Revolution massively decreased the likelihood of lodging. These genes were selectively bred into wheat and reduced sensitivity to gibberellins, a group of plant hormones involved regulating growth. Gibberellins are essential for stem elongation and determining plant height and therefore the new dwarf cultivars were much shorter, with height reductions of up to 30% (Hedden, 2003; Wilhelm et al., 2013; Wurschum, Langer, & Longin, 2015). Due to their shortened stature, the new dwarf cultivars had much lower likelihoods of lodging. They proportioned fewer resources into stem elongation, and therefore more resources were dedicated to ear development, resulting in larger ears containing a higher number of grains. Furthermore, the dwarf plants were stronger and able to support more weight without an increased risk of lodging. Moreover, this meant that high fertiliser inputs no longer posed an increased risk of lodging (Berry et al., 2004).

Carbon dots (CDs) have been shown to increase plant height across a variety of different plant species, from mung beans and lettuce through to rice and water spinach (Aji et al., 2020; Li et al., 2020a; Li et al., 2021b; Wang et al., 2018b). Given the complex relationship between plant height and yield in wheat, the impacts of CDs on plant height will be critical to their suitability as a biostimulant candidate.

4.1.2. Nitrogen status and fertilisers use

Nitrogen fertilisers are essential for crops, required for photosynthesis and yield. Furthermore, in cereal crops such as wheat, nitrogen is essential for maintaining the protein content of grain (Hawkesford, 2014). However, the excess use of fertilisers causes severe environmental problems and nitrogen in particular has been shown to severely pollute rivers and water bodies (Tian et al., 2007; Zhao et al., 2012). Furthermore, excess fertiliser applications can reduce the fertiliser use efficiency in plants, rendering them less effective (Cabrera, 2003). Therefore, fertiliser application must be closely monitored to prevent excessive use.

The nitrogen content of leaves is directly related to the level of nitrogen fertiliser application. In turn, the chlorophyll and flavonoid contents of leaves are dependent on the nitrogen content, with chlorophylls being positively associated and flavonoids being negatively associated. Therefore, by measuring the levels of chlorophylls and flavonoids within leaves, it is possible to monitor the nitrogen content (Martinon, 2010).

The DUALEX is a non-invasive, optical leafclip meter which can be used to measure the levels of chlorophylls and flavanols within leaves. By measuring these parameters, the DUALEX is then able to calculate a parameter called the Nitrogen Balance Index (NBI). NBI is a measurement of the ratio of chlorophylls to flavanols within leaves, and it is used to indicate the nitrogen status of a plant. To calculate NBI, the DUALEX measures the absorbance of the epidermis when exposed to wavelengths of UV and red visible light (ForceA, 2010). A higher NBI indicates a higher level of chlorophylls and a lower level of flavanols, and therefore indicates plentiful nitrogen resources within a leaf. On the other hand, a lower NBI indicates a lack of nitrogen, and this can be used to pre-empt nitrogen deficiencies in the field (Cartelat et al., 2005; Cerovic et al., 2012; Tremblay, Wang, & Bélec, 2009). The NBI can also be used to measure stress, nutritional quality, and water deficiencies (Bürling et al., 2013; ForceA, 2010; Li, 2015; Martinon, 2010). In sum, the NBI is a valuable measurement which can be used as an early warning system for nitrogen deficiencies, to enable tailored application of fertilisers and prevent excess applications.

Due to their ability to bind water and nutrients to their cell surface, CDs are able to uptake and transport fertilisers from the soil into plants, slowly dispersing them as they travel around the plant's vascular system (Saxena et al., 2014; Tripathi & Sarkar, 2014). Furthermore, CDs have been shown to increase the chlorophyll content of leaves in rice and mung beans by 34.67% and 14.8% respectively (Li et al., 2020b; Wang et al., 2018c). If CDs increase nitrogen uptake and transport, then their use as a biostimulant could reduce nitrogen fertiliser requirements.

4.1.3 Assessing plant height and nitrogen status: chapter aims

Plant height and nitrogen are vital factors in determining yield in wheat. Therefore, the impacts of CDs on both of these parameters will be critical in assessing their suitability as a biostimulant candidate. Herein, this chapter aims to assess the impacts of CDs on plant height and nitrogen status in two cultivars of common wheat, *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon.

4.2. Methods

4.2.1. Plant height measurement

Two cultivars of common wheat, *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon, were grown and treated as outlined in the 'Materials and Methods' chapter. Plant height was measured at harvest. In the autumn-sown wheat, plants were harvested at 5 weeks post-germination in *Triticum aestivum* cv. Apogee and at 8 weeks post-germination in *Triticum aestivum* cv. Paragon. In the spring-sown wheat, plants were harvested at 10 weeks post-germination in both the *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon wheat. Plant height was quantified as the distance between the base of the stem at the soil to the bottom of the ear. A clothing tape was used to take measurements along the stem to allow for any bends in the stem to be accounted for.

4.2.2. Nitrogen status measurement

Two cultivars of common wheat, *Triticum aestivum* cv. Apogee and Paragon, were grown as outlined in the 'Materials and Methods' chapter. Using a DUALEX leafclip optical meter, mature flag leaves were measured at six weeks post-germination, after three weeks of treatment, once development had reach Zadoks stage 6 (Zadoks et al., 1974). As pigment concentration increases along the flag leaf, the DUALEX was used at a constant point of two inches down the leaf from the stem in order to prevent confounding factors (Cartelat et al., 2005; Cerovic, 2005). For each leaf, both the adaxial and abaxial leaf surfaces were measured and an average of the two was calculated for each leaf.

The following parameters were measured using the DUALEX:

1. *Chlorophyll content* – measured in relative units, ranging from 0-150.
2. *Flavonol content* – measured in relative units, ranging from 0-3.
3. *Nitrogen Balance Index (NBI)* – the ratio of chlorophyll content to flavonol content; measured in relative units, ranging from 0-999 (ForceA, 2010).

4.2.3. Statistical analysis

Data were statistically analysed as outlined in the 'Materials and Methods' chapter.

4.2.4. Covid adjustment

Measuring the internode lengths along the stem would have been a more informative way to assess impacts on plant height (O'Dogherty et al., 1995). Similarly, methods like high-performance liquid chromatography could have been an effective way to quantitatively investigate the impact of CDs on the contents of leaves (Meyer, 2013). However, due to the constraints outlined in the Covid Statement, a simplified method was chosen. For this reason, the DUALEX was used to provide brief insights into the impact of CDs on leaves.

4.2. Results

4.2.1. Plant height in *T. aestivum* cv. Apogee

In both the autumn-sown and spring-sown *T. aestivum* cv. Apogee wheat there were no significant differences in plant height between groups, as presented in Figure 4.1 below.

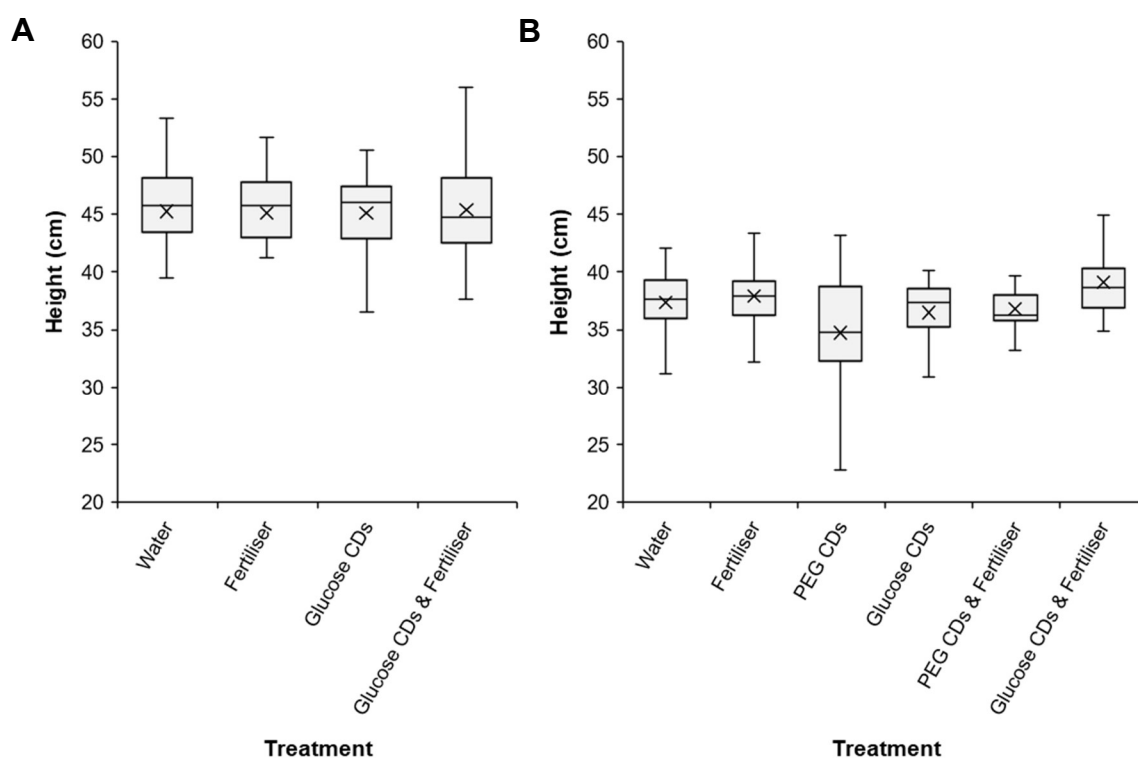


Figure 4.1 – Average plant height in the (A) autumn-sown and (B) spring-sown *Triticum aestivum* cv. Apogee wheat. Plant height was measured at harvest and quantified as the distance between the base of the stem at the soil and the bottom of the ear. In the autumn-sown wheat, plants were harvested at 5 weeks post-germination and in the spring-sown wheat, plants were harvested at 10

weeks post-germination. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. There are no statistically significant differences between groups.

4.2.2. Plant height in *T. aestivum* cv. Paragon

In the autumn-sown *T. aestivum* cv. Paragon wheat, plant height was significantly increased in the dual glucose CD & fertiliser-treated plants with respect to the water-, fertiliser-, and glucose CD-treated plants, as presented in Figure 4.2 below. When compared to the water-treated plants, the glucose CD & fertiliser-treated plants were 54.10% taller at harvest. By contrast, in the spring-sown wheat plant height was significantly decreased in the fertiliser- and PEG CD-treated plants with respect to the water-treated plants, showing deductions of 7.43% and 6.88% respectively.

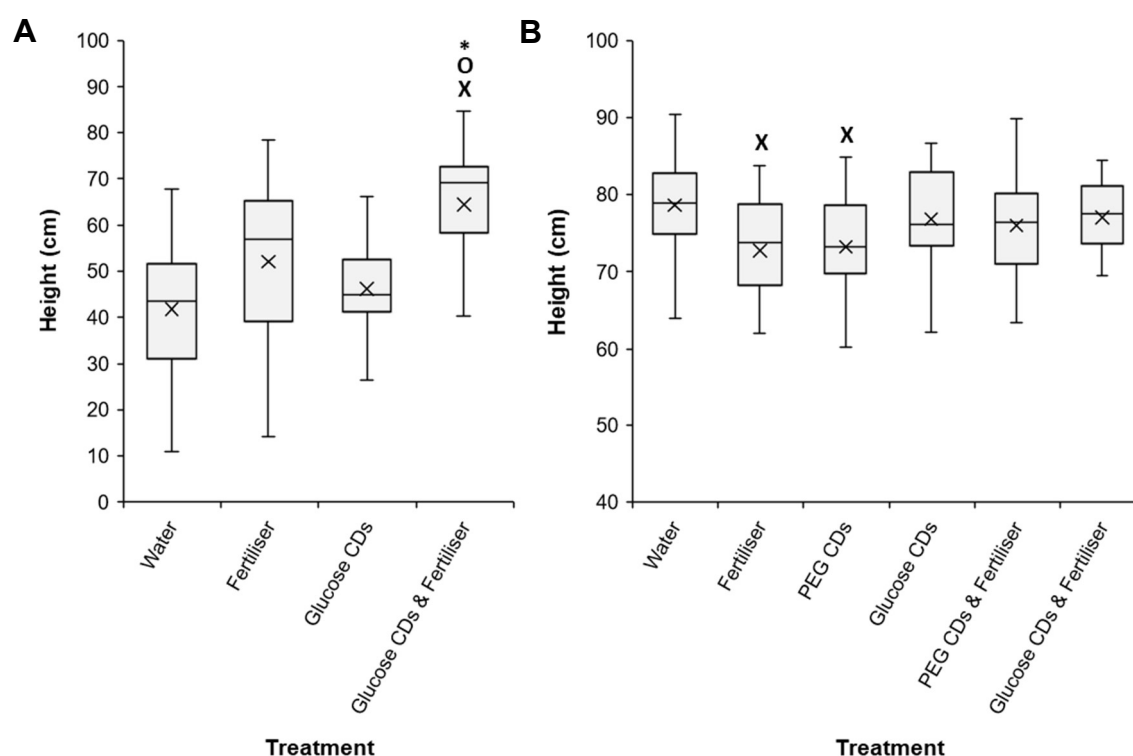


Figure 4.2 – Average plant height in the (A) autumn-sown and (B) spring-sown *Triticum aestivum* cv. Paragon wheat. Plant height was measured at harvest and quantified as the distance between the base of the stem at the soil and the bottom of the ear. In the autumn-sown wheat, plants were harvested at 8 weeks post-germination and in the spring-sown wheat, plants were harvested at 10 weeks post-germination. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values

respectively, excluding outliers. Statistically significant differences are marked on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (*) denoting a result significantly different to the glucose CD treatment.

4.2.3. Chlorophyll, flavanol, and nitrogen contents in *T. aestivum* cv. Apogee

In the autumn-sown wheat, chlorophyll content was significantly increased in the glucose CD & fertiliser-treated plants relative to the water-treated plants, as presented in Figure 4.3 below.

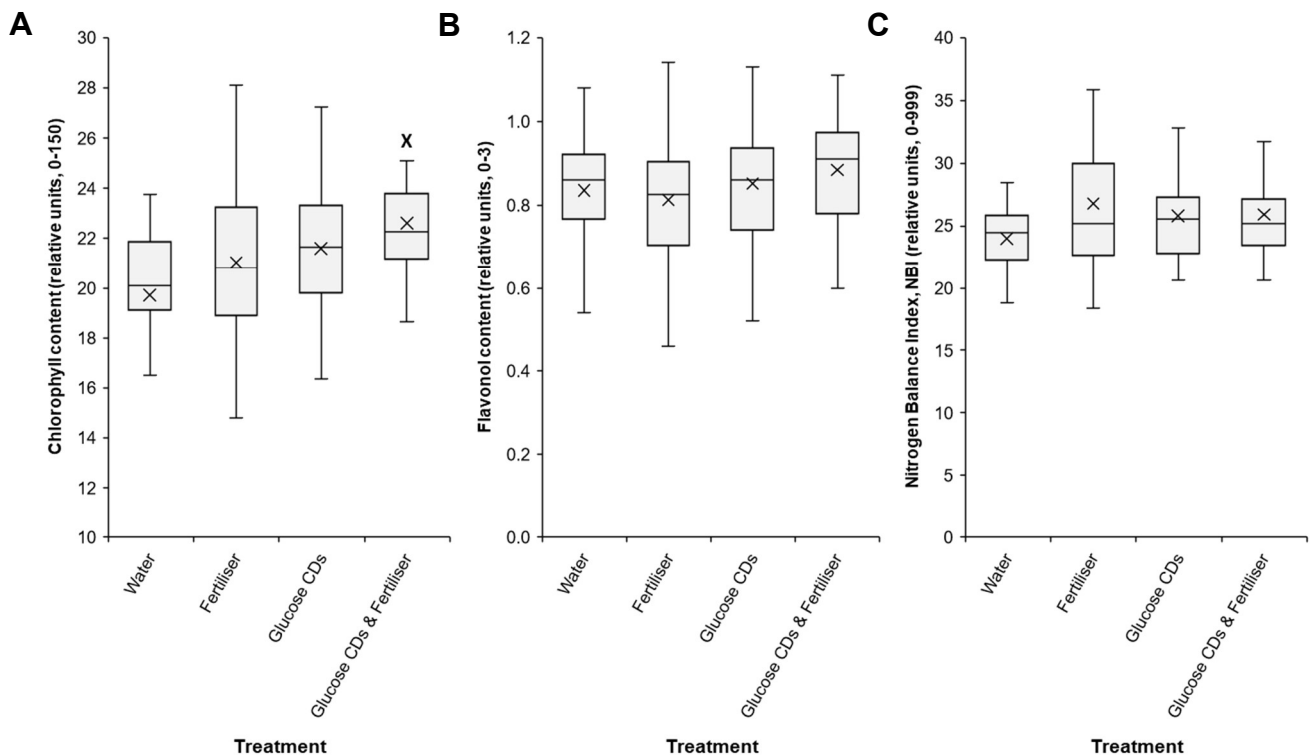


Figure 4.3 – The (A) chlorophyll content, (B) flavanol content, and (C) nitrogen balance index of mature flag leaves in autumn-sown *Triticum aestivum* cv. Apogee, as measured by a DUALEX leafclip optical meter. Measurements were taken six weeks post-germination at a constant point of two inches down the flag leaf from the stem. For each leaf, both the adaxial and abaxial leaf surfaces were measured and an average of the two was calculated for each leaf. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are marked on the graph, with (X) denoting a result significantly different to the water treatment.

There were no significant differences in the spring-sown wheat, as presented in Figure 4.4 below.

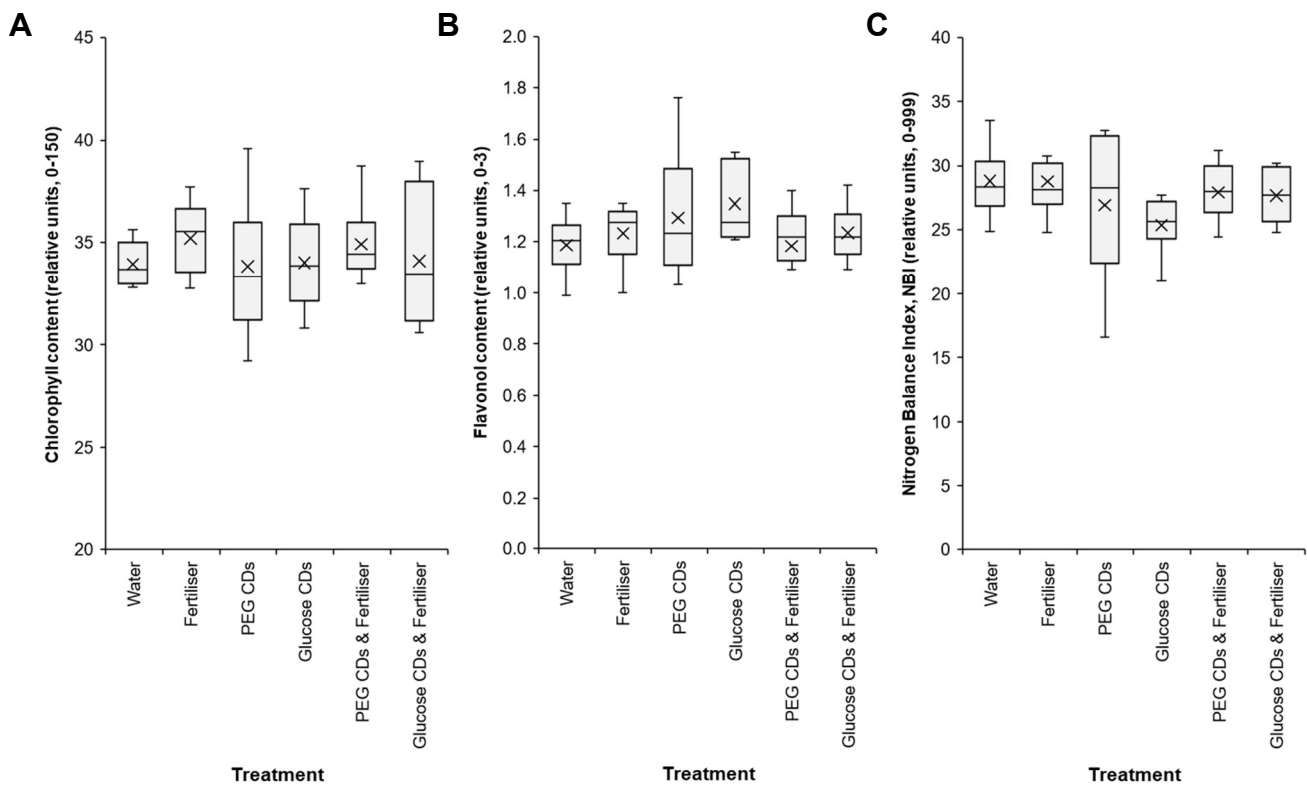


Figure 4.4 – The (A) chlorophyll content, (B) flavonol content, and (C) nitrogen balance index of mature flag leaves in spring-sown *Triticum aestivum* cv. Apogee, as measured by a DUALEX leafclip optical meter. Measurements were taken six weeks post-germination at a constant point of two inches down the flag leaf from the stem. For each leaf, both the adaxial and abaxial leaf surfaces were measured and an average of the two was calculated for each leaf. N = 30 for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. There are no statistically significant differences between groups.

4.2.4. Chlorophyll, flavanol, and nitrogen contents in *T. aestivum* cv. Paragon

In the autumn-sown wheat, chlorophyll content was significantly increased in the glucose CD & fertiliser-treated plants relative to the water-treated plants, as presented in Figure 4.5 below. Similarly, flavonol content was significantly increased in the glucose CD-treated plants relative to the water-treated plants.

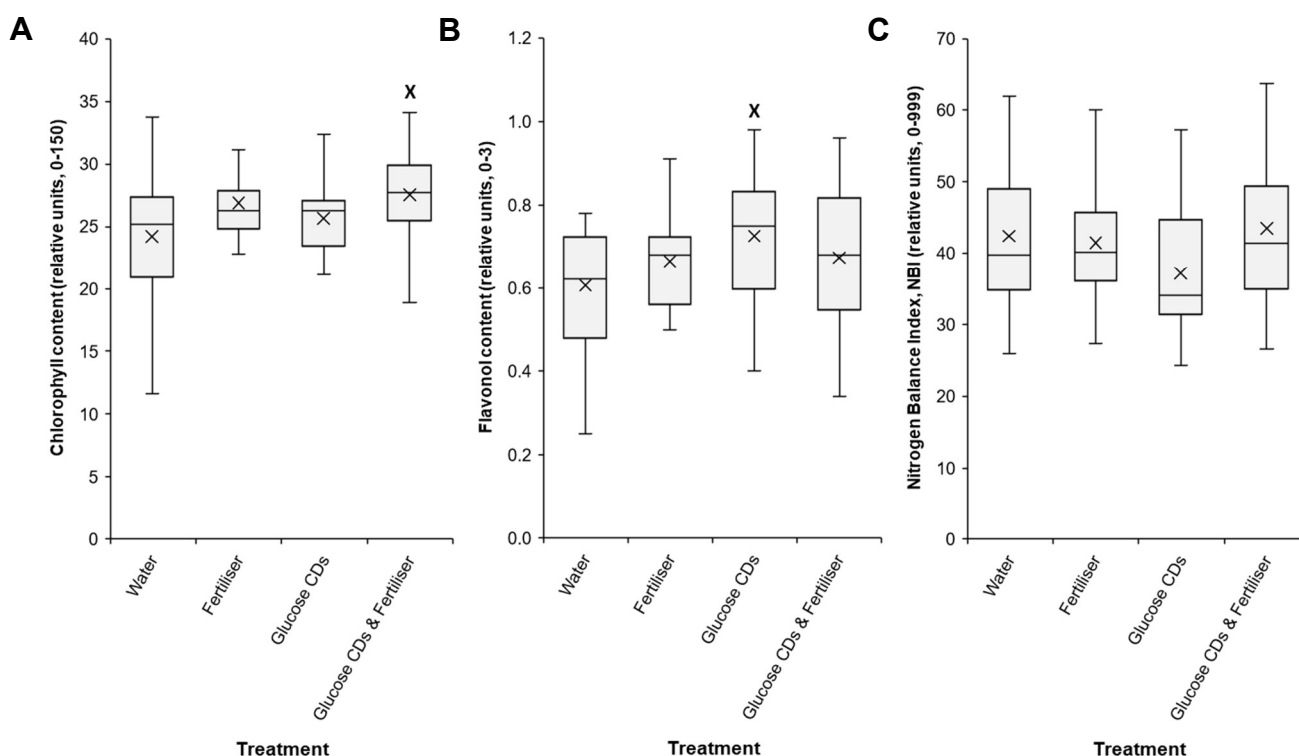


Figure 4.5 – The (A) chlorophyll content, (B) flavonol content, and (C) nitrogen balance index of mature flag leaves in autumn-sown *Triticum aestivum* cv. Paragon, as measured by a DUALEX leafclip optical meter. Measurements were taken six weeks post-germination at a constant point of two inches down the flag leaf from the stem. For each leaf, both the adaxial and abaxial leaf surfaces were measured and an average of the two was calculated for each leaf. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are marked on the graph, with (X) denoting a result significantly different to the water treatment.

In the spring-sown wheat, flavanol content was significantly decreased in the PEG CD-treated plants relative to the water-treated plants, as presented in Figure 4.6 below. By contrast, the nitrogen balance index was significantly increased in the PEG-treated plants relative to the water-treated plants. Furthermore, the PEG-treated plants had a significantly higher nitrogen balance index compared to the PEG CD & fertiliser-treated plants.

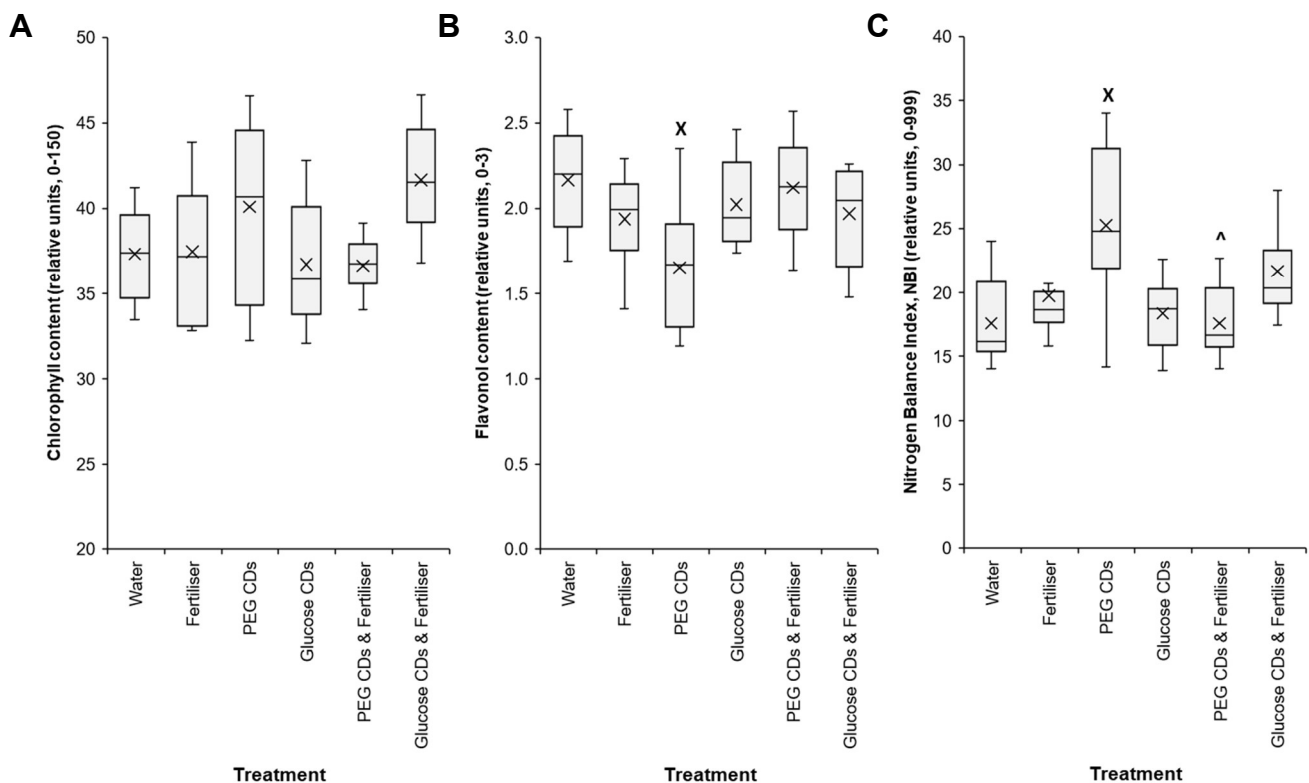


Figure 4.6 – The (A) chlorophyll content, (B) flavonol content, and (C) nitrogen balance index of mature flag leaves in spring-sown *Triticum aestivum* cv. Paragon, as measured by a DUALEX leafclip optical meter. Measurements were taken six weeks post-germination at a constant point of two inches down the flag leaf from the stem. For each leaf, both the adaxial and abaxial leaf surfaces were measured and an average of the two was calculated for each leaf. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are marked on the graph, with (X) denoting a result significantly different to the water treatment and (^) denoting a result significantly different to the PEG CD treatment.

4.3. Discussion

There was no significant impact on plant height in *T. aestivum* cv. Apogee. An extremely fast growing full-dwarf wheat cultivar, ear emergence can occur in *T. aestivum* cv. Apogee wheat after just 23 days in optimum conditions (Koerner, 1997). In this experiment, treatment began three weeks post-germination. In wheat, the bulk of stem elongation is completed before heads emerge (Simmons, Oelke, & Anderson, 1985). Therefore, it is possible that treatment started after stem elongation had occurred, therefore having no impact on plant height. With increased plant height in dwarf cultivars

linked to increased risks of lodging and reduced yields, a lack of impact on plant height could be beneficial if CDs still provide benefits elsewhere.

There were a number of significant impacts on *T. aestivum* cv. Paragon wheat. *T. aestivum* cv. Paragon wheat is a non-dwarf, photoperiod-sensitive cultivar which is slower growing but higher yielding in shorter photoperiods (Shaw et al., 2012). In the autumn-sown wheat, which had 5 less hours of natural light on average compared to the June wheat, the glucose CD & fertiliser-treated plants were 54.10% taller than the water-treated plants. CDs are thought to enhance stem elongation in a number of different ways. Firstly, the CD surface features hydrophilic hydroxyl and carboxyl groups which enables them to adhere water and nutrients to their surface. In this way, once taken up by the roots the CDs are able to transport and gradually release water and nutrients throughout the plant, which promotes growth (Li et al., 2019; Li et al., 2018a). Secondly, CDs promote root elongation, which increases the surface area available for the uptake of nutrients and water from the soil (Li et al., 2018a). This has been seen in mung beans, where CDs significantly increased root growth by 30% and root vigour by 36% which, in turn, led to 18% increased stem lengths (Wang et al., 2018b). Similar effects have been seen in water spinach, rice, and lettuce (Aji et al., 2020; Li et al., 2020; Li, et al., 2021b). Thirdly, CDs increase the light harvesting and photosynthetic capacity of plants, which similarly promotes growth (Chandra et al., 2014; Swift et al., 2021).

Overall, by increasing the uptake capacity and transport of water and nutrients around the plant, and by increasing the photosynthetic capacity of plants, CDs promote growth, and this is seen in the 54.10% increase in plant height of the glucose CD & fertiliser-treated plants when compared to plants treated with water alone. However, increases in plant height will increase the risk of lodging. The glucose CD and the fertiliser treatments did not significantly impact on plant height, and so this elongation effect may be the result of the dual treatment alone. With CDs able to adhere and transport nutrients from the soil, and with synthetic fertiliser application increasing the numbers of these nutrients within the soil, the dual treatment may facilitate the highest possible nutrient uptake and promote maximal stem elongation. Given the increased risk of lodging, the dual application of CDs and synthetic fertiliser may be detrimental to wheat yields if used on non-dwarf cultivars, and so the use of CDs as a biostimulant may require tailoring depending on the cultivar (Berry et al., 2004).

On the other hand, in the spring-sown *T. aestivum* cv. Paragon wheat the fertiliser- and PEG CD-treated plants were 7.43% and 6.88% shorter than the water-treated plants respectively. As a cultivar, *T. aestivum* cv. Paragon is known to flower earlier in longer photoperiods, and in the spring-sown wheat the plants were grown in a photoperiod with 5 more hours of daylight on average than

in the autumn-sown wheat, as outlined in the 'Materials and Methods' chapter. Therefore, it is likely that in the spring-sown wheat a larger proportion of assimilates were partitioned into grain development earlier than in the autumn-sown wheat, shortening the stage of stem elongation (Foulkes et al., 2010; Pérez-Gianmarco, Slafer, & González, 2018). With CDs able to increase nutrient uptake and transport, the application of fertiliser and PEG CDs may have facilitated increased nutrient uptake and in turn increased partitioning of nutrients for grain development, resulting in the decreases in height seen (Foulkes et al., 2010).

In both the autumn-sown *T. aestivum* cv. Apogee and *T. aestivum* cv. Paragon wheat, the glucose CD & fertiliser treatment significantly increased the chlorophyll content of the flag leaves. As light-harvesting pigments, chlorophylls play an extremely important role in photosynthesis and the levels of chlorophylls directly relate to the light-harvesting capacity of a plant. As the levels of chlorophylls increase, more light can be absorbed. Up to a certain point, this can increase the rate of photosynthesis, although too much light can induce photodamage (Mackinney, 1941; Ruban, 2016; Smith & Benitez, 1955). The increase in chlorophyll content corroborates the findings of other studies, with CDs having been shown to increase chlorophyll levels in mung beans, rice, and wheat (Li et al., 2021a; Swift et al., 2021; Wang et al., 2018b). CDs increase the capacity of plants to uptake and transport water and nutrients, and nitrogen is an essential nutrient for maintaining the chlorophyll content of leaves and is strongly associated with the photosynthetic capacity of a leaf (Ercoli et al., 1993; Evans, 1989; Li et al., 2019; Li et al., 2018b). With CDs able to increase the uptake and transport of nitrogen, and with the application of nitrogen from the synthetic fertiliser, the glucose CD & fertiliser treatment may facilitate the highest possible nitrogen uptake from the soil and in turn maximise the chlorophyll content of the leaf.

In *T. aestivum* cv. Paragon, flavonol levels were significantly increased in the autumn-sown glucose CD-treated plants, but were significantly decreased in the spring-sown PEG CD-treated plants. Flavonols are a sub-group of flavonoids, a group of polyphenolic secondary metabolites found in plants (Manach et al., 2004). Flavonols are involved in a number of processes including plant defense and stress tolerance (Chen et al., 2020; Kiani, Arzani, & Maibody, 2021; Treutter, 2006). Flavonol production is light-dependent, with higher light levels being associated with increased flavonol production (Herrmann, 1988). Furthermore, the flavonol content of a leaf is inversely related to nitrogen content, and under nitrogen deficiency flavonol biosynthesis is upregulated (Lillo, Lea, & Ruoff, 2008; Stewart et al., 2001). CDs increase the ability of plants to uptake and transport water and nutrients (Li et al., 2019; Li et al., 2018a). Given that decreased flavonol levels are linked to plentiful nutrient reserves, the ability of CDs to increase nutrient uptake could explain the significant decrease in the flavonol contents of the spring-sown PEG CD-treated *T. aestivum* cv. Paragon wheat

However, the autumn-sown glucose CD-treated *T. aestivum* cv. Paragon wheat had significantly increased flavanol levels, contrary to what would be expected from an increased level of nutrient uptake. Under high light levels flavanols are upregulated, and it has been shown that flavonols provide protection from photodamage in these conditions (Guidi et al., 2016; Merzlyak, Melø, & Naqvi, 2008; Ryan et al., 2002). CDs have strong light harvesting properties and are able to easily exchange electrons once photoexcited (Choi et al., 2018; Li et al., 2020a; Li et al., 2018b; Swift et al., 2018). Therefore, it is possible that glucose CD-application in the autumn-sown wheat increased the light-harvesting capacity of the plants. If so, flavonol production would be expected to increase in order to provide photoprotection. Similarly, the NBI was significantly increased in the PEG CD-treated spring-sown *T. aestivum* cv. Paragon wheat, indicating an increased ratio of chlorophyll to flavanols. Higher nitrogen levels are strongly correlated with photosynthetic capacity (Song et al., 2013). Lastly, the PEG-treated plants had a significantly higher nitrogen balance index compared to the PEG CD & fertiliser-treated plants. If the PEG CDs were increasing nitrogen primarily by increasing nutrient uptake, it would be expected that the PEG CD & fertiliser-treated plants would have a nitrogen balance index than PEG CDs. Therefore, it is possible that the effects of PEG CDs on photosynthetic capacity are more impactful on the nitrogen balance index than their ability to transport nutrients. This will be explored further in Chapters 5 and 7, where the impacts of CDs on photosynthesis will be investigated.

4.4. Plant height and nitrogen status – concluding remarks

In *T. aestivum* cv. Apogee, there were no significant impacts on plant height, whereas in *T. aestivum* cv. Paragon plant height was greatly increased, which greatly increases the risk of lodging and could reduce yields in the field. This could be due to the increased nutrient uptake facilitated by CDs, as seen in the increased nitrogen and chlorophyll contents. Therefore, CD application may need to be tailored depending on the cultivar. While the increased nutrient uptake facilitated by CDs may increase yields in dwarf wheat cultivars, it could decrease yields in non-dwarf cultivars, and so this will be explored in later chapters.

5. The impacts of carbon dots on photosynthesis

5.1 Introduction

5.1.1. Photosynthesis: a brief history and summary

Photosynthesis is the most essential process for life on Earth, historically transforming the prehistoric atmosphere and facilitating the evolution of the lifeforms seen on Earth today (Fischer, Hemp, & Johnson, 2016; Hohmann-Marriott & Blankenship, 2011). Simply, photosynthesis converts sunlight energy into electrical energy, which is then converted into chemical energy (Segalla et al., 2005). Photosynthesis is composed of two core reactions – a primary light-dependent reaction (LDR), and a secondary light-independent reaction (LIR). Both reactions occur within chloroplasts, organelles with endosymbiotic origins that historically integrated into host cells during the evolutionary history of photosynthesis (McFadden, 2001; Waters & Langdale, 2009). The chloroplast internal membrane consists of structures called thylakoids, which themselves consist of stacks of membrane discs called grana. Interconnected by structures called lamellae, thylakoids make up a continuous membrane network within chloroplasts and this is the site of the LDR. Internal to thylakoids is the lumen, and external to thylakoids is the chloroplast stroma; the latter is the site of the LIR (Pribil, Labs, & Leister, 2014).

In the LDR, light energy drives the transport of electrons along a series of protein complexes. There are four multi-subunit proteins involved in the LDR: photosystem I (PSI), photosystem II (PSII), cytochrome b_6f , and F-ATPase (Nelson & Yocum, 2006). The reaction begins in PSII, a protein complex composed of three parts – a central reaction centre (RCII), an oxygen-evolving complex (OEC), and light-harvesting complexes (LHCII) (Nelson & Yocum, 2006). The LHCII are made up of proteins bound to light-harvesting chlorophyll pigments (Senge et al., 2014). When LHCII absorb light, the chlorophylls become excited and initiate a chain of down-stream electron transport (Nelson & Yocum, 2006). Water is split, producing electrons and oxygen, in a reaction catalysed by the OEC (Murchie & Niyogi, 2010; Nelson & Yocum, 2006). The electrons are transported to cytochrome b_6f , a transmembrane protein complex, which transports the electrons to PSI. Simultaneously, this process transports protons across the thylakoid membrane, producing a proton gradient across the membrane which drives the production of ATP by ATP synthase (Nelson & Yocum, 2006). Like PSII, PSI is a protein complex; however, it is made up of only two parts – a central reaction centre (RCI), and light-harvesting complexes (LHCI). When the LHCI absorb light, electrons are donated to the enzyme ferredoxin NADP reductase, reducing NADP^+ to NADPH (Nelson &

Yocum, 2006). The end products of the LDR, ATP and NADPH, are then taken forward into the LIR (Murchie & Niyogi, 2010).

In the LIR, ATP and NADPH are involved in CO₂ fixation in a cyclic reaction catalysed by the enzyme Rubisco. The products of this reaction are used to synthesise useful carbohydrates such as glucose, which are either exported to the cytosol for immediate use or stored as starch within chloroplasts (Paul, 2012).

The rates of electron transport and CO₂ fixation are key factors in determining the rate of photosynthesis. The actual rate of photosynthesis often underperforms the maximum theoretical rate. Many factors contribute to this, including environmental factors such as light levels, nutrient availability, and temperature, as well as architectural factors such as plant height, leaf angle, and leaf surface area (Anten, 2005; Murchie & Niyogi, 2010). While light levels are a significant limiting factor for photosynthesis, the relationship between light and photosynthesis is not completely linear; while low light levels decrease the rate of photosynthesis, increasing light levels will only increase the photosynthetic rate up to a certain threshold point. There is a maximum amount of light that can be safely used to drive photosynthesis, and any light absorbed above this threshold can cause photodamage, decreasing not only the photosynthetic rate but also growth and yield. Non-regulated energy dissipation (NO) is the result of energy lost between LHCII and RCII under high light levels, and this energy is lost in the form of unsafe, unregulated heat dissipation. Under normal light levels, NO is maintained at a minimum level; however, under high light levels, NO is increased and this results in the production of harmful reactive oxygen species (ROS) (Samson, Bonin, & Maire, 2019). While ROS are important components of many plant signalling pathways, they are harmful to the photosynthetic apparatus of plants and can damage proteins such as PSI and PSII, decreasing photosynthesis, growth, and yield (Choudhury et al., 2017; Gill & Tuteja, 2010; Murchie & Niyogi, 2010).

Light levels naturally fluctuate over the course of a day, and plants have evolved a number of adaptive mechanisms to optimise light absorption and prevent photodamage (Murchie & Niyogi, 2010). Under high light levels, photoprotective mechanisms aim to prevent increased ROS production; they do this by regulating the absorption and dissipation of excess excitation energy. To regulate excess light absorption, plants can change the angle of leaves, move chloroplasts deeper within leaves, and adjust the sizes of light-harvesting complexes (Fujita et al., 1989; Johnson et al., 2011; Lovelock & Clough, 1992; Wada, Kagawa, & Sato, 2003). To regulate the dissipation of excess energy, plants can increase the rate of safe thermal dissipation, increase the number of electron

sinks, and increase ROS scavenging (de Bianchi et al., 2010; Murchie & Niyogi, 2010; Neely, Martin, & Barker, 1988; Streb et al., 2005).

Increasing the rate of safe thermal dissipation is the main mechanism by which excess energy is safely dissipated in plants. This process is called non-photochemical quenching (NPQ), and it is a photoprotective mechanism which minimizes photodamage under high light levels (de Bianchi et al., 2010). The rate of NPQ is regulated by the proton concentration in the lumen. Under high light levels, excess energy absorption leads to proton accumulation in the lumen. This increases the pH of the lumen, triggering NPQ once a pH threshold is reached (Pospíšil, 1998). PSII is particularly susceptible to photodamage, and although there are repair mechanisms for photodamage, NPQ remains the most rapid and effective solution (Ruban, 2016). Therefore, NPQ is essential in regulating PSII activity, and thus the photosynthetic rate as a whole.

5.1.2. Measuring photosynthesis: chlorophyll fluorescence

To measure photosynthesis, a technique called chlorophyll fluorescence can be used. A relatively simple technique, chlorophyll fluorescence relies on the principle that any light absorbed by a leaf must either be (a) used in photosynthesis, (b) dissipated as heat, or (c) emitted as fluorescence. From this principle, by exposing leaves to known wavelengths of light and measuring the resultant fluorescence, it is possible to measure a number of different parameters which provide insights into the photosynthetic activity of the plant (Maxwell & Johnson, 2000; Schreiber, Schliwa, & Bilger, 1986).

Chlorophyll fluorescence measurements follow a characteristic trace, as presented in Figure 5.1. Measurements are taken using a measuring light, with the first measurement recording a value of minimum fluorescence, represented by the symbol F_0 . Then, a brighter, saturating light is applied, which allows for the measurement of maximum fluorescence in the dark, as represented by the symbol F_m . Then, photosynthetically active light is applied, in order to begin driving photosynthesis. This light is followed by another pulse of saturating light, which allows measurement of the maximum fluorescence in the light, represented by the symbol F_m' . Lastly, the measurement of fluorescence between the application of the photosynthetically active light and the saturating pulse can be measured, which is represented by F_t (Maxwell & Johnson, 2000; Schreiber, Schliwa, & Bilger, 1986).

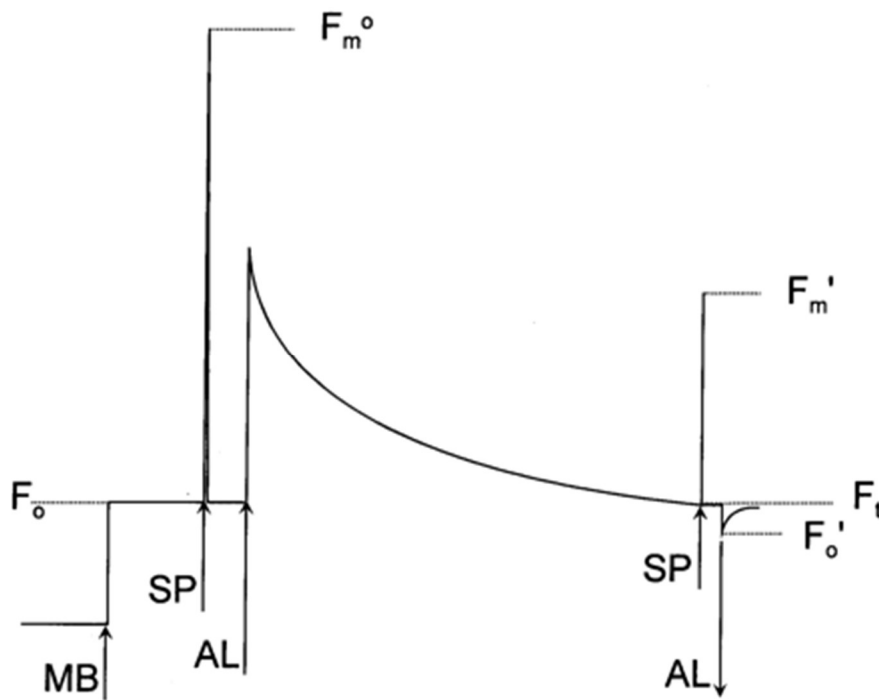


Figure 5.1 Example of a chlorophyll fluorescence trace, where (MB) marks the point at which the measuring light is switched on, (SP) marks the application of a saturating pulse, (F_o) represents the minimum chlorophyll fluorescence value, and (F_m') represents the maximum chlorophyll fluorescence value. Taken from Maxwell and Johnson, 2000.

Together, these measurements allow for the calculation of a number of fluorescence parameters.

These include, but are not limited to:

1. Φ_{PSII} , or $Y(II)$; effective quantum yield of PSII; calculated by $F'_m - F_t / F'_m$
2. F_v/F_m ; the maximum quantum yield of PSII; calculated by $F_m - F_o / F_m$
3. NPQ; non-photochemical quenching; calculated by $F_m - F'_m / F'_m$
4. $Y(NPQ)$; quantum yield of non-photochemical quenching; calculated by $1 - Y(NO) - Y(II)$
5. $Y(NO)$; quantum yield of non-regulated energy dissipation; calculated by $1 / (NPQ + 1 + q_L \cdot (F_m / F_o - 1))$ (Maxwell & Johnson, 2000; Schreiber, Schliwa, & Bilger, 1986).

Fluorescence parameters measured in this chapter are outlined in the Methods section to follow.

5.1.3. Thermography, stomatal conductance, and photosynthesis

Another way to investigate photosynthesis is to look at leaf temperature. Leaf temperature is an extremely important factor for many physiological processes within plants, including photosynthesis, respiration, and growth. Leaf temperature is determined by the flow of energy into and out of the leaf. The maintenance of this energy flow is vital for maintaining stable leaf temperatures. Energy flow into the leaf is derived from the energy from sunlight absorbed by the leaf. Some of this energy

will be partitioned for use in photosynthesis, but the majority of light energy absorbed must be dissipated out of the leaf (Gates, 1968).

One route of energy dissipation is the evaporation of water vapour out of the leaf, which is facilitated by a process called transpiration. Here, the evaporation of water has a cooling effect, helping to dissipate energy out of the leaf. As well as helping cool the leaves, transpiration facilitates the transport of nutrients and assimilates up from the roots. It also helps regulate the levels of oxygen (O₂) and carbon dioxide (CO₂) within leaves (Gates, 1968; Kumar & Arakeri, 2020).

Transpiration occurs out of the stomata, small openings on the leaf surface that can open and close in response different stimuli. In doing so, they regulate the rate of transpiration out of leaves (Gates, 1968; Kumar & Arakeri, 2020). The rate of transpiration out of the stomata can be quantified, and this measurement is called the stomatal conductance. Due to its role in regulating the levels of O₂ and CO₂ in leaves, stomatal conductance plays an essential role in regulating the rate of photosynthesis (Urban et al., 2017). The rate of photosynthesis is positively associated with stomatal conductance, and consequently increased rates of stomatal conductance are associated with increased yields (Lu et al., 1998).

Infrared thermography is a technique by which the surface temperature of leaves can be measured (Harrap et al., 2018). Increased leaf temperatures are inversely related to stomatal conductance (Bajons, Klinger, & Schlosser, 2005; Vialet-Chabrand & Lawson, 2019). Therefore, by measuring leaf temperature it is possible to make inferences about photosynthetic activity.

5.1.4. Photosynthesis and carbon dots: chapter aims

Carbon dots (CDs) have been shown to increase the rate of photosynthesis in plants, by increasing the rate of electron transport, chlorophyll levels, rubisco activity, ATP production, and carbohydrate production (Chandra et al., 2014; Li et al., 2018a; Li et al., 2018b; Swift et al., 2021; Wang et al., 2018b). With the rate of photosynthesis shown to be positively associated with yields, the impact of CDs on photosynthesis in wheat will have vast economic implications given its status as a key cereal crop (Jiang et al., 2003; OECD/FAO, 2020; Zelitch, 1982). Herein, the impacts of CDs on photosynthesis, as measured by chlorophyll fluorescence and leaf temperature, is investigated in two cultivars of common wheat, *T. aestivum* cv. Apogee and Paragon.

5.2. Methods

5.2.1 Plant growth and sampling

Two cultivars of common wheat, *Triticum aestivum* cv. Paragon and Apogee, were grown and treated as outlined in the 'Materials and Methods' chapter. Three plants per treatment from the spring-sown wheat were randomly selected when the plants had reached Zadoks stage 7, which was approximately seven weeks post-germination (Zadoks et al., 1974). This was done to allow for comparison of results to previous works of others in the lab group (Swift et al., 2021). A summary of the Zadoks growth stages is presented in Table 2.1 in the 'Materials and Methods' chapter.

5.2.2 Chlorophyll fluorescence

To measure chlorophyll fluorescence, a Pulse Amplitude Modulated (PAM) fluorometer was used (Walz IMAGING-PAM M-Series MAXI). The flag leaves from mature wheat leaves were measured, with 10 measurements taken at random points along each leaf. With three plants measured per treatment group, this resulted in 30 measurements taken per treatment. Plants were dark-adapted for 45 minutes before measurement. To produce the light curves, the actinic light step length was set to 30 seconds. Six levels of photosynthetically active radiation (PAR) were used, ranging from 0-1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, as wheat plants have been shown to respond well to intermediate PAR values (Shikhov, Nesterenko, & Tikhomirov, 2016).

Chlorophyll fluorescence was used record a number of measurements. Firstly, fluorescence yield parameters were used to measure the partitioning of energy in PSII (Genty et al., 1989; Klughammer and Schreiber, 2008; Kramer et al., 2004). The parameters measured were:

1. $Y(II)$ – The effective quantum yield of PSII
2. $Y(NPQ)$ – The quantum yield of regulated energy dissipation
3. $Y(NO)$ – The quantum yield of non-regulated energy dissipation

Next, electron transport rate (ETR) was calculated by using the equation $ETR = Y(II) \times PAR \times 0.5$ (Genty et al., 1989; Maxwell & Johnson, 2000). Then, F_v/F_m values were calculated (Genty *et al.*, 1989). F_v/F_m is a measurement of the maximum quantum efficiency of PSII, and is sensitive to plant stress. By calculating F_v/F_m , the impacts of CDs on plant stress can be evaluated (Maxwell & Johnson, 2000).

CDs have been shown to increase the rate of photosynthesis in plants in a number of different ways, including by increasing light harvesting and electron transport (Chandra et al., 2014; Li et al., 2018a; Li et al., 2018b; Swift et al., 2021; Wang et al., 2018b). However, too much light can induce

photodamage and cause stress (Samson, Bonin, & Maire, 2019). While plants have developed mechanisms to protect themselves from photodamage, in order to assess the suitability of CDs as a biostimulant it is essential to clarify: a) whether CDs increase the rate of photosynthesis, b) whether CDs increase the rate of safe, regulated energy dissipation, or c) whether CDs increase the rate of non-regulated energy dissipation and cause stress to the plant.

5.2.3. Infrared thermography

Two cultivars of common wheat, *Triticum aestivum* cv. Paragon and Apogee, were grown and treated as outlined in the 'Materials and Methods' chapter. Leaf temperature measurements were made using infrared thermography at six weeks post-germination, after three weeks of treatment. At this point, wheat development had reached Zadoks stage 6, as outlined in the 'Methods and Materials' chapter (Zadoks et al., 1974). A FLIR E60bx infrared camera was used to photograph the wheat. Each tray of 10 plants was photographed three times over the course of a day, at 9am, 1pm, and 5pm, to account for the circadian cycle.

Images were analysed using FLIR Tools. For each tray of plants photographed, 20 randomly distributed wheat leaves within each photograph were selected and used to take leaf temperature measurements. This resulted in 60 total measurements per treatment group.

For image analysis in FLIR Tools, a number of settings were used:

1. Emissivity – a measurement of the ability of an object to radiate energy as heat, ranging from 0-1; for image analysis, emissivity was set to 0.98 (Harrap et al., 2018; French, Schmugge, & Kustas, 2000).
2. Distance was set to 0m, as photographs were taken from less than 1m away
3. Atmospheric temperature was set to 22°C, as this was the constantly controlled temperature within the glasshouse
4. Reflected temperature – a measurement of reflected background radiation; to calculate reflected temperature each photograph, a multidimensional mirror was placed in frame when photographing the wheat. Calculating the surface temperature of the multidimensional mirror provided a value for reflected temperature (Harrap et al., 2018).
5. Humidity was set to 70%, as this was the constantly controlled humidity within the glasshouse

An example of the image analysis carried out in FLIR Tools is presented in Figure 5.2, below.

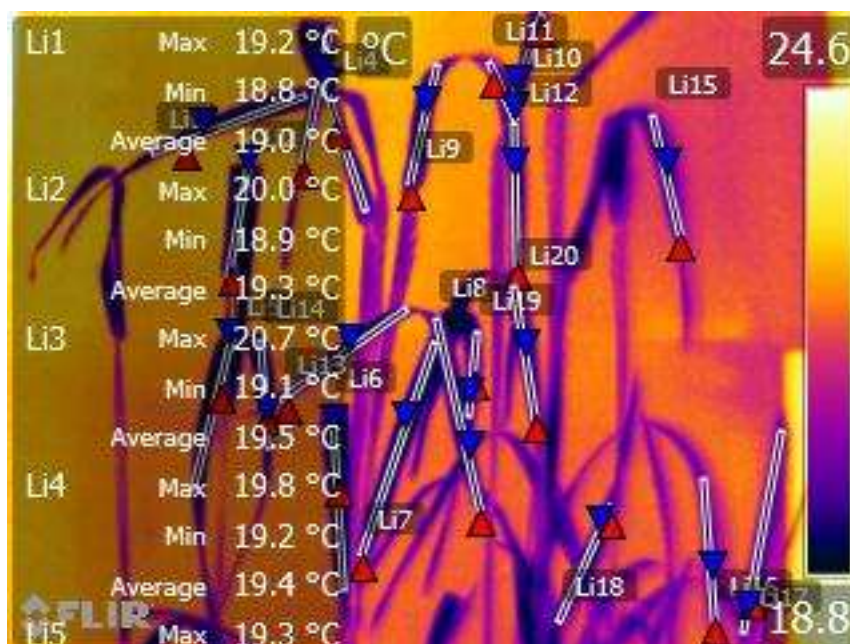


Figure 5.2 – Example of image analysis in FLIR Tools

5.2.4. Statistical Analysis

Data were statistically analysed as outlined in the ‘Materials and Methods’ chapter.

5.2.5. Covid adjustment

When measuring chlorophyll fluorescence, it would have been useful to measure the kinetics of photosynthetic performance to provide more detailed insights into the impacts on photosynthesis. Similarly, it would have been beneficial to test a larger sample size and repeat measurements at different developmental stages. However, due to the timing and training constraints outlined in the Covid Statement, this was not possible. Likewise, gas-exchange analysis would have been an excellent way to analyse the real-time uptake and release of CO₂ and water by the processes of photosynthesis and transpiration (Bernacchi, Diaz-Espejo, & Flexas, 2012). However, as outlined in the Covid Statement, time constraints and a lack of training availability meant that infrared thermography was used instead to provide a brief insight into the impacts on leaf temperature.

5.3. Results

5.3.1 Chlorophyll fluorescence analysis of *T. aestivum* cv. Apogee

Chlorophyll fluorescence analysis revealed a number of statistically significant differences between groups, as displayed in Figure 5.3 below.

For $Y(II)$ and ETR, there were identical significant differences between groups. Compared to the water-treated plants, $Y(II)$ and ETR were significantly increased in the PEG CD-, fertiliser-, glucose CD & fertiliser-, and glucose CD-treated plants at four, two, two, and one light levels respectively. In the PEG CD-treated plants, $Y(II)$ and ETR were also significantly increased relative to the PEG CD & fertiliser treatment.

Compared to the water treatment, $Y(NPQ)$ was significantly decreased in the PEG CD, fertiliser, glucose CD, and glucose CD & fertiliser treatments, at five, three, one, and one light levels respectively. Similarly, $Y(NPQ)$ was significantly lower in PEG CD-treated plants than in fertiliser-treated plants at two light levels. The PEG CD & fertiliser-treated plants had a significantly higher $Y(NPQ)$ than the PEG CD-treated plants but were not significantly different to the water treatment.

$Y(NO)$ was significantly increased by the PEG CD treatment, at four light levels when compared to the water treatment, and at two light levels when compared to the fertiliser treatment. The PEG CD-treated plants also had a significantly increased $Y(NO)$ compared to the PEG CD & fertiliser-treated plants at one light level.

On the other hand, $Y(NO)$ was significantly decreased by the glucose CD & fertiliser treatment, at two light levels when compared to the water treatment, and at three light levels when compared to the fertiliser treatment. Furthermore, the glucose CD & fertiliser-treated plants had a significantly decreased $Y(NO)$ compared to the glucose CD-treated plants at five light levels.

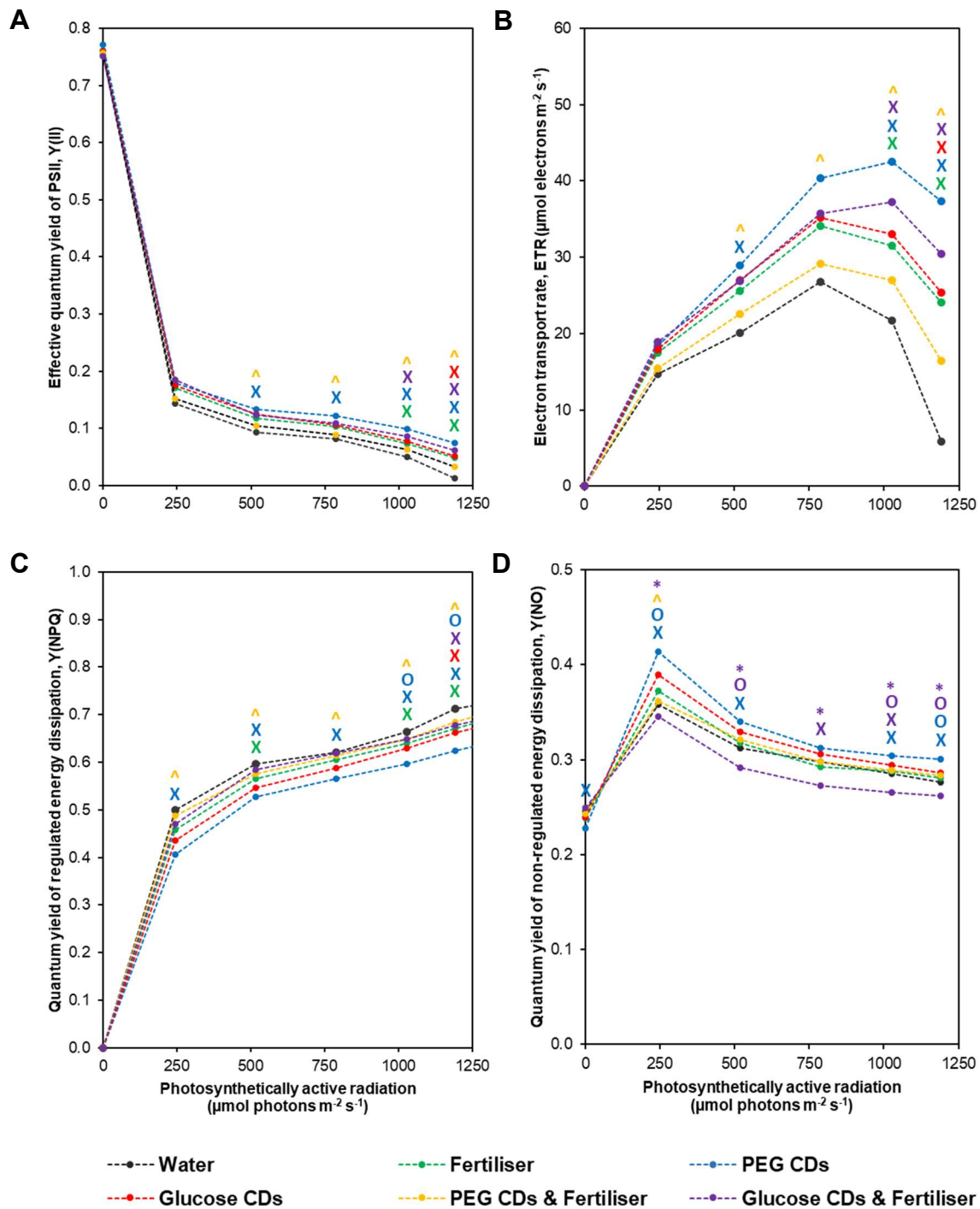


Figure 5.3 Chlorophyll fluorescence measurements in *Triticum aestivum* cv. Apogee, as shown by (A) light curve of effective quantum yield of PSII, $Y(II)$, (B) light curve of electron transport rate, (ETR), (C) light curve of quantum yield of regulated energy dissipation, $Y(NPQ)$, and (D) light curve of quantum yield of non-regulated energy dissipation, $Y(NO)$. $N = 30$ for all groups. Measurements were taken at seven weeks post-germination. Statistically significant differences are marked on the figure. Symbols are categorised into treatment group by colour as per the key. Symbol shape denotes a significant difference from the (X) water treatment, (O) fertiliser treatment, (*) glucose CD treatment, and (^) PEG CD treatment.

There were no statistically significant differences between groups for Fv/Fm, as presented in Figure 5.4 below.

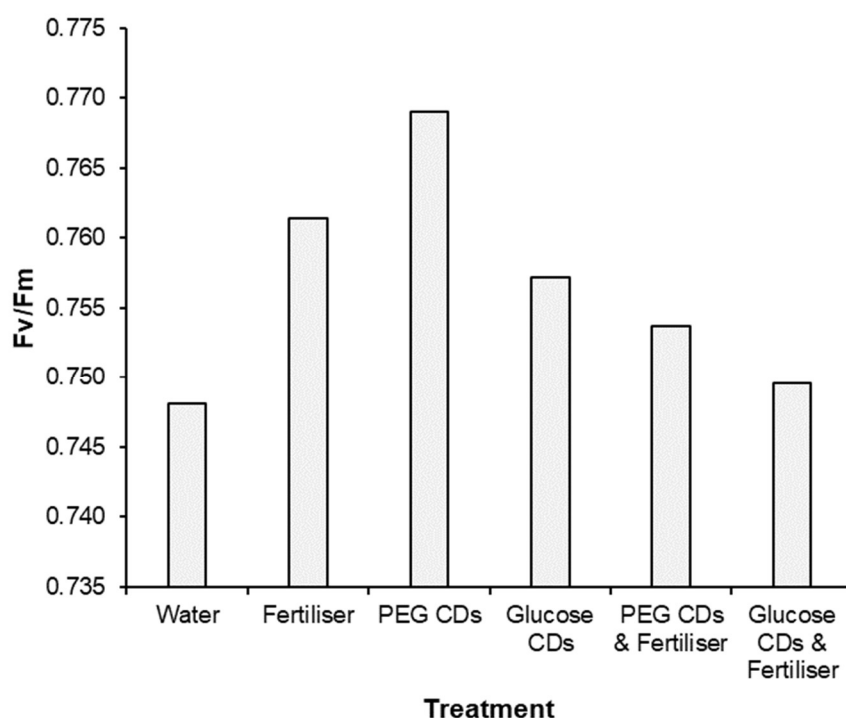


Figure 5.4 – Fv/Fm values in *Triticum aestivum* cv. Apogee wheat. Values were calculated from chlorophyll fluorescence measurements taken at seven weeks post-germination. N = 30 for all groups. There are no statistically significant differences between groups.

5.3.2 Chlorophyll fluorescence analysis of *T. aestivum* cv. Paragon

Multiple statistically significant differences were revealed by chlorophyll fluorescence analysis, as displayed in Figure 5.5 below.

Compared to the water treatment, Y(II) was significantly increased by the glucose CD treatment at three light levels. Similarly, Y(II) was significantly increased by the glucose CD treatment at one light level when compared to the fertiliser treatment. On the other hand, glucose CD & fertiliser treatment significantly decreased Y(II) at two light levels when compared to the water treatment, and at five light levels when compared to the fertiliser and glucose CD treatments. Y(II) was significantly increased in the PEG CD treatment at one light level relative to the PEG CD & fertiliser treatment. The PEG CD treatment also significantly increased Y(II) at one light level when compared to the water treatment.

The results for ETR were similar to Y(II), but not identical as they were for *T. aestivum* cv. Apogee. ETR was significantly increased by the glucose CD treatment at two light levels when compared to

the water treatment, and at two light levels when compared to the fertiliser treatment. ETR was significantly increased by the PEG CD treatment at one light level relative to the water treatment and was significantly increased compared to the PEG CD & fertiliser treatment at one light level as well. Conversely, glucose CD & fertiliser treatment significantly decreased ETR at three light levels compared to the water treatment, and at five light levels compared to the and glucose CD treatments.

$Y(NPQ)$ was significantly decreased in the glucose CD-treated plants at four light levels relative to the water treatment, and at five light levels relative to the fertiliser and glucose CD & fertiliser treatments. Similarly, $Y(NPQ)$ was significantly decreased in the PEG CD-treated plants at three light levels relative to the water treatment, at four light levels relative to the fertiliser treatment, and at five light levels relative to the PEG CD & fertiliser treatment.

PEG CD treatment significantly increased $Y(NO)$ at five light levels relative to the fertiliser treatment, at one light level relative to the water treatment, and at four light levels relative to the PEG CD & fertiliser treatment. Likewise, $Y(NO)$ was significantly increased in the glucose CD-treated plants at two light levels relative to the fertiliser treatment. $Y(NO)$ was significantly decreased in fertiliser at one light level relative to the water treatment. Lastly, $Y(NO)$ was significantly increased in the glucose CD & fertiliser treated plants at five light levels relative to the fertiliser treatment and at two light levels relative to the water treatment.

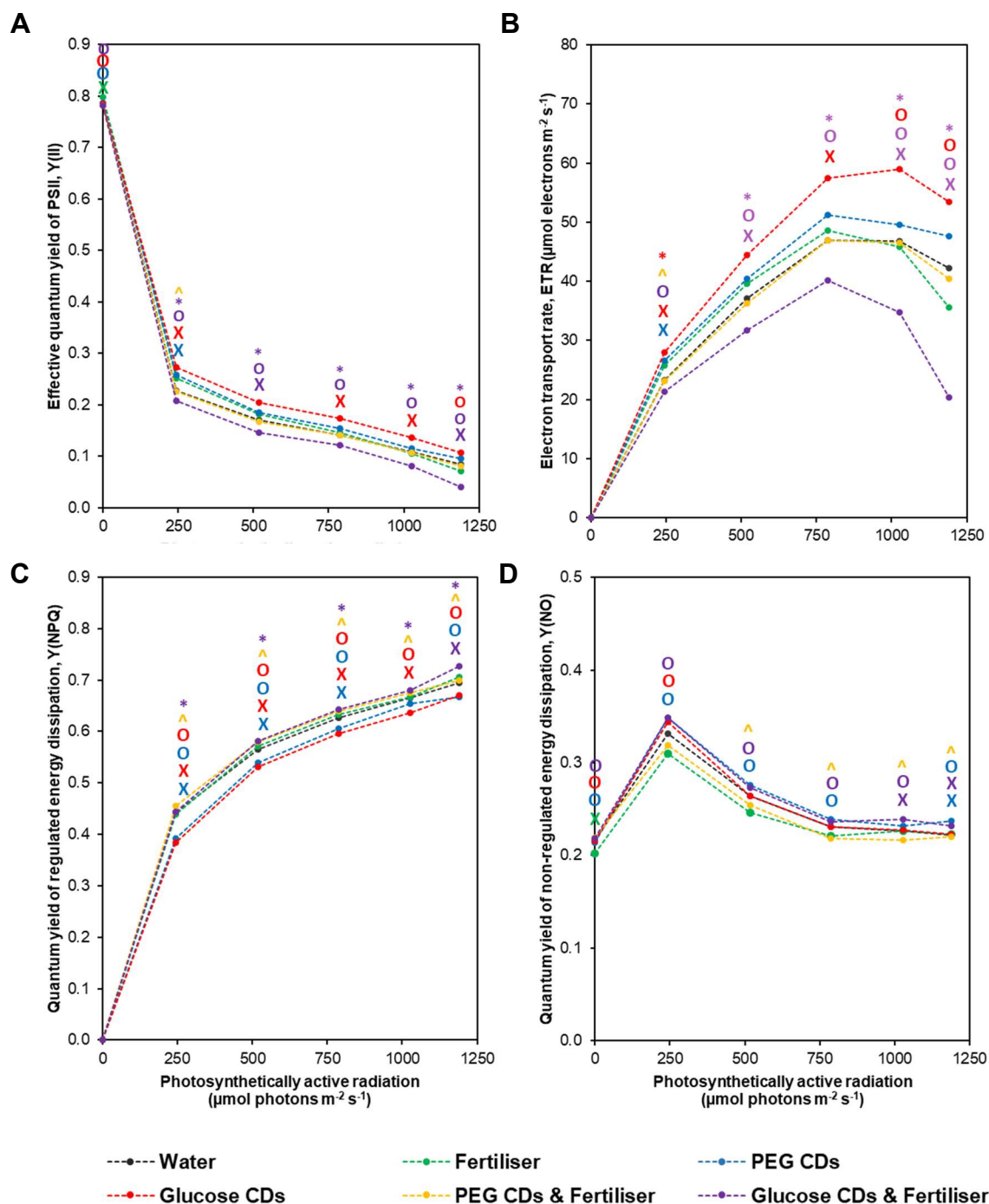


Figure 5.5 Chlorophyll fluorescence measurements in *Triticum aestivum* cv. *Paragon*, as shown by (A) light curve of effective quantum yield of PSII, $Y(II)$, (B) light curve of electron transport rate, (ETR), (C) light curve of quantum yield of regulated energy dissipation, $Y(NPQ)$, and (D) light curve of quantum yield of non-regulated energy dissipation, $Y(NO)$. $N = 30$ for all groups. Measurements were taken at seven weeks post-germination. Statistically significant differences are marked on the figure. Symbols are categorised into treatment group by colour as per the key. Symbol shape denotes a significant difference from the (X) water treatment, (O) fertiliser treatment, (*) glucose CD treatment, and (^) PEG CD treatment.

Some statistically significant differences were found between groups for Fv/Fm, as presented in Figure 5.6 below. In all treatment groups, Fv/Fm was significantly decreased when compared to the fertiliser treatment.

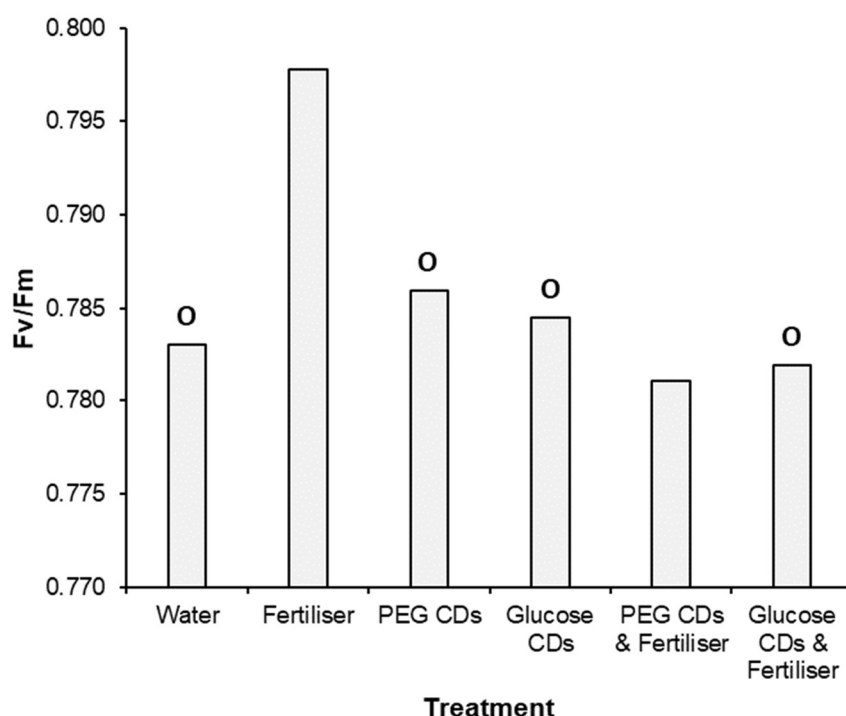


Figure 5.6 – Fv/Fm values in *Triticum aestivum* cv. Paragon wheat. Values were calculated from chlorophyll fluorescence measurements taken at seven weeks post-germination. N = 30 for all groups. Statistically significant groups are marked on the graph, with (O) representing a significant difference relative to the fertiliser treatment.

5.3.3 Leaf temperature analysis of *T. aestivum* cv. Apogee

Leaf temperature varied significantly between groups in the autumn-sown wheat, as presented in Figure 5.7 below. Leaf temperature was significantly decreased at 9am in the glucose-CD treatment compared to the water and fertiliser treatments, at 9am in the glucose CD & fertiliser-treated plants when compared to the water treatment, and at 1pm when compared to both the fertiliser and glucose CD treatments. The only group to have a significantly different leaf temperature over the whole day average was in the glucose CD & fertiliser treatment, where leaf temperature was significantly decreased compared to the fertiliser treatment.

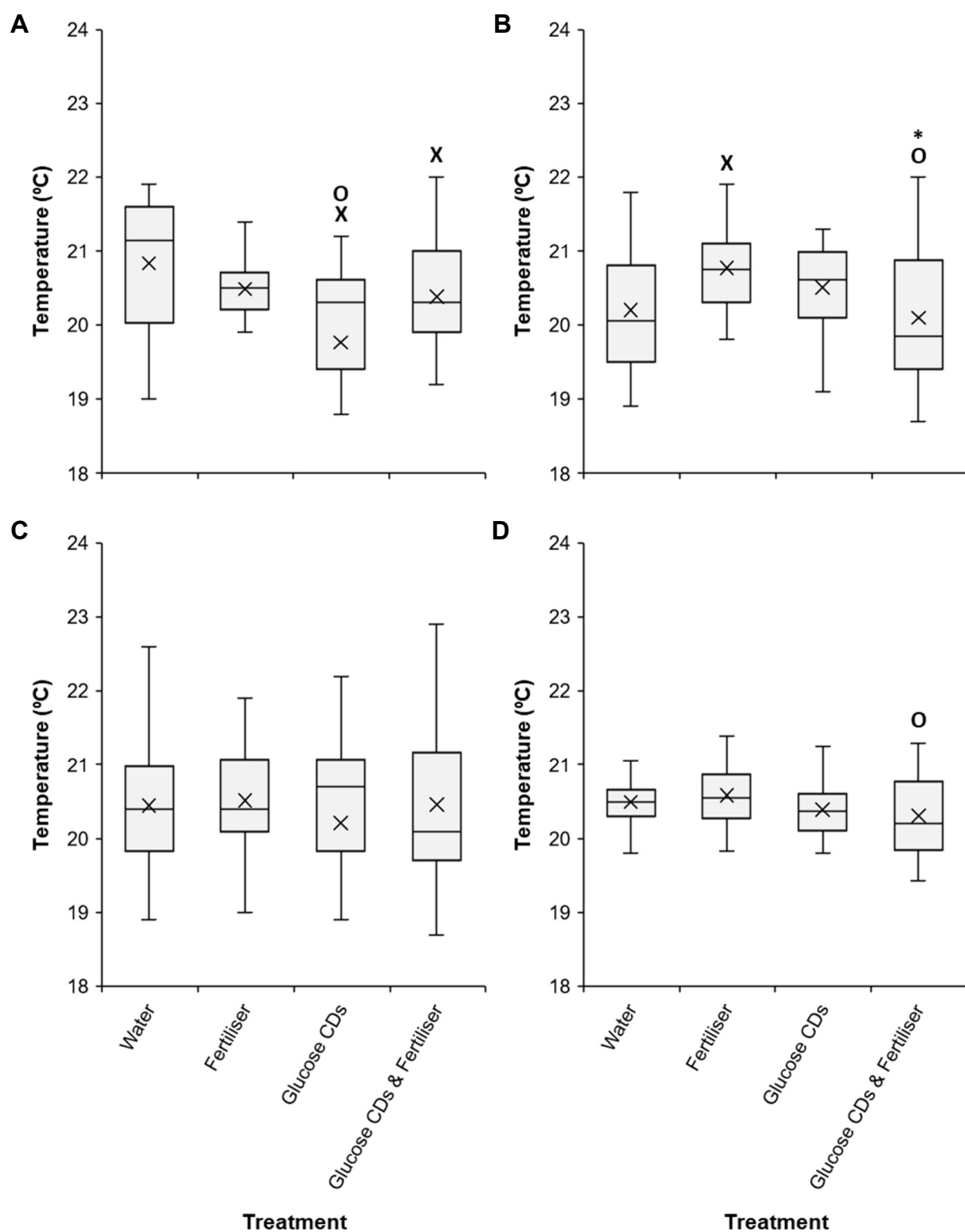


Figure 5.7 Leaf temperature in autumn-sown *Triticum aestivum* cv. Apogee wheat over the course of a day, as shown by (A) leaf temperature at 9am, (B) leaf temperature at 1pm, (C) leaf temperature at 5pm, and (D) average daily leaf temperature. Leaf temperature was measured using infrared thermography at six weeks post-germination. $N = 60$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and

75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and () denoting a result significantly different to the glucose CD treatment.*

Like in the autumn-sown wheat, leaf temperature differed significantly between groups in the spring-sown wheat, as presented in Figure 5.8 below. Leaf temperature was significantly decreased by the fertiliser treatment at 9am when compared to the water treatment. PEG CD treatment significantly increased leaf temperature at 9am when compared to the water treatment, and at both 1pm and over the whole day average when compared to both the water and fertiliser treatments. Glucose CD treatment significantly increased leaf temperature at 1pm but significantly decreased leaf temperature at 5pm relative to the water control. PEG CD & fertiliser treatment significantly decreased leaf temperature at 9am relative to the fertiliser and PEG CD treatments, significantly increased leaf temperature at 1pm relative to the water treatment, significantly decreased leaf temperature at 5pm relative to the water treatment, and significantly decreased leaf temperature across the whole day average relative to the PEG CD treatment. Lastly, glucose CD & fertiliser treatment significantly increased leaf temperature at 9am relative to the water treatment, significantly decreased leaf temperature at 1pm relative to the glucose CD treatment, significantly decreased leaf temperature at 5pm relative to the water treatment, and significantly decreased leaf temperature across the whole day average when compared to both the water and glucose CD treatments.

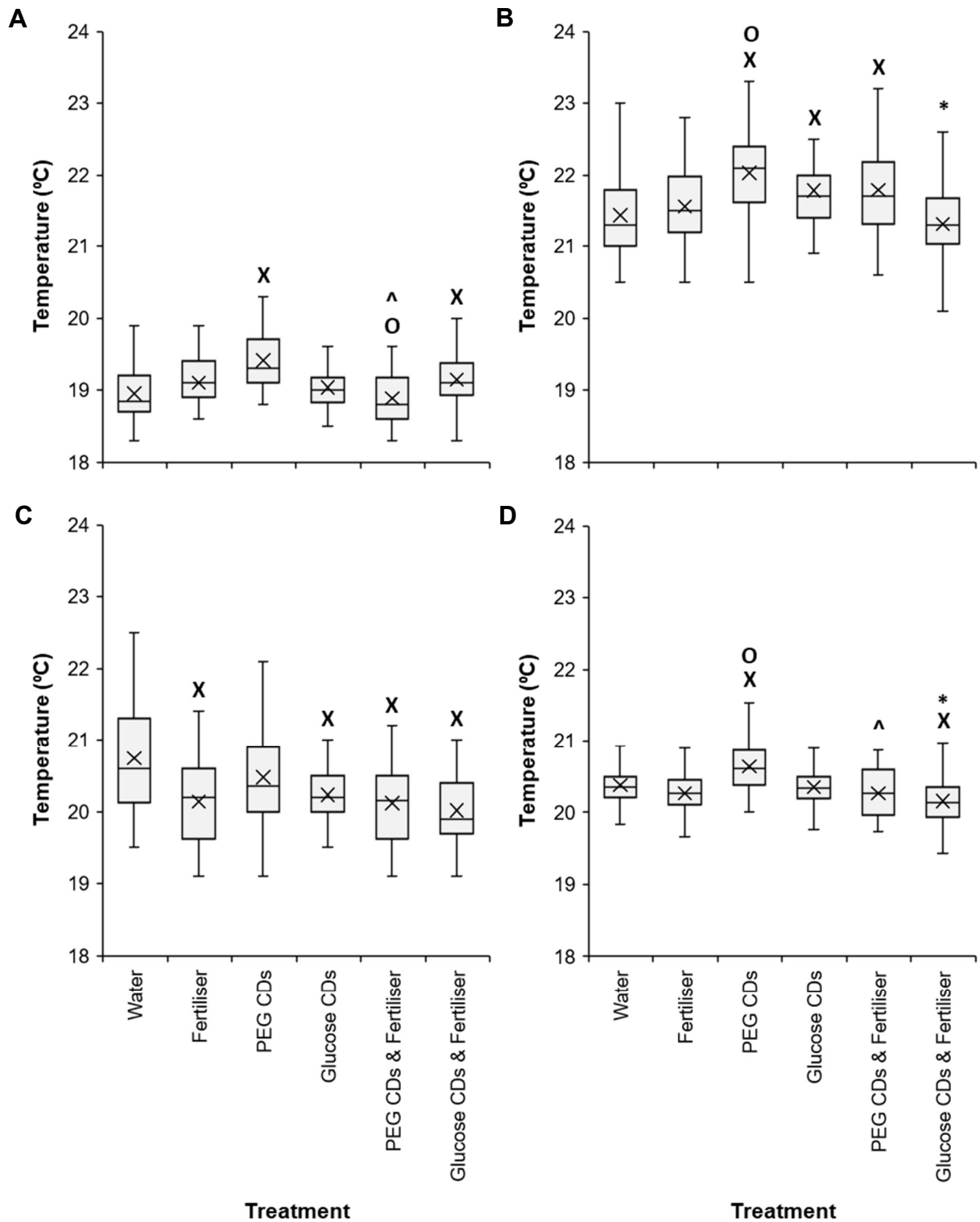


Figure 5.8 Leaf temperature in spring-sown *Triticum aestivum* cv. *Apogee* wheat over the course of a day, as shown by (A) leaf temperature at 9am, (B) leaf temperature at 1pm, (C) leaf temperature at 5pm, and (D) average daily leaf temperature. Leaf temperature was measured using infrared thermography at six weeks post-germination. $N = 60$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and

75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, (*) denoting a result significantly different to the glucose CD treatment, and (^) denoting a result significantly different to the PEG CD treatment.

5.3.4 Leaf temperature analysis of *T. aestivum* cv. Paragon

In the autumn-sown wheat, leaf temperature varied statistically significantly between groups, as presented in Figure 5.9 below. Leaf temperature was significantly increased at 9am in the glucose CD-treated plants relative to the fertiliser-treated plants. On the other hand, at 1pm leaf temperature was significantly decreased in glucose CD-treated plants relative to the water, fertiliser, and glucose CD & fertiliser treatments.

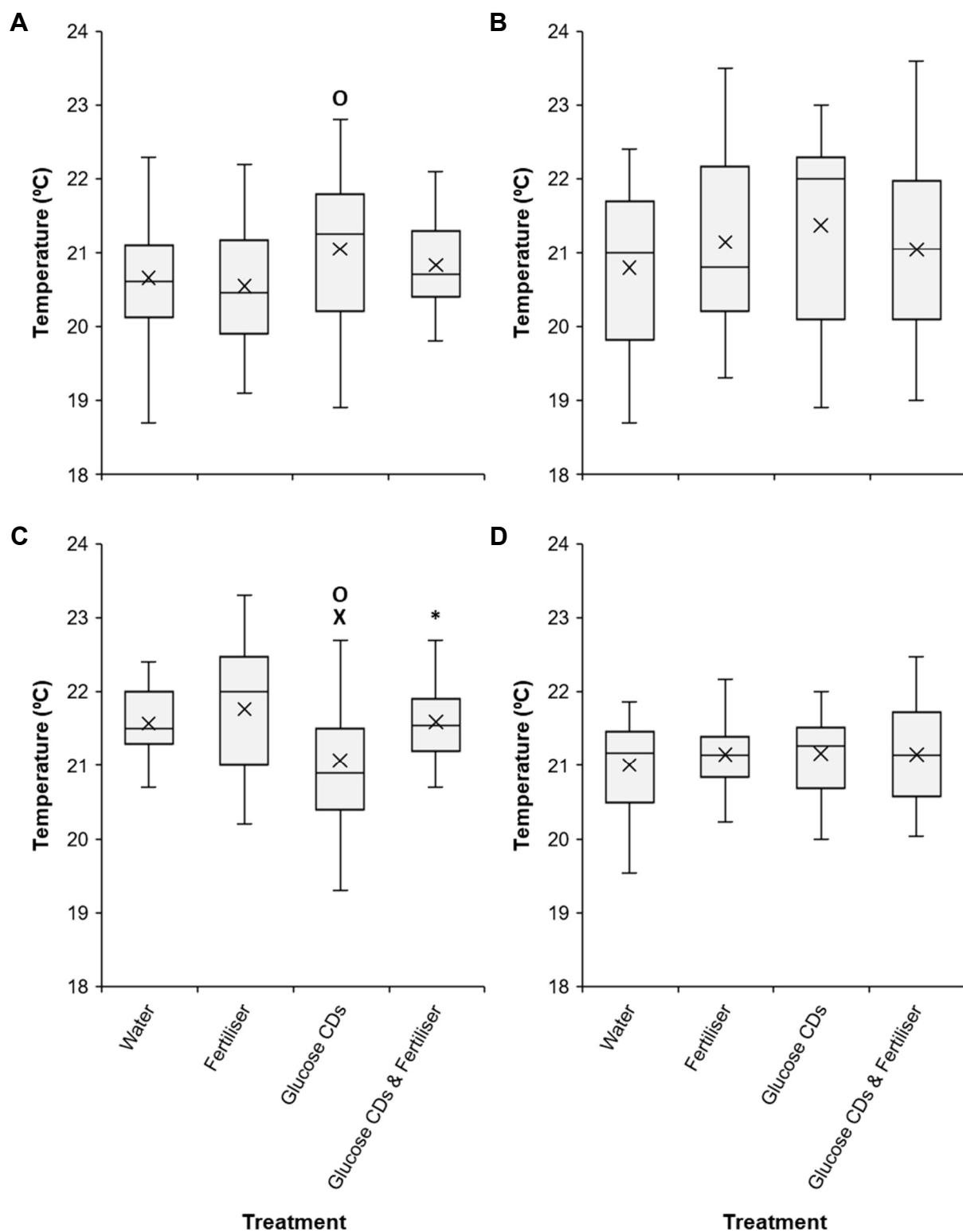


Figure 5.9 Leaf temperature in autumn-sown *Triticum aestivum* cv. *Paragon* wheat over the course of a day, as shown by (A) leaf temperature at 9am, (B) leaf temperature at 1pm, (C) leaf temperature at 5pm, and (D) average daily leaf temperature. Leaf temperature was measured using infrared thermography at six weeks post-germination. $N = 60$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and

75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and () denoting a result significantly different to the glucose CD treatment.*

Leaf temperature differed significantly between groups in the spring-sown wheat, as presented in Figure 5.10 below. Fertiliser treatment significantly decreased leaf temperature at 9am but significantly increased leaf temperature at 1pm when compared to the water treatment. PEG CDs significantly increased leaf temperature at 9am relative to the water treatment and significantly decreased leaf temperature at 1pm relative to the fertiliser treatment. Glucose CD treatment significantly increased leaf temperature at 1pm and across the whole day average when compared to the water treatment. PEG CDs & fertiliser treatment significantly increased leaf temperature at 5pm when compared to the water, fertiliser, and PEG CD treatments. Lastly, glucose CD & fertiliser treatment significantly increased leaf temperature at 9am when compared to the water, fertiliser, and glucose CD treatments, but significantly decreased leaf temperature at 1pm when compared to the fertiliser and glucose CD treatments.

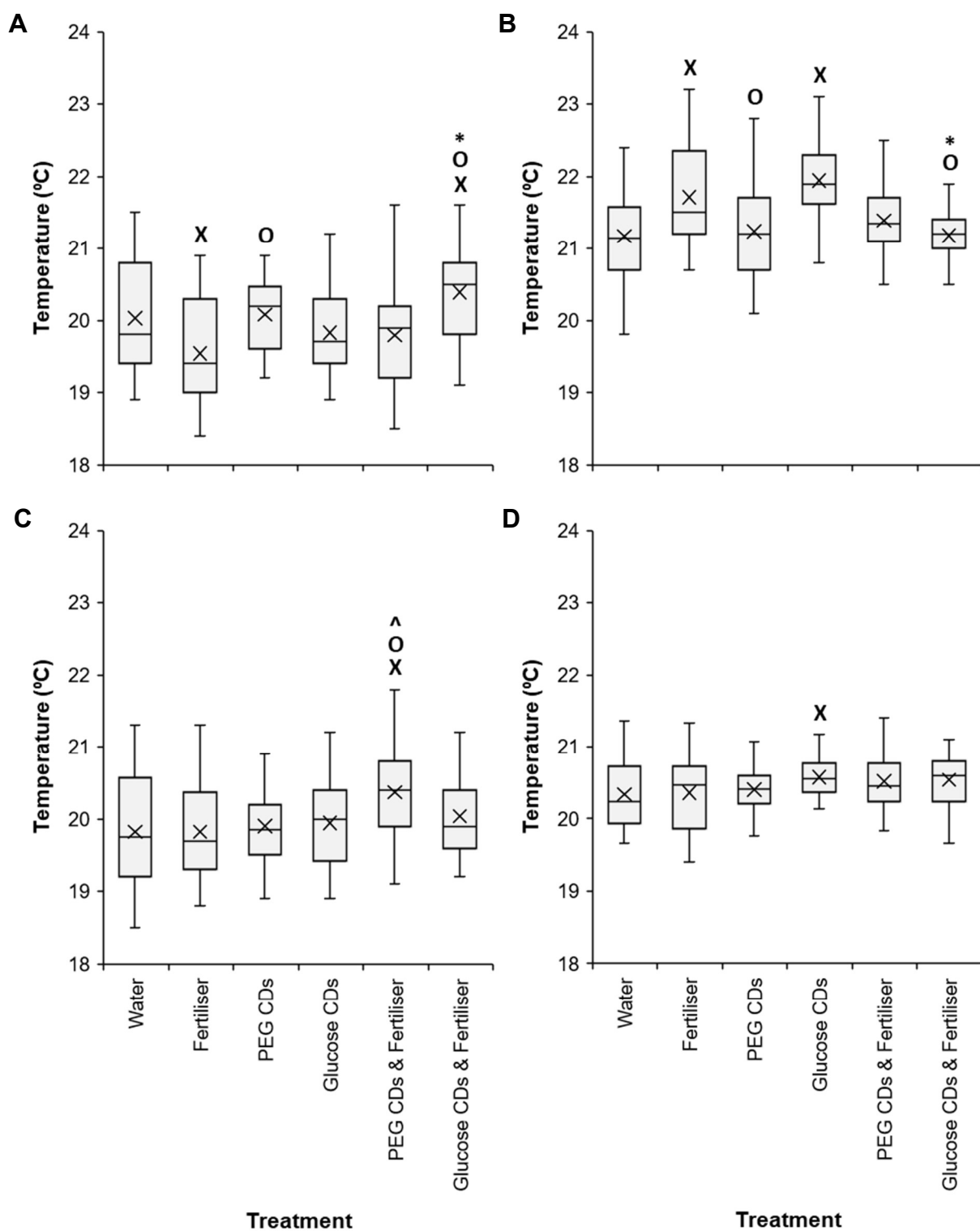


Figure 5.10 Leaf temperature in spring-sown *Triticum aestivum* cv. *Paragon* wheat over the course of a day, as shown by (A) leaf temperature at 9am, (B) leaf temperature at 1pm, (C) leaf temperature at 5pm, and (D) average daily leaf temperature. Leaf temperature was measured using infrared thermography at six weeks post-germination. $N = 60$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and

75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (*) denoting a result significantly different to the glucose CD treatment.

5.4 Discussion

In *T. aestivum* cv. Apogee, both PEG CDs and glucose CDs significantly increased ETR and Y(II) compared to the water treatment. Similarly, in *T. aestivum* cv. Paragon glucose CDs significantly increased ETR and Y(II) compared to the water-treated plants. CDs show strong electron accepting and donating properties, and have been shown to conjugate to the chloroplast surface in plants; furthermore, CDs absorb light in the UV region and emit blue light, which matches the absorption spectrum of chloroplasts (Ambrosi et al., 2014; Chandra et al., 2014; Li et al., 2018b; Swift et al., 2018; Xu et al., 2020). In this way, CDs act as efficient light harvesters. By increasing light-harvesting capacity, CDs increase the rate of electron transport and, subsequently, the rate of photosynthesis. This corroborates the significant increases seen in ETR and Y(II).

Fertiliser application also increased ETR and Y(II) in *T. aestivum* cv. Apogee relative to the water treatment. The relative concentrations of nutrients in soils are considered one of the major factors limiting photosynthetic rates worldwide, with nutrient deficiencies decreasing photosynthetic yields (Jain et al., 1999; Maire et al., 2015; Walker et al., 2014; Wang et al., 2018c). Furthermore, as discussed in Chapter 4, nitrogen is a particularly important nutrient for photosynthesis, with nitrogen content directly related to the photosynthetic capacity of a leaf (Chtouki et al., 2021; Conroy et al., 1986; Ercoli et al., 1993; Evans, 1989; Hikosaka, 2004). The application of fertiliser treatment increased nutrient availability, which in turn facilitated the significant increases in ETR and Y(II).

The glucose CD & fertiliser treatment also significantly increased Y(II) and ETR relative to the water treatment. As discussed in previous chapters, CDs increase nutrient uptake capacity (Li et al., 2019; Li et al., 2018a). With fertiliser application increasing nutrient availability and CDs increasing nutrient uptake capacity, the glucose CD & fertiliser plants benefitted not only from maximal nutrient uptake but also from the impact of CDs on light harvesting and electron transport. This may also have been seen in the leaf temperature data. In the autumn-sown *T. aestivum* cv. Apogee wheat, leaf temperature was significantly decreased over the whole day by the glucose CD & fertiliser treatment relative to the water treatment. A lower leaf temperature indicates a higher rate of stomatal conductance, and in turn this indicates an increased rate of photosynthesis (Bajons, Klinger, &

Schlosser, 2005; Violet-Chabrand & Lawson, 2019). However, these leaf temperature results were seen in the autumn-sown wheat, whereas chlorophyll fluorescence measurements were made on the spring-sown wheat. Further work measuring chlorophyll fluorescence in wheat over different time periods would be beneficial here. Overall, CDs act as an effective biostimulant in *T. aestivum* cv. Apogee plants when combined with fertilisers.

However, in the *T. aestivum* cv. Paragon plants, the glucose CD & fertiliser-treated plants had a decreased rate of ETR and Y(II) relative to the water treatment. Photosynthesis is dependent on nutrients such as nitrogen, with the ratio of nitrogen to phosphorous being essential for the regulation of photosynthesis. Furthermore, high fertiliser inputs can decrease this ratio (Carstensen et al., 2018; Mu & Chen, 2021; Rivas-Ubach et al., 2012). Although maximal nutrient uptake benefited the glucose CD & fertiliser-treated plants in *T. aestivum* cv. Apogee, in *T. aestivum* cv. Paragon this could have transported an excess of nutrients into the leaf, decreasing the nitrogen to phosphorous ratio and subsequently decreasing ETR and Y(II). As previously discussed in Chapter 4, the application of CDs as a biostimulant may therefore require tailoring according to the cultivar. As shown in Chapter 4, there were no impacts on the chlorophyll, flavonol, or nitrogen contents of leaves in the spring-sown, glucose CD & fertiliser-treated plants. Therefore, further work is required to assess the impacts of CDs on leaf contents and how this impacts photosynthesis. In-depth analysis of the nutrient contents of leaves would allow for a quantitative look at the impacts on the ratios of nutrients, such as nitrogen and phosphorous, and technologies like hyperspectral imaging could be used (Christensen et al., 2004; Liu et al., 2015).

In *T. aestivum* cv. Apogee, the PEG CD treatment significantly increased Y(II) and ETR relative to the PEG CD & fertiliser treatment, and the latter treatment had no significant impact on Y(II) or ETR relative to any other group. As a biostimulant candidate, it may be the case that PEG CDs act more effectively alone than when combined with fertiliser. Similarly, in *T. aestivum* cv. Paragon, the glucose CD & fertiliser treatment significantly decreased Y(II) and ETR relative to the glucose CD and fertiliser treatments. Again, it would appear that glucose CDs act as a better biostimulant in *T. aestivum* cv. Paragon when they are applied without fertiliser, which is contrary to their action in *T. aestivum* cv. Apogee. Once again, it would appear that the application of CDs as a biostimulant requires tailoring according to the cultivar.

In *T. aestivum* cv. Apogee, PEG CD treatment significantly decreased Y(NPQ) compared to the water. Likewise, in *T. aestivum* cv. Paragon, both PEG and glucose CD treatment significantly decreased Y(NPQ) relative to the water treatment. CDs are very effective light harvesters, and have been shown to increase the rates of electron transport and photosynthesis in plants by increasing light

harvesting capacity (Ambrosi et al., 2014; Chandra et al., 2014; Li et al., 2018a; Swift et al., 2018; Xu et al., 2020). Even though CDs significantly increased ETR and Y(II), likely due to their light-harvesting properties, Y(NPQ) was significantly decreased. If more light was being absorbed than could be used for photosynthesis, protons would accumulate in the lumen and trigger NPQ, which would be seen as an increased Y(NPQ) measurement. However, the opposite was seen in the results (Derks, Schaven, & Bruce, 2015; Pospíšil, 1998; Ruban, 2016; Xu, Roy, & Croce, 2017). This corroborates the findings of Swift *et al.* (2021) who found that glucose CDs increased the rate of electron transport and photosynthesis but decreased the rate of NPQ. In this paper, it was concluded that glucose CDs increased the proportion of electrons partitioned for photochemistry, and therefore although light harvesting and electron transport were increased, NPQ was also decreased; this was corroborated by demonstrating that ROS levels had not increased, as would be expected if too much light was being absorbed (Swift et al., 2021).

There was no significant impact on Fv/Fm in the *T. aestivum* cv. Apogee wheat, with all values ranging between 0.74-0.76. In the *T. aestivum* cv. Paragon wheat, all Fv/Fm values were significantly decreased relative to the fertiliser treatment, but all values ranged from 0.78-0.79. As a parameter, Fv/Fm is a measurement sensitive to plant stress. At peak photosynthetic performance, the value of Fv/Fm is 0.83 (Maxwell & Johnson, 2000). In the *T. aestivum* cv. Apogee wheat, although the Fv/Fm measurements were lower than 0.83, they did not differ significantly from each other. Therefore, it can be assumed that any stresses exposed to the plant were environmental, rather than being a result of the treatments. Similarly, in the *T. aestivum* cv. Paragon wheat, there were no significant differences in Fv/Fm compared to the water treatment, and so the plants appear to not have been stressed. However, all treatment groups had a significantly decreased Fv/Fm value when compared to the fertiliser treatment, and so it can be inferred that the application of fertiliser provided some relief from environmental stress. This corroborates the findings of Swift *et al.* (2021), wherein CDs were not found to cause stress and induce photodamage despite decreasing NPQ.

In *T. aestivum* cv. Apogee, PEG CD treatment significantly increased Y(NO) relative to the water treatment. Similarly, in *T. aestivum* cv. Paragon PEG CD treatment significantly increased Y(NO) relative to the water treatment. Increases in Y(NO) would ordinarily be expected to induce photodamage in PSII, but this was not seen in the Y(II) results. To prevent uncontrolled ROS cascades, plants utilise ROS-scavenging antioxidant defense pathways (Gill & Tuteja, 2010). Furthermore, CDs themselves show ROS-scavenging properties, with increasing concentrations of CDs directly correlating with decreases in ROS. The ROS-scavenging properties of CDs are thought to be due to their strong reducing activity, whereby they can reduce free radicals and subsequently reduce oxidative stress (Das et al., 2014; Zhao et al., 2015). This would corroborate the findings that

although CDs increased $Y(NO)$, $Y(II)$ was not negatively impacted. If CDs possess the innate ability to scavenge ROS, then their ability to increase light harvesting comes at a much lower risk of photodamage. It could also explain why, despite $Y(NPQ)$ being decreased and $Y(NO)$ being increased, F_v/F_m was not increased, as would be expected if unregulated energy dissipation was occurring. Further work investigating the impacts of CDs on ROS species would be valuable, in order to quantify this.

On the other hand, in *T. aestivum* cv. Paragon, the glucose CD & fertiliser treatment significantly increased $Y(NO)$ while it significantly decreased $Y(II)$. However, in *T. aestivum* cv. Apogee, the same treatment significantly decreased $Y(NO)$. Once again, the impacts of the combined CD and fertiliser treatment is very different between the two cultivars, and this needs to be taken into account when considering their use as a biostimulant.

5.5. Carbon dots and photosynthesis – concluding remarks

CDs significantly increase the rates of electron transport and photosynthesis, due to their abilities to harvest light and increase water and nutrient uptake. However, the photosynthetic enhancements of the CD and synthetic fertiliser dual treatment may be cultivar- and concentration-dependent. Further work to investigate this could provide clarity on the impacts of CDs across different cultivars and assess how to tailor CD application for optimal impacts. CDs were also shown to reduce levels of NPQ, but plants were not found to be stressed. Therefore, it is likely that CDs are accelerating downstream electron transport and inducing ROS-scavenging, which act in a photoprotective way. Further work to investigate and quantify this would be useful in assessing the impacts of CDs on plants. As with CDs alone, the combined treatment of CDs and fertiliser can show beneficial biostimulating effects in one cultivar, but show opposite effects in another. Therefore, while CDs appear to have a stronger biostimulating effect on wheat than fertilisers alone, their use as a biostimulant in wheat, with or without accompanying fertilisers, will be highly dependent on the cultivar.

6. The impact of carbon dots on ear development and biomass

6.1. Introduction

6.1.1. The importance of flowering time on yields in wheat

The demand for crops is projected to double by 2050, yet current and projected crop yield increases will not meet this demand (FAO, 2009b; Ray et al., 2013; Tilman et al., 2011). Wheat yield is influenced by many different factors, from photoperiod and stress to photosynthetic yield and nutrient uptake capacity (Curtis & Halford, 2014). An important factor in determining yield in wheat is flowering time. Wheat cultivars are selectively bred to maximise environmental adaptation and it is vital that flowering occurs at the optimum time in order to maximise yields. If flowering time does not occur optimally, then environmental damage is much more likely and yields will decrease.

Flowering time is controlled by a number of genetic factors, particularly genes controlling vernalization and photoperiod sensitivity. These genes act in combination as protective mechanisms to prevent flowering over winter, to prevent frost damage and maintain high yields (Worland, 1996). Similarly, the number of ears per plant is an important factor in determining yield, and there is a direct relationship between the number of ears and yield, and ear number itself is directly affected by factors such as nitrogen uptake and the availability of water and light (Austin, Ford, & Morgan, 1989; Gales, 1983; Hsu & Walton, 1971).

6.1.2. Carbon dots, flowering time, and yield: chapter aims

Carbon dots (CDs) have been shown to increase yields of mung beans by 14.9%, lettuce by 48.1%, and wheat by 18.0% (Swift et al., 2018; Wang et al., 2018b; Zheng et al., 2017). However, little work has been done to investigate the impacts of CDs on flowering time in wheat. If CDs were able to decrease flowering time as well as increase yields, this would majorly impact on their suitability as a biostimulant candidate; if crops were to yield higher, in a shorter space of time, this would have major economic implications and be extremely beneficial. With this in mind, the impacts of CDs on the flowering time and biomass of two cultivars of common wheat, *T. aestivum* cv. Apogee and Paragon, is presented here.

6.2. Methods

6.2.1. Ear development measurements

Two cultivars of common wheat, *Triticum aestivum* cv. Apogee and Paragon, were grown and treated as outlined in the 'Materials and Methods' chapter. Ear development was investigated using a number of different measurements. Firstly, the number of days taken for ears to emerge, noted as

the emergence of visible awns or kernels in *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon respectively, was recorded. Then, the total number of ears per plant at harvest was recorded. In the autumn-sown wheat, additional measurements were made to track the length of ears, and the rate of elongation of the ears. To do this, the lengths of the ears were measured three times a week on Mondays, Wednesdays, and Fridays, from when treatment started at three week post-germination through to harvest, which took place at 5 and 8 weeks post-germination in the *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon wheat respectively. In the spring-sown wheat, both wheat cultivars were harvested at 10 weeks post-germination. These measurements were then used to calculate the average daily growth rate of ears and calculate the average length of ears at harvest. In the spring-sown wheat, additional measurements were made to measure the time taken to flower. To measure this, the number of days taken for visible awns or kernels to emerge in *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon respectively was recorded, as was the number of days taken for these ears to flower.

6.2.2. Biomass measurements

To measure biomass, in the autumn-sown wheat, plants were harvested at 5 and 8 weeks post-germination in the *Triticum aestivum* cv. Apogee and *Triticum aestivum* cv. Paragon wheat respectively. In the spring-sown wheat, both wheat cultivars were harvested at 10 weeks post-germination. For harvesting, the ears were cut at the base of the ear, and the rest of the shoot was cut at the soil. These were then separated into two measurements – the ‘ear’ mass, and the ‘stem and leaf’ mass. The sum of these two measurements was labelled as ‘total shoot’ mass.

In the autumn-sown wheat, the plants were measured when fresh from harvesting and these masses labelled the ‘wet mass’. The samples were then dried for 72 hours at 60°C in a drying oven before being weighed again, and these masses labelled as the ‘dry mass’. The difference between these two measurements was labelled as ‘water retention’, being a measurement of the water lost through the drying process. In the spring-sown wheat, the plants had grown at a much faster rate and so were already dried out at the time of harvest. Therefore, a single ‘dry mass’ was recorded for each plant.

6.3.3. Statistical analysis

Data were statistically analysed as outlined in the ‘Methods and Materials’ chapter.

6.2.4. Covid adjustment

There are many other developmental and biomass measurements that would have been beneficial to record. It would have been useful to track measurements such as the numbers and weights of grains per ear, the number and lengths of awns, and the individual flag leaf lengths and biomasses,

amongst others. However, due to the time constraints outlined in the Covid Statement a simplified experimental protocol was used, as presented here.

6.3. Results

6.3.1. Ear length and elongation in autumn-sown *T. aestivum* cv. Apogee

Ear length and elongation differed significantly between groups in the autumn-sown wheat, as presented in Figure 6.1 below. Glucose CD & fertiliser treatment significantly decreased ear length and ear elongation rate when compared to the water and fertiliser treatments. Relative to the water treatment, the glucose CD & fertiliser treatment resulted in a 22.8% decreased rate of ear elongation.

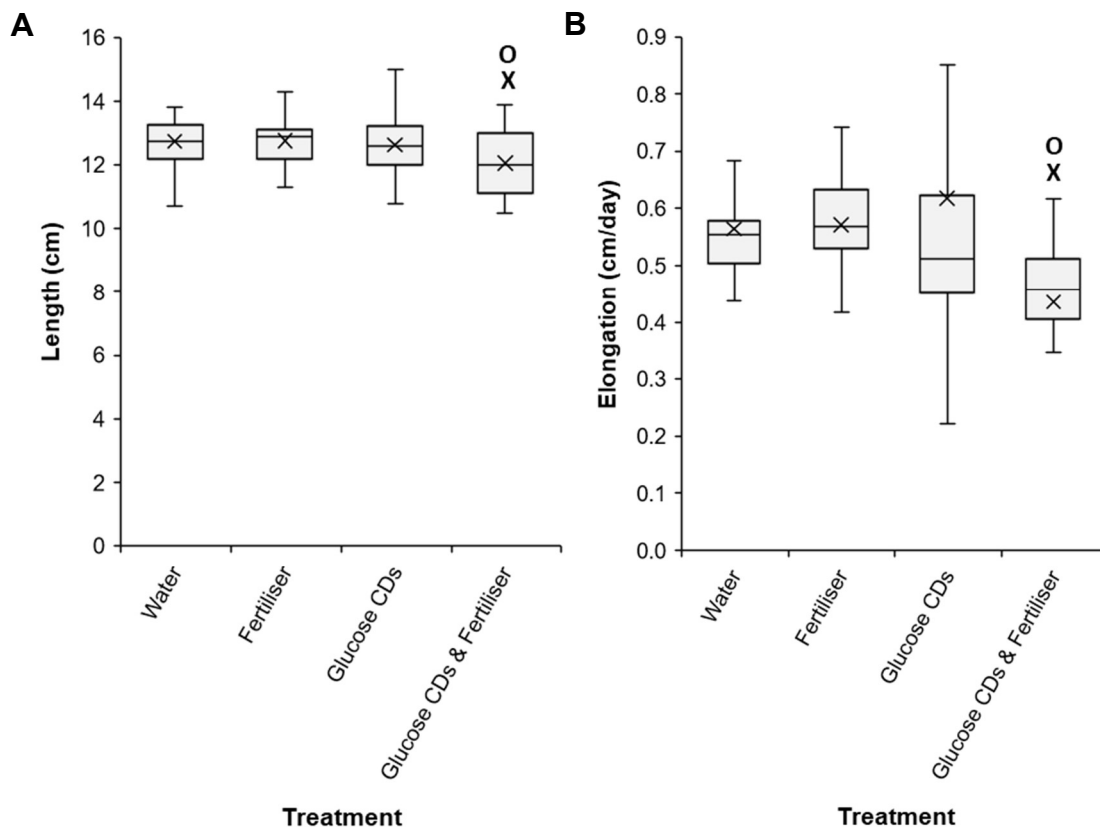


Figure 6.1 Ear development in autumn-sown *Triticum aestivum* cv. Apogee wheat, as shown by (A) the length of ears at harvest, and (B) the rate of ear elongation. The first ear to emerge per plant was measured. Ear length was measured from the start of treatment at 3 weeks post-germination through to harvest at 5 weeks post-germination, and these values were used to calculate the rate of ear elongation and length of ears at harvest. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the

graph, with (X) denoting a result significantly different to the water treatment, and (O) denoting a result significantly different to the fertiliser treatment.

6.3.2. Ear length and elongation in autumn-sown *T. aestivum* cv. Paragon

There were no significant differences in the length of ears at harvest between groups, as presented in Figure 6.2 below. However, the glucose CD & fertiliser treatment significantly decreased the rate of ear elongation relative to the water treatment, with a decrease of 64.51%.

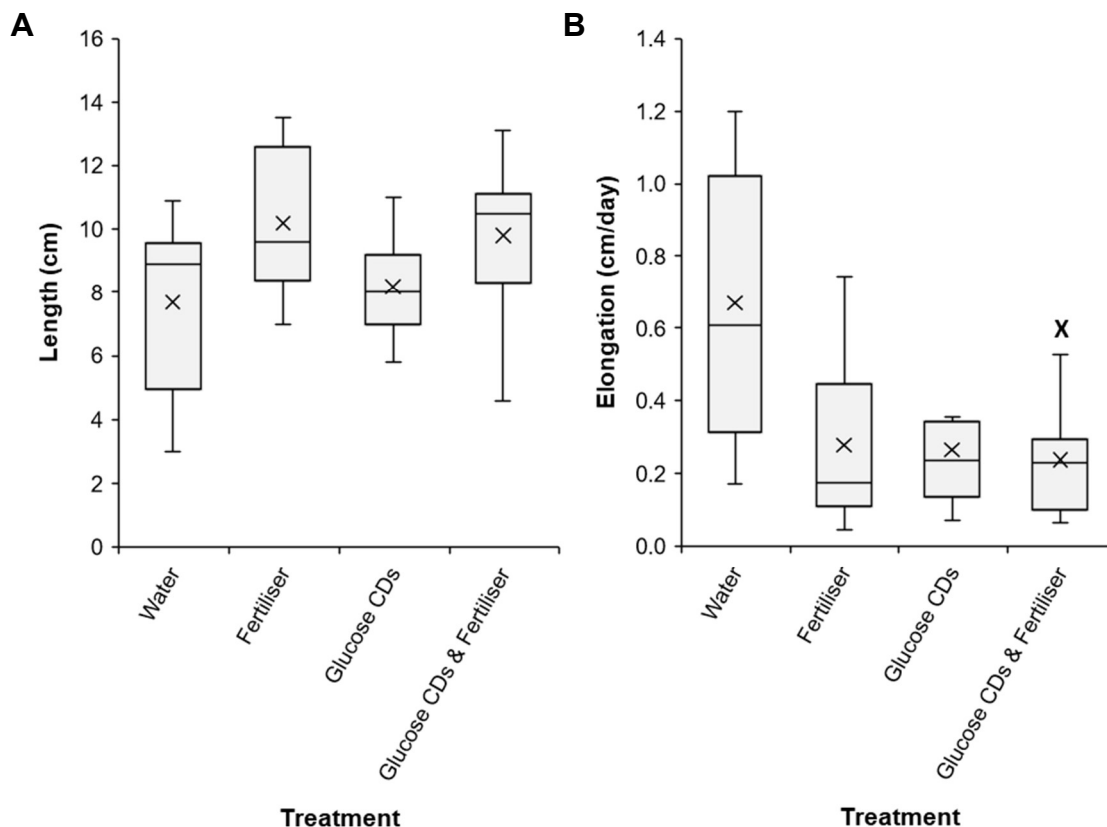


Figure 6.2 Ear development in autumn-sown *Triticum aestivum* cv. Paragon wheat, as shown by (A) the length of ears at harvest, and (B) the rate of ear elongation. The first ear to emerge per plant was measured. Ear length was measured from the start of treatment at 3 weeks post-germination through to harvest at 8 weeks post-germination, and these values were used to calculate the rate of ear elongation and length of ears at harvest. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment.

6.3.3. Ear emergence in *T. aestivum* cv. Apogee

As presented in Figure 6.3 below, there were significant differences between groups in the ear emergence of the autumn-sown wheat. Fertiliser treatment significantly increased the time taken for the first ear to emerge relative to the water treatment. On the other hand, the glucose CD & fertiliser treatment significantly decreased the time taken for the second ear to emerge relative to the fertiliser and glucose CD treatments.

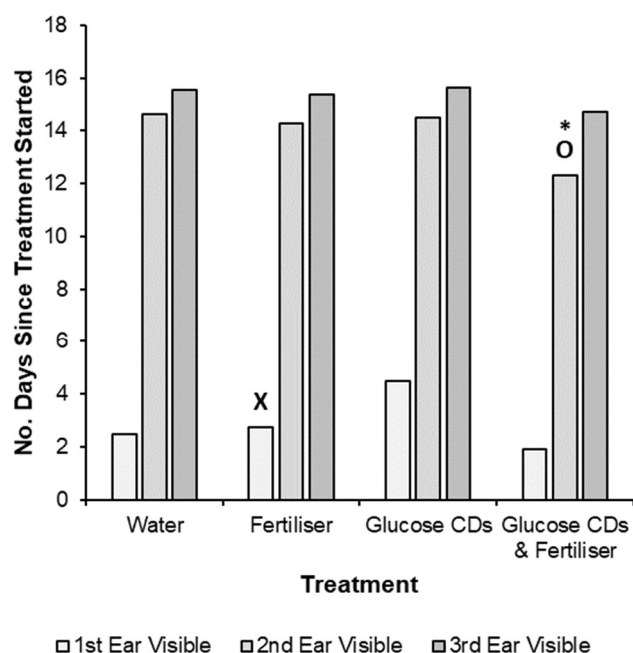


Figure 6.3 Ear emergence in autumn-sown *Triticum aestivum* cv. Apogee wheat. Ear emergence was calculated as the average number of days taken for ears to emerge, as quantified by the appearance of visible spikelets. Day number was counted from the point treatment began, at 3 weeks post-germination. $N = 30$ for all groups. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (*) denoting a result significantly different to the glucose CD treatment.

In the spring-sown wheat, there were also significant differences between groups, as presented in Figure 6.4 below. Fertiliser treatment significantly increased the time taken for 1st ears to flower and the time taken for 2nd ears to emerge relative to the water treatment. Likewise, PEG CD treatment significantly increased the time taken for 2nd ears to emerge with respect to the water and fertiliser treatments, and significantly increased the time taken for the 2nd ears to flower relative to the water treatment. The PEG CD & fertiliser treatment significantly decreased the time taken for the 2nd ears

to emerge and flower compared to the PEG CD treatment. Lastly, glucose CD & fertiliser treatment significantly decreased the time taken for 1st ears to flower relative to the fertiliser control.

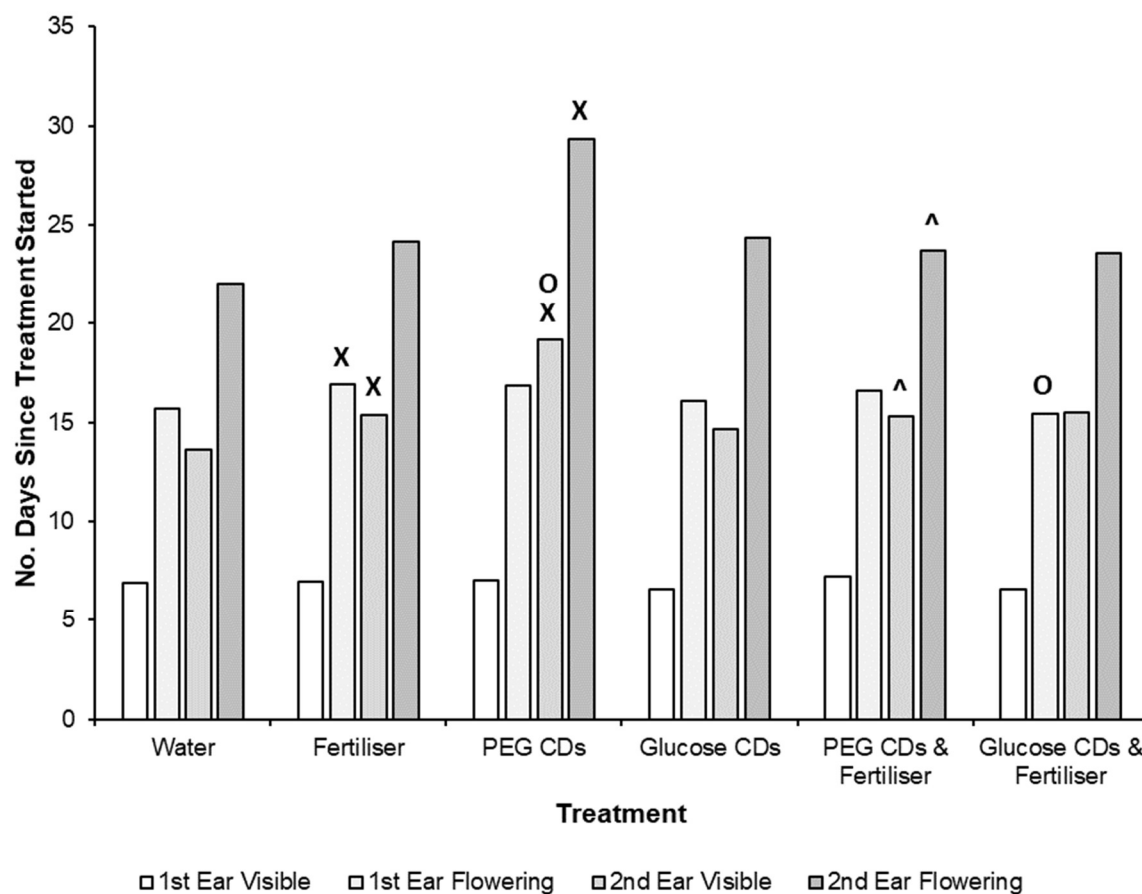


Figure 6.4 Ear emergence and flowering time in spring-sown *Triticum aestivum* cv. Apogee wheat. Ear emergence was calculated as the average number of days taken for ears to emerge, as quantified by the appearance of visible spikelets. Flowering time was calculated as the average number of days taken for ears to flower, as quantified by the appearance of visible flowers on each ear. Day number was counted from the point treatment began, at 3 weeks post-germination. $N = 30$ for all groups. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (^) denoting a result significantly different to the PEG CD treatment.

6.3.4. Ear emergence in *T. aestivum* cv. Paragon

There was a significant difference between groups in the ear emergence of the autumn-sown wheat, as presented in Figure 6.5 below. In the glucose CD & fertiliser-treated plants, the 2nd ears took significantly less time to emerge than the water-treated plans, with a decrease of 7.4 days on average.

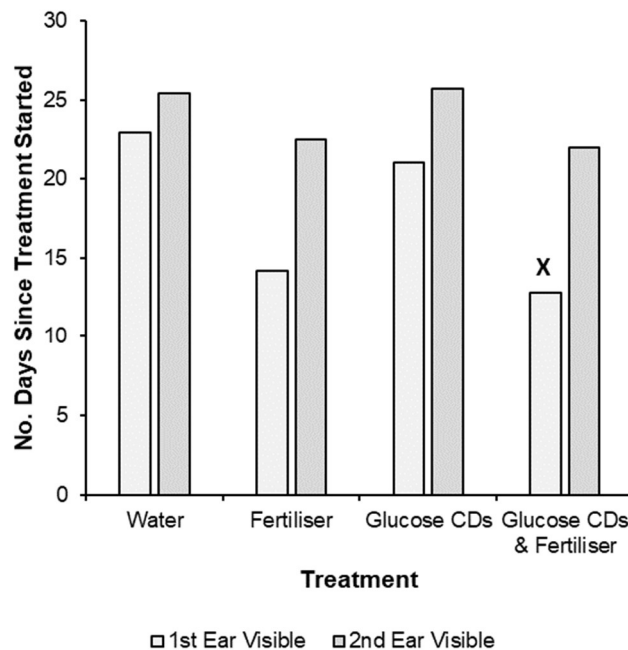


Figure 6.5 Ear emergence in autumn-sown *Triticum aestivum* cv. Paragon wheat. Ear emergence was calculated as the average number of days taken for ears to emerge, as quantified by the appearance of visible spikelets. Day number was counted from the point treatment began, at 3 weeks post-germination. $N = 30$ for all groups. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment.

In the spring-sown wheat, there was no significant difference in ear emergence between groups, as shown in Figure 6.6 below.

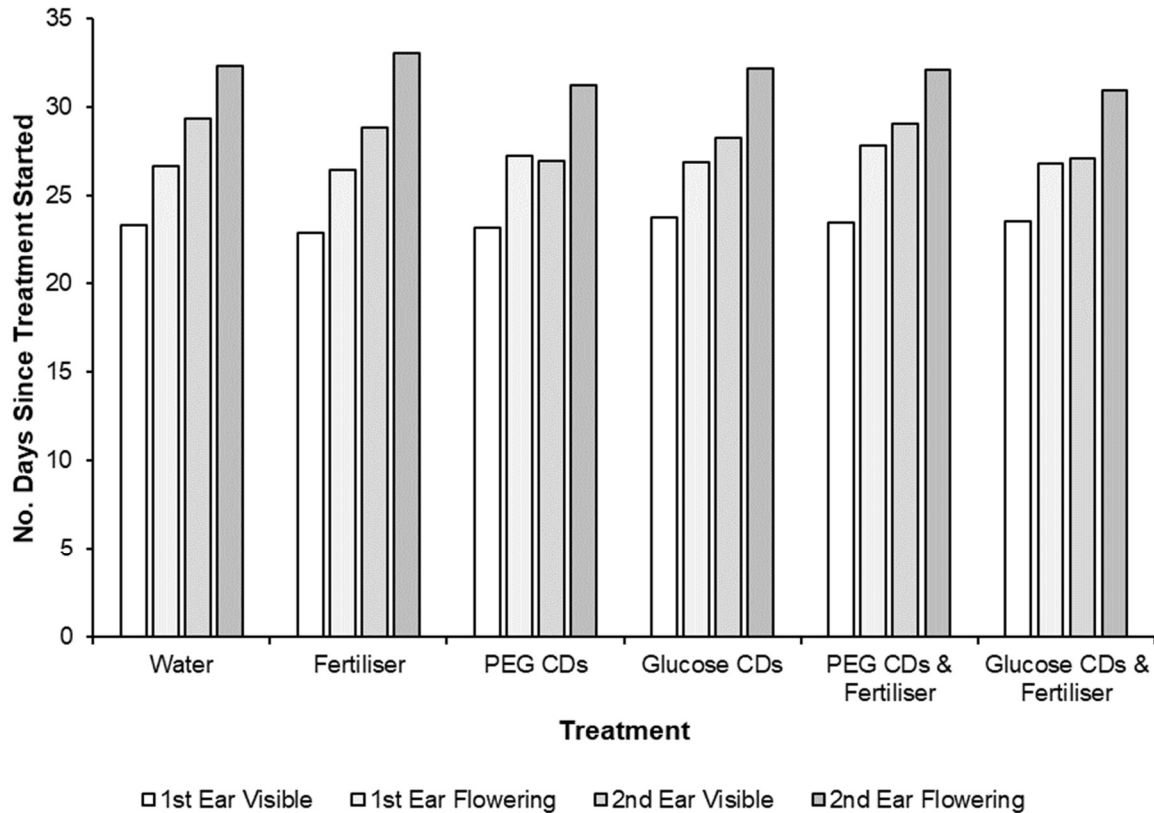


Figure 6.6 Ear emergence and flowering time in spring-sown *Triticum aestivum* cv. *Paragon* wheat. Ear emergence was calculated as the average number of days taken for ears to emerge, as quantified by the appearance of visible spikelets. Flowering time was calculated as the average number of days taken for ears to flower, as quantified by the appearance of visible flowers on each ear. Day number was counted from the point treatment began, at 3 weeks post-germination. $N = 30$ for all groups. There were no statistically significant differences between groups.

6.3.5. Number of ears at harvest in *T. aestivum* cv. *Apogee*

In both the autumn-sown and spring-sown wheat, there were no significant differences in the number of ears at harvest, as presented in Figure 6.7 below.

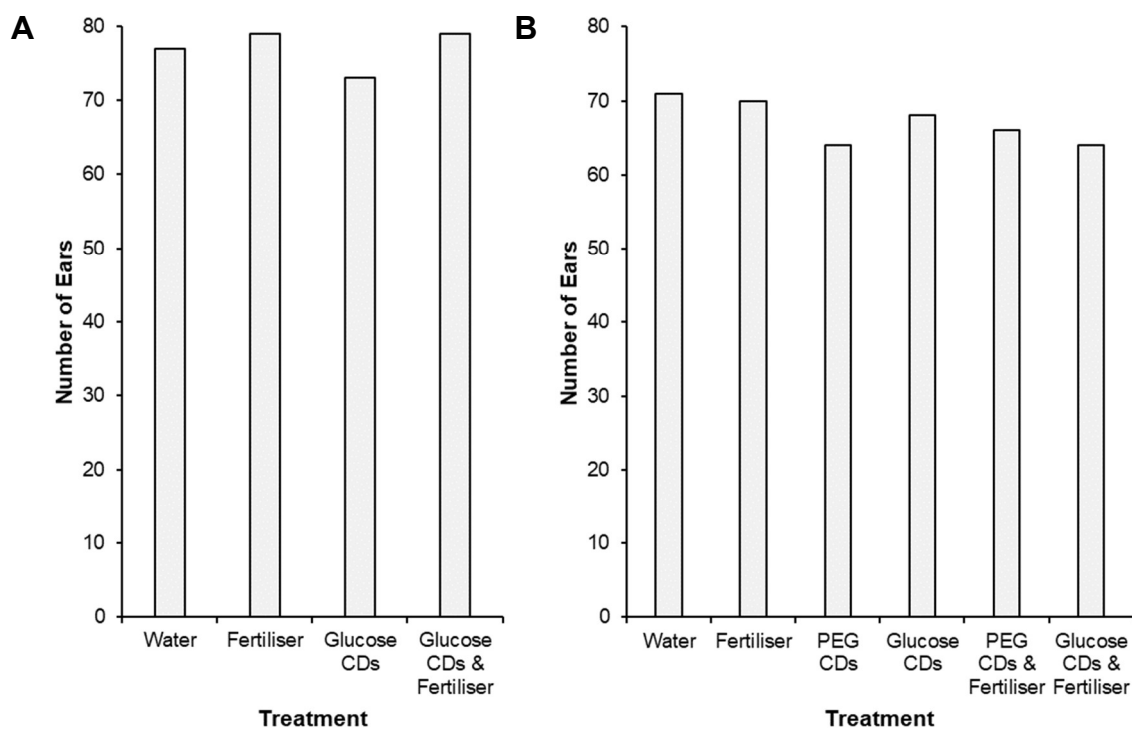


Figure 6.7 Total number of ears at harvest in (A) autumn-sown and (B) spring-sown *Triticum aestivum* cv. Apogee wheat. Number of ears was measured at harvest; plants were harvested at 5 weeks post-germination in the autumn-sown wheat and at 10 weeks post-germination in the spring-sown wheat. $N = 30$ for all groups. There were no statistically significant differences between groups.

6.3.5. Number of ears at harvest in *T. aestivum* cv. Paragon

In the autumn-sown wheat, glucose CD & fertiliser treatment significantly increased the number of ears at harvest relative to the water, fertiliser, and glucose CD treatments. Compared to the water-treated plants, the glucose CD & fertiliser-treated plants were 141.2% taller.

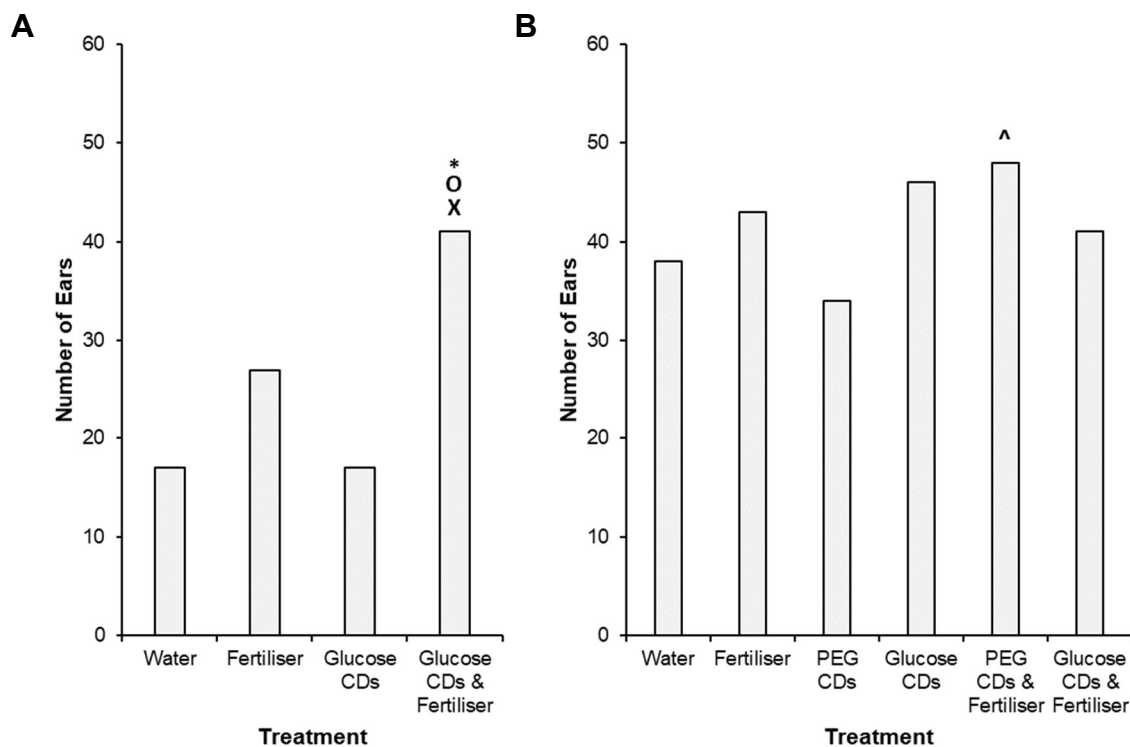


Figure 6.8 Total number of ears at harvest in (A) autumn-sown and (B) spring-sown *Triticum aestivum* cv. Paragon wheat. Number of ears was measured at harvest; plants were harvested at 8 weeks post-germination in the autumn-sown wheat and at 10 weeks post-germination in the spring-sown wheat. $N = 30$ for all groups. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, (*) denoting a result significantly different to the glucose CD treatment, and (^) denoting a result significantly different to the PEG CD treatment

6.3.5. Biomass measurements in *Triticum aestivum* cv. Apogee

In the autumn-sown wheat, biomass was significantly different between groups, as displayed in Figure 6.9 below. In the glucose CD & fertiliser treatment, stem and leaf dry biomass and total shoot dry biomass were significantly decreased relative to the water treatment. Similarly, water retention by weight was significantly decreased in the ears relative to the fertiliser and glucose CD treatments, in the stems and leaves relative to the water treatment, and in the total shoot relative to the water, fertiliser, and glucose CD treatments.

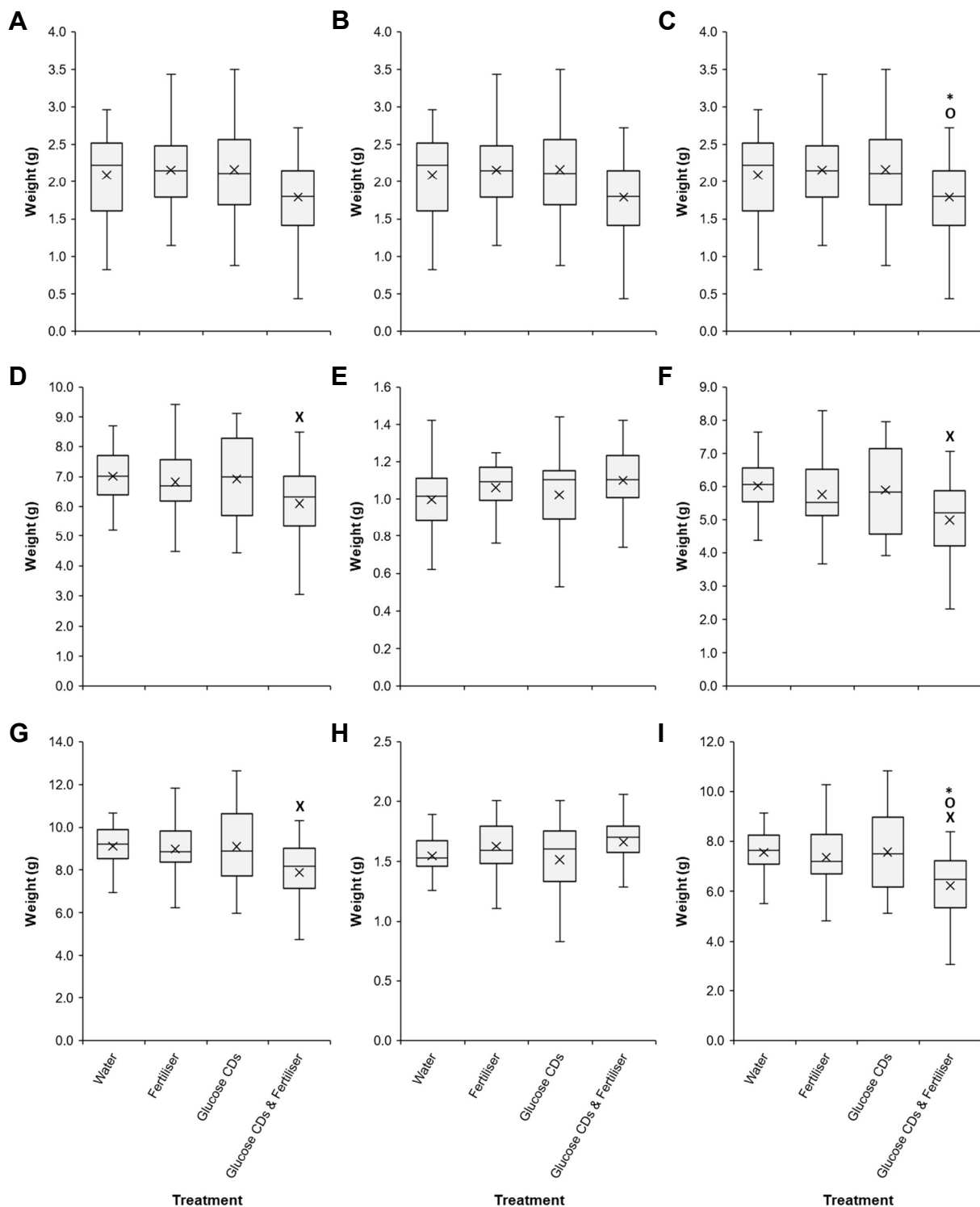


Figure 6.9 Biomass values for autumn-sown *Triticum aestivum* cv. Apogee wheat, shown as (A) ear wet mass, (B) ear dry mass, (C) ear water retention by weight, (D) stem and leaf wet mass, (E) stem and leaf dry mass, (F) stem and leaf water retention by weight, (H) total shoot wet mass, (G) total shoot dry mass, and (I) total shoot water retention by weight. Biomass measurements were taken when plants were harvested at 5 weeks post-germination. $N = 30$ for all groups. For each box, the

central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (*) denoting a result significantly different to the glucose CD treatment.

In the biomasses of the spring-sown wheat, a number of significant differences were found between groups, as presented in Figure 6.10 below. Ear dry mass was significantly decreased by the PEG and glucose CD treatments relative to both the water and fertiliser treatments. The stem and leaf dry mass was significantly decreased in the fertiliser, PEG CD, and PEG CD & fertiliser treatments relative to the water treatment. Lastly, the total shoot dry mass was significantly decreased in the glucose CD and PEG CD & fertiliser treatments relative to the water control.

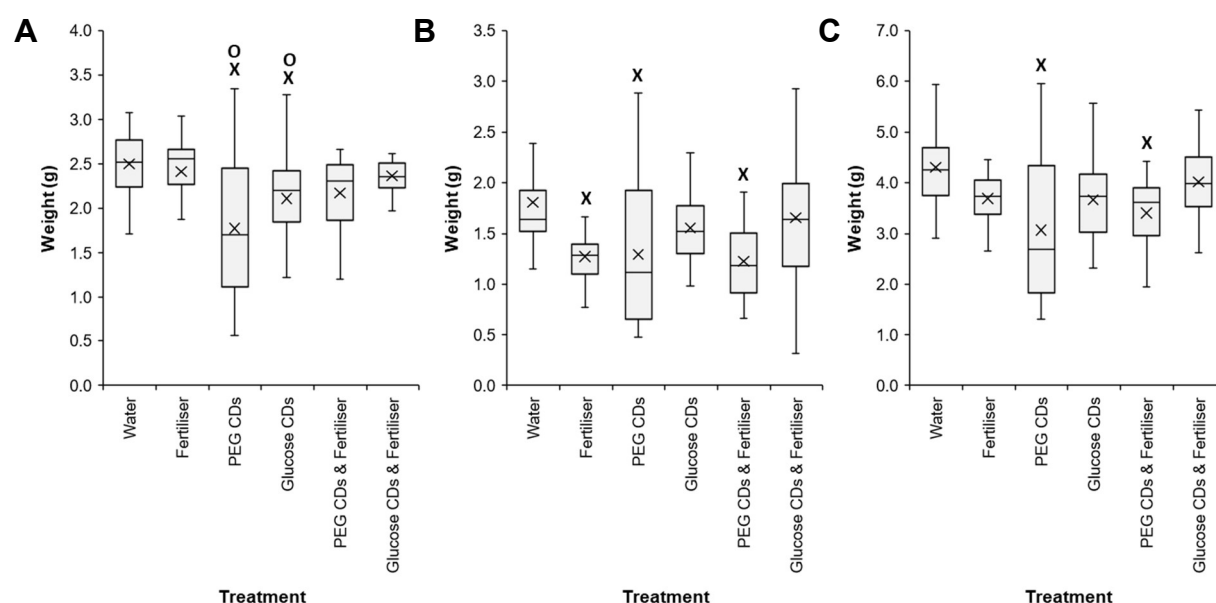


Figure 6.10 Biomass values for spring-sown *Triticum aestivum* cv. Apogee wheat, shown as (A) ear dry mass, (B) stem and leaf dry mass, and (C) total shoot dry mass. Biomass measurements were taken when plants were harvested at 10 weeks post-germination. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, and (O) denoting a result significantly different to the fertiliser treatment.

6.3.5. Biomass measurements in *Triticum aestivum* cv. Paragon

A number of significant differences were found in biomass between groups in the autumn-sown wheat, as presented in Figure 6.11 below. Ear wet mass and ear water retention by weight were both significantly increased by the glucose CD & fertiliser treatment relative to the water, fertiliser, and glucose CD treatments. Likewise, ear dry mass was significantly increased by the glucose CD & fertiliser treatment relative to the water and glucose CD treatments. On the other hand, the stem and leaf water retention by weight was significantly decreased by the glucose CD & fertiliser treatment when compared to the water, fertiliser, and glucose CD treatments.

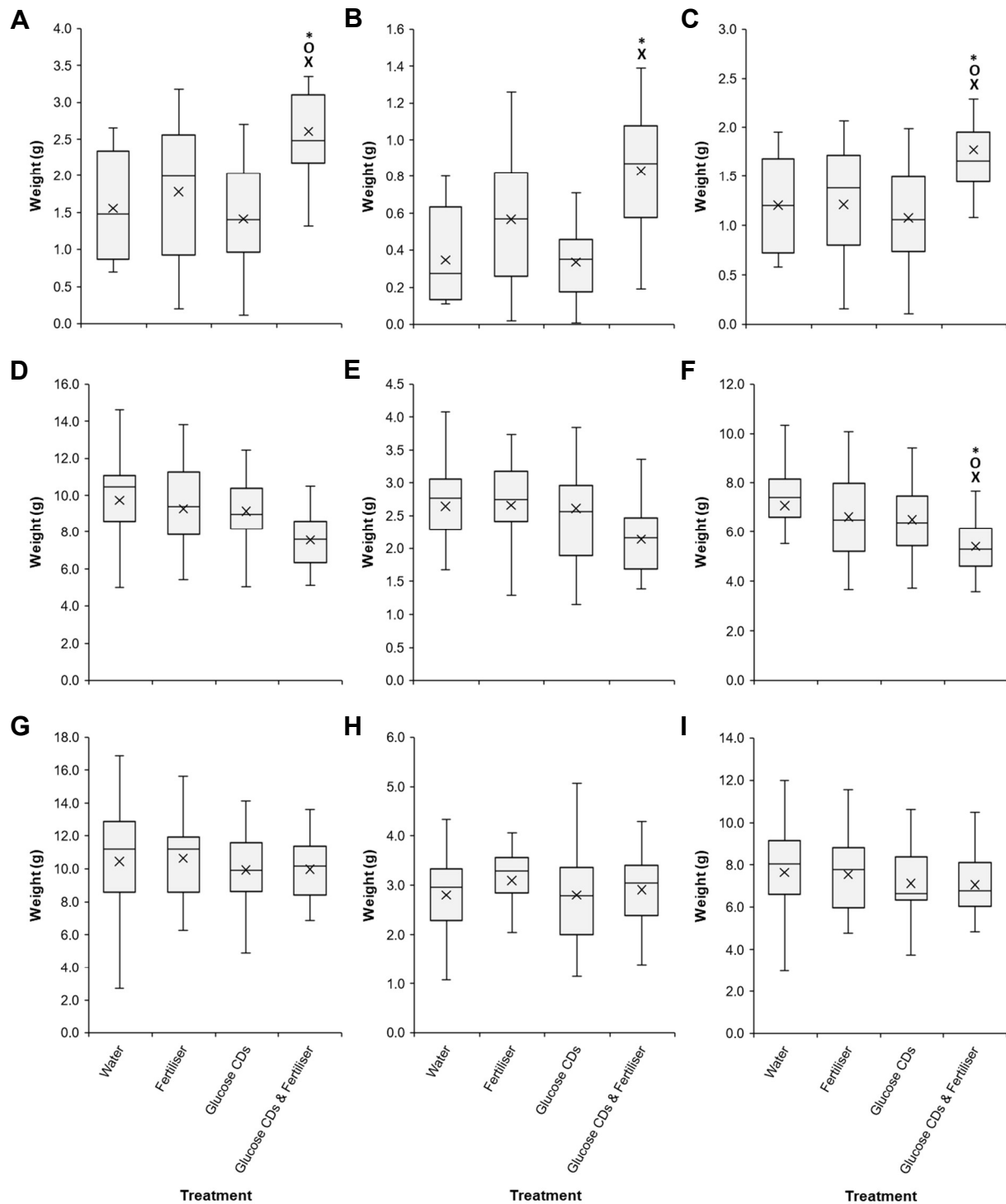


Figure 6.11 Biomass values for autumn-sown *Triticum aestivum* cv. *Paragon* wheat, shown as (A) ear wet mass, (B) ear dry mass, (C) ear water retention by weight, (D) stem and leaf wet mass, (E) stem and leaf dry mass, (F) stem and leaf water retention by weight, (H) total shoot wet mass, (G) total shoot dry mass, and (I) total shoot water retention by weight. Biomass measurements were taken when plants were harvested at 8 weeks post-germination. $N = 30$ for all groups. For each box, the central line indicates the median, X indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (*) denoting a result significantly different to the glucose CD treatment.

There were significant differences in the biomass values of the spring-sown wheat, as shown in Figure 6.12 below. Stem and leaf biomass was significantly increased in the fertiliser-treated plants relative to the water treatment, and was significantly increased in the PEG CD & fertiliser-treated plants relative to the water and PEG CD treatments. On the other hand, stem and leaf biomass was significantly decreased in the PEG CD, glucose CD, and glucose CD & fertiliser treatments relative to the fertiliser-treated plants. Total shoot biomass was significantly increased in the fertiliser-treated plants relative to the water treatment, and was significantly increased in the PEG CD & fertiliser-treated plants relative to the water and PEG CD treatments. On the other hand, stem and leaf biomass was significantly decreased in the PEG CD and glucose CD treatments relative to the fertiliser-treated plants.

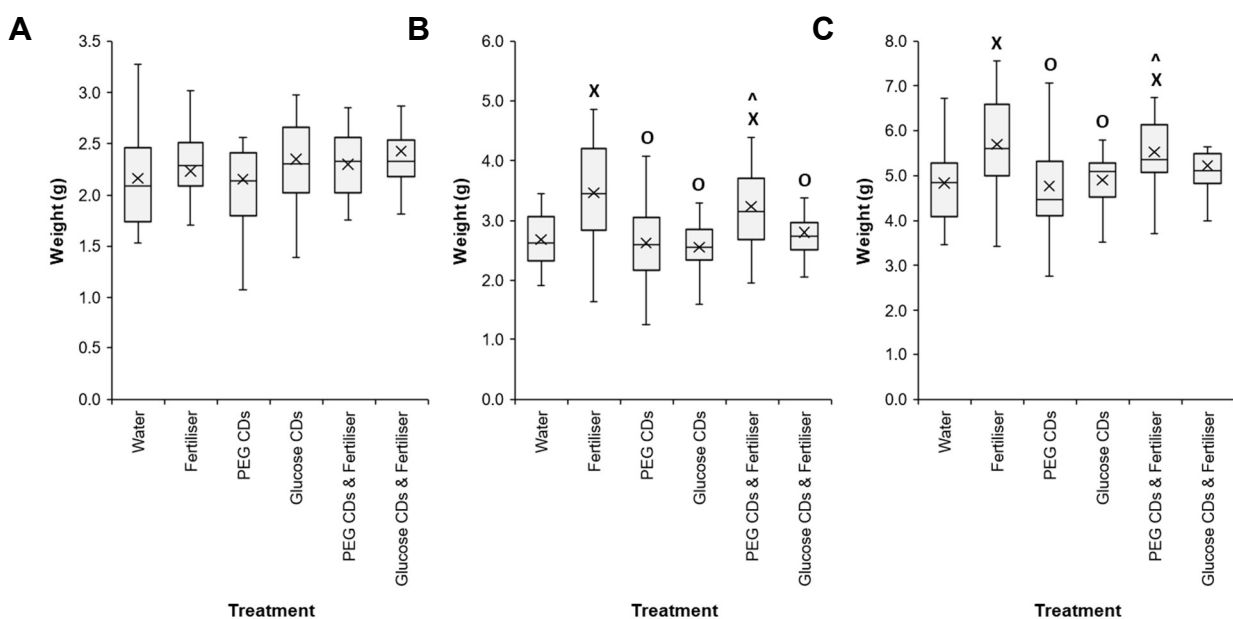


Figure 6.12 Biomass values for spring-sown *Triticum aestivum* cv. Paragon wheat, shown as (A) ear dry mass, (B) stem and leaf dry mass, and (C) total shoot dry mass. Biomass measurements were taken when plants were harvested at 10 weeks post-germination. $N = 30$ for all groups. For each box, the central line indicates the median, \bar{X} indicates the mean, and the top and bottom edges represent the 25th and 75th percentiles respectively. The top and bottom whiskers represent the maximum and minimum range values respectively, excluding outliers. Statistically significant differences are shown on the graph, with (X) denoting a result significantly different to the water treatment, (O) denoting a result significantly different to the fertiliser treatment, and (^) denoting a result significantly different to the PEG CD treatment

6.4. Discussion

The glucose CD and fertiliser treatment significantly enhanced ear development and yield in the autumn-sown *T. aestivum* cv. Paragon wheat when compared to the water treatment. Similarly, in the spring-sown *T. aestivum* cv. Paragon wheat the PEG CD & fertiliser treatment significantly increased the number of ears at harvest relative to the water control, although this did not increase the grain yield. CDs are able to adhere water and nutrients to their surface, and therefore are able to transport and gradually release water and nutrients inside plants (Li et al., 2019; Li et al., 2018a). Furthermore, CDs have been shown to elongate roots, thus increasing the surface area available for water and nutrient uptake (Li et al., 2018a). Similarly, CDs have strong light-harvesting properties and have been shown to increase the photosynthetic activity of various plant species (Ambrosi et al., 2014; Chandra et al., 2014; Li et al., 2018b; Swift et al., 2018; Xu et al., 2020). Overall, by increasing the uptake capacity and transport of water and nutrients around the plant, and by increasing the photosynthetic capacity of plants, CDs promote growth and grain development. Similarly, fertiliser application is directly related to both yield and the number of ears (Khan et al., 2007; Le Gouis et al., 2000; Römer & Schilling, 1986; Singh & Agarwal, 2001). With CDs increasing nutrient uptake and with synthetic fertiliser application increasing the nutrient levels in the soil, the glucose CD & fertiliser, and PEG CD & fertiliser treatments may facilitate the highest possible nutrient uptake which, in turn, increases the rate of ear development and yield. In this way, CDs have beneficial biostimulating effects when applied alongside fertilisers.

These impacts were not seen in the autumn-sown *T. aestivum* cv. Apogee wheat. *T. aestivum* cv. Apogee is an extremely fast growing full-dwarf wheat cultivar, with ears emerging after just 23 days in optimum conditions (Koerner, 1997). In this experiment, treatment began three weeks post-germination, in lieu of the work of others in the lab group (Swift et al., 2021). In wheat, the bulk of stem elongation is completed before heads can emerge (Simmons et al., 1985). Therefore, it is highly

likely that treatment started after grain development had begun. The lack of ear biomass increase in the *T. aestivum* cv. Apogee is contradictory to the findings of Swift *et al.*, who found that CDs increase *T. aestivum* cv. Apogee ear biomass by 18%. Although treatment began at the same time, the concentration used was a third of that used in the work by Swift *et al.* (2021). The impact of CDs has been shown to be highly concentration dependent, with lower concentrations showing an enhancement effect but high concentrations showing an inhibition effect (Chen *et al.*, 2018; Wang *et al.*, 2018b). From these results, there is potentially a minimum concentration threshold above which the enhancement effect begins. With *T. aestivum* cv. Apogee being a rapidly developing wheat, a higher concentration may be required in order to see any significant increases in ear development and yield. Similarly, treatment may need to begin earlier in order to show any significant impacts. Therefore, further work is needed to quantify both the threshold and optimum concentrations, and to calculate the optimum time to begin treatment. In the spring-sown *T. aestivum* cv. Apogee, ear development was significantly slowed and yields were decreased in CD-treated and fertiliser-treated plants. These results are contradictory to both the work of Swift *et al.* and the positive impacts on photosynthesis presented in Chapter 5. Therefore, further work is needed to clarify the impacts on ear development and yield in *T. aestivum* cv. Apogee, in order to determine a) optimal CD concentration, and b) optimal timing of CD application.

6.5. Flowering time and yield – concluding remarks

CD and fertiliser combined treatments significantly enhanced yield and ear development in *T. aestivum* cv. Paragon wheat, indicating that CDs provide a significant enhancement effect when used as a biostimulant alongside fertilisers. However, the impacts of both CDs and the combined CD and fertiliser treatments in *T. aestivum* cv. Apogee wheat were not consistent and were contrary to the impacts seen in *T. aestivum* cv. Paragon. Further work is required to determine the optimum concentration and timing of CD application, and this is very likely to be cultivar-dependent.

7. The next step: carbon dots and the genome

7.1 Introduction

Carbon dots (CDs) have been shown to have significant impacts on plant physiology. From facilitating root and stem elongation and increasing rates of photosynthesis, through to increasing carbohydrate production and increasing biomass, CDs show great promise for use as a biostimulant in agriculture (Chandra et al., 2014; Swift et al., 2021; Wang et al., 2018b). However, while there is ample physiological data on the impacts of CDs in plant systems, there is very little in the way of genomic data. Therefore, in the present study, a transcriptomics analysis was carried out, to gain an insight into the transcriptomic impacts of CDs on wheat plants with comparison to an NPK fertiliser. To carry out the transcriptomics analysis, Oxford Nanopore MinION sequencing was used. In MinION sequencing, a strand of DNA is passed through a biological pore. As the strand passes through the pore, changes in electrical conductivity are measured and used to identify the base sequence, allowing for rapid genomic sequencing (Lu, Giordano, & Ning, 2016).

7.2 Methods

7.2.1. Plant growth and treatment

A cultivar of common wheat, *T. aestivum* cv. Paragon, was grown and treated as outlined in the 'Materials and Methods' chapter.

7.2.2. Sample harvesting

Mature flag leaves were harvested from the spring-sown *T. aestivum* cv. Paragon wheat at seven weeks post-germination. The leaves were harvested at the same time as the chlorophyll fluorescence measurements were taken, as presented in Chapter 5, to allow for cross-comparison of results. For each treatment group, the flag leaves were harvested from three randomly selected plants and snap frozen in liquid nitrogen. The samples were then stored at -80°C until RNA extraction took place.

7.2.3. RNA extraction

To extract RNA, the frozen samples were lysed until a fine powder was formed. The samples were then vortexed with TRIzol, and then again with chloroform. Then, the resulting supernatant from each sample was treated following the Qiagen RNeasy Kit (starting from page 65, step 6). An extra drying step was carried out at the end of the extraction. The resulting samples were analysed using a

Nanodrop spectrophotometer to quantify the concentration of RNA, and a small amount of each sample was run on an agarose gel to check RNA quality.

7.2.4. RNA sequencing

Once extracted the RNA samples were handed over to Gilda Varliero, who used the samples to produce cDNA. This cDNA was then used for the MinION sequencing.

7.2.5. Transcriptomics analysis

Once sequenced, the transcriptomics data was handed over to two master's students – Ellie Carr and Nicole Kilgour. They analysed the data to look for differentially expressed genes (DEGs) between samples, relative to the water control treatment. From this, a list of DEGs was produced.

7.2.6. Analysing differentially expressed genes

The list of DEGS was produced by Ellie Carr and Nicole Kilgour. With their permission, I used this list to:

1. Produce a table displaying the total number of DEGS, and a graph showing the overlap of the top up-regulated and down-regulated DEGs between groups
2. Identify photosynthesis DEGs
3. Produce a figure to display the up-regulated and down-regulated photosynthesis DEGs identified

The figures produced using the list of DEGS are presented in the results section to follow.

7.3 Results

7.3.1. Number of differentially expressed genes

In total, 687 differentially expressed genes (DEGs) were identified by Ellie Carr and Nicole Kilgour, as presented in Table 7.1 below.

Table 7.1 – The total number of differentially expressed genes found following transcriptomics analysis of Triticum aestivum cv. Paragon, relative to the water treatment. Of these total numbers, the numbers of up-regulated and down-regulated genes are shown. Samples were harvested at seven weeks post-germination and underwent RNA extraction and MinION sequencing, to produce a transcriptome for analysis. Transcriptomics analysis was carried out by Ellie Carr and Nicole Kilgour.

Treatment	Total no. up-regulated genes	Total no. down-regulated genes	Total no. differentially expressed genes
Fertiliser	325	362	687
Glucose CDs	313	335	648
Glucose CDs & Fertiliser	310	349	659

Out of the 687 DEGs, the top 20 most up-regulated genes and top 20 most down-regulated genes were identified by Ellie Carr and Nicole Kilgour. There was some overlap between treatment groups, as shown in Figure 7.1 below.

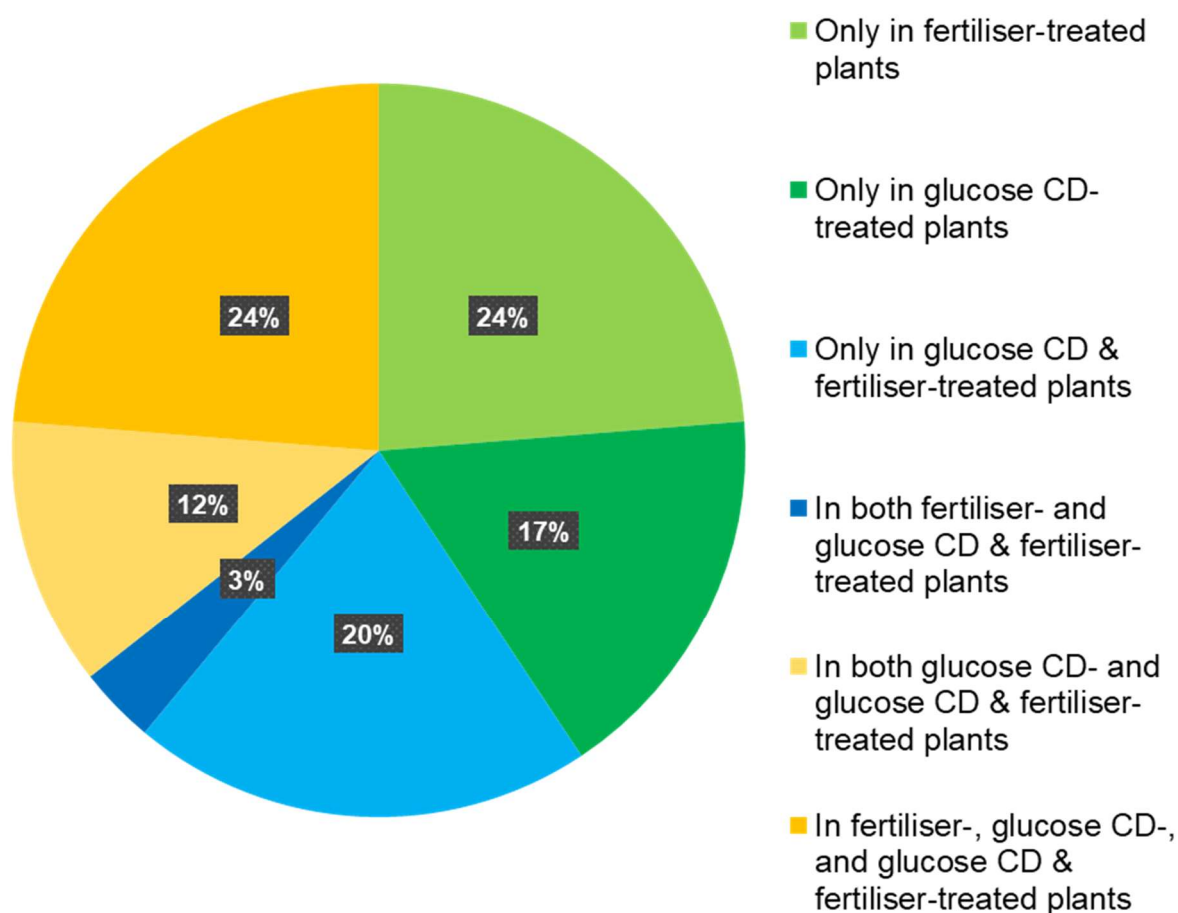


Figure 7.1 – The top 20 up-regulated and top 20 down-regulated differentially expressed genes (DEGs) found following transcriptomics analysis of *Triticum aestivum* cv. Paragon. DEGs are grouped by the treatment group in which they were found, relative to the water treatment. Samples were harvested at seven weeks post-germination and underwent RNA extraction and MinION sequencing, to produce a transcriptome for analysis. Transcriptomics analysis was carried out by Ellie Carr and Nicole Kilgour.

7.3.2. Differentially expressed photosynthesis genes

From the list of DEGs, genes related to photosynthesis were identified, as displayed in Figure 7.2 below. Three main groups of DEGs were found:

1. Photosystem protein genes
2. Oxygen-evolving enhancer protein genes
3. Chlorophyll binding protein genes

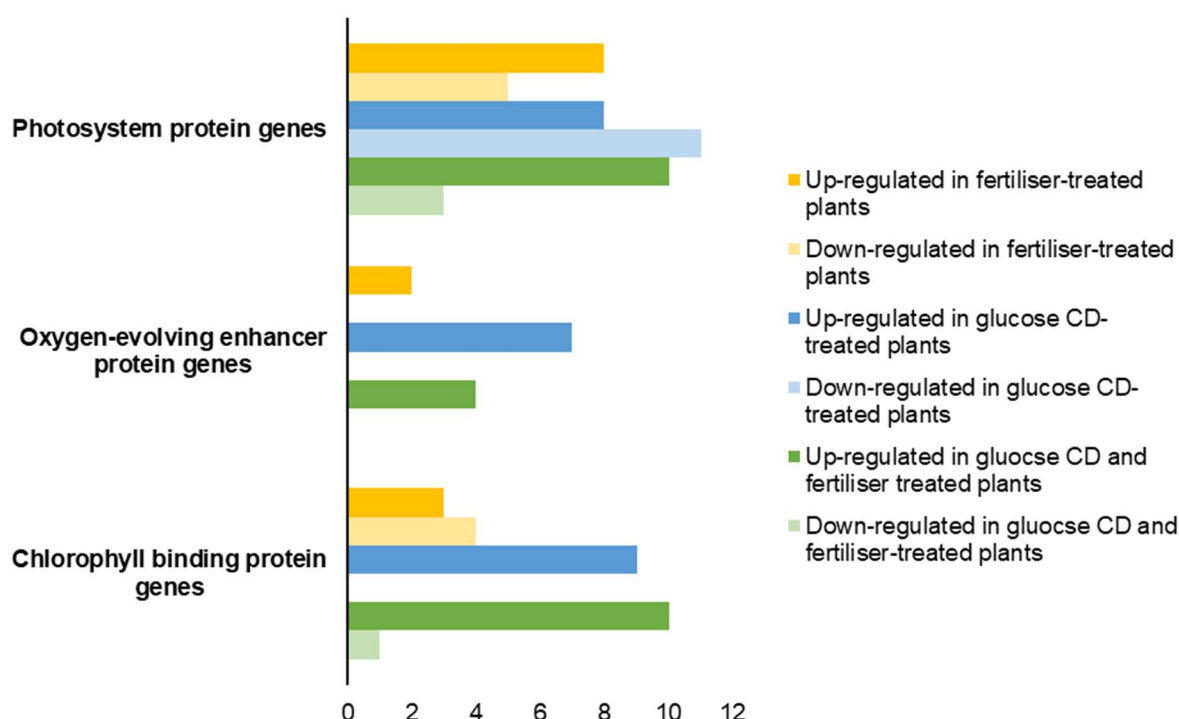


Figure 7.2 – A summary of differentially expressed photosynthesis genes found following transcriptomics analysis of *Triticum aestivum* cv. Paragon, relative to the water treatment. Samples were harvested at seven weeks post-germination and underwent RNA extraction and MinION sequencing, to produce a transcriptome for analysis. Transcriptomics analysis was carried out by Ellie Carr and Nicole Kilgour.

7.4. Discussion

As discussed in Chapter 5, it was shown that the CDs significantly increased $Y(II)$ and ETR relative to the water treatment, likely by increasing the light harvesting capacity of plants and by increasing nutrient uptake. However, glucose CD & fertiliser-treated plants had significantly decreased $Y(II)$ and ETR, which was likely due to excess fertiliser uptake decreasing the ratio of nitrogen and phosphorus. It was shown that CDs significantly decreased $Y(NPQ)$, significantly increased $Y(NO)$, but

were not found to stress the plants, as shown by the Fv/Fm values, likely by increasing ROS scavenging.

Chlorophyll a-b binding protein genes were differentially expressed in all three treatment groups, with the most genes up-regulated in the glucose CD and glucose CD and fertiliser-treated plants, and the most down-regulated genes in the fertiliser-treated plants. Chlorophyll a-b binding proteins are vital in regulating the rate of photosynthesis and play important roles in harvesting light, in balancing excitation energy across photosystems, and in regulating the quantum efficiency of PSII (Andrews, Fryer, & Baker, 1995; Brestic et al., 2015; Liu et al., 2013; Pichersky & Green, 1990). Compared to the fertiliser-treated plants, the glucose CD- and glucose CD & fertiliser-treated plants had at least triple the number of up-regulated chlorophyll a-b binding proteins. This would suggest a genetic mechanism by which CDs increase the light harvesting capacity of plants.

Oxygen-evolving enhancer (OEE) protein genes were up-regulated in all three treatment groups. The OEE proteins are essential in splitting water in PSII, a reaction which produces electrons and oxygen and initiates the linear electron transport chain (Momonoki, Yamamoto, & Oguri, 2009). Compared to the fertiliser-treated plants, the glucose CD- and glucose CD & fertiliser-treated plants had triple and double the number of up-regulated OEE genes compared to the fertiliser treatment respectively. This would suggest a genetic mechanism by which CDs upregulate electron transport and increase the amount of energy partitioned for photochemistry, as proposed by Swift *et al.*, (2021).

Many photosystem protein genes were up- and down-regulated. The ratio of PSII to PSI, known as the photosystem stoichiometry, is a critical factor in the rate of electron transport and dynamically adapts to the environment. As light levels fluctuate, photosystem stoichiometry optimally adapts to compensate for varying levels of light. Under low light levels, this maximises the absorption of light and increases the rate of electron transport. On the other hand, under high light levels this helps regulate the flow of excess excitation energy, balancing energy between the photosystems and preventing photodamage. In this way, photosynthetic rates are maintained under high light levels that would, under any other circumstances, be damaging (Chow, Melis, & Anderson, 1990; Terao et al., 1996; Yokono et al., 2019). In general, CD treatment appeared to up-regulate PSI genes, but down-regulate PSII genes. In this way, CDs appear to be altering photosystem stoichiometry. As a photoprotective mechanism, the impact of CDs on photosystem stoichiometry presents a route by which CDs are able to facilitate increased rates of electron transport without inducing photodamage and also demonstrates how a larger proportion of excitation energy can be safely partitioned for photochemistry, as theorised by Swift *et al.* (2021).

Lastly, two chloroplastic protein genes were found to be down-regulated in the glucose CD-treated plants. These genes are known to play a role in NPQ (Wang & Portis, 2007). This corroborates the findings of both the chlorophyll fluorescence results presented in Chapter 5 and the work of Swift *et al.*, whereby glucose CD application was found to decrease NPQ (Swift *et al.*, 2021). This provides a genetic mechanism by which CDs decrease NPQ in wheat.

7.5. Carbon dots and the genome – concluding remarks

CDs upregulate the expression of both chlorophyll a-b binding proteins and oxygen-evolving enhancer proteins. This increases the light-harvesting capacity of plants and increases the rate of electron transport. CDs alter photosystem stoichiometry, a photoprotective mechanism. This provides a genetic basis for the biostimulant enhancement effect of CDs on photosynthesis. Furthermore, these effects were much stronger in CD-treated plants than in fertiliser-treated plants.

8. Conclusions and further work

From the results presented in this body of work, it is evident that carbon dots (CDs) enhance wheat growth in a number of ways.

Out of all the physiological impacts presented in this body of work, it is evident that the most significant impact of CDs on wheat plants is on photosynthesis. Furthermore, these photosynthetic impacts were multi-faceted, increasing photosynthetic activity in a variety of different ways. Firstly, CDs significantly increase the light-harvesting capacity of leaves. This was demonstrated by increases in leaf chlorophyll content and the up-regulation of chlorophyll a-b binding proteins. Secondly, CDs increase the rate of electron transport. This was shown by increases in ETR as measured by chlorophyll fluorescence, in the up-regulation of oxygen-evolving enhancer proteins, and in the decrease of Y(NPQ) and down-regulation of NPQ genes, which allows for the partitioning of more energy into photochemistry. Lastly, and perhaps most importantly, CDs increase photoprotection. This is vital in protecting the plants from photodamage under the increased levels of light-harvesting and electron transport, and was shown by the increase in the production of photoprotective flavonols and the modulation of photosystem stoichiometry, as demonstrated by the up-regulation of PSI genes and down-regulation of PSII genes.

When applied in conjunction with synthetic NPK fertilisers, CDs enhance ear development and yield. Therefore, CDs can show a strong biostimulating effect when applied alongside fertilisers. However, at other times the opposite is true, with the dual treatment showing an inhibition effect rather than an enhancement effect. It is also evident that at times CDs alone have a stronger biostimulating effect than when applied with fertilisers. Therefore, further work is needed to assess the biostimulant suitability of CDs, both alone and in relation to fertilisers. Moreover, the impacts of CDs are evidently cultivar-dependent, and so testing CDs over a wide range of cultivars and plant species will be a vital part of this.

Given the wide-ranging dataset, there are many areas where future work would be beneficial. Firstly, the results presented in this body of work focus on the impacts of CDs on the light-dependent reaction (LDR) of photosynthesis, the rate of which determines the light-independent reaction (LIR). CDs have been shown to increase the activity of Rubisco, the enzyme which catalyses the LIR. With Rubisco activity directly impacting on yield, and with CDs being shown to increase Rubisco activity by 30.9%, it would be beneficial to investigate the impacts of CDs on Rubisco in *T. aestivum* cv. Apogee and *T. aestivum* cv. Paragon, to investigate the impacts of CDs on all stages of photosynthesis, from light-harvesting at the start through to carbohydrate production at the end (Andersson & Backlund,

2008; Wang et al., 2018b). Next, throughout this body of work the impacts of CDs on roots was regularly referenced, as in other works the uptake of CDs via roots has been suggested to be the primary mechanism by which CDs facilitate increased plant growth and yield (Aji et al., 2020; Li et al., 2020; Xu, et al., 2021; Wang et al., 2018b). Although roots were not investigated in this work due to the time constraints outlined in the Covid Statement, this would provide valuable information into the impacts of CDs on wheat. This could be done by growing and treating wheat hydroponically, for example, so that roots are visible and quantifiable.

There are many other areas where future work could be focused; the impacts of CDs on ROS production and the ROS-scavenging activity of CDs would be valuable to measure, particularly when discussing their impacts on NPQ and photoprotection; testing a wider variety of wheat cultivars would be highly beneficial, especially when considering the huge genetic variety between different cultivars; testing the optimum time to begin treatment would be valuable, especially in very fast-growing cultivars where CDs will have a very limited time of action, or in very slow-growing cultivars where CDs will inevitably accumulate over time; the impacts of CDs on wheat grown hydroponically, for example in vertical farming, would be very useful; the impacts of CDs on stomatal conductance could be quantified, as this will have many indirect knock-on effects on other physiological processes in plants; and lastly, the impacts of CDs over a range of environmental temperatures and CO₂ concentrations would be vital in assessing their suitability as a biostimulant in the face of climate change.

In sum, it is evident that CDs present a highly versatile, sustainable biostimulant candidate capable of increasing photosynthetic activity, enhancing ear development, and increasing yield. By decreasing NPK fertiliser requirements, CDs may well present a solution to the problem of sustainably intensifying agriculture in an increasingly unpredictable climate.

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