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Investigating the Effect of Putative Cytokinin Antagonists on Root Growth in Rice, and their Efficacy in Mitigating Stress

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Abstract

There is a plethora of challenges that must be addressed this century to ensure the demand for food, fodder and biofuel is met. Feeding 9 billion people whilst counteracting the negative effects that erratic and more severe weather events are having due to climate change is a challenge that requires innovative approaches. Drought and salinity are significant limiting factors to crop yields, and modifying plant traits to avoid these stresses has been identified as a method of improving crop productivity.

This study investigated the ability of putative root-specific cytokinin antagonists, molecules that block activity of the plant hormone cytokinin, to promote root growth in rice as a mechanism for improving crop abiotic stress tolerance. In addition to the parent compound, four novel compounds synthesised by Globachem Discovery Ltd. were found to promote root growth of the rice variety, Nipponbare, in liquid media. Subsequently, seed priming was established as a way of applying the compounds, significantly reducing preparation time and the quantity of product required. The long-term effects of priming were found to not affect aboveground biomass but did confer a negative effect to yield.

The compounds were also tested for their ability to promote root growth in commercially relevant rice varieties and growth settings under drought and salt stress. However, the increase in root length found in Nipponbare was not observed in a commercial setting or commercially used rice varieties under optimum or stress conditions, highlighting the high specificity of the compounds. These findings show that whilst there is potential for these compounds to promote root growth, their use must be further optimised for agricultural purposes.

In parallel to the lab-based studies, three models were designed and implemented in Chapters 2, 4 and 5. A machine learning technique was used to predict the likelihood of a compound having biological activity, based on its chemical properties. In a subsequent chapter the effects of spatial heterogeneity within a glasshouse were quantified and accounted for statistically. Finally, geospatial modelling was used to identify key regions where plant growth regulators could be applied most effectively. These models allow the optimisation of current practice, from agrochemical design to dissemination of a product, thereby contributing to a more robust agricultural system.

The lab-based assays and different modelling approaches used in this study highlight the multi-faceted and collaborative approaches that are required to tackle the pressing humanitarian and environmental challenges of this century. This study goes some way to addressing these challenges.

Acknowledgements

The opportunities I've had throughout the last four and a half years have been brilliant, and I have many people to thank for that. The Centre for Global Eco-Innovation, the European Regional Development Fund and Globachem Discovery Ltd. funded the project and made this all possible, for which I'm very grateful. Secondly, I'd like to thank my supervisors, Dr Gabriela Toledo-Ortiz and Professor Martin McAinsh, who have supported me throughout, challenged me at times, and spent many unsociable hours providing feedback to help improve my research and writing skills, all of which have allowed me to develop professionally. I hope I can one day emulate the unwavering positivity, sage advice, eloquence and astuteness that has been imparted upon me throughout this project.

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List of Abbreviations and Acronyms

ABA	Abscisic acid
Acetyl-CoA	Acetyl coenzyme A
AHK	Arabidopsis kistidine kinase
AHP	Arabidopsis histidine phosphotransfer proteins
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARR	Arabidopsis response regulators
C	Control
CAT	Catalase
CK	Cytokinin
CKX	Cytokinin oxidases/dehydrogenases
CRE1	Cytokinin response 1
DMAPP	dimethylallyl pyrophosphate
DMSO	Dimethyl sulfoxide
FAO	Food and Agriculture Organization
h	hours
ha	hectares
HS	High stress
iP	isopentenyladenine
IPP	isopentenyl pyrophosphate
IPT	Isopentenyltransferases
IQR	Interquartile range
LS	Low stress
min	minutes
*OsHK*s	Oryza sativa histidine kinases
*OsHP*s	Oryza sativa histidine phosphotransfer proteins
*OsRR*s	Oryza sativa response regulators
PCA	Principal component analysis
PGR	Plant growth regulator
PEG	Polyethylene glycol
RGP	Root growth promoter
ROS	Reactive oxygen species
RR	Response regulators
RSA	Root system architecture
SEM	Standard error of the mean
SOD	Superoxide dismutase
SVI	Seed vigour index
WW	Well-watered

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1 Introduction and Literature Review

1.1 Introduction

The rising global population and increasingly conspicuous effects of climate change mean that increases in food production of around 60% must be achieved by 2050 through sustainable intensification of agriculture (Alexandratos and Bruinsma, 2012). In addition, global warming is changing weather patterns and climate throughout the world, and extreme weather events are increasing in both intensity and frequency. Climate models predict an average increase in global temperatures between 2.5-5.4°C by 2100 coupled with a decrease in precipitation of 15% (Ciscar, 2012, Tadross et al., 2007). The changes in weather and climate often impose abiotic stresses on crops that negatively impact yields and in turn, food availability, with declines in yields of major crops (corn, wheat and rice) anticipated (Mickelbart et al., 2015). Abiotic stresses often occur simultaneously (e.g. heat and drought) or in succession (e.g. flooding and then drought), and the timing and duration of each abiotic stress can result in synergistic effects (Mittler, 2006, Zandalinas et al., 2021). The order, frequency and longevity of each stress plays a role in the impact of the stress. For example, a concurrent occurrence of drought and heat is more destructive to crops than the two stresses occurring separately (Prasad et al., 2011). As climate change events increase in both frequency and severity, so too will the effects of abiotic stresses on plants, severely affecting both growth and yield (Godoy et al., 2021).

Root systems are central to the acquisition of water and nutrients by plants and have, therefore, attracted interest from agrochemical companies and crop improvement programmes which aim to improve food security (Gamuyao et al., 2012, Ju et al., 2015, Gowda et al., 2011, Jeong et al., 2010, Arai-Sanoh et al., 2014). Of particular interest are traits such as root length and structure which determine the extent and distribution of root surface area within the soil profile where resources are unevenly distributed (Fromm, 2019). Water and nitrate, for example, are highly mobile within the soil and tend to accumulate in the deeper layers (Fromm, 2019). Therefore, a steep rooting angle and longer roots have become recognised as a high-value crop improvement target associated with the improved performance of crops at lower levels of irrigation and nitrate fertiliser application (Ju et al., 2015, Gamuyao et al., 2012).

1.2 Demand for abiotic stress mitigation

At present, agricultural production is neither sustainable nor able to meet the needs of the current population (FAO, 2018); in 2020, 768 million people were undernourished and nearly one in three people (2.37 billion globally) did not have access to adequate food (FAO, 2021b). The pressure to increase food production and security will be exacerbated as the population increases towards the 9.7 billion predicted by 2050 (FAO, 2018). The population increase naturally means more people to feed, but there are also secondary factors that put further pressure on agriculture, such as increased wealth leading to a diet shift with greater demand for meat, the growing biofuel industry, urbanisation and the surge in behaviours that accelerate climate change (FAO, 2018). Demand for grain increased 2.2-fold from 1970 to 2020, though the amount of cultivated land globally has remained largely unchanged since 1965. Given that access to suitable land is finite, and the population is still increasing, cultivated land area per person will continue to decline worldwide. Therefore, it is essential that agricultural land becomes more productive and that the global food system is optimised (Nishimoto, 2019, FAO, 2009b).

To date, much work has been carried out on the impact of biotic stress on crops; the herbicide, fungicide and insecticide agrochemical industry is worth an estimated £187bn (Fernández, 2021). The effect of abiotic stress on crops includes the effects of drought, salinity, flooding, extreme temperatures, and a gradual rise in tropospheric ozone (Wang and Frei, 2011), all of which are anticipated to become more severe due to climate change (Wang and Frei, 2011). Abiotic stresses result in extensive loss of crop yields, resulting in large financial penalties for farmers (Mittler, 2006). Despite this, the market for abiotic stress mitigation is largely under-developed and currently lacks any applications to reduce the impact of abiotic stress on crops. Abiotic stress management is currently heavily reliant on either breeding programmes or genetic modification (Bansal et al., 2014), which is often met with resistance from consumers (Gaskell et al., 2004). Therefore, new approaches are urgently required to increase crop yields to feed a growing population in increasingly stressful environmental conditions.

1.2.1 Globachem Discovery Ltd.

Agrochemical applications are used predominantly for biotic stress mitigation and macronutrient enrichment of soils (McKinlay et al., 2012). They are typically applied by foliar spray, application of fertilisers to the ground, or, more recently, by the chemical priming of seeds (see section 3.1.1) (Rhaman et al., 2021). Application of a wide range of natural and synthetic growth regulators has been found to mitigate the adverse effects of abiotic stress such as drought stress (Farooq et al., 2009a, Lipiec et al., 2013, Brownfield et al., 2008), salinity and heavy metals (Wani et al., 2016). Plant hormone based abiotic stress defence is, therefore, emerging as a promising research area (Nishiyama et al., 2011, Colebrook et al., 2014, Rhaman et al., 2021).

In order to achieve greater food security, Globachem Discovery Ltd. synthesises novel fungicides, insecticides and herbicides for optimising agricultural yields (Globachem, 2021a). Compounds synthesised early in the agrochemical discovery process are analogues of compounds with known biologically active status, minimising cost and development time for new products. One aspect of their research focusses on abiotic stress mitigation, with the objective of increasing yields in challenging environments.

1.3 Rice: a model crop species

Rice, *Oryza sativa*, is one of the most important food crops; more than half of the global population are dependent on rice to meet their daily calorific needs and it accounts for 20% of all calories consumed worldwide (Fukagawa and Ziska, 2019). Over 90% of rice is grown in Asia, where 60% of the global population live and much of the projected population growth will occur (Khush, 2005). Rice production underpins food security and provides an important source of vitamins (such as thiamine, niacin and folic acid) and minerals (such as magnesium, manganese, selenium, iron and phosphorus) in many low- and middle-income countries. Given its global significance, rice is a major component in the Food and Agriculture Organization's (FAO) goal to end undernourishment by 2030 (FAO, 2021b, Fukagawa and Ziska, 2019). As the model crop species for monocotyledonous plants, rice is of enormous interest globally for scientific, humanitarian and commercial purposes (FAO, 2018, Fukagawa and Ziska, 2019, Khush, 2005).

Rice is highly sensitive to both drought and salt stress (Uga et al., 2013, Zeng and Shannon, 2000), susceptibility to which has grown, in part, due to higher sowing densities which increases competition for resources (Mickelbart et al., 2015). Rice sensitivity to salt and drought stress and the losses incurred as a result creates vulnerabilities for the future of food security that need to be addressed urgently (Haefele and Bouman, 2009, Zeng and Shannon, 2000, FAO, 2021a).

1.4 Cytokinins

Plant growth, development and responses to external stimuli are regulated by very low concentrations of plant hormones (Ciura and Kruk, 2018). Plant hormones include auxin, cytokinins (CKs), gibberellins, abscisic acid (ABA), salicylic acid, ethylene, brassinosteroids, jasmonates and peptides (Khan et al., 2014). Signals involving plant hormones are spatiotemporally diverse; short-term responses such as closure of stomata are elicited in a matter of minutes and are largely dependent on ABA, whereas long term responses such as CK-mediated temperature acclimation happens over a period of days (Escandón et al., 2016).

CK and ABA interact directly through their signalling components and are known to behave antagonistically (Pavlů et al., 2018). Their antagonistic behaviour plays a role in both drought and stress tolerance. The Arabidopsis histidine kinase (AHK) 1 receptor, a known CK receptor, has been found to be a positive regulator of drought and salt stress responses, in addition to ABA signalling, whereas CK receptors AHK2, AHK3 and CRE1 are negative regulators of ABA signalling (Tran et al., 2007). Interestingly, under drought stress, plants with decreased levels of CK or reduced CK signalling have lower levels of ABA, but a higher sensitivity to ABA and greater drought tolerance (Pavlů et al., 2018). CK is also known to behave antagonistically with auxin to control meristem development (Moubayidin et al., 2009), this interaction plays an important role in modulating root development (Ioio et al., 2008). CK controls the rate of cell differentiation by repressing auxin signalling and transport, hence, the co-ordination of these two hormones is essential for controlling root size (Moubayidin et al., 2009). Simultaneous adjustments of plant hormones, their crosstalk with other signalling molecules and the knock-on effect this has on gene expression plays a crucial role in stress tolerance and elicits an optimised response to stress (Tuteja and Mahajan, 2007, Sewelam et al., 2016, Santner and Estelle, 2009).

1.4.1 Cytokinin structure, synthesis and signalling

CKs are a class of plant hormone that play a key role in almost all stages of plant growth and development (Werner and Schmülling, 2009, Kieber and Schaller, 2014). Naturally occurring CKs are classed as either aromatic CKs or isoprenoid CKs, depending on whether they have an aromatic or isoprene-derived side chain at the N^6 terminus, respectively (Sakakibara, 2006) (see Figure 1.1 for side chain configurments). CK was originally thought to be synthesized solely in roots and transported to shoots; however, genes that encode enzymes involved in CK biosynthesis have since been found throughout the plant and it is now known that CKs are also synthesised in shoots and transported to the roots, though to a lesser extent than root to shoot CK signalling. (Sakakibara, 2006).

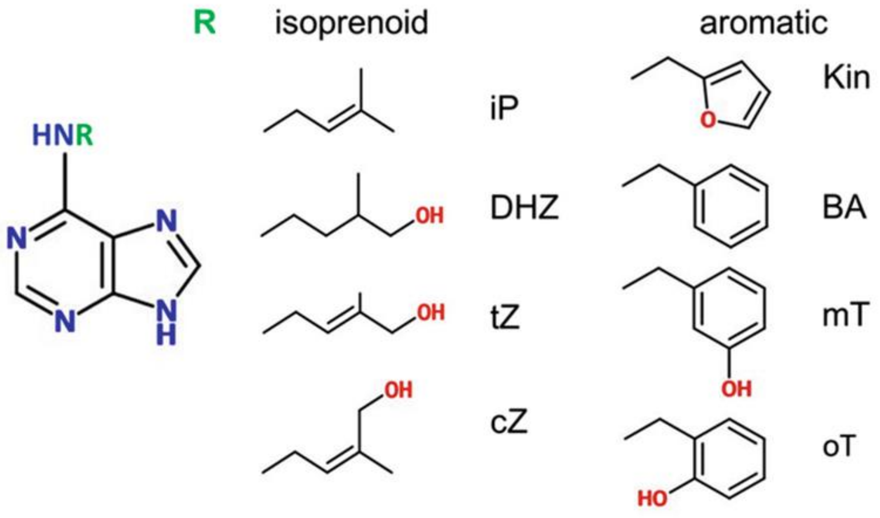


Figure 1.1. The naturally occurring CKs, consisting of an adenine base with an isoprenoid or aromatic-derived side chain. The CKs with an isoprenoid side chain are: isopentenyladenine (iP), Dihydrozeatin (DHZ), trans-Zeatin (tZ) or cis-Zeatin (cZ). The aromatic CKs are Kinetin (kin), Benzyladenine (BA), meta-Topolin (mT), ortho-Topolin (oT). Adapted from (Pekarova et al., 2018).

The isoprenoid precursors to CK, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are produced by one of two pathways, the methylerythritol phosphate (MEP) pathway in plastids and the mevalonate (MVA) pathway in the cytosol (Kieber and Schaller, 2014, Kasahara et al., 2004). The isoprenoid side chain of isopentenyladenine (iP), a CK, can be hydroxylated on the *cis* or *trans* methyl group, forming *cis*-zeatin or *trans*-zeatin. The conformation strongly influences biological activities and the function of the CK, with *cis*-zeatin typically having lower biological activity than *trans*- (Schafer et al., 2015). The free bases that convert iP to *cis*- or *trans*-zeatin are released from CK ribotides by the LONELY GUY (LOG) protein family, a single step reaction that converts inactive CK nucleotides into active free bases, allowing rapid conversion of inactive CK into an active form (Seo et al., 2016). Prenyl groups are hydrophobic molecules that are added to a protein or compound. The prenyl group of tZ and iP is most commonly produced via the MEP pathway, whilst the prenyl group of cZ is produced via the transfer of DMAPP from the MVA pathway to tRNA in the cytosol (Záveská Drábková et al., 2021, Kasahara et al., 2004).

Plants respond to CKs via a multistep phosphorelay system; the first step of the CK signalling pathway is the perception of CKs by receptors (Hutchison and Kieber, 2002). The perception of CK by receptors prompts the transfer of phosphate from a histidine kinase to a histidine phosphotransferase which translocates through the nuclear membrane and amplifies transcription, leading to a biological response (Hutchison and Kieber, 2002) (Figure 1.2). Rice has 11 histidine kinases (OsHKs), 5 histidine phosphotransferases (OsHPT) and 36 response regulators (OsRRs), though little is known about how they interact and what their signalling cascades entail (Sharan et al., 2017).

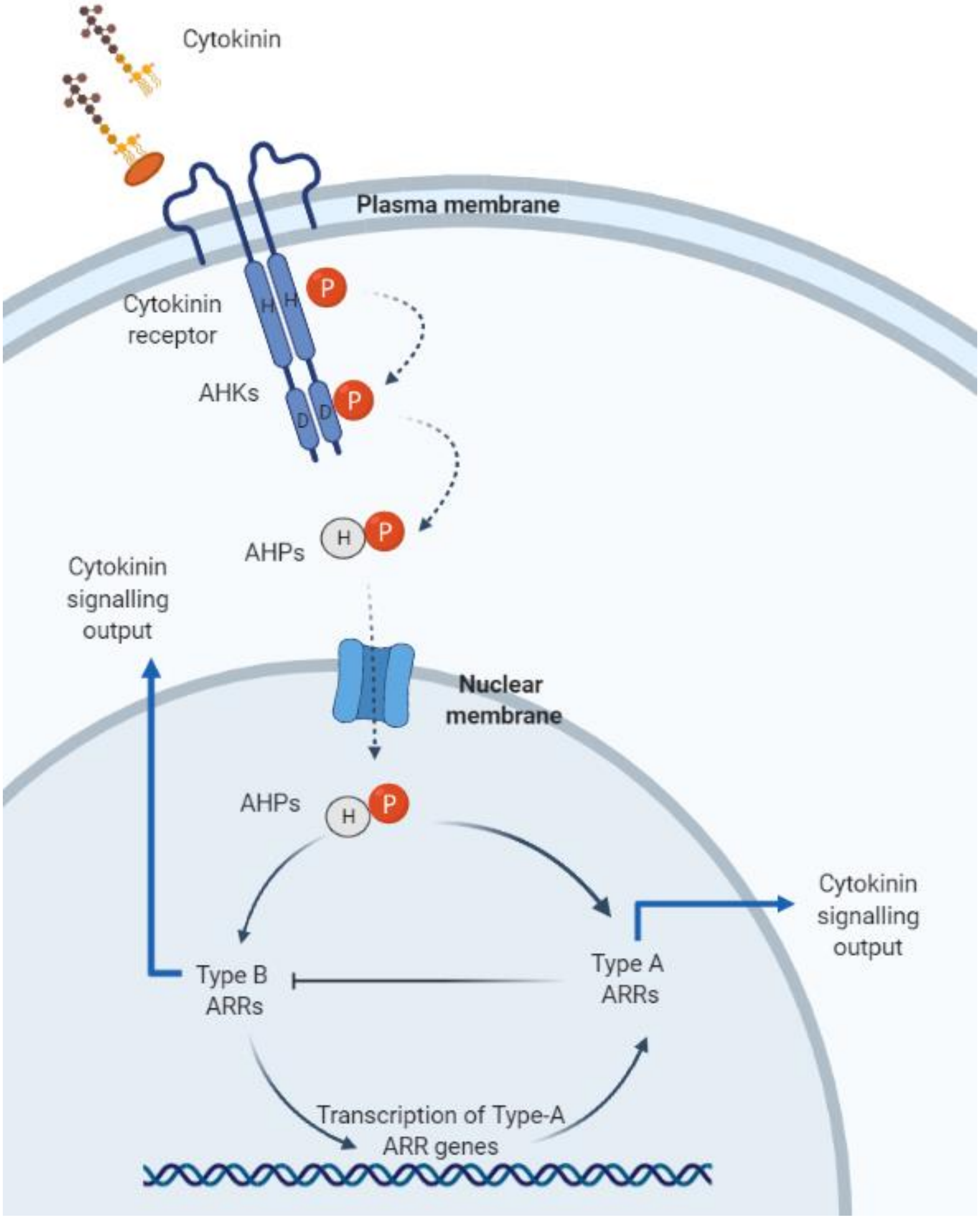


Figure 1.2. CK receptors, which are histidine kinases (AHK), initiate the phosphorelay when CK binds to cytokinin response 1 (CRE1), thought to be located in the plasma membrane. The binding of CK causes the transmitter domain to autophosphorylate on a His (H). Phosphate is then transferred to an Asp-residue (D) within the receiver domain. The phosphate is then transferred to an AHP protein which translocated to the nucleus, where type-B ARRs are activated. Activation of type-B ARRs causes increased transcription of type-A ARRs which in turn feed back and inhibit their own transcription. The signal is then outputted, but the pathway is mostly unknown. Image produced in BioRender, adapted from Schaller et al. (2008).

1.4.2 Effect of cytokinin in the root and shoot

CKs are involved, in varying abundance, in the growth and development of a plant, such as delaying leaf senescence (Gan and Amasino, 1995) and increased grain yield (Ashikari et al., 2005). In the roots, CKs create positional cues for lateral root formation to ensure adequate spacing between neighbouring roots, and are also involved in inhibiting lateral root formation (Laplaze et al., 2007b, Julkowska, 2018). A plants' response to the environment is shaped by CK abundance, such as shade avoidance (Carabelli et al., 2007) and light responses (Boonman et al., 2007, Zubo et al., 2008) in leaves; and drought stress (Ha et al., 2012), nitrate, sulphate, and phosphate assimilation in the roots (Rubio et al., 2008). The presence of CK and the resulting interactions between different molecules is complex, and its role varies according to location and abundance in the plant.

CKs are negative regulators of root development, inhibiting formation of adventitious roots, lateral roots and root elongation (Ramireddy et al., 2018) (Figure 1.3). Various methods have been implemented to reduce the amount of CK in the roots to promote root growth, including overexpression of CK oxidase/dehydrogenase(CKX) to degrade CK (Ramireddy et al., 2018), mutation of isopentenyltransferase genes (responsible for the rate-limiting step of CK biosynthesis, *atipt3 5 7* triple and the *atipt1357* quadruple mutants (Miyawaki et al., 2006)) to inhibit CK synthesis, mutation of CK receptor genes (AHK2,AHK3 and CRE1/AHK4) (Riefler et al., 2006), or suppressing the CK signalling pathway by a CK antagonist binding to the CK receptor and preventing signal transduction (Arata et al., 2010).

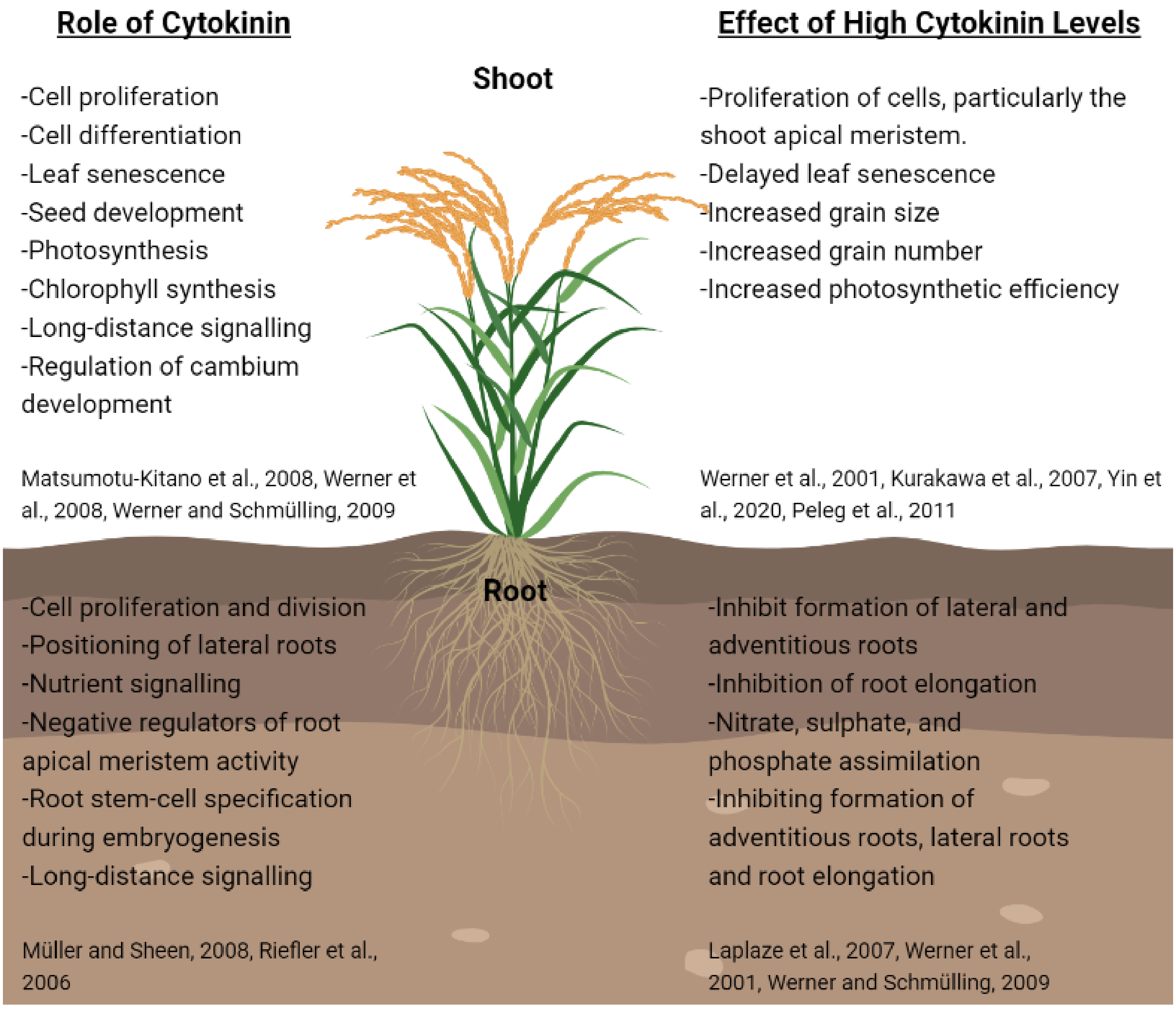


Figure 1.3. CK has many roles in the root and shoot of plants involved in growth and development, some of the effects of high CK levels on root and shoot development are highlighted (Matsumoto-Kitano et al., 2008, Werner et al., 2008, Werner and Schmülling, 2009, Müller and Sheen, 2008, Riefler et al., 2006, Werner et al., 2001, Kurakawa et al., 2007, Yin et al., 2020, Peleg et al., 2011, Laplaze et al., 2007b). Image produced in BioRender.

1.4.3 Cytokinin antagonists

Structural analogues of CKs compete with CKs in signal transduction systems within a plant, affecting biological processes and gene expression (Gregorini and Laloue, 1980). Analogues that exhibit this behaviour are known as CK antagonists and inhibit activity of CK within cells (Gregorini and Laloue, 1980). The potential value for modifying plant traits by application of a CK antagonist has been recognised for several decades, but optimum utilization is yet to be achieved (Gregorini and Laloue, 1980, Hecht et al., 1971, Arata et al., 2010)

Early studies using CK antagonists did not yield the crucial receptor-specific activity that is required for CK antagonists to be suitable for trait modification for agricultural purposes (Spíchal et al., 2009, Nisler et al., 2010). CK antagonist activity was reported at the receptor level in Arabidopsis by Spíchal et al. (2009); a purine derivative compound, PI-55, competitively inhibited binding of *trans*-zeatin to receptor cytokinin response 1 (CRE1, also referred to as Arabidopsis histidine kinase 4 (AHK4)), but also exhibited agonist activity with two other receptors: Arabidopsis histidine kinase 2 and 3 (AHK2 and AHK3) (Spíchal et al., 2009). This specificity was improved somewhat by Nisler et al. (2010), in which a CK receptor antagonist, LGR-991, derived from 6-benzylaminopurine interacted with CK receptors AHK3 and AHK4 and exhibited reduced agonist activity compared to PI-55. Application of LGR-991 resulted in more rapid germination and increased hypocotyl length of dark-grown seedlings (Nisler et al., 2010). Subsequently, a non-competitive CK antagonist was identified by Arata et al. (2010) which bound exclusively to CRE1 in the roots of Arabidopsis and rice with no agonistic interactions, resulting in an increase in root growth in both species. Further research into receptor-specific CK antagonists that lower CK levels exclusively in the root offers a promising mechanism to promote root growth (Ramireddy et al., 2018, Nehnevajova et al., 2019).

1.5 Signalling response to stress

Multiple signalling pathways can be activated during exposure to stress, which has been found to cause an overlap in gene expression (Zandalinas et al., 2021). This can lead to the initiation of similar biological processes and molecular functions to different triggers (Zandalinas et al., 2021). Stress responses vary in their spatiotemporal scales; a plants' response to stress can be localised (cellular) or systemic (whole plant), short term responses can occur within minutes of initiation of the stress, whereas a long-term stress (days or weeks) results in a long -term response (Heap et al., 2020, Gilroy et al., 2016, Das and Roychoudhury, 2014).

Rice plants have developed complex, targeted responses to stress; regulatory networks within the plant allow the fitness cost associated with responding to the stress to be minimised (Wang et al., 2019, Kim et al., 2020b, Horie et al., 2012). Signalling pathways are a multi-level signalling network, beginning with the sensing of an exogenous signalling molecule, often a ligand binding to a receptor protein on the cell surface (McAinsh and Taylor, 2017). This is followed by signal transduction in which the signalling molecule binds to a receptor, eliciting a change in structure and initiating the process of signal transduction. Signal transduction is followed by signal amplification and response of the plant which often involves transcription factors, secondary metabolites and hormones (Heap et al., 2020).

1.5.1 Involvement of cytokinin in abiotic stress signalling pathways

During drought stress, the CK levels in rice roots and shoots decreases (Kim et al., 2020b, Todaka et al., 2017, Maruyama et al., 2014). Under salt stress, the generally recognised pattern for CK levels in plants is to decrease (Albacete et al., 2008, Ghanem et al., 2008, Kudoyarova et al., 2007). Interestingly, however, the one study that has been published to date on the effects of salt stress on CK levels in rice found a decrease of CK in the shoots but an increase of CK in the roots (Yin et al., 2020) which may have complications for engineering trait modifications. Nevertheless, the modulation of CK metabolism and signalling has been shown to improve drought and salt tolerance, whether through inhibiting CK activity in the roots or increasing the abundance of CK in the shoots (Yin et al., 2020, Nehnevajova et al., 2019, Ramireddy et al., 2018).

Aboveground, CK-related mechanisms contribute to increased plant tolerance to water deficit conditions via increased expression of genes involved in photosynthesis, chlorophyll abundance, photochemical efficiency and quenching, electron transport rates, and CO_2 assimilation (Pavlů et al., 2018). Modulation of CK metabolism enhances antioxidant systems which protect cells from stress-induced ROS accumulation, in turn protecting chloroplast integrity and reducing electrolyte leakage (Nguyen et al., 2016a, Rivero et al., 2007). Plants with low levels of CK or weak CK signalling, akin to having a CK antagonist active, have improved water regulation and balance, typically having higher water contents during drought stress than plants with high CK content or stronger signalling (Pavlů et al., 2018). Higher water content is thought to be a result of having longer roots, and is complemented with reductions in transpiration rate and stomatal aperture which are thought to occur to reduce water loss during stress (Nguyen et al., 2016a). CK oxidase enhances root growth, nutrient uptake, drought tolerance and improves recovery post-drought stress with no cost to shoot growth (Ramireddy et al., 2018). It is therefore hypothesised that a CK antagonist would confer the same benefits to a drought-exposed plant.

1.6 Drought stress

1.6.1 Physiological and biochemical effects of drought

Drought stress reduces leaf water potential and turgor, induces stomatal closure, and decreases the rate of cell growth (Farooq et al., 2009b) which results in reduced leaf size, root growth and yield (Kim et al., 2020a). A reduction of physiological and biochemical functions, such as photosynthesis, respiration, and the metabolism of nutrient and carbohydrates also occurs under drought stress (Hussain et al., 2018, Yordanov et al., 2000, Kumar et al., 2018) (Figure 1.4).

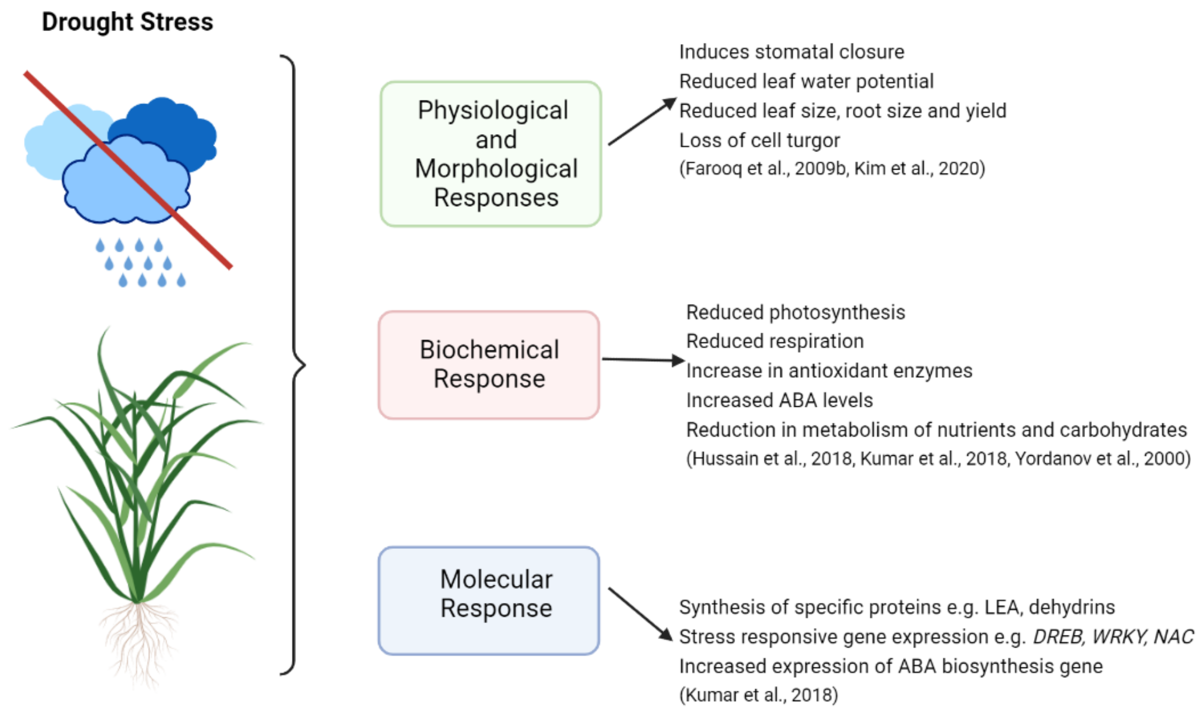


Figure 1.4. Depiction of a plants' physiological, morphological, biochemical and molecular response to drought (Farooq et al., 2009b, Kim et al., 2020b, Hussain et al., 2018, Kumar et al., 2018, Yordanov et al., 2000). Image produced in BioRender.

Plants adapt to drought stress using a range of mechanisms depending on the severity and duration of the drought and their acclimation and adaptation capacity (Farooq et al., 2009b). Drought tolerance occurs when a plant has adapted to endure low water availability via osmotic adjustment of cell turgor and cellular elasticity (Farooq et al., 2009b). Drought avoidance relates to the plants' ability to maintain high water potential despite the low water potential of the soil; this is achieved by either minimizing water loss by techniques, such as reducing transpiration or by maximising water uptake via methods such as increased rooting (Basu et al., 2016).

Changes in the water potential gradient between soil and root prompts the production of ABA, a long-distance signalling molecule which triggers the closure of stomata and induces the expression of an array of stress-related genes and signalling molecules and transcription factors (Hussain et al., 2018), preventing further water loss through transpiration, but also preventing photosynthesis in the process. Consequently, overexpression of ABA receptors has been reported to increase drought tolerance but also to negatively affect yield under non-drought conditions (Hussain et al., 2018). This highlights the necessity of promoting development of traits that are beneficial under both optimum and stress conditions.

1.6.2 The effect of drought stress on rice productivity

Access to freshwater varies spatially and temporally, leading to droughts which result in two thirds of the global population experiencing severe water scarcity for at least one month of the year (Mekonnen and Hoekstra, 2016). Globally, around 151 million hectares (ha) of land is dedicated to rice-growing, one third of which is grown in rain-fed lowlands in non-irrigated conditions solely dependent on rainfall. In South and Southeast Asia 45 million ha of land is used for rice cultivation, of which 19 to 23 million ha are estimated to be frequently and severely affected by drought (Haefele and Bouman, 2009). There are many drought-risk areas beyond this region, their risk determined by topography, soil characteristics and local weather patterns (Haefele and Bouman, 2009). As a result of drought, rice yields are typically low in these regions and there is great opportunity for increasing yields (Tsubo et al., 2009).

Low yields due to rainfed lowlands experiencing drought is a common problem throughout Asia, with an estimated 45% of the total rice area having no irrigation input, with up to 35% of yield lost due to droughts in rainfed regions of Thailand (Fukai et al., 1999). Traditional rice cultivation in flooded paddy fields have higher water demand than other major crops, making the effects of drought more pronounced (Datta et al., 2017). Yields in rainfed rice systems are typically much smaller than those of irrigated systems due to poor growing conditions, limited resources and lack of development of suitable cultivars or products (Fukai et al., 1999). Despite irrigated land typically having higher yield, these areas are prone to yield losses as a result of salinity (Asch et al., 2000) (see section 1.7.2).

1.6.3 Engineering drought tolerance in rice

It has been forecast that changes in precipitation will result in more frequent droughts and floods (Trenberth, 2011). Both outcomes present a different array of problems: increased flooding will lead to issues such as increased runoff and in turn, increased leaching of soil nutrients; whereas increased drought will decrease the amount of freshwater available for irrigation of crops (Trenberth, 2011, Scalenghe et al., 2012). With the discovery of *Sub1A* gene that confers submergence tolerance to rice, genetic engineering of rice has gone some way to mitigating the effects of flooding on rice crops (Xu et al., 2006). Deep roots are the most widely accepted target trait for improved drought resistance (Gowda et al., 2011), and roots engineered to be longer by root-specific expression of *OsNAC10,* a gene involved in hormone signalling pathways and cell development, have been found to have improved drought tolerance (Jeong et al., 2010). Additionally, elongation of the rice root has been found to aid phosphorus acquisition (Gamuyao et al., 2012) and nitrogen use efficiency (Ju et al., 2015). In all instances the increased root length and improved nutrient acquisition or drought tolerance resulted in higher grain yield (Gamuyao et al., 2012, Ju et al., 2015, Jeong et al., 2010).

Drought tolerance has been achieved in rice by the overexpression of the *DRO1* gene, a gene that controls gravitropic curvature in rice roots, by enhancing deep rooting and auxin-regulated asymmetric root growth and modulating CK flux from the root (Arai-Sanoh et al., 2014, Uga et al., 2013). Hence, modulating root growth by altering root-specific CK activity could be a promising avenue for increasing drought tolerance in rice and making agricultural land more productive. As plant hormones are critical components of modulating physiological and molecular responses to abiotic stress, manipulation of their signalling has been identified as having the potential to be a key player to produce abiotic stress-tolerant crops (Wani et al., 2016). However, drought stress remains the key limiting factor to crop productivity, with rice growth and productivity severely affected by drought stress.

1.7 Salinity stress

1.7.1 Physiological and biochemical effects of salt

Accumulation of salt within cells causes disruption to osmotic and ionic equilibriums which leads to changes in metabolism, stunted growth and cell or plant death (Singh et al., 2018). The presence of salt within the plant exerts an osmotic and ionic stress. The change in osmotic pressure between the plant root cells and the surrounding water results in inhibition of water uptake, cell expansion and lateral bud development (Horie et al., 2012). The osmotic stress that arises due to salt stress reduces leaf growth, root growth, stomatal conductance and in turn, photosynthesis (Horie et al., 2012). The accumulation of Na^+ inside plant cells, particularly in leaves, imposes an ionic stress and increases the rate of chlorosis. The increased chlorosis is coupled with a reduction in photosynthesis and increased cell death (Munns, 2002). In addition, salt stress can inhibit enzyme function, decrease carbon use efficiency and cause decomposition of proteins and membranes. Salt stress also generates reactive oxygen species (ROS) which accumulate and inhibit plant growth (Lee et al., 2001) (Figure 1.5).A negative correlation exists between Na^+ accumulation in leaves and survival of rice plants under salt stress, understandably therefore, one plant adaptation to salt stress is by preventing Na^+ reaching the intracellular space (Yeo et al., 1990). Indeed, most plants can effectively exclude Na^+ from the roots whilst uptaking water from the soil (Munns, 2005). Alternative adaptations to salt stress include osmotic stress tolerance and the tolerance of tissue that has accumulated to Na^+ (Munns and Tester, 2008).

The root system architecture of a plant can adapt to saline conditions by reducing primary root growth and initiating lateral root development (Rahnama et al., 2011, Jung and McCouch, 2013). Soil salinity tends to increase with depth, this increase in lateral root development is, therefore, thought to be a method of exploiting non-saline areas within the soil (Munns and Gilliham, 2015). Therefore, manipulation of CK abundance within a root may offer a method to further exploit the upper part of the soil by increasing lateral root abundance.

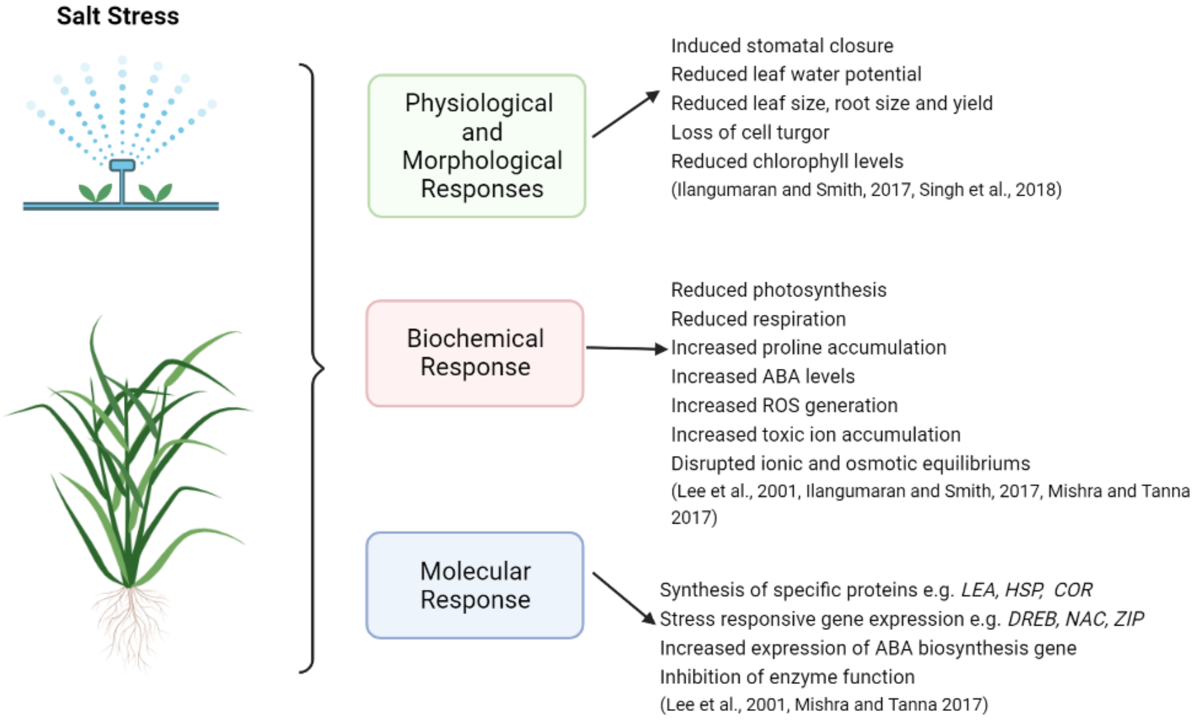


Figure 1.5. Depiction of a plants' physiological, morphological, biochemical and molecular response to salt (Ilangumaran and Smith, 2017, Singh et al., 2018, Mishra and Tanna, 2017, Lee et al., 2001). Image produced in BioRender.

1.7.2 The effect of salt stress on rice productivity

It is estimated that 19.5% of irrigated land and 2.1% of dry land agriculture is affected by salinity (FAO, 2021a). Salt stress is common in rice paddies in delta and coastal areas, but is also common in other irrigation water due to the high solubility of salts in water (Tack et al., 2015). Furthermore, rising sea levels due to climate change are leading to further degradation of soil by salinity (Reddy et al., 2017).

Rice is a highly salt-sensitive crop, therefore, irrigating rice fields with water with high salt content creates a major obstacle to increasing rice yields globally (Zeng and Shannon, 2000). Studies have shown that salinity at 1.9 dS m^{-1} significantly reduces rice seedling shoot dry weight and reduces seedlings survival at 3.4 dS m^{-1} (Zeng and Shannon, 2000). Rice harvest at 3.4 dS m^{-1} revealed leaf chlorosis and significantly reduced spikelets per panicle, grain dry weight per panicle and grain dry weight per plant (Zhou and Zeng, 2018). These data are particularly alarming as water used for flood irrigation practices has been found to typically have an electrical conductivity of 0.5 to 3.5 dS m^{-1} (Zeng and Shannon, 2000).

1.7.3 Engineering salt tolerance in rice

Salt tolerance in rice has mainly been attributed to the plants' ability to control the amount of Na^+ reaching leaf tissue (Singh et al., 2018). This can be achieved by ion exclusion, tissue tolerance or osmotic tolerance (Rajendran et al., 2009). Ion exclusion involves the retrieval of Na^+ from the xylem and the efflux of ions back into the soil. Tissue tolerance occurs when Na^+ is sequestered into cell vacuoles in root tissue as osmoticum, another component of tissue tolerance involves enzyme synthesis that catalyses reactions to detoxify ROS (Rajendran et al., 2009, Singh et al., 2018). Chen et al. (2007) engineered increased salt tolerance in rice by overexpressing a vacuolar Na^+/H^+ antiporter gene which removes Na^+ from the cytosol and compartmentalize it in vacuoles in order to maintain a low intracellular Na^+ concentration, amplifying the tissue tolerance response found in rice. The mechanisms involved in osmotic tolerance result in the plants' ability to tolerate the drought effects of salinity stress and maintain leaf expansion and stomatal conductance (Rajendran et al., 2009). Fast growing genotypes with early plant vigour allow plants to avoid salt toxicity during the highly salt-sensitive seedling stage and provide another strategy for improving plant growth and yield under salt stress (Reddy et al., 2017).

Manipulating CK distribution has been identified as a strategy to improve grain yield and salt tolerance in rice. Yin et al. (2020) found that overexpression of ARGONAUTE2 (AGO2) improved salt tolerance in rice, in addition to increasing grain yield. The improved salt tolerance was achieved by activation of a putative CK transporter (BIG GRAIN3) (Yin et al., 2020). Given that stresses often occur consecutively or simultaneously, this highlights the care that must be taken when modifying plant stress signalling.

1.8 The hidden half of plant biology: the roots

1.8.1 Desirable phenotypic root traits

Abiotic stress tolerance, including tolerance to drought, flooding, nutrient deficiency and toxicity, is frequently dependent on the adaptation of roots (Mickelbart et al., 2015). Root size and morphology govern the uptake of water and nutrients to the plant (Arai-Sanoh et al., 2014, Gowda et al., 2011). A deep rooting phenotype has been found to maintain high yields under prolonged soil water deficit whilst also enhancing nutrient uptake and yield under non-drought conditions in the field, illustrating that a deep root confers benefits under abiotic stress and optimum conditions (Arai-Sanoh et al., 2014). Although a deep root phenotype prior to onset of stress is typically beneficial, the critical root length density will depend on soil conditions, particularly moisture (Gowda et al., 2011). Importantly, deep rooting rice genotypes are associated with improved productivity under stress (Gowda et al., 2011).

There are several different mechanisms to cope with drought stress: drought escape, tolerance, recovery and avoidance (Gowda et al., 2011). Roots are associated with drought avoidance; deep, dense, thick roots with high branching and penetration ability having a competitive advantage over other roots (Gowda et al., 2011). The ability of a plant to grow deep roots is currently the most targeted trait for improving drought avoidance, and has been shown to be a beneficial trait for drought avoidance in rice (Henry et al., 2011). The efficacy of deep roots in drought conditions depends on the duration of the drought period, the availability of water at depth and the rate of water uptake (Gowda et al., 2011). Initially, it was hypothesised that thick roots would contribute to drought resistance, though this hypothesis was later rejected in favour of small, fine root diameters (Yambao et al., 1992, Comas et al., 2013). Other traits associated with drought avoidance include: high elasticity in leaf rolling, early stomatal closure, high cuticular resistance, and high root to shoot ratio (Kadioglu and Terzi, 2007, O'Toole et al., 1979, Xu et al., 2015). However, these drought avoidance traits often come with trade-offs; for example leaf curling and early stomatal closure result in reduced photosynthesis (Kadioglu and Terzi, 2007).

1.8.2 Rice roots

Rice has a fibrous, shallow root system which has limited water extraction capacity below 60cm, its length and mass decreasing exponentially with depth (Gowda et al., 2011). Rice roots have a shorter root length and lower ability to take up water per unit of root length compared to other cereal crops, such as maize (Lesschen et al., 2004). Therefore, rice is a good candidate for modifying root structure to access water lower down in the water table. Modifications that increase root length have been found to confer additional benefits, such as an increase in grain yield (Gamuyao et al., 2012, Ju et al., 2015, Jeong et al., 2010). Several other traits have been selected to benefit rice under drought stress, these are: high maximum rooting depth, high root: shoot ratio and high dry weight (Gowda et al., 2011).

1.8.3 Methods for analysing root system architecture

Roots have been termed the ‘hidden half’ of plant biology due the difficulties involved with non-invasive imaging and the intricacies of extracting and cleaning root tissue (Mairhofer et al., 2012). Despite the complexities, various methods have been developed to study root development and architecture, these include: soil-based growth and extraction, rhizotrons, X-ray microcomputer tomography (µCT), agar assays and hydroponics (Mairhofer et al., 2012, Clark et al., 1999, Richard et al., 2015, Smit et al., 2000). The soil-based method of extracting and washing the root is inexpensive and requires little technology, but can result in an underestimation of root length as there are often breakages of fine roots (Smit et al., 2000). One method to avoid such breakages is to use a rhizotron, this allows root growth in soil without any handling or damage to the root, though the spatial distribution of roots is restricted and the extent to which roots can be seen and analysed is limited. A recent development to eliminate the negative effects of both soil-based techniques is µCT; allowing non-invasive, in-situ visualisation of roots (Mairhofer et al., 2012). µCT scans offer a 3D visualisation of roots and is an exciting new tool, but the costs associated with processing can render µCT unfeasible for high-throughput studies (Mairhofer et al., 2012).

Growth in agar and hydroponics enables visibility of the root and makes extraction easier, typically with less damage to roots than extraction from a soil-based system. Due to the differences in mechanical impedance between agar, hydroponics and soil, root physiology varies and conclusions drawn in non-soil experiments should not be assumed in soil (Clark et al., 1999). Most techniques for phenotyping root traits are low throughput, lab-based, and have limited ability to reproduce field-like conditions. However, lab assays have the advantage of being able to be conducted out of season, are less time consuming and are conducted in a more controlled, homogenous environment which provide more reproducible results (Richard et al., 2015).

1.9 Opportunities and thesis aims

There are significant challenges to be addressed this century if everyone is to have access to safe, sufficient, and nutritious food (FAO, 2021b). Many of the low- and middle-income countries, and much of the forecast population growth, will depend on rice as a source of nutrition and to meet their daily calorific needs (Fukagawa and Ziska, 2019). Climate change adds additional complexity to these challenges. Salt and drought stress are two of the major abiotic stresses which account for significant losses in rice yields (Wade et al., 1999, Hussain et al., 2018), for that reason, targeting these areas could make significant contributions towards reaching the goal of eradicating undernourishment by

2030 (FAO, 2021b). Whilst rice is short-rooting, longer roots are a recognised acclimation to drought stress (Gowda et al., 2011, Basu et al., 2016, Samson et al., 2002), which can be modulated by manipulation of CK abundance (Ramireddy et al., 2018). This suggests that manipulation of rice plant root length by modulation of CK levels and/or CK signalling may provide a mechanism for improving the drought and salt tolerance of rice, leading to improved rice yields. Hence, the overall aim of this thesis is to investigate the efficacy of CK modulation in modifying root traits, and exploring whether this makes rice more resilient to abiotic stress. Specifically, the objectives of this project are to:

1. Establish whether analogues of a known CK antagonist promote root growth in *Oryza sativa* cv. Nipponbare in optimum conditions using a hydroponic system.
2. Use machine learning to establish whether the RGP behaviour of a compound can be predicted using its chemical structure data which could reduce the time and resources used in the preliminary stages of agrochemical discovery.
3. Transfer the novel compounds into use in a commercial setting using commercially researched rice varieties, and establish whether the root growth promoters (RGPs) confer benefits to rice seedlings under salt or drought stress.
4. Establish whether seed priming is a viable method of application of the novel compounds.
5. Establish whether the short-term benefits of seed priming confer any long-term benefits to mature rice plants by conducting a yield harvest analysis.
6. Quantify the effects of spatial heterogeneity within a glasshouse and incorporate these data into weighted statistical tests for improved glasshouse trial analyses.
7. Create a novel agricultural toolkit, using global datasets to identify regions where the optimum conditions for the compounds are likely to be found.

2. Do novel cytokinin antagonists act as root growth promoters in the model monocot species, Oryza sativa?

2.1 Introduction

The global population is expected to grow by more than a third between 2009 and 2050, with almost all of this growth forecast to occur in low- and middle-income countries where access to nutritious, safe, and sufficient food is already limited (FAO, 2009b). The continued rise in population and the increased demand for meat, dairy and biofuels is increasing the pressure on agricultural systems. In addition to this, climate change is increasing the severity and frequency of stresses on crops, leading to unreliable yields and losses. These two substantial issues represent a complex challenge for global food security during the 21st Century (FAO, 2009b).

Although a 28% increase in crop production was achieved between 1985 and 2005 (Foley et al., 2011), many of these initial yield increases are now stagnating or declining (Ray et al., 2012). Globally, over half of the population is reliant on rice for their primary source of calories. It provides an important source of carbohydrates, minerals and vitamins and ensures food security in low- and middle-income countries. However, rice yields are only increasing by 1% annually, failing to meet the 2.4% yield improvement needed to meet the requirement to increase food production by 60% between 2007 and 2050. Rice producing regions in Asia and Africa are fraught with issues relating to drought, nutrient deficiency, and poor irrigation, all of which are likely to be exacerbated by climate change.

2.1.1 Root system architecture

Over 30% of the variability in global crop yields can be explained by climatic irregularities (Ray et al., 2015). One method to reduce this variability and improve food security is to increase crop resilience to abiotic stress, leading to a more productive crop and more consistent yields; it is anticipated that 85% of the required increases in crop production can be achieved as a result of improved yields (FAO, 2018). Water and nutrient availability are both major limitations on crop productivity (Lynch et al., 2014, Gamuyao et al., 2012). Gamuyao et al. (2012) found that overexpression of *PSTOL1* in rice enhances early root growth, resulting in enhanced phosphorus and nutrient uptake which in turn enhances grain yield in phosphorus deficient soil. Discovery of the *PSTOL1* gene offers an opportunity to revolutionise farming and significantly enhance productivity in poor soils (which account for 60% of rain-fed lowland rice production), in the same way that discovery of the submergence-tolerance gene *SUB1A* revolutionised rice growing in flood-prone areas (Gamuyao et al., 2012, Xu et al., 2006). Therefore, increasing a plants' ability to access water and nutrients by engineering plants with longer roots, whether through breeding, genetic modification or agrochemical application, may similarly offer an efficient solution for improving crop yields and limiting crop losses due to water or nutrient scarcity and crop lodging (Lynch et al., 2014).

Deep root systems allow plants greater access to water and nutrients compared to plants with more shallow root systems (Gamuyao et al., 2012), and concurrently minimise leaching to the soil (Thorup-Kristensen and Kirkegaard, 2016). These benefits allow increases in productivity to be achieved without using more land or resources, particularly in water-limited environments (Thorup-Kristensen et al., 2020). For wheat, maize and barley, three of the major crops grown globally, most of the roots are concentrated in soil depth 50-100cm below ground, indicating this is a key depth for water and nutrient acquisition (Fan et al., 2016). In contrast, the maximum root depth of rice is typically 0.5m (Figure 2.1). The shallow rooting depth suggests that there is scope to increase the root length of rice to a length comparable to other major cereals crops, and do so before soil depth leads to compaction that would impede root growth and limit water and nutrient uptake (Chapman et al., 2012, Pandey et al., 2021). Interestingly, deeper roots have been identified as an important trait to achieve higher grain yield and nitrogen use efficiency in rice (Ju et al., 2015). Hence, deep-rooting could be a key player in sustainable intensification of agriculture and improving resource use efficiency in rice (Gamuyao et al., 2012, Thorup-Kristensen and Kirkegaard, 2016).

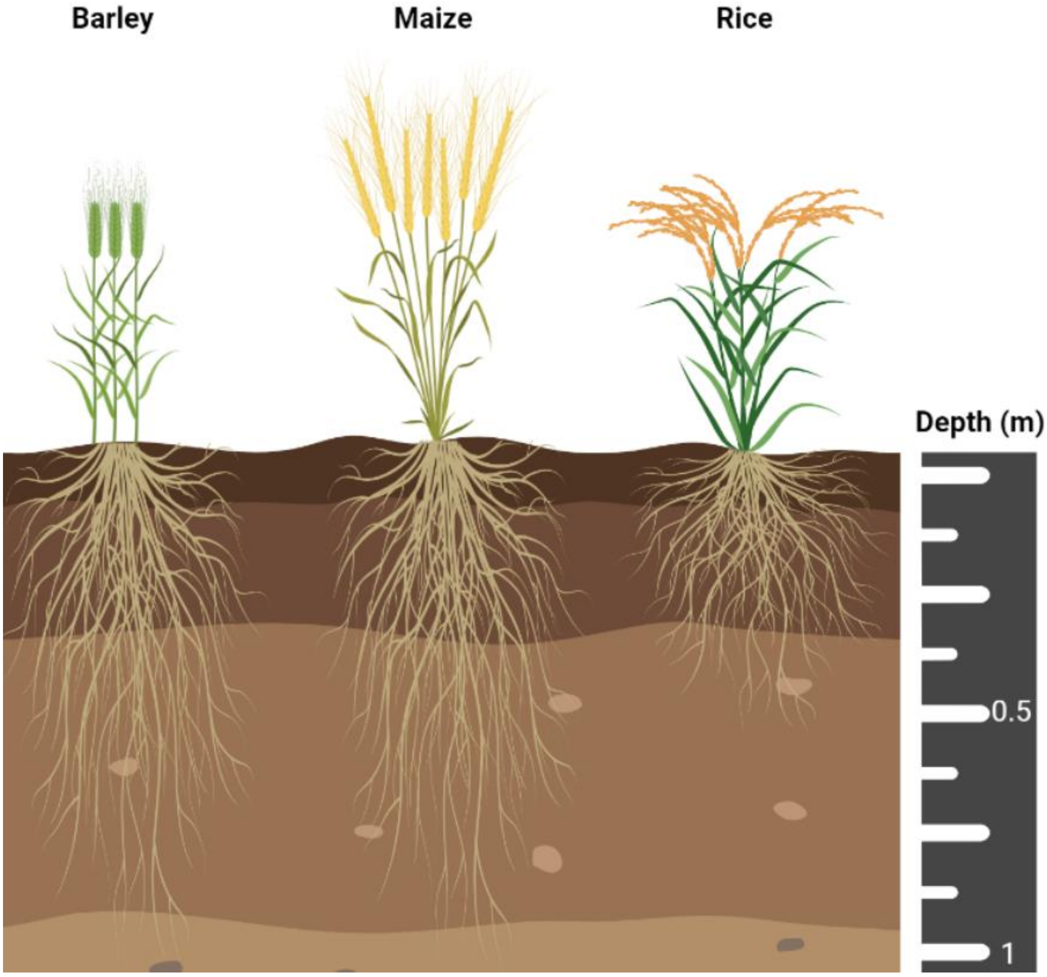


*Figure 2.1. Rooting depths of major crop types. *minimum value of maximum rooting depth data range used to prevent overestimation. Image created in BioRender. Adapted from Lesschen et al. (2004).*

In rainfed lowland regions of rice production, shallow rooting is common (Samson et al., 2002). Rice cultivars with deeper roots able to penetrate the hardpan layer of soil, which is typically shallow in rainfed lowland regions where alternate flooding and drying is common, have greater access to water and are more drought tolerant (Clark et al., 2002). Maximum rooting depth has been found to be highly dependent on environmental conditions; Champoux et al. (1995) found that rice varieties with the shallowest roots under well drained conditions had the deepest roots under flooded conditions. This suggests that rice in rainfed lowlands could benefit from having longer roots from the offset and be more resilient to the fluctuating environmental conditions.

Plants have evolved a high degree of phenotypic plasticity in the roots due to the spatiotemporal heterogeneity of water and nutrients in the soil (Fromm, 2019). The ability of roots to reach deeper soil with lower water content variability has been associated with a method of drought tolerance (Fromm, 2019). Additionally, lateral roots can be produced at almost any position along a main root, dependent on internal and external signalling (Motte and Beeckman, 2019). As water deficiency is emerging as one of the prevalent abiotic stresses occurring as a result of climate change (Lynch et al., 2014), this phenotypic plasticity offers an exciting opportunity for 'engineering' the water and nutrient use efficiency of rice. Interestingly, xerotrophism, a phenomenon by which roots exhibit an enhanced response to gravity, is mediated by auxin. This occurs when the top soil layer is dry and deep soil retains water (Fromm, 2019). CK and auxin are known to behave antagonistically (Moubayidin et al., 2009). This raises the intriguing possibility that application of a CK antagonist could mediate the same effect as enhanced auxin levels in the root, initiating a xerotropic effect.

2.1.2 Hydroponic systems

Studying root system architecture (RSA) and root physiology in soil poses major research challenges. The heterogeneity of the below ground environment prompts a myriad of environmental signals in a plant in response to the natural variability of water, fungi and nutrients – primarily nitrogen and phosphate (Morris et al., 2017). Heterogeneity of nutrients arises due to the uneven input and solubility variation within the soil. Phosphate rapidly forms insoluble complexes, reducing its mobility, whereas nitrate is highly water soluble and is therefore a highly mobile nutrient (Morris et al., 2017). Water availability varies according to soil pore size, compaction, and location; topsoil dries more rapidly than the soil lower down in the soil profile (Morris et al., 2017). Hydroponic systems allow the study of whole plant physiology, particularly root morphology, in a simplified growth environment thereby mitigating these effects (Nguyen et al., 2016b). Benefits of a hydroponic system include the ability to control the nutrient input to the plant in a homogenous environment, and the subsequent extraction of clean roots for analysis, eliminating the root damage that frequently arises from cleaning roots that have been grown in soil (Nguyen et al., 2016b).

2.1.3 Target molecules

CK plays a key role in the development and architecture of roots creating positional cues for new lateral roots by mediating the spacing between lateral roots and reducing lateral root formation (Laplaze et al., 2007b). Although the regulatory role of CK in roots is crucial, however, under standard growth conditions CK levels in roots limit root growth (Julkowska, 2018). Previously, a novel synthetic compound with a 4-phenylquinazoline structure (Figure 2.2) has been shown to have root growth promoting activities by non-competitively inhibiting binding of the CK 2-isopentenyladenine to a CK receptor, cytokinin response 1 (CRE1) in the dicotyledonous and monocotyledonous model crop species, *Arabidopsis thaliana* and *Oryza sativa* (Arata et al., 2010). The synthesised compound was found to promote root growth in both species. CK antagonists that bind competitively to the receptor have also been reported in an Arabidopsis binding assay (Nisler et al., 2010). This raises the possibility of developing novel CK antagonists that promote root growth by releasing the CK brakes on root growth, thereby improving the yields and resource use efficiency of crops.

A technique called structure activity relationship analysis is used for drug discovery purposes and for the development of novel bioactive agrochemicals (Kim et al., 2015). Using this technique, small changes are made to a lead molecule with the objective of increasing the activity of the molecule, in turn increasing the effect of the favourable properties. Some structural modifications yield positive effects, others negative. Feedback from experiments testing the biological activity of compounds enables the molecular structure to be refined and establishes what changes to the molecule are beneficial or detrimental to plant development, for example root growth. This is an iterative process, each iteration increases knowledge and feeds into the subsequent molecular changes. Using synthetic chemistry makes targeting a specific location on the molecule easier than traditional methods (Guha, 2013).

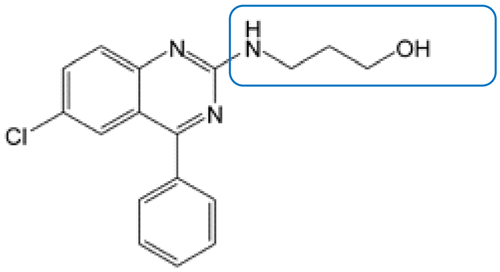


Figure 2.2. The 4- phenylquinazoline structure identified as a CK antagonist in a large-scale yeast screening in Arata et al. (2010). Hydroxy propylamine, highlighted in blue, can be changed for other amines, alcohols and esters to vary the solubility of the molecule and the physical properties to aid bioavailability.

2.2 Aims and Objectives

The aim of this chapter is to explore the potential of 16 analogues of the 4-phenylquinazoline structure as candidates for novel root growth promoters (RGPs), on the physiology of rice seedlings. The analogues are derived from compound S-4893 in Arata et al. (2010) and later synthesised by Globachem Discovery Ltd. The objectives of the chapter are:

1. To establish and optimise a robust hydroponic assay for measuring root length in rice.
2. To establish the concentration ranges at which the 16 Globachem analogues can be used without their carrier solvents impacting physiological processes.

3. To explore the relationship between compound application and growth of the shoot and root.
4. To determine suitable bio-active candidate compounds for subsequent detailed analysis.

2.3 Materials and Methods

2.3.1 Plant and growth conditions

Rice seeds (*Oryza sativa* cv. Nipponbare) were sterilised using 100% (v/v) ethanol for 2 min and 20% (v/v) sodium hypochlorite solution for 10 min and rinsed six times with sterile water. The seeds were then placed on moist filter paper in a Petri dish and sealed with 3M micropore tape. The Petri dish was covered in aluminium foil in a controlled environment at 30°C:26°C (11h:13h day:night). After 48h the aluminium foil was removed, and the Petri dish returned to the controlled environment. Once germinated, seeds with the most homogenous growth were selected for use in the assay and placed on a polystyrene float with a hole cut into it to place the seed in. The seed was then cultivated hydroponically in a test tube using 50ml liquid medium consisting of Murashige and Skoog medium (0.22% w/v) at half strength (2.2 g/L) and 0.5% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Atom Scientific, Kimble, Borosilicate Glass 25mm x 200mm buffer (Atom Scientific, Kimble, Borosilicate Glass 25mm x 200mm). Test tubes were placed in a wooden box to prevent light exposure of the root zone (Figure 2.3). Growth assays were performed in a controlled environment at 30°C:26°C (11h:13h day:night) for 10 days.

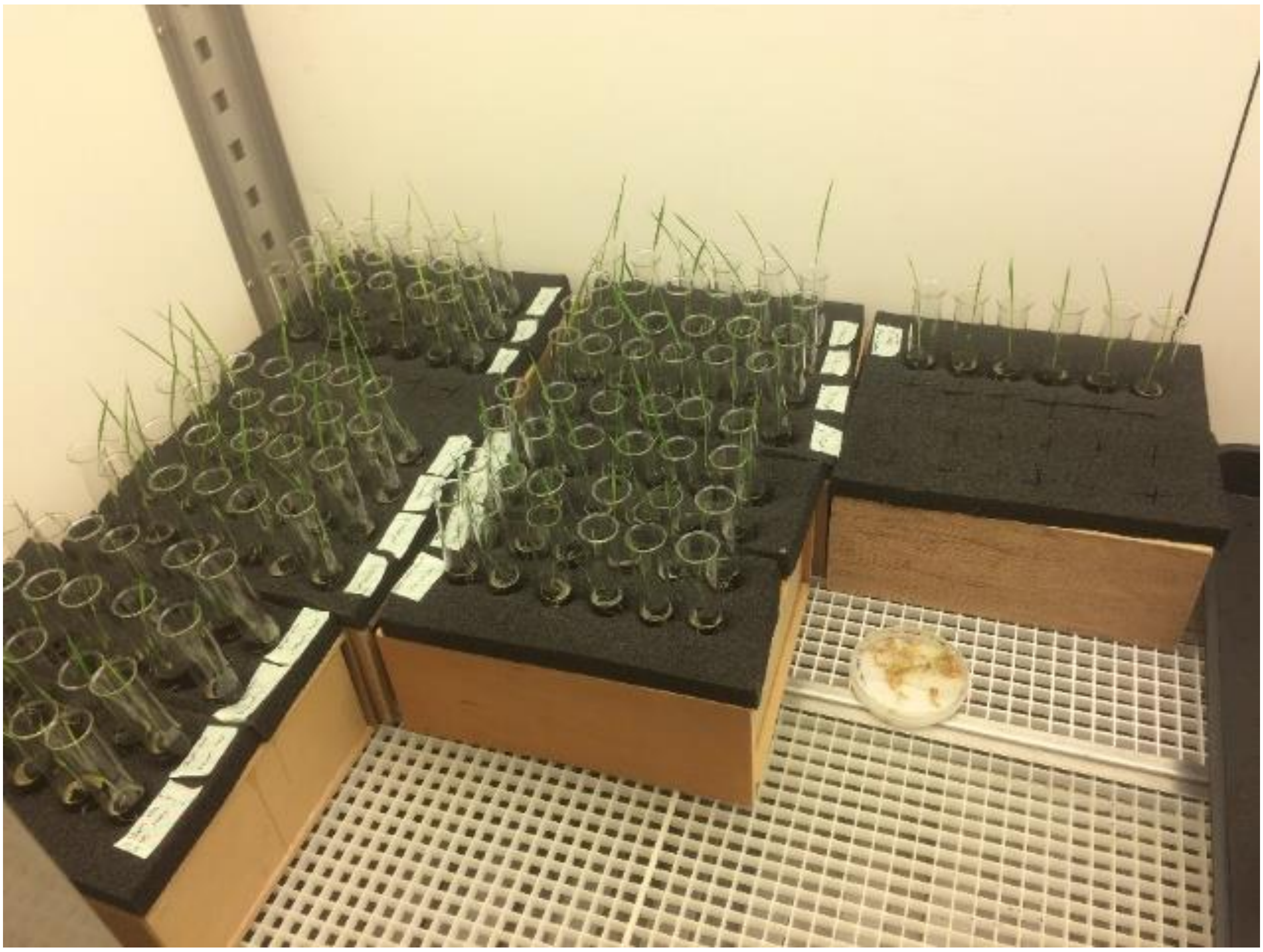


Figure 2.3. Boxes with covered sides and a foam top to create a narrow opening for the test tubes, limiting the exposure of roots to light.

2.3.2 Chemical application

2.3.2.1 Validation of compound 974 as a root growth promoter in *O.sativa*

The parent compound used was referred to as S-4893 in Arata et al. (2010) and will subsequently be referred to as compound 974 herein. Compound 974 was obtained from Globachem Discovery Ltd and was a 4-phenylquinazoline structure, molecular formula: $C_{17}H_{16}ClN_3O$. Previously, Arata et al. (2010) published an optimum concentration of 32µM, with a typical bell curve effect observed. In the present study, concentrations ranged from 0.0096µM to 96µM; much lower concentrations were used to assess whether a double bell curve response or complex non-monotonic dose response to the parent compound existed. A stock solution was prepared in DMSO and acetone (1:4 [v/v], DMSO: acetone) and the nutrient solution was supplemented with the stock solution to give final test concentrations of 0.0096µM to 96µM and a maximum final concentration of DMSO and acetone of 0.05% and 0.2% (v/v), respectively.

2.3.2.2 Screening for novel *O.sativa* root growth promoting chemicals

The chemical library screened was obtained from Globachem Discovery Ltd. and consisted of 16 novel candidate RGPs. Compounds were all analogues of the 4-phenylquinazoline parent compound. Stock solutions of test compounds were prepared in DMSO and acetone (1:4 [v/v], DMSO: acetone). Nutrient solution was supplemented with stock solution of the compound to give final test concentrations of 0.32, 0.96, 3.2, 9.6, and 32µM and a maximum final concentration of DMSO and acetone of 0.05% and 0.2% (v/v), respectively. In all cases, no significant difference in root growth was observed between the control with or without DMSO-acetone at any concentration used. Therefore, for subsequent experiments the control was performed without the inclusion of DMSO-acetone. Experiments were repeated in triplicate where the quantity of the compound allowed with six biological replicates per experiment.

2.3.3 Root imaging

The root phenotype was imaged 10 days after transfer to the test tubes using a high-resolution scanner (EPSON Expression 11000XL), measurements were obtained using WinRhizo software (Figure 2.4). Primary (consisting of seminal and crown) and lateral root growth were measured, diameter classes were optimised, a diameter of <0.02cm selected for the lateral roots whilst a root diameter of ≥0.02cm selected for primary roots.

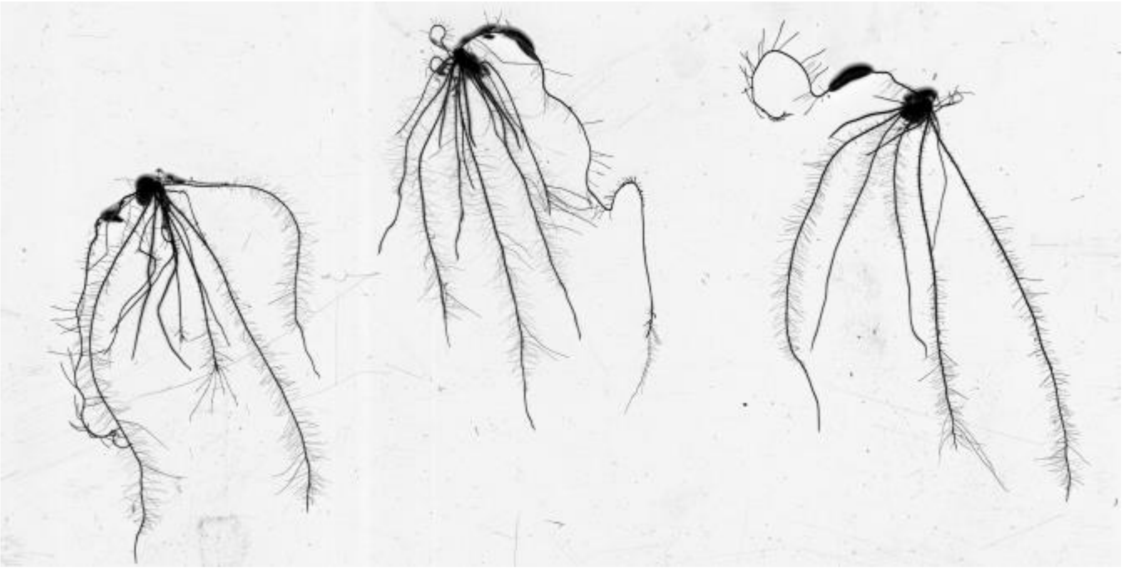


Figure 2.4. Sample root images of roots scanned on a flatbed scanner in preparation for analysis with WinRhizo software.

2.3.4 Statistical analysis

All statistical analyses were carried out in R (R Core Team, 2021). To identify if the root and shoot length of treated plants were significantly different to the control, independent t-tests were performed. Analysis of variance (ANOVA) was performed to identify when a significant difference between concentrations existed within each treatment. ANCOVA was performed on lateral, primary and total root length, p=0.05. Linear regression was used to explore the relationships between lateral, primary and total root length. The model selection for all regression analyses was loess, the shaded area represents a 95% confidence interval. Principal component analysis (PCA) was carried out to establish whether the chemical properties of a compound could predict whether they were likely to be an RGP or not.

Using R package 'corrplot' the relationship between each root variable was analysed. Rose (2017) found that ignoring diameter heterogeneity and clustering the root types lead to an overestimation of length and an underestimation of volume. To avoid this, data for each boundary classification were split into primary, and lateral. Total values were calculated accounting for each root type, taking into account the diameter heterogeneity within each root type.

2.4 Results

2.4.1 Identification of root growth promoting chemicals

Of the 16 chemicals in the chemical library obtained from Globachem Discovery Ltd. only 10 remained in solution when added to the growth medium and were subsequently screened for their root growth promoting activity (Figure 2.5, Table 2.1). An ANOVA was performed to identify whether intra-compound variation was statistically significant. All compounds selected as RGPs produced an ANOVA p-value of <0.05. To identify results that were statistically significant from the control, independent t-tests compared each intra-compound concentration to the control of that group. Compounds 974 and 1027 showed a positive correlation between root length and concentration. Compounds 1026 and 991 had a lower optimum concentration, with root length peaking at 3.2µM and 0.96µM respectively (Figure 2.5). Compound 1070 showed significant RGP activity at each concentration (Table 2.1), though with some variation in significance between experimental replicates. The following compounds were identified as RGPs due to one or more of the concentrations significantly increasing root length: 974, 1027, 1070, 1093 and 1131 (Figure 2.5). The remaining treatments had either no significant effect on root growth or an inhibitory effect. Mean total root length for seedlings treated with a RGP was 153cm, compared to a total root length of 78.9cm for seedlings treated with compounds that did not have RGP properties (SE ± 14.6 and 5.02 respectively). Total root length was significantly influenced by whether a compound showed RGP activity or not $F(1,420)=20.31$, $p<.0001$.

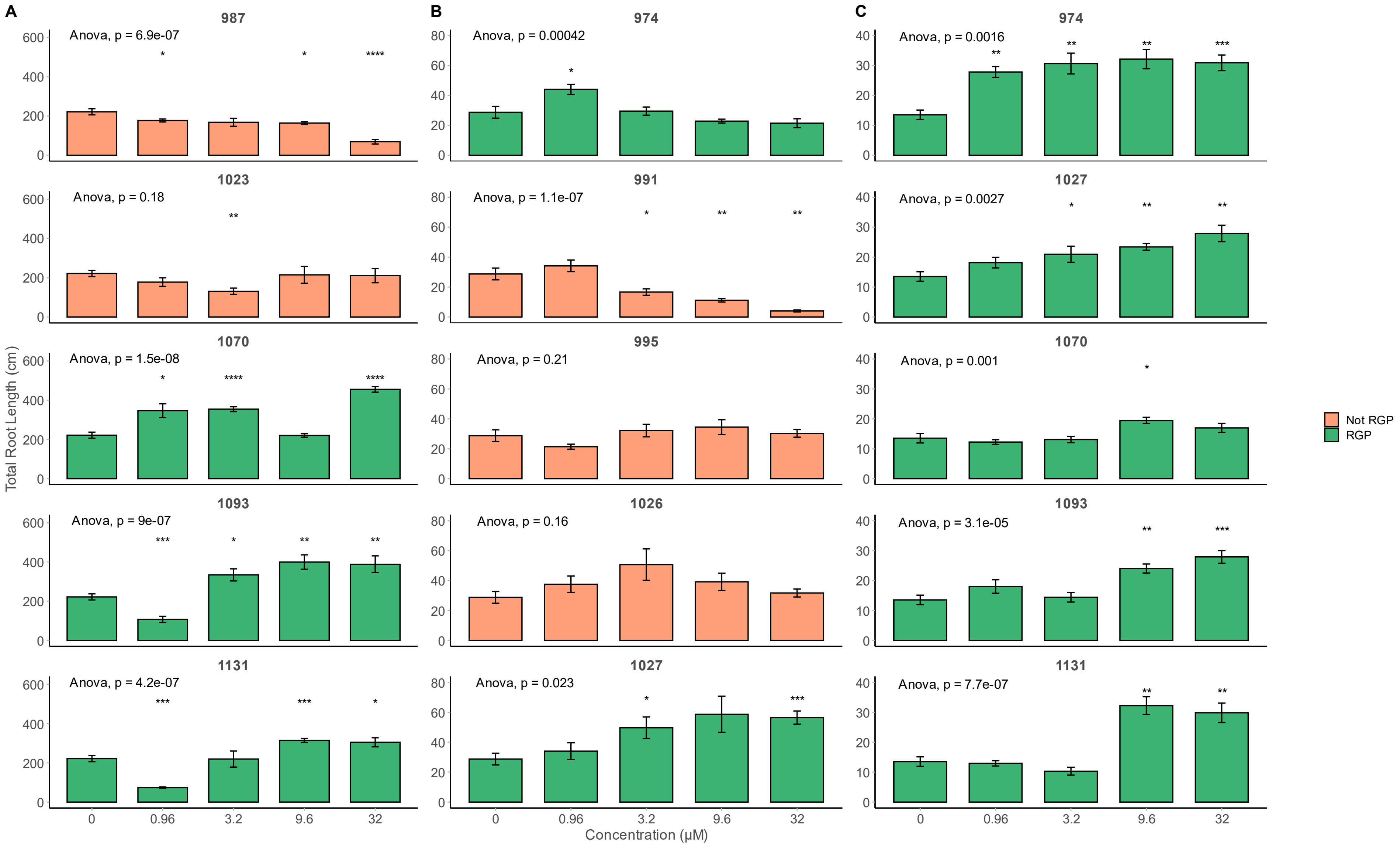
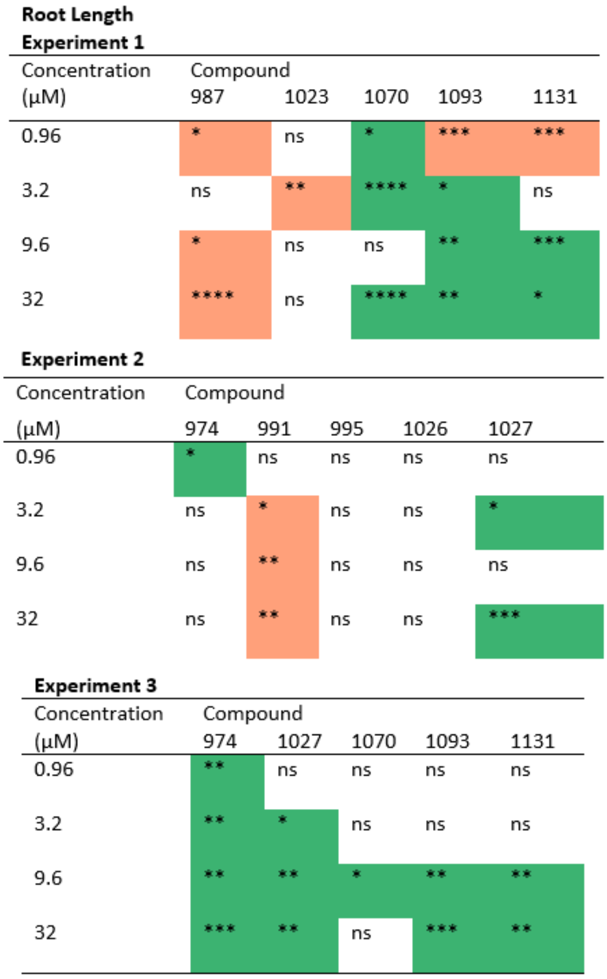


*Figure 2.5. The effects of putative root growth promoting chemicals on the root length of O.sativa seedlings. Experiments were performed in triplicate where quantity of compound provided was sufficient. Panel A and B show data from two different experiments, testing all ten compounds. Panel C shows a third experiment to verify that the root growth promotion in the first two experiments was repeatable. (Data are also shown in Table 2.1). A,B and C correspond with Experiment 1,2 and 3 in Table 2.1. Values are the means +/- SEM (n=6). Statistical significance from the control was determined by independent t-tests: * = p ≤ 0.05, **= p ≤ 0.01, ***= p ≤ 0.001, ****= p ≤ 0.0001.*

*Table 2.1. Results for the independent t-tests for total root length for each concentration of each compound, compared to the control for that experiment. Green shading indicates a significant increase in root length, red shading indicates a significant decrease in root length. Experiments 1, 2 and 3 are the results for each of the three independent repeats. * = p ≤ 0.05, **= p ≤ 0.01, ***= p ≤ 0.001, ****= p ≤ 0.0001.*



Root Length
Experiment 1

Concentration (µM)	Compound 987	1023	1070	1093	1131
0.96	🟥 *	ns	🟩 *	🟥 ***	🟥 ***
3.2	ns	🟥 **	🟩 ****	🟩 *	ns
9.6	🟥 *	ns	ns	🟩 **	🟩 ***
32	🟥 ****	ns	🟩 ****	🟩 **	🟩 *

Experiment 2

Concentration (µM)	Compound 974	991	995	1026	1027
0.96	🟩 *	ns	ns	ns	ns
3.2	ns	🟥 *	ns	ns	🟩 *
9.6	ns	🟥 **	ns	ns	ns
32	ns	🟥 **	ns	ns	🟩 ***

Experiment 3

Concentration (µM)	Compound 974	1027	1070	1093	1131
0.96	🟩 **	ns	ns	ns	ns
3.2	🟩 **	🟩 *	ns	ns	ns
9.6	🟩 **	🟩 **	🟩 *	🟩 **	🟩 **
32	🟩 ***	🟩 **	ns	🟩 ***	🟩 **

2.4.2 Effect of putative root growth promoting compounds on root system architecture

The total root length and the proportion of lateral or primary roots was analysed in response to treatment with each of the 10 compounds. For the compounds that showed RGP activity, lateral roots were more predominant than the control in that category (Appendix Figure 1). Interestingly, a linear relationship was observed between lateral and total root length. The development of lateral roots is an essential component of increasing root length, this is likely due to the fibrous root structure (Meng et al., 2019). This is conveyed in the results, in which having a higher proportion of total root length deriving from lateral roots is strongly positively correlated to increased root length R^2 =0.98. At shorter root lengths, lateral roots account for around half of the total root length, with primary roots accounting for the other half, see Figure 2.6C (correlation coefficient y=+0.49x). At longer root lengths, lateral roots are responsible for most of the total root length (y=+0.93x). (Figure 2.6B). (R^2=0.82 and 0.99, Figure 2.6B and C, respectively). Therefore, lateral roots play a more important role in creating longer roots than primary roots.

Where linear regressions were independently created for each compound, there was no clear discrimination between RGP and non-RGP compounds for the relationship between lateral root length and total root length (Appendix Figure 2 and Appendix Table 1). Despite this, the two highest correlation coefficients, indicating lateral roots being responsible for the greatest amount of total root length, were attributable to the RGP compounds: 1093 and 1070, which were both identified as RGPs in Figure 2.5 (Appendix Figure 2 and Appendix Table 1).

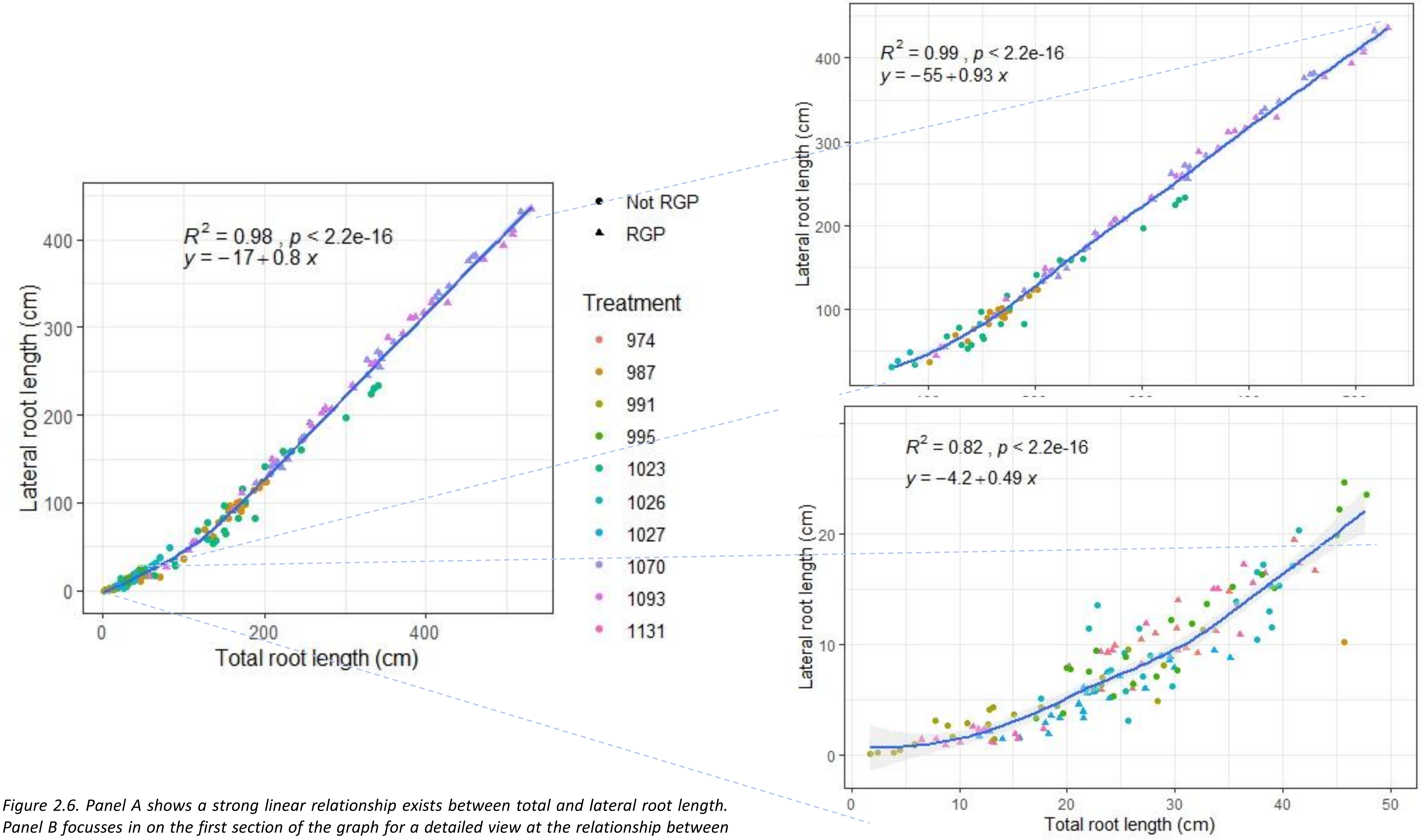


Figure 2.6. Panel A shows a strong linear relationship exists between total and lateral root length. Panel B focusses in on the first section of the graph for a detailed view at the relationship between lateral and total root length. In Panel B, lateral root length is responsible for around half of the total root length, with primary roots responsible for the other half. Panel C shows a focussed in view of the higher values on the graph, lateral root length account for almost all of the total root length. All figures are linear regression loess models with 95% confidence interval.

To assess whether treatments had a significant effect on lateral roots only, or on both primary and lateral roots, an analysis of covariance (ANCOVA) was performed using the ‘car’ package in R. This analyses the impact of the treatment on the total root length, whilst controlling for the effect of primary root length. Primary root length, the covariant, was significantly related to the total root length ($F(1,233)=115$, $p<.001$). There was also a significant effect of the treatment on total root length after controlling for the effect of primary root length ($F(49,233)=20.2$, $p<.001$). When the impact of the treatment on total root length was assessed, whilst controlling for lateral root length, lateral root length was significantly related to the total root length ($F(1,233)=9797$, $p<.001$). There was also a significant effect of the treatment on total root length after controlling for the effect of lateral root length ($F(49,233)=38.5$, $p<.001$).

Interestingly, whilst the treatments that showed significant increases in root length have a greater proportion of their total root length from lateral roots (Appendix Figure 1), the results of the ANCOVA show that increases in total root length are due to increases in both lateral and primary root lengths. Therefore, the increase in total root length due to the active compounds result from increases in lateral and primary roots, and do not affect one root type alone; lateral root length, primary root length and treatment all have a significant effect on total root length.

2.4.3 Effect of putative root growth promoting compounds on root to shoot ratio

Figure 2.7 and Table 2.2 show the effects of the 10 compounds on the root to shoot (R/S ratio) of *O.sativa* seedlings. A R/S ratio lower than 1 indicates that the shoot mass is greater than its root mass. Therefore, where the R/S ratio is lower than the control, this is indicative that the effect of the compound has had a greater positive effect on shoot growth than root growth.

There was a significant difference between R/S ratios of RGP and non-RGP compounds, $F(1,420)=11.72$, $p=<.001$ (ANOVA). The average R/S ratio of compounds that are RGP is 0.521 (SE± 0.0116), whereas for non-RGP the average ratio is 0.582 (SE± 0.0107). Compounds identified as promoting root growth (compounds 974, 1027,1070, 1093 and 1131, Table 2.1), all had R/S ratios lower than the control in Experiment 3. The R/S ratio for the remaining treatments showed no significant differences compared to the control except for 1093 and 1131, 0.96µM (Table 2.2). This suggests that application of the RGP has no inhibitory effect on shoot growth. Therefore, the increase in root length does not come at a cost to shoot growth and there appears to be no negative effect of altered resource allocation from the shoot to the root.

Only three treatments had a R/S ratio significantly greater than the control, compound 991:32μM, 1093:0.96μM and 1131:0.96μM (Table 2.2). The application of these treatments had a detrimental effect on both the root and the shoot (Figure 2.5 and Table 2.1, Figure 2.7 and Table 2.2). Importantly, the negative effect of the compound on the root length (Figure 2.5), and not an increase in shoot growth, was responsible for the significant R/S ratio. Consequently 991:32μM, 1093:0.96μM and 1131:0.96μM were not used in further root growth assays.

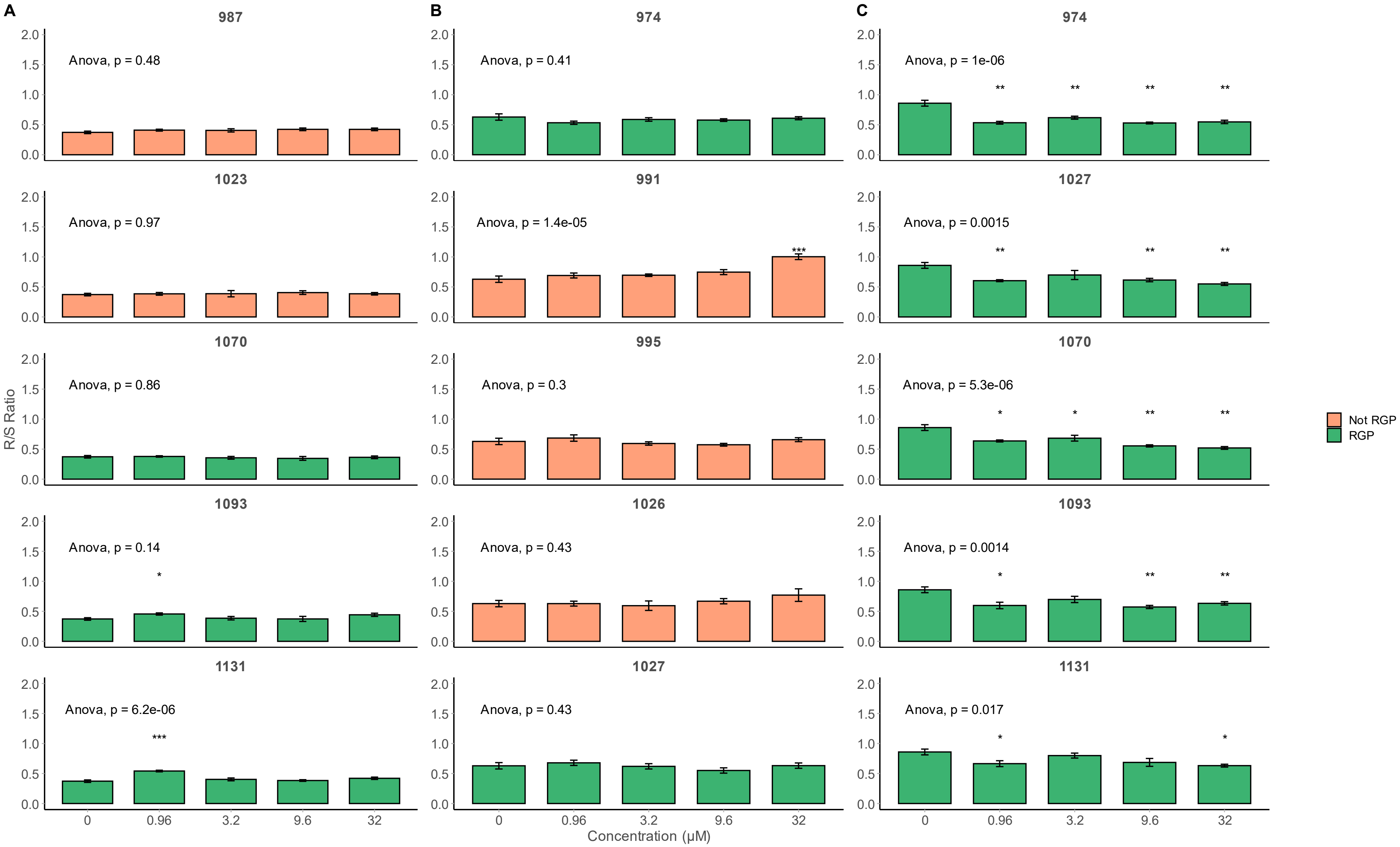


Figure 2.7. Root/shoot ratio of dry mass determined that an increase in root length did not come at a cost to shoot growth. Experiments were repeated three times where quantity of compound provided was sufficient. Panel A and B show data from two different experiments, testing all ten compounds. Panel C shows a third experiment to verify that the root growth promotion in the first two experiments was repeatable (data are also shown in Table 2.1).

*Table 2.2. Results for the independent t-tests for each compound. R/S ratio for seedlings of each concentration of each compound, compared to the control for that experiment. Green shading indicates a significant increase in the R/S ratio, red shading indicates a significant decrease in R/S ratio. Experiments 1, 2 and 3 are the results for each of the three independent repeats. * = p ≤ 0.05, **= p ≤ 0.01, ***= p ≤ 0.001.*

R/S Ratio

Experiment 1

Concentration (µM)	Compound				
	987	1023	1070	1093	1131
0.96	ns	ns	ns	*	***
3.2	ns	ns	ns	ns	ns
9.6	ns	ns	ns	ns	ns
32	ns	ns	ns	ns	ns

Experiment 2

Concentration (µM)	Compound				
	974	991	995	1026	1027
0.96	ns	ns	ns	ns	ns
3.2	ns	ns	ns	ns	ns
9.6	ns	ns	ns	ns	ns
32	ns	***	ns	ns	ns

Experiment 3

Concentration (µM)	Compound				
	974	1027	1070	1093	1131
0.96	**	**	*	*	*
3.2	**	ns	*	ns	ns
9.6	**	**	**	**	ns
32	**	**	**	**	*

2.4.4 Effect of putative root growth promoting compounds on root tips

The impact of the RGP activity of compounds on total root length and the number of root tips was assessed using a two-way ANOVA. There was a significant interaction between total root length and number of tips (*F*(1,418)=888.47, *p*=<.0001); Figure 2.8 and Figure 2.9 both show a positive correlation between the two. However, overall there was no significant effect of whether or not a compound was an RGP on number of tips (*F*(1,418)=2.104, *p*=0.523).

2.4.5 The effect of a compound's RGP activity on the relationship between root traits

The correlation between each parameter of root and shoot growth was measured, and the impact of a compound's RGP activity on this, was visualised by means of a correlogram (Figure 2.8) and scatterplot matrix (Figure 2.9) produced using the 'corrplot' package in R. These reveal a strong positive relationship between root length, area, volume and tips and an inverse relationship between the aforementioned variables and R/S ratio and diameter. A weak positive correlation between R/S ratio and root diameter was observed (see Figure 2.8 and Figure 2.9). Whether or not a compound was an RGP had a significant effect on root diameter (*F*(1,292)=209.16, *p*<.0001). There was a strong negative correlation between root length and diameter (*F*(1,292)=266.15, *p*<.0001, R=-0.76). These results suggest that the resource allocation to make longer roots arises from producing roots with a smaller diameter.

*Table 2.3. Results for the independent t-tests for each compound. Number of tips for seedlings of each compound, compared to the control for that experiment. Green shading indicates a significant increase in the number of tips, red shading indicates a significant decrease in the number of tips. Experiments 1, 2 and 3 are the results for each of the three independent repeats. * = p ≤ 0.05, **= p ≤ 0.01, ***= p ≤ 0.001.*

Tips
Experiment 1

Concentration (µM)	Compound 987	1023	1070	1093	1131
0.96	ns	ns	*	ns	ns
3.2	ns	ns	**	ns	ns
9.6	ns	ns	**	*	ns
32	**	ns	**	ns	ns

Experiment 2

Concentration (µM)	Compound 974	991	995	1026	1027		
0.96	ns	ns	ns	ns	ns		
3.2	ns	ns	ns	ns	*		
9.6	ns	*	ns	ns	ns		
32			ns	*	ns	ns	**

Experiment 3

Concentration (µM)	Compound 974	1027	1070	1093	1131
0.96	*	ns	ns	ns	ns
3.2	*	ns	ns	ns	ns
9.6	*	**	**	**	**
32	**	**	**	***	***

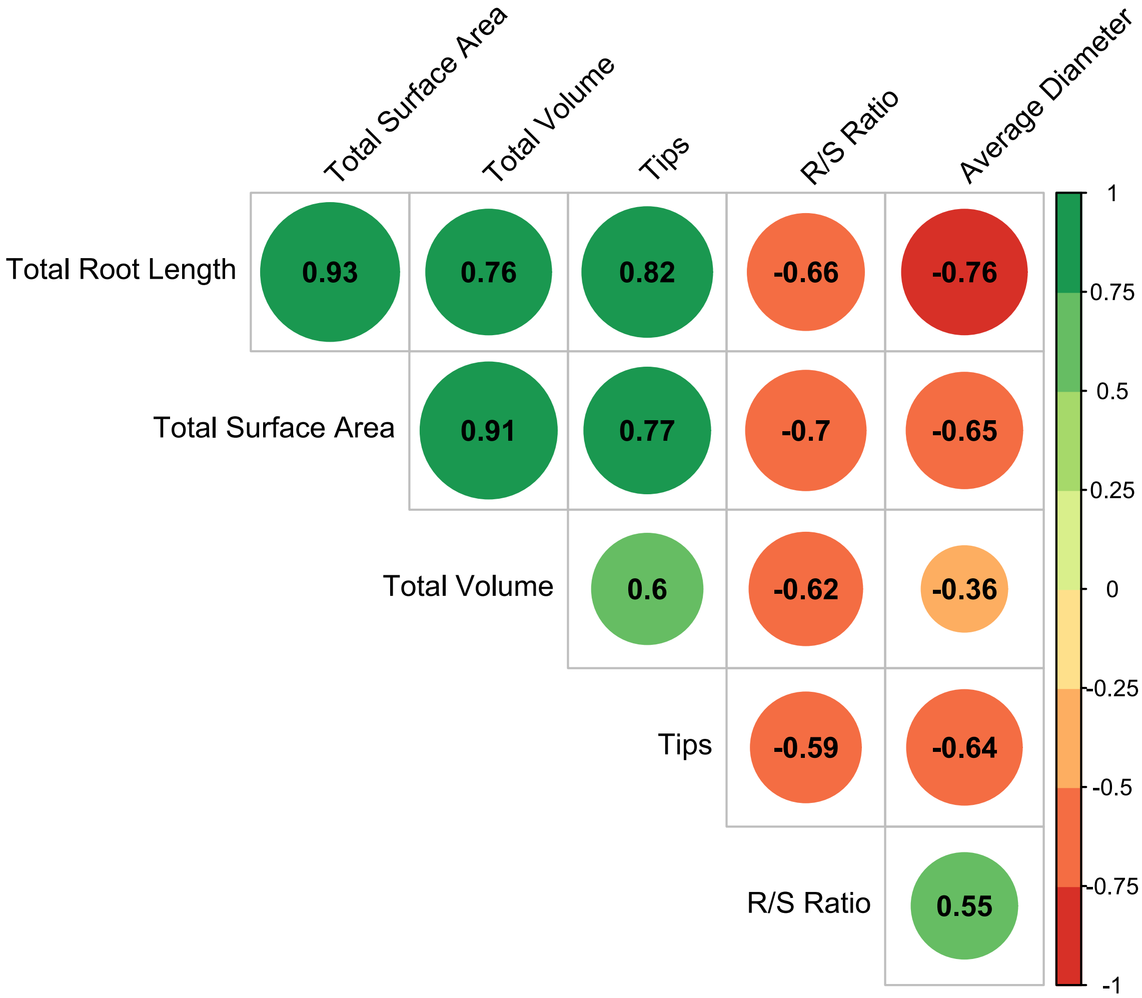


Figure 2.8. There is a strong positive relationship between root length, area, volume and tips and an inverse relationship between the variables and R/S ratio and diameter. Circle size corresponds to the correlation coefficient (R) value, the value within each circle is the R value. All values are statistically significant (p<0.05). Green colour indicates a positive correlation whilst red indicates a negative correlation.

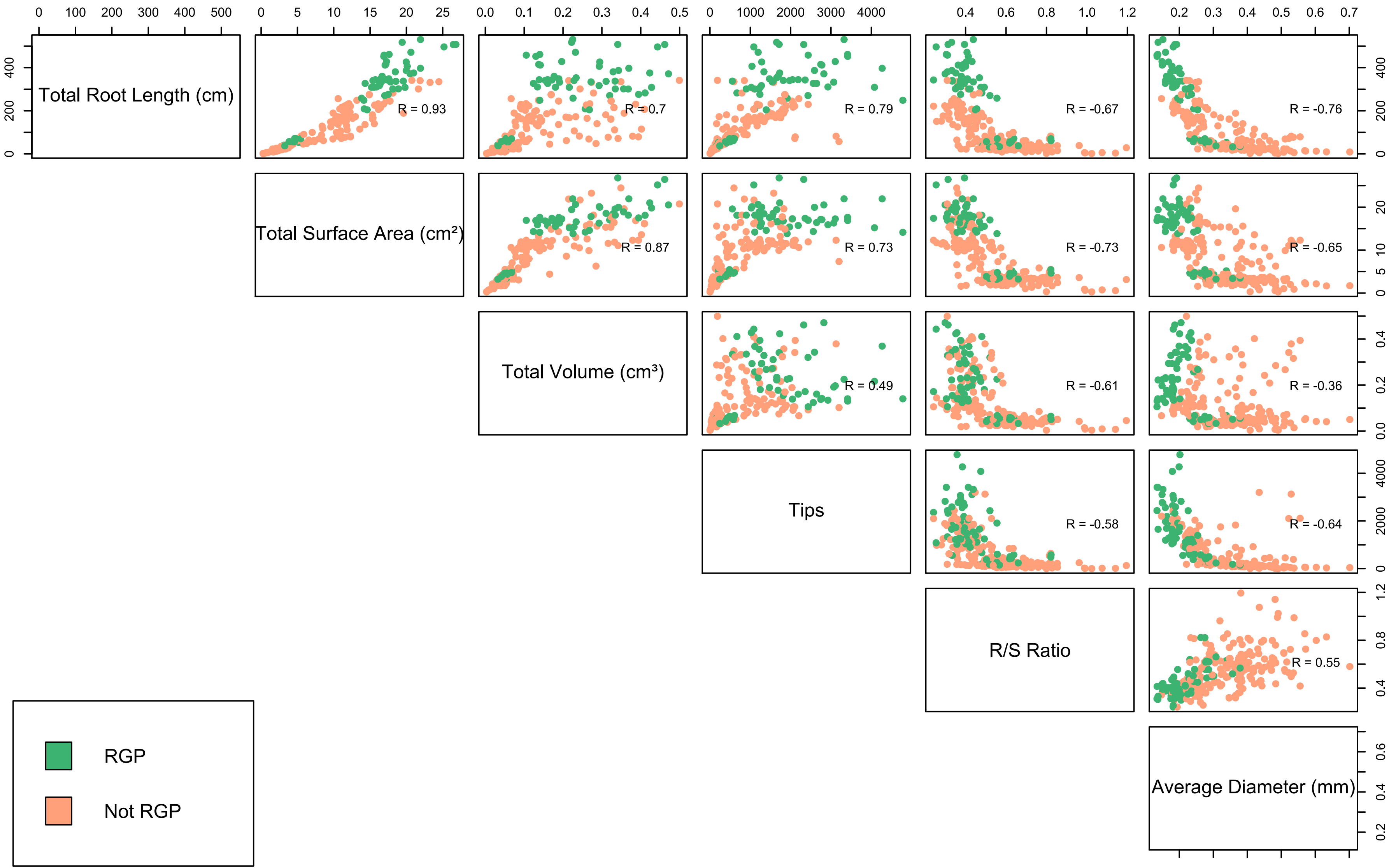


Figure 2.9. The scatterplot matrix shows the positive relationship between root length, surface area, volume and tips and a negative relationship between the aforementioned variables and R/S ratio and root diameter. Values from RGP and non-RGP compounds are in green and orange respectively.

2.4.6 Using machine learning for agrochemical discovery

The agrochemical discovery process involves screening chemical libraries in high-throughput assays (Fozard and Forde, 2018), a process which is costly and laborious (Burrell et al., 2017, Smith, 2003). The use of machine learning to predict the likelihood of a compound being biologically active before performing lab experiments has been used for pharmaceutical drug discovery (Lavecchia, 2015, Vamathevan et al., 2019) and, more recently, herbicide discovery (Oršolić et al., 2021). Its potential use for RGP discovery in this study was carried out by using principal component analysis (PCA). PCA is an unsupervised machine learning technique and classification designed to reduce the dimensionality of multivariate data, enabling both comparative data visualisation and exploratory data analysis with minimal loss of information. The total data capture for each dimension of the PCA for each variable is shown in Table 2.4. 71% of the overall data variance is captured in dimensions 1 and 2 (Dim1 and Dim2).

Table 2.4. The calculated Eigenvalues measure the amount of variation retained by each principal component. The first principal component (Dim1) is the largest and retains the maximum variation in the dataset.

	Dim1 (%)	Dim2 (%)	Total (%)
log P	91.2	33.8	95
log S	76.0	0.7	76.7
pKa	6.4	0.4	6.8
pKa (Basic)	29.2	45.9	75.1
CNS MPO score	92.0	1.9	93.9
Topological polar surface area (Ã…Â²)	38.6	22.0	60.6
Mass (g/mol)	19.6	68.5	88.1
Fsp3	2.6	48.1	50.7
Heavy atom count	14.2	80.4	94.6

PCA can also be used to predict categories; in this case, the likelihood of a compound being an RGP or not. Data for the ten compounds were split into training and validation datasets with a 60:40 split. The following chemical properties were inputted into the PCA, which became predictor variables: log P, log S, pKa, pKa (Basic), CNS MPO score, topological polar surface area (Ã…Â²), mass (g/mol), Fsp3, and heavy atom count. When doing PCA analysis, the grouping variables, in this case whether a compound was an RGP or not, are not inputted into the model. Using the training dataset, the PCA clustered RGPs and non-RGPs correctly without ‘knowledge’ of which compound were which. The four compounds used for the validation data set were 50% RGPs (compound 1027 and 974, points 9 and 10 in Figure 10) and 50% non-RGPs (compounds 991 and 987, points 7 and 8 in Figure 10). The PCA correctly clustered compounds 991 and 987 near to the other non-RGPs, and compounds 1027 and 974 near to the RGPs. The close proximity indicates a likelihood of belonging to the same cluster.

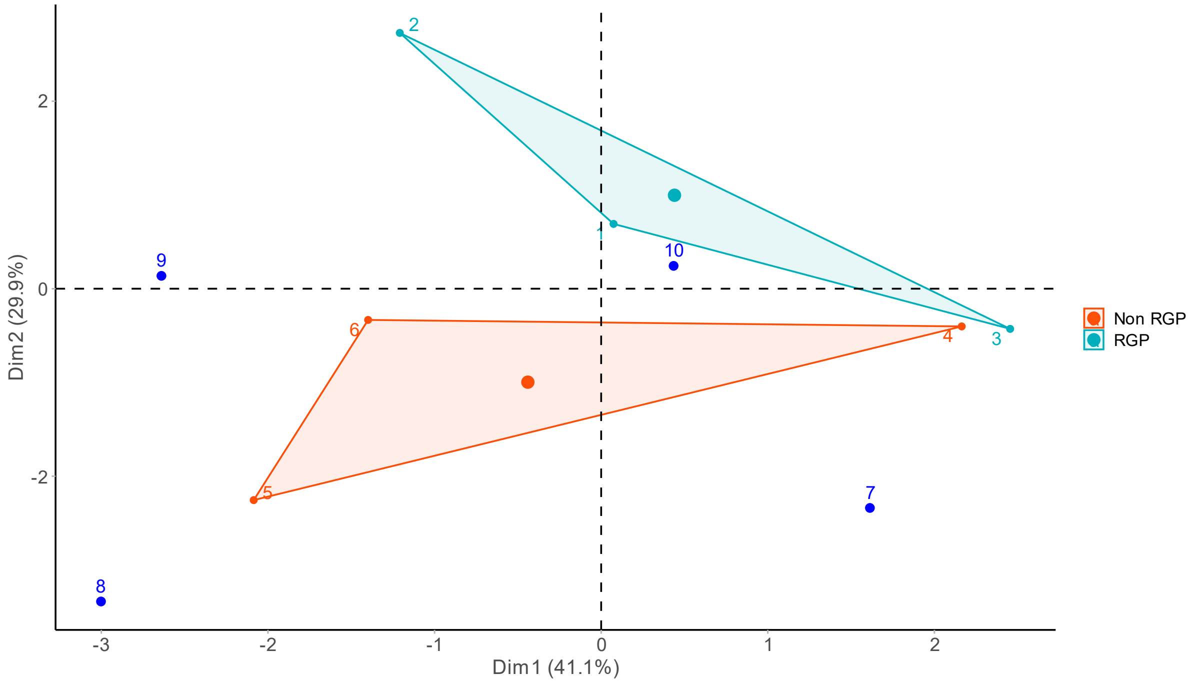


Figure 2.10. The PCA correctly grouped RGP and non-RGP compounds used in this study, inputting only chemical structure data and no experimental results. Using the training dataset, the PCA correctly grouped compounds 991 and 987 (points 7 and 8) with the non RGPs and compound 974 and 1027 (points 9 and 10) near to the known-RGPs. The first principal component captures most of the variance (41.1%), with 71% of the total variance being retained in this PCA.

Of the 16 compounds that were initially provided by Globachem Discovery Ltd, only 10 were tested due to low quantities or being unable to get the compound to stay in solution. Using the predictive capacity of PCA, the chemical data for the remaining six compounds were inputted to establish whether they shared the chemical traits of the known RGPs. Figure 2.11 shows the results for the predictive PCA. The chemicals representing points 7-11 have chemical similarities to the known RGPs, whereas point 12 shares few and is more unlikely to have RGP properties. Point 9 and 10 share the most similar chemical traits and could therefore be considered most likely to behave as an RGP.

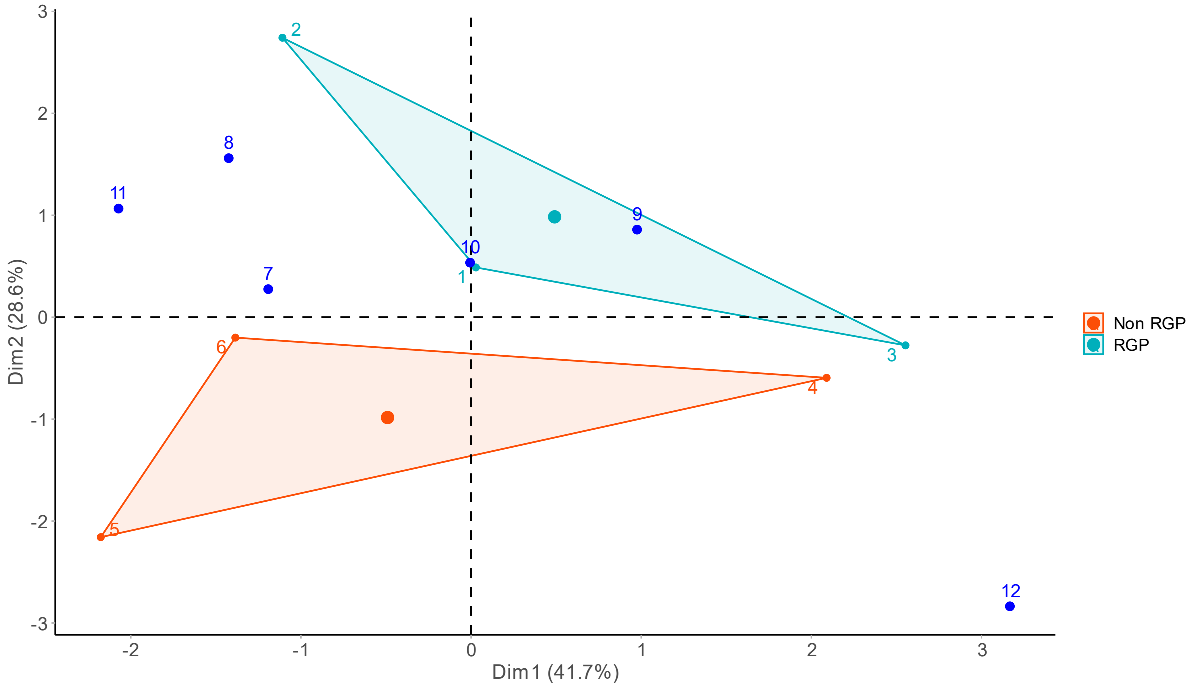


Figure 2.11. The PCA predictive data suggests that 5 compounds (points 7-11) may behave as RGPs, with points 9 and 10 sharing many chemical similarities to the RGPs. Point 12 is not likely to be an RGP as it is chemically dissimilar to the RGPs. Points 7-10 in this figure are different compounds to 7-10 in Figure 11.

2.5 Discussion

2.5.1 Root growth promoting compounds increased the length of both primary and lateral roots

It is clear that techniques for modifying the root structure of crops plants can contribute to achieving different ideotypes, such as improved root lodging resistance or enhanced nutrient acquisition (Meister et al., 2014). The preliminary screening of the Globachem chemical library presented in this work revealed four candidate compounds (1027, 1070, 1093 and 1131) that promoted root growth, in addition to the patented parent compound (974) (Figure 2.5 and Table 2.2). There was a significant interaction between treatment and lateral and primary roots independently ($p<0.001$), showing that increases in root length are attributed to increases in both primary and lateral root lengths, not just one root type alone, and that application of the novel RPG compounds has the potential to increase rooting depth and branching. The resultant increased surface area and anchoring ability conferred to plants by longer roots, in addition to enhanced access to the water table, gives plants a competitive advantage particularly during periods of drought stress and reduced nutrient availability (Maeght et al., 2013, Gamuyao et al., 2012). Deep roots generally confer an advantage to plants growing in water limited soils, and may be particularly advantageous for crops with a shallow root system, such as rice, to facilitate water acquisition when the water table is low (Comas et al., 2013).

Previous studies have analysed abundance, spatial distribution and soil contact of wheat roots in order to determine whether incomplete extraction of water was due to limitations of the root system (White and Kirkegaard, 2010). They concluded that increased root-soil contact by denser root hairs or increased root proliferation would likely increase water uptake from soil pores. The increases in lateral roots and tips as a result of application of the novel compounds in this study is a form of root proliferation and could therefore contribute to increased water uptake capacity. In rice, drought sensitivity is at its height during the reproductive stage, during seed set and grain filling (Zhang et al., 2018). During these periods, access to the water table becomes crucial and as such, efforts to make better use of soil water in the form of trait selection for deeper roots, established prior to the onset of stress, could help to increase yields. Additionally, in situations where the subsoil is unused by the plants, which is likely to occur more frequently in plants with shallow roots such as rice, the stagnant subsoil water has been found to exacerbate drainage and salinity issues (White and Kirkegaard, 2010). The incentive to improve resource acquisition from the soil is therefore twofold: to increase resilience to abiotic stresses by using deep water resources and to improve drainage and salinity difficulties, eliminating the occurrence of another abiotic stress before it has a detrimental effect on plant development.

The effects of different growing medium on root growth is well documented (Kuijken et al., 2015). The homogenous hydroponic medium alters root morphology when compared to growth in soil. There is less of a tendency towards deep-angled roots, as water is readily available and hydrotropic effects on the root are absent. Despite the modification to root growth, it is widely accepted that hydroponic systems offer benefits that outweigh the negatives associated with hydroponic research (Nguyen et al., 2016b). However, the possibly cannot be excluded that the increased number of tips and increased branching observed in this experiment (Table 2.3) could in part be due to the growth in hydroponics. Nevertheless, by comparing each treatment to the control as in this study, a meaningful comparison on the effect of treatment can be drawn.

2.5.2 Root growth promoting compounds do not affect root system architecture at the expense of shoots

Changes in the R/S ratio of plants is indicative of whether an increase in root length is achieved at a cost to the shoot. Importantly when modifying root system architecture with the aim of improving crop productivity and resource use efficiency, the shoot should not be negatively affected by the modification of the root system architecture (Pospíšilová et al., 2016, Mrízová et al., 2013). An increase in R/S ratio results in leaves having more non-photosynthetic tissue to sustain, reducing overall plant growth (Lynch et al., 2014). In this study, increases in root length were complemented by a decrease in R/S ratio (Table 2.1 and Table 2.2) indicating the observed root increase came at no cost to shoot growth. In some cases, an increase in root mass was complemented by an increase in shoot mass (Table 2.1 and Table 2.2, Experiment 3), indicating that the overall plant development benefited from compound application. However, there were some inconsistencies between the effects of compounds on R/S ratio (Table 2.2), therefore, additional studies in this area are required before firm conclusions are drawn.

Interestingly, the Arabidopsis *ahk2 ahk3* loss of function CK receptor double mutant exhibits an enhanced root system with more rapid growth of primary root and increased branching of lateral roots, with opposing regulatory functions in roots and shoots (Riefler et al., 2006), similar to that observed with the RGP compounds identified in Table 2.2. However, they also displayed reduced chlorophyll content and CK-dependent inhibition of dark-induced chlorophyll loss. This indicates that these receptors play an important role in leaf development. If the RGP compounds identified in Table 2.2 are acting as CK antagonists, thereby affecting development in a similar manner to the CK receptor mutants (Riefler et al., 2006), it would be expected that the growth of shoots would have been adversely affected resulting in a higher R/S ratio in Figure 2.7. The disparity in effect on shoot mass, however, suggests that the RGP compounds may have a root-specific mechanism of action, similar to the parent compound (974) had in Arata et al. (2010).

2.5.3 Increasing root length has a negative effect on root diameter

This study shows that treatment with Globachem's compounds had no effect on the number of root tips overall (p=0.523) irrespective of the compound's PGR activity, although plants with longers roots did have significantly more tips (p<.0001). Therefore, RGP treatment increased root length and increased the number of roots overall. In addition to increasing root length, the ability to penetrate hard or deep soil, is a facet of a crop's roots system that increases a plants' access to water and makes plants less susceptible to drought (Samson et al., 2002). This ability correlates strongly with root diameter; thicker roots are less likely to buckle or deflect when in hard soil (Lynch et al., 2014). The compounds that were identified as promoting root growth in this chapter showed a negative correlation (R = -0.76) between root length and root diameter (p<0.0001). Whilst this may be the result of growth in a hydroponic medium (Kuijken et al., 2015), further research into the effects of the RGP compounds on mechanical impedance is required.

2.5.4 Unsupervised machine learning can be used to predict whether a compound is likely to promote root growth

Using machine learning to predict whether a compound was likely to be an RGP or not using only its chemical data allowed the correct differentiation between RGP and non-RGP, despite the small training dataset used for the PCA (Section 2.4.6). Experimental results from this chapter were used as a validation dataset to check the extent to which the model could correctly predict RGP activity. The PCA correctly predicted the regions of the plot that the validation data belonged in; two RGPs and two non-RGP were split into the correct regions of the plot (Figure 2.10). This highlights the opportunity to use machine learning for PGR discovery purposes, in a similar way to which it has been used in the pharmaceutical industry (Vamathevan et al., 2019, Lavecchia, 2015) and for herbicide discovery (Oršolić et al., 2021). Utilising such a method would allow screening of large chemical libraries in minutes, unlike the time-consuming and costly processes currently involved in product discovery.

Using the predictive PCA to analyse the chemical structure data of the six compounds that were unable to be tested, two (points 9 and 10) showed strong positive correlation, and hence strong chemical similarities to the known RGPs. Points 7, 8 and 11 also clustered towards the known RGPs, suggesting further analysis in this area could be beneficial (Figure 2.11). By using machine learning to assess the likelihood that a compound will exhibit RGP activity, based on data acquired from an initial small, lab-based study, there is potential for the financial and time investment of discovery of PGRs to be significantly reduced (Oršolić et al., 2021).

2.5.5 Conclusion

Four novel compounds (1027, 1070, 1093 and 1131), in addition to the parent compound (974), have been established as promoting root growth of rice seedlings, at no cost to shoot growth. Root growth was attributable to an increase in the length of both the primary and lateral roots and an increase in the total number of roots. Whilst this yields promising results for production of a marketable RGP, several avenues of research need to be developed in order to transfer this knowledge into a commercially relevant application. Firstly, a more practical and efficient technique for chemical application, such as seed priming, is required. Secondly, whether the activity of the compound is cultivar-specific needs to be established. Thirdly, whilst the compounds identified as having root growth promoting properties offer a new avenue of exploration in order to achieve sustainable intensification of crop yields, the compounds would offer additional commercial and environmental benefits if they also increased plant resilience to some of the major environmental pressures that are encountered in agriculture, such as drought and salinity stress. These issues are examined in Chapter

3, where the efficacy of the compounds by priming of different cultivars and their resilience to salt and drought stresses will be explored.

3 The Effects of Seed Priming with RGPs on the Growth of Rice Varieties Under Controlled and Commercially Relevant Conditions

3.1 Introduction

Global food production and security are limited by the effects of abiotic stress on crops, which result in vast yield losses (Boyer, 1982, Mittler, 2006). Whilst breeding technologies go some way to mitigating these losses (Xu et al., 2006, Tester and Langridge, 2010), these research efforts can be complemented by techniques that prime plants' tolerance to stress (Rhaman et al., 2021, Borges et al., 2014), such as the root growth promoters (RGPs) identified in Chapter 2.

3.1.1 Seed priming

Plants can be primed against stress by applying a physical pre-germinative treatment such as heat, cold or ultraviolet (Lutts, 2016). Another common method of priming involves soaking the seed in water, osmotic agents, hormones or chemicals (Savvides et al., 2016). An array of chemical or biological priming agents, such as water, sugars, hormones, rhizobacteria and inorganic salts can contribute to increased agricultural productivity (Sher et al., 2019). These treatments prime against abiotic stress such as low temperature (Hussain et al., 2016), salinity (Afzal et al., 2012), drought (Goswami et al., 2013) and some biotic stresses (Joe et al., 2012, Reddy, 2013) in rice by generating a faster and/or stronger response against a stress (Lutts, 2016). In addition, seed priming promotes uniform seedling emergence, high germination rate and improved seed vigour (Lutts, 2016) and can also improve crop productivity, such as grain yield (Paparella et al., 2015).

Seed priming has advantages over other methods to improve stress tolerance, such as conventional breeding, which is slow, and transgenic crops, which are met with resistance in some countries (Savvides et al., 2016). Priming treatments that target improvements in root size are commercially available but currently have high species-specificity (Paparella et al., 2015). The use of seed priming to alleviate the impact of abiotic stress is a key research area, though knowledge-gaps and opportunities for development still remain (Amritha et al., 2021, Borges et al., 2014, Wargent et al., 2013). The novel RGPs discovered in Chapter 2 therefore provide opportunities for investigating the efficacy of these compounds as growth regulators and their ability to prime a plant against stress.

3.1.2 The effect of salt and drought stress on rice productivity

Throughout Asia there are large differences in rice yield between irrigated and rainfed areas (Fukai et al., 1999). Many of the rice-producing regions globally are dependent on rainfall (Heap et al., 2021), hence, the productivity of these regions is severely limited by water availability. Yields in rainfed lowland regions are 50% lower than in irrigated areas, whilst the yields in rainfed upland regions are 75% lower than in irrigated areas (Wade et al., 1999). In addition to water-accessibility, salinity is a major limiting factor to agriculture. It is anticipated that 50% of land suitable for cultivation will be affected by salt by 2050 (Mahajan and Tuteja, 2005). Soil salinity is affected by the precipitation rate, a high rate lowers salinity, and evaporation, which increases salinity (Mahajan and Tuteja, 2005). In arid areas, 48% of irrigation water is lost by evaporation, leading to an accumulation of sodium ions (Na^+) which increases over time (Mahajan and Tuteja, 2005, Russo and Callegarin, 1997). Rice is a glycophyte, a plant with high salt sensitivity, yet it grows in areas requiring irrigation and, consequently, is prone to salinization (Horie et al., 2012). These arid areas that are prone to salt stress from irrigation, are otherwise prone to drought stress from lack of water input (Horie et al., 2012). Hence, there is great scope for improving yields of water-limited and salinity-prone rice growing regions. Additionally, the water requirements of rice fields represent a significant financial cost to farmers, as well as there being increasing concerns over water quality from pollution (Russo and Callegarin, 1997). Therefore, a method to reduce the need for irrigation input, alongside reducing the negative effects of salinity on rice growth, would be highly beneficial to farmers.

Salt-tolerant plants minimise accumulation of Na^+ in shoots by excluding Na^+ from the cytosol and sequestering it into the vacuole (Singh et al., 2018). The mechanisms of salt and drought stress and responses they elicit are somewhat similar (Horie et al., 2012). Both alter the osmotic balance by decreasing the water potential gradient between the soil and the root, limiting water availability for plants, and ultimately resulting in dehydration of cells (Horie et al., 2012). Salt and drought stress results in similar physiological conditions within a plant, increasing production of ROS, reducing photosynthesis, and disrupting some enzyme functions and cell metabolism (Mahajan and Tuteja, 2005). These responses lead to inhibition of growth and sometimes death of the cell or plant (Figure 1) (Singh et al., 2018).

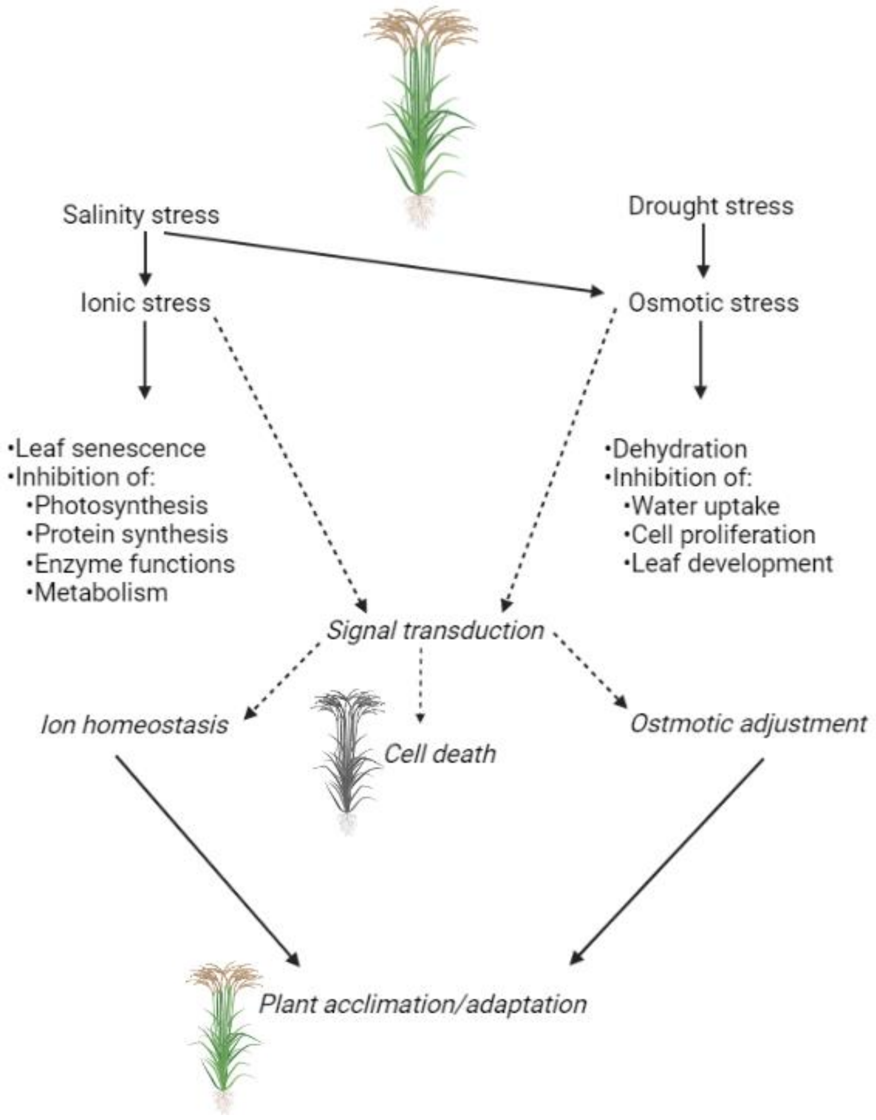


Figure 3.1. Plant and drought stress exert similar effect and initiate some similar responses within a plant. Salt stress exerts an ionic stress in addition to an osmotic stress. A summary of the effects of salt and drought stress on a plant, and the plant response to survive the stress are shown. Image created in BioRender and adapted from Horie et al. (2012).

3.1.3 Cytokinins play a crucial role in stress-response signalling

CKs are plant hormones that are principally involved in developmental processes such as shoot and root growth (Werner et al., 2008). They are also involved in responses to external stimuli, such as light conditions in the shoot and water and nutrient availability in the root, they therefore have an important role in the response to abiotic stress (Werner and Schmülling, 2009). CKs regulate cell proliferation and senescence; an abundance of CKs in the shoot delays leaf senescence (Gan and Amasino, 1995), promotes growth of the shoot apical meristem (Werner and Schmülling, 2009, Talla et al., 2016), and increases grain size and/or quantity (Ashikari et al., 2005, Jameson and Song, 2015, Yin et al., 2020). In the roots, CKs are involved with lateral root positioning, root development and prevention of overcrowding (Laplaze et al., 2007b). Typically, CK levels in the roots are too high for maximal root growth (Julkowska, 2018). In addition, when CK levels are artificially increased, there is an inhibition of root formation and branching (Laplaze et al., 2007b). Conversely, when CK levels are artificially decreased there is an increase in root growth (Ramireddy et al., 2018).

3.1.4 Crosstalk within cytokinin signalling networks

CKs form part of complex signalling networks involving other plant hormones, enzymes (namely: adenosine phosphate-isopentenyltransferases (*IPT*s) and CK oxidases/dehydrogenases (CKX)), sensor histidine kinases (*HK*s), histidine phosphotransfer proteins (*HP*s) and response regulators (*RR*s). Abiotic stress signalling and tolerance are mediated by these signalling networks, and there is strong evidence in support of crosstalk existing between CKs, ABA and stress signalling pathways (Nishiyama et al., 2011).

The complexity of the signalling network is in part due to CKs pleiotropic nature, in addition to gene expression for synthesis and metabolism of CKs belonging to multiple gene families, which themselves are spatially and temporally diverse (Jameson and Song, 2015). The effects of CK in plants are heavily dependent on external conditions (Pavlů et al., 2018), plant species, age of the seed and site of activity within the plant (Werner et al., 2001). In addition, signalling molecules have been found to have positive or negative regulatory roles, depending on the abundance of CK (Nishiyama et al., 2011). For example, histidine kinases are transmembrane proteins that play a role in signal transduction across cell membranes (Wolanin et al., 2002). In Arabidopsis, Arabidopsis histidine kinase 4 (AHK4) has a dual function: phosphorylating Arabidopsis histidine phosphotransfer proteins (AHPs) when CK is present, and dephosphorylating them in the absence of CK. When CK is present, AHK4 acts as a negative regulator in stress responses. Arabidopsis histidine kinase 2, 3 and 4 (AHK2, AHK3 and AHK4) are also negative regulators of abscisic acid (ABA) signalling and osmotic stress signalling, both of which involve cross-talk with CK signalling (Nishiyama et al., 2011).

Rice responds to water-limiting conditions by modulating CK biosynthesis or signalling, resulting in reduced CK activity (Todaka et al., 2017, Maruyama et al., 2014, Kim et al., 2020b). The decrease in plant productivity in response to drought is, in part, due to this decrease in CK levels that occurs at the onset of stress (Pavlů et al., 2018). CK synthesis and signalling has also been shown to be a key limiting factor for photosynthesis and above-ground function during salt stress (Yin et al., 2020, Wang et al., 2019, Joshi et al., 2018) and drought stress (Peleg et al., 2011). Overexpression of CK biosynthesis enzymes is detrimental to the roots, resulting in a decrease in root biomass in control conditions (Ghanem et al., 2010). However, increased CK biosynthesis results in improved plant longevity, delayed stomatal closure and leaf senescence, and almost double the shoot biomass (Ghanem et al., 2010). In addition, several key factors in the alleviation of salt and drought stress, such as the maintenance of a plant's ionic and osmotic equilibrium, the control of oxidative damage by antioxidant signalling, and signalling to coordinate cell division, require the action of CK (Mahajan and Tuteja, 2005).

The reduction of CK can be achieved via modulation of CK metabolism or the regulation of CK receptor expression and/or negative regulation of CK signalling by Arabidopsis histidine phosphotransfer proteins (AHP6) and Arabidopsis response regulator 5 (ARR5) (Pavlů et al., 2018). Artificial modulation of CK abundance and signalling can improve drought and salt tolerance by mechanisms such as: protecting photosynthetic processes, boosting antioxidant systems, modulating plant growth or by modulating the balance between other phytohormones, for example ABA, that are also involved in stress signalling (Pavlů et al., 2018). Typically, these modifications are a result of increased CK levels in the leaves which increases aboveground biomass (Pavlů et al., 2018, Peleg et al., 2011, Joshi et al., 2018, Wang et al., 2019, Yin et al., 2020).

Importantly, however, an abundance of CK in the roots leads to an inhibition of root growth (Julkowska, 2018). Research examining the effects of CK in roots has found that lowering CK levels in the roots by overexpressing cytokinin dehydrogenase (CKX), a CK degradation enzyme, increases the number and length of lateral roots, whilst improving drought tolerance and post-drought recovery in barley (Ramireddy et al., 2018, Pospíšilová et al., 2016). The higher tolerance to drought stress was mostly caused by altered root morphology resulting in better dehydration avoidance (Pospíšilová et al., 2016). Therefore, whether CK abundance is high or low and whether the associated signalling is beneficial or not differs with respect to the aerial and below ground biomass accumulation.

Given the differential impacts of CK on the growth of the shoots and roots of plants (Werner and Schmülling, 2009), it is important to be able to target the effects of CKs within a plant for agricultural and plant breeding purposes. Although a challenging task, there are instances where this has been achieved successfully for above or below ground specific CK manipulation. For example, Ashikari et al. (2005) reduced the expression of the *Gn1a* gene which encodes for CK dehydrogenase synthesis, an enzyme that degrades CK, resulting in increased CK accumulation. The increased CK accumulation resulted in an increase in the grain number per plant and therefore an increase in yield and benefits to the aerial part of the plant. Root-specific reduction of CK, resulting in enhanced root growth, has to-date been achieved in maize (Ramireddy et al., 2018), oilseed rape (Nehnevajova et al., 2019), Arabidopsis and tobacco (Werner et al., 2010).

3.2 Aims and Objectives

The aim of this chapter is to investigate the potential of the parent compound and four novel RGPs studied in Chapter 2 to prime a seedling against stress in a commercially relevant application process and setting. Specially, this chapter will achieve this aim by:

1. Investigating whether the novel RGPs from Chapter 2 are as effective at promoting root growth when applied as a seed priming treatment, rather than applied to the root via liquid media.
2. Determining if priming with the compounds confers any benefits to seedlings under drought or salt stress.
3. Determining if the benefits conferred in Chapter 2 are as effective at promoting root growth in soil as they are in liquid media.

3.3 Methods

3.3.1 Laboratory priming methods

3.3.1.1 Chemical preparation

The RGP chemicals used were obtained from Globachem Discovery Ltd. and were derivatives of a 4-phenyl quinazoline structure, as in Arata et al (2010), molecular formula: $C_{17}H_{16}ClN_3O$. A 25.5mM stock solution was prepared in 1:4 DMSO:acetone and diluted with sterile water accordingly.

3.3.1.2 Assay preparation

Rice seeds (*Oryza sativa* cv Nipponbare) were sterilised using 100% ethanol for 2min and 20% (v/v) sodium hypochlorite solution for 10min, then rinsed 6 times with sterile water. Seed priming treatments were applied by shaking the seeds in a test tube placed on a shaker with a final concentration of 0.96, 3.2, 9.6, 32 and 96µM of the RGPs for 3.5h The length of time seeds were exposed to the RGPs was determined by a preliminary experiment in which the time for seed saturation to be achieved with water was calculated (Appendix Figure 3). Seeds were then placed on moist filter paper on a Petri dish, sealed with 3M micropore tape and placed in the dark in a controlled environment at 30°C:26°C (11h:13h day:night) for 48h.

3.3.1.3 Plant and growth conditions

Seeds were removed from the dark and transferred to a controlled environment at 30°C:26°C (11h:13h day:night) to allow germination. Five days after the seed treatment, percentage germination, root length and shoot length were measured. The seed vigour index (SVI) is an indicator of a seed's potential to perform well during germination and emergence and was calculated according to Abdul-Baki and Anderson (1973) using the following equation, where RL is root length, SL is shoot length and GP is germination percentage:

$$SVI = RL + SL \times GP$$

3.3.2 Commercial setting priming method

3.3.2.1 Chemical and assay preparation

Chemical preparation and assay preparation were the same as in Section 3.3.1.

3.3.2.2 Plant and growth conditions

Rice seeds (*O. sativa* cv Loto and Selenio) were soaked in the previously identified optimum concentration from Figure 3.6 for each RGP compound for 3.5h. Primed seeds were sown in trays in a 4x4 arrangement containing 50% peat, 50% perlite (v/v) (Figure 3.2) and grown in either a glasshouse or growth chamber for 13 days. Glasshouse conditions had a temperature range of 22.5-34.1°C and relative humidity range of 23-97.2%. Growth chambers assays were carried out in a Snijder growth chamber, 30°C:26°C (11h:13h day:night). The conditions of each were reflective of the commercially relevant growing environments at Globachem.

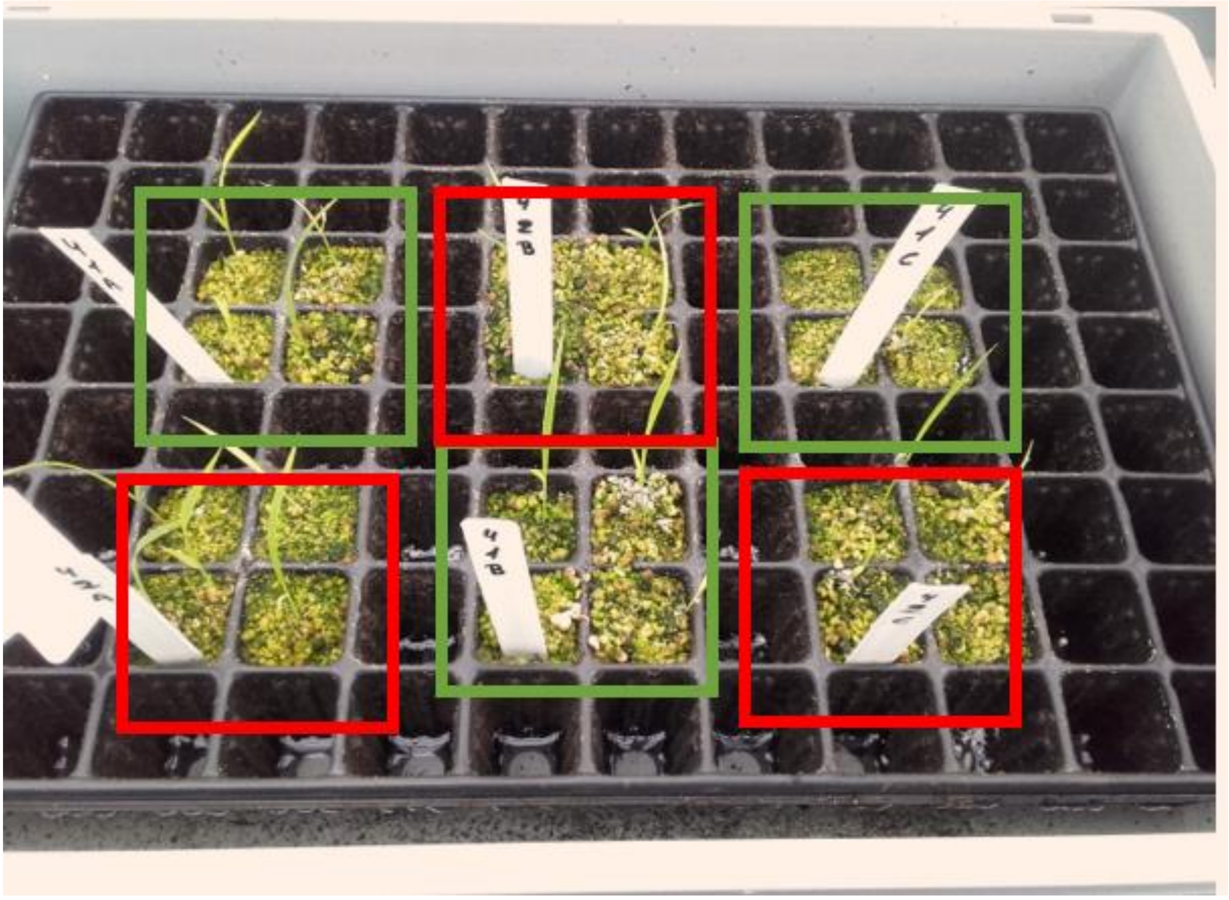


Figure 3.2. Experimental design for assays with and without the parent compound, compound 974. Green boxes are control plants and red have compound 974 applied. Salt or drought treatments were applied to the base to ensure even application.

For the drought treatment all seeds were well watered (water always in the base) until germination. Trays in the glasshouse then had the following treatments applied: water always in the base (control (C)), watered three times per week (mild), water withheld from when the shoot was 4cm (moderate) or water withheld from when the shoot was 2cm (severe). The latter was deemed to be the most severe stress, as these plants went the longest without water. For the WW treatment in the growth chambers, plants were given water once a week. Plants were watered from the base to ensure even uptake and availability. Salt stress was applied during the watering regime at concentration of 50mM, 75mM or 100mM NaCl, with water always in the base when the first leaf was visible, to ensure salt stress had no effect on germination. For each experiment, there were two sets of controls. One control was included in all watering and salt treatment regimes with no compound added, but seeds had been soaked in a DMSO:acetone solution at a final concentration of 0.05% (v/v). For the second control, the salt treatment control had no salt added to the water and the drought stress control always had water in the base of the tray. A summary of each experiment and the conditions is provided in Table 1 (n=12 for each experiment).

3.3.2.3 Quantification of physiological traits

Measurements were taken when the second true leaf had emerged, after approximately 11 days of growth. Chlorophyll fluorescence (Fm) was measured using a mini Plant Photosynthesis Meter (EARS). Shoot length was measured as the height from the base of the plant to the top of the shoot. The fresh weight of the root and shoot were weighed using a microbalance. Dry weights of the shoot were measured after samples had been in a drying oven for seven days at 30°C.

3.3.2.4 Quantification of root system size and biomass

The root phenotype was imaged when the second true leaf had emerged, after approximately 11 days of growth, using a high-resolution scanner (EPSON Expression 11000XL). Measurements were obtained using WinRhizo (Regent Instruments Inc, 1991). The data shown are the mean from eight seedlings. Primary and lateral root growth were measured, differentiated in WinRhizo by creating two diameter classes.

3.3.3 Statistical analysis

Statistical analysis was carried out in R (R Core Team, 2021). Independent t-tests were performed between the control and each concentration of S-4893, and between the control and the optimum concentration of each compound, where indicated. A two-way ANOVA and a Tukey Kramer post-hoc test was performed for each experiment to see the effect of treatment and/or compound, and whether or not there was an interaction, significance $p < 0.05$.

Table 3.1 A summary of experiments carried out at Globachem. Two rice varieties, Loto and Selenio, had a mild, moderate and severe stress imposed, plus a control for each (0mM NaCl for salt stress or 'water every day' for drought stress).

Location	Variety	Stress	Compound	Data Available
Glasshouse	Loto	Drought	Optimum concentration of each compound, as decided in Figure 6	Figure 8, Figure 9 and Figure 10
		Salt	Compound 974, optimum concentration	Figure 13
	Selenio	Drought	Compound 974, optimum concentration	Figure 11
Growth Chamber	Loto	Drought	Compound 974, optimum concentration	Figure 12
	Selenio	Salt	Compound 974, optimum concentration	Figure 14

3.4 Results

3.4.1 Laboratory priming studies

3.4.1.1 Seed priming promotes root and shoot growth in *O.sativa* cv. Nipponbare seedlings

In order to test the effect of seed priming on *O.sativa* cv Nipponbare with the RGP compounds, shoot length, root length and germination rate were measured. Incorporating the data from root length (Figure 3.3), shoot length (Figure 3.4) and germination rate (Figure 3.5) allowed the production of a seed vigour index (SVI) (Figure 3.6), which is a measure of seed viability and health (Abdul-Baki and Anderson, 1973). Application of the compounds by seed priming increased root length (Figure 3.3) and shoot length (Figure 3.4) in a similar trend to the results for each RGP and concentration applied via hydroponics (Figure 2.5).

Root length tended to increase as the concentration of each compound increased, particularly for compounds 974, 1027 and 1070 (Figure 3.3). At high concentrations there was a tendency for the compound to decrease root length, this was most severe for compounds 974 and 1027. There was a slight trend of increasing root length at concentrations 9.6 and 32µM for 1093, though this was not significant. For each priming treatment, shoot length was strongly correlated to root length (Figure 3.4). Compounds 1027 and 1070 had the most positive effect on shoot length (between concentrations 0.96-32µM and 3.2-96µM, respectively). No priming treatment decreased the seedling shoot length when compared to the control; the highest concentration of the compounds (96µM) produced shoot lengths comparable to the control for 974 and 1027 but did not inhibit length as these concentrations did for root length in the respective compounds.

Germination rates were very high for most treatments, including the control achieving 100% germination (Figure 3.5). Germination rates were slightly lower, between 70-95% for the moderate to high concentrations of 974 and 1027, though this germination rate is still considered good. The concentration with the highest SVI value for each compound was selected for subsequent experiments. The concentrations selected for each compound were: 974:3.2µM, 1027:32µM, 1070:32µM, 1093:32µM and 1131:9.6µM.

Pictures of the effect of seed priming with the parent compound, 974, on *O.sativa* cv. Nipponbare are available in Figure 7. For 3.2µM compound 974, there is a clear promotion of root growth. Root growth is then stunted slightly at concentration 32µM compared to the control, correlating well with the root length data in Figure 3.3. The effects on shoot length are less clear to see, which is reflected in there being no significant differences in shoot length for 974 control (0), 3.2µM or 32µM (Figure 3.4).

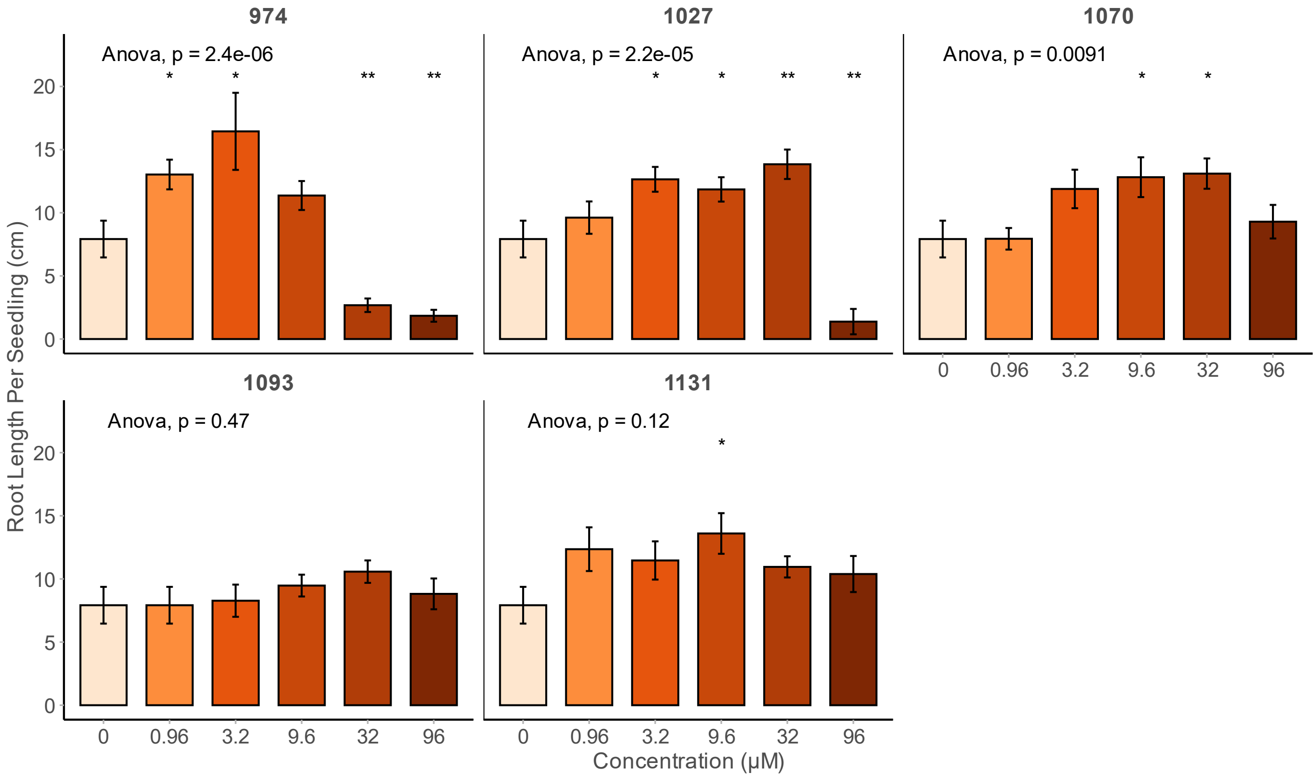


*Figure 3.3. The effect of RGP compounds was dose-dependent. All compounds exerted a positive effect on root length at an intermediate concentration, followed by root inhibition or return to a similar length to the control at higher concentrations. Significance values indicate the results of independent t tests carried out between the control and each concentration of the compound. *p ≤ 0.05, **p ≤ 0.01. Values are means ± SEM (n = 6).*

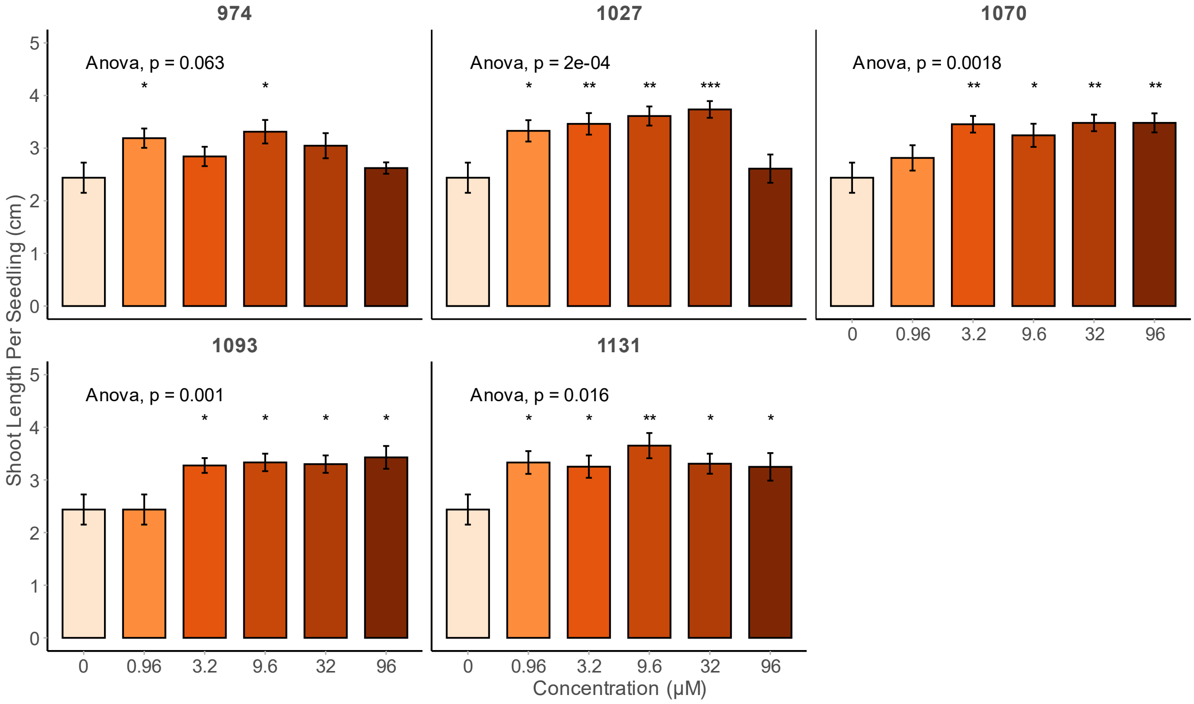


*Figure 3.4. Shoot lengths followed a similar positive trend to root length data for the array of concentrations and compounds. No shoot lengths were decreased by the seed priming compound application. Significance values indicate the results of independent t tests carried out between the control and each concentration of the compound. *p ≤ 0.05, **p ≤ 0.01, ***p ≤0.001. Values are means ± SEM (n = 6).*

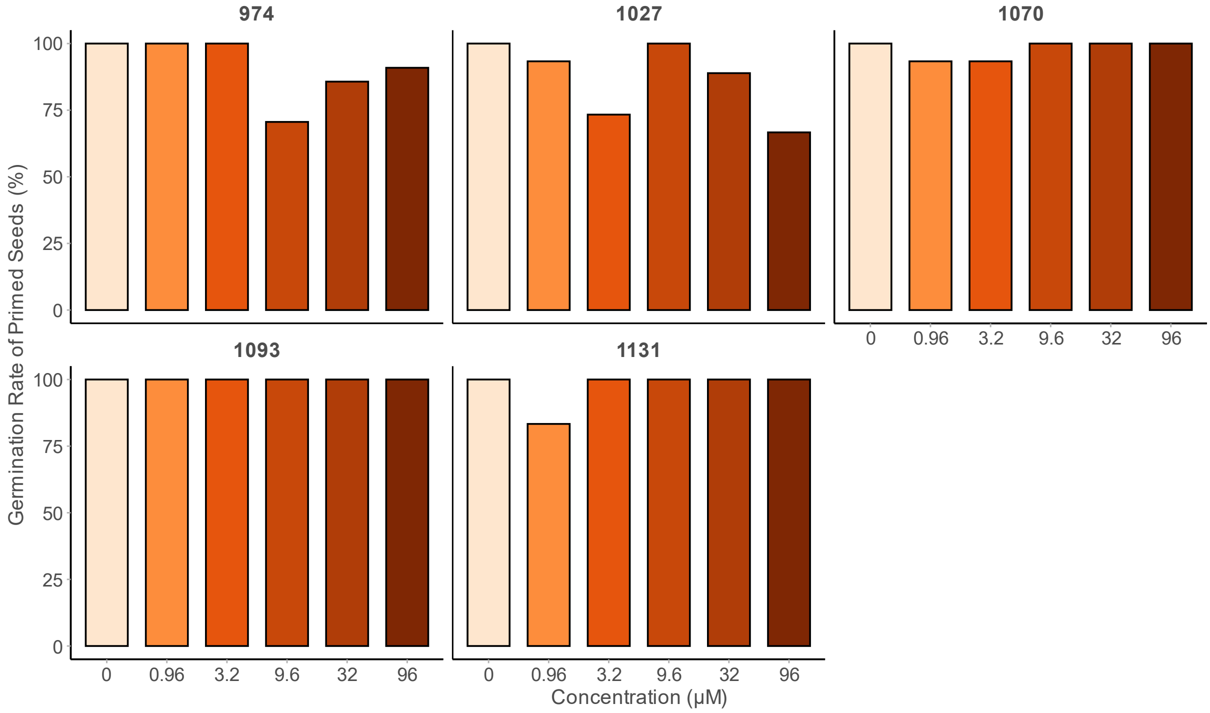


Figure 3.5. Germination rates were consistently high for all concentrations and compounds, as well as the control. Germination was most affected by priming with compounds 974 and 1027, though germination rate remained relatively high. Values are the percentage of the six seedlings that germinated (n=6).

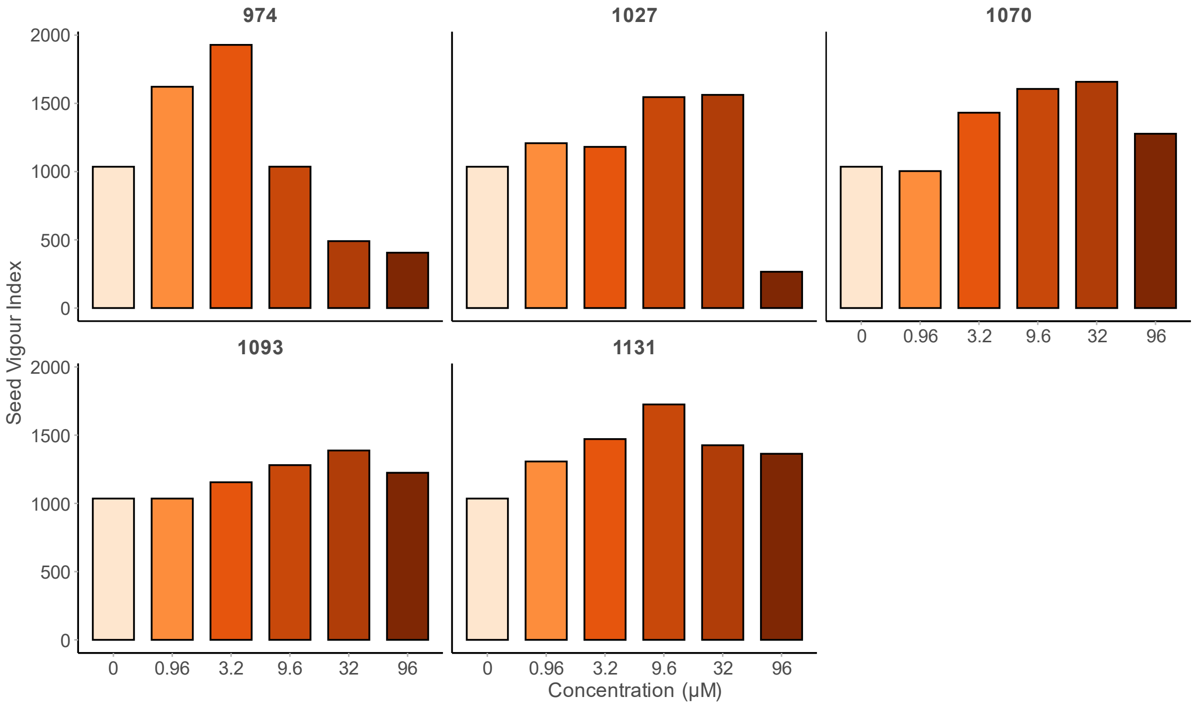


Figure 3.6. The Seed Vigour Index (SVI) revealed an optimum concentration for the effects of individual RGPs. SVI was calculated by the equation: root length + shoot length × germination percentage. The following optimum RGP concentration for each compound was used subsequently in translational experiments in a commercial setting: 974:3.2µM, 1027:32µM, 1070:32µM, 1093:32µM and 1131:9.6µM (n=6).

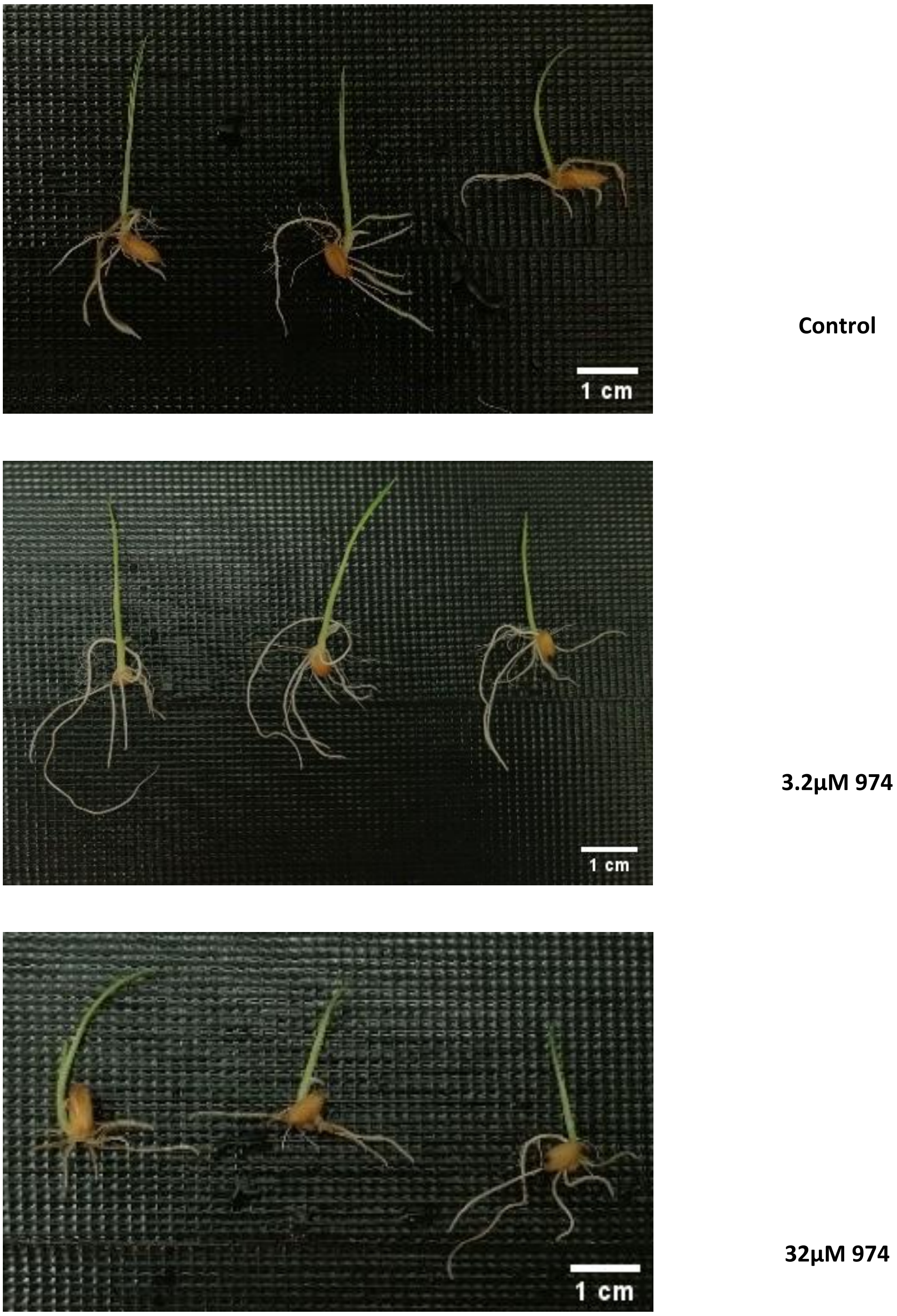


Figure 3.7. Representative images showing the positive effect of compound 974 on root growth at 3.2µM; and the slight inhibitory effect of compound 974 at 32µM.

3.4.2 Seed priming in a commercial setting

In order to establish whether seed priming with the RGP compounds is a viable method of application in a commercial setting, two rice cultivars, Loto and Selenio, used by Globachem were primed using the optimum concentrations for seed vigour that were established previously (see Figure 3.6). The efficacy of the compounds under standard glasshouse conditions at Globachem were tested, along with the efficacy of the compounds under salt and drought stress at three levels of severity.

3.4.2.1 Priming of *O. sativa* cv Selenio and Loto offers no benefit to seedlings under drought stress or optimum conditions under commercial growing conditions

Seedling growth from primed seeds of Loto, a lowland rice seed variety, that had been treated with the optimum concentration for each compound derived (Figure 3.6), exhibited few differences from control seeds under drought conditions (Figure 3.8, Figure 3.9 and Figure 3.10). The parent compound (974), and compound 1093 had longer roots than the control plants under the control water regime ($p<0.05$) (Figure 3.8). The increase in root length for compounds 974 and 1093 under optimum conditions came at no cost to shoot length (Figure 3.10), although it didn't confer any significant benefits to shoot length or biomass (Figure 3.9). The varying degrees of drought stress exerted no notable change in root length (Figure 3.8).

Dry weights and shoot lengths were lowest for the seedlings that had had water withheld from 2cm shoot, indicating that a drought stress had been achieved with this watering regime. However, for this watering regime, treated compounds behaved very similarly to the control (ANOVA=0.49) and treatment conferred no benefit to shoot length (Figure 3.10). Under the HS treatment, compounds 974 and 1093 had similar root and shoot lengths to the control, and a tendency towards greater dry weights (Figure 3.8, Figure 3.10 and Figure 3.9, respectively).

Compound 1027 performed consistently worse than the control, with significant reductions in dry weight of shoot (Figure 3.9) and corresponding shoot length (Figure 3.10) under the moderate treatment. Whilst these reductions could have been indicative of a reallocation of resources to root growth, this was not reflected in the root length data (Figure 3.8).

The same environmental conditions and water deficit control were applied to an upland rice cultivar, Selenio (Figure 3.11). The water deficit stress had a significant effect on dry weight ($p<0.001$), shoot length ($p<0.001$) and root length ($p<0.01$), Figure 3.11A, B and C, respectively. However, the application of compound 974 (the parent compound) conferred no benefit to the dry weight or root length of seedlings under control or any water deficit conditions. Data for seedlings with and without seed priming with compound 974 showed very similar responses to each of the stresses imposed (Figure 3.11A and C). Application of the parent compound inhibited shoot length at the severe treatment (Figure 3.11B, $p<0.05$), though there was no knock-on effect for root length or shoot dry weight. The compounds had no overall effect on dry weight of shoot, shoot length or root length (ANOVA $p>0.05$ for all), nor was there an interaction between compound application and water deficit application (ANOVA $p>0.05$ for all).

Experiments reported in Figure 3.8 to Figure 3.11 and Figure 3.13, were conducted in October when large temperature fluctuations in the glasshouse may have resulted in plants becoming temperature stressed. Therefore, in order to assess the impact of RGPs under stable temperature conditions, the upland rice cultivar, Selenio was grown inside a temperature-controlled growth chamber and the effects of the parent compound (compound 974) on plant responses to water deficit stress determined (Figure 3.12).

Chlorophyll fluorescence increased as water stress increased, though not to a significant extent. Interestingly, chlorophyll fluorescence was highest for the severe treatment (Figure 3.12A). This raises the question as to whether the severe treatment had indeed imposed a marked water stress on plants. However, the dry weight of the shoot was negatively affected by increasing stress severity ($p<0.001$), as was shoot length ($p<0.001$), which is indicative of an increasing stress (Figure 3.12B and C). Roots of seedlings treated with compound 974 were slightly longer under control and moderate, though this was not significant (Figure 3.12D).

Application of compound 974 decreased chlorophyll fluorescence compared to the untreated control, however, this was only significant when under moderate ($p<0.05$), and is indicative that the effect on CK signalling was not root-specific. Surprisingly, the dry weight of seedlings treated with compound 974 under moderate was greater than the untreated seedlings with the same stress applied ($p<0.05$, see Figure 3.12B). For all other water regimes, the seedlings treated with compound 974 had slightly lower dry weights than their untreated counterparts, though this was not significant (see Figure 3.12B). The shoot length of treated and untreated seedlings was very similar for each water regime (Figure 3.12C), following a very similar pattern to that of the dry weight data.

The median root length for seedlings treated with compound 974 was higher for all water regimes except the most severe (Figure 3.12D), this however, was not significant (ANOVA $p>0.05$). Physiological responses for treated and untreated seeds were very similar throughout, with no clear trends or benefits of the seed priming treatment conferred under optimum or drought stress conditions.

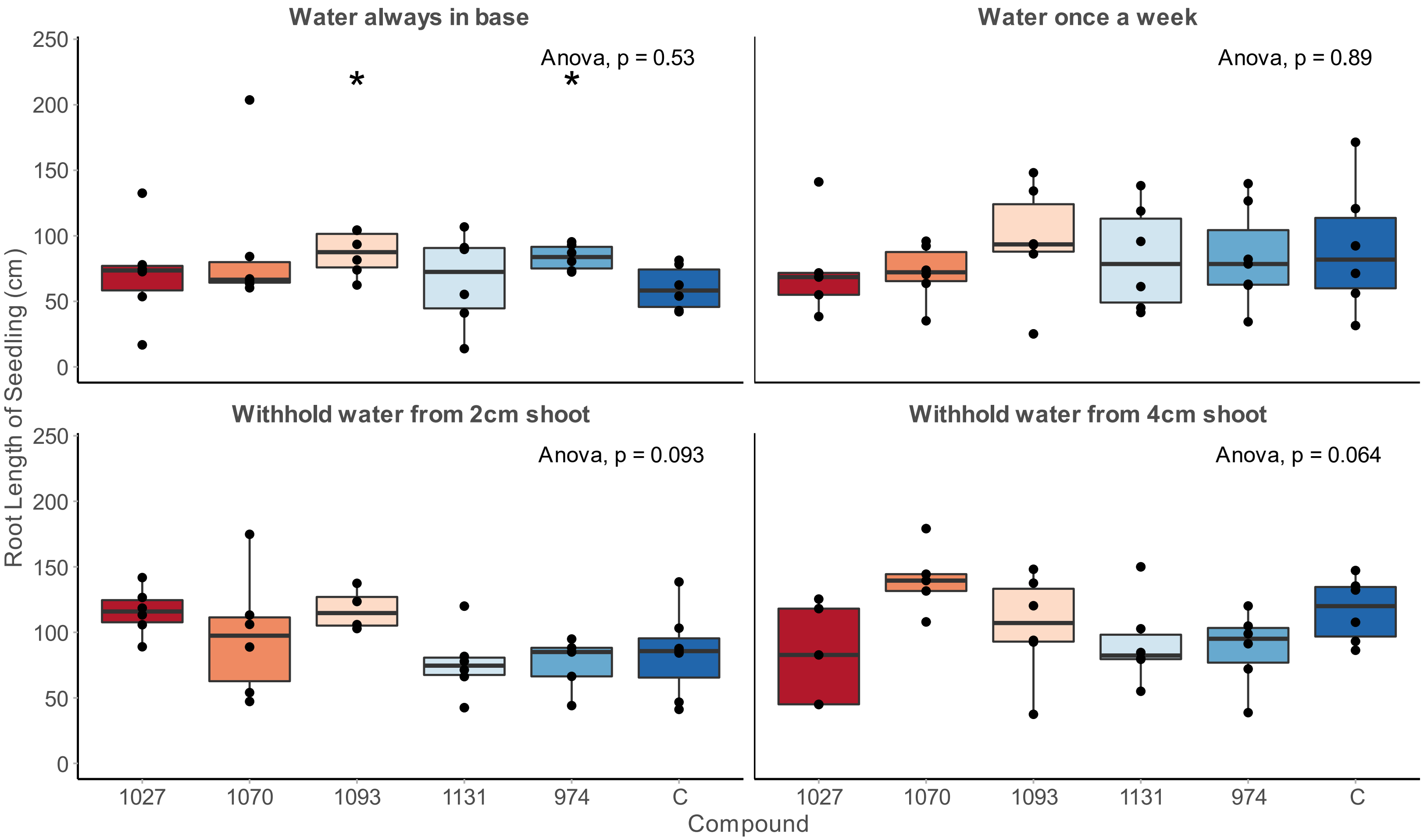


*Figure 3.8. Root length of glasshouse-grown Loto, a lowland rice variety, exposed to drought stress. Treatments clockwise from top-left represent control, mild, moderate and severe stress. The boxplot displays the median, and the upper and lower quartiles. The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Significance values indicate the results of independent t tests carried out between the control and each concentration of compound 974. *p ≤ 0.05 (n=6), 'C' on the x-axis represents the control.*

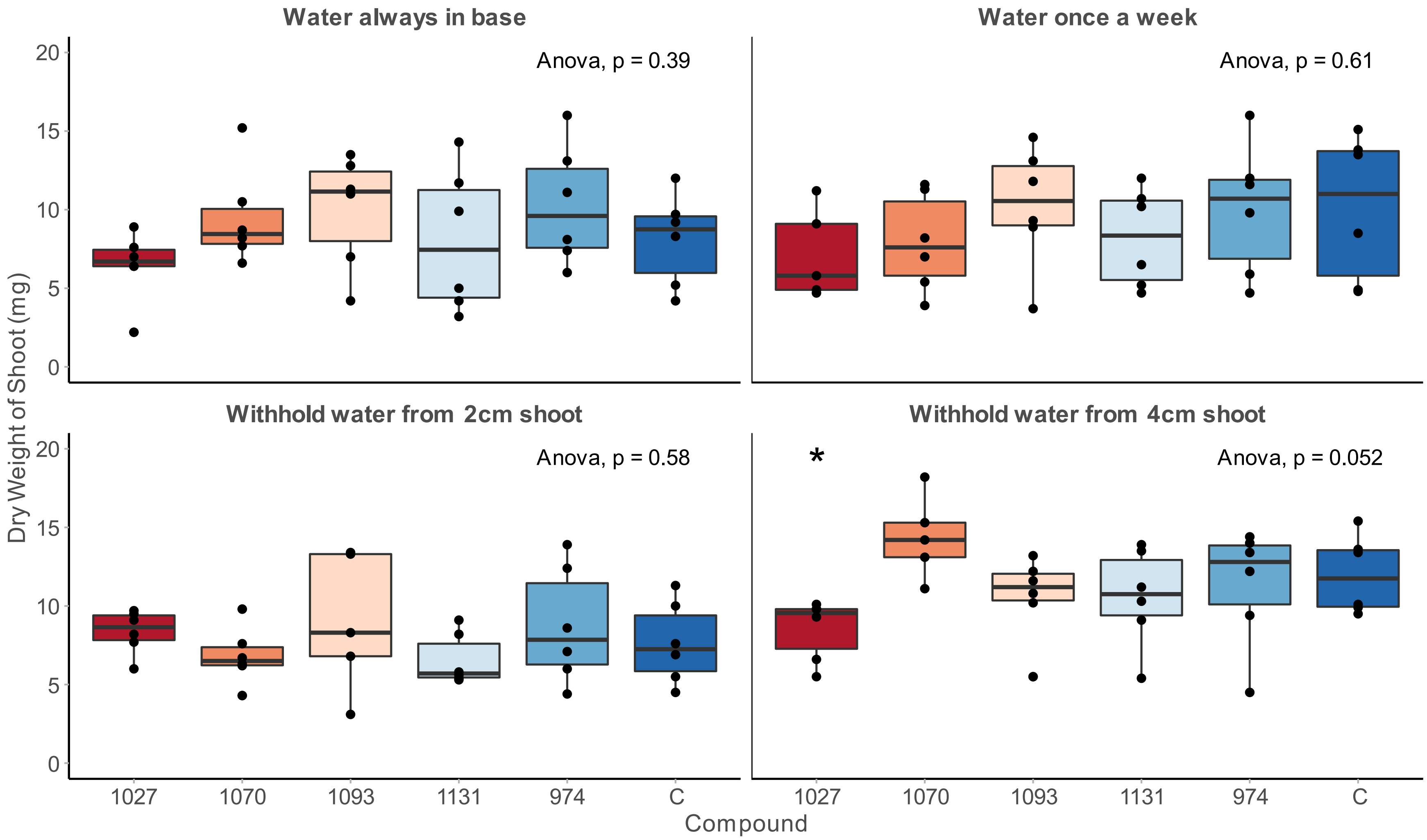


*Figure 3.9. Root length of a lowland rice variety, Loto, exposed to varying levels of drought stress, grown in a glasshouse. Treatments clockwise from top-left represent control, mild, moderate and severe stress. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Significance values indicate the results of independent t tests carried out between the control and each concentration of compound 974. *p ≤ 0.05 (n=6), 'C' on the x-axis represents the control.*

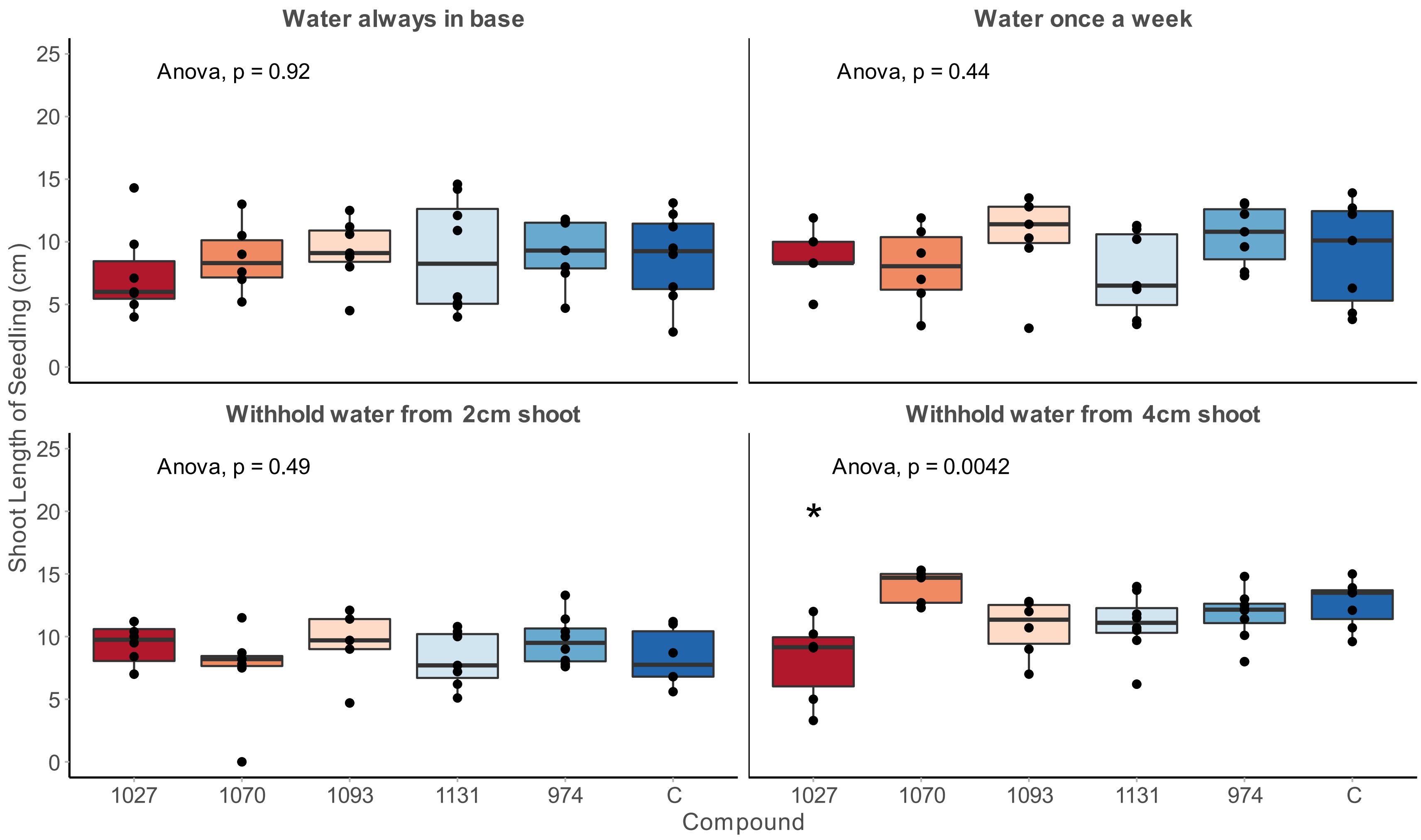


*Figure 3.10. Root length of a lowland rice variety, Loto, exposed to varying levels of drought stress, grown in a glasshouse. Treatments clockwise from top-left represent control, mild, moderate and severe stress. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Significance values indicate the results of independent t tests carried out between the control and each concentration of compound 974. *p ≤ 0.05 (n=6), 'C' on the x-axis represents the control.*

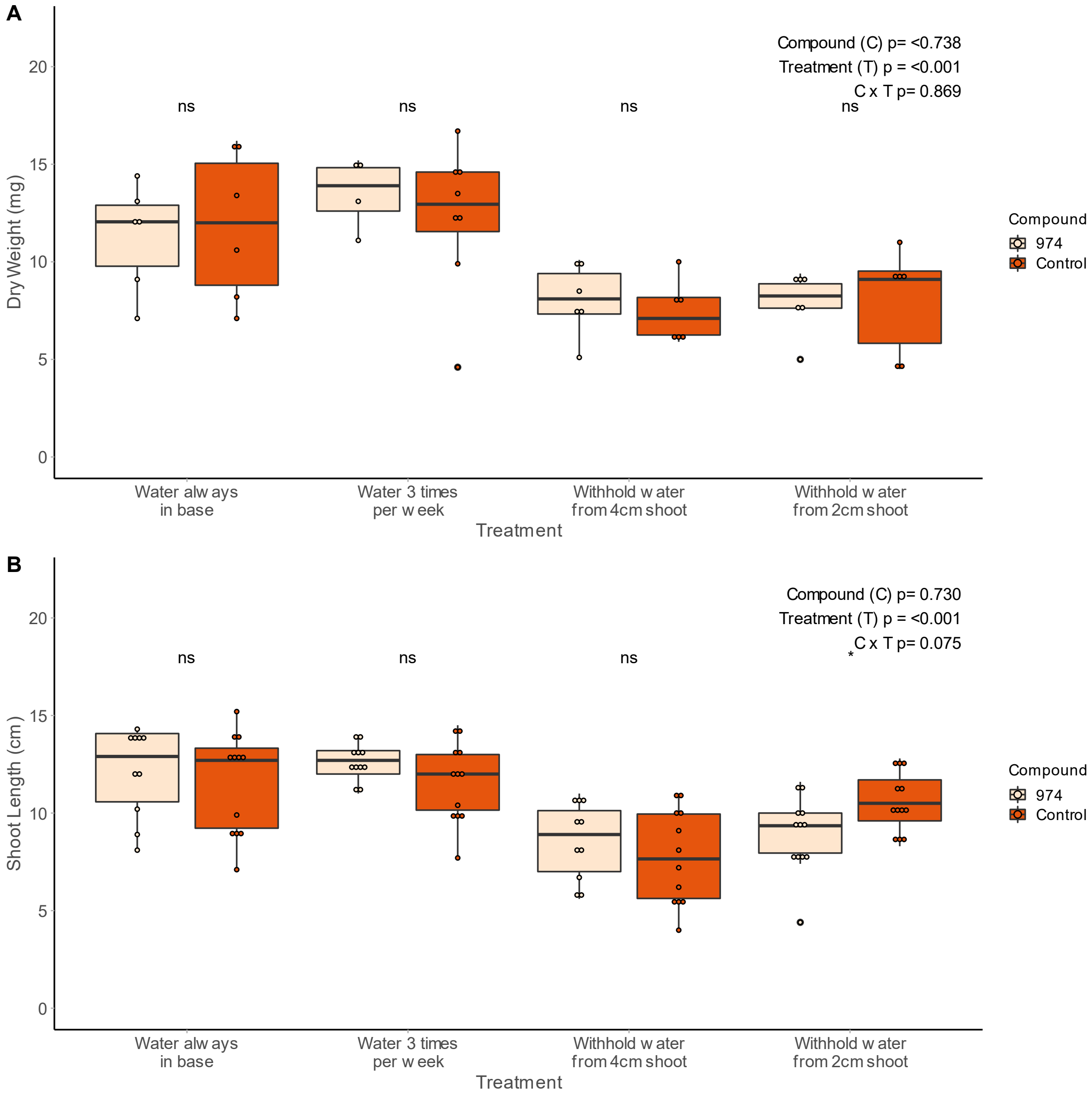


Figure 3.11. (continued on next page, legend follows).

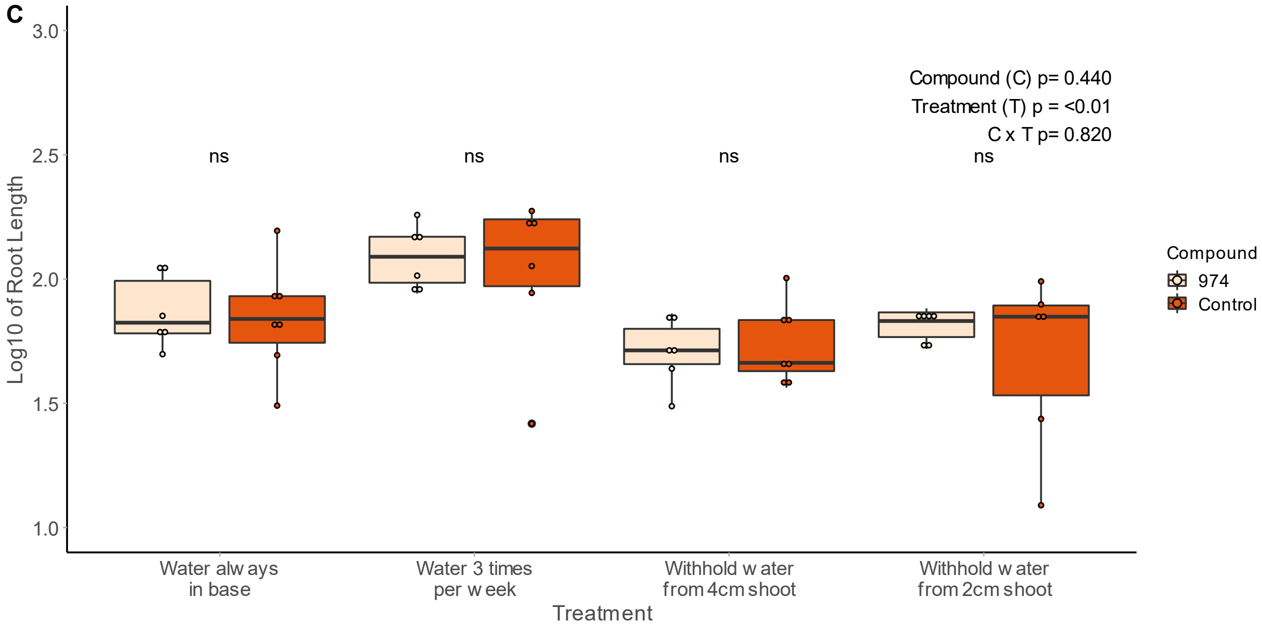


*Figure 3.11. Application of drought stress, or the CK antagonist, to the upland rice cultivar, Selenio, had no effect on shoot or root growth under glasshouse conditions. Panel A represents the shoot dry weight. Treatments (left to right) represent a control, mild, moderate and severe water-deficit stress, respectively. Significance values indicate the results of an independent t test carried out between treated and untreated seedlings for each drought stress level. *p ≤ 0.05 (n = 6). Root length values are log10 to normalise data. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Statistics for the effect of compound application and drought stress are the results of a two-way ANOVA.*

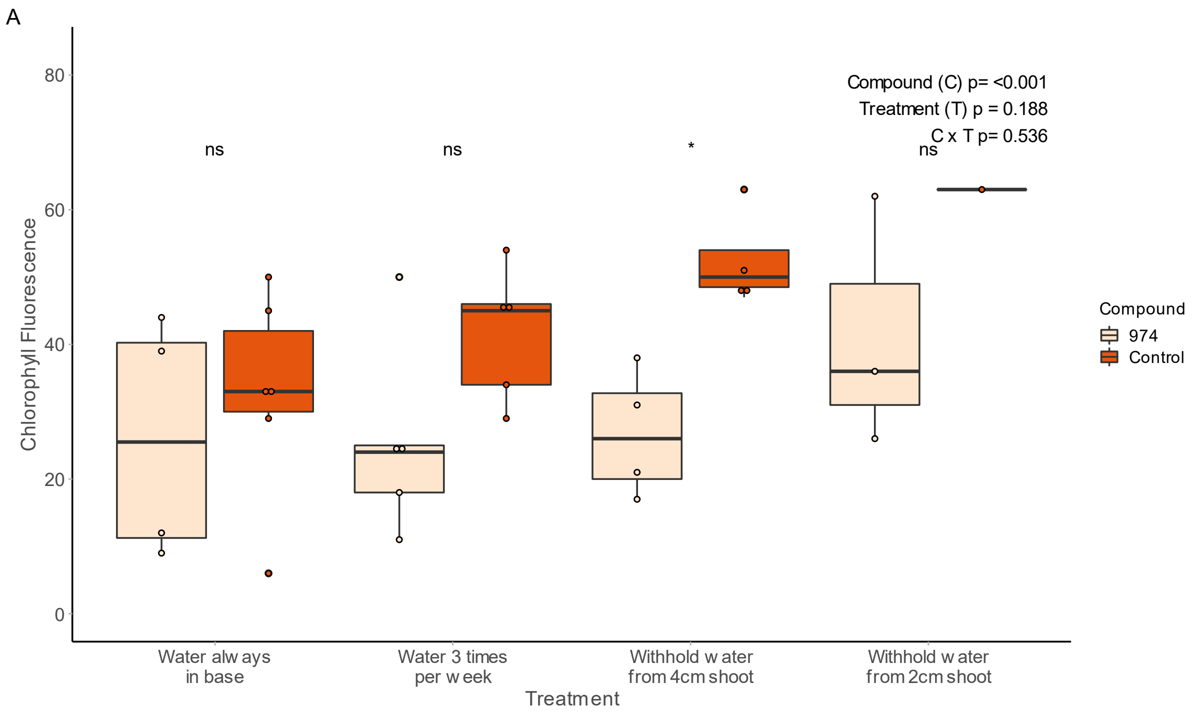


Figure 3.12. (continued on next page, legend follows).

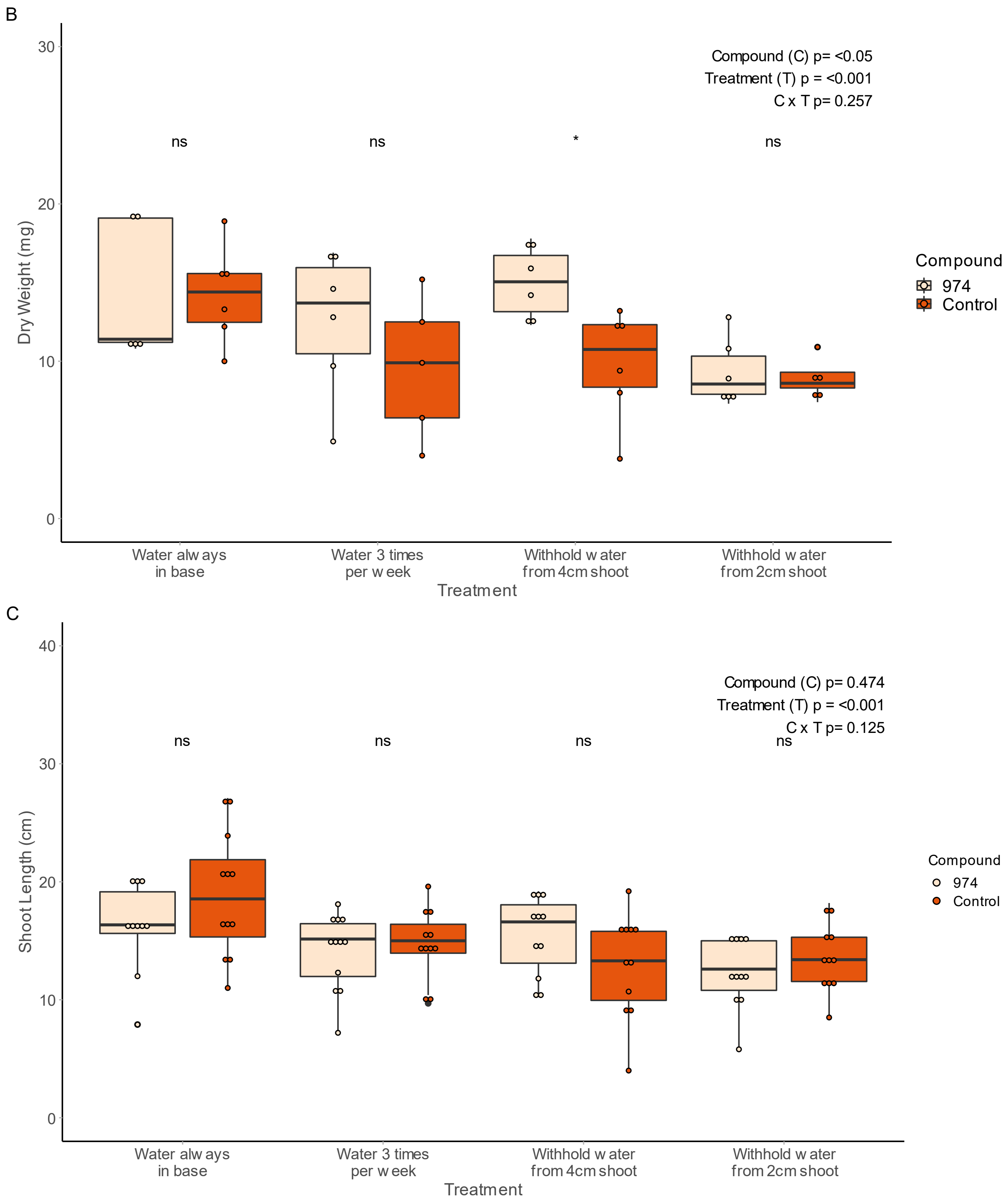


Figure 3.12. (continued on next page, legend follows).

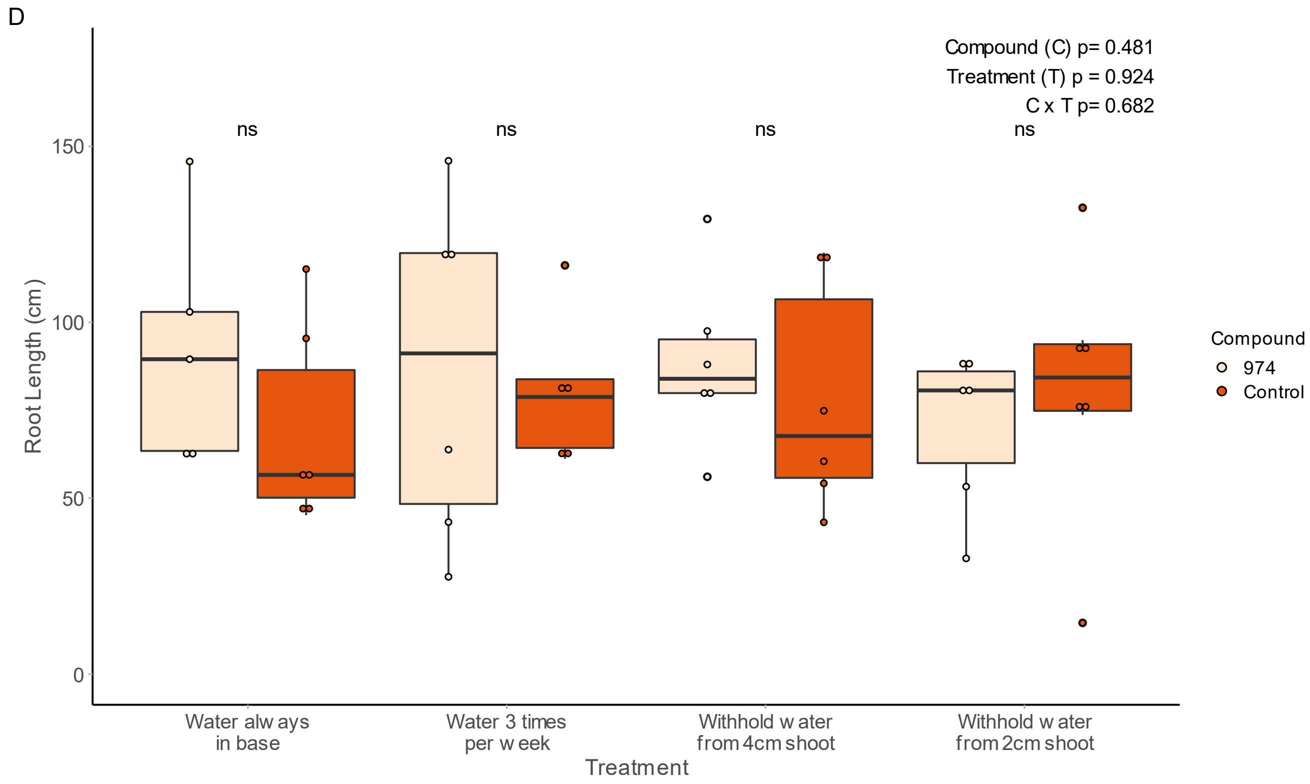


*Figure 3.12. Application of drought stress, or the CK antagonist, to the lowland rice cultivar, Loto, had no effect on shoot or root growth under growth chamber conditions. Panel B represents the shoot dry weight. Treatments (left to right) represent a control, mild, moderate and severe water-deficit stress, respectively. Significance values indicate the results of an independent t test carried out between treated and untreated seedlings for each drought stress level. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. *p ≤ 0.05. Values are means ± SEM (n = 6). Statistics for the effect of compound application and drought stress are the results of a two-way ANOVA.*

3.4.2.2 Priming of *O.sativa* cv Selenio and Loto seeds with compound 974 offers no benefit to seedlings under salt stress

The effect of salt stress on seeds of a lowland rice cultivar, Loto, primed with compound 974 was investigated in the glasshouse. Preliminary testing to establish a salt stress found that 100mM NaCl treatment significantly inhibited root growth (Appendix Figure 4). However, in the glasshouse trial, there was no effect of salt treatment on dry weight, shoot length or root length (Figure 3.13A, B and C, respectively, ANOVA p>0.05). Interestingly, median dry weight was highest at the 100mM NaCl for both seeds treated with and without compound 974 (Figure 3.13A). Median shoot length and root length also increased for seedlings treated with 974 from concentrations 50mM to 100mM (Figure 3.13B and C), though the effect of the salt treatment was not significant, nor was the interaction between salt stress and priming (two-way ANOVA results Compound x Treatment p>0.05 for both root length and shoot length).

There were no benefits conferred to dry weight of shoot, shoot length or root length by applying compound 974 (ANOVA p>0.05 for dry weight of shoot, shoot length and root length). Preliminary testing of compound 974 with Nipponbare under salt stress found that seeds primed with compound 974 had slightly longer roots under control, 75mM and 100mM conditions, however, this was not significant (Appendix Figure 5 and Appendix Figure 6). When no salt stress was applied, seedlings primed with 974 had slightly longer root lengths than the control, though this was not significant. The slight increase in root length observed in the preliminary experiments (Appendix Figure 5) and slight increase observed from treating with 974 under no salt stress, in which median root length was slightly higher for seedlings primed with 974 under no salt stress, (Figure 3.13C) was not reflected in the more severe salt stress treatments (Figure 3.13C), as seedlings treated with compound 974 performed slightly worse and had medians equal to or lower than their counterparts that had not been primed with 974. Overall, application of compound 974 resulted in no significant difference in response at each stress severity for dry weight of shoot, shoot length, or root length (Figure 3.13A, B and C, respectively).

It is possible, as in the drought stress experiments (Figure 3.8 to Figure 3.12), that the effects of RGPs on plant salt stress responses were affected by temperature fluctuation in the glasshouse. To eliminate this possibility seeds of the upland rice variety, Selenio were primed with and without compound 974 and were grown in a temperature-controlled growth chamber with 50, 75 or 100mM salt regime applied. There was no overall effect of salt stress on chlorophyll fluorescence, shoot length or root length (ANOVA p>0.05 for all, Figure 3.14A, B and C, respectively), suggesting the salt concentrations were not high enough for the Selenio rice variety.

Similarly to Loto grown in the glasshouse (Figure 3.13C), the root length of seedlings treated with compound 974 was slightly longer than untreated seedlings under control conditions (Figure 3.14C), however, this was not significant, and any small benefit conferred by applying compound 974 diminished as the severity of the stress increased. The slight promotion of root growth with treated seeds when no salt stress was applied was not reflected by an alteration in shoot growth. Shoot lengths were very similar for all treatments and conditions imposed. Treating seeds with compound 974 conferred no significant benefits to chlorophyll fluorescence, both treated and untreated seeds gave diverse readings with no significant difference, though seeds primed with compound 974 performed notably worse at 100mM NaCl stress.

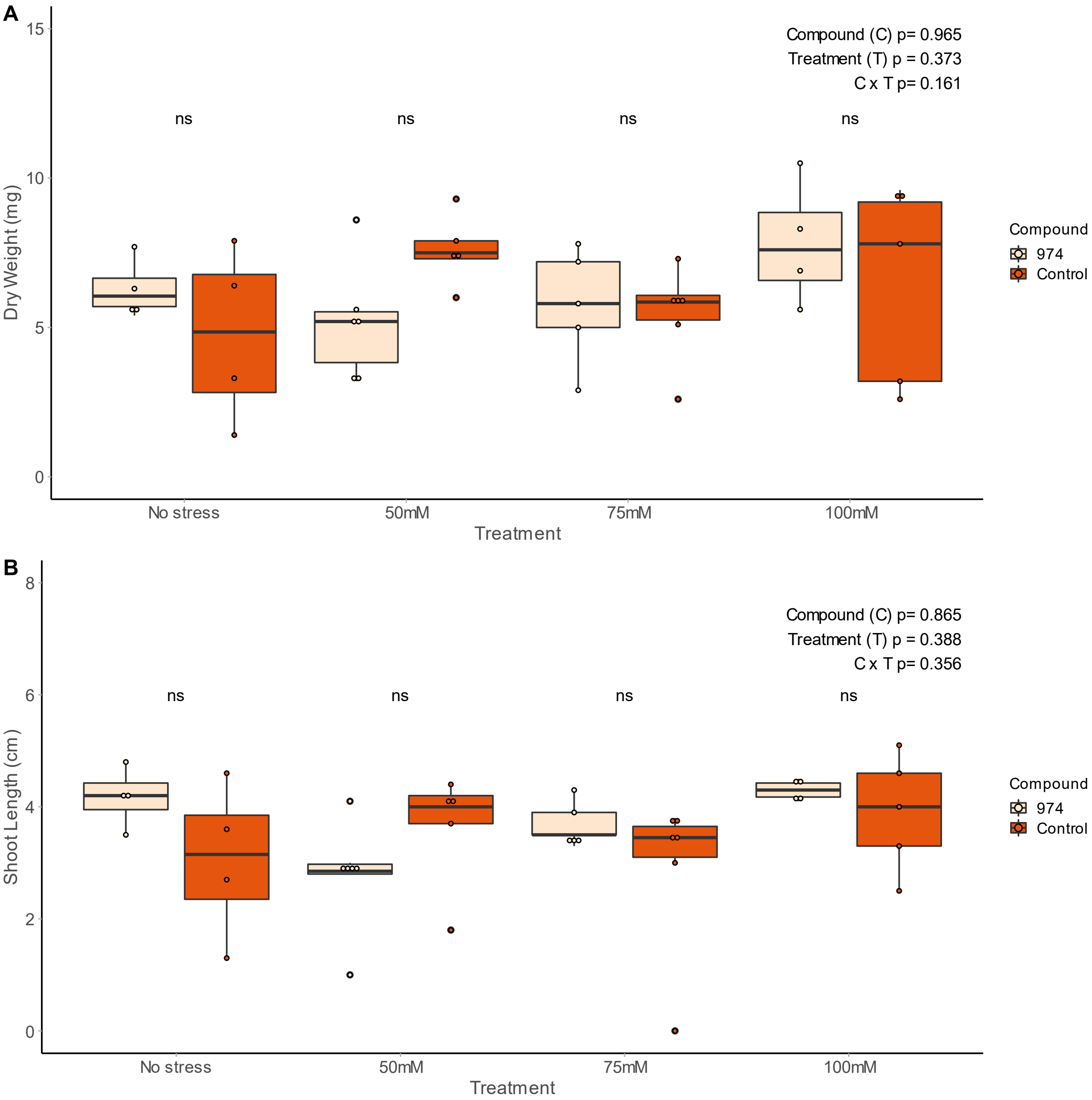


Figure 3.13. (continued on next page, legend follows).

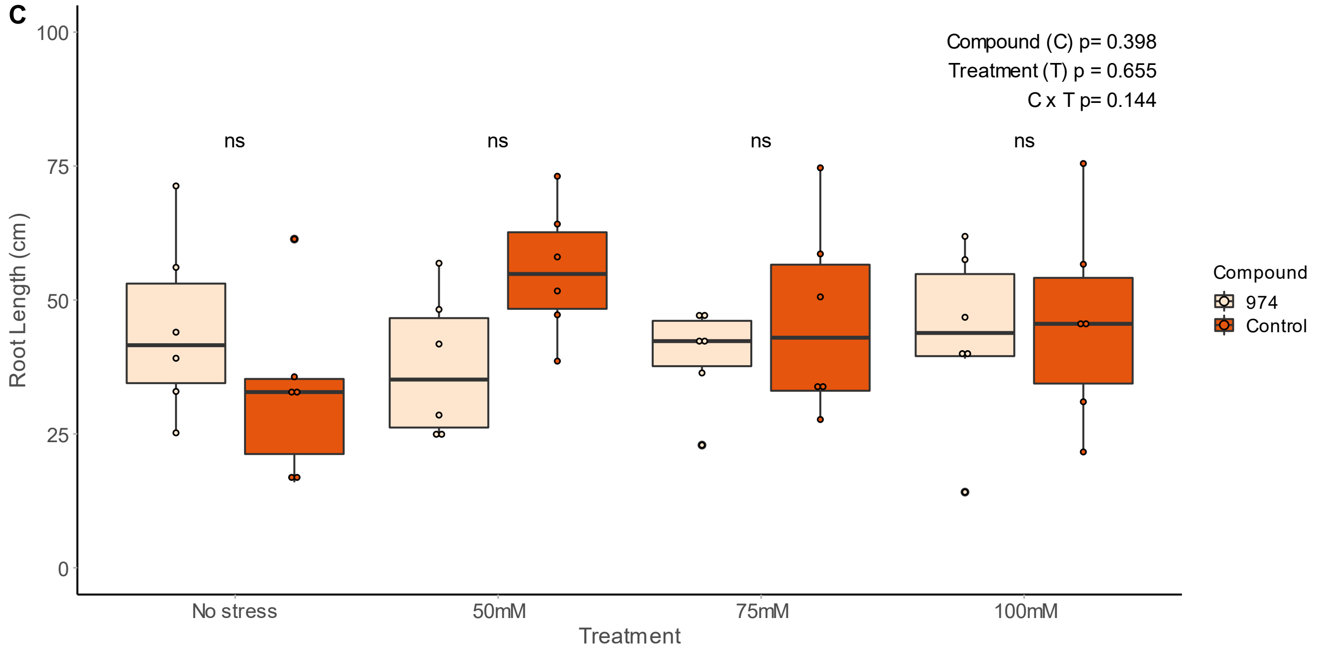


*Figure 3.13 Seed priming with compound 974 had no effect on shoot or root growth of the lowland rice cultivar, Loto, in response to salt stress under glasshouse conditions. Panel A represents the shoot dry weight. 50, 75 and 100mM NaCl represent mild, moderate and severe salt stress, respectively. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Significance values indicate the results of an independent t test carried out between treated and untreated seedlings for each salt stress level. *p≤0.05. Values are means ± SEM (n = 6). Statistics for the effect of compound application and salt stress are the results of a two-way ANOVA.*

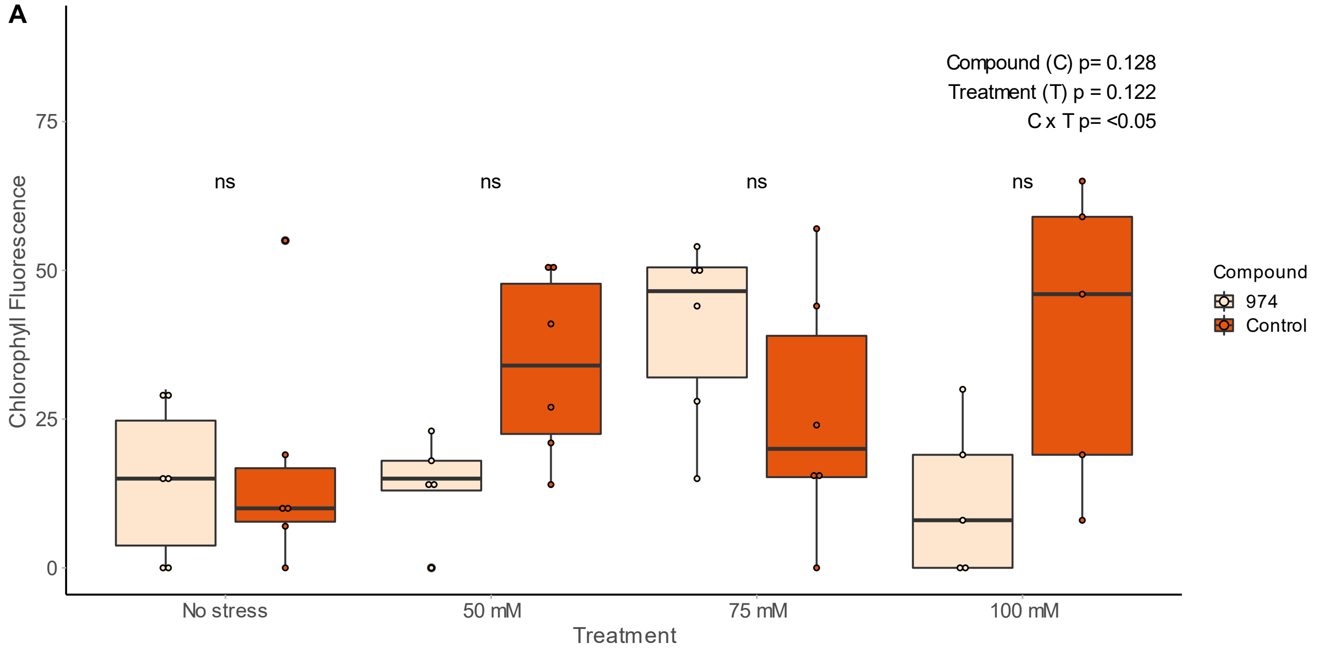


Figure 3.14. (continued on next page, legend follows).

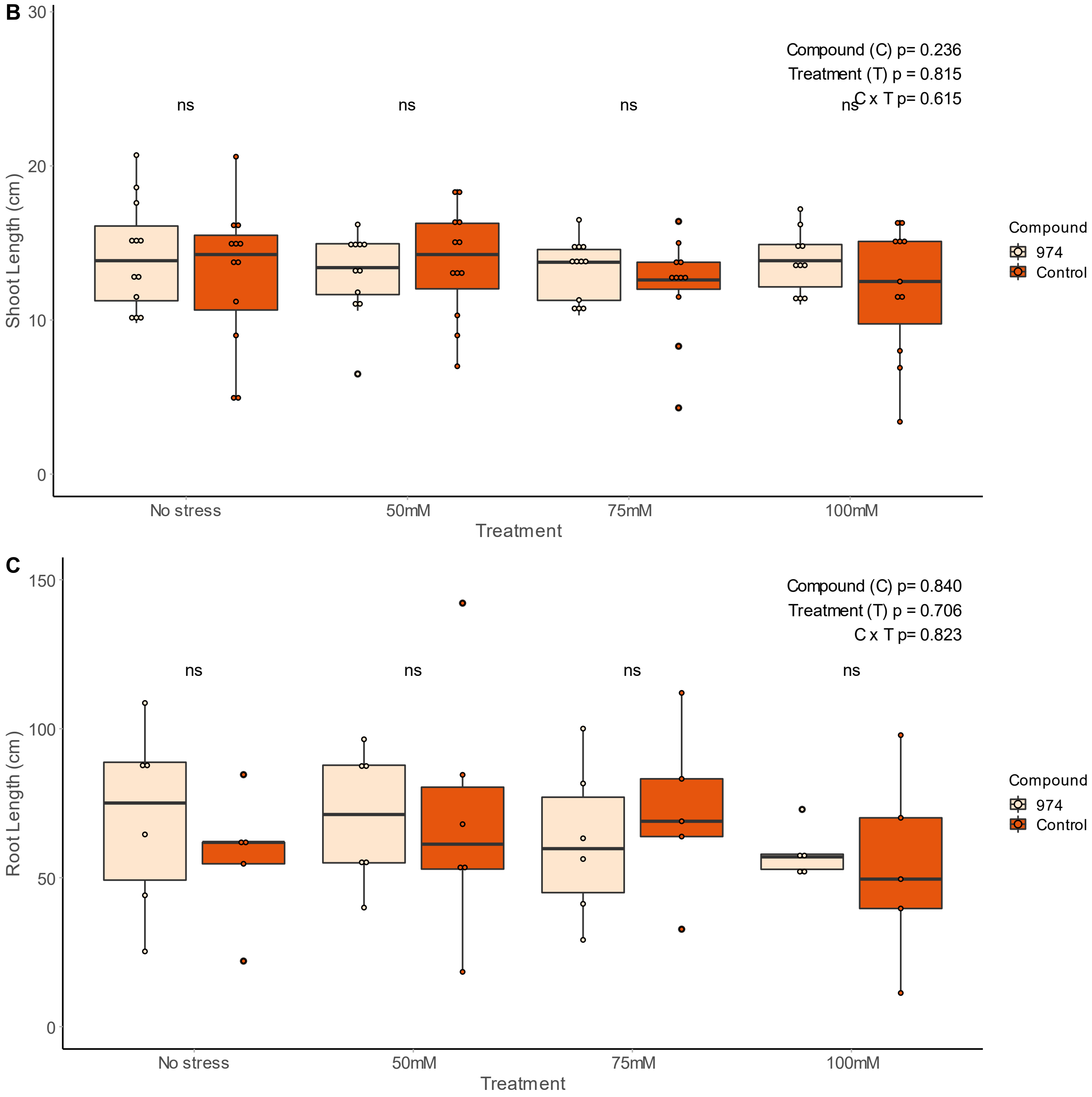


*Figure 3.14. Application of salt stress, or the CK antagonist, to the upland rice cultivar, Selenio, had no effect on shoot or root growth in growth chamber conditions. 50, 75 and 100mM NaCl represent mild, moderate, and severe salt stress, respectively. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the IQR from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual data points. Significance values indicate the results of an independent t test carried out between treated and untreated seedlings for each salt stress level. *p ≤ 0.05. Values are means ± SEM (n = 6). Statistics for the effect of compound application and salt stress are the results of a two-way ANOVA.*

3.5 Discussion

Whilst there are efforts to implement water-saving strategies in rice, such as the water-saving ground rice systems (Li et al., 2017) and alternative wetting and drying practices (Norton et al., 2017), socio-economic factors can limit farmer’s willingness to adopt such practices and more complementary measures must be sought (Howell et al., 2015, Enriquez et al., 2021). Seed priming with novel RGPs offers an exciting approach to improve water use in rice growing systems, requiring minimal input from farmers who can utilise existing agrochemical business models and practices for effective dissemination.

3.5.1 Seed priming is an effective method of applying the RGP compounds

Applying the RGP compounds via seed priming increased root length (Figure 3.3) and shoot length (Figure 3.4) compared to the untreated control. Interestingly, most treatments had very high germination rate (Figure 3.5), this is inclusive of the control which was soaked in only DMSO:acetone and water. Soaking in water, a technique known as hydropriming, has been found to promote high germination and seed vigour (Lutts, 2016) which could account for the high germination rates observed in this study. The seed soaking method used for seed priming resulted in less compound being used and a less laborious set up process than the methods in Chapter 2, suggesting seed priming would lend itself to a commercial, high-throughput setting.

3.5.2 Cytokinin signalling is typically repressed by abiotic stress in rice, although trends vary between environments and species

The parent compound 974 was identified as a root-specific CK antagonist by Arata et al. (2010), therefore, seed priming with this compound and applying a salt stress would have been expected to dampen the decrease in root length exerted by high presence of CK. However, no effect on the root or shoot length under salt stress was observed (see Figure 3.13 and Figure 3.14). This could have been due to the glasshouse studies performed at Globachem HQ in Belgium (Figure 3.9 - Figure 3.11 and Figure 3.13), being subject to a wide variation in temperature. Whilst this is reflective of commercially relevant conditions, this may have been an inhibitory factor for the novel RGP compounds used which appear to have a very specific window of activity, both in terms of active concentration and environmental conditions, and that the response of CK to low temperature is complex and not yet fully understood (Pavlů et al., 2018). Given that under cold and drought stress CK signalling decreases in rice (Maruyama et al., 2014), and that the reduction of CK signalling as a result of cold treatment lowers plant tolerance to cold stress (Jeon et al., 2010), it would be expected that the effects of CK and therefore a CK antagonist would also decrease. This may go some way to explaining the minimal effects observed under drought stress (see Figure 3.8 to Figure 3.12).

Yin et al. (2020) found CK levels increased in the roots and shoots in response to salt stress in rice, which helped to alleviate salt stress damage. This, combined with CK abundance leading to shoot proliferation (Werner and Schmülling, 2009) suggests that the negative aspects of an abundance of CK (namely, a decrease in root length (Werner et al., 2010)) are outweighed by the benefits such as delayed leaf senescence, enhanced shoot proliferation and increased grain yield (Jameson and Song, 2015, Werner et al., 2001). Shoot growth is modulated, in part, by root-derived CKs which act as long-distance signalling molecules, channelling information relating to root nitrogen status (Pavlů et al., 2018). Based on the findings by Pavlů et al. (2018) application of the compounds, which are putative CK antagonists, could be expected to alter shoot growth; however, there was no difference in shoot growth between the control and those with the compounds applied under control conditions (Figure 3.10, Figure 3.11B, Figure 3.12C, Figure 3.13B and Figure 3.14B).

There are a plethora of conflicting reports on the effects of salt stress on CK levels. Ghanem et al. (2010) found salt stress caused a decrease in CK of 20-50% within a tomato plant. However, CK levels remained high under salt stress in the rootstock of apple trees (Feng et al., 2019), tomato seedlings (Liu et al., 2020) and rice (Yin et al., 2020). These contradictory experimental results highlight the species-specific responses to CK signalling. In the current study, the activity of the applied compounds was observed to be cultivar specific, with an effect elicited in Nipponbare under optimum conditions (see Chapter 2), yet none observed in Selenio or Loto. The reports of CK both increasing and decreasing abiotic stress tolerance (Ghanem et al., 2010, Feng et al., 2019, Liu et al., 2020, Yin et al., 2020), and of CK signalling also being both repressed and stimulated by abiotic stress (Zwack and Rashotte, 2015, O'Brien and Benkova, 2013), along with the lack of root promotion from Loto and Selenio in this study, make it clear that the commercial use CK-related compounds for cultivation may not be a viable option to improve plant stress responses.

Studies have shown that overproduction of CK in some species, such as Arabidopsis, decreases plant tolerance to salt and water deficit stress due to the down-regulation of stress-responsive genes, reduction in chlorophyll content, or alterations in expression of ROS producing genes and ROS scavenging enzymes (Liu et al., 2020, Wang et al., 2015). ROS production occurs mainly in the chloroplasts, mitochondria and peroxisome, with photosystems I and II (PSI and PSII) being the main sites of ROS production in the chloroplast (Heap et al., 2020). Current evidence suggests that CK signalling during abiotic stress leads to malfunction of ROS production in the PSI and PSII by altering of gene expression in the subunits (Kobayashi et al., 2012). Additionally, it has been suggested that there is complex interplay between ROS, CK signalling and abiotic stress (Wang et al., 2015). A decrease in ROS scavenging gene expression has been reported in response to salt stress leading to greater inhibitory effects of the stress (Wang et al., 2015) whilst overexpression of ROS scavenging enzymes, such as superoxide dismutase (SOD) and catalase (CAT) has been shown to lead to an increased tolerance to abiotic stress, resulting in improved plant survival rate and yield (Apel and Hirt, 2004).

Reduced levels of CK in rice are associated with higher stress tolerance (Liu et al., 2020, Yin et al., 2020). In addition, low CK abundance and/or signalling has been associated with higher water contents during drought stress due to increased root growth and the associated increased water uptake (Ramireddy et al., 2018). The increase in root growth under low CK levels is accompanied by reduced stomatal apertures and transpiration rates, suggesting a cohesive mechanism for limiting aboveground water loss and maximising belowground water uptake during periods of drought stress (Nishiyama et al., 2011, Pavlů et al., 2018). Therefore, low CK abundance is a useful tool for limiting water loss during short-term stress, though long-term inhibition of photosynthesis would have negative long-term effects. However, no negative effects of drought stress or of the CK antagonist on chlorophyll fluorescence were identified in this study (Figure 3.13A). The lack of effect of the CK antagonist on chlorophyll fluorescence or root growth (Figure 3.11C, Figure 3.12A and D) compared to the control in optimum and stress conditions in these experiments suggests that CK levels could have already been low in control conditions, and were not further lowered by the addition of the CK antagonist. However, hormone analysis, which lies outside of the scope of the present study, would be required to test this hypothesis experimentally.

Response regulators (RRs) are involved in signal transduction systems and are highly expressed in rice roots, where they mediate a cell's response to environmental changes as part of the two-step phosphorelay system (Du et al., 2007). They are categorised into four groups: type-A, type-B, type-C and pseudo-response regulators (PRRs). Type-A RRs are negative regulators of CK signalling, whereas type-B RRs are positive regulators of CK signalling. Specifically in rice, 15 type-A RRs (*OsRR1-15),* 7 type-B RRs *(OsRR16-22)* and 5 predicted pseudo-response regulators *(OsPRR1-5)* have been identified that are components of the two-step phosphorelay system for CK signalling (Du et al., 2007). Expression of *OsRR*s have been found to increase 20-fold after treatment with exogenous CK. Their genes are also expressed in response to environmental stresses; *OsRR* transcript levels increased 4-

fold in response to salt stress and 2-fold in response to dehydration, indicating they are involved in cross-talk between a rice plants' response to CK signalling and abiotic stress (Jain et al., 2006).

The increase in expression of *OsRR* genes that is induced by cold and water deficit stress has a negative impact on yield harvest traits that are of commercial value, such as impaired panicle development and grain sterility in rice (Pandey, 2020). These genes are also type-A RRs and are therefore negative regulators of CK signalling. The increased expression of the type-A RRs when the salt or drought stress and the subsequent prevention of CK signalling within the plant is likely to have dampened the effect of the novel compounds and any root growth promotion that would have occurred from their activity as CK antagonists (Figure 3.8 to Figure 3.14).

The large fluctuation in temperature and the use of an unheated glasshouse in October may have inflicted an unintentional cold stress to the rice seedlings (Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.13). Low temperatures can cause ROS accumulation and inhibition of ROS scavenging enzymes, leading to cell metabolism malfunctioning and cell death (Petrov et al., 2015). Cold stress has been mitigated by the application of CKs (Liu et al., 2020). Hence, the application of a CK antagonist during a cold stress would be likely to have a synergistically negative effect on seedling development. and may have initiated expression of the type-A RRs, leading to decreased CK signalling within the rice plants. This would go some way in explaining the lack of differences between the control conditions and imposed stress conditions in the glasshouses (Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.11 and Figure 3.13). Given that no differences were observed between the control and stress treatments, or the control and CK antagonist application in the growth chambers (Figure 3.12 and Figure 3.14), this also suggests that other variables in the experiments may not have been optimised for the rice varieties used – such as the growth media. The lack of effect of the salt or drought stress in these experiments is unexpected, as this conflicts with the data from preliminary experiments and in the literature. Indeed, Almeida et al. (2016) state that the developmental stage of the rice plant strongly influences plant sensitivity to stress, with rice plants having high susceptibility to abiotic stress during the seedling stage.

Root-specific reduction of CK has been found to increase drought tolerance and root length (Werner et al., 2010), as has root-specific overexpression of cytokinin dehydrogenase (Ramireddy et al., 2018). This suggests that root-specific alteration to CK activity could be a viable solution, but the interplay between signalling molecules and the pleotropic nature of CK makes CK signalling modification a complex research area that must be refined.

3.5.3 Areas for further research

Although glasshouse experiments performed at Globachem in Belgium provide tantalising glimpses into the effects of seed priming with RGPs in rice, data collection was limited to three weeks in October 2019. In addition, access to glasshouse space was restricted limiting the overall number of experiments that could be performed during this period. Therefore, whilst these data represent an initial starting point for exploring the application of the RGPs under commercially relevant conditions, further glasshouse and field trials are necessary for a more extensive analysis.

Importantly, several of the methods used in Chapter 2 were unsuitable for use in a commercial glasshouse environment at Globachem Headquarters in Belgium due to the time and space constraints of the commercial growing environment. These include the selection of uniformly germinated seed for experiments and the establishment of the optimum concentration of RGPs for Loto and Selenio. Whilst it was not possible to investigate this, and the effects of other variables within the scope of the present study, these should be considered as suitable areas for further investigation in subsequent work.

3.5.4 Conclusion

The experiments in this chapter assayed whether compounds which decrease root-specific CK activity led to an increase in abiotic stress tolerance. Whilst this was found not to be the case, the findings by Wang et al. (2015) and Liu et al. (2020) illustrate the relationship and fine balance that exists between CK signalling, ROS and abiotic stress application. These studies, and the results from this chapter, illustrate the delicate nature and complexity of modifying hormone abundance in plants in a commercially viable and robust way.

An interesting avenue for further development would be to explore the mechanism behind the differential responses to CK for the different rice cultivars. Cultivars are well known to display variation under a variety of environmental conditions (Kakade et al., 2017, Gupta and Ahmad, 2014, Wade et al., 1999), yet the differential response to CK signalling in different rice cultivars is yet to be elucidated. Another promising area for development would be to establish an inert seed coating that made the product more robust to environmental variation, such products have been developed to help priming compounds bind and persist on the seed (Amirkhani et al., 2014), but have not been optimised for this study.

4 Exploring the long-term efficacy of priming *Oryza sativa* with novel root growth promoters

4.1 Introduction

4.1.1 Modifying root growth could help to meet challenging agricultural targets

Rice is the primary calorie source for over half of the global population (Huang et al., 2013). There is, therefore, great interest in achieving high rice yields. In order to meet the fuel, fodder and biofuel demands of an increasing global population, the productivity of agricultural land must be optimized (Pan et al., 2013). Novel solutions must be developed in order to meet these demands and increase crop productivity (Ray et al., 2012). One approach which has recently come to the fore is the use of plant growth regulators (PGRs) to modify root growth and potentially improve crop stress tolerance, productivity and yield (Fahad et al., 2016). Cytokinins (CKs) are implicated in the control of root development (Werner and Schmülling, 2009) and have been suggested as potential root growth promoters (RGPs). Several studies have examined the effects of altering the activity of CK in the root, either genetically (Pospíšilová et al., 2016) or through the application of a synthetic CK antagonist (Arata et al., 2010), to promote root growth. However, when manipulating CK abundance or CK perception in the roots, whether via transgenic or chemical methods, it is essential that the effects are root-specific and that there are no negative effects on aboveground biomass and yield (Pospíšilová et al., 2016).

It is estimated that 10% of crop yields are lost due to drought stress (Kim et al., 2019). Climate models predict that severe drought events will increase in frequency in major crop producing regions, many of which are reliant on rain-fed systems with low irrigation input (Fukai et al., 1999, Ramireddy et al., 2018). Another limiting factor to plant yields is often nutrient acquisition capacity (Chapman et al., 2012), which is counteracted by application of fertilizers. Application of artificial nutrient addition using fertilizers is costly, laborious and has a negative effect on the environment through leaching and surface runoff (Syers et al., 2008). This in turn causes a surge of nutrients in the water table and leads to events such as eutrophication (Syers et al., 2008). Understandably, these harmful losses must be minimised.

Transgenic barley with root-specific CK degradation has been found to maintain higher water content than control plants under drought stress, in addition to improved recovery and yield post-drought stress (Pospíšilová et al., 2016, Ramireddy et al., 2018). The improved drought tolerance was caused by the modified root morphology allowing dehydration avoidance (Pospíšilová et al., 2016). The findings of Pospíšilová et al. (2016), together with those of Arai-Sanoh et al. (2014) in deep rooting transgenic rice, show that plants with longer roots have the capacity to access more water and nutrients than their unmodified counterparts, offering the potential to reduce fertilizer application and make crops more resilient to drought. This suggests that the manipulation of root CK levels and/or root CK signalling has the potential to improve the drought tolerance of crops by promoting increased root growth.

In Chapter 2, five compounds were identified which promote root growth in rice. Interestingly, these RGPs were found to be as effective at promoting root growth by seed priming as they were when the compounds were suspended in liquid media, using similar proportions for each treatment and concentration (Figure 2.5 and Figure 3.3). This raises the intriguing possibility of delivering RGPs in a commercial growing context via seed priming. A key component for exploiting the root growth promotion effects of PGRs and the benefits of priming conferred, however, is to ensure that the positive effects on root growth do not occur at the expense of aboveground biomass and productivity in the form of negatively affecting yield harvest. Previously, Ramireddy et al. (2018) genetically modified barley to overexpress cytokinin oxidase, resulting in an increase in root mass of up to 47%. Yield-related traits of the transgenic plants were not significantly different from those of the wild type at $p<0.05$. Additionally, Arai-Sanoh et al. (2014) produced transgenic rice that had deep rooting and an increased yield, confirming that increased root biomass can be achieved without a reduction in shoot biomass. Therefore, it is important to also demonstrate that the priming effects of RGPs on root growth do not confer a negative effect on aboveground biomass and productivity and yield in this study.

4.1.2 Spatial heterogeneity severely affects the results of glasshouse trials

When studying the effects of RGPs on root growth and morphology and plant yield under glasshouse conditions it is essential to be able to decouple the effect of climatic spatial heterogeneities from the effect of the RGP treatments. The environmental conditions in glasshouses are typically controlled to improve crop yield and quality; and involve modifying conditions such as temperature, CO_2, light intensity or humidity (Xin et al., 2019). Glasshouse controls can improve the productivity of a crop; however, they tend to cause spatial heterogeneity leading to microclimates within the glasshouse. The fluctuation in growth conditions in glasshouses is a common phenomenon which is well documented (Kimura et al., 2020, Teitel et al., 2010, Boulard et al., 2002, Zhao et al., 2001), the solution, to which, has been the source of debate for many decades (Kempthorne, 1957, Brien et al., 2013). Potvin et al. (1990) found three distinct types of spatiotemporal heterogeneity: within growth facilities of the same specification, fluctuations in programmed conditions over time within a growth chamber, and consistent spatial variability within a growth chamber, the latter referring to the middle of the growth facility being consistently warmer with the best growing plants.

Whilst there has been extensive modelling on glasshouse-specific variables, little research addresses how data for the plants growing in research glasshouses should be handled (Kimura et al., 2020, Teitel et al., 2010, Boulard et al., 2002, Zhao et al., 2001). There are two generally accepted approaches to mitigate the effect of spatial heterogeneity. One of which is to implement a 'conveyor system' where plants are moved, though this has been found to result in increased plant injury, significantly increases labour and may result in unobserved bias. The conveyor system requires each plant to spend equal time in each position during the same growth stages. It has been found to increase error of variance, though one perceived benefit of this treatment is that error variance is increased equally for each treatment type (Brien et al., 2013). In a similar approach, the implementation of randomised block design is a method of mitigating edge effects; the edge effect is still present, but it is equally distributed throughout the treatments. Neyman and Fisher (1992) were amongst the first to develop an experimental design to account for spatial heterogeneity in agricultural experiments. Neyman's model is based on completely randomized experiments and has subsequently been extended to look at the effect of causation in observational and experimental studies and outputs an average causal effect of a variable, such as location (Rubin, 1990). Similarly, Fisher's experimental design, coined a 'Latin square', is an *n* x *n* array in which treatments are placed so that each treatment occurs once in each row and each column (Fisher, 1992). These well-known and adopted approaches have been scrutinized and optimised for more complex models in recent years (Bailey and Druilhet, 2014, Bailey, 2017, Sabbaghi and Rubin, 2014).

Despite the logistical challenges of the conveyor system approach, Brien et al. (2013) found no evidence to suggest it is more precise than accounting for heterogeneity in the statistical analysis. Glasshouse trials are an essential aspect of plant research, and the spatiotemporal heterogeneity within these controlled environments are widely acknowledged, anecdotally and in literature (Boulard et al., 1999, Majdoubi et al., 2009, Hansen and Høgh-Schmidt, 1996, Ma et al., 2019). Despite this, and despite statistical analysis being acknowledged as the preferred method for correcting this heterogeneity (Brien et al., 2013), a robust method to identify and account for microclimates within a space has not been incorporated into routine practice. Therefore, in the present study, it is important to develop a spatial heterogeneity analysis to understand whether trends in data are attributable to the plants' location or treatment or to the effects of the applied RGPs.

Spatial autocorrelation analysis has been implemented for agricultural field data in order to apply agronomic inputs more efficiently (Koutsos et al., 2021). Whether locally or globally, this is a powerful statistical method of identifying relationships between data and understanding whether this relationship is due to its location or another factor. Spatial autocorrelation methods include Global Moran's I tests whether neighbouring geographic units are more similar than would be expected under the null hypothesis (Bivand et al., 2009) and Local Getis-ord Gi* which produces a p and z-value for each data and identifies whether high or low values are clustered together, producing 'hotspots' and 'coldspots' (Getis and Ord, 1992). This method allows the effect of location to be calculated and used as a weighting factor for statistical analyses. The method not only quantifies the effect of location, it also provides a simple visual cue of the effect of location on each variable. This method removes the need for discounting locations using basic elimination techniques which are frequently practised in glasshouse experiments.

4.2 Aims and objectives

This chapter aims to investigate the effects of priming on aboveground physiological and yield-related traits of glasshouse-grown rice plants. Specifically, this chapter will address the following objectives, which are to:

1. Create a novel method for quantifying the extent to which spatial heterogeneity affects different plant traits.
2. Create a method to weight data, to allow the incorporation of spatial heterogeneity data into statistical analyses.
3. Use techniques developed in points 1 and 2 to decouple the effect of climatic spatial heterogeneities from the effect of root growth promoter treatment and establish whether the tangible benefits of priming in seedlings are conferred to aboveground traits of mature plants under glasshouse conditions.

4.3 Materials and Methods

4.3.1 Seed preparation and plant growth

O.sativa cv. Nipponbare seeds were sterilised using the methods described in section 2.3.1 and primed using the methods described in section 3.3.1. For each compound that was identified as a root growth promoter (RGP) in Chapter 1 (compounds 974, 1027, 1070, 1093 and 1131), 12 replicates for each concentration (0.96, 3.2, 9.6, 32, 96µM) of each compound were pre-germinated and the seed vigour index for each was calculated (see section 3.4.1 for results). These seedlings were then transferred to deep pots (12 x 12 x 20cm, W x D x H) filled with compost (Potting Supreme No.2, Petersfield). The control seeds had no compound applied and were prepared as described in section 3.3.1.

Pots were placed in a glasshouse and arranged in a block design with 12 replicates per treatment (Figure 4.1). Supplementary lighting was provided 0600-1800 GMT to maintain the light intensity in the glasshouse at 450-500 W/m^2 controlled by an automated glasshouse climate system (Ridder). Temperature within the glasshouse varied depending on the time of year (Table 4.1). Plants were watered daily and grown until they had reached maturity.

Table 4.1. Ridder climate system temperature data for the duration of the experiment.

Date	Minimum Temperature (°C)	Maximum Temperature (°C)	Average Temperature (°C)
August 2019	17.5	39.1	24.6
September 2019	17.3	34.8	24.5
October 2019	17.2	30.2	23.1
November 2019	16.9	28.3	22.1
December 2019	17.1	28.2	21.9
January 2020	17	27.5	21.8
February 2020	16.9	28.1	21.8
March 2020	16.6	34	22.1

4.3.2 Glasshouse trial and trait evaluation

Table 4.2 shows the list of traits determined at the end of the experiment. Yield harvest data were collected when plants reached maturity. The methods outlined were adapted from the yield harvest analysis method detailed by Xiao et al. (1998). Plant height was calculated as the average number of centimetres from the ground to the tip of the tallest panicle (excluding the awn). Panicle length was measured as the average number of centimetres from the panicle neck to the panicle tip (excluding the awn) based on an evaluation of three panicles from three plants. The panicle neck, which has a cylindrical structure, was treated as the dividing point between the stem and the panicle. Panicles per plant was the average number of panicles on all plants for each treatment (n=12). Number of tillers was the average number of tillers on all plants for each treatment (n=12). Spikelets per panicle and flag leaf length were calculated by counting the total number of spikelets and length of flag leaf from three panicles from three plants, divided by the nine panicles sampled from the three plants from each treatment.

Grains per panicle was the average number of filled spikelets from the three plants divided by the number of panicles sampled from the three plants (n=9). Seed set rate/spikelet fertility was calculated as a percentage: the number of filled spikelets per panicle divided by the number of spikelets per panicle. Grains per plant was the average number of filled spikelets on each of the three plants analysed. Grain morphological traits such as: filled and unfilled spikelet number (to determine spikelet fertility), spikelet length and width were calculated using the calibrated SeedCounter App (Komyshev et al., 2017).

Table 4.2 All physiological measurements taken from O.sativa cv. Nipponbare primed with different seed treatments. Group 1 measurements taken for every plant (n=12). Group 2: three measurements taken per plant, for three plants per treatment (n=9). Group 3: grain morphological traits taken from three panicles on a plant, for three plants per treatment (n=9).

	Plant morphological traits measured	
Group 1	**Group 2**	**Group 3**
Plant height	Spikelets per panicle	Spikelet length
Panicles per plant	Flag leaf length	Spikelet width
Number of tillers	Spikelet fertility (%)	Spikelet area
	Panicle length	Spikelet rugosity
		Spikelet roundness

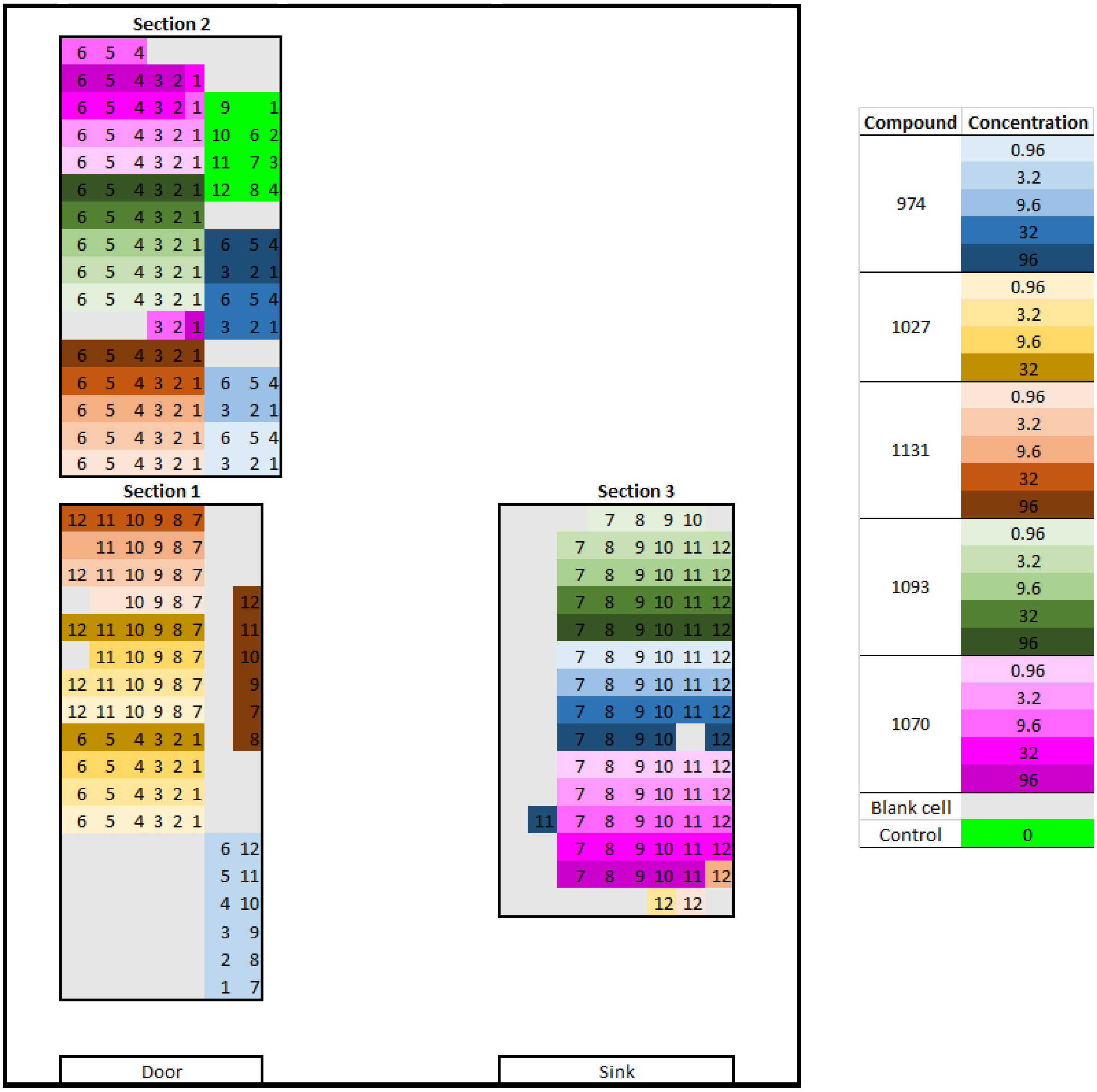


Figure 4.1. Diagrammatic representation of the experimental design in the glasshouse. Plants were arranged in a block design with 12 replicates per treatment. Treatments are colour coded by compound and concentration.

4.3.3 Local spatial autocorrelation analysis

A Global Moran's I analysis was carried out in R to identify spatial autocorrelation (the tendency for variables to cluster based on location) (R Core Team, 2021). Observed values that are significantly greater than the expected value indicate that the values of the dependent variable are positively autocorrelated. In contrast, if the observed value is less than the expected value, this is indicative of negative autocorrelation. A positive Moran's I index value indicates tendency toward clustering, while a negative Moran's I index value indicates tendency toward dispersion (Figure 4.2).

Global Moran's I is designed to reject the null hypothesis of spatial randomness in favour of clustering. When the Global Moran's I rejects the null hypothesis, the identified clustering is a characteristic of the complete spatial pattern and does not provide an indication of the location of the clusters (Bivand et al., 2009). To identify cluster locations, a local indicator of spatial association (LISA) must be performed. LISA provides a significance value for each location and a proportional relationship between the sum of local statistics and a corresponding global statistic, allowing identification of which areas are positively or negatively affected by location, and the statistical significance of the effect (Bivand et al., 2009).

Local Getis-ord Gi* hotspot analysis, a local spatial autocorrelation analysis established by Getis and Ord (1992), was performed in QGIS 3.10 to identify where hotspots and coldspots in the data existed. The ability to calculate hotspots often produces qualitative, visual results rather than statistically robust results. The analysis created by Getis and Ord (1992) uses a quantitative measure of spatial autocorrelation, and the calculation of statistically significant hotspots. The p-value for each data point provides a value to allow weighting of each plant in the subsequent analyses.

Local Getis-ord Gi* was used as a method to weight variability that occurred due to location for all variables except spikelet fertility. Spikelet fertility was not included due to the data needing prior manipulation. Spikelet fertility is a percentage for each panicle (filled spikelets/total spikelets x 100), for which the average of 9 panicles was taken. Additionally, spikelet fertility data for compound 1070 had to be imputed. Given these caveats, it was deemed inappropriate to apply a further level of manipulation to the data.

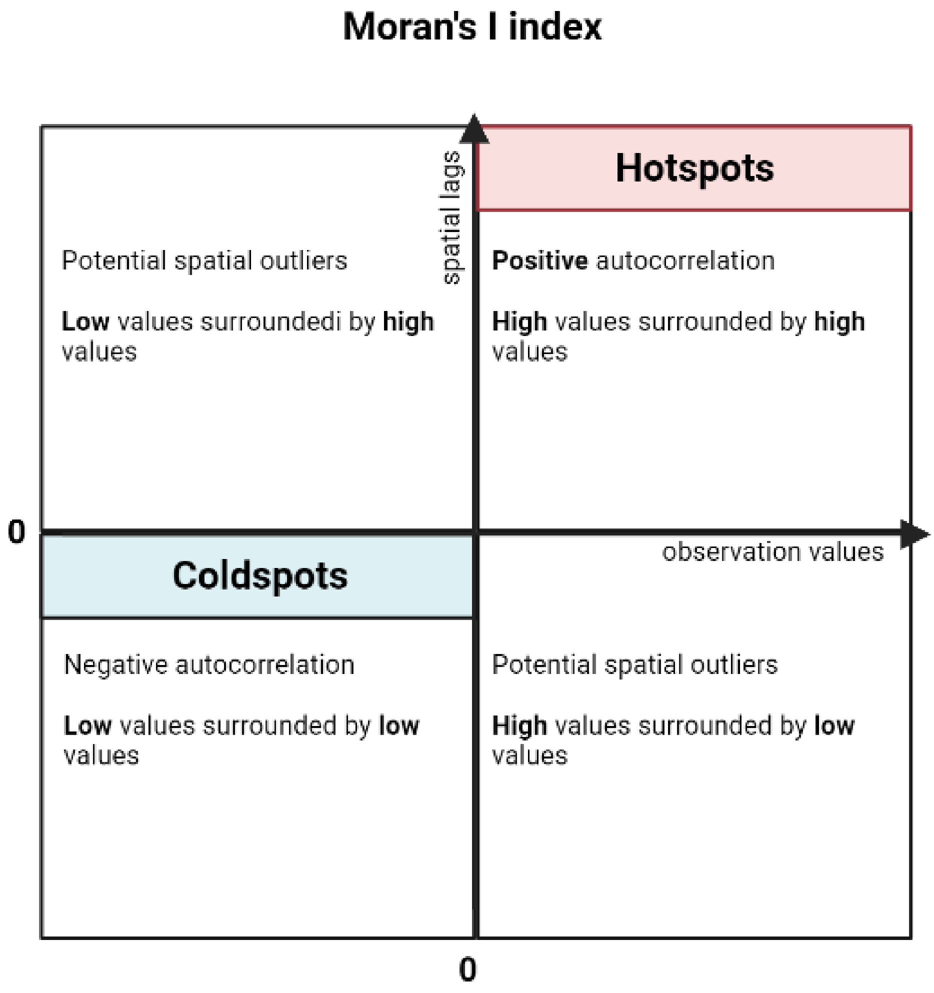


Figure 4.2. The quadrants of a Moran Scatterplot, modified from Koutsos et al. (2021), provide a visual representaton of the parameters which dictate whether data are positively or negatively autocorrelated.

4.3.4 Statistical tests

All Group 1 and Group 2 variables (see Table 4.2), except for panicle length, had skewed data distribution that was not normalised by using the squared, square root or log value. A Wilcoxon test was used to establish whether there was a significant difference between each concentration of a compound and the control. The test was weighted by the hotspot value and p-value obtained from the Getis-Ord Gi* values, those that had a small p-value were given greater weighting by using the calculation:

Weighting = dependent variable x (1/z score) x (1- p-value)

4.3.5 Imputation: predictive modelling to replace missing data

Two groups, compounds 974:3.2µM and 1027:96µM, were missing from the database. Using the data obtained from the other compounds, missing data were imputated using the Hmisc package in R. The package predicts values to substitute missing data using the existing data from other compounds and variables. Missing data are replaced with predicted values and an uncertainty of the values (R^2) is produced. Muiltiple iterations are run until the highest R^2 value is achieved; in this instance, seven iterations produced the highest R^2 value. By combining the results from the iterations, a single value for each missing data point is produced.

The role of imputation is to replace missing values with a set of plausible values with natural variability and uncertainty. The imputed data eliminates bias that would result from non-substitution of data and allows the assumptions of tests to analyse variance to be met. The weighting used to adjust for hotspots or coldspots (weighting = dependent variable x (1/z score) x (1- p-value)) was used as a covariate for the imputation so that estimates are not disproportionately influenced by samples that were in a hotspot or coldspot (Rosenbaum and Rubin, 1983).

4.4 Results

4.4.1 Global Moran's I statistical analysis of plant height, number of tillers and number of panicles shows that these traits are affected by the plants' location in the glasshouse

Throughout the course of the experiment, it became evident that a plant's location within the glasshouse strongly affected its morphology. To determine the effect of climatic spatial heterogeneities within the glasshouse on the effects of RGPs, a Global Moran's I statistical analysis was performed for plant height, number of tillers and number of panicles. Table 4.3 shows that the observed Moran's I value is greater than the values expected under the null hypothesis, indicating that values are positively spatially autocorrelated. Therefore, the location of a plant within the glasshouse has a significant influence on plant height, number of tillers and number of panicles.

Table 4.3. Results from Global Moran's I statistical analysis. Observed values indicate the computed Moran's I. Expected values are those expected under the null hypothesis. Observed values are greater than expected values. There is a tendency towards clustering of data for plant height, number of tillers and number of panicles and the location has a significant influence on each variable (p<0.001).

	Plant height	**Number of tillers**	**Number of panicles**
Observed	0.15840	0.08065	0.12784
Expected	-0.00310	-0.00310	-0.00310
Standard Deviation	0.00482	0.00481	0.00483
p-value	0.001	0.001	0.001

4.4.2 Coldspots occur in a column on the outer edge, whereas hotspots occur in a central column

To identify where the differences lie within the glasshouse, further statistical analysis was required. Local Getis-ord Gi* Hotspot Analysis is a local spatial autocorrelation test which produces a z-value, indicating deviation from the mean, and p-value for each data point and variable. The p-value is derived from the z-value, indicating whether the deviation from the mean is statistically significant. A positive z-value refers to the location under study which has similarly high or low values as its neighbours, such a location is called a "spatial cluster". Conversely, a negative value indicates a potential spatial outlier which is different from the values of its surrounding locations.

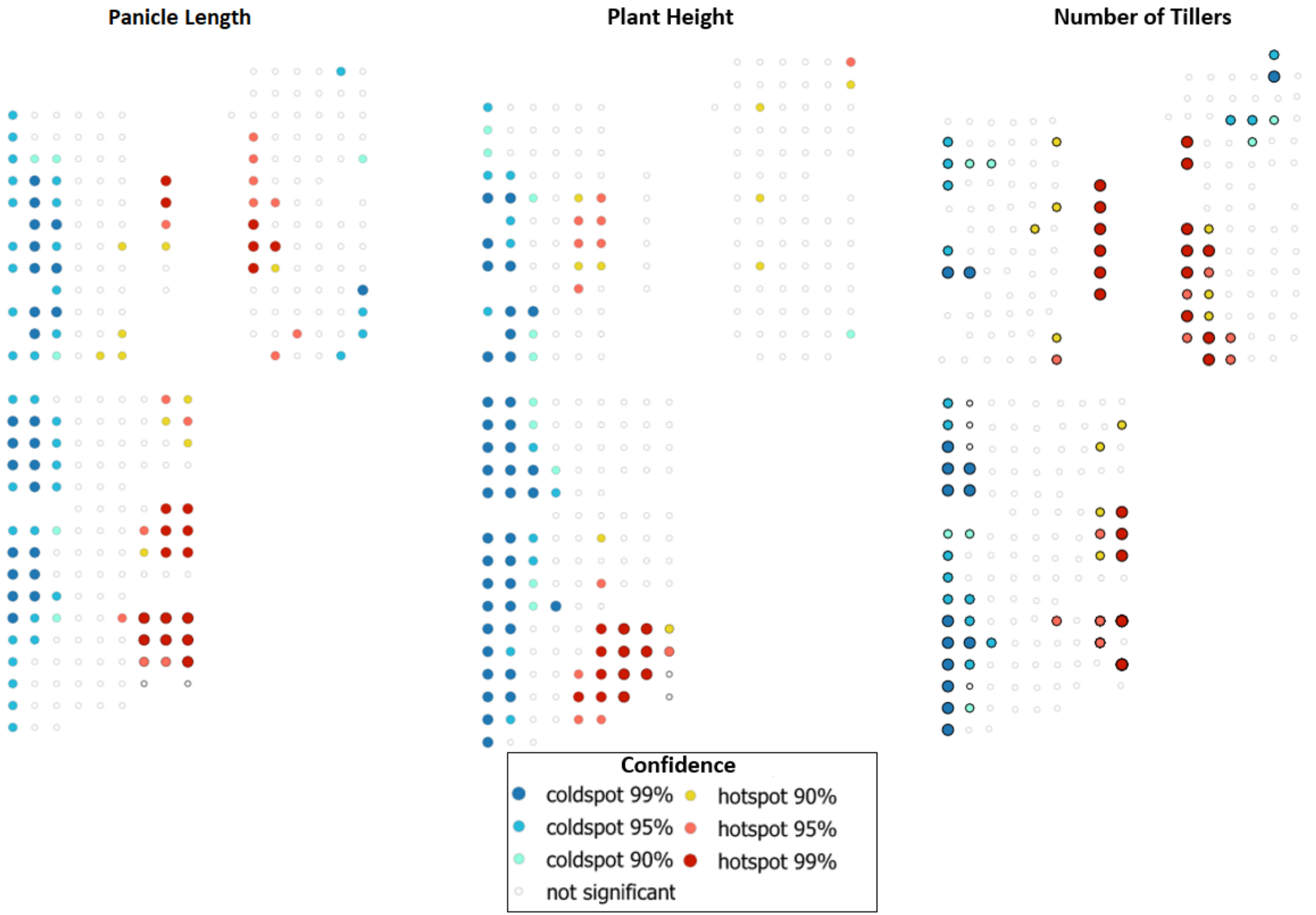


Figure 4.3. Visual data from the Local Getis-ord Gi shows the degree to which positioning of a plant within the glasshouse influenced panicle length, plant height and number of tillers. The perimeters of the glasshouse, particularly on the left-hand side, tended to be negatively affected by their positioning, whereas being in the central column had a positive influence on plant variables. Each circle represents a plant, glasshouse layout is the same as detailed in Figure 4.1.*

4.4.3 Priming with novel RGPs has no significant effect on aboveground biomass development

Aboveground biomass parameters were analysed to establish whether the initial benefits on the root and shoot that were conferred by priming seeds with the RGPs (see Chapter 3) influenced mature plant morphology or yield. RGP treatments had no significant overall effect on plant height or tiller quantity ($p>0.05$) except for the height of plants treated with compound 1131, where plants were significantly smaller than the control (Kruskal-Wallis, $p=0.02$; Wilcoxon-test, 3.2, 32 and 96µM significance $p<0.05$, <0.05 and <0.001, respectively) (Figure 4.4 and Figure 4.5). Compound 974 concentration 0.96µM had a significant increase in tiller quantity (Figure 4.5), however, this increase did not affect panicle quantity (Figure 4.7).

There was no significant effect of RGP treatment on flag leaf length between the treated rice plants and the control (Figure 4.6). Only the 96µM compound 1070 treatment showed a significantly longer flag leaf length ($p<0.05$), however, this increase did not affect panicle length or spikelet number. There was little variation in flag leaf length for any treatment or concentration, with responses comparable to the control for all treatments except compound 1070, concentration 96µM, which had a significant increase in flag leaf length ($p<0.05$, Figure 4.6). After weighting panicle length data to account for spatial heterogeneity within the glasshouse (see section 4.4.2), there was no clear response of panicle length to treatments with RGPs. 1070 concentrations 0.96-32µM had a decrease in panicle length compared to the control (Figure 4.7). However, panicle lengths for rice treated with 1070 concentration 96µM were slightly longer than the control. The decrease in panicle length for compound 1070:0.96-32µM, 974:0.96µM and 1093:9.6µM caused a decrease in spikelet number. Though there was a clear correlation between the two, the decrease in spikelet number was not significantly different to the control in each case (Figure 4.11).

The minimal effect on plant height, tiller quantity and flag leaf length and variable effect on panicle length suggests that the RGPs are mostly root-specific and neither positively nor negatively influence aboveground plant growth. Spikelet number was weakly correlated with flag leaf length ($R^2=0.309$) and spikelet fertility ($R^2=0.247$) and strongly positively correlated with panicle length ($R^2=0.690$) (Figure 4.8). Taken together, the application of the CK antagonists do not significantly affect shoot growth and aboveground biomass development.

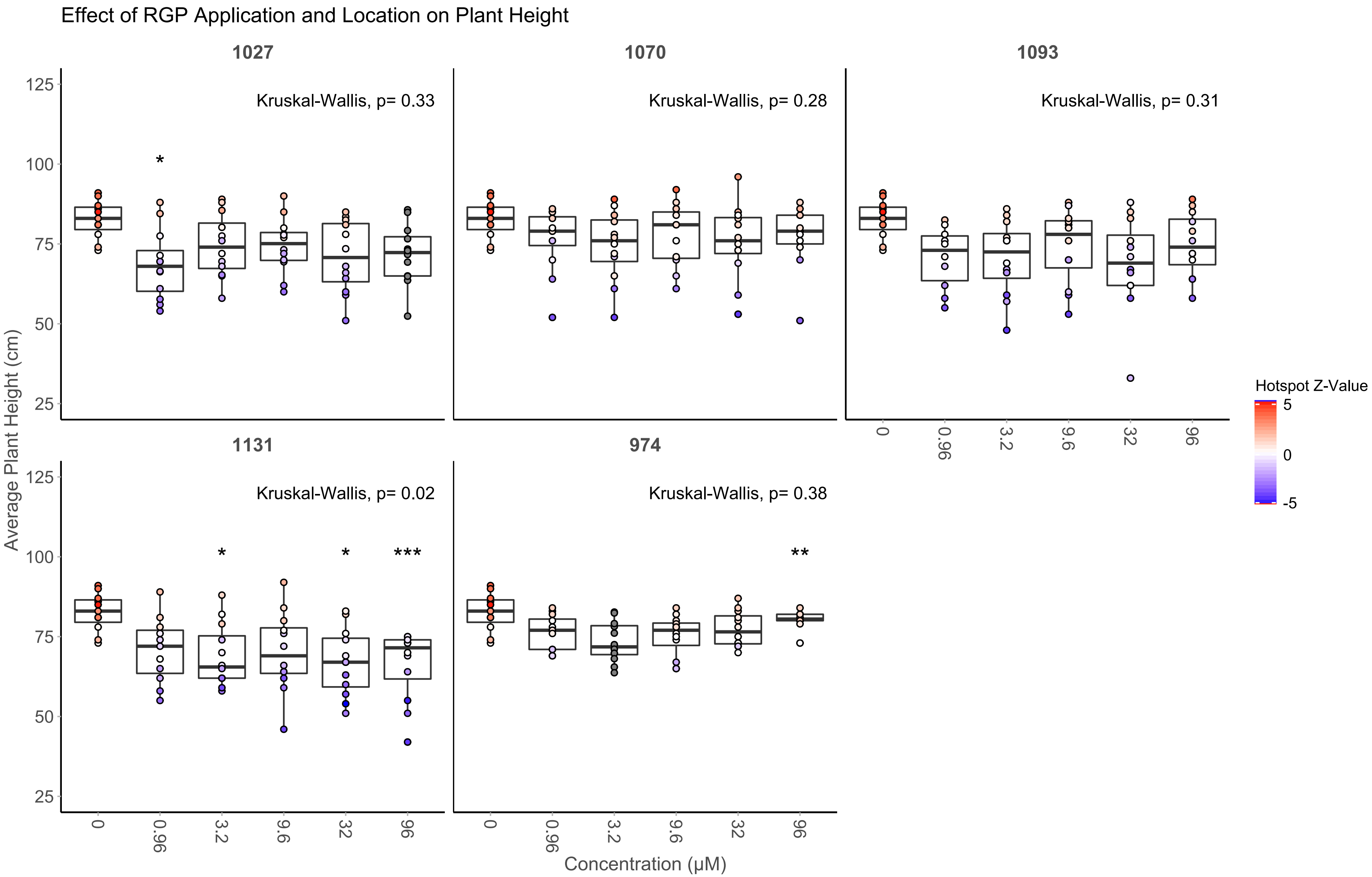


*Figure 4.4 The effect of RGP on plant height, weighted by the extent to which a plants' location affected its growth. Significance values indicate the results of independent Wilcox tests carried out between the control and each concentration of each compound. * = p < 0.05, **=p < 0.01, ***= p< 0.001. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-values indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values (n=12).*

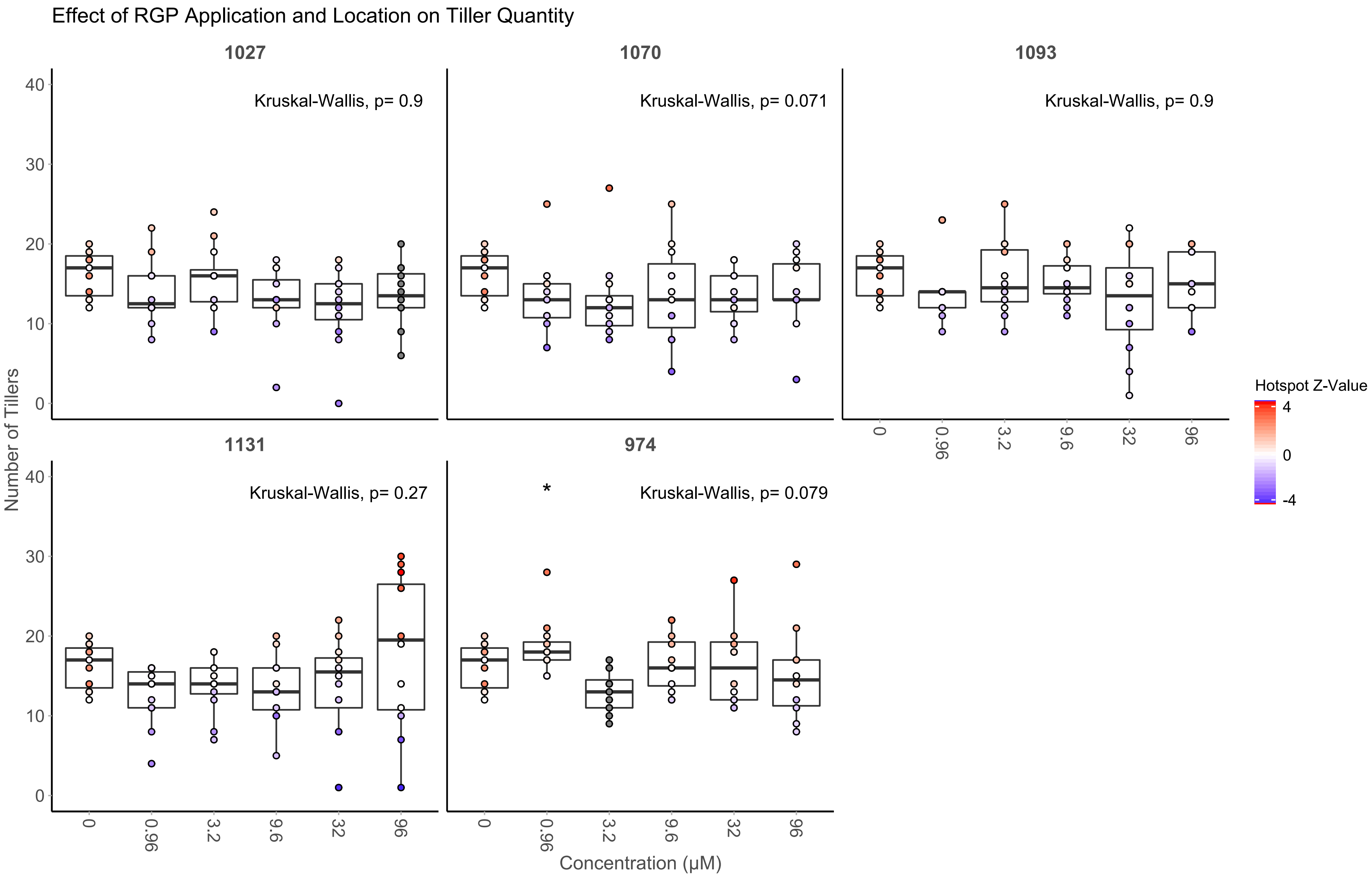


*Figure 4.5. The effect of RGP on tiller number, weighted by the extent to which a plants' location affected its growth. Significance values indicate the results of independent Wilcox tests carried out between the control and each concentration of each compound. * = p < 0.05. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-values indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values (n=12).*

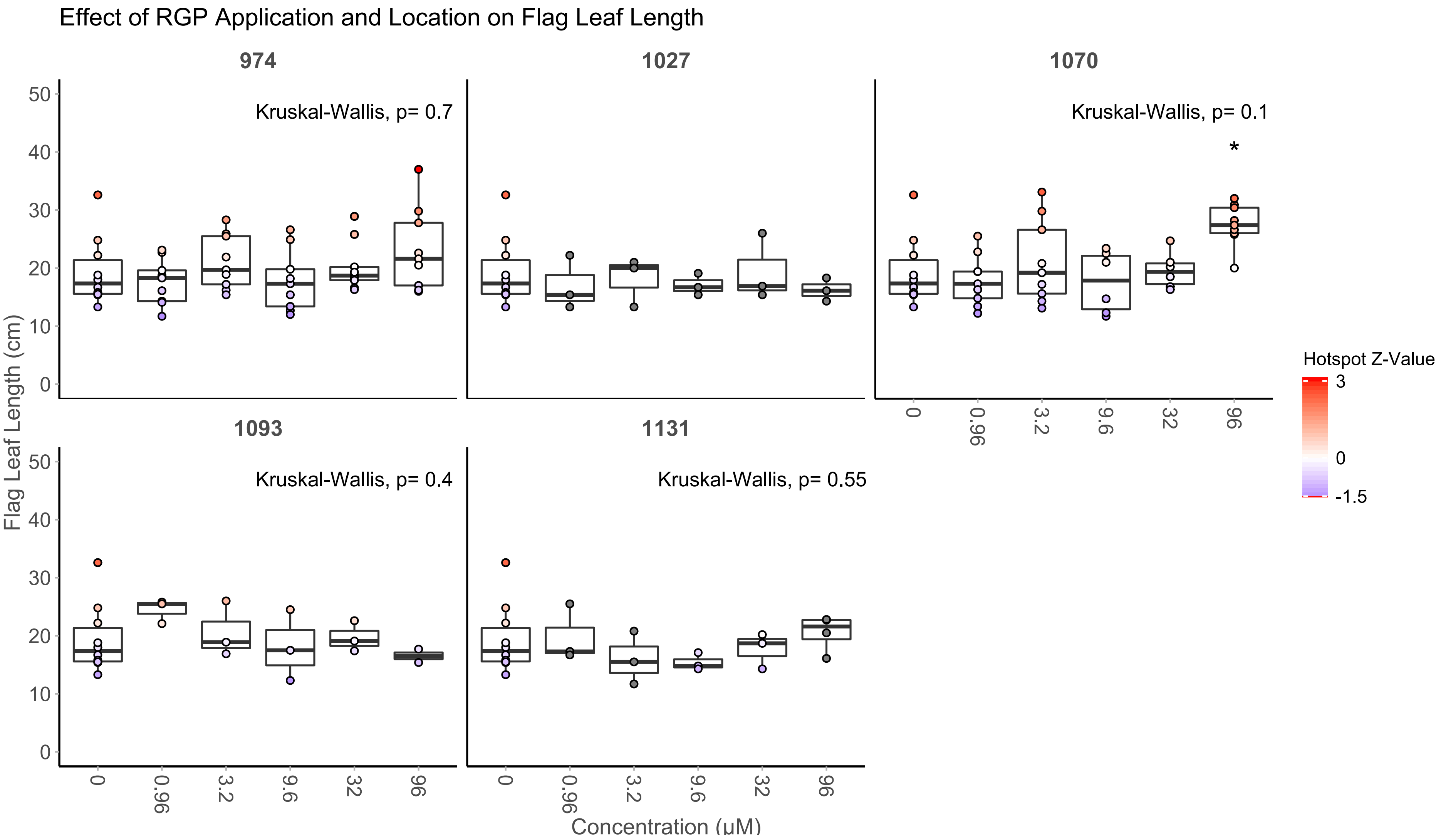


*Figure 4.6. The effect of RGP on flag leaf length, weighted by the extent to which a plants' location affected its growth. Significance values indicate the results of independent t-tests carried out between the control and each concentration of each compound. * = p < 0.05. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-values indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values, compound 1027 has no Kruskal-Wallis statistics due to the values being imputed (n=12).*

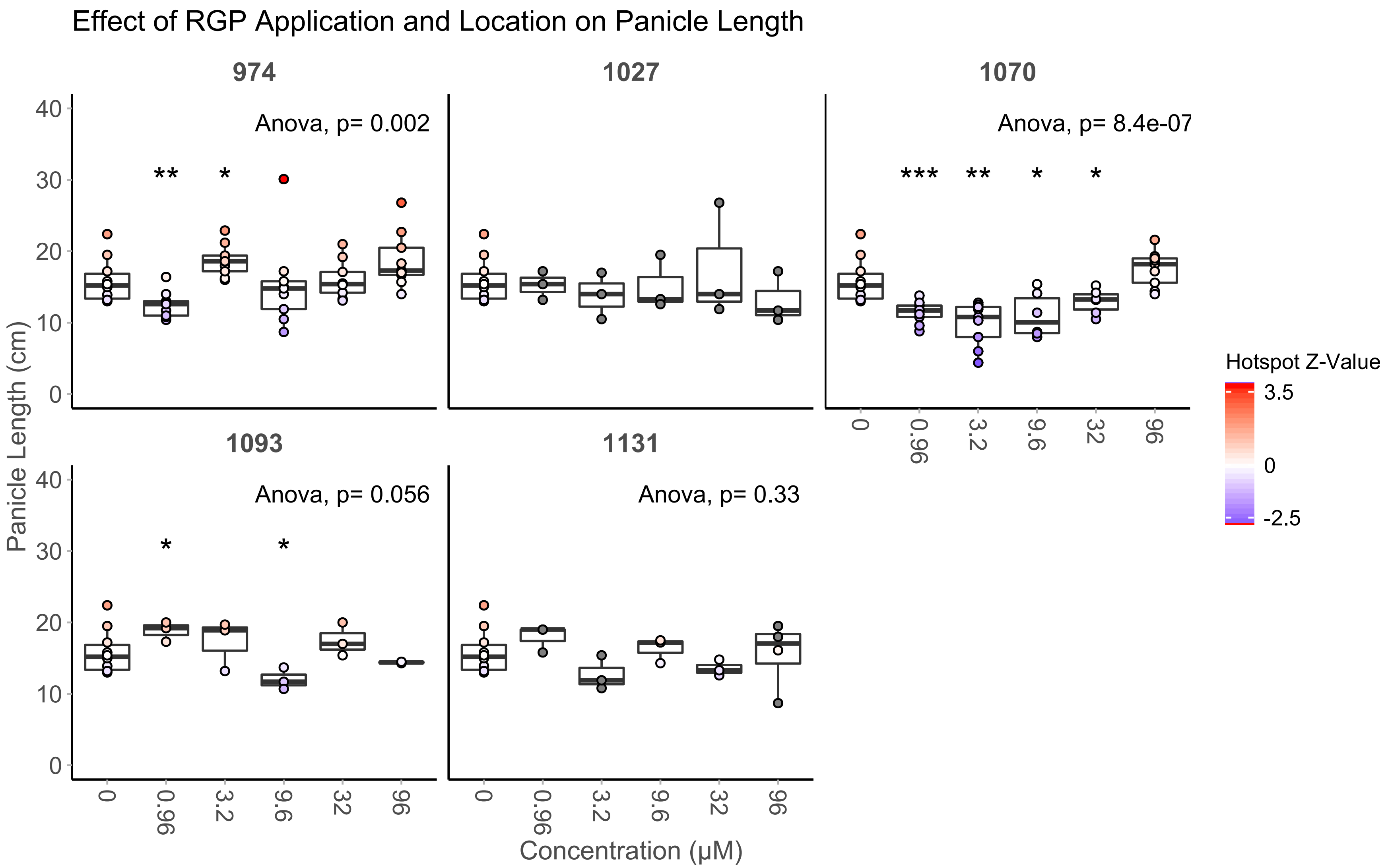


*Figure 4.7. The effect of RGP on panicle length, weighted by the extent to which a plants' location affected its growth. Significance values indicate the results of independent t-tests carried out between the control and each concentration of each compound. * = p < 0.05, **=p < 0.01, ***= p < 0.001. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-values indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values (n=12).*

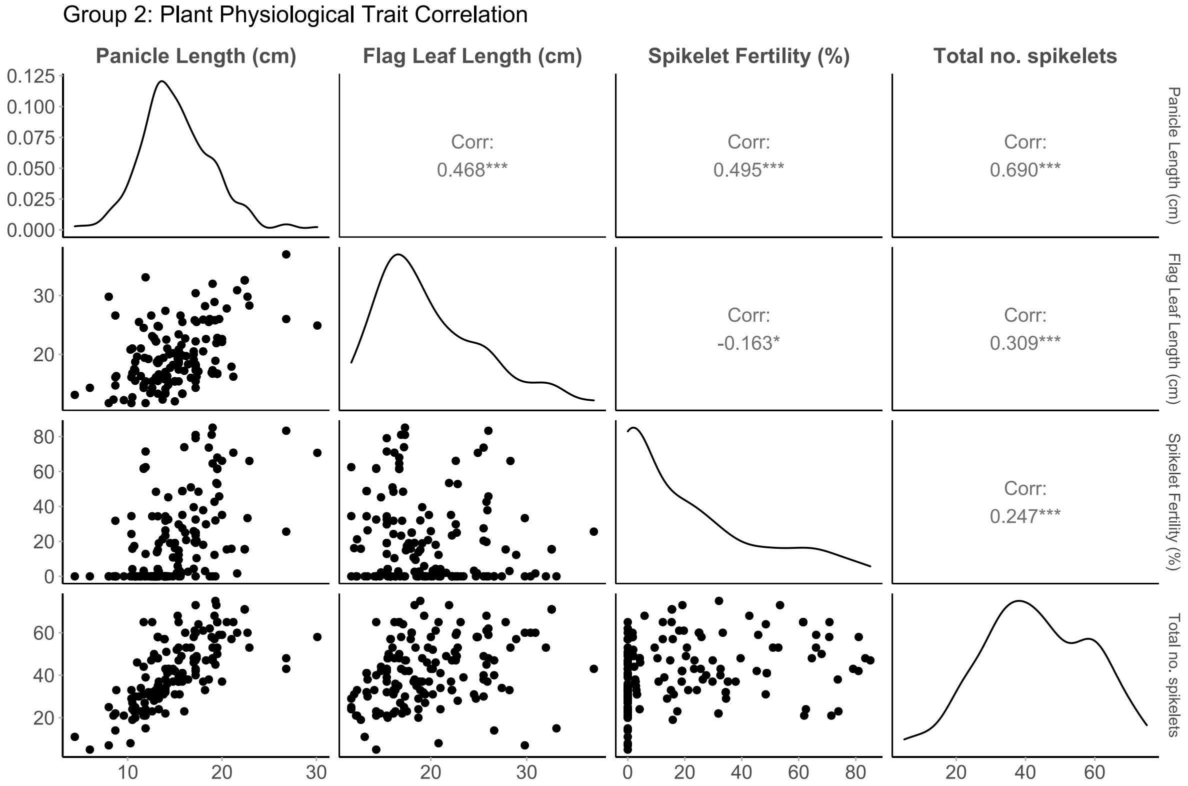


*Figure 4.8 Scatterplot matrix showing correlations from Group 2 variables. There is a weak positive correlation between all variables; except for spikelet fertility and flag leaf length, which have a weak negative correlation. Upper right panels show correlation (R^2 values) and level of significance * = $p < 0.05$, **=$p < 0.01$, ***= $p < 0.001$. Bottom left panels are scatterplots between each variable. Central diagonal panels show the distribution of the data for each variable.*

4.4.4 Priming with novel RGPs negatively affects the reproductive capacity of rice

After weighting the data based on its hotspot value to account for positioning having a detrimental or advantageous effect on growth (see Figure 4.3), a Wilcoxon-test was performed between the control and the different concentration for each treatment. Overall, there is a negative effect on panicle production, however, panicle quantity is only significantly lower for three treatments: 1027 and 1070 concentration 0.96µM and treatment 1093, concentration 96µM ($p< 0.05$) (Figure 4.9). This trend correlates with plant height (R^2=0.636, $p< 0.01$) and number of tillers (R^2= 0.758, $p< 0.01$) (Figure 4.10). This suggests that plant height could be a good indicator or tiller number, which in turn affects panicle quantity and the reproductive potential of the rice plant.

Application of the RGP had no significant effect on spikelet number ($p > 0.05$ for all treatments) (Figure 4.11). These data suggest that when the panicle develops, there is no effect on the number of spikelets produced on that panicle, however, the negative effect on panicle quantity would affect the overall yield. Interestingly, spikelets treated with compound 1070 were completely sterile, with no filled panicles for any treatment (Figure 4.12). This was correlated with a significant decrease in panicle length for concentrations 0.96-32µM (Figure 4.7), indicating that panicle length is a strong indicator of spikelet fertility. For the remaining compounds, a slight bell-curve effect was observed, with significant increases in spikelet fertility for compound 973, concentration 3.2µM and 1131, concentration 9.6µM (Figure 4.12) compared to the control. This increase in spikelet fertility was correlated with an increase in panicle length for compound 974:3.2µM (Figure 4.7).

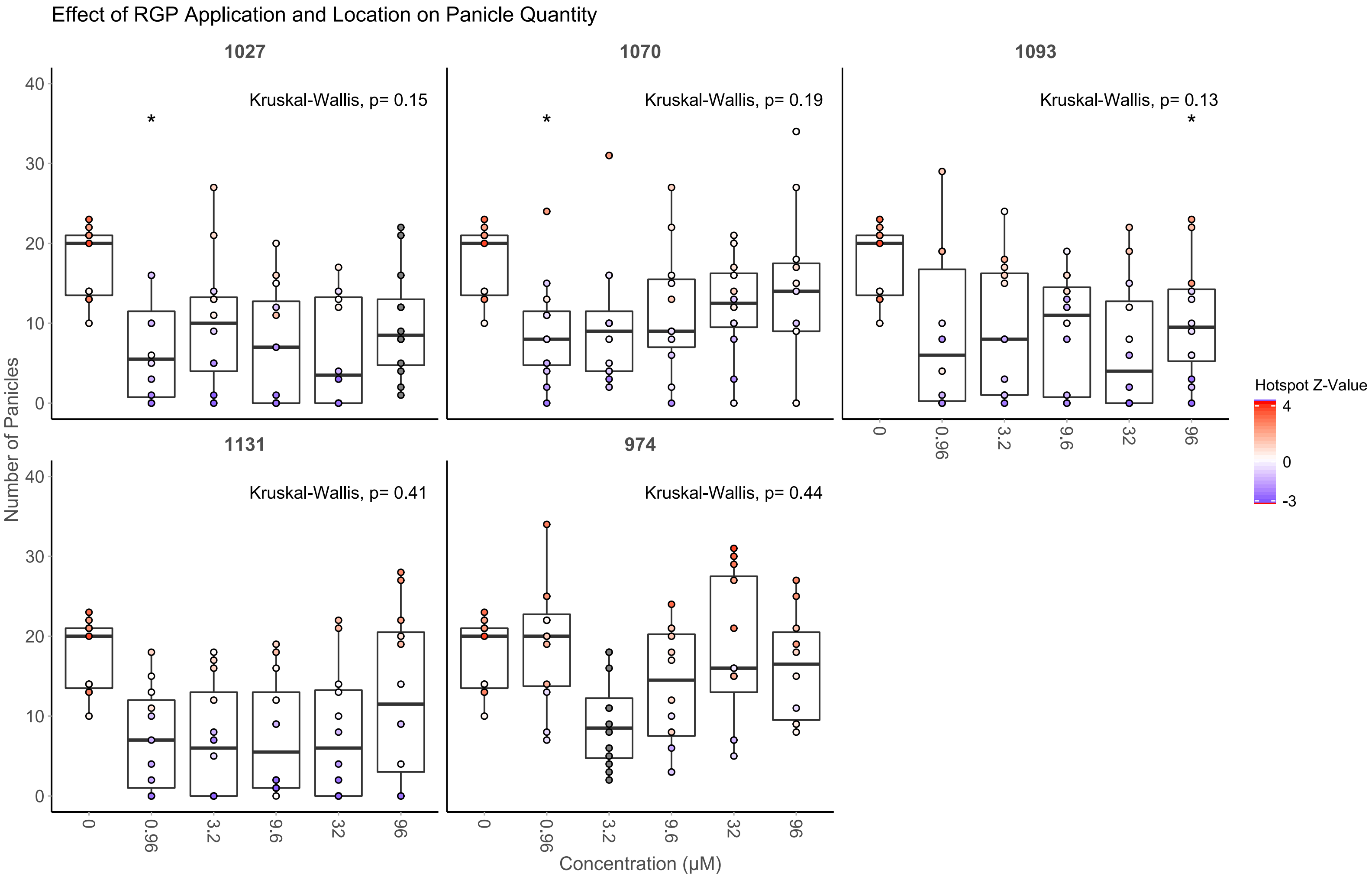


*Figure 4.9. The effect of RGP on number of panicles, weighted by the extent to which a plants' location affected its growth. Significance values indicate the results of independent Wilcox tests carried out between the control and each concentration of each compound. * = p ≤ 0.05. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-value indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values (n=12).*

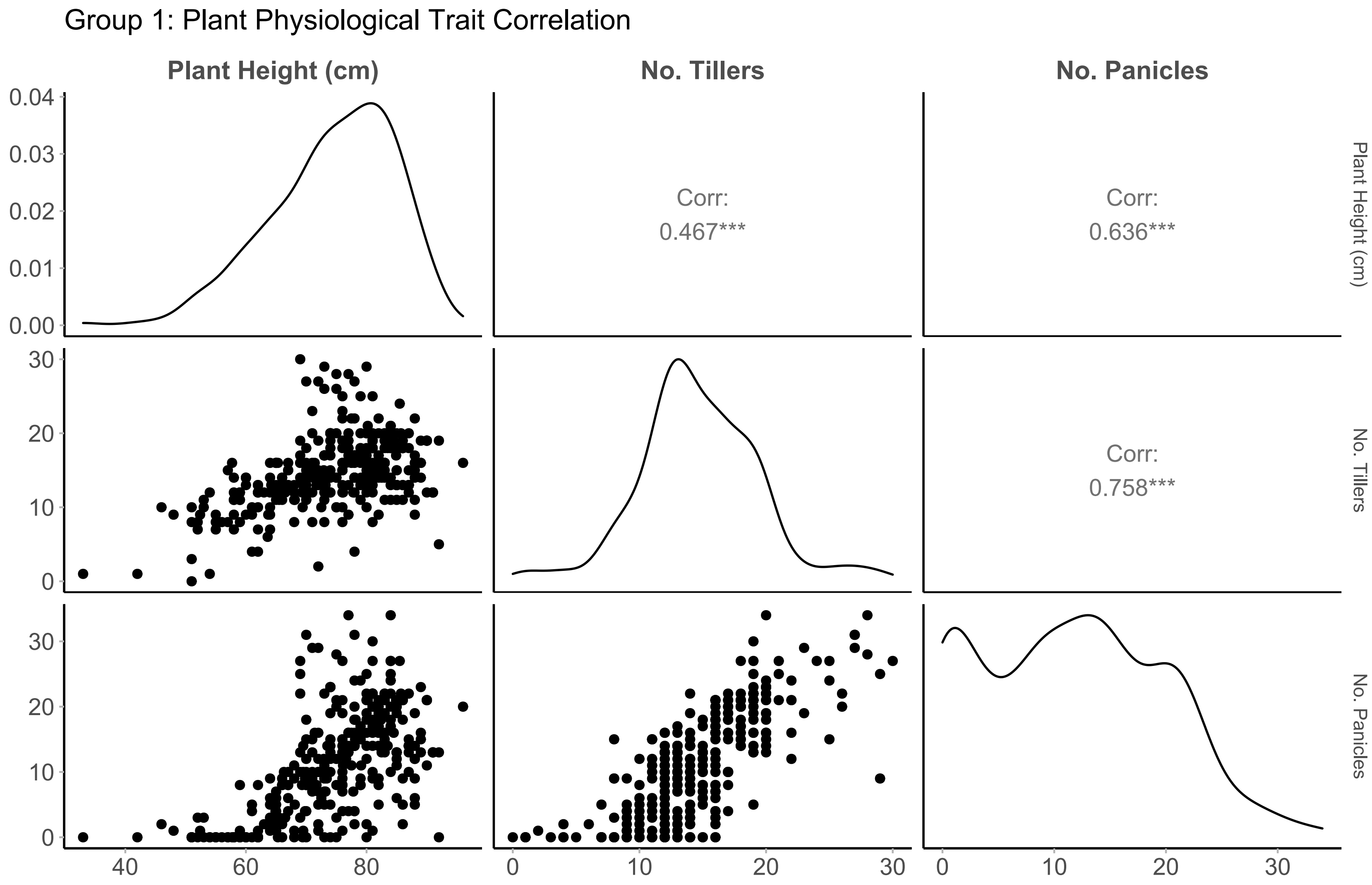


*Figure 4.10. Scatterplot matrix for Group 1 variables. All were positively correlated, p<0.001. The central diagonal column shows the frequency distribution of the data for each variable. Upper right panels show correlation (R^2 values) and level of significance * = p < 0.05, **=p < 0.01, ***= p < 0.001. Bottom left panels are scatterplots between each variable.*

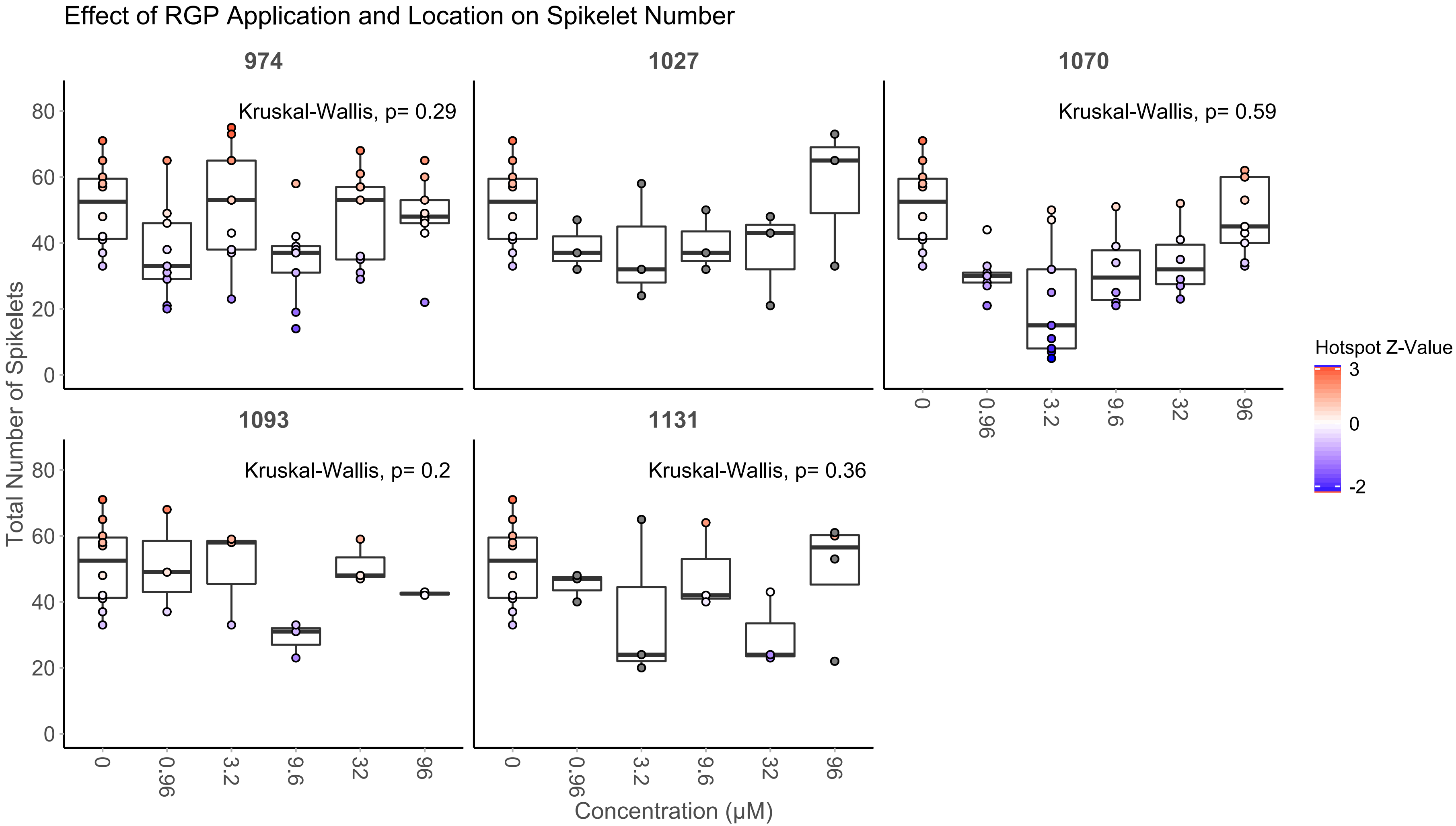


Figure 4.11. The effect of RGP on spikelet number per panicle, weighted by the extent to which a plants' location affected its growth. A Wilcox test was carried out between the control and each concentration of each compound, no concentrations were significantly different from the control. The boxplot displays the median, and the upper and lower quartiles (25th and 75th percentiles). The whiskers extend from the upper and lower quartiles to the smallest and largest values that are no greater than 1.5 times the interquartile range (IQR) from the quartile. Data beyond the end of the whiskers are outliers. Dots represent individual plants. Positive z-value indicate growth higher than expected due to the plants' location, whilst negative z-values indicate growth worse than expected due to the plants' location. Grey dots represent imputed values, compound 1027 has no Kruskal-Wallis statistics due to the values being imputed. Total number of spikelets indicates the number of spikelets on three panicles of three plants (nine panicles in total).

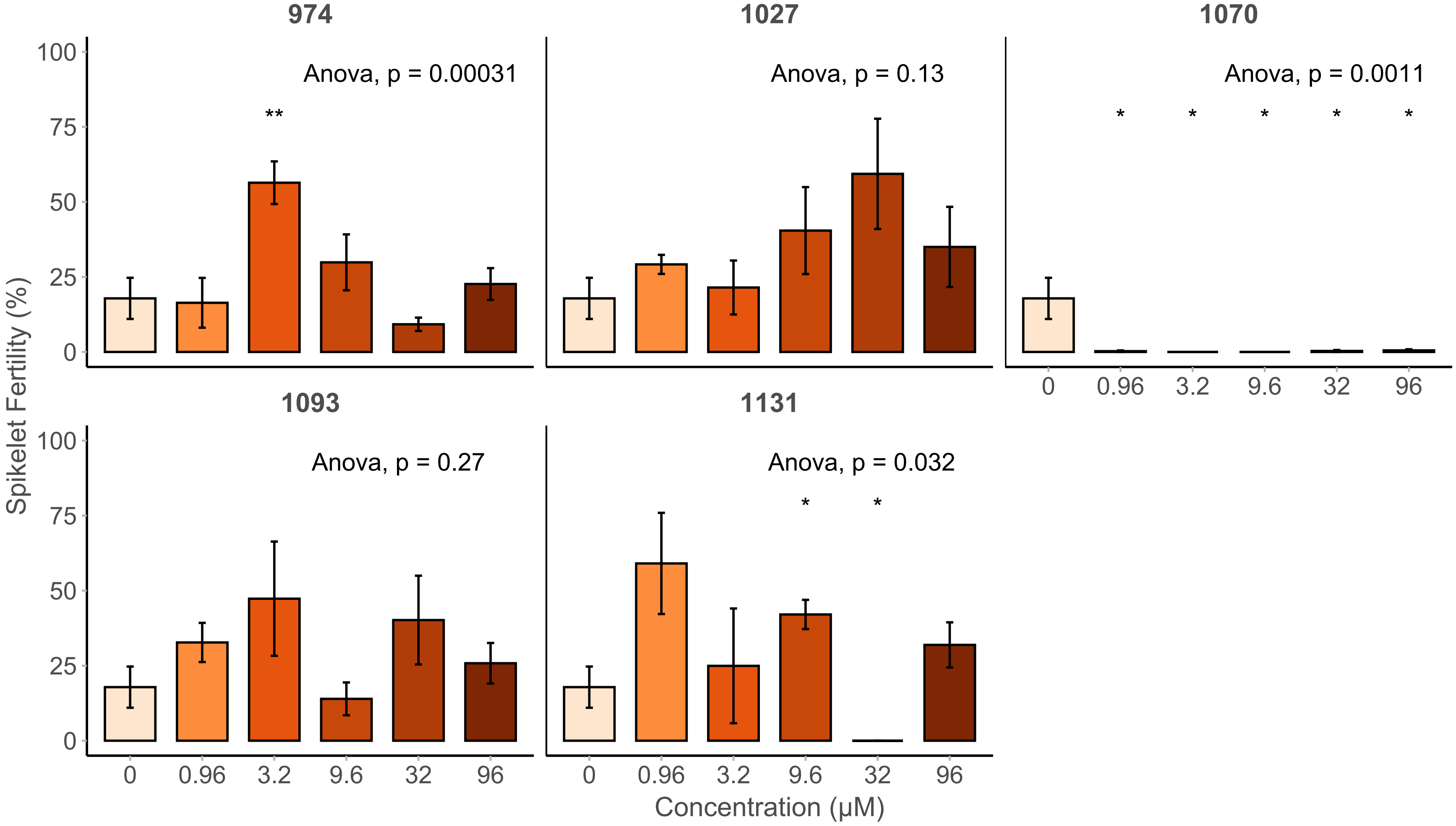


*Figure 4.12. RGP application on spikelet fertility conferred benefits at some concentrations but severely inhibited grain formation for seeds treated with compound 1070. *p ≤ 0.05, **p ≤ 0.01. Compound 1027, concentrations 0.96-96µM are imputed values. Data are the results from total number of filled or unfilled spikelets for 9 panicles per treatment. Values are means ± SEM (n = 9)*

4.4.5 RGP effects on grain size correlates with panicle length but not other preferential grain characteristics

The decrease in panicle length for compound 1070 (Figure 4.7) correlates with a lower mean grain width and length than the control (Table 4.4) suggesting a yield penalty as a result of application of the compound. The decrease in grain width and length is correlated to low rugosity, resulting in both favourable and unfavourable characteristics of a grain (Table 4.4). Two treatments (974:3.2µM and 1093:0.96µM) resulted in an increased panicle length with corresponding increases in width and length of grains, greater than the control (Figure 4.7 and Table 4.4). Despite an increase in width and length of grains for 974:3.2µM, these grains had a higher average rugosity and lower roundness, both of which are characteristic of poor seed quality (Zhou et al., 2019), resulting in both favourable and unfavourable characteristics of a grain (Table 4.4).

The relationships between grain variables; for example, the positive correlation between panicle length and width and length of grain (see Figure 4.7 and Table 4.4), the negative correlation between the length of grain and the rugosity (R^2= -0.111, Figure 4.13), and the weak correlation between seed length and width with roundness (R^2= 0.011 and 0.502 respectively) suggests in this instance that a longer panicle length leads to larger, lower quality seeds (Figure 4.13). These contradictory effects suggest application of the compound would lead to lower quality seed.

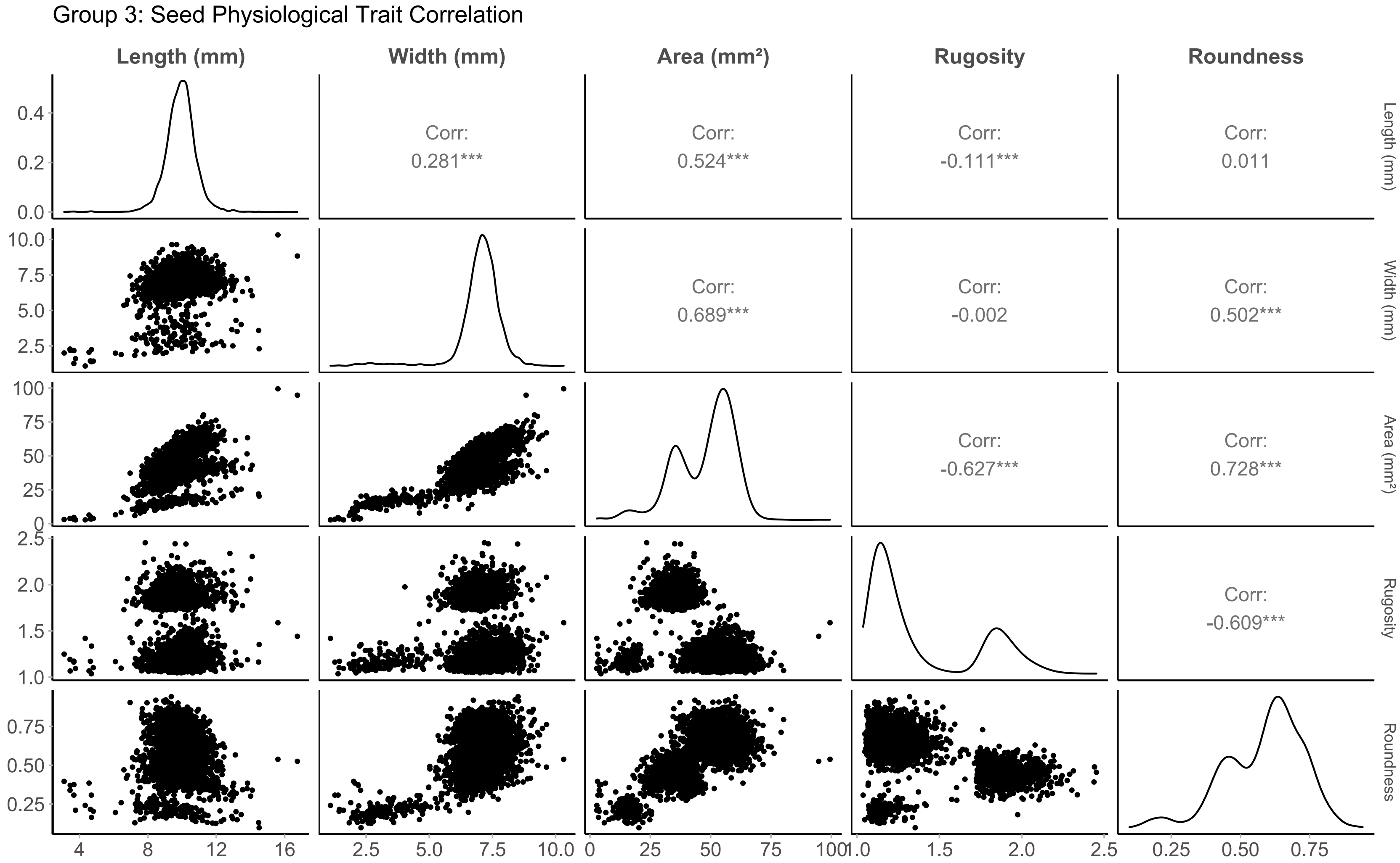


Figure 4.13. The correlation between seed physiological traits, based on data obtained from the SeedCounter app. Data collected are for all seeds collected from three panicles for each treatment. Upper right panels show correlation (R^2 values) and level of significance * = $p < 0.05$, **=$p < 0.01$, ***= $p < 0.001$. Bottom left panels are scatterplots between each variable. Central diagonal panels show the distribution of the data for each variable.

Table 4.4. Grain morphology data for primed seeds show that most seeds were shorter and narrower than the control. The transition from red, yellow and green represent low, medium and high values for each variable, compared to the control in that group. The total number of samples per treatment varies due to every grain that was produced being included in the analysis. Data were collected using the SeedCounter app.

		Length (mm)		**Width (mm)**		**Area (mm^2)**		**Rugosity**		**Roundness**	
Treatment	**Concentration (µM)**	**Mean**	**+/- SE**	**Mean**	**+/- SE**	**Mean**	**+/- SE**	**Mean**	**+/- SE**	**Mean**	**+/- SE**
Control		10.230	0.043	7.143	0.045	49.258	0.595	1.417	0.014	0.578	0.006
974	0.96	9.961	0.043	7.053	0.051	47.618	0.646	1.418	0.018	0.586	0.007
	3.2	10.180	0.043	7.175	0.043	48.102	0.642	1.471	0.018	0.554	0.007
	9.6	9.604	0.039	6.972	0.035	47.200	0.584	1.341	0.018	0.606	0.007
	32	9.748	0.037	6.832	0.039	46.343	0.504	1.344	0.016	0.595	0.006
	96	10.021	0.044	6.868	0.050	45.811	0.611	1.425	0.017	0.559	0.007
1070	0.96	9.395	0.050	6.874	0.050	45.960	0.657	1.305	0.016	0.628	0.008
	3.2	9.834	0.070	6.817	0.097	47.361	1.076	1.346	0.021	0.591	0.011
	9.6	9.529	0.079	6.796	0.071	44.446	0.997	1.405	0.024	0.583	0.010
	32	10.045	0.044	7.315	0.035	53.964	0.602	1.248	0.016	0.652	0.007
	96	9.981	0.044	7.136	0.038	51.484	0.524	1.283	0.014	0.623	0.006
1093	0.96	10.228	0.078	7.138	0.045	51.983	0.993	1.301	0.034	0.604	0.013
	3.2	9.749	0.053	6.934	0.031	43.237	0.800	1.541	0.030	0.555	0.010
	9.6	9.696	0.059	6.901	0.047	50.919	0.667	1.150	0.019	0.659	0.010
	32	10.113	0.076	6.753	0.094	41.847	0.989	1.568	0.030	0.502	0.012
	96	10.033	0.061	7.205	0.076	50.373	0.928	1.353	0.025	0.606	0.011
1131	9.6	9.992	0.072	7.194	0.054	45.058	0.938	1.589	0.030	0.559	0.011
	32	9.904	0.136	7.107	0.126	48.177	1.463	1.401	0.033	0.574	0.015

4.5 Discussion

4.5.1 The effect of spatial heterogeneity within a glasshouse can be quantified and accounted for statistically

Spatial heterogeneity is a well-known phenomenon in glasshouses and growth chambers (Potvin et al., 1990, Edmondson, 1989). Brien et al. (2013) compared the two competing approaches to deal with spatial variation in glasshouses: regular re-arrangement of plants or to keep plants in the same place and adjust for their positioning in the analysis (Hardy and Blumenthal, 2008, Cox and Cochran, 1946, Brien et al., 2013). The latter was found to be preferential as it accounted for variance without increasing standard error of variance. Whilst Brien et al. (2013) acknowledge the opportunity for using spatial analysis designs in glasshouses, this has not been explored in detail until this experiment.

The negative effects of growth experienced by perimeter plants, as detailed by Potvin et al. (1990) were also observed in this study (Figure 4.3). To quantify the effects of positioning on plant growth and yield traits in a glasshouse, local and global spatial autocorrelation analyses were performed and incorporated into the statistical analysis by colour-coding each data point according by their tendency to be positively or negatively affected by positioning (Figure 4.4 to Figure 4.7, Figure 4.9 and Figure 4.11). Adjusted results were obtained from the data that were previously heavily skewed by plant positioning by collecting physiological measurements of the rice plants and weighting them in the statistical analysis according to how strongly that plants' growth had been affected by its location. In addition, this novel method provides a map output for each variable (Figure 4.3) to allow glasshouse users to visualise the extent to which a sample is affected by positioning. This study shows that this novel use of local and global autocorrelation analysis is a useful tool for glasshouse experiments which is preferential to crude techniques such as omitting samples around the edges or laborious, commonly used processes such as moving plants around glasshouses (Brien et al., 2013). Applying this technique for future glasshouse studies would help to reduce the error of variance in data for studies that use a randomized block design and in experiments that rely on plants being moved frequently (Hardy and Blumenthal, 2008, Cox and Cochran, 1946).

4.5.2 Application of the RGPs did not affect aboveground growth but did affect yield

The application of the RGPs did not negatively affect aboveground growth of a plant (Figure 4.4 to Figure 4.7). Despite no severe effects on aboveground plant development, there was a yield penalty for plants that had had the RGP applied (Figure 4.9 and Figure 4.11). In addition, traits that are usually closely correlated, such as flag leaf length and seed fertility (Yoshida, 1981), were found to have a weak negative correlation in this instance (R^2=-0.163 p<0.05, Figure 4.8). These results suggest trends that may negatively affect plant growth in rice nurseries or fields in an agricultural setting (Yoshida, 1981) and raise doubt as to whether these RGPs are suitable for commercial application. Specifically, as discussed in Chapter 1, whilst an increase in root growth would be advantageous to a rice plant, it is imperative that this advantage does not come at a disadvantage to the harvested part of the crop, i.e. the grain (Arai-Sanoh et al., 2014, Gowda et al., 2011, Jeong et al., 2010).

Interestingly, the present study shows there is a slight tendency for the higher concentrations of the RGP compounds to be more efficacious than the lower concentrations, particularly for compound 1070 (Figure 4.6, Figure 4.7 and Figure 4.11). This suggests that whilst lower concentrations are optimum for experiments with young plants (Figure 3.3), a higher dose could have been more suitable for a long-term experiment. Seed coating can be used to aid the slow release of the compounds as in Afzal et al. (2020). Therefore, the use of seed coating in combination with priming with RGPs may be beneficial in longer-term and field-based experiments.

Shading negatively affects root growth, when rice plants produce a large number of tillers this exacerbates the shading, leading to a short root system (Abd Allah et al., 2010). Hence, low tillering is a desirable trait when the plant may be exposed to a drought stress, as the longer roots can access water stored in deep soil (Abd Allah et al., 2010). Unfortunately, low tillering capacity limits the yield potential when grown under optimal conditions. In the present study, tiller quantity was unaffected by application of the RGP compounds (Figure 4.5), suggesting that the root growth promotion activity of the compounds could still occur and be unaffected by tillering.

The length of the rice panicle is reported to be strongly correlated to the number of spikelets per panicle, which in turn directly influences rice yield (Huang et al., 2013). As a result, panicle length is of key interest in rice research to control dependent traits. The strong correlation between panicle length and number of spikelets was confirmed in this experiment (R^2= 0.690, p < 0.001, Figure 4.8). Panicle lengths were similar for each compound compared to the control, except for 1070 which caused a decrease in panicle length (Figure 4.7). The results for the other four compounds suggest that there may be no cost to panicle development and, as this is closely linked to yield, may not negatively affect yield under optimum conditions but may offer the plant increased access to water lower in the water table when under drought conditions.

4.5.3 Conclusion

Pot-based trials pose experimental constraints, particularly when working with RGPs. In the present study, root length variability, and the resulting increased access to nutrients was not possible due to the development of a dense root system, the structure and size of which was affected by the pot. Consequently, rather than attempting to measure root length in the mature plants, the objective of the present study was to establish whether the novel RGP compounds conferred any effects on yield in addition to positive effects on root growth, rather than as a consequence of enhanced root growth, similarly to Ramireddy et al. (2018). In addition, it was important to establish whether compound application to enhance the root development resulted in a cost to aboveground plant traits or seed development, as experienced in other root assays (Mrízová et al., 2013).

This study offers preliminary findings to suggest that whilst application of the RGP does not severely affect aboveground physiological development, it does not confer any beneficial traits towards aboveground physiology or yield. Whilst there is no severe cost to shoot growth, some preferential traits, such as an increase in seed width or length, come at a cost to overall seed quality. Therefore, further work is required to establish whether there are nutritional costs or benefits as a result of applying the compounds. Additionally, it would be interesting to establish whether there is a specific developmental stage at which RGPs are most influential, in order to establish the conditions under which they could be best utilised.

In order to develop this study further without the negative effects inherent in pot-based glasshouse trials it is necessary to conduct a field trial using seedlings primed with the compounds. A field trial would establish whether there were any practical benefits to application of an RGP, and whether root enhancement is a viable method of increasing water and nutrient acquisition and greater anchoring for shallow-rooted rice systems.

This study shows that the novel use of local and global autocorrelation analysis is a useful tool for glasshouse experiments. However, it is important to ensure that the method is transferable to other environments and glasshouse spaces by studying a single uniform treatment. This would ensure that the observed effects are entirely due to positioning and that the spatial data is independent of any other effect. The method of performing a Local Getis-ord Gi* hotspot analysis and incorporating the data into a weighted statistical analysis, such as in this chapter, offers an exciting new method to counter a widespread problem throughout plant biology glasshouses.

The capacity to collect and interpret data has increased rapidly in recent years, which has facilitated novel spatial analyses such as the hotspot analysis in this chapter. As a result of increased data acquisition, opportunities for new multi-sector collaborations have arisen. One opportunity for knowledge to be disseminated in a novel and effective way is by merging multiple sources and types of data, such as merging maps with environmental and agronomic data. This technique will be explored further in Chapter 5.

RootTarget: A dynamic model enabling the targeted application of plant growth regulators for rice

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5.1 Societal Impact Statement

The rising global population and the increasingly conspicuous effects of climate change are putting growing pressure on the agricultural industry. Food production must increase significantly with little or no access to extra land; therefore, new approaches are urgently required to increase crop productivity. The strategic application of plant growth regulators is one method of achieving this, made possible by RootTarget. RootTarget maps regional land use and resource availability, identifying locations where crops could benefit from the application of plant growth regulators. Such targeted application can contribute to creating a more resilient and sustainable farming system.

5.2 Summary

- Developing novel strategies for optimising crop productivity is essential to meet the requirement of doubling food production by 2050. Geographic information system (GIS) software provides the opportunity to develop models for targeted application of plant growth regulators in rice-growing regions based on water availability and agricultural practices, in order to optimise yields.

- GIS was used to identify rice-growing areas with adequate irrigation, a high evapotranspiration index or suitable sowing technique for site-specific application of plant growth regulators. Rule-based decisions were incorporated into a GIS model in order to identify key areas where plant growth regulators should be applied. This approach allows information sharing between multiple stakeholders including researchers, companies and farmers with the aim of maximising crop productivity.

- A root growth promoter (RGP) was identified that promotes root growth under optimum conditions but not when applied concurrently with a drought stress. The effect of the RGP diminishes as the severity of the stress increases. These data were used to execute queries to identify regions with a suitable drought index, growing practice and adequate irrigation. Two hundred and twenty-nine million hectares of land, spanning four continents, were identified as having the potential to benefit from application of the RGP.

- Significant areas of land used for rice production were identified that could benefit from application of the RGP. This study highlights the potential to incorporate academic research into models for research dissemination to the agricultural industry and increase crop productivity.

5.3 Introduction

The challenges of meeting the agricultural requirements of the future are complex. World population is projected to reach almost 10 billion by 2050, with most of this growth forecast to occur in low- and middle-income countries where access to safe, sufficient and nutritious food is already limited (FAO, 2009b). Meeting the food requirements of a growing population must occur with little or no access to extra land; therefore, increasing the productivity of existing agricultural land is essential. In addition, alterations in global weather patterns due to climate change and the increased frequency and intensity of extreme weather events imposes abiotic stresses on crops, putting further pressure on agricultural production (Ciscar, 2012). The resultant negative impact on yields has a marked impact on food availability, with anticipated yield declines of major crops (corn, wheat and rice) that are relied upon for a large proportion of daily calorific consumption (Mickelbart et al., 2015).

The rising global population and increasingly conspicuous effects of climate change mean that food production must increase by 60% from 2007 to 2050 by sustainable intensification of agriculture (Alexandratos and Bruinsma, 2012). Therefore, new approaches are urgently required to increase crop productivity. Plant growth regulators are one method of achieving these aims; they are chemical products used to enhance yields and promote growth of crops, within which the root growth promoters (RGPs) are a subgroup. Their mode of action is based on regulating hormone levels, including those of cytokinin, and modulating signal transduction pathways within a plant (Neill et al., 2019).

Despite the significance of the global issues affecting agriculture, a key component required to address these is nevertheless missing. An adaptable model to highlight areas prone to stress and identify where specific plant growth regulators would increase yields is currently lacking and is urgently needed. Existing models are not tailored to the strengths or requirements of plant growth regulators, crucially missing the multidisciplinary nature required for their effective use (van Oort, 2018, van Oort and Zwart, 2018). Therefore, a model that takes this into account would be a valuable tool to different stakeholders, from small-scale farmers to large industry. This research transforms multiple sources of open access data and academic research into a cohesive model for knowledge exchange. The model could be used in commercial applications for region-specific marketing of products or crops engineered for traits resistant to drought stress. Farmers could buy products specific to their requirements, resulting in less waste and increased yields, which could ultimately increase their income and quality of life. As such, the model provides a precision agriculture toolkit specific to RGPs, by which site-specific crop management can be recommended to address regional variability. Because plant growth regulators need to be applied prior to the onset of stress, real-time data collection and analysis are not appropriate, making the use of historic data imperative.

Rice is the staple food crop for over half of the global population, providing an important source of minerals, vitamins, fibre and carbohydrates and ensuring food security in many low- and middle-income countries. As the model species for monocotyledonous plants, rice is of enormous scientific and commercial interest (Mordor Intelligence, 2019). Globally, around one third of rice is grown in rainfed lowlands in nonirrigated conditions solely dependent on rainfall. In South and Southeast Asia, 45 million hectares of land is used for rice cultivation, of which 19 to 23 million hectares is estimated to be frequently and severely affected by drought, their risk determined by topography, soil characteristics and local weather patterns, with many drought-risk areas extending beyond this region (Haefele and Bouman, 2009). Low yields due to rain-fed lowlands experiencing drought is a common problem throughout Asia, with an estimated 45% of the total rice area having no irrigation input (Fukai et al., 1999, Haefele and Bouman, 2009). Yield increases in rain-fed systems are typically much smaller than that of irrigated rice which is attributed to poor growing conditions, limited resources and lack of development of suitable cultivars or products (Fukai et al., 1999). Consequently, there is great opportunity for developing strategies for optimising growth in these regions (Tsubo et al., 2009).

Cytokinin is a plant hormone involved in almost all stages of plant growth and development, an abundance of cytokinin in the roots inhibits growth (Ferreira and Kieber, 2005). Interestingly, a 4-phenylquinazoline compound has been shown to cause an increase in root length under optimum conditions. This compound behaves as a cytokinin antagonist that noncompetitively binds to the cytokinin receptor (CRE1), blocking the activity of the cytokinin (Arata et al., 2010). When rice is grown in a nursery prior to transplanting to the field, there is scope to apply RGPs under controlled conditions using better crop management practices (Vareed Thomas, 2002). The growth of new roots is a key factor in the establishment of transplanted rice seedlings (Mishra and Salokhe, 2008). In addition, pruning of the root system prior to transplanting reveals a positive correlation between the size of the root system prior to transplantation and the shoot and root dry matter, P concentration in leaves at panicle initiation, straw dry matter and grain yield at maturity (Ros et al., 2003). Crops with longer roots have increased surface area and anchoring ability in addition to enhanced access to the water table which gives them a competitive advantage during periods of drought stress and nutrient availability (Maeght et al., 2013). Deep roots generally confer an advantage to plants growing in water-limited soils but may be particularly important for crops such as wheat and rice to facilitate water acquisition when the water table is low (Comas et al., 2013). This study provides experimental validation of the effects of a novel patented cytokinin antagonist (Patent No. WO2008062907), as a RGP for root growth in rice. A geographic information system (GIS) model, RootTarget, has been developed using: rice-growing areas, methods of planting, growing season in that area, historic data indicating drought, and irrigation capacity of the area. This model allows inferences about areas that are drought prone without possibility for irrigation to be drawn, allowing recommendations for targeting sales of RGPs and their application to be made.

5.4 Materials and Methods

5.4.1 Validation of S-4893, a 4-phenylquinazoline structure, as a RGP in Oryza sativa

5.4.1.1 Plant and growing conditions

Rice seeds (O. sativa cv Nipponbare) were sterilised using 100% (v/v) ethanol for 2 min and 20% (v/v) sodium hypochlorite solution for 10 min and rinsed six times with sterile water. Seeds were then placed on moist filter paper in a Petri dish and sealed with 3 M micropore tape. The Petri dish was covered in aluminium foil in a controlled environment at 30°C:26°C (11 h:13 h day:night). Rice is typically considered to be a short-day plant; studies have found a day length between 8 and 12 h to be optimum although rice is photoperiod insensitive during the juvenile phase (Vergara, 1985). After 48 h, the aluminium foil was removed, and the Petri dish returned to the controlled environment. Once germinated, seeds with the most homogenous growth were selected for use in the assay and placed on a polystyrene float with a hole cut into it. The seed was then cultivated hydroponically in a test tube containing 50 ml liquid medium consisting of Murashige and Skoog medium (0.22% w/v) at half strength (2.2 g/L) and 0.5% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Atom Scientific, Kimble, Borosilicate Glass 25 mm 200 mm). Test tubes were placed in a wooden box to provide support and to prevent light exposure to the root zone. Growth assays were performed in a controlled environment at 30°C:26°C (11 h:13 h day:night) for 10 days.

5.4.1.2 Chemical and drought stress application

The chemical used (S-4893) was a 4-phenylquinazoline structure, as in Arata et al. (2010), molecular formula: $C_{17}H_{16}ClN_3O$. A stock solution of S-4893 was prepared in 100% DMSO (v/v). Preliminary tests were performed to establish the active range of the compound. Nutrient solution was supplemented with stock solution of the compound to give final test concentrations of 0.96, 3.2, 9.6 and 32µM and a final DMSO and acetone concentration of 0.05% (v/v). Six biological replicates per experiment were conducted. The experiment was repeated in triplicate. For stress assays, the liquid media was supplemented with 10, 20 or 30% (w/v) of polyethylene glycol (PEG) 8000 to impose a water-deficit stress on the seedlings. PEG concentrations were based on preliminary testing and concentrations used in literature (Fozard and Forde, 2018, Shereen et al., 2019).

5.4.1.3 Root imaging and statistical analysis

The root phenotype was imaged 10 days after transfer to the test tubes using a high-resolution scanner (EPSON Expression 11000XL). Measurements were obtained using WinRhizo (Regent Instruments Inc, 1991). The data shown are the mean from the six seedlings. Seminal, crown and lateral root growth were measured. Statistical analysis was carried out in R. A one-way ANOVA and independent t tests were performed between the control and each concentration of S-4893. A two-way ANOVA and a Tukey Kramer post hoc test were performed for the water-deficit analysis, significance $p \leq 0.05$.

5.4.2 GIS modelling

5.4.2.1 Study area

Data for rice production, potential evapotranspiration (PET), rainfall and irrigation were obtained using the resources from Table 5.1 to create RootTarget. Data and their corresponding shape files were overlaid using QGIS 3.14.16. The coordinate reference system used was EPSG:4326-WGS 84. Spatial detail varies across maps, continents and countries due to variation in data availability. The most comprehensive rice calendar to date was used, providing extensive rice production data for 115 countries (Laborte et al., 2017). The Standardised Precipitation-Evapotranspiration Index (SPEI) is a drought index based on global climatic data and is based on monthly precipitation and PET, based on the FAO-56 Penman–Monteith estimation of PET which is considered the best available for long-term climatological analysis (Vicente-Serrano et al., 2010).

5.4.2.2 Rice data

Areas were divided by their sowing method into directly sown and transplanted. Seven methods were listed in the RiceAtlas database. For clarity, areas referred to as ‘tankfed’, ‘sowing/planting’, ‘sowing’ and ‘planting’ in Laborte et al. (2017) were omitted; ‘direct sowing’ and ‘direct seeding’ were treated as areas with direct sowing; ‘transplanting’ was categorised as transplanted, that is, areas where rice is grown in nurseries and then transplanted into the field when several weeks old. SPEI values and irrigation capacity were not taken into consideration for transplanting data, due to the more controlled conditions in a nursery. In areas where drought stress was considered likely due to low precipitation and high evapotranspiration, the stress was deemed circumvented by having an adequate irrigation supply. Adequate irrigation was defined as over 50% of the land area being ‘actually irrigated’, poor irrigation was defined as less than 50% of the land area being ‘actually irrigated’ (Siebert et al., 2013a).

5.4.2.3 SPEI data

Using the most recent data available, a 5-year average (2013–2018) of the SPEI value of the peak planting month for each region was calculated (see Figure 5.1). The SPEI raster layer was converted into vector data to allow merging of attribute tables and analysis of the results. Drought prone was defined by an area having a negative SPEI value.

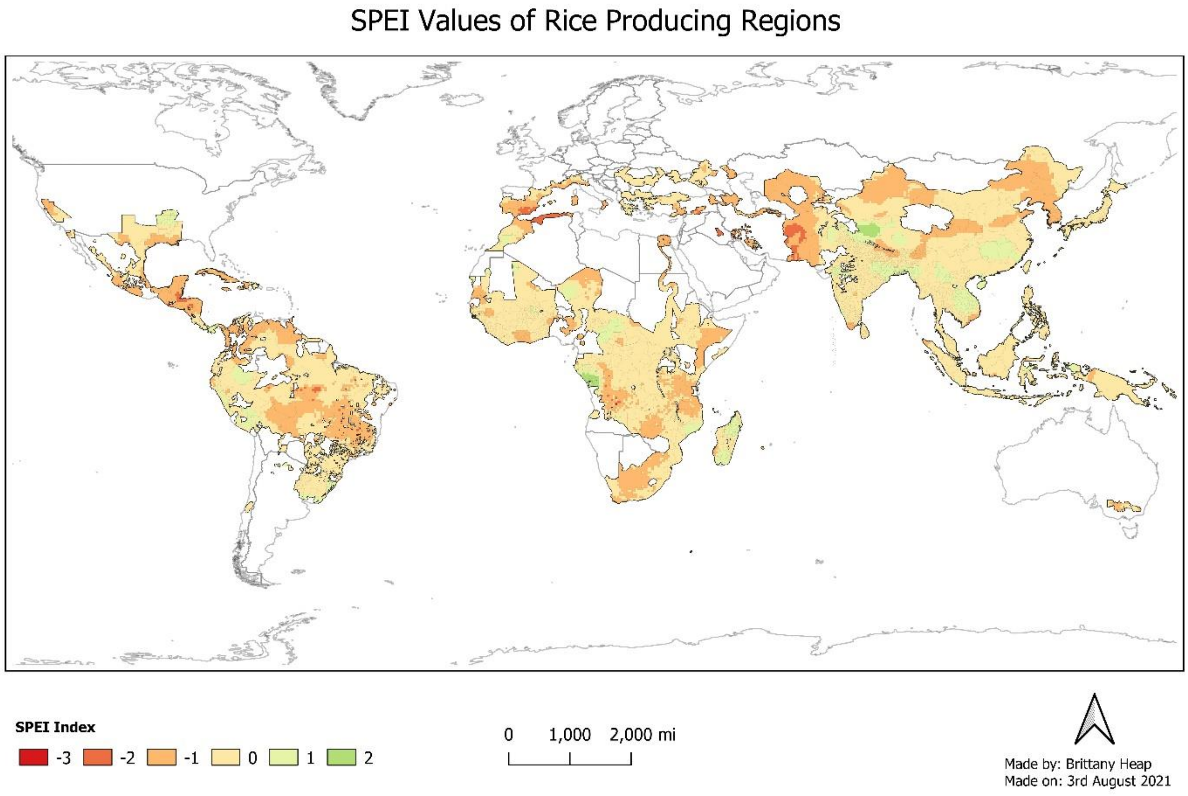


Figure 5.1. Historic data from the peak planting month for each area, from 2013 to 2018. Data are averaged over the 5 years. SPEI indices are z values that represent the standard deviation of the data from the mean. Negative values indicate dry conditions, and a positive z value indicates the data are higher than the mean. The SPEI values for all rice producing regions globally have been displayed

Table 5.1. Data sources inputted into QGIS to create RootTarget.

Input data	Map Name	Reference
Rice production	Rice Atlas	Laborte et al. (2017)
Irrigation	AQUASTAT	Siebert et al. (2013b)
SPEI Global Drought Monitor	SPEI Global Drought Monitor	Vicente-Serrano et al. (2010)

5.5 Results

5.5.1 Root growth promotion of the 4-phenylquinazoline structure S-4893 in rice

The ability of the 4-phenylquinazoline structure S-4893 to promote root growth in a monocot species was examined in vivo in a hydroponic rice assay with roots submerged in liquid media with varying concentrations of the compound. Root length was analysed 10 days after treatment application using WinRhizo root imaging analysis software; images of treated roots are available in Appendix Figure 7. Total root length increased when grown in concentrations ranging from 0.96 to 32µM, with root length more than twice as long as the control (Figure 5.2). Root:shoot ratio decreased for all concentrations of S-4893 indicating that S-4893 caused a proliferation of aboveground biomass in addition to belowground (Appendix Figure 8).

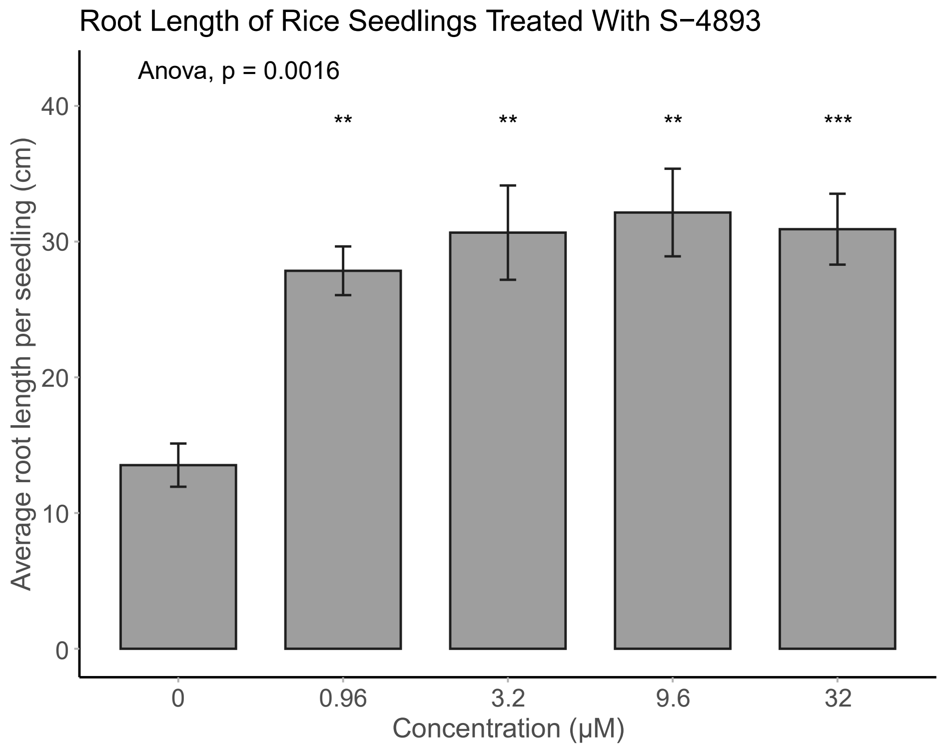


*Figure 5.2. RGP properties of the cytokinin antagonist S-4893 under optimum conditions under five different test concentrations. Significance values indicate the results of independent t-tests carried out between the control and each concentration of S-4893. * = P ≤ 0.05, **=P ≤ 0.01, ***= P ≤ 0.001. Values are means +/- SEM (n=6).*

5.5.2 Effect of the 4-phenylquinazoline S-4893 on root growth under water-deficit stress

The efficacy of the 4-phenylquinazoline structure S-4893 as a RGP was established by using the same method as under optimum conditions. The optimum concentration, 0.96µM, of the 4-phenylquinazoline structure S-4893 was established by performing the experiment in triplicate, and used in subsequent stress assays; 10%, 20% or 30% w/v PEG 8000 was added to the liquid media to induce a water-deficit stress response in the seedlings. As seen in Figure 5.3, shoot and root length decreased as water-deficit stress increased. This trend was seen for treated and untreated seedlings, possibly due to cytokinin levels decreasing in roots under stress, thus the cytokinin antagonist having a less pronounced effect. The seedling treated with the 4-phenylquinazoline structure S-4893 had slightly longer roots under all stress conditions, but this trend was only significant under mild stress, and the increase in root length became less pronounced as the severity of the stress increased. Images of the rice seedlings from each treatment are available in Appendix Figure 9.

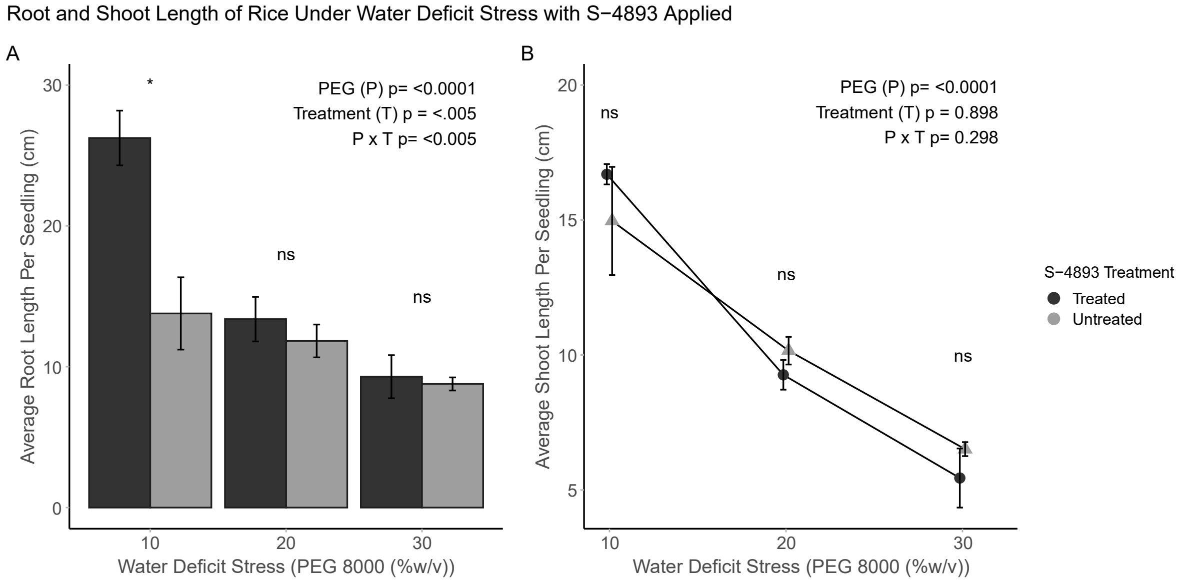


*Figure 5.3. The positive effect of the cytokinin antagonist S-4893 is reduced in roots (A) and shoots (B) as water deficit stress increases. 0.96µM of S-4893 produced the most significant root growth promotion when repeated in triplicate, hence this was the concentration used for the water deficit assay. 10, 20 and 30% PEG 8000 (%w/v) represent mild, moderate and severe water deficit stress, respectively. Significance values indicate the results of an independent t-test carried out between treated and untreated seedlings for each water deficit stress level. * = P ≤ 0.05. Values are means +/- SEM (n=6). Statistics for the effect of treatment and water-deficit stress are the results of a two-way ANOVA.*

5.5.3 RootTarget modelling outputs

A stepwise decision process for when the 4-phenylquinazoline structure S-4893 could be applied for a positive effect on root growth was created (Figure 5.4), based on the results from Figure 5.2 and Figure 5.3, for agrochemical companies and farmers to follow in order to identify locations for optimum product sales and application. The decision tree incorporates each section of the methods to form a cohesive output.

Root length increased when conditions were optimum (Figure 5.2); these conditions could be mimicked in a nursery setting for transplanted seedlings. Optimum conditions are also likely to be found where there is adequate irrigation, or low SPEI value (based on evapotranspiration and rainfall data), each of these factors forms one step of the decision tree (Figure 5.4). This decision tree instructs the layering of the queries built in QGIS, which will generate recommendations for agrochemical companies and farmers to follow in order to identify locations for optimum product application. The objectives of this analysis, relating to the decision tree in Figure 5.4, are twofold: first, to identify areas that use the transplanting method for rice, and second, areas where rice is grown via direct sowing with 'optimum conditions' for rice growth. Optimum conditions were defined as an area having an SPEI ≥ 0 during the peak sowing period or having adequate irrigation practices in place.

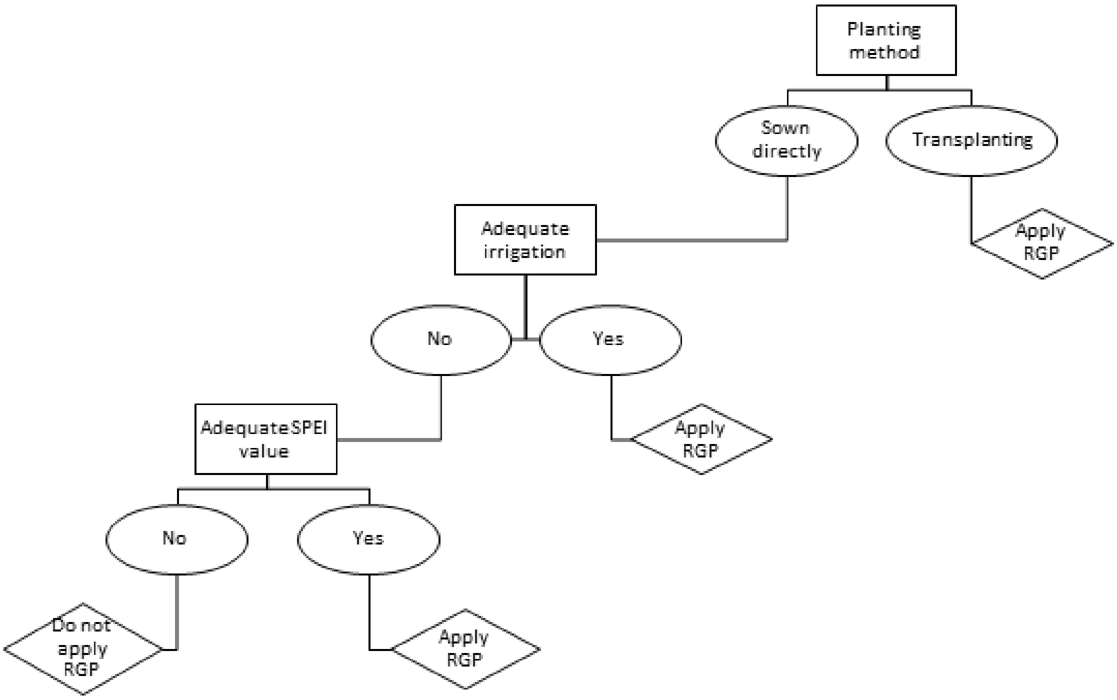


Figure 5.4. A decision tree illustrating the criteria that must be met for the model to suggest application of the 4-phenylquinazoline structure S-4893.

5.5.4 Transplanting

One thousand two hundred sixty-four regions were identified as using the transplanting method, located predominantly in China (Figure 5.5). The number of distinct crop seasons ranged from 1 to 3, with a mean of 1.56 (SD = 0.66). The first growing season accounted for 75.7% of the annual yield and 73.3% of the total rice-growing area.

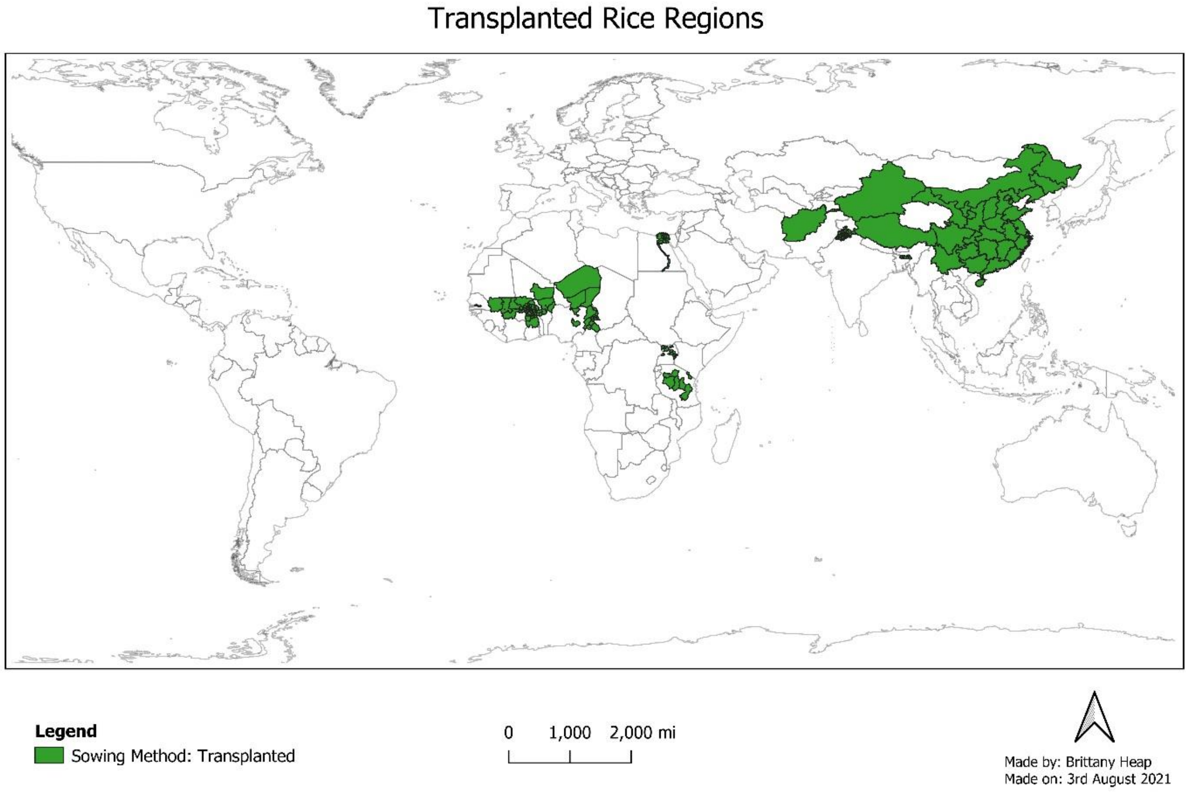


Figure 5.5. Areas that use the transplanting method for rice production are highlighted in green. Transplanting is currently the most common method of rice production and is used throughout much of Asia and some countries within Africa.

5.5.5 Direct sowing and over 50% of area irrigated

Five thousand five hundred and fifty-nine regions used the direct sowing method for rice and had between 50.01% and 100% of the land irrigated (Figure 5.6). The mean area irrigated was 81.4% (SD = 14.49). Most areas only had one cropping season, with a mean of 1.03 and a maximum crop season number of 2. During the peak cropping season, land area cover ranges from 10 to 943,714 ha, with a mean of 32,384 ha (SD = 71,749). The harvest ranges from 26 to 2,126,106 t with a mean of 237,217 (SD = 489,737). The large range in land area and yield suggests there is good data capture from small- and large-scale farmers. Yield from the first harvest accounted for 99.3% of the total rice harvested.

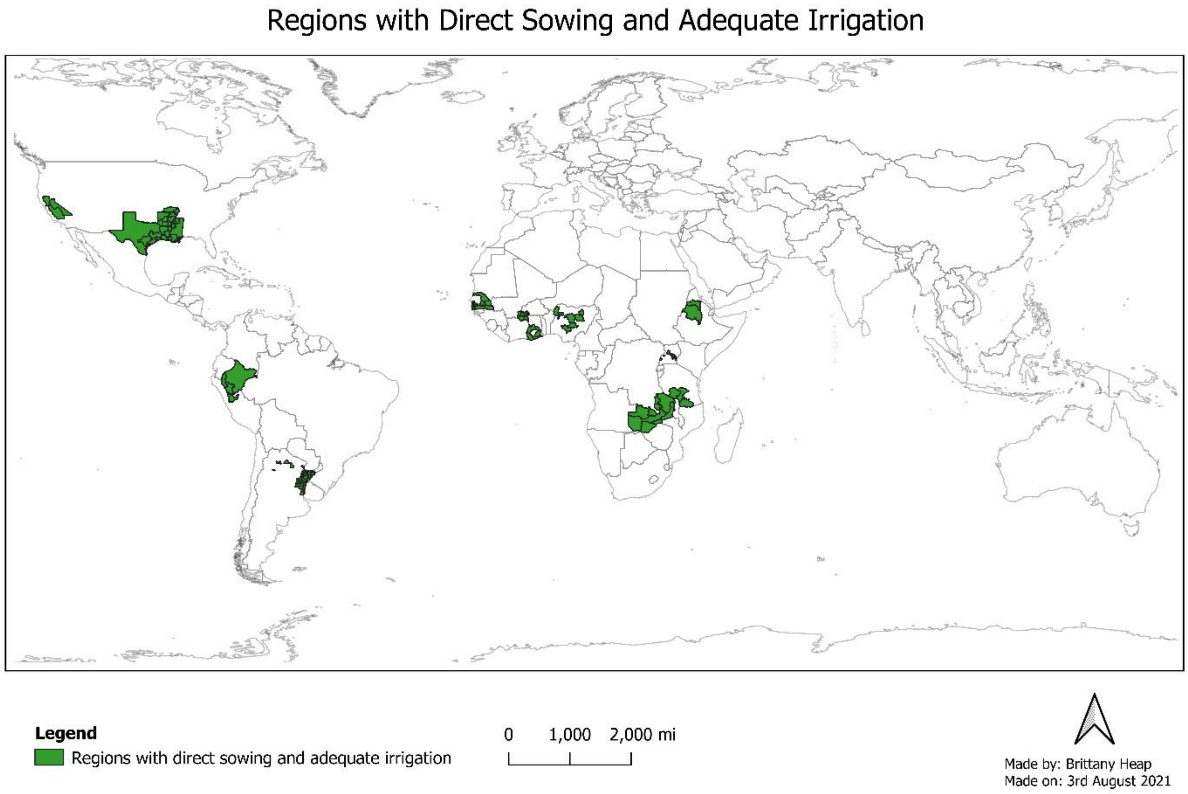


Figure 5.6. Regions with direct sowing of rice and over 50% of the area irrigated are highlighted in green.

5.5.6 Direct sowing, less than 50% of area irrigated and an SPEI value of ≥ 0

Of the 1411 regions that used the direct sowing method with less than 50% of the area irrigated, 759 locations had an SPEI value of equal to or greater than 0. All regions were in the United States: Arkansas, California, Louisiana, Mississippi, Missouri and Texas (Figure 5.7). These areas were surrounded by areas that were better equipped with irrigation (>50%). Despite covering a much smaller area than the land identified by the first two criteria, the locations had a mean area of 10,390 ha and produced a total of 122.61 million tonnes of rice (Table 5.2).

*Table 5.2. Data from each selection stage of the decision tree. * Represents the average t/ha for all land classifications in this study.*

	Number of distinct crop seasons	Total rice production (Mt)	Total rice area (million ha)	t/ha	Number of regions	Peak growing season production (Mt)	Peak growing season area total (million ha)
Transplanting	1-3	227.16	35.59	6.38	1264	171.99	26.07
Direct sowing and over 50% of area irrigated	1-2	1318.69	184.68	7.14	5559	1309.39	180.03
Direct sowing, under 50% of area irrigated and SPEI value of ≥0	1	122.61	16.29	7.52	759	122.61	16.29
Total	NA	1608.13	229	7.01*	6483	1543.66	214.83

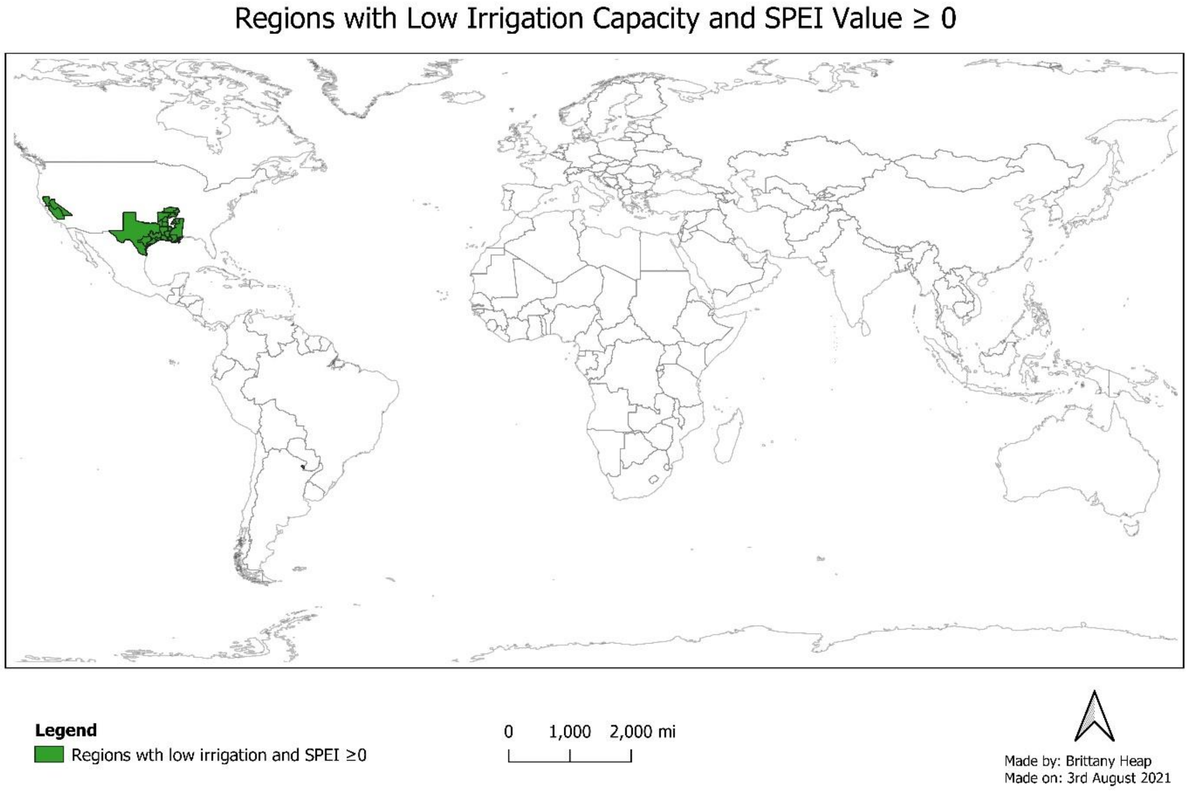


Figure 5.7. Regions with direct rice sowing, less than 50% of land area irrigated and an SPEI value of ≥0. All 759 locations were in North America.

5.6 Discussion

Based on the decision tree (Figure 5.4), large areas of land where the RGP was deemed suitable for application were identified (Table 5.2 and Appendix Figure 10). The incorporation of data for methods to avoid drought; such as a good irrigation system, and indices to predict drought, such as the SPEI index, allowed significant proportions of rice-growing land in America, Asia and Africa to be identified as having optimum conditions for the application of RGP S-4893. This study therefore helps to bridge the gap between research and industry and helps target areas where the yield increases could be achieved with relative ease with the correct product. However, when identifying drought-prone areas, short-scale topographic data have been found to be a better determinant of drought risk than large-scale climatic variation, such as the SPEI index (van Oort, 2018). High-resolution mapping that accounts for groundwater depth and duration of flooding is needed to identify hotspots suitable for rice cultivation in arid regions, such as Africa. Local data collection of groundwater and flood data is therefore necessary although currently unfeasible on a global scale.

van Oort and Zwart (2018) simulated production in irrigated and nonirrigated, water-limited (rain-fed) regions. Similarly to RootTarget, their model does not take into consideration soil fertility, biotic stresses and abiotic stresses with the exception of water limitation. Hence, the parameters of this model are not exhaustive but go some way towards indicating regions of interest for agrochemical companies to liaise with local industry. (van Oort, 2018) overlaid different rice production maps of Africa and revealed some discrepancy between rain-fed and irrigated rice-growing areas, highlighting issues with inconsistent data quality across countries and a lack of current global data. To minimise this issue, the present study has overlaid the most comprehensive rice map available with irrigation data from the FAO, the two most widely accepted and comprehensive datasets available.

While models such as the Global Agro-Ecological Zones (GAEZ) consider more parameters (such as soil moisture, radiation, temperature used to calculate potential biomass production and yield), they fail to identify crucial differences in sowing technique, that is, whether a crop is sown in a nursery or transplanted which has a strong influence on the recommendations. Consequently, the potential to incorporate more variables into RootTarget remains, with the possibility of increasing still further benefits of this approach to achieve a sustainable farming system.

5.6.1 Conclusions

In 2018, \$5.9bn was spent on agrochemicals for rice production. Of this, \$5.8bn was spent on herbicides, insecticides and fungicides. Just \$65m was spent on ‘other’; a subset of this includes plant growth regulators (Amis®Agriglobe®, 2018). In an agrochemical market worth over $237.8bn, this is an underdeveloped area that could increase profits in the agricultural sector while increasing plants' access to increasingly limited resources, thereby increasing yields (Research Dive, 2020). While the Green Revolution vastly improved global yields, these improvements in yield are stagnating and need an additional science-based approach to optimising yields, in addition to the existing commercially based strategies.

This study shows that the RGP activity of the 4-phenylquinazoline structure S-4893 was significant under optimum conditions, with the benefits of product application diminishing as drought stress increased in severity (Figure 5.2 and Figure 5.3). Therefore, the decision tree was built to identify areas where no stress was likely to occur (Figure 5.4). Where conditions are suboptimal, application of the 4-phenylquinazoline structure S-4893 must occur before transferring into the field. Sowing technique dictates whether seedlings are initially grown in more controlled conditions, such as transplanting seedlings, or sown directly into the ground. If it is the latter, a water deficit is considered more likely. Transplanting, the most common method of rice sowing (He et al., 2018), enables seedlings to avoid a possible drought stress at their most drought-sensitive time. Locations using the transplanting method had more rice seasons than those using direct sowing (Table 5.2). Despite the increased number of growing seasons, production per hectare was lower for transplanted rice (6.38 t/ha), compared with direct sowing and over 50% of area irrigated (7.14 t/ha), and direct sowing with under 50% of area irrigated and SPEI value of ≥0 (7.13 t/ha) (Table 5.2). The transplanting method is a labour-intensive method yet still produces a lower yield per hectare but may be used to grow in areas that would otherwise be unsuitable for rice production and offers an opportunity to apply the 4-phenylquinazoline structure S-4893 to act as a RGP in favourable conditions.

Taking into consideration the SPEI of an area (Figure 5.1) may be a particularly useful tool for rice due to its typically shallow rooting system which provides access to a relatively small volume of soil. Application of a RGP in areas with a low SPEI value (before the onset of drought) would increase the plants' access to soil area and increase access to water when the groundwater-table is low (Comas et al., 2013, Henry et al., 2011). Drought sensitivity increases once again during the reproductive stage, during seed set and grain filling (Zhang et al., 2018). During these periods, access to the water table becomes crucial and as such, efforts to make better use of soil water in the form of trait selection for deeper roots, established prior to the onset of stress, could help to increase yields.

This model has explored the effect of a single stress on a single compound which acts as a RGP and has identified 229 million hectares of land with a yield of 1608.13 Mt that could benefit. The three selection criteria (Table 5.2) provide the initial criteria to allow a targeted approach for product application. RootTarget therefore acts as a novel proof of concept model, highlighting the potential for widespread use of more targeted applications of existing growth promoters. Integrating the effects of other abiotic stresses, such as salt stress, on the RGP properties of the 4-phenylquinazoline structure S-4893 and identifying further areas that could benefit from application of S-4893 is an important next step for the RootTarget model. Further experimental work should include measuring the effect of product application on yield and exploring model accuracy and precision in greater detail. A financial feasibility assessment of the model recommendations would be an interesting addition, increasing the interdisciplinary nature of this project further. There are multiple opportunities in this area for future developments beyond this proof of concept study. In future, modelling to target the application of other RGPs and exploring the effects of S-4893 in other crop species will help generate a more cohesive model and ensure products are applied in an effective manner to bridge the gap between yield potential and actual yield.

6 Discussion

6.1 Summary of results

The overarching aim of this project was to use novel innovations in the agrochemical sector through Globachem Discovery Ltd.'s innovative crop protection chemistry to address the twin challenges climate change and population growth have on access to sufficient food (FAO, 2009a, Globachem, 2021b). To meet the target of increasing food production by 60% by 2050 (Alexandratos and Bruinsma, 2012) with access to little additional agricultural land (FAO, 2009b), most of this growth must come from increasing productivity of existing agricultural land. Currently, over 30% of the variability of crop yields is attributed to climatic variation (Ray et al., 2015), hence, making plants more resilient to fluctuations in climate would make a significant contribution to meeting the food security requirements of the near future. However, globally, rice yields are only increasing at the rate of 1% annually, well below the 2.4% required in order to achieve the target of increasing food production by 60% between 2007 and 2050 (Ray et al., 2012). These factors, combined with rice's high sensitivity to stress means there are significant losses that represent opportunities for improvement (Zeng and Shannon, 2000, Uga et al., 2013).

Rice cultivation is strongly influenced by the quantity and quality of the water input (Haefele and Bouman, 2009, Zeng and Shannon, 2000). In Asia, rainfed regions account for an estimated 45% of the total rice area; the yield of these rainfed lowland rice systems is 50% lower than in irrigated areas, and yields in rainfed upland rice systems are 75% lower than their irrigated counterparts (Wade et al., 1999, Fukai et al., 1999). Whilst irrigation practices increase yield and reduce farmers' reliance on rainfall, which is anticipated to alter in intensity and frequency in the coming years (FAO, 2018), there is a tendency for irrigated agricultural land to be affected by salinity; an estimated 50% of land suitable for cultivation will be affected by saltwater by 2050 (Mahajan and Tuteja, 2005). Salt stress is common in rice paddies near deltas and coastal areas, but is also common in other areas that rely on irrigation as a main water source due to the high solubility of salt in water (Tack et al., 2015). The effect of salt on agricultural land is increased further by the negative effects of rising sea levels (Reddy et al., 2017).

Advances in genetic engineering, such as the discovery and dissemination of the flood-tolerant rice genotype Swarna-Sub1 (Xu et al., 2006) contribute to mitigating losses encountered by climate change. Additionally, breeding programmes have successfully produced rice with desirable traits in rice, such as drought resistance and higher yields (Kumar et al., 2014). However, a multi-faceted approach that incorporates advances in genetic engineering, breeding and agrochemical application is likely needed in order to reach these challenging targets in the face of increasing pressures from climate change. In 2018, \$5.9bn was spent on agrochemicals for rice production. Of this, \$5.8bn was spent on herbicides, insecticides and fungicides. In contrast, less than \$65m was spent on plant growth regulators (Amis®Agriglobe®, 2018). In an agrochemical market worth over $237.8bn, this is an underdeveloped area that could increase profits in the agricultural sector while increasing plants' access to increasingly limited resources, thereby increasing yields (Research Dive, 2020). These statistics highlight that the agrochemical market for mitigating abiotic stress is largely underdeveloped, this presents an opportunity to reduce the losses incurred by abiotic stress using novel strategies and compounds (Godoy et al., 2021).

Manipulation of plant hormones, which are an inherent part of a plants' development and response to stress, offers a promising technique to modify growth and promote favourable traits within a plant (Nehnevajova et al., 2019, Pospíšilová et al., 2016, Yin et al., 2020). CKs, a class of plant hormone, play an intrinsic role in the growth of a plant (Werner et al., 2001, Werner and Schmülling, 2009). In the roots, CK inhibits formation of lateral root and limits root elongation whereas in the shoots CKs promote cell division and differentiation (Riefler et al., 2006, Werner et al., 2001, Laplaze et al., 2007a). In shoots, high levels of CK delays leaf senescence (Gan and Amasino, 1995) and improves the size and number of seeds (Ashikari et al., 2005). In contrast, in the roots, CK levels tend to be too high for maximal root growth (Julkowska, 2018), and therefore lowering the CK activity in the root increases root length (Ramireddy et al., 2018, Riefler et al., 2006, Arata et al., 2010).

Roots play a key role in water and nutrient acquisition and help to anchor the plant (Gamuyao et al., 2012). It is widely accepted that deep roots are a key trait that can be targeted for improving drought resistance (Gowda et al., 2011) and increasing the roots depth of plants has been found to increase plant tolerance to drought (Jeong et al., 2010, Ramireddy et al., 2018, Arai-Sanoh et al., 2014, Uga et al., 2013), increase phosphorus acquisition and nutrient use efficiency (Gamuyao et al., 2012, Ju et al., 2015), and increase grain yield (Gamuyao et al., 2012, Ju et al., 2015, Jeong et al., 2010). Cytokinin levels are typically too high to allow maximum root growth (Julkowska, 2018), reducing cytokinin in the roots increases root length, and manipulating this mechanism can be used as a tool to promote root growth and potentially increase drought tolerance (Pospíšilová et al., 2016).

6.1.1 Identifying novel root growth promoters and their effect on root traits

16 putative CK antagonists were synthesised by Globachem Discovery Ltd., the industry partner for this project. Of these, five compounds, including the parent compound, were found to promote root growth in rice seedlings (see Chapter 2). This increase in root length was due to increases in both primary and lateral roots rather than an increase in one root type alone (see section 2.4.2). Crucially, this increase in root length did not come at a cost to the aboveground biomass (Figure 2.7 and Table 2.2). A positive correlation existed between total root length, volume, and number of tips.

Interestingly, a negative correlation existed between the aforementioned variables and root diameter (Figure 2.8 and Figure 2.9), indicating that the application of the root growth promoter (RGP) resulted in longer, thinner roots. Hydroponic growth systems offer little resistance to growing roots and so, in this instance, the longer, thinner roots did not affect the plants' ability to increase root length. However, the roots of plants grown hydroponically have been shown to differ significantly to those of soil-grown plants (Clark et al., 2002, Clark et al., 1999). Plant have been found to increase root diameter in order to penetrate denser soil (Hargreaves et al., 2009), hence, any negative effect of reduced diameter is an important area for further study and there is a need to explore the effect of applying the RGPs in a soil-based growth system (Hargreaves et al., 2009, Kuijken et al., 2015).

6.1.2 Applying the RGPs in a commercial setting

It is essential to establish whether the RGP properties of the compounds identified under controlled conditions are transferrable to a commercial setting. To this end, the study initially investigated the use of seed priming as an alternative method for commercial delivery of the compounds (section 3.4.1.1. The seed priming method was as effective at promoting root growth as when the roots of the seedlings were constantly in contact through inclusion in the hydroponic nutrient solution (Figure 3.3). Importantly, this approach markedly reduced the quantity of each compound required and also significantly reduced the setup time for growth experiments. Therefore, seed priming was established as a viable technique for commercial use of the five RGP compounds identified in Chapter 2 and was used for compound delivery in subsequent studies (see Chapters 3 and 4).

The effects of the five RGP compounds were tested on two rice cultivars used in Globachem's research: Loto and Selenio, a lowland and upland rice, respectively Table 3.1. Primed seeds were grown in a soil-based glasshouse growth system used by Globachem and a salt or drought stress applied to each rice variety. Surprisingly, the five compounds showed no RGP properties when applied to Loto and Selenio in the soil-based growth system, either with or without a salt or drought stress applied (Figure 3.8 to Figure 3.14). Whilst time and space constraints at Globachem prohibited further studies to investigate the reasons underlying this observation there are number of possible explanations for why the compounds may not have had an any affect under the commercial growing conditions used by Globachem. Cytokinin responses are species specific (Ghanem et al., 2008, Feng et al., 2019, Yin et al., 2020) and therefore it is possible that the RGP response was cultivar-specific in this instance (Syngenta, 2021). Alternatively, the concentration range at which the compounds are active might be different in Loto and Selenio, as is known to be the case with PGR application (Syngenta, 2021). In addition, the peat and perlite mixture used in the soil-based growth system might impede root growth (Clark et al., 2002) which, combined with the decreased root diameter observed in Chapter 2, might override the effects of the compounds. Furthermore, CK antagonists have been shown at times to also behave as agonists; if this was the case for the novel RGPs the antagonist effect would have been cancelled out or reduced by the agonist effect (Nisler et al., 2010, Spíchal et al., 2009).

6.1.3 Establishing the long-term effects of RGP priming

To explore whether priming of seeds had a long-term effect on aboveground plant traits and yield, Nipponbare seeds primed with the five RGPs from Chapter 2 were grown to maturity in soil. At maturity, plants whose seeds had been treated had no penalty to aboveground traits such as plant height and number of tillers (see Figure 4.4 and Figure 4.5). However, seed priming with the five RGPs negatively affected the reproductive capacity of rice by reducing the number of panicles and spikelets (Figure 4.9 and Figure 4.11 respectively). Additionally, traits have been reported to have a strong correlation in the literature, such as flag leaf length and seed fertility (Yoshida, 1981) had a weak negative correlation in this study (Figure 4.8). Whilst an increase in root length would confer advantages to the plant, particularly under drought stress (Ramireddy et al., 2018), it is crucial that this increase does not affect the development of the reproductive part of the plant. Based on this observation, further studies are required to establish whether the use of seed priming to deliver the compound is suitable for commercial application. However, based on the results from Chapter 3 and 4, four novel compounds have been found that promote root growth in the rice cultivar, Nipponbare, demonstrating that priming is an effective method of applying this CK antagonist treatment for a positive root growth effect in seedlings (Figure 3.4 to Figure 3.6). Throughout the experimental process, several novel strategies for incorporating newer technologies or statistical techniques were identified and explored.

6.1.4 Using machine learning for agrochemical discovery

The use of chemical libraries has contributed significantly to the identification of putative agrochemicals in recent years (Smith, 2003). Nevertheless, the process still requires significant inputs of time, funding and resources (Burrell et al., 2017). In this study, an unsupervised machine learning technique using chemical data for the compounds supplied by Globachem Discovery Ltd., segregated the compounds into two distinct groups based on their chemical profiles (see Section 2.4.6). Despite the small training dataset, the PCA correctly grouped the RGP and non-RGP compounds into separate sections of the PCA (Figure 2.10). The chemical data for six compounds not used in the experiments were inputted, identifying five which were deemed to be worth further investigation and one compound which was deemed unlikely to have RGP properties given the dissimilarity of the chemical profile to the other RGPs (Figure 2.11). Importantly, whilst this machine learning technique used only a small dataset, it highlights the potential of using data science approaches to effectively analyse data and to predict the efficacy of novel compounds, once a training dataset has been creating by using laboratory data. This represents a significant saving in terms of time and the resources required to identify potential new agrochemicals.

6.1.5 Quantifying spatial heterogeneity within a growth space

Whilst the effects of heterogeneities within growth environments are well-known (Kimura et al., 2020, Teitel et al., 2010, Boulard et al., 2002, Zhao et al., 2001), robust approaches to quantify and account for the effect of spatial layout within a glasshouse are lacking. Current practice is to rotate plants, which can be demanding on time and/or resources (Brien et al., 2013) and has a tendency to inflict damage, or to use a randomised block design (Fisher, 1992, Rubin, 1990). In both methods, the effects of environmental heterogeneity are spread equally among all treatments, though this is coupled with an increase in the error of variance and crucially does not account for the variance. Hence, employing these methods means the degree to which a plants' location affects growth remains unquantified. Using Local Getis-ord Gi*, the spatial heterogeneity of the glasshouse on each variable was quantified and mapped (Figure 4.3). This enabled the quantification and visualisation of hotspots and coldspots within a glasshouse environment at a level which has not previously been achieved. The approach allowed the influence of a plant's location on growth to be assessed, i.e. whether growth was positively or negatively affected by location, and whether this influence was statistically significant. The subsequent analysis of the data was weighted such that variables which were significantly affected by location had a lower weighting in the analysis, relative to the p-value. This offers a novel method to account for heterogeneity within a growth space; quantitively discriminating between viable and unviable samples rather than traditional methods for discounting data based on arbitrary values which have the potential to lead to bias, plant injury or poor discrimination and/or selection criteria (Brien et al., 2013).

6.1.6 Development of a research-based geospatial toolkit

At present, there is no framework for incorporating results obtained in the lab or field into a user-friendly toolkit for end-users i.e., agrochemical companies and farmers. Consequently, the effectiveness of both the dissemination of applied research to farmers and the application of agrochemical products is not optimised to the unique regional requirements and crop or sowing practices of different growing regions; RootTarget was developed to bridge this gap (Heap et al., 2021). The focus of Chapter 5 was to transfer the information gained from lab experiments and identify regions globally where rice is grown and conditions are likely to be optimum for product application, given what is known about how the Globachem compounds investigated in this study behave under optimum and stress conditions. Using only the parent compound (referred to as 974 in Chapters 2-4 and compound S-4893 in Chapter 5) for this proof-of-concept model, data showed that the compound promoted root growth under optimum conditions but not under drought stress (Figure 5.2 and Figure 5.3). The model, RootTarget, incorporated rice growing areas and techniques (Figure 5.5) with global evapotranspiration data for each peak planting month globally (Figure 5.1) and the irrigation capacity of each region, to identify where rice could be grown in non-drought conditions. RootTarget identified a total area of 229 million hectares that could benefit from application of the parent compound (Table 5.2) with just one compound and three selection criteria (planting method, evapotranspiration index value and irrigation capacity of an area, see Figure 5.4). Targeting the application of products to the places that need it the most could help to make the application of products more efficient, reducing negative environmental impact of unnecessary product application and making application more cost-effective for farmers. Adoption of this method would result in a more environmentally and economically efficient food system. Although currently the number of variables inputted into the RootTarget model is limited, there is opportunity for further development by addition of more compounds, species and/or environmental variables.

6.1.7 Conclusions and outcomes

As illustrated, effectively incorporating machine learning and modelling into agricultural research can contribute to increasing the efficiency of the process. There is the potential to speed up the discovery process for new chemicals by using the predictive power of machine learning (Chapter 2) to predict potentially active analogues of a compound before moving to time consuming and costly lab experiments. Additionally, modelling where implementation of the products would be most effective would make the dissemination of knowledge and products more effective and has the potential to reduce waste. The methods outlined in this thesis provide an opportunity for further drug discovery methods and modelling to be built upon and strengthened.

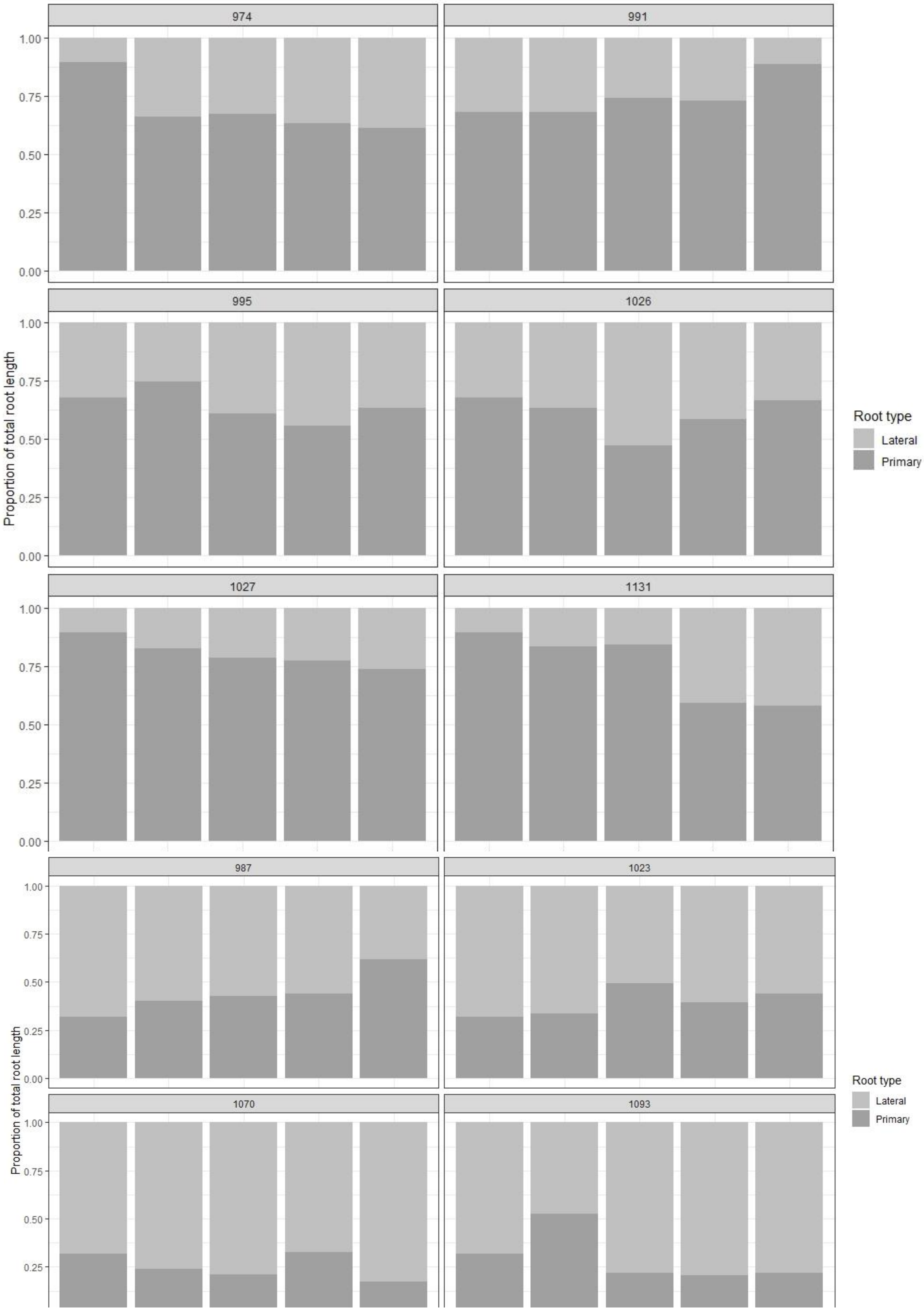
There is significant further work that must be done in order to develop the five RGPs into products that reliably promote root growth across cultivars, as the variability between conditions and cultivars was demonstrated in Chapter 2 and 3. In addition to identifying novel RGPs and determining their efficacy in different cultivars and conditions, three methodologies were created and explored in Chapters 2, 4 and 5: using machine learning for predicting activity of compounds, quantifying the effect of spatial heterogeneity within a glasshouse and using mapping software to identify regions that would likely benefit from plant growth regulators (PGRs) with specific optimum ranges of activity.

These methodologies are transferable and can be applied to different agrochemical discovery libraries, glasshouse setups and PGRs with different optimum ranges and target species and highlight the importance of collaborations between sectors and the opportunity to use modelling and machine learning in plant science to optimise processes relating to increasing the efficiency of the global food production system.

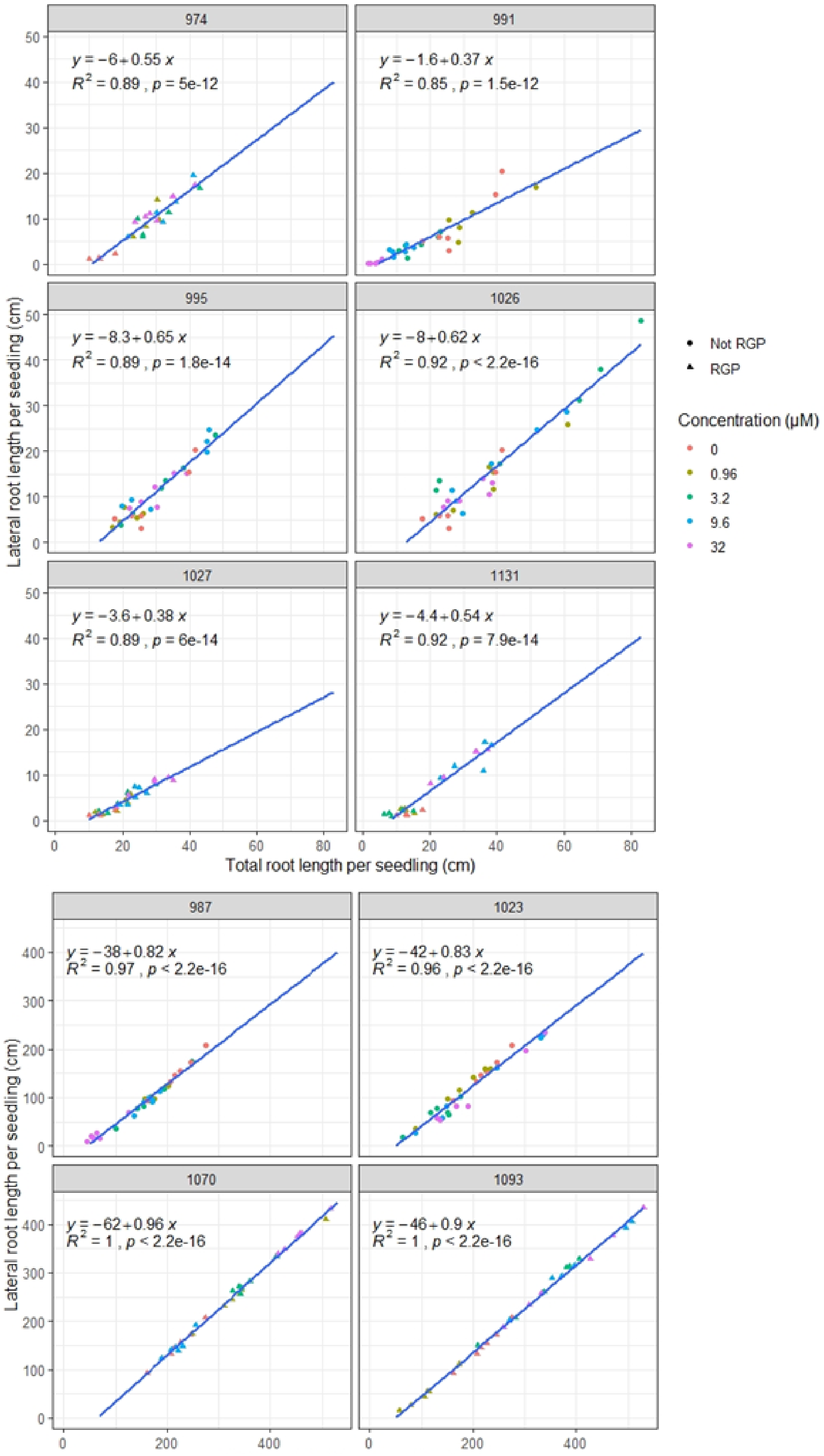
The key findings of this research are:

1. Four analogues of a CK antagonist, in addition to the parent compound, were found to promote root growth in the rice cultivar Nipponbare under optimum conditions using a simple hydroponic system.
2. Using an unsupervised machine learning technique, a training dataset was inputted with existing data of the structural properties of the putative RGPs. The predictions were then corroborated using the results from experimental data and the algorithm had successfully split known RGP and non-RGPs. Using the training dataset, 5 untested compounds were identified as being likely to have RGP properties, and 1 was discounted as unlikely to be an RGP based on their chemical data alone.
3. Seed priming, in addition to the hydroponic delivery of RGPs, was found to promote root growth in seedlings, suggesting a more cost- and time-efficient mode of delivery for the commercial adoption of RGPs.
4. The five RGPs were applied in a commercially relevant environment and species with salt and drought stresses applied. No positive effects were identified under optimum or stressed conditions, highlighting the specificity of action of hormones and the need to investigate the viability of CK antagonists as PGRs in a commercial setting.
5. The long-term effect of priming Nipponbare was investigated. Priming did not affect above ground plant development, but had a negative effect on key reproductive traits, such as panicle and grain number.
6. A novel method to quantify the effect of spatial heterogeneity within a glasshouse was developed, in addition to a spatial weighting system to ensure plants whose growth is strongly affected by location have lower weighting in the statistical analysis.
7. A geospatial agricultural toolkit, RootTarget, was developed to identify regions that grow specific crops and have key environmental conditions for optimising application of PGRs globally.

7 Appendix



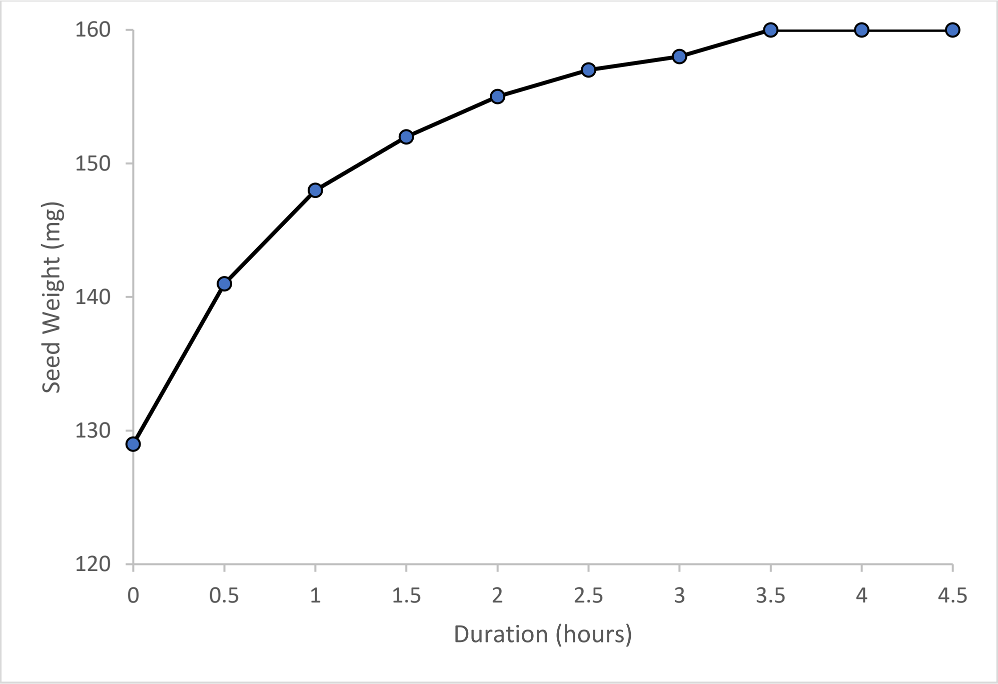
Appendix Figure 1. Stacked barplot indicating the proportion of total root length that the primary and lateral roots accounted for. Lateral roots accounted for a greater proportion of total root length for the putative RGPs: 974,1027,1070,1093 and 1131 in their active ranges compared to the control (“0”).



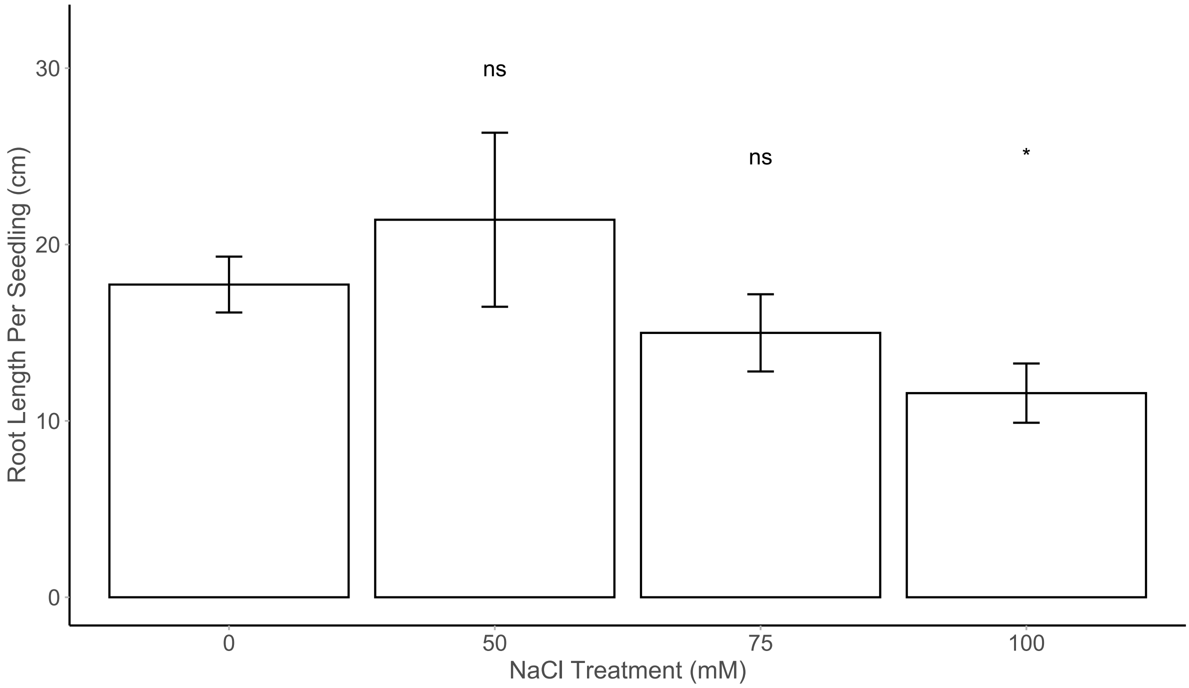
Appendix Figure 2. The two highest correlation coefficient for the linear regressions t were for RGPs 1093 and 1070, indicating that having a high lateral root value is an important trait for developing increased root growth.

Appendix Table 1. Grouping the intercepts and correlation coefficients from the scatterplots in Appendix Figure 2 by RGP and non-RGP shows no clear trend that allows compounds to be discriminated by y-value

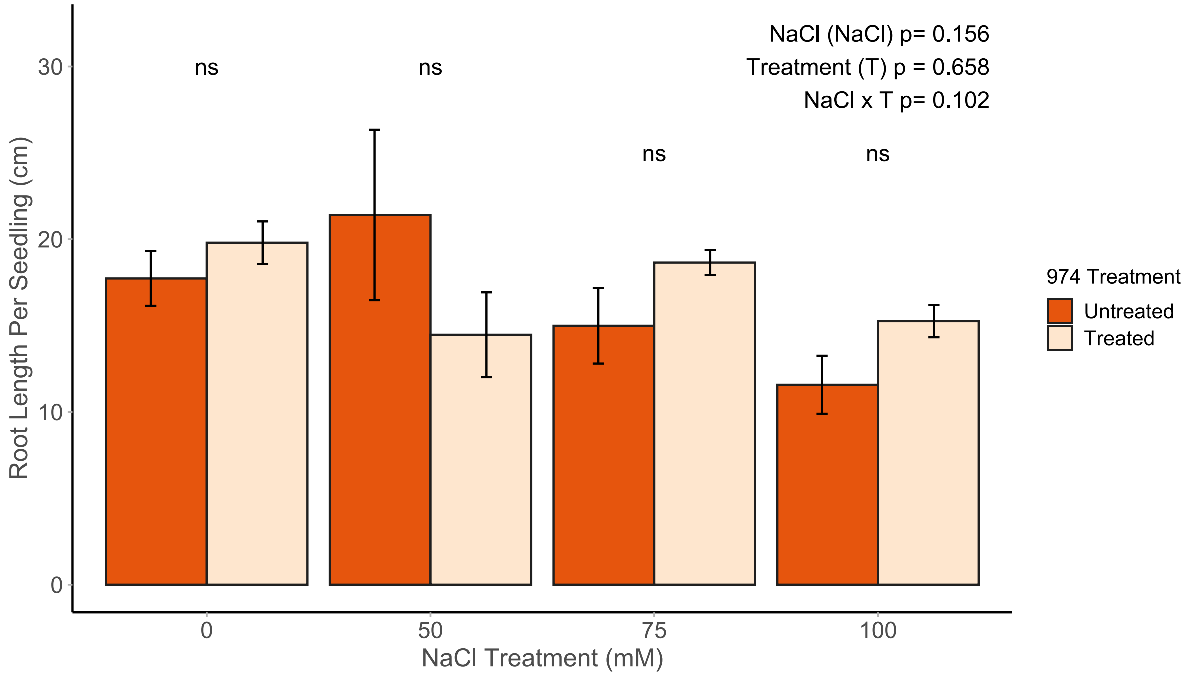
	Compound	***y*-value**
RGP	974	$-6+0.55x$
	1027	$-3.6+0.38x$
	1131	$-4.4+0.54x$
	1093	$-46+0.9x$
	1070	$-62+0.96x$
Not RGP	98	$-38+0.82x$
	1023	$-42+0.83x$
	991	$1.6+0.37x$
	995	$-8.3+0.65x$
	1026	$-8+0.62x$



Appendix Figure 3. Seed soaking, time taken for 5 seeds to reach constant weight and full saturation.

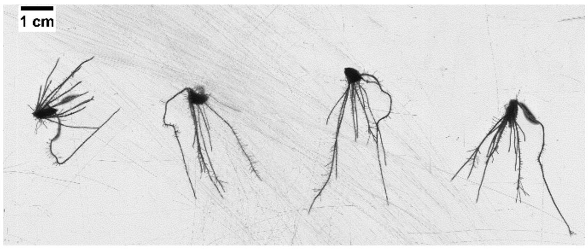
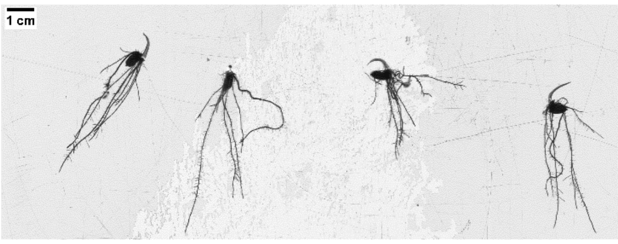
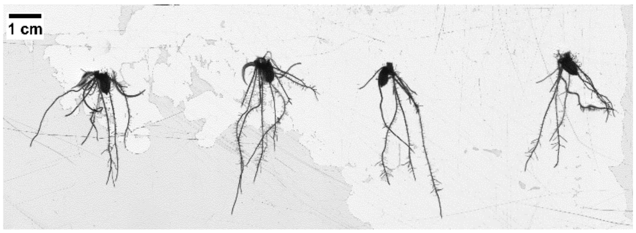


*Appendix Figure 4.Root length of Nipponbare is significantly inhibited by 100mM NaCl treatment. Significance values indicate the results of independent t tests carried out between the control and each salt concentration. *p ≤ .05. Values are means ± SEM (n = 6).*

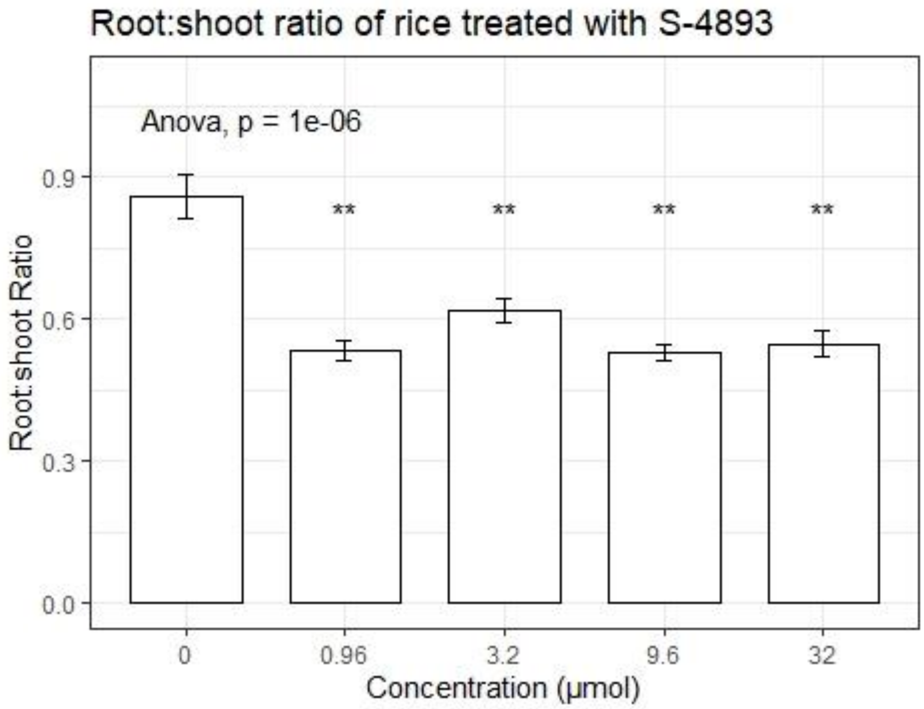


Appendix Figure 5. Treatment of Nipponbare with 3.2uM 974 increased root growth slightly under no salt stress, 75mM and 100mM salt stress, though this was not significant. Significance values indicate the results of an independent t test carried out between treated and untreated seedlings for each salt stress level. Values are means ± SEM (n = 6). Statistics for the effect of treatment and salt stress are the results of a two-way ANOVA.

Appendix Figure 6. Images of the root and shoot of O.sativa cv. Nipponbare treated with compound 974 and salt stress show root length decreasing slightly as salt stress increases for both control and 974 seedlings.

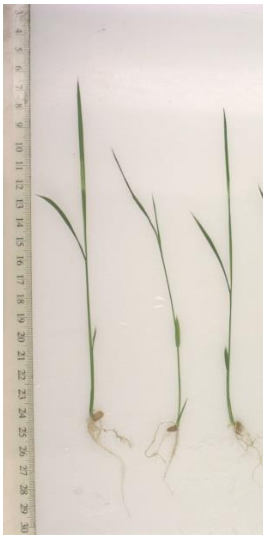
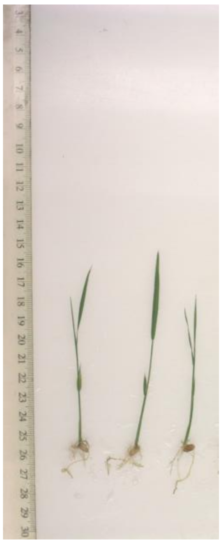
Treatment	WinRhizo Root Image
Control	
0.96	
3.2	
9.6	
32	

Appendix Figure 7. Images of the roots and shoots grown under varying concentrations of S-4893.

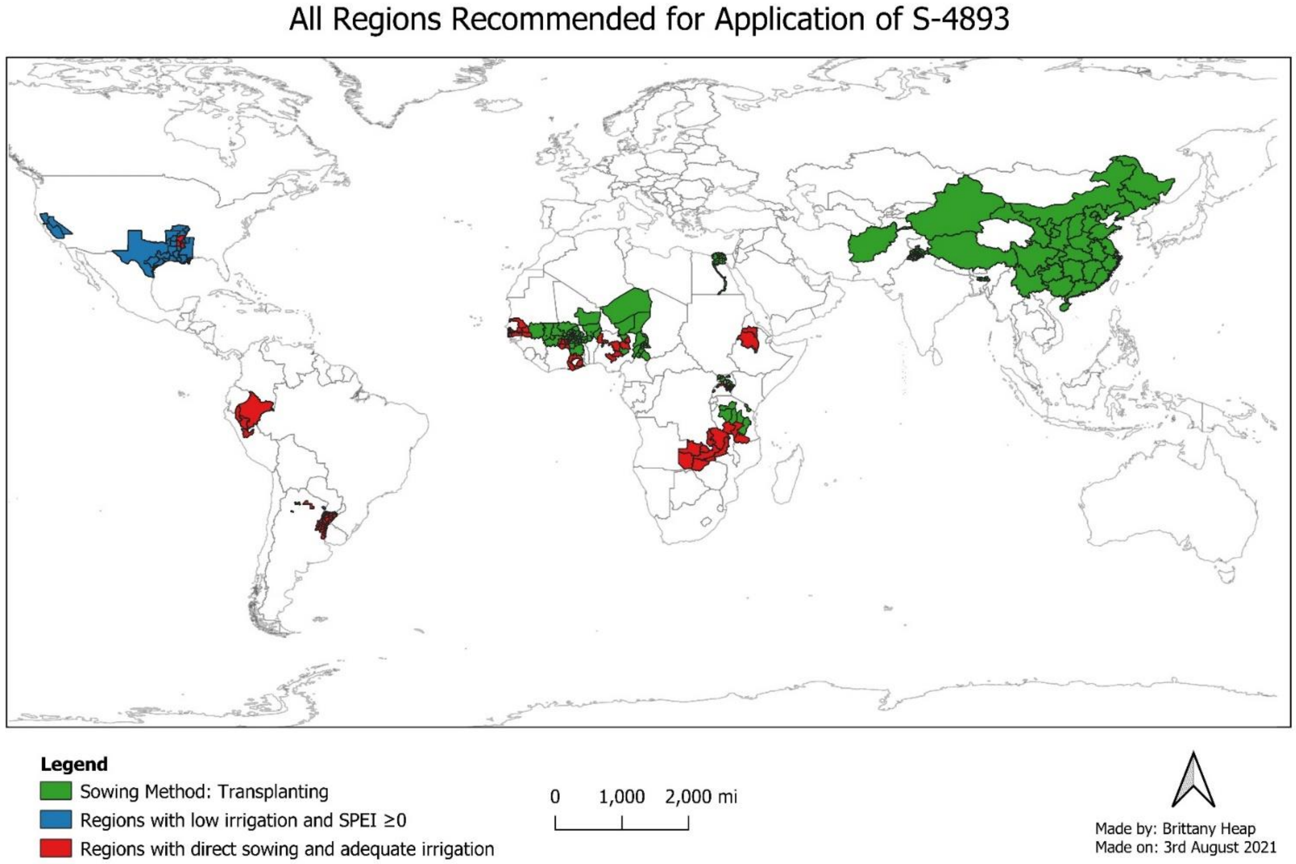


Appendix Figure 8. Root:shoot ratio of RGP S-4893

PEG

	10% (mild)	20% (moderate)	30% (severe)
CONTROL			
S-4893			

Appendix Figure 9. Images of the root and shoot of plants treated with RGP S-4893 and water deficit stress.



Appendix Figure 10. Map illustrating all areas that product application is recommended, taking into account transplanting method, irrigation availability and the 5-year average evapotranspiration value for the peak planting month in that area.

8 References

ABD ALLAH, A. A., BADAWY, S. A., ZAYED, B. A. & EL-GOHARY, A. A. 2010. The role of root system traits in the drought tolerance of rice (Oryza sativa L.). *Journal of Plant Production,* 1**,** 621-631.

ABDUL-BAKI, A. A. & ANDERSON, J. D. 1973. Vigor Determination in Soybean Seed by Multiple Criteria1. *Crop Science,* 13**,** cropsci1973.0011183X001300060013x.

AFZAL, I., BUTT, A., REHMAN, H. U., BASRA, A. B. A. & AFZAL, A. 2012. *Alleviation of salt stress in fine aromatic rice by seed priming*, Southern Cross Journals.

AFZAL, I., JAVED, T., AMIRKHANI, M. & TAYLOR, A. G. 2020. Modern Seed Technology: Seed Coating Delivery Systems for Enhancing Seed and Crop Performance. *Agriculture,* 10.

ALBACETE, A., GHANEM, M. E., MARTÍNEZ-ANDÚJAR, C., ACOSTA, M., SÁNCHEZ-BRAVO, J., MARTÍNEZ, V., LUTTS, S., DODD, I. C. & PÉREZ-ALFOCEA, F. 2008. Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (Solanum lycopersicum L.) plants. *Journal of Experimental Botany,* 59**,** 4119-4131.

ALEXANDRATOS, N. & BRUINSMA, J. 2012. *World Agriculture Towards 2030/2050: The 2012 Revision*.

ALMEIDA, D. M., ALMADANIM, M. C., LOURENÇO, T., ABREU, I. A., SAIBO, N. J. M. & OLIVEIRA, M. M. 2016. Screening for Abiotic Stress Tolerance in Rice: Salt, Cold, and Drought. *In:* DUQUE, P. (ed.) *Environmental Responses in Plants: Methods and Protocols.* New York, NY: Springer New York.

AMIRKHANI, M., MORTAZ, M., NETRAVALI, A. & TAYLOR, A. 2014. *Seed Coating Technologies Employing a Plant-based Green Binder*.

AMIS®AGRIGLOBE®. 2018. *Plant growth regulators (PGR)/value ($ m)* [Online]. [Accessed 08/03 2020].

AMRITHA, M. S., SRIDHARAN, K., PUTHUR, J. T. & DHANKHER, O. P. 2021. Priming with Nanoscale Materials for Boosting Abiotic Stress Tolerance in Crop Plants. *Journal of Agricultural and Food Chemistry,* 69**,** 10017-10035.

APEL, K. & HIRT, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol,* 55**,** 373-99.

ARAI-SANOH, Y., TAKAI, T., YOSHINAGA, S., NAKANO, H., KOJIMA, M., SAKAKIBARA, H., KONDO, M. & UGA, Y. 2014. Deep rooting conferred by DEEPER ROOTING 1 enhances rice yield in paddy fields. *Scientific Reports,* 4**,** 5563.

ARATA, Y., NAGASAWA-IIDA, A., UNEME, H., NAKAJIMA, H., KAKIMOTO, T. & SATO, R. 2010. The phenylquinazoline compound S-4893 is a non-competitive cytokinin antagonist that targets Arabidopsis cytokinin receptor CRE1 and promotes root growth in Arabidopsis and rice. *Plant Cell Physiol,* 51**,** 2047-59.

ASCH, F., DINGKUHN, M., DÖRFFLING, K. & MIEZAN, K. 2000. Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica,* 113**,** 109.

ASHIKARI, M., SAKAKIBARA, H., LIN, S., YAMAMOTO, T., TAKASHI, T., NISHIMURA, A., ANGELES, E. R., QIAN, Q., KITANO, H. & MATSUOKA, M. 2005. Cytokinin Oxidase Regulates Rice Grain Production. *Science,* 309**,** 741-745.

BAILEY, R. A. 2017. Inference from Randomized (Factorial) Experiments. *Statistical Science,* 32**,** 352-355, 4.

BAILEY, R. A. & DRUILHET, P. 2014. Optimal cross-over designs for full interaction models. *The Annals of Statistics,* 42**,** 2282-2300, 19.

BANSAL, K. C., LENKA, S. K. & MONDAL, T. K. 2014. Genomic resources for breeding crops with enhanced abiotic stress tolerance. *Plant Breeding,* 133**,** 1-11.

BASU, S., RAMEGOWDA, V., KUMAR, A. & PEREIRA, A. 2016. Plant adaptation to drought stress. *F1000Research,* 5**,** F1000 Faculty Rev-1554.

BIVAND, R., MÜLLER, W. G. & REDER, M. 2009. Power calculations for global and local Moran's I. *Computational Statistics & Data Analysis,* 53**,** 2859-2872.

BOONMAN, A., PRINSEN, E., GILMER, F., SCHURR, U., PEETERS, A. J. M., VOESENEK, L. A. C. J. & PONS, T. L. 2007. Cytokinin Import Rate as a Signal for Photosynthetic Acclimation to Canopy Light Gradients. *Plant Physiology,* 143**,** 1841-1852.

BORGES, A. A., JIMÉNEZ-ARIAS, D., EXPÓSITO-RODRÍGUEZ, M., SANDALIO, L. M. & PÉREZ, J. A. 2014. Priming crops against biotic and abiotic stresses: MSB as a tool for studying mechanisms. *Frontiers in plant science,* 5**,** 642-642.

BOULARD, T., HAXAIRE, R., LAMRANI, M. A., ROY, J. C. & JAFFRIN, A. 1999. Characterization and Modelling of the Air Fluxes induced by Natural Ventilation in a Greenhouse. *Journal of Agricultural Engineering Research,* 74**,** 135-144.

BOULARD, T., KITTAS, C., ROY, J. C. & WANG, S. 2002. SE—Structures and Environment: Convective and Ventilation Transfers in Greenhouses, Part 2: Determination of the Distributed Greenhouse Climate. *Biosystems Engineering,* 83**,** 129-147.

BOYER, J. S. 1982. Plant Productivity and Environment. *Science,* 218**,** 443-448.

BRIEN, C. J., BERGER, B., RABIE, H. & TESTER, M. 2013. Accounting for variation in designing greenhouse experiments with special reference to greenhouses containing plants on conveyor systems. *Plant Methods,* 9**,** 5.

BROWNFIELD, D. L., TODD, C. D. & DEYHOLOS, M. K. 2008. Analysis of Arabidopsis arginase gene transcription patterns indicates specific biological functions for recently diverged paralogs. *Plant Mol Biol,* 67**,** 429-40.

BURRELL, T., FOZARD, S., HOLROYD, G. H., FRENCH, A. P., POUND, M. P., BIGLEY, C. J., JAMES TAYLOR, C. & FORDE, B. G. 2017. The Microphenotron: a robotic miniaturized plant phenotyping platform with diverse applications in chemical biology. *Plant Methods,* 13**,** 10.

CARABELLI, M., POSSENTI, M., SESSA, G., CIOLFI, A., SASSI, M., MORELLI, G. & RUBERTI, I. 2007. Canopy shade causes a rapid and transient arrest in leaf development through auxin-induced cytokinin oxidase activity. *Genes Dev,* 21**,** 1863-8.

CHAMPOUX, M. C., WANG, G., SARKARUNG, S., MACKILL, D. J., O'TOOLE, J. C., HUANG, N. & MCCOUCH, S. R. 1995. Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. *Theoretical and Applied Genetics,* 90**,** 969-981.

CHAPMAN, N., MILLER, A. J., LINDSEY, K. & WHALLEY, W. R. 2012. Roots, water, and nutrient acquisition: let's get physical. *Trends Plant Sci,* 17**,** 701-10.

CHEN, H., AN, R., TANG, J.-H., CUI, X.-H., HAO, F.-S., CHEN, J. & WANG, X.-C. 2007. Over-expression of a vacuolar Na+/H+ antiporter gene improves salt tolerance in an upland rice. *Molecular Breeding,* 19**,** 215-225.

CISCAR, J.-C. 2012. The impacts of climate change in Europe (the PESETA research project). *Climatic Change,* 112**,** 1-6.

CIURA, J. & KRUK, J. 2018. Phytohormones as targets for improving plant productivity and stress tolerance. *Journal of Plant Physiology,* 229**,** 32-40.

CLARK, L. J., COPE, R. E., WHALLEY, W. R., BARRACLOUGH, P. B. & WADE, L. J. 2002. Root penetration of strong soil in rainfed lowland rice: comparison of laboratory screens with field performance. *Field Crops Research,* 76**,** 189-198.

CLARK, L. J., WHALLEY, W. R., LEIGH, R. A., DEXTER, A. R. & BARRACLOUGH, P. B. 1999. Evaluation of agar and agarose gels for studying mechanical impedance in rice roots. *Plant and Soil,* 207**,** 37-43.

COLEBROOK, E. H., THOMAS, S. G., PHILLIPS, A. L. & HEDDEN, P. 2014. The role of gibberellin signalling in plant responses to abiotic stress. *Journal of experimental biology,* 217**,** 67-75.

COMAS, L., BECKER, S., CRUZ, V. M., BYRNE, P. F. & DIERIG, D. A. 2013. Root traits contributing to plant productivity under drought. *Frontiers in Plant Science,* 4.

COX, G. M. & COCHRAN, W. 1946. Designs of greenhouse experiments for statistical analysis. *Soil Science,* 62**,** 87-98.

DAS, K. & ROYCHOUDHURY, A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science,* 2.

DATTA, A., ULLAH, H. & FERDOUS, Z. 2017. Water management in rice. *Rice production worldwide.* Springer.

DU, L., JIAO, F., CHU, J., JIN, G., CHEN, M. & WU, P. 2007. The two-component signal system in rice (Oryza sativa L.): A genome-wide study of cytokinin signal perception and transduction. *Genomics,* 89**,** 697-707.

EDMONDSON, R. N. 1989. Glasshouse Design for Repeatedly Harvested Crops. *Biometrics,* 45**,** 301-307.

ENRIQUEZ, Y., YADAV, S., EVANGELISTA, G. K., VILLANUEVA, D., BURAC, M. A. & PEDE, V. 2021. Disentangling Challenges to Scaling Alternate Wetting and Drying Technology for Rice Cultivation: Distilling Lessons From 20 Years of Experience in the Philippines. *Frontiers in Sustainable Food Systems,* 5.

ESCANDÓN, M., CAÑAL, M. J., PASCUAL, J., PINTO, G., CORREIA, B., AMARAL, J. & MEIJÓN, M. 2016. Integrated physiological and hormonal profile of heat-induced thermotolerance in Pinus radiata. *Tree Physiology,* 36**,** 63-77.

FAHAD, S., HUSSAIN, S., SAUD, S., HASSAN, S., IHSAN, Z., SHAH, A. N., WU, C., YOUSAF, M., NASIM, W., ALHARBY, H., ALGHABARI, F. & HUANG, J. 2016. Exogenously Applied Plant Growth Regulators Enhance the Morpho-Physiological Growth and Yield of Rice under High Temperature. *Frontiers in Plant Science,* 7.

FAN, J., MCCONKEY, B., WANG, H. & JANZEN, H. 2016. Root distribution by depth for temperate agricultural crops. *Field Crops Research,* 189**,** 68-74.

FAO 2009a. Global Agriculture Towards 2050.

FAO 2009b. Global agriculture towards 2050. .

FAO 2018. The future of food and agriculture – Alternative pathways to 2050. Rome. . 224 pp.

FAO. 2021a. *FAO Soils Portal* [Online]. Available: https://www.fao.org/soils-portal/soil-management/manejo-de-suelos-problematicos/suelos-afectados-por-salinidad/more-information-on-salt-affected-soils [Accessed 30/10/2021].

FAO 2021b. *The State of Food Security and Nutrition in the World 2021: Transforming food systems for food security, improved nutrition and affordable healthy diets for all*, FOOD & AGRICULTURE ORGN.

FAROOQ, M., AZIZ, T., WAHID, A., LEE, D.-J. & SIDDIQUE, K. H. M. 2009a. Chilling tolerance in maize: agronomic and physiological approaches. *Crop and Pasture Science,* 60**,** 501-516.

FAROOQ, M., WAHID, A., KOBAYASHI, N., FUJITA, D. & BASRA, S. 2009b. Plant drought stress: Effects, mechanisms and management. *Agronomy for sustainable development,* 29**,** 185-212.

FENG, Y., LIU, J., ZHAI, L., GAN, Z., ZHANG, G., YANG, S., WANG, Y., WU, T., ZHANG, X., XU, X. & HAN, Z. 2019. Natural variation in cytokinin maintenance improves salt tolerance in apple rootstocks. *Plant, Cell & Environment,* 42**,** 424-436.

FERNÁNDEZ, L. 2021. *Agricultural chemicals market value worldwide in 2018 and 2019 with a forecast from 2020 to 2025* [Online]. Available: https://www.statista.com/statistics/311943/agrochemical-market-value-worldwide/ [Accessed 27th October 2021].

FERREIRA, F. J. & KIEBER, J. J. 2005. Cytokinin signaling. *Current Opinion in Plant Biology,* 8**,** 518-525.

FISHER, R. A. 1992. The Arrangement of Field Experiments. *In:* KOTZ, S. & JOHNSON, N. L. (eds.) *Breakthroughs in Statistics: Methodology and Distribution.* New York, NY: Springer New York.

FOLEY, J. A., RAMANKUTTY, N., BRAUMAN, K. A., CASSIDY, E. S., GERBER, J. S., JOHNSTON, M., MUELLER, N. D., O'CONNELL, C., RAY, D. K., WEST, P. C., BALZER, C., BENNETT, E. M., CARPENTER, S. R., HILL, J., MONFREDA, C., POLASKY, S., ROCKSTRÖM, J., SHEEHAN, J., SIEBERT, S., TILMAN, D. & ZAKS, D. P. M. 2011. Solutions for a cultivated planet. *Nature,* 478**,** 337-342.

FOZARD, S. & FORDE, B. G. 2018. Novel Micro-Phenotyping Approach to Chemical Genetic Screening for Increased Plant Tolerance to Abiotic Stress. *In:* FAUSER, F. & JONIKAS, M. (eds.) *Plant Chemical Genomics: Methods and Protocols.* New York, NY: Springer New York.

FROMM, H. 2019. Root Plasticity in the Pursuit of Water. *Plants (Basel, Switzerland),* 8**,** 236.

FUKAGAWA, N. K. & ZISKA, L. H. 2019. Rice: Importance for Global Nutrition. *Journal of Nutritional Science and Vitaminology,* 65**,** S2-S3.

FUKAI, S., PANTUWAN, G., JONGDEE, B. & COOPER, M. 1999. Screening for drought resistance in rainfed lowland rice. *Field Crops Research,* 64**,** 61-74.

GAMUYAO, R., CHIN, J. H., PARIASCA-TANAKA, J., PESARESI, P., CATAUSAN, S., DALID, C., SLAMET-LOEDIN, I., TECSON-MENDOZA, E. M., WISSUWA, M. & HEUER, S. 2012. The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency. *Nature,* 488**,** 535-539.

GAN, S. & AMASINO, R. M. 1995. Inhibition of Leaf Senescence by Autoregulated Production of Cytokinin. *Science,* 270**,** 1986-1988.

GASKELL, G., ALLUM, N., WAGNER, W., KRONBERGER, N., TORGERSEN, H., HAMPEL, J. & BARDES, J. 2004. GM foods and the misperception of risk perception. *Risk Anal,* 24**,** 185-94.

GETIS, A. & ORD, J. K. 1992. The Analysis of Spatial Association by Use of Distance Statistics. *Geographical Analysis,* 24**,** 189-206.

GHANEM, M. E., ALBACETE, A., MARTÍNEZ-ANDÚJAR, C., ACOSTA, M., ROMERO-ARANDA, R., DODD, I. C., LUTTS, S. & PÉREZ-ALFOCEA, F. 2008. Hormonal changes during salinity-induced leaf senescence in tomato (Solanum lycopersicum L.). *Journal of Experimental Botany,* 59**,** 3039-3050.

GHANEM, M. E., ALBACETE, A., SMIGOCKI, A. C., FRÉBORT, I., POSPÍŠILOVÁ, H., MARTÍNEZ-ANDÚJAR, C., ACOSTA, M., SÁNCHEZ-BRAVO, J., LUTTS, S., DODD, I. C. & PÉREZ-ALFOCEA, F. 2010. Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (Solanum lycopersicum L.) plants. *Journal of Experimental Botany,* 62**,** 125-140.

GILROY, S., BIAŁASEK, M., SUZUKI, N., GÓRECKA, M., DEVIREDDY, A. R., KARPIŃSKI, S. & MITTLER, R. 2016. ROS, Calcium, and Electric Signals: Key Mediators of Rapid Systemic Signaling in Plants. *Plant Physiology,* 171**,** 1606.

GLOBACHEM. 2021a. *Crop Protection* [Online]. Available: https://www.globachem.com/en/agrochemicals# [Accessed 12/11 2021].

GLOBACHEM. 2021b. *Globachem Discovery* [Online]. Available: https://www.globachem.com/en/globachem-discovery# [Accessed 22/11 2021].

GODOY, F., OLIVOS-HERNÁNDEZ, K., STANGE, C. & HANDFORD, M. 2021. Abiotic Stress in Crop Species: Improving Tolerance by Applying Plant Metabolites. *Plants (Basel, Switzerland),* 10**,** 186.

GOSWAMI, A., BANERJEE, R. & RAHA, S. 2013. Drought resistance in rice seedlings conferred by seed priming : role of the anti-oxidant defense mechanisms. *Protoplasma,* 250**,** 1115-29.

GOWDA, V. R. P., HENRY, A., YAMAUCHI, A., SHASHIDHAR, H. E. & SERRAJ, R. 2011. Root biology and genetic improvement for drought avoidance in rice. *Field Crops Research,* 122**,** 1-13.

GREGORINI, G. & LALOUE, M. 1980. Biological Effects of Cytokinin Antagonists 7-(Pentylamino) and 7-(Benzylamino)-3-Methylpyrazolo(4,3-d)Pyrimidines on Suspension-cultured Tobacco Cells. *Plant physiology,* 65**,** 363-367.

GUHA, R. 2013. On exploring structure-activity relationships. *Methods in molecular biology (Clifton, N.J.),* 993**,** 81-94.

GUPTA, M. & AHMAD, M. A. 2014. Arsenate induced differential response in rice genotypes. *Ecotoxicol Environ Saf,* 107**,** 46-54.

HA, S., VANKOVA, R., YAMAGUCHI-SHINOZAKI, K., SHINOZAKI, K. & TRAN, L. S. P. 2012. Cytokinins: metabolism and function in plant adaptation to environmental stresses. *Trends in Plant Science,* 17**,** 172-179.

HAEFELE, S. & BOUMAN, B. 2009. Drought-prone rainfed lowland rice in Asia: limitations and management options.

HANSEN, J. & HØGH-SCHMIDT, K. A computer controlled chamber system designed for greenhouse microclimatic modelling and control. International Symposium on Plant Production in Closed Ecosystems 440, 1996. 310-315.

HARDY, E. M. & BLUMENTHAL, D. M. 2008. An efficient and inexpensive system for greenhouse pot rotation. *HortScience,* 43**,** 965-966.

HARGREAVES, C. E., GREGORY, P. J. & BENGOUGH, A. G. 2009. Measuring root traits in barley (Hordeum vulgare ssp. vulgare and ssp. spontaneum) seedlings using gel chambers, soil sacs and X-ray microtomography. *Plant and Soil,* 316**,** 285-297.

HE, H., YOU, C., WU, H., ZHU, D., YANG, R., HE, Q., XU, L., GUI, W. & WU, L. 2018. Effects of Nursery Tray and Transplanting Methods on Rice Yield. *Agronomy Journal,* 110**,** 104-114.

HEAP, B., HOLDEN, C., TAYLOR, J. & MCAINSH, M. 2020. ROS Crosstalk in Signalling Pathways. *eLS.*

HEAP, B., MCAINSH, M. & TOLEDO-ORTIZ, G. 2021. RootTarget: A dynamic model enabling the targeted application of plant growth regulators for rice. *PLANTS, PEOPLE, PLANET*.

HECHT, S. M., BOCK, R. M., SCHMITZ, R. Y., SKOOG, F. & LEONARD, N. J. 1971. Cytokinins: development of a potent antagonist. *Proceedings of the National Academy of Sciences,* 68**,** 2608-2610.

HENRY, A., GOWDA, V. R. P., TORRES, R. O., MCNALLY, K. L. & SERRAJ, R. 2011. Variation in root system architecture and drought response in rice (Oryza sativa): Phenotyping of the OryzaSNP panel in rainfed lowland fields. *Field Crops Research,* 120**,** 205-214.

HORIE, T., KARAHARA, I. & KATSUHARA, M. 2012. Salinity tolerance mechanisms in glycophytes: An overview with the central focus on rice plants. *Rice,* 5**,** 11.

HOWELL, K. R., SHRESTHA, P. & DODD, I. C. 2015. Alternate wetting and drying irrigation maintained rice yields despite half the irrigation volume, but is currently unlikely to be adopted by smallholder lowland rice farmers in Nepal. *Food and Energy Security,* 4**,** 144-157.

HUANG, C., YANG, W., DUAN, L., JIANG, N., CHEN, G., XIONG, L. & LIU, Q. 2013. Rice panicle length measuring system based on dual-camera imaging. *Computers and Electronics in Agriculture,* 98**,** 158-165.

HUSSAIN, H. A., HUSSAIN, S., KHALIQ, A., ASHRAF, U., ANJUM, S. A., MEN, S. & WANG, L. 2018. Chilling and Drought Stresses in Crop Plants: Implications, Cross Talk, and Potential Management Opportunities. *Frontiers in Plant Science,* 9.

HUSSAIN, S., KHAN, F., HUSSAIN, H. A. & NIE, L. 2016. Physiological and Biochemical Mechanisms of Seed Priming-Induced Chilling Tolerance in Rice Cultivars. *Frontiers in Plant Science,* 7.

HUTCHISON, C. E. & KIEBER, J. J. 2002. Cytokinin signaling in Arabidopsis. *The Plant cell,* 14 Suppl**,** S47-S59.

ILANGUMARAN, G. & SMITH, D. L. 2017. Plant Growth Promoting Rhizobacteria in Amelioration of Salinity Stress: A Systems Biology Perspective. *Frontiers in Plant Science,* 8.

IOIO, R. D., NAKAMURA, K., MOUBAYIDIN, L., PERILLI, S., TANIGUCHI, M., MORITA, M. T., AOYAMA, T., COSTANTINO, P. & SABATINI, S. 2008. A genetic framework for the control of cell division and differentiation in the root meristem. *Science,* 322**,** 1380-1384.

JAIN, M., TYAGI, A. K. & KHURANA, J. P. 2006. Molecular characterization and differential expression of cytokinin-responsive type-A response regulators in rice (Oryza sativa). *BMC plant biology,* 6**,** 1-1.

JAMESON, P. E. & SONG, J. 2015. Cytokinin: a key driver of seed yield. *Journal of Experimental Botany,* 67**,** 593-606.

JEON, J., KIM, N. Y., KIM, S., KANG, N. Y., NOVÁK, O., KU, S.-J., CHO, C., LEE, D. J., LEE, E.-J., STRNAD, M. & KIM, J. 2010. A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in Arabidopsis. *The Journal of biological chemistry,* 285**,** 23371-23386.

JEONG, J. S., KIM, Y. S., BAEK, K. H., JUNG, H., HA, S.-H., DO CHOI, Y., KIM, M., REUZEAU, C. & KIM, J.-K. 2010. Root-Specific Expression of OsNAC10 Improves Drought Tolerance and Grain Yield in Rice under Field Drought Conditions *Plant Physiology,* 153**,** 185-197.

JOE, M. M., ISLAM, M. R., KARTHIKEYAN, B., BRADEEPA, K., SIVAKUMAAR, P. K. & SA, T. 2012. Resistance responses of rice to rice blast fungus after seed treatment with the endophytic Achromobacter xylosoxidans AUM54 strains. *Crop Protection,* 42**,** 141-148.

JOSHI, R., SAHOO, K. K., TRIPATHI, A. K., KUMAR, R., GUPTA, B. K., PAREEK, A. & SINGLA-PAREEK, S. L. 2018. Knockdown of an inflorescence meristem-specific cytokinin oxidase – OsCKX2 in rice reduces yield penalty under salinity stress condition. *Plant, Cell & Environment,* 41**,** 936-946.

JU, C., BURESH, R. J., WANG, Z., ZHANG, H., LIU, L., YANG, J. & ZHANG, J. 2015. Root and shoot traits for rice varieties with higher grain yield and higher nitrogen use efficiency at lower nitrogen rates application. *Field Crops Research,* 175**,** 47-55.

JULKOWSKA, M. 2018. Releasing the Cytokinin Brakes on Root Growth. *Plant physiology,* 177**,** 865-866.

JUNG, J. K. H. M. & MCCOUCH, S. R. M. 2013. Getting to the roots of it: genetic and hormonal control of root architecture. *Frontiers in plant science,* 4**,** 186.

KADIOGLU, A. & TERZI, R. 2007. A Dehydration Avoidance Mechanism: Leaf Rolling. *Botanical Review,* 73**,** 290-302.

KAKADE, D., SINGH, J., WALLALWAR, M., JANJAL, A., GUPTA, A., RAGHUVANSHI, R., KONGBRAILATPAM, M., VERULKAR, S. & BANERJEE, S. 2017. Differential Response of Root Morphology of Rice (Oryza sativa L.) Genotypes under Different Phosphorus Conditions. *International Journal of Current Microbiology and Applied Sciences,* 6**,** 149-160.

KASAHARA, H., TAKEI, K., UEDA, N., HISHIYAMA, S., YAMAYA, T., KAMIYA, Y., YAMAGUCHI, S. & SAKAKIBARA, H. 2004. Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in Arabidopsis. *J Biol Chem,* 279**,** 14049-54.

KEMPTHORNE, O. 1957. 126. Query: Arrangements of Pots in Greenhouse Experiments. *Biometrics,* 13**,** 235-237.

KHAN, M., ROZHON, W. & POPPENBERGER, B. 2014. The Role of Hormones in the Aging of Plants - A Mini-Review. *Gerontology,* 60**,** 49-55.

KHUSH, G. S. 2005. What it will take to Feed 5.0 Billion Rice consumers in 2030. *Plant Molecular Biology,* 59**,** 1-6.

KIEBER, J. J. & SCHALLER, G. E. 2014. Cytokinins. *The arabidopsis book,* 12**,** e0168-e0168.

KIM, J. H., LIM, S. D. & JANG, C. S. 2020a. Oryza sativa drought-, heat-, and salt-induced RING finger protein 1 (OsDHSRP1) negatively regulates abiotic stress-responsive gene expression. *Plant Molecular Biology,* 103**,** 235-252.

KIM, S., HAN, L., YU, B., HÄHNKE, V. D., BOLTON, E. E. & BRYANT, S. H. 2015. PubChem structure–activity relationship (SAR) clusters. *Journal of Cheminformatics,* 7**,** 33.

KIM, W., IIZUMI, T. & NISHIMORI, M. 2019. Global Patterns of Crop Production Losses Associated with Droughts from 1983 to 2009. *Journal of Applied Meteorology and Climatology,* 58**,** 1233-1244.

KIM, Y., CHUNG, Y. S., LEE, E., TRIPATHI, P., HEO, S. & KIM, K.-H. 2020b. Root Response to Drought Stress in Rice (Oryza sativa L.). *International Journal of Molecular Sciences,* 21**,** 1513.

KIMURA, K., YASUTAKE, D., KOIKAWA, K. & KITANO, M. 2020. Spatiotemporal variability of leaf photosynthesis and its linkage with microclimates across an environment-controlled greenhouse. *Biosystems Engineering,* 195**,** 97-115.

KOBAYASHI, K., BABA, S., OBAYASHI, T., SATO, M., TOYOOKA, K., KERÄNEN, M., ARO, E.-M., FUKAKI, H., OHTA, H., SUGIMOTO, K. & MASUDA, T. 2012. Regulation of Root Greening by Light and Auxin/Cytokinin Signaling in Arabidopsis *The Plant Cell,* 24**,** 1081-1095.

KOMYSHEV, E., GENAEV, M. & AFONNIKOV, D. 2017. Evaluation of the SeedCounter, A Mobile Application for Grain Phenotyping. *Frontiers in Plant Science,* 7.

KOUTSOS, T. M., MENEXES, G. C. & MAMOLOS, A. P. 2021. The Use of Crop Yield Autocorrelation Data as a Sustainable Approach to Adjust Agronomic Inputs. *Sustainability,* 13.

KUDOYAROVA, G. R., VYSOTSKAYA, L. B., CHERKOZYANOVA, A. & DODD, I. C. 2007. Effect of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (Solanum lycopersicum L.) xylem sap and leaves. *Journal of experimental botany,* 58**,** 161-168.

KUIJKEN, R. C. P., VAN EEUWIJK, F. A., MARCELIS, L. F. M. & BOUWMEESTER, H. J. 2015. Root phenotyping: from component trait in the lab to breeding. *Journal of Experimental Botany,* 66**,** 5389-5401.

KUMAR, A., DIXIT, S., RAM, T., YADAW, R. B., MISHRA, K. K. & MANDAL, N. P. 2014. Breeding high-yielding drought-tolerant rice: genetic variations and conventional and molecular approaches. *Journal of Experimental Botany,* 65**,** 6265-6278.

KUMAR, S., SACHDEVA, S., BHAT, K. V. & VATS, S. 2018. Plant Responses to Drought Stress: Physiological, Biochemical and Molecular Basis. *In:* VATS, S. (ed.) *Biotic and Abiotic Stress Tolerance in Plants.* Singapore: Springer Singapore.

KURAKAWA, T., UEDA, N., MAEKAWA, M., KOBAYASHI, K., KOJIMA, M., NAGATO, Y., SAKAKIBARA, H. & KYOZUKA, J. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature,* 445**,** 652.

LABORTE, A. G., GUTIERREZ, M. A., BALANZA, J. G., SAITO, K., ZWART, S. J., BOSCHETTI, M., MURTY, M. V. R., VILLANO, L., AUNARIO, J. K., REINKE, R., KOO, J., HIJMANS, R. J. & NELSON, A. 2017. RiceAtlas, a spatial database of global rice calendars and production. *Scientific Data,* 4**,** 170074.

LAPLAZE, L., BENKOVA, E., CASIMIRO, I., MAES, L., VANNESTE, S., SWARUP, R., WEIJERS, D., CALVO, V., PARIZOT, B. & HERRERA-RODRIGUEZ, M. B. 2007a. Cytokinins act directly on lateral root founder cells to inhibit root initiation. *The Plant Cell,* 19**,** 3889-3900.

LAPLAZE, L., BENKOVA, E., CASIMIRO, I., MAES, L., VANNESTE, S., SWARUP, R., WEIJERS, D., CALVO, V., PARIZOT, B., HERRERA-RODRIGUEZ, M. B. A., OFFRINGA, R., GRAHAM, N., DOUMAS, P., FRIML, J., BOGUSZ, D., BEECKMAN, T. & BENNETT, M. 2007b. Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation. *The Plant Cell,* 19**,** 3889-3900.

LAVECCHIA, A. 2015. Machine-learning approaches in drug discovery: methods and applications. *Drug discovery today,* 20**,** 318-331.

LEE, D. H., KIM, Y. S. & LEE, C. B. 2001. The inductive responses of the antioxidant enzymes by salt stress in the rice (Oryza sativa L.). *Journal of Plant Physiology,* 158**,** 737-745.

LESSCHEN, J. P., STOORVOGEL, J. J. & SMALING, E. M. A. 2004. Scaling soil nutrient balances. FAO.

LI, S., ZUO, Q., WANG, X., MA, W., JIN, X., SHI, J. & BEN-GAL, A. 2017. Characterizing roots and water uptake in a ground cover rice production system. *PloS one,* 12**,** e0180713-e0180713.

LIPIEC, J., DOUSSAN, C., NOSALEWICZ, A. & KONDRACKA, K. 2013. Effect of drought and heat stresses on plant growth and yield: a review. *International Agrophysics,* 27**,** 463-477.

LIU, Y., ZHANG, M., MENG, Z., WANG, B. & CHEN, M. 2020. Research Progress on the Roles of Cytokinin in Plant Response to Stress. *International journal of molecular sciences,* 21**,** 6574.

LUTTS, S. B., P.; WOJTYLA, L.; KUBALA, S.; PACE, R.; LECHOWSKA, K.; QUINET, M.; AND GARNCZARSKA, M. 2016. *Seed Priming: New Comprehensive Approaches for an Old Empirical Technique*.

LYNCH, J. P., CHIMUNGU, J. G. & BROWN, K. M. 2014. Root anatomical phenes associated with water acquisition from drying soil: targets for crop improvement. *Journal of Experimental Botany,* 65**,** 6155-6166.

MA, D., CARPENTER, N., MAKI, H., REHMAN, T. U., TUINSTRA, M. R. & JIN, J. 2019. Greenhouse environment modeling and simulation for microclimate control. *Computers and Electronics in Agriculture,* 162**,** 134-142.

MAEGHT, J.-L., REWALD, B. & PIERRET, A. 2013. How to study deep roots-and why it matters. *Frontiers in plant science,* 4**,** 299-299.

MAHAJAN, S. & TUTEJA, N. 2005. Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics,* 444**,** 139-158.

MAIRHOFER, S., ZAPPALA, S., TRACY, S. R., STURROCK, C., BENNETT, M., MOONEY, S. J. & PRIDMORE, T. 2012. RooTrak: automated recovery of three-dimensional plant root architecture in soil from x-ray microcomputed tomography images using visual tracking. *Plant physiology,* 158**,** 561-569.

MAJDOUBI, H., BOULARD, T., FATNASSI, H. & BOUIRDEN, L. 2009. Airflow and microclimate patterns in a one-hectare Canary type greenhouse: an experimental and CFD assisted study. *Agricultural and Forest Meteorology,* 149**,** 1050-1062.

MARUYAMA, K., URANO, K., YOSHIWARA, K., MORISHITA, Y., SAKURAI, N., SUZUKI, H., KOJIMA, M., SAKAKIBARA, H., SHIBATA, D., SAITO, K., SHINOZAKI, K. & YAMAGUCHI-SHINOZAKI, K. 2014.

Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. *Plant Physiol,* 164**,** 1759-71.

MATSUMOTO-KITANO, M., KUSUMOTO, T., TARKOWSKI, P., KINOSHITA-TSUJIMURA, K., VÁCLAVÍKOVÁ, K., MIYAWAKI, K. & KAKIMOTO, T. 2008. Cytokinins are central regulators of cambial activity. *Proceedings of the National Academy of Sciences,* 105**,** 20027-20031.

MCAINSH, M. & TAYLOR, J. 2017. Cell Signalling Mechanisms in Plants. *eLS.*

MCKINLAY, R., DASSYNE, J., DJAMGOZ, M., PLANT, J. A. & VOULVOULIS, N. 2012. Agricultural pesticides and chemical fertilisers. *Pollutants, human health and the environment: a risk based approach, 1st edn. Wiley, West Sussex***,** 181-206.

MEISTER, R., RAJANI, M. S., RUZICKA, D. & SCHACHTMAN, D. P. 2014. Challenges of modifying root traits in crops for agriculture. *Trends in Plant Science,* 19**,** 779-788.

MEKONNEN, M. M. & HOEKSTRA, A. Y. 2016. Four billion people facing severe water scarcity. *Science Advances,* 2**,** e1500323.

MENG, F., XIANG, D., ZHU, J., LI, Y. & MAO, C. 2019. Molecular Mechanisms of Root Development in Rice. *Rice,* 12**,** 1.

MICKELBART, M. V., HASEGAWA, P. M. & BAILEY-SERRES, J. 2015. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nature Reviews Genetics,* 16**,** 237-251.

MISHRA, A. & SALOKHE, V. 2008. Seedling characteristics and the early growth of transplanted rice under different water regimes. *Experimental Agriculture,* 44**,** 365-383.

MISHRA, A. & TANNA, B. 2017. Halophytes: Potential Resources for Salt Stress Tolerance Genes and Promoters. *Frontiers in Plant Science,* 8.

MITTLER, R. 2006. Abiotic stress, the field environment and stress combination. *Trends in Plant Science,* 11**,** 15-19.

MIYAWAKI, K., TARKOWSKI, P., MATSUMOTO-KITANO, M., KATO, T., SATO, S., TARKOWSKA, D., TABATA, S., SANDBERG, G. & KAKIMOTO, T. 2006. Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences,* 103**,** 16598-16603.

MORDOR INTELLIGENCE. 2019. *RICE MARKET - GROWTH, TRENDS, AND FORECAST (2020 - 2025)* [Online]. [Accessed 10/11 2021].

MORRIS, E. C., GRIFFITHS, M., GOLEBIOWSKA, A., MAIRHOFER, S., BURR-HERSEY, J., GOH, T., VON WANGENHEIM, D., ATKINSON, B., STURROCK, C. J., LYNCH, J. P., VISSENBERG, K., RITZ, K., WELLS, D. M., MOONEY, S. J. & BENNETT, M. J. 2017. Shaping 3D Root System Architecture. *Current Biology,* 27**,** R919-R930.

MOTTE, H. & BEECKMAN, T. 2019. The evolution of root branching: increasing the level of plasticity. *Journal of Experimental Botany,* 70**,** 785-793.

MOUBAYIDIN, L., DI MAMBRO, R. & SABATINI, S. 2009. Cytokinin-auxin crosstalk. *Trends Plant Sci,* 14**,** 557-62.

MRÍZOVÁ, K., JISKROVÁ, E., VYROUBALOVÁ, Š., NOVÁK, O., OHNOUTKOVÁ, L., POSPÍŠILOVÁ, H., FRÉBORT, I., HARWOOD, W. A. & GALUSZKA, P. 2013. Overexpression of Cytokinin Dehydrogenase Genes in Barley (Hordeum vulgare cv. Golden Promise) Fundamentally Affects Morphology and Fertility. *PLOS ONE,* 8**,** e79029.

MÜLLER, B. & SHEEN, J. 2008. Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature,* 453**,** 1094-1097.

MUNNS, R. 2002. Munns, R.: Comparative Physiology of Salt and Water Stress. Plant Cell Environ. 28, 239-250. *Plant, cell & environment,* 25**,** 239-250.

MUNNS, R. 2005. Genes and salt tolerance: bringing them together. *New phytologist,* 167**,** 645-663.

MUNNS, R. & GILLIHAM, M. 2015. Salinity tolerance of crops – what is the cost? *New Phytologist,* 208**,** 668-673.

MUNNS, R. & TESTER, M. 2008. Mechanisms of Salinity Tolerance. *Annual Review of Plant Biology,* 59**,** 651-681.

NEHNEVAJOVA, E., RAMIREDDY, E., STOLZ, A., GERDEMANN-KNÖRCK, M., NOVÁK, O., STRNAD, M. & SCHMÜLLING, T. 2019. Root enhancement in cytokinin-deficient oilseed rape causes leaf mineral enrichment, increases the chlorophyll concentration under nutrient limitation and enhances the phytoremediation capacity. *BMC Plant Biology,* 19**,** 83.

NEILL, E. M., BYRD, M. C. R., BILLMAN, T., BRANDIZZI, F. & STAPLETON, A. E. 2019. Plant growth regulators interact with elevated temperature to alter heat stress signaling via the Unfolded Protein Response in maize. *Scientific Reports,* 9**,** 10392.

NGUYEN, K. H., VAN HA, C., NISHIYAMA, R., WATANABE, Y., LEYVA-GONZÁLEZ, M. A., FUJITA, Y., TRAN, U. T., LI, W., TANAKA, M. & SEKI, M. 2016a. Arabidopsis type B cytokinin response regulators ARR1, ARR10, and ARR12 negatively regulate plant responses to drought. *Proceedings of the National Academy of Sciences,* 113**,** 3090-3095.

NGUYEN, N. T., MCINTURF, S. A. & MENDOZA-CÓZATL, D. G. 2016b. Hydroponics: A Versatile System to Study Nutrient Allocation and Plant Responses to Nutrient Availability and Exposure to Toxic Elements. *Journal of visualized experiments : JoVE***,** 54317.

NISHIMOTO, R. 2019. Global trends in the crop protection industry. *Journal of pesticide science,* 44**,** 141-147.

NISHIYAMA, R., WATANABE, Y., FUJITA, Y., LE, D. T., KOJIMA, M., WERNER, T., VANKOVA, R., YAMAGUCHI-SHINOZAKI, K., SHINOZAKI, K., KAKIMOTO, T., SAKAKIBARA, H., SCHMÜLLING, T. & TRAN, L.-S. P. 2011. Analysis of Cytokinin Mutants and Regulation of Cytokinin Metabolic Genes Reveals Important Regulatory Roles of Cytokinins in Drought, Salt and Abscisic Acid Responses, and Abscisic Acid Biosynthesis *The Plant Cell,* 23**,** 2169-2183.

NISLER, J., ZATLOUKAL, M., POPA, I., DOLEZAL, K., STRNAD, M. & SPÍCHAL, L. 2010. Cytokinin receptor antagonists derived from 6-benzylaminopurine. *Phytochemistry,* 71**,** 823-30.

NORTON, G. J., SHAFAEI, M., TRAVIS, A. J., DEACON, C. M., DANKU, J., POND, D., COCHRANE, N., LOCKHART, K., SALT, D., ZHANG, H., DODD, I. C., HOSSAIN, M., ISLAM, M. R. & PRICE, A. H. 2017. Impact of alternate wetting and drying on rice physiology, grain production, and grain quality. *Field Crops Research,* 205**,** 1-13.

O'BRIEN, J. & BENKOVA, E. 2013. Cytokinin cross-talking during biotic and abiotic stress responses. *Frontiers in Plant Science,* 4.

O'TOOLE, J. C., CRUZ, R. T. & SEIBER, J. N. 1979. Epicuticular Wax and Cuticular Resistance in Rice. *Physiologia Plantarum,* 47**,** 239-244.

ORŠOLIĆ, D., PEHAR, V., ŠMUC, T. & STEPANIĆ, V. 2021. Comprehensive machine learning based study of the chemical space of herbicides. *Scientific reports,* 11**,** 1-12.

PAN, S., RASUL, F., LI, W., TIAN, H., MO, Z., DUAN, M. & TANG, X. 2013. Roles of plant growth regulators on yield, grain qualities and antioxidant enzyme activities in super hybrid rice (Oryza sativa L.). *Rice,* 6**,** 9.

PANDEY, B. K., HUANG, G., BHOSALE, R., HARTMAN, S., STURROCK, C. J., JOSE, L., MARTIN, O. C., KARADY, M., VOESENEK, L., LJUNG, K., LYNCH, J. P., BROWN, K. M., WHALLEY, W. R., MOONEY, S. J., ZHANG, D. & BENNETT, M. J. 2021. Plant roots sense soil compaction through restricted ethylene diffusion. *Science,* 371**,** 276-280.

PANDEY, G. K. 2020. *Protein Kinases and Stress Signaling in Plants: Functional Genomic Perspective*, Wiley.

PAPARELLA, S., ARAÚJO, S. S., ROSSI, G., WIJAYASINGHE, M., CARBONERA, D. & BALESTRAZZI, A. 2015. Seed priming: state of the art and new perspectives. *Plant Cell Reports,* 34**,** 1281-1293.

PAVLŮ, J., NOVÁK, J., KOUKALOVÁ, V., LUKLOVÁ, M., BRZOBOHATÝ, B. & ČERNÝ, M. 2018. Cytokinin at the Crossroads of Abiotic Stress Signalling Pathways. *International journal of molecular sciences,* 19**,** 2450.

PEKAROVA, B., SZMITKOWSKA, A., HOUSER, J., WIMMEROVA, M. & HEJÁTKO, J. 2018. Cytokinin and Ethylene Signaling. *In:* HEJÁTKO, J. & HAKOSHIMA, T. (eds.) *Plant Structural Biology: Hormonal Regulations.* Cham: Springer International Publishing.

PELEG, Z., REGUERA, M., TUMIMBANG, E., WALIA, H. & BLUMWALD, E. 2011. Cytokinin-mediated source/sink modifications improve drought tolerance and increase grain yield in rice under water-stress. *Plant Biotechnology Journal,* 9**,** 747-758.

PETROV, V., HILLE, J., MUELLER-ROEBER, B. & GECHEV, T. S. 2015. ROS-mediated abiotic stress-induced programmed cell death in plants. *Frontiers in Plant Science,* 6.

POSPÍŠILOVÁ, H., JISKROVÁ, E., VOJTA, P., MRÍZOVÁ, K., KOKÁŠ, F., ČUDEJKOVÁ, M. M., BERGOUGNOUX, V., PLÍHAL, O., KLIMEŠOVÁ, J., NOVÁK, O., DZUROVÁ, L., FRÉBORT, I. & GALUSZKA, P. 2016. Transgenic barley overexpressing a cytokinin dehydrogenase gene shows greater tolerance to drought stress. *New Biotechnology,* 33**,** 692-705.

POTVIN, C., LECHOWICZ, M. J., BELL, G. & SCHOEN, D. 1990. Spatial, temporal, and species-specific patterns of heterogeneity in growth chamber experiments. *Canadian Journal of Botany,* 68**,** 499-504.

PRASAD, P. V. V., PISIPATI, S. R., MOMČILOVIĆ, I. & RISTIC, Z. 2011. Independent and Combined Effects of High Temperature and Drought Stress During Grain Filling on Plant Yield and Chloroplast EF-Tu Expression in Spring Wheat. *Journal of Agronomy and Crop Science,* 197**,** 430-441.

R CORE TEAM 2021. R: A language and environment for statistical computing. . R Foundation for Statistical

Computing.

RAHNAMA, A., MUNNS, R., POUSTINI, K. & WATT, M. 2011. A screening method to identify genetic variation in root growth response to a salinity gradient. *Journal of experimental botany,* 62**,** 69-77.

RAJENDRAN, K., TESTER, M. & ROY, S. J. 2009. Quantifying the three main components of salinity tolerance in cereals. *Plant, Cell & Environment,* 32**,** 237-249.

RAMIREDDY, E., HOSSEINI, S. A., EGGERT, K., GILLANDT, S., GNAD, H., VON WIRÉN, N. & SCHMÜLLING, T. 2018. Root Engineering in Barley: Increasing Cytokinin Degradation Produces a Larger Root System, Mineral Enrichment in the Shoot and Improved Drought Tolerance. *Plant Physiology,* 177**,** 1078-1095.

RAY, D. K., GERBER, J. S., MACDONALD, G. K. & WEST, P. C. 2015. Climate variation explains a third of global crop yield variability. *Nat Commun,* 6**,** 5989.

RAY, D. K., RAMANKUTTY, N., MUELLER, N. D., WEST, P. C. & FOLEY, J. A. 2012. Recent patterns of crop yield growth and stagnation. *Nature Communications,* 3**,** 1293.

REDDY, I. N. B. L., KIM, B.-K., YOON, I.-S., KIM, K.-H. & KWON, T.-R. 2017. Salt Tolerance in Rice: Focus on Mechanisms and Approaches. *Rice Science,* 24**,** 123-144.

REDDY, P. P. 2013. Bio-priming of Seeds. *Recent advances in crop protection.* New Delhi: Springer India.

RESEARCH DIVE. 2020. *Agrochemicals Market, by Type (Fertilizers and Pesticides), Regional Outlook (North America, Europe, Asia-Pacific, and LAMEA), Global Opportunity Analysis and Industry Forecast, 2018-2026.* [Online]. Available: https://www.researchdive.com/61/agrochemicals-market#:~:text=The%20Agrochemicals%20Market%20size%20was,reach%20%24%20176.8%20Billion%20by%202026 [Accessed 22/11 2021].

RHAMAN, M. S., IMRAN, S., RAUF, F., KHATUN, M., BASKIN, C. C., MURATA, Y. & HASANUZZAMAN, M. 2021. Seed Priming with Phytohormones: An Effective Approach for the Mitigation of Abiotic Stress. *Plants,* 10**,** 37.

RICHARD, C. A. I., HICKEY, L. T., FLETCHER, S., JENNINGS, R., CHENU, K. & CHRISTOPHER, J. T. 2015. High-throughput phenotyping of seminal root traits in wheat. *Plant Methods,* 11**,** 13.

RIEFLER, M., NOVAK, O., STRNAD, M. & SCHMÜLLING, T. 2006. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *The Plant cell,* 18**,** 40-54.

RIVERO, R. M., KOJIMA, M., GEPSTEIN, A., SAKAKIBARA, H., MITTLER, R., GEPSTEIN, S. & BLUMWALD, E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. *Proceedings of the National Academy of Sciences,* 104**,** 19631-19636.

ROS, C., BELL, R. & WHITE, P. 2003. Seedling vigour and the early growth of transplanted rice (Oryza sativa). *Plant and soil,* 252**,** 325-337.

ROSE, L. 2017. Pitfalls in Root Trait Calculations: How Ignoring Diameter Heterogeneity Can Lead to Overestimation of Functional Traits. *Frontiers in Plant Science,* 8.

ROSENBAUM, P. R. & RUBIN, D. B. 1983. The central role of the propensity score in observational studies for causal effects. *Biometrika,* 70**,** 41-55.

RUBIN, D. B. 1990. [On the Application of Probability Theory to Agricultural Experiments. Essay on Principles. Section 9.] Comment: Neyman (1923) and Causal Inference in Experiments and Observational Studies. *Statistical Science,* 5**,** 472-480.

RUBIO, V., BUSTOS, R., IRIGOYEN, M. L., CARDONA-LÓPEZ, X., ROJAS-TRIANA, M. & PAZ-ARES, J. 2008. Plant hormones and nutrient signaling. *Plant Molecular Biology,* 69**,** 361.

RUSSO, S. & CALLEGARIN, A. M. 1997. Rice production and research in Italy. *In:* CHATAIGNER, J. (ed.) *Activités de recherche sur le riz en climat méditerranéen.* Montpellier : CIHEAM.

SABBAGHI, A. & RUBIN, D. B. 2014. Comments on the Neyman–Fisher Controversy and Its Consequences. *Statistical Science,* 29**,** 267-284, 18.

SAKAKIBARA, H. 2006. Cytokinins: activity, biosynthesis, and translocation. *Annu Rev Plant Biol,* 57**,** 431-49.

SAMSON, B. K., HASAN, M. & WADE, L. J. 2002. Penetration of hardpans by rice lines in the rainfed lowlands. *Field Crops Research,* 76**,** 175-188.

SANTNER, A. & ESTELLE, M. 2009. Recent advances and emerging trends in plant hormone signalling. *Nature,* 459**,** 1071.

SAVVIDES, A., ALI, S., TESTER, M. & FOTOPOULOS, V. 2016. Chemical Priming of Plants Against Multiple Abiotic Stresses: Mission Possible? *Trends in Plant Science,* 21**,** 329-340.

SCALENGHE, R., EDWARDS, A. C., BARBERIS, E. & AJMONE-MARSAN, F. 2012. Are agricultural soils under a continental temperate climate susceptible to episodic reducing conditions and increased leaching of phosphorus? *Journal of Environmental Management,* 97**,** 141-147.

SCHAFER, M., BRUTTING, C., MEZA-CANALES, I. D., GROSSKINSKY, D. K., VANKOVA, R., BALDWIN, I. T. & MELDAU, S. 2015. The role of cis-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. *J Exp Bot,* 66**,** 4873-84.

SCHALLER, G., KIEBER, J. & SHIU, S.-H. 2008. Two-Component Signaling Elements and Histidyl-Aspartyl Phosphorelays. *The Arabidopsis book / American Society of Plant Biologists,* 6**,** e0112.

SEO, H., KIM, S., SAGONG, H. Y., SON, H. F., JIN, K. S., KIM, I. K. & KIM, K. J. 2016. Structural basis for cytokinin production by LOG from Corynebacterium glutamicum. *Sci Rep,* 6**,** 31390.

SEWELAM, N., KAZAN, K. & SCHENK, P. M. 2016. Global Plant Stress Signaling: Reactive Oxygen Species at the Cross-Road. *Frontiers in Plant Science,* 7.

SHARAN, A., SONI, P., NONGPIUR, R. C., SINGLA-PAREEK, S. L. & PAREEK, A. 2017. Mapping the 'Two-component system' network in rice. *Scientific Reports,* 7**,** 9287.

SHER, A., SARWAR, T., NAWAZ, A., IJAZ, M., SATTAR, A. & AHMAD, S. 2019. Methods of Seed Priming. *In:* HASANUZZAMAN, M. & FOTOPOULOS, V. (eds.) *Priming and Pretreatment of Seeds and Seedlings: Implication in Plant Stress Tolerance and Enhancing Productivity in Crop Plants.* Singapore: Springer Singapore.

SHEREEN, A., ASMA, A. & KHANZADA, M. A. 2019. Effects of PEG induced water stress on growth and physiological responses of rice genotypes at seedling stage. *Pakistan Journal of Botany,* 51**,** 2013-2021.

SIEBERT, S., HENRICH, V., FRENKEN, K. & BURKE, J. 2013a. Update of the digital global map of irrigation areas to version 5. *Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany and Food and Agriculture Organization of the United Nations, Rome, Italy*.

SIEBERT, S., HENRICH, V., FRENKEN, K. & BURKE, J. 2013b. *Update of the digital global map of irrigation areas to version 5*.

SINGH, V., SINGH, A. P., BHADORIA, J., GIRI, J., SINGH, J., T. V, V. & SHARMA, P. C. 2018. Differential expression of salt-responsive genes to salinity stress in salt-tolerant and salt-sensitive rice (Oryza sativa L.) at seedling stage. *Protoplasma,* 255**,** 1667-1681.

SMIT, A., BENGOUGH, A., ENGELS, C., VAN NOORDWIJK, M., PELLERIN, S. & GEIJN, S. 2000. *Root Methods: A Handbook*.

SMITH, S. 2003. Combinatorial chemistry in the development of new crop protection products. *Pesticide Outlook,* 14**,** 21-26.

SPÍCHAL, L., WERNER, T., POPA, I., RIEFLER, M., SCHMÜLLING, T. & STRNAD, M. 2009. The purine derivative PI-55 blocks cytokinin action via receptor inhibition. *The FEBS Journal,* 276**,** 244-253.

SYERS, J., JOHNSTON, A. & CURTIN, D. 2008. Efficiency of soil and fertilizer phosphorus use. *FAO Fertilizer and plant nutrition bulletin,* 18.

SYNGENTA. 2021. *Plant Growth Regulators: everything you need to know* [Online]. Available: https://www.syngenta.co.uk/blog/ornamentals-advisory-blog/plant-growth-regulators-everything-you-need-know [Accessed 28/11 2021].

TACK, J., SINGH, R. K., NALLEY, L. L., VIRAKTAMATH, B. C., KRISHNAMURTHY, S. L., LYMAN, N. & JAGADISH, K. S. V. 2015. High vapor pressure deficit drives salt-stress-induced rice yield losses in India. *Global Change Biology,* 21**,** 1668-1678.

TADROSS, M., SUAREZ, P., LOTSCH, A., HACHIGONTA, S., MDOKA, M., UNGA-NAI, L., LUCIO, F., KAMDONYO, D. & MUCHINDA, M. 2007. Changes in growing-season rainfall characteristics and downscaled scenarios of change over southern Africa: Implications for growing maize. *IPCC Regional Expert Meeting on Regional Impacts, Adaptation, Vulnerability, and Mitigation*.

TALLA, S. K., PANIGRAHY, M., KAPPARA, S., NIROSHA, P., NEELAMRAJU, S. & RAMANAN, R. 2016. Cytokinin delays dark-induced senescence in rice by maintaining the chlorophyll cycle and photosynthetic complexes. *Journal of Experimental Botany,* 67**,** 1839-1851.

TEITEL, M., ATIAS, M. & BARAK, M. 2010. Gradients of temperature, humidity and CO2 along a fan-ventilated greenhouse. *Biosystems Engineering,* 106**,** 166-174.

TESTER, M. & LANGRIDGE, P. 2010. Breeding Technologies to Increase Crop Production in a Changing World. *Science,* 327**,** 818-822.

THORUP-KRISTENSEN, K., HALBERG, N., NICOLAISEN, M., OLESEN, J. E., CREWS, T. E., HINSINGER, P., KIRKEGAARD, J., PIERRET, A. & DRESBØLL, D. B. 2020. Digging Deeper for Agricultural Resources, the Value of Deep Rooting. *Trends in Plant Science,* 25**,** 406-417.

THORUP-KRISTENSEN, K. & KIRKEGAARD, J. 2016. Root system-based limits to agricultural productivity and efficiency: the farming systems context. *Annals of botany,* 118**,** 573-592.

TODAKA, D., ZHAO, Y., YOSHIDA, T., KUDO, M., KIDOKORO, S., MIZOI, J., KODAIRA, K. S., TAKEBAYASHI, Y., KOJIMA, M., SAKAKIBARA, H., TOYOOKA, K., SATO, M., FERNIE, A. R., SHINOZAKI, K. & YAMAGUCHI-SHINOZAKI, K. 2017. Temporal and spatial changes in gene expression, metabolite accumulation and phytohormone content in rice seedlings grown under drought stress conditions. *Plant J,* 90**,** 61-78.

TRAN, L. S., URAO, T., QIN, F., MARUYAMA, K., KAKIMOTO, T., SHINOZAKI, K. & YAMAGUCHI-SHINOZAKI, K. 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proc Natl Acad Sci U S A,* 104**,** 20623-8.

TRENBERTH, K. E. 2011. Changes in precipitation with climate change. *Climate Research,* 47**,** 123-138.

TSUBO, M., FUKAI, S., BASNAYAKE, J. & MAKARA, O. 2009. Frequency of occurrence of various drought types and its impact on performance of photoperiod-sensitive and insensitive rice genotypes in rainfed lowland conditions in Cambodia. *Field Crops Research,* 113.

TUTEJA, N. & MAHAJAN, S. 2007. Calcium signaling network in plants: an overview. *Plant signaling & behavior,* 2**,** 79-85.

UGA, Y., SUGIMOTO, K., OGAWA, S., RANE, J., ISHITANI, M., HARA, N., KITOMI, Y., INUKAI, Y., ONO, K., KANNO, N., INOUE, H., TAKEHISA, H., MOTOYAMA, R., NAGAMURA, Y., WU, J., MATSUMOTO, T., TAKAI, T., OKUNO, K. & YANO, M. 2013. Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions. *Nature Genetics,* 45**,** 1097-1102.

VAMATHEVAN, J., CLARK, D., CZODROWSKI, P., DUNHAM, I., FERRAN, E., LEE, G., LI, B., MADABHUSHI, A., SHAH, P. & SPITZER, M. 2019. Applications of machine learning in drug discovery and development. *Nature Reviews Drug Discovery,* 18**,** 463-477.

VAN OORT, P. A. J. 2018. Mapping abiotic stresses for rice in Africa: Drought, cold, iron toxicity, salinity and sodicity. *Field Crops Research,* 219**,** 55-75.

VAN OORT, P. A. J. & ZWART, S. J. 2018. Impacts of climate change on rice production in Africa and causes of simulated yield changes. *Global Change Biology,* 24**,** 1029-1045.

VAREED THOMAS, E. 2002. Development of a mechanism for transplanting rice seedlings. *Mechanism and Machine Theory,* 37**,** 395-410.

VERGARA, B. S. C., T.T 1985. *The Flowering Response Of The Rice Plant To Photoperiod*, International Rice Research Institute.

VICENTE-SERRANO, S. M., BEGUERÍA, S. & LÓPEZ-MORENO, J. I. 2010. A Multiscalar Drought Index Sensitive to Global Warming: The Standardized Precipitation Evapotranspiration Index. *Journal of Climate,* 23**,** 1696-1718.

WADE, L. J., FUKAI, S., SAMSON, B. K., ALI, A. & MAZID, M. A. 1999. Rainfed lowland rice: physical environment and cultivar requirements. *Field Crops Research,* 64**,** 3-12.

WANG, W.-C., LIN, T.-C., KIEBER, J. & TSAI, Y.-C. 2019. Response Regulators 9 and 10 Negatively Regulate Salinity Tolerance in Rice. *Plant and Cell Physiology,* 60**,** 2549-2563.

WANG, Y. & FREI, M. 2011. Stressed food – The impact of abiotic environmental stresses on crop quality. *Agriculture, Ecosystems & Environment,* 141**,** 271-286.

WANG, Y., SHEN, W., CHAN, Z. & WU, Y. 2015. Endogenous Cytokinin Overproduction Modulates ROS Homeostasis and Decreases Salt Stress Resistance in Arabidopsis Thaliana. *Frontiers in plant science,* 6**,** 1004-1004.

WANI, S. H., KUMAR, V., SHRIRAM, V. & SAH, S. K. 2016. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal,* 4**,** 162-176.

WARGENT, J. J., PICKUP, D. A., PAUL, N. D. & ROBERTS, M. R. 2013. Reduction of photosynthetic sensitivity in response to abiotic stress in tomato is mediated by a new generation plant activator. *BMC Plant Biology,* 13**,** 108.

WERNER, T., HOLST, K., PÖRS, Y., GUIVARC'H, A., MUSTROPH, A., CHRIQUI, D., GRIMM, B. & SCHMÜLLING, T. 2008. Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. *Journal of Experimental Botany,* 59**,** 2659-2672.

WERNER, T., MOTYKA, V., STRNAD, M. & SCHMÜLLING, T. 2001. Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 10487-10492.

WERNER, T., NEHNEVAJOVA, E., KÖLLMER, I., NOVÁK, O., STRNAD, M., KRÄMER, U. & SCHMÜLLING, T. 2010. Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and tobacco. *Plant Cell,* 22**,** 3905-20.

WERNER, T. & SCHMÜLLING, T. 2009. Cytokinin action in plant development. *Current Opinion in Plant Biology,* 12**,** 527-538.

WHITE, R. G. & KIRKEGAARD, J. A. 2010. The distribution and abundance of wheat roots in a dense, structured subsoil--implications for water uptake. *Plant Cell Environ,* 33**,** 133-48.

WOLANIN, P. M., THOMASON, P. A. & STOCK, J. B. 2002. Histidine protein kinases: key signal transducers outside the animal kingdom. *Genome biology,* 3**,** REVIEWS3013-REVIEWS3013.

XIAO, J., LI, J., GRANDILLO, S., AHN, S. N., YUAN, L., TANKSLEY, S. D. & MCCOUCH, S. R. 1998. Identification of trait-improving quantitative trait loci alleles from a wild rice relative, Oryza rufipogon. *Genetics,* 150**,** 899-909.

XIN, P., LI, B., ZHANG, H. & HU, J. 2019. Optimization and control of the light environment for greenhouse crop production. *Scientific Reports,* 9**,** 8650.

XU, K., XU, X., FUKAO, T., CANLAS, P., MAGHIRANG-RODRIGUEZ, R., HEUER, S., ISMAIL, A. M., BAILEY-SERRES, J., RONALD, P. C. & MACKILL, D. J. 2006. Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature,* 442**,** 705-708.

XU, W., CUI, K., XU, A., NIE, L., HUANG, J. & PENG, S. 2015. Drought stress condition increases root to shoot ratio via alteration of carbohydrate partitioning and enzymatic activity in rice seedlings. *Acta Physiologiae Plantarum,* 37**,** 9.

YAMBAO, E. B., INGRAM, K. T. & REAL, J. G. 1992. Root xylem influence on the water relations and drought resistance of rice. *Journal of Experimental Botany,* 43**,** 925-932.

YEO, A., YEO, M., FLOWERS, S. & FLOWERS, T. 1990. Screening of rice (Oryza sativa L.) genotypes for physiological characters contributing to salinity resistance, and their relationship to overall performance. *Theoretical and Applied Genetics,* 79**,** 377-384.

YIN, W., XIAO, Y., NIU, M., MENG, W., LI, L., ZHANG, X., LIU, D., ZHANG, G., QIAN, Y., SUN, Z., HUANG, R., WANG, S., LIU, C.-M., CHU, C. & TONG, H. 2020. ARGONAUTE2 Enhances Grain Length and Salt Tolerance by Activating BIG GRAIN3 to Modulate Cytokinin Distribution in Rice[OPEN]. *The Plant Cell,* 32**,** 2292-2306.

YORDANOV, I., VELIKOVA, V. & TSONEV, T. 2000. Plant Responses to Drought, Acclimation, and Stress Tolerance. *Photosynthetica,* 38**,** 171-186.

YOSHIDA, S. 1981. *Fundamentals of Rice Crop Science*, International Rice Research Institute.

ZANDALINAS, S. I., SENGUPTA, S., FRITSCHI, F. B., AZAD, R. K., NECHUSHTAI, R. & MITTLER, R. 2021. The impact of multifactorial stress combination on plant growth and survival. *New Phytologist,* 230**,** 1034-1048.

ZÁVESKÁ DRÁBKOVÁ, L., HONYS, D. & MOTYKA, V. 2021. Evolutionary diversification of cytokinin-specific glucosyltransferases in angiosperms and enigma of missing cis-zeatin O-glucosyltransferase gene in Brassicaceae. *Scientific Reports,* 11**,** 7885.

ZENG, L. & SHANNON, M. C. 2000. Salinity Effects on Seedling Growth and Yield Components of Rice. *Crop Science,* 40**,** 996-1003.

ZHANG, J., ZHANG, S., CHENG, M., JIANG, H., ZHANG, X., PENG, C., LU, X., ZHANG, M. & JIN, J. 2018. Effect of Drought on Agronomic Traits of Rice and Wheat: A Meta-Analysis. *International journal of environmental research and public health,* 15**,** 839.

ZHAO, Y., TEITEL, M. & BARAK, M. 2001. SE—Structures and Environment: Vertical Temperature and Humidity Gradients in a Naturally Ventilated Greenhouse. *Journal of Agricultural Engineering Research,* 78**,** 431-436.

ZHOU, B. & ZENG, L. 2018. Immunity-Associated Programmed Cell Death as a Tool for the Identification of Genes Essential for Plant Innate Immunity. *Methods Mol Biol,* 1743**,** 51-63.

ZHOU, H., XIA, D. & HE, Y. 2019. Rice grain quality—traditional traits for high quality rice and health-plus substances. *Molecular Breeding,* 40**,** 1.

ZUBO, Y. O., YAMBURENKO, M. V., SELIVANKINA, S. Y., SHAKIROVA, F. M., AVALBAEV, A. M., KUDRYAKOVA, N. V., ZUBKOVA, N. K., LIERE, K., KULAEVA, O. N., KUSNETSOV, V. V. & BÖRNER, T. 2008. Cytokinin Stimulates Chloroplast Transcription in Detached Barley Leaves. *Plant Physiology,* 148**,** 1082-1093.

ZWACK, P. J. & RASHOTTE, A. M. 2015. Interactions between cytokinin signalling and abiotic stress responses. *Journal of Experimental Botany,* 66**,** 4863-4871.