CONFERMENT OF THERMOTOLERANCE:
PHYSIOLOGY, PRODUCTIVITY, QUALITY AND
POST-HARVEST QUALITY OF SUBTROPICAL AND
TEMPERATE VEGETABLE CROPS EXPOSED TO
SUB-LETHAL HEAT STRESS

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National Institute of Education

Nanyang Technological University

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Lai Cheng Hsiang
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National Institute of Education
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Doctor of Philosophy

2019
Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution. In addition, I declare that to the best of my knowledge, this thesis is free of plagiarism, and contains no material previously published or written by another person, except where due reference has been made in the text.

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This thesis contains material from a paper published in a peer-reviewed journal where I was the first and/or corresponding author.


The contributions of the co-authors are as follows:

- I prepared the manuscript drafts. The manuscript was revised by A/P He Jie.
- I co-designed the study with A/P He Jie and performed all the laboratory work at the Plant Physiology Laboratory, NIE. I also analyzed the data.

[Signature]
Lai Cheng Hsiang

[Date]
22nd Nov 2019
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List of Abbreviations

\[ A_{\text{sat}} \quad \text{Light saturated photosynthetic CO}_2 \text{ assimilation rate} \]
\[ A \quad \text{CO}_2 \text{ assimilation rate} \]
Car \quad \text{Carotenoid} \\
Chl \quad \text{Chlorophyll} \\
CO_2 \quad \text{Carbon dioxide} \\
DAT \quad \text{Days after transplant} \\
EC \quad \text{Electrical conductivity} \\
ETC \quad \text{Electron Transport Chain} \\
ETR \quad \text{Electron Transport Rate} \\
\[ F_v/F_m \quad \text{Chl variable to maximal fluorescence ratio} \]
FW \quad \text{Fresh weight} \\
g_s \quad \text{Stomatal conductance rate} \\
g_{s\text{ sat}} \quad \text{Light saturated stomatal conductance rate} \\
hsc \quad \text{Constitutive heat shock protein} \\
hsf \quad \text{Heat shock factor} \\
hsp \quad \text{Heat shock protein} \\
hsp\text{s} \quad \text{Heat shock proteins} \\
hsr \quad \text{Heat shock response} \\
LHC \quad \text{Light harvesting complex} \\
LHCl \quad \text{Light harvesting complex I} \\
LHClII \quad \text{Light harvesting complex II} \\
LT_{50} \quad \text{Temperature at which an observed trait decreased by 50\%} \\
N \quad \text{Nitrogen} \\
NPQ \quad \text{Non-photochemical quenching} \\
O_2 \quad \text{Oxygen} \\
PAL \quad \text{Phenylalanine ammonia-lyase} \\
P_{\text{max}} \quad \text{Light- and CO}_2\text{-saturated photosynthetic oxygen evolution rate} \\
POD \quad \text{Peroxidase} \\
PPFD \quad \text{Photosynthetic photon flux density} \\
PPO \quad \text{Polyphenol oxidase} \\
PS \quad \text{Photosystem} \\
PSI \quad \text{Photosystem I} \\
PSII \quad \text{Photosystem II} \\
qP \quad \text{Photochemical quenching} \\
RIL \quad \text{Recombinant inbred lines} \\
ROS \quad \text{Reactive oxygen species} \\
\text{Rubisco} \quad \text{ribulose 1,5-bisphosphate carboxylase/oxygenase} \\
RZ \quad \text{Root Zone} \\
RZT \quad \text{Root zone temperature} \\
TRN \quad \text{Total reduced nitrogen} \]
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List of Publications Arising from this Study

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**Journal Papers**


Summary

In face of imminent climate change and its catastrophic effects on crop yield and food security, it is imperative that the thermal limits of food crops should be explored to elucidate their optimal physiological thermal window and thermal limits. This research was carried out in 3 phases: 1) to inaugurate a dose-response of temperate vegetables to heat stress and therein determine the thermal biology, optimal thermal window for physiological and photosynthetic performances, and the critical temperature at which irreparable failure of physiological processes occur; 2) to determine the kinetics and magnitude of heat shock responses of the vegetables via the heat shock protein (hsp70) quantification and 3) to design sub-lethal heat priming regimes to boost productivity and/or qualities of food crops in response to heat stress. Six temperate vegetables (*Lactuca sativa* ‘Canasta’, *Eruca sativa* “Arugula”, *L. serriola*, *L. sativa* L. ‘Salinas’ and RILs 141 and 192) were stressed with high temperature and their physiological and photosynthetic responses were recorded. Dose relationships were observed in shoot FW, $A_{\text{sat}}$, $g_s$, $P_{\text{max}}$, ETR, $qP$ & NPQ with significant decrease in performance at 28°C (versus 25°C) for selected species. Through these relationships of physiological performances with response to temperature, $LT_{50}$, for each stress index was calculated. This is the temperature at which the measured parameter would differ from that of control plants (25°C RZT) by 50%. It is expected that $LT_{50}$ value of maximum quantum yield, of photosystem II, $F_v/F_m$ ratio (~46°C) will approximate the critical (pessimum) temperature of the plant species. Heat shock protein (hsp70) analysis of plants grown in constant 25, 28, 32, and 36°C RZT showed that hsp70 level in the shoot tissue increased with increasing RZT when analysed at noon time. Acute heat shock experiment showed that Canasta and RIL192 had peak hsp70 at 38°C while Arugula and RIL 142 at 42°C with hsp70 attenuated at 46°C for all four vegetables. Temporal kinetics of hsp70 synthesis showed that 30 min heating at 38°C was enough to induce hsp70 synthesis in all four vegetables. From the hsp70 kinetic experiment, it was determined
that heat shock response could be elucidated at ~38°C for most of the vegetables. From these two findings, heat priming regime at 38°C was designed, and both control and heat primed plants were later subjected to 46°C heat shock to test the effects of heat priming. It was discovered that heat primed vegetables could have productivity comparable to control plants that were kept constantly in cool RZTs and yet have higher nutritional value in terms of ascorbic acid content and phenolic compound levels. Conversely, plants that were not heat primed and constantly kept in 25°C RZT had much lower shoot productivity when subjected to 46°C heat shock 21~30 DAT compared to both heat primed vegetables and plants kept in constant 25°C RZT. This study further suggested that aeroponic plants could be kept in ambient RZT after 11 DAT, thus conserving greenhouse energy expenditure.
CHAPTER 1

Introduction
Sessile plants have evolved to possess special adaptations in response to the ever-changing environmental conditions (Sudan et al., 2018). In face of high and/or low temperature (Hadi et al., 2018), hypersalinity (Rasool et al., 2017), heavy metal contamination (Nissim et al., 2018) or drought conditions (Wang et al., 2018b), plants have adapted to respond to the various abiotic stressors where the option or ability to move away from the stress origin is not available. Each of these abiotic conditions affect plants in varying degrees (Dubois et al., 2018), dependent on the stressor’s intensity and quantity (Joshi et al., 2018). Through these adaptations, plants also became dependent on a certain quantity of each of the environmental cues for optimal growth (Bray et al., 2000). When the magnitude of these environmental cues exceeds or falls short of the accustomed level required for optimal development, growth and/or productivity, it is considered an environmental stressor and termed as abiotic stress (Sudan et al., 2018). Plant survival and healthy growth thus depend on its intricate network of stress response system (Nover et al., 1999). Stress response to mitigate stress will thus be activated through a cascade of signal transduction originating from the detection of the stress signal (Dubois et al., 2018).

Vegetables such as lettuce and spinach (leaves), carrots and turnips (roots), celery (petiole), cauliflower (flower bud), tomato (fruit) or beans (seeds) are herbaceous plants, essential to the human diet (Gruda, 2005). About 90% and 60% of human dietary Vitamin C and A respectively are from plant sources according to the Food and Agriculture Organization statistics (FAO, 2017). While vegetables may contain less protein or fat compared to animal products, they possess a huge suite of other beneficial products or metabolites (fibre, minerals, vitamin) on top of phytochemicals heralded to provide protection against or prevention of cancer formation and viral-, fungal- and bacterial-infections (Osagie and Eka, 1998).

Modern agriculture emphasises the growth of high-quality vegetables. Given the imminent climate change events and global warming, extra challenge is posed for productivity
and quality of temperate vegetables (Makinen et al., 2018). Photosynthesis could be limited by stomatal closure under high temperatures (Vishwakarma et al., 2017; Wu et al., 2017), which results in depletion of CO₂ at the chloroplast level for photosynthesis. Vegetables grown in high root zone temperatures (RZTs) also exhibited transient water deficit and midday stomatal closure characteristic of droughted plants despite continuous water supply in greenhouse conditions (He et al., 2013; Choong et al., 2016) due to alterations in root morphology (He et al., 2009; Srikanth et al., 2016). Plants grown in heat stress conditions had lowered anthocyanin (Niu et al., 2017; Koski and Galloway, 2018), polyphenol contents (Antillotti et al., 2015) and antioxidant activities (Boo et al., 2011) while heat stress of 1~5 min could significantly increase the overall browning potential of broccoli (Ansorena et al., 2014).

Among the vegetables, Arugula or Rocket (Eruca sativa Mill.) is a vegetable found in the Mediterranean countries but is now gaining popularity as a salad component in European countries (Doležalová et al., 2013; Tsirogiannis et al., 2013) and around the world (Bell and Wagstaff, 2014; Raffo et al., 2018). The slightly bitter vegetable is full of phytonutrients beneficial to consumers (Gutiérrez et al., 2016: vitamin A, vitamin C; Gutiérrez et al., 2018: flavonoids, sulphur; Nunes et al., 2013: potassium and fibre). Lettuce, another temperate/subtropical vegetable of the genus Lactuca, consists of about 100 species (Simko and Hu, 2008). One of the species, L. serriola L., is a winter annual tolerant to drought (Werk and Ehleringer, 1985). The cultivated lettuce (Lactuca sativa) is descended from the wild-type ancestor L. serriola L. and is a major horticultural crop (Simko and Hu, 2008). The heat sensitive Lactuca sativa L. ‘Salinas’ was crossed with the heat tolerant L. serriola accession UC96US23 (Argyris et al., 2005) at the Michelmore lab (Genome Centre, UC Davis, USA) and F9 RIL seeds were obtained. Selected RILs were examined together with the commercial cultivars Canasta (L. sativa) and Arugula (E. sativa). Using these genotypes and commercial
cultivars, this research attempts to identify the thermal windows of these vegetables and if thermotolerance could be induced/conferred through physiological hardening events.

Climate change affects all aspects of plant biological processes and interaction with the environment and therein poses a serious challenge to sustainable food supply and crop yield; urgency of agricultural improvement and development is further augmented by the trend of global population growth rate (e.g. Thermotolerance in winter wheat: Chen et al., 2018; Drought resistance in Arabidopsis: Sakuma et al., 2006 and maize: Li et al., 2008; abiotic stress tolerance in transgenic rice: Oh et al., 2007; for reviews see Lavana et al., 2015 and Dhankher and Foyer, 2018). Of the various abiotic stressors encountered by plants in nature, high temperature, partly resulting from global warming, acts independently on the physiological and metabolical processes of plant cells. Heat stress thus seriously threaten worldwide crop yield (Lobell et al., 2011; Teixeira et al., 2013), adding further challenge to global food securities already compromised by global population increase (Nelson et al., 2018). Human activities often emit CO2, methane, chlorofluorocarbons and nitrous oxides that augment the existing concentrations of greenhouse gases. As a result, global mean temperature has been predicted to increase 0.3°C per decade by different global circulation models (Lavana et al., 2015), culminating in approximately 1.5 to about 4°C rise in the global mean temperature by 2025 and 2100. This could translate to geographical displacement and different planting season for food crops, even affecting the time of crop harvest (Porter, 2005). With that in mind, elucidating the thermal threshold and determining how food crops respond to heat stress is both urgent and imperative today.

In addition to the imminent climate change and rise in ambient temperature, crop cultivation has also been affected by the problems of pollution issues, water resources depletion, and hypersalinity (Shabbaz and Ashraf, 2013). About 20% of cultivated and 33% of irrigated agricultural lands are already negatively impacted by these factors, and a further 50%
of farmable area would be affected by hypersalinity by 2050 (Jamil et al., 2007), especially in the Mediterranean countries from where these vegetables originate (Polemio, 2016). This results in overall reductions in agricultural land area, productivity and food crop quality (Shahbaz and Ashraf, 2013). Considering the economic importance of these vegetables and the predicted reduced productivity and crop yield in their traditional cultivation areas, controlled environment horticulture has been proposed as an innovative method for maximization of food production (Vadiee and Martin, 2014; Tsitsimpelis et al., 2016). One such horticulture method is the aeroponics systems.

Although the tropical warm and humid climate of Singapore is not conducive for growth of temperate crops, Lee et al. (1994), He and Lee (1998) and He et al (2001) all reported success in growing lettuce in aeroponics system by just cooling the roots (15–25°C) while shoots are exposed to hot ambient temperatures. Plants with cooled roots but exposed to full sunlight had higher photosynthetic O₂ evolution in comparison to vegetables grown under temperate conditions with reduced sunlight (He and Lee, 1998), where photoinhibition of PS II was alleviated (He and Lee, 2004). For example, He et al. (2009) reported success in growing temperate crops in 28–42°C ambient temperatures by simply providing RZ cooling at 15–25°C. Similar reports was observed with Chinese cabbage (Qin et al., 2002), capsicum (Dodd et al., 2000), Chinese broccoli (He et al., 2008) and tomato (Choong, 1998). Growing of temperate and subtropical vegetable crops in tropical greenhouses were thus made possible via aeroponics developments providing independent root-zone cooling despite hot ambient temperatures, while tropical irradiation promoted faster shoot growth compared to temperate conditions (Lee, 1993; Choong, 1998), made possible largely by the reduction of photoinhibition and of stomatal closure. Cool RZ conditions have positive effects on the development and growth of roots, e.g. root elongation and increased number of tips (Tan et al., 2002; He at al., 2009), in the aeroponics system which has a direct effect in alleviating water deficit problems.
manifesting in plants grown in ambient RZT (He et al., 2001). By balancing water loss from the leaves and water uptake from the roots (He et al., 2001), stomatal limitations of photosynthesis are thus circumvented via the maintenance of relative water content in the leaf/shoots and photosynthetic gas exchange. Non-stomatal limitations of photosynthesis such as PS II photochemistry, Rubisco protein and activity are also mitigated by proper root development in cool RZTs (He and Lee, 2001; 2004, He et al., 2013).

Although production yield of commercial greenhouses has been heralded to potentially be 10 times higher than the corresponding open field cultivation (Vadiee and Martin, 2014; Tsitsimpelis et al., 2016), they are also known as the most energy consuming cultivation method (Vadiee et al., 2016) due to the constant need to maintain optimal conditions of light, temperature and ventilation for maximum yield (Vox et al., 2010; Giacomelli et al., 2012). To that effect, this research is two pronged: (i) identifying the basal and acquired thermotolerance in selected plants and (ii) adopting sub-lethal heat stress regimes to boost adaptive thermotolerance in plants in agricultural applications. Commercial cultivars Arugula and Canasta, together with lettuce (Lactuca sativa L.) recombinant inbred lines (RILs) would be examined for their basal thermotolerance by determining dose-response relationships of physiological and photosynthetic performances to heat stress. This will be examined alongside exploration of heat shock protein synthesis with response to heat stress.

Heat shock proteins (hsp) are highly conserved and present in all plants (Vierling, 1991) and animals (Feder and Hoffman, 1999). Some hsp can confer acquired thermotolerance in that they provide protein stabilization and maintenance of cellular functions by repairing heat-damaged proteins (Efeoğlu. 2009). The expression of heat-induced protein is thus a vital adaptive mechanism in this respect (Feder and Hofmann, 1999). Heat-shock proteins are highly conserved in all living organisms from bacteria to human beings (Kalmar and Greensmith, 2009). Several of these stress proteins are ATP-dependent both in terms of synthesis and
functionality in binding to target protein (Wang et al., 2004). Thus, there is the need to shed more light on the various mechanisms on how plants respond to stress, such as heat, and the role they play with regards to stress tolerance (Goraya et al., 2017). Upon establishing the physiological responses of plants to RZ heat stress, regimes of sub-lethal heat stress would then be designed to boost thermotolerance in the crops. This is designed to firstly increase productivity under elevated temperature and light exposure, and secondly to reduce energy costs of environmental and RZ temperature maintenance in greenhouse conditions.

Given that thermotolerance conferment often comes at a great energetic cost to the organism (Wahid et al., 2007), commercial values of the crops would be compromised if productivity and photosynthetic capabilities of hardened plants are drastically reduced compared to plants kept in cool RZTs. On the other hand, postharvest qualities of hardened plants are enhanced if nutritional contents are increased (e.g. ascorbic acid and phenolic compounds: Nunes et al., 2013) and/or antioxidants levels are elevated to increase shelf life of a crop (e.g. resistance to browning: Kim et al., 2014).

Hence, the objectives of this proposed project are as follows:

1. To develop stress indices in heat stressed plants to qualify and quantify heat tolerant and heat sensitive traits (Chapter 3)
2. To study the profile and kinetics of heat shock proteins in heat shocked plants (Chapter 4)
3. To determine the optimal sub-lethal heat shock regime to acquire thermotolerance in crop cultivation and maintenance (Chapter 5)
4. To ascertain productivity and post-harvest quality of vegetables subjected to the sub-lethal heat shock regimes (Chapter 5)
CHAPTER 2

Literature review
2.1. Climate warming and plant responses to heat stress

Global food security has been severely affected by climate change in recent years through direct inhibition of plant growth and productivity (Deligios et al., 2019; Fahad et al., 2017) and through effects on soil mineral and nutrient levels, carbon and nitrogen sequestration, and changes in soil microbial diversity, abundance and activity (Dhankher and Foyer, 2018). Predicted average temperature elevation, tropical extreme temperature event frequencies and intensities, compounded by increasing hypersalinity and heavy metals concentrations in soils, are expected to drastically affect crop yields (Long et al., 2015), especially in broad leaved crops (Reckling et al., 2018). With crop cultivation forced to shift to non-optimal grounds and non-arable lands, stress tolerant crops must be developed for sustainable crop harvesting (Kathuria et al., 2007). Agricultural transformation is largely needed to cater to the global food demand growth of 70–85% as the population increases (FAO, 2017; Ray et al., 2013).

Abiotic stress is the greatest threat to food security in that crop productivity can be affected at the molecular, cellular and physiological levels, resulting in plant architecture and crop yield changes (Asada, 2006). Arguably, plants have evolved to possess an arsenal of adaptive mechanisms in response to the different environmental stressors by rapid and synchronized changes at gene expression and protein translation levels (Boyko and Kovalchuk, 2008). For example, detrimental abiotic conditions, such as water deficit, high temperature, heavy metal toxicity, and high light result in photoinhibition due to ETC over-reduction (Kangasjarvi et al., 2012; Nishiyama and Murata, 2014). One of the several mechanisms to overcome this problem would be reducing the rate of electron transport by converting the excessively absorbed light into thermal energy. NPQ is the plant mechanism to alleviate this situation: excess excitation energy is converted to heat and dissipated (Rochaix, 2011; Spetea et al., 2014).
Of the various environmental stressors, temperature permeates all levels of biological functions and significantly affects the interconnected cellular stress response in plants, where elevated temperatures can induce a dramatic re-strategizing of energy allocation to physiological and molecular mechanisms for survival (Mittler, 2006). With imminent climate change and events of extreme temperatures, the escalation of thermal stress may be beyond the coping mechanisms of plants, therein threatening agricultural yield (Dhankher and Foyer, 2018). Although agriculture yield could be affected by both abiotic and biotic stresses, abiotic stresses are the major limiting factor affecting the spread of plant species across geographical zones (Gillham et al., 2017). It is predicted that by 2100, environmental temperatures will rise by 1.5-5.8°C. As it stands, wheat and maize yield has already decreased by 3.8% and 5.5% respectively since 1980s (Lobell et al., 2011). With low crop yields due to increased environmental stress, compounded with the trend of global population growth, understanding conferment of thermotolerance and cultivation of stress tolerant food crop is of particular urgency (Gillham et al., 2017).

Heat stress affects the whole plant through various stages of plant development, starting from seed germination to flowering. High temperature could impair seed quality, affecting germination rates (Bita and Gerats 2013), scorch leaves and stems (Sharma et al., 2016), cause leaf abscission and senescence (Chen et al., 2015), growth inhibition (Tovar et al., 2018) or affect reproductive stages like pollen tube growth and causing pollen infertility, fruit damage, therein reducing or inhibiting crop yield (Hemantaranjan et al., 2014). High temperatures affect cellular functions by causing protein denaturation, aggregation of damaged proteins and reducing membrane integrity (Vandereyken et al., 2018). Heat stress affects subcellular processes in chloroplast and mitochondria by directly affecting metabolic and enzymatic reaction rates, disturbing transcription and translation activities, thereby causing disruption to metabolic homeostasis (Mittler et al., 2012; Kumar and Rao 2013).
2.1.1 Heat stress effects on shoot productivity

Temperature, especially RZT, had been reported to directly affect shoot development (Choong et al., 2013; He, 2009; 2010; 2015). Optimal temperatures differ for different plants species: for example, although *Capsicum annum* is reported to be sensitive to cold temperatures and leaf initiation and growth is inhibited by night temperatures below 20°C (Mercado et al., 1997), Dodd et al. (2000) still observed greater growth in the 20°C RZT plants as compared to the ambient RZT plants in Singapore. That said, the authors acknowledge that “an optimal RZT for pepper under tropical aerial conditions is yet to be determined”. Nodulation, nitrogen fixation, and plant growth of soybean are also reported to be reduced at soil temperatures below 25°C (Schmidt et al., 2015). Low RZT also decreased shoot growth of maize (Walker, 1969) due to possible alteration in the supply of phytohormones (Atkin et al., 1973), water (Barlow and Boersma, 1976) or nutrients from the roots (Engels and Marschner, 1990). Conversely, tomato plants grown at 36°C RZT had reduced shoot growth compared to their 25°C RZT counterparts (Klock et al., 1997). Wheat (*Triticum aestivum*) was reported to have significantly reduced shoot and root dry weight when grown under 30°C RZT compared to plants grown under 23°C RZT (Al-Hamdani et al., 1990). Haque et al. (2014) observed reduced plant height and leaf area 15 days after planting for spring wheat grown in 30°C RZT compared to 24°C RZT, but this was only observed on 22 DAT for 28°C RZT plants, thus showing that temperature affects shoot productivity (Farrar, 1999).

For vegetables, iceberg lettuce grown in 25°C (versus 13°C) exhibited stem elongation and bolting without head formation (Al-Said et al., 2018). Butterhead lettuce grown in 20°C RZT also had higher shoot and root FW compared to those grown at hot ambient-RZT (A-RZT). (Qin et al., 2007). Shoot FW was similarly higher for plants grown in lower RZT (25°C) for the Chinese broccoli (*Brassica alboglabra* cv. Bailey) compared to plants grown in 30°C RZT (Choong, 1998; He et al., 2009). Shoot FW under 25°RZT was seven times heavier
compared to plants grown in ambient RZT (25~43°C) at 40 DAT. Shoot FW of plants transferred to ambient RZT after two weeks in 25°C was diminished by more than ten times when compared to plants transferred after 4 weeks under 25°C RZT (Choong 1998). Shoot and root FW, and highest leaf number, was observed at 20°C RZT for Panama lettuce (He et al., 2009). Shoot FW of lettuce decreased by 50% when transferred to ambient RZT after three weeks of growing in cool RZT, compared to those left growing in optimal cool RZT (He et al., 2013). Typically, lettuce grown in tropical ambient RZT, which occasionally exceeds 40°C, tends to decrease growth and yield (Choong et al., 2013). Rocket Salad *Eruca sativa* also had significantly higher biomass of shoot and root at 20°RZT as compared to ambient (He et al., 2016).

### 2.1.2 Heat stress effects on root development

While optimal shoot growth could be obtained within thermal deviations of ± 5°C from the optimal window (tomatoes, cucumbers, tobacco and soybean: Cooper, 1973; Klock 1995), thermal variation is a lot smaller for optimal RZT for seedling growth (maize: Walker, 1969; Engels and Marschner, 1990; snake tomato: Adebooye et al., 2009). Root development precedes shoot development (Weaver and Himmel, 1929), and has been reported to affect shoot growth and senescence more than shoot temperature does (e.g. *Triticum aestivum* L.: Kuroyanagi and Paulsen, 1988). Rachmilevitch et al. (2006) also reported lower shoot productivity in turf-type *Agrostis* species exposed to 37°C RZT, due to increased respiratory costs.

Root morphology could be very dependent on abiotic conditions (Choong et al., 2016). Generally, length of roots decreases with increasing environmental stress (Nagel et al., 2009). Given that roots are more susceptible to high temperatures than shoots (Ingram et al., 2015), high RZT could compromise root functions (e.g. water uptake), thereby causing decreased shoot and root development (e.g. Lettuce in He and Lee, 1998; He et al., 2001; He et al., 2009).
High RZT and the increased root respiration could have caused reduced root length (Ingram et al., 2015) resulting in increased carbon demand (Rachmilevitch et al., 2006). It is conjectured that with increased RZT there will be decreased respiration in the roots due to the disruption of the respiratory pathway components or the lack of respirable substrate (Ingram et al., 2015). High RZT compounded with low substrate oxygen levels would thus further attenuate plant growth (Mathus et al., 2014) as poorly developed root system would in turn affect efficiency of water and nutrient uptake (Minchin et al., 1994; Klock et al., 1997), further decreasing available photosynthetic for root growth (Ganmore-Neumann and Kafkafi, 1983). This is reflected in the works of He et al. (2009) where roots of plants in ambient RZT have less newly fixed 14C-photoassimilate and root total insoluble sugar content compared to plants in cool RZT. In general, plant roots are more thermal sensitive than shoots, and a large proportion of heat responses of plants could be attributed to thermal stress to the root systems (Du and Tachibana, 1994; Ingram et al., 2015): high RZT (35 - 40°C) will inhibit root growth and decrease root/shoot ratio (Ingram et al., 2015).

Interestingly, plants given a recovery period of benign RZT at night were observed to perform better than plants exposed to constant heat stress RZTs. Pardales et al. (1991) observed that sorghum grown in day/night 40°C/25°C RZTs had much better root growth than plants kept in constant 40°C-RZT. This was attributed to the prevention of excessive root respiration during the lower night temperatures which increased availability of carbohydrates for continued root growth. The fact that optimal RZTs can offset shoot damage from detrimental ambient temperatures (Kuroyanagi and Paulsen, 1988) forms the basis for the aeroponics systems used in recent studies (He et al., 2013).

2.2 Heat stress effects on photosynthesis

Photosynthesis is a cascading process of consecutive redox reactions that occur when the photonic energy gets absorbed by LHCs and transferred to PS reaction centres via excitons
(Baker, 2008). Photooxidation occurs when the ETC is over-reduced as a result of adverse environmental stress like high temperature (Nishiyama and Murata, 2014). High temperature has been shown to decrease photosynthesis (e.g. Hurkman et al., 2009) and reduce chl synthesis (Huang et al., 2017), decrease sucrose content in leaves and increase leaf soluble sugars (Tan et al., 2011).

2.2.1 Heat stress effects on photoinhibition

Photosynthesis consists of two distinct phases, light-dependent reactions and the Calvin cycle. Very briefly, light energy is converted into ATP and NADPH in the first phase (with O₂ evolution) while CO₂ is reduced by ATP and NADPH into energy rich carbon compounds in the second phase (Padmasree et al., 2002). Output of these phases must be balanced not only for initiation of photosynthesis, but also for optimisation of the whole process to avoid photoinhibition under the influence of the multiple environmental cues (Sunil et al., 2013). If CO₂ assimilation is reduced as a result of environmental stress (e.g. heat stress leading to stomata closure, \( A_{sat} \) decrease and stroma CO₂ depletion), NADPH would be in excess (Ort and Baker, 2002) thereby causing over-reduction of ETC and generation of ROS, leading to photoinhibition (Yoshida et al., 2007). Therefore, the synthesis and utilization of reducing equivalents in the stroma must be balanced to avoid photoinhibition (Walker et al., 2014).

Of the whole photosynthesis process, PSII is the most thermal sensitive and photosynthesis compromises are often due to photoinhibition of PSII (Feng et al., 2014). PSII functionality inhibition leads to variations in the variable chl fluorescence, which traditionally has been used as a stress indicator for heat stress (Greer and Weedon, 2012). However, heat stress in itself does not cause photoinhibition. Photodamage to PSII is only related to the dosage of incident light (Chow et al., 2005), and without light stress, the other environmental stresses would not cause quite the same extent of damage to PSII functionality (Demmg-Adams and Adams, 2018; Malnoe, 2018). Photoinhibition increases when the energy absorbed by LHCII
exceeds energy consumption, resulting in severe damage to PSII (Tikkanen and Aro, 2014). ROS like the superoxide anion radical (O$_2^-$) or singlet oxygen is produced when ETC is over-reduced by the excess electrons that are not utilized in the Calvin cycle. If the enzyme superoxide dismutase (SOD) is available, the superoxide anion can be catalysed into H$_2$O$_2$ (Nishiyama and Murata, 2014), which is in turn catalysed into the highly reaction hydroxyl free radical OH$^+$. Accumulation of the OH$^+$ and singlet oxygen would eventually damage PSII and cause photoinhibition (Nishiyama and Murata, 2014). Other abiotic stresses like hypersalinity (Ohnishi and Murata, 2006), extreme temperatures (Takahashi et al., 2010), and limited CO$_2$ fixation (Takahashi and Murata, 2005, 2006; Wang et al., 2014), play a role in photoinhibition via inhibiting repair of damaged PSII (Nishiyama and Murata, 2014). It is believed that presence of these environmental stressors inhibits the translation of PsbA mRNA, thus inactivating the PSII repair process (Nishiyama and Murata, 2014).

For continued photosynthesis, reducing equivalents NADPH must be balanced in the production and consumption in the light-dependent and independent stages respectively. For continual photosynthesis in strong irradiance, ROS production must be balanced by ROS-scavenging enzymes to avoid photoinhibition (Shikanai et al., 1998). Al-Taweel et al. (2007) advocate for the active production of anti-oxidant enzymes like catalase (Miyagawa et al., 2000) and APX (Maruta et al., 2007) in higher plants to reduce photoinhibition via decreased stroma ROS. Another potential candidate to reduce ROS is the vitamin E: alpha tocopherol (Demmig-Adams et al., 2013) and carotenoids (Ramel et al., 2012). It is believed, based on these findings, that the inhibiting effects of abiotic stress on PSII repair (synthesis of D1 protein) could be achieved by increasing enzymatic activities of ROS-scavenging chaperone molecules and/or overexpression of the synthesis and accumulation of anti-oxidants.

PSII, despite its reputation as the “engine of life”, is the most thermal labile complex in the whole photosynthesis process (Nath et al., 2013a). As such, plants have adapted in
evolutionary history to cope with the frequent photodamage to PSII for effective continuation of photosynthesis (Yamamoto et al., 2011). The extent of high irradiance induced photodamage in plants could be gauged by impeding the repair process of PSII via chemical application of lincomycin or chloramphenicol to exposed plant cells, thus blocking D1 protein translation (Murata et al., 2007). PSII repair cycle is a multistep process involving: (a) resceindable phosphorylation of PSII proteins (Bonardi et al., 2005); (b) partial breakdown of the PSII complex (Nixon et al., 2010); (c) sequestering and breaking down damaged D1 protein (Samol et al., 2012); (d) replacement of damaged D1 protein with new copy (Nath et al., 2013b) and (e) re-formation of the PSII complex (Tikkanen et al., 2014). Recent works by Chen et al. (2017) and Li et al. (2018) has shown that controlled phosphorylation of PSII under moderate light could help acclimate PSII-LHCII super-complex to decrease PSII damage through regulated energy transfer from LHCII to PSI. PSI could migrate to the grana margins with moderate phosphorylation of LHCII and facilitate energy transfer to PSI to prevent photodamage in PSII (Tikkanen et al., 2010). This phosphorylation rate intensifies under high irradiance, however, and PSII-LHCII’s structural integrity becomes compromised and energy transfer to PSI becomes unchecked (Grieco et al., 2012). When high irradiance persists, dephosphorylation of LHCII occurs and energy transfer to PSI ceases (Tikkanen and Aro, 2014). D1 protein is the crux of the PSII repair process (Li et al., 2018; Malnoë, 2018). Breakdown and production of D1 protein is regulated by chloroplastic proteases (Che et al., 2013; Pribil et al., 2014).

PSII phosphorylation regulates the functional folding and macroscopic structure of plant thylakoid membranes (Fristedt and Vener, 2011; Nath et al., 2013; Pribil et al., 2014). STN7-dependent phosphorylation appears to provide adequate levels of excitation energy to PSI to accomplish an efficient electron transfer from PSII to PSI (Grieco et al., 2012). In addition, as a retrograde signalling kinase, STN7 is known to trigger the phosphorylation
cascade, and thus regulating the expression of photosynthesis related genes and assembly of the photosynthetic machinery (Tikkanen et al., 2012). The functional characterization of Arabidopsis stn8 single and stn7 3 stn8 double mutants indicated that STN7 can phosphorylate LHCII, as well as PSII proteins to some extent, because complete inhibition of PSII phosphorylation was only observed in the stn7 3 stn8 double mutant (Bonardi et al., 2005; Fristedt and Vener, 2011). In contrast, studies of a rice stn8 mutant revealed that Osstn8 alone can produce all of the phenotypes observed in Arabidopsis stn7 3 stn8 mutants, indicating that distinctly specific regulatory mechanisms involving STN7 and STN8 exist in monocots versus dicots (Nath et al., 2013). Studies on TAP38/PPH1 have indicated that PPH1 may have other unknown function(s) besides STN7-mediated dephosphorylation of LHCII, as co-expression of PPH1 and STN7 was not detected (Obayashi et al., 2009; Nath et al., 2013). Recent studies using Arabidopsis mutants with inactivated protein phosphatase 2C (PP2C)-type PBCP revealed that dephosphorylation of PSII subunits is crucial for efficient degradation of D1 (Puthiyaveetil et al., 2014). In addition, PBCP has been found to regulate thylakoid stacking (Bonardi et al., 2005). Although the role of LHCII phosphorylation in PSII repair is still not clear, the phosphorylation of CP29, a minor subunit of LHCII, appears to be essential for the disassembly of the LHCII-PSII super-complex (Fristedt and Vener, 2011).

Reaction-centre D1 protein is a key player in the PSII repair cycle (Nath et al., 2013; Nishiyama and Murata, 2014; Rochaix, 2014; Tikkanen and Aro, 2014; Li et al., 2018; Malnoë, 2018). The proteolysis and de novo synthesis of D1 protein during the PSII repair cycle are facilitated by chloroplastic proteases in the lumen, stroma, and the thylakoid envelope (Kapri-Pardes et al., 2007; Sun et al., 2007; Kato et al., 2009; Schuhmann and Adamska, 2012; Pribil et al., 2014). Serine-, metallo- and putative cysteine- and aspartic acid-proteases have been identified, some of which require ATP (Sakamoto, 2006; van der Hoorn, 2008; Pribil et al., 2014). Presumably, many other proteases remain to be discovered. Known protease functions
include chloroplastic biogenesis, degradation of signalling components, maintenance of chloroplastic homeostasis, and degradation of damaged proteins (Yin et al., 2008; Sun et al., 2010a, 2010b).

Chloroplastic degradation proteases have received significant attention primarily due to their role in the degradation of photodamaged PSII proteins. The function of many of these proteases, however, remains to be determined. In plants, periplasmic degradation proteases are ATP-independent serine endopeptidases that are involved in the degradation of photodamaged proteins (Haussuhl et al., 2001; Sakamoto, 2006; van der Hoorn, 2008; Sun et al., 2010a; Schuhmann and Adamska, 2012). Although these proteases were first discovered in *Escherichia coli*, they are present in most organisms (Schuhmann and Adamska, 2012). *Arabidopsis* contains 16 degradation proteases (Deg1–16) that, with the exception of Deg5, possess a C-terminal protease domain and an N-terminal PDZ domain (Haussuhl et al., 2001). Degradation proteases are present in chloroplasts, mitochondria, peroxisomes, and nuclei (Schuhmann and Adamska, 2012). Of the five known chloroplastic degradation proteases, Deg1, Deg5, and Deg8 are attached to the luminal side of thylakoid membranes, and Deg2 and Deg7 are in the stroma. *Arabidopsis* mutants with reduced levels of Deg1 exhibit enhanced sensitivity to photoinhibition and an increased accumulation of D1 protein; indicating that Deg1 plays a role in the degradation of D1 protein (Kapri-Pardes et al., 2007). In addition, recent studies in *Arabidopsis* have indicated the involvement of Deg1 in the degradation of CP29 and CP26 proteins under photoinhibition (Zienkiewicz et al., 2012), and of Deg2 in degradation of CP24 under various abiotic stress conditions (Lucinski et al., 2011). The Deg2 protease has been associated with abiotic stress responses in plants. *Arabidopsis* deg2 mutants showed impaired activity to degrade CP24 in response to various abiotic stresses, such as high salinity, HL, and wounding, indicating that Deg2 protease is required for plant growth and development under optimal as well as stressed conditions (Lucinski et al., 2011). Interestingly,
the effect of different abiotic stresses on the accumulation of proteases is distinctive. For instance, two- to four-fold increase in the amount of Deg2 was observed in *Arabidopsis* leaves treated with high salinity, desiccation, or high irradiance for 2 h, while heat-treated leaves exhibited only trace amounts of Deg2 in the thylakoid membranes (Haussuhl et al., 2001).

There is inherent difference between PSI and PSII, starting with structural difference in their respective LHCl and LHClI (Rochaix, 2014): LHCl contains Chl a and a little Chl b while LHClI contains Chl a and Chl b in comparable amounts (Xu et al., 2012). In addition, PSII–LHClI super-complex contains a D1–D2 dimer (Che et al., 2013). The entire PSII–LHClI super-complex is embedded in the granum, while PSI is localized in the stromal lamellae of the chloroplasts (Rochaix, 2014). Unlike PSII, PSI is very well protected against photodamage (Sonoike, 2011) but recovery of PSI is very slow if damage ensues when electron supply from PSII exceeds the PSI electron accepting capacity (Tikkanen et al., 2014).

Several studies have indicated that the proton gradient-dependent or Cytochrome b6f-mediated slowdown of ETC and LHClI-mediated excitation of PSII and PSI via NPQ and LHClI phosphorylation regulate the photoprotection of PSI in higher plants (Sonoike, 1995; Joliot and Johnson, 2011; Suorsa et al., 2012; Grieco et al., 2012). Recently, Demmig-Adams and Adams (2018) reviewed that in addition to the above-mentioned mechanisms, controlled photoinhibition of PSII regulates the ETC and prevents the formation of ROS and photodamage to PSI. Increased PSI-mediated cyclic electron flow was reported in *Secale cereale* plants subjected to chilling and high light stress, indicating the role of temperature/light-dependent acclimation in the induction of selective tolerance to PSI photoinhibition (Ivanov et al., 1998). Similarly, it has been reported that cyclic electron flow around PSI is required to produce a proton gradient that in turn leads to efficient NPQ under heat stress in *Ficus concinna* trees (Jin et al., 2009).
Synchronizing the photosynthetic apparatus requires a balance between the excitation energies driving PSII and PSI. This balance has been attributed to the ability to regulate the level of phosphorylation and dephosphorylation of LHCII and PSII proteins (Tikkanen et al., 2010; Grieco et al., 2012; Tikkanen et al., 2014). Phosphorylation of LHCII and PSII proteins in *Arabidopsis* is facilitated by the state transition kinases, STN7 and STN8, respectively (Bonardi et al., 2005), while their dephosphorylation is triggered by thylakoid-associated phosphatase 38 (TAP38 or PPH1) (Pribil et al., 2010; Shapiguzov et al., 2010) and Psb core phosphatase (PBCP) (Samol et al., 2012), respectively. A recent study has demonstrated that TAP38/PPH1 phosphatase was required to prevent canonical state transition upon increase in light intensity (Nageswara et al., 2015).

### 2.2.2 Heat stress and photosynthetic CO₂ assimilation and stomatal conductance

It has been demonstrated that plant heat tolerance is a major factor in maintaining *A* and leaf gas exchange (He et al., 2001). Under heat stress conditions, stomata of leaves will close, thereby limiting intracellular CO₂ concentrations and resulting in further impairment of the photosynthesis process (Du and Tachibana, 1994). *Prunus mira* (Koehne) seedlings exposed to high RZT were also reported to have low leaf water content and compromised photosynthesis (Hao et al., 2012). Tomatoes grown in 24°C had the highest CO₂ uptake and photosynthesis rate compared to plants grown in 12, 18, 30 and 36 °C (Gosselin and Trudel, 1994). He et al. (2001) reported low internal CO₂ concentration in lettuce exposed to high light and high RZT. Working with the apple cultivars Royal Gala and McIntosh *Malus domestica*, Behboudian et al. (1994) reported low *gₛ,sat* and *Aₛat* for plants in hot RZT. In conclusion, high temperature causes low *A* as a result of the closing of stomata (He et al., 2001) which in turn limits the intracellular CO₂ (Vogelmann, 1993). With limitation of CO₂ assimilation, photoinhibition eventually occurs (Walker et al., 2014) and plant growth rate is reduced (Tan et al., 2002).
In general, plants play a daily balancing game of somatic growth and stress avoidance via stomatal aperture control: transpiration and photosynthesis would be enhanced with stomata opening, but water loss would be accelerated (Tardieu et al., 2011). When water uptake is compromised as a result of high RZT (e.g. He et al., 2009), stomata closure would result in fewer endogenous electron acceptors (Valentini et al., 1995), diminished efficiency in photosynthesis, and increase rates of photorespiration (Osmond and Björkman, 1972) and Mehler reaction (Schreiber and Neubauer, 1990). H$_2$O$_2$ formed via the Mehler reaction is toxic to surrounding biological processes (Cornic and Fresneau, 2002) and together with ROS formation under chronic high light stress (Baker, 1991), compounded by stroma CO$_2$ limitation, would eventually cause photoinhibition. For example, lettuce plants grown in A-RZT has about 30% of $g_{s\text{ sat}}$ and $A_{\text{ sat}}$ compared to plants grown in 20°C-RZT (He et al., 2009). Using ponderosa pine seedlings, Hubbard et al. (2001) demonstrated $g_s$ and $A$ are directly affected by root hydraulic conductance. Although other factors could also play a part in stomata regulation, (e.g. Abscisic acid from the root, Dodd et al., 2003), strong correlations between RWC and $g_{s\text{ sat}}$ (e.g. lettuce: He et al., 2001) suggest a strong relationship between stomata closure and reduced shoot turgor and water potential.

2.2.3 Heat stress and photosynthetic pigments

Heat stress reduces the amount of Chl a, Chl b and total Chl levels (Hanif and Wahid, 2018) and a major effect of heat stress is the enhanced activity of chlorophyllase enzyme leading to the degradation of chlorophylls (Rossi et al., 2017) and causing photodamage to PSII (Havaux, 1993; Havaux and Tardy, 1996). PSII damage as a result of Chl breakdown is irrevocable (Fracheboud and Leipner, 2003). Increase in Chl b levels typically signify thermal tolerance in plants due to the protective effects of Chl b on PSII from photodamage (Sakuraba et al., 2010). This is also demonstrated in the recent works of Choong et al. (2016) where lettuce Lactuca genotypes grown in tropical greenhouse with higher shoot FW had corresponding
lower Chl a/b ratios. Conversely, Dinc et al. (2012) demonstrated that heat stressed plants had extremely low Chl a/b ratios. Considering that Car also protect against photodamage (Filella et al., 2009), Chl/Car might be a better stress indicator (Hendry and Price, 1993).

2.2.4 Heat stress and Chl fluorescence

When a plant in darkness is suddenly exposed to light, Chl fluorescence will be induced through fluorescence radiation from the absorbed light energy (Papageorgiou et al., 2007). In a nutshell, light energy absorbed by Chl will be (1) used in driving of photosynthesis, (2) dissipated as heat via NPQ in a regulated manner, and (3) dissipated as heat in a constitutive, relatively unregulated manner and as Chl fluorescence qP. Fluorescence induction is characterized by two segments: a fast and a slow induction phase. The former reflects the photochemistry of PSII while the latter reflects the complicated interplay of processes within the thylakoids and the stroma, with particular links to the Calvin cycle (Krause and Weis, 1991). In other words, the fast and slow Chl fluorescence induction is related to the light-dependent and light-independent phases of photosynthesis respectively. Information from the fast phase derives primarily from processes of electron accepting in the ETC, but analysis of the slow phase is a lot more complicated due to the interplay of processes like NPQ, ATP synthesis and the initiation of the Calvin cycle (Stirbet, 2011) but is useful in quantifying electron transfer and proton movement (Goltsev et al., 2009). It is also very useful in assessing the effects of environmental stress on plant performance. Itoh (1980) and Bilger et al. (1987) worked on the effects of low and high temperatures on Chl fluorescence. Valikhanov et al. (2002) and Zhang et al. (2007a) explored the effects of high irradiance of visible light and UV light respectively. Zhang and Xing (2008), Plekhanov and Chemeris (2003) and Mladenova et al. (1998) investigated the effects of hypersalinity, heavy metal contamination and water deficiency on plant performance and Chl fluorescence, the three main reason for the decrease in suitable farmland area. Zhang et al. (2007b) proposed that slow fluorescence estimates
photosynthetic potential and Chl content. Conversely, fast fluorescence could reflect PSII efficiency via chl a transient kinetics (Gururani et al., 2013, 2015), revealing information like structural integrity of the PSII when under heat stress.

Fluorescence (F) increases sharply from F_0 (minimum) to F_m (maximum) in dark adapted plants exposed to a strong saturating light pulse (3000–12000 mmol photons m^{-2}s^{-1}, 200–1000 ms). Recent findings show that the increase from F_0 to F_m may indicate the reduction of quinone, the primary electron acceptor of PSII (Schansker et al., 2014). Under high temperature stress, CO_2 assimilation and electron transport were progressively inhibited, F_0 increased and F_m and F_v/F_m decreased in relation to PSII inactivation (Gorbe and Calatayud, 2012). While mild heat stress resulted in down-regulation of PSII and in protection against damage by excess of light (i.e., stimulation of non-photochemical quenching), strong heat stress inhibited the protection mechanism reflected by NPQ (Schreiber and Klughammer, 2008).

2.2.5 Heat stress, nitrogen metabolism and Rubisco protein

Rubisco is arguably the most important protein in the world for it is the key element in driving photosynthesis (Yasumoto et al., 2018). It is, however, also the major limiting factor in photosynthesis due to its inefficiency in binding to CO_2 and competitive preference to bind to O_2 resulting in photorespiration (Suganami et al., 2018), resulting in a net 25% loss in photosynthesis rate in C_3 plants (Sharkey, 2005). Plants were thought to have evolved against this by over accumulation of Rubisco protein in photosynthetic apparatus (Woodrow and Berry, 1988). With further consideration of the highly variable environmental conditions, plants strategize to amass large amounts of Rubisco protein as a failsafe plan to boost photosynthetic outcome (Stitt and Schulze, 1994). This strategy, however, heavily taxes the energy budget and protein pool of the plant (Fukayama et al., 2018). For example, He et al. (2013) demonstrated strong correlation between leaf Rubisco levels with both TRN and total
soluble protein levels, and $P_{\text{max}}$ will ultimately be affected by all three factors. Makino et al. (1997) suggested that Rubisco accounts for about 27% of total leaf N and practically all leaf N depletion would result in Rubisco level decrease (Long et al., 2004). Makino and Mae (1999) also demonstrated that $A$ is directly affected by the amount of Rubisco available. This is further demonstrated in the works of He et al. (2001), where lettuce plants transferred to high RZT from 20°C-RZT reacted immediately by closing their stomata, but $g_s$ increase was not accompanied by $P_{\text{max}}$ increase when plants grown in high RZT was transferred to 20°C-RZT, suggesting a Rubisco limiting condition where A-RZT plants often suffer from N deficiency which in turn affected available Rubisco amount (He and Lee, 2001). Generally, high amounts of TRN, leaf soluble protein and Rubisco protein result in higher $A$ (Li et al., 2009), which in turn increases $P_{\text{max}}$ (Makino et al., 2003).

2.3 Heat stress and plant proteins

Amino acid and protein metabolism are interrelated, and both play important roles in plant adaptation to heat stress. For example, Wang et al. (2018) reported that hard fescue (Festuca trachyphylla) in response to heat stress, increased total amino acid content but decreased total soluble protein content. Total soluble protein also decreased in two cultivars of Rhododendron with heat stress probably due to protein hydrolysis to scavenge reactive oxygen species and maintain cell stability (Shen et al., 2017).

2.3.1 Total soluble protein determination

For proteomic studies, total protein concentration must first be determined (Peterson, 1983). Among the colorimetric methods conventionally employed, the methodology advocated by Bradford (1976) is the most frequently utilized for it is simple, fast and sensitive (Sapan et al., 1999) compared with other methods such as the Biuret reaction (Gornall et al., 1949), Lowry method (Lowry et al., 1951) or Bicinchoninic Acid Assay (Smith et al., 1985).
The Bradford assay relies on the binding of the dye Coomassie Blue G-250 to proteins, therefore giving rise to a dye–protein complex with increased molar absorbance (Chial et al., 1993). The free dye can exist in four ionic forms, of which three predominate in the acidic pH range of the assay reagent solution (Sapan et al., 1999). Of these three forms, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively (Compton and Jones, 1985). In comparison, the more anionic blue form of the dye, which binds to protein, has an absorbance maximum at 590 nm (Congdon et al., 1993). The Bradford assay therefore estimates the quantity of protein by determining the amount of dye in the blue ionic form.

Sedmak and Grossberg (1977) have reported that upon binding to the protein, there will be a metachromatic response, and the absorbance maximum of the dye–protein complex will shift to a range between 595 nm and 620 nm, depending on the dye source. Although it would appear sensible to measure the absorbance at the higher wavelength, the presence of the green form of the dye (max = 650 nm) will interfere with absorbance measurement of the dye–protein complex at 620 nm. Therefore, measurement at 595 nm represents the best compromise between maximizing the absorbance due to the dye-protein complex and minimizing the interference caused by the green form of the free dye (Compton and Jones, 1985; Chial et al., 1993; Congdon et al., 1993).

2.3.2 Heat stress and heat shock proteins

Environmental stress conditions like extreme temperatures and pH changes could alter protein configuration and structural integrity, causing protein to unfold and thus lose its functionality due to positional displacement of active binding sites (Vandereyken et al., 2018). Aggregation of such damaged or denatured proteins may eventually kill the cells. Heat stress is defined as temperatures far exceeding the coping mechanisms of plants such that normal functions like development and growth are discontinued (Zhou et al., 2011). Extreme unchecked heat stress in plants could result in cascading events of over production of ROS and
metabolically toxic compounds that impede photosynthetic processes, leading to plant starvation and somatic growth cessation (Haworth et al., 2018) and eventual cell death and structural collapse in various plant architecture organizations (Shen et al., 2017). Although heat shock response and hsp synthesis could offer defence against heat stress, effectiveness of the chaperone molecules depends on the intensity and severity of the heat stress event and inherent energy budget and well-being of the plant upon heat exposure (Goloubinoff et al., 2018). In general, despite recent efforts in elucidating the effects of heat stress, how plants sense and mitigate heat stress is still poorly understood (Silva et al., 2018).

Thermotolerance in plants could be acquired to resist environmental heat stress (Vierling, 1991) which is defined as a cellular priming with short exposure to sub-lethal heat stress which conditions the plant and cellular system to survive a subsequent heat shock exposure that would be lethal to non-conditioned plants (Wang et al., 2014). Membrane lipid saturation is considered as an important element in high temperature tolerance. In a mutant wheat line with increased heat resistance, heat treatment increased relative quantities of linolenic acid (among galactolipids) and trans-3-hexadecanoic acid (among phospholipids), when compared with the wild type (Behl et al., 1996). Currently, it is unknown whether a higher or a lower degree of membrane lipid saturation is beneficial for high-temperature tolerance (Klueva et al., 2001). The contribution of lipid and protein components to membrane function under heat stress needs further investigation. Localization of low molecular weight hsp70s with chloroplastic membranes upon heat stress suggests that they play a role in protecting photosynthetic electron transport (Heckathorn et al., 1998). An important component of thermotolerance is changes in gene expression. Heat stress swiftly alters patterns of gene expression (Yang et al., 2009). This transitory shift in gene expression in response to higher temperature is termed a heat shock response (hsr), which is normally induced 5-10°C above the homeostatic plant temperature (Schöfl et al., 1999) resulting in preferential downstream
production of stress mediating molecules and pathway initiation. Several pathways, for example ROS-scavenging antioxidants, Abscisic acid and Salicylic acid, all play a part in the acquisition and maintenance of thermotolerance (Larkindale and Huang, 2005; Charng et al., 2006), but hsp synthesis is the most conserved and discernible (Hupalo et al., 2018).

Hsp are preferentially over-expressed in heat shock cells, but they are also constitutively expressed in low amounts as housekeeping proteins for cellular function regulation (Zafar et al., 2016). Based on the general molecular weight, hsps are divided into five groups (Zhou et al., 2011), of which plants have a huge diversity of within each plant species, reflecting the evolutionary history of plant adaptation to environmental heat stress (Swindell et al., 2007). Hsp transcription are regulated by heat shock factors (hsfs) found in cell cytoplasm (Qu et al., 2013). Heat stress signals transferred to hsfs triggers hsp production (Larkindale and Huang, 2005) and each major hsp works primarily by targeting heat damaged and aggregated proteins to (i) repair and fold the damaged protein to its native state or (ii) sequester irreparable protein to facilitate cellular disposal or degradation (Oliverio et al., 2018). Hsp also works in synergy with other stress mitigation pathways (Wang et al., 2004) through protein breakdown to osmolytes (amino acids) for cellular osmosis regulation (Diamant et al., 2001) and cellular redox reaction regulation (Arrigo, 1998) and induction of ROS-scavenging enzymes (Panchuk et al., 2002). Under chronic heat stress, the hsp trigger is inactivated (Schöffl, 2005). In summary, plants acquire thermotolerance to cope with heat stress by differentially expressing gene which, though energetically costly and intensive, offers rapid protection against otherwise lethal heat stress.

**2.3.2.1 Protein separation**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) are the two most popular electrophoretic techniques used to separate protein molecules (Righetti, 2004). SDS-PAGE separates proteins using the differences in the
molecular weight of the proteins (Righetti, 2005). This separation technique is performed on a polyacrylamide gel containing the detergent sodium dodecyl sulphate (SDS). SDS is an anionic detergent that denatures proteins and makes them negatively charged by wrapping around the polypeptides, thus giving equal charge densities per unit length (~1.4 g of SDS per gram of polypeptide; Reynolds and Tanford, 1970). In addition, SDS disrupts hydrogen bonds, blocks hydrophobic interactions and partially unfolds the protein, thus eliminating secondary and tertiary structures for electrophoresis (Kaufman et al., 1995).

SDS-PAGE is usually performed with a discontinuous buffer system. In this system, the proteins migrate in the order of their mobility in tight zones, called the stack, between the leading and trailing ions of the discontinuous buffer (Laemmli, 1970). The pore size and thus the sieving properties of the polyacrylamide gel can be controlled by altering the amounts of acrylamide and cross-linker in the gel (see Simpson, 2003). In summary, acrylamide and cross-linker concentrations are described in terms of % T and % C respectively. % T is the total weight of acrylamide while % C is the weight of cross-linker expressed as the percentage of total weight of both acrylamide and cross-linker. For example, a 1-litre stock solution of acrylamide at 30% T and 2.5% C would contain 292.5 g of acrylamide and 7.5 g of cross-linker. When % C remains constant and % T is increased, the pore size of the gel decreases. When % T remains constant and % C is increased, the pore size of the gel follows a parabolic function, where at high and low values of C, the pores are large. The minimum pore size is at C = 4%. Simpson (2003) provides recommended acrylamide concentrations for a variety of protein molecular-weight ranges (Table 1).

Discontinuous buffer systems (Laemmli, 1970), as opposed to continuous gels (Weber and Osborn, 1969), are a composite of short wide-pore “stacking gel” (3-4% T) layered on top of a long, small-pore “resolving gel” (5-25%T). The stacking and resolving gels in the discontinuous buffer system are also discontinuous with respect to buffer composition, both in
terms of their pH and ionic composition. These two parameters, i.e., different buffer and acrylamide compositions, allow very diluted samples to be concentrated in the upper stacking gel prior to their separation upon entering the lower resolving gel by shrinking the original sample into very thin, highly concentrated starting zones, so that all of the protein molecules begin the electrophoretic separation at very nearly the same point (Chrambach and Rodbard, 1971). In contrast, continuous gels utilising monophasic buffers are limited, as they do not allow separation of samples with low concentrations and large volumes (Simpson, 2003).

Table 1 Recommended acrylamide concentrations for a variety of protein molecular-weight ranges (after Simpson, 2003).

<table>
<thead>
<tr>
<th>% Acrylamide in resolving gel</th>
<th>Separation size range (molecular weight × 10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>36-200</td>
</tr>
<tr>
<td>7.5</td>
<td>24-200</td>
</tr>
<tr>
<td>10</td>
<td>14-200</td>
</tr>
<tr>
<td>12.5</td>
<td>14-100</td>
</tr>
<tr>
<td>15</td>
<td>14-60</td>
</tr>
</tbody>
</table>
2.3.2.2 Western blot analysis

Western blotting is a protein/protein hybridization technique that is used for immunodetection of specific antigen(s) of interest in a complex mixture of proteins. This technique first separates a protein mixture according to molecular size by using SDS-PAGE. The separated protein molecules are then immobilized onto a nitrocellulose membrane or its equivalent. Hybridisation is then performed using the membrane and a specific antibody raised against the specific antigen. This will allow specific binding to the antigen of interest in the protein mixture that is immobilized on the membrane. The antibody-antigen complex is then typically detected by a second antibody to which an enzyme is attached, which in subsequent reaction with a substrate, will give a visualised endpoint of the antibody-antigen reaction (see Durrant and Fowler, 1994).

Nitrocellulose, polyvinylidene difluoride (PVDF), activated paper or activated nylon have all been used successfully to bind transferred proteins (Gershoni and Palade, 1983; Renart et al., 1979; Towbin et al., 1979). Although nitrocellulose is the most commonly used membrane support, it has several shortcomings (Too et al., 1994). First, the proteins are not covalently bound and therefore are subject to signal loss. Second, it is brittle when dry and hence is not robust enough for frequent handling. Third, small proteins tend to move through nitrocellulose membranes and only a small fraction of the total protein actually binds to the membrane.

In comparison, using PVDF membranes offers several advantages, including its high protein binding capacity, physical strength and chemical stability (Mansfield, 1994). Another advantage of electroblotting proteins onto PVDF membranes is that replicate lanes from a single gel can be used for various purposes such as N-terminal sequencing (Baker et al., 1991), C-terminal sequencing (Zvaritch et al., 1990), peptide mapping (Iwamatsu, 1992) and amino acid analyses (Yuen et al., 1989), as PVDF is amenable to Coomassie Brilliant Blue staining.
(Christiansen and Houen, 1992). More pertinent to this study, PVDF is a good medium for various methods of protein quantification such as colourimetric technique (Pryor et al., 1992), radioactive technique (Gultekin and Heermann, 1988) and chemiluminescence method (Sandhu et al., 1991; Bronstein et al., 1992). It also has high binding capacity for proteins (130-400 µg cm⁻²; Mansfield, 1994).

In contrast to amino acid sequencing, amino acid analyses and peptide mapping, blocking is essential for immunodetection of bound protein on PVDF membrane post transfer, so as to block unoccupied binding sites on the membrane to avoid non-specific interactions of the antibody probe (a protein in itself) with other proteins or with the membrane (Maio, 1994). Typically, a blocking solution would contain two components, an “inert” protein and a non-ionic detergent (Bartles, 1984). Choice of the protein blocker is largely based on availability since there is no truly “inert” protein, and diluted non-fat dry milk has been very popular primarily due to its cost and accessibility (Johnson et al., 1984). Tween 20 is typically used as a non-ionizing detergent to prevent binding of proteins to the membrane. However, concentration > 0.5% Tween 20 is discouraged because this would cause protein elucidation from the membrane and decrease signal strength in protein detection (Batteiger et al., 1982).

Two methods are commonly used for detecting proteins: (1) radioactive and (2) enzyme-linked reagents (Simpson, 2003). For radioactive reagents, a radiolabeled protein, either iodinated (125I) staphylococcal protein A (Dimond and Loomis, 1976) or streptococcal protein G (Harper et al., 1988), is bound to the primary antibody that, in turn, binds to the antigen on the membrane blot, thus enabling detection of the antigen of interest with autoradiography (Laskey, 1994). Enzyme-labelled antibodies are typically visualised in two ways: a direct colourimetric detection (Pryor et al., 1992) and a chemiluminescence method of detection (Sandhu et al., 1991). Colourimetric detection involves using antibody coupled with horseradish peroxidase or alkaline phosphatase along with a range of soluble substrates that
will concomitantly yield insoluble coloured products (Knecht and Dimon, 1984). This effect is visible to the naked eye and therefore does not require any sophisticated equipment and offers the advantage of providing quick results. The major drawback is that quantitative results cannot be obtained. In addition, the poor contrast between the colour of the reaction product and membrane (background) can often make it difficult to obtain good photographs of enzyme developed blots (Kurien and Scofield, 2003). Substrates that can be triggered to produce light exist for both alkaline phosphatase (Bronstein et al., 1989) and horseradish peroxidase (Whitehead et al., 1983) and have been used for the detection of protein bands on X-ray film as with radiolabeled probes. Although both alkaline phosphatase and horseradish peroxidase systems are sensitive, interpretation of the former results can sometimes be hindered by high background levels (Durrant and Fowler, 1994). The horseradish peroxidase system, in contrast, offers excellent signal to noise ratio, is extremely rapid and is commercially available as the ECLTM (Enhanced ChemiLuminescence) Western blotting system (Durrant, 1990; Pollard-Knight et al., 1990).

2.4 Strategies for improving plant tolerance to abiotic stress

Crop yield reductions are largely due to abiotic stresses like extreme temperature and water deficit or flood conditions (Loreti et al., 2016). Strategies to boost tolerance to environmental stresses offers a relatively rapid and simpler solution to maintain sustainable crop yield (Zhao et al., 2018). ‘Priming’ is a preferred solution for heat-tolerant plants for there is no genetic modification involved, no additional chemicals involved, and only involves tolerance acquirement via preferential shift in intrinsic gene expression for stress mediating pathways at the cost of energy budget reserves of the plant and optimizing the priming conditions (Lämke et al., 2016).
2.4.1 General induced resistance in plants

Both biotic and abiotic stresses can induce resistance in plants (Pieterse et al., 2014) via rapid activation of defence pathways (Fu and Dong, 2013). Induced resistance in plants could be differentiated as systemic acquired resistance or induced systemic resistance, both of which confer tolerance to stress and regulated by phytohormones like ethylene, jasmonic acid and salicylic acid (Walters et al., 2013). Systemic acquired resistance requires the salicylic acid signalling pathway (Durrant and Dong, 2004) whereas induced systemic resistance is dependent on jasmonic acid and ethylene (Van Wees et al., 1999). These act by priming the plant to pre-emptive preparedness for future stress events, but do not directly induce genetic expression or pathway initiation of stress defence (Romanazzi et al., 2016). Ruthenburg et al. (2007) reported chromatin modifications in heat primed *Arabidopsis* that could be one of the potential stress memory modifications (Conrath et al., 2015).

2.4.2 Heat priming and heat shock response

Heat priming of plants principally involves a priming event which exposes plants to a sub-lethal heat stress and allowing thermotolerance to be acquired (Mittler, 2002). Heat-stress memory must be established post priming (Lämke et al., 2018). Although the mechanisms of heat priming is well established, the characterization of the heat stress memory is lacking (Balmer et al., 2015). It is, however, established that the heat stress memory genes would ensure transcription and translation of relevant proteins (Meiri and Breiman, 2009).

Heat priming could confer thermotolerance to plants under conditions of extreme high heat (Larkindale and Huang, 2004) with protective mechanisms mainly applied to the photosynthetic apparatus (Berry and Bjorkman, 1980). Normally under extreme heat stress when PSII is photodamaged, D1 protein repair is inhibited, causing ROS to build up in plant cells (Melis, 1999). Thermotolerance primarily works via enhancement of ROS detoxification efforts (Suzuki and Mittler, 2006), maintenance of membrane structural integrity (Foyer et al.,
1994) and maintenance of low cellular ROS concentrations (Xu et al., 2006) due to increased ROS-scavenging activities compared to non-primed plants (Shi et al., 2001). Mitochondria and chloroplasts of heat primed plants would thus be less susceptible to ROS damage under heat stress compared to non-primed plants (Wang et al., 2014).

2.5 Heat priming and post-harvest qualities of vegetables

It is difficult to devise a simple definition for quality of fruits and vegetables for there are frequent changes of consumer needs and preferences with changing lifestyles and perception of quality (Jabs and Devine, 2006). When comparing products with price variations within an acceptable range, decisions are often based on the perceived intrinsic values rather than price (Harker et al., 2003).

In this study, heat primed vegetables would be compared to plants grown in cool RZTs in terms of productivity and post-harvest quality. It is hypothesized that with a sub-lethal heat priming, even if productivity of the heat primed vegetables is less than that of the control plants, the heat primed vegetables might have higher nutritional values and higher concentrations of anti-browning agents to prolong shelf life. To that effect, phenolic compounds, ascorbic acids, and selected browning enzymes of vegetables would be analyzed.

2.5.1 Post-harvest quality: Vegetable nutritional values

Environmental stress in plants could often generate secondary products, which though not essential for survival, can play important and primary roles in adaptation mechanisms. For example, lignins, which comprise an integral part of secondary cell walls, serve as a physical barrier against invading organisms (Barros-Rios et al., 2011). Additionally, polyphenols, including flavonoids and simple phenolic acids, have anti-oxidant functions, which are useful and effective for protection against ultraviolet (UV) light-induced oxidative damage, because those phytochemicals possess biological chromophores that specifically absorb UV light. Moreover, terpenoids, especially volatile ones abundantly present in herbs and citrus, have
unique roles as ‘infochemicals’. These are produced in response to feeding damage caused by insects and can convey a danger sign to neighboring plants (Arimura et al., 2005). Conversely, the phytochemicals in lettuce, such as polyphenols, have a positive effect on the prevention of cardiovascular disease (Lee and Aedin, 2006). Among the various anti-browning agents that had been evaluated for their influence on phenolic compounds in lettuce. Oxalic and ascorbic acids were more effective in preserving the phenolic compounds in comparison with cysteine and citric acid (Altunkaya and Gökmen, 2009).

Phenolic and flavonoid compounds in plants allegedly have many health benefits like cancer and allergy prevention, antioxidant effects like acting against inflammation, and boosting the immune system again microbial infections (Islam and Khan, 2011), and are excellent sources of dietary antioxidants (El-Zaeddi et al., 2017). Potentially, these plant sourced secondary metabolites have a preventive effect on human health against cardiovascular diseases and chronic diseases (El-Zaeddi et al., 2017; Yashin et al., 2017).

One of the most important plant antioxidants is ascorbic acid (Vitamin C) which ironically is the simplest vitamin in terms of chemical structures. Plants can synthesise and accumulate huge amounts of ascorbic acid, but actual ascorbate content is species specific (Ren et al., 2013), can vary between cultivars of the same species (Koh et al., 2012), or even between development stages of the same species (spinach: Bergquist et al., 2006; and celery: Huang et al., 2016) where younger plant tissue tend to have higher levels of ascorbic acid. Having higher ascorbic acid content in vegetables not only increases nutritional values, but can result in environmental stress tolerant plants due to increases in the antioxidant concentrations (Hancock and Viola, 2005). Food crops rich in vitamin C contents are desirable to consumers for although Vitamin C could be easily manufactured, the general dietary trend nowadays avoids artificially synthesised compounds. As such, close to 90% of our daily ascorbic acid
consumption relies on plant (natural) sources (Lee and Kader, 2000) and naturally ascorbic acid rich vegetables would be highly desirable.

2.5.2 Post-harvest quality: Browning enzyme levels.

Browning reactions are generally detrimental in food crops due to the unattractive pigmentation and emergence of undesirable flavours like bitterness (Altunkaya and Gökmén, 2009). Polyphenol oxidase (PPO) is a catalyst that oxidises phenol into quinine which is a pigment directly responsible for the browning in fruits and vegetables (Sanches-Ferrer et al., 1990). PPO naturally occurs in many fruits and vegetables, but browning reaction is induced only when PPO accumulates beyond a threshold concentration (Mayer, 1987; Vamos-Vigyazo, 1981). As such, PPO is a very useful indicator for browning potential in fruits and vegetables in laboratory assays and immunoassays due to its sensitivity (Siers, 1991). Aside from PPO, peroxidases (POD) is another important enzyme linked to the browning reaction of fruits and vegetables (Thypayong et al., 1995). Kwak et al. (1996) reported that cellular levels of PPO and POD increase proportionally with biotic and abiotic stress levels. Phenylalanine ammonia-lyase (PAL) is a major plant enzyme involved in the production of phenolic compounds like tannic, gallic, caffeic, chlorogenic and cinnamic acids (Ju et al., 1992). Trans-cinnamic acid, a product of PAL, regulates PAL levels (Basha et al., 2006): cinnamic accumulation inhibits PAL activities (Fujita et al., 2006) while removal of cinnamic acid (metabolic breakdown) induces over expression of PAL (Gerrish et al., 1985). While anthocyanin production is correlated with PAL activities, Ju et al. (1992) reported that PAL activity is more linked with simple phenol production. While PAL does not cause browning reaction by itself, the phenolic substrate it produces induces browning potential (Kim et al., 2014) while also adding a bitterness to the food crop taste which may not be desirable depending on situations. PPO, POD and PAL levels are inversely proportional to storage life of food crop (Min et al, 2017) so vegetables with inherent anti-browning properties would have a better commercial quality.
CHAPTER 3

Plant growth, productivity and photosynthetic performances of vegetables with response to chronic and acute heat stress
3.1 Abstract

To understand the thermal biology of food crops, their thermal limits and thermal window for optimal physiological processes should be examined. This research aimed to establish: 1) if a dose-response to increasing chronic (constant root-zone temperature (RZT) over 30 days after transplantation (DAT)) and acute (1 h exposure) heat stress existed for plant stress indices; 2) if a thermotolerance hierarchy exists among the examined parameters; and 3) the optimal thermal range and critical temperatures of the six vegetables. Dose responses were observed in shoot FW, P<sub>max</sub>, ETR, qP and NQPr with some parameters showing significant differences at temperatures as low as 28°C for selected species. Conversely, F<sub>v</sub>/F<sub>m</sub> ratio did not change with increasing chronic heat stress and only showed a decrease at 46°C in acute heat stress. Through comparison of LT<sub>50</sub>, it could be seen that shoot FW decreased at the lowest temperature range (28–30°C), followed by P<sub>max</sub> (35–45°C) while chl fluorescence like qP and NQPr are much higher (47–60°C), which mirrors the biological hierarchy of these performance parameters: productivity is the integrated product of photosynthesis and would be the highest level of complexity in a biological system. P<sub>max</sub> in comparison to ETR, qP and NQPr processes would be more complex and thus be inhibited by a lower temperature. By examining the temperature relationships of the various stress indices, it can be inferred that Arugula, RIL 141 and the parental plants Lactuca serriola (Paternal plant) and L. sativa L. ‘Salinas’ (Maternal plant) have a larger thermal buffer zone for acclimation compared to Canasta and RIL192.
3.2 Introduction

Heat stress affects plant growth and survival by inhibiting photosynthesis, which is thought to be the most heat labile plant function (Berry and Björkman, 1980; Kim and Portis, 2005). Both the light-dependent and light-independent phases of photosynthesis have heat sensitive components, namely PSII (Heckathorn et al., 2002) and Rubisco activase (Crafts-Brandner and Salvucci, 2002) respectively. While photodamage to PSII is a major manifestation of heat stress (Wise et al., 2004), chl fluorescence and Fv/Fm ratio also correlate with heat stress (Yamada et al., 1996). High heat stress increases rates of dark- and photorespiration, wherein resulting in an overall decrease in photosynthesis. Photosynthesis would also be affected by enzyme inactivation and protein denaturation under extreme temperatures (Nakamoto and Hiyama, 1999). However, the magnitude of photosynthetic compromise is species specific (McDonald and Paulsen, 1997).

Climate change’s negative effects on global food security has exacerbated in recent years (Dhankher and Foyer, 2018). Predicted elevation of temperature, especially frequencies and intensities of extreme temperature events in the tropics, are expected to drastically affect crop yields (Long et al., 2015), especially in broad leaved crops (Reckling et al., 2018). With food crop yields dropping and global population rising, development of stress-resilient crops is a matter of great urgency (Kathuria et al., 2007). Agricultural transformation is largely needed to cater to the increased global food demand of 70–85% by 2050 (Ray et al., 2013; FAO, 2017).

Temperate and subtropical crop plants tend to exhibit inhibited growth under tropical conditions due to compromised physiological performances, especially in photosynthesis, as a result of the constant exposure to a high mean temperature (He and Lee, 2004; He, 2009). Sakamoto and Suzuki (2015) reported that optimal thermal range for root development is a lot narrower than that for shoots. Tropical greenhouses have reported success in growing
temperate vegetables by applying cool RZT while plant shoots were left in ambient conditions (Lee et al., 1994; He et al., 2001). Instead of cooling the whole greenhouse, only a small volume of nutrient solution is needed to be cooled to lower RZT (Lee, 1993). Conversely, temperate and sub-tropical vegetables exposed to local ambient RZTs had diminished productivity and generally showed impaired photosynthetic performance compared to plants grown at constant 20°C RZT (He et al., 2001). These in turn have been attributed eventually to impaired root development due to high RZT (Tan et al., 2002) where root elongation was severely compromised and root diameter significantly thickened (Qin et al., 2002; Tan et al., 2002; Qin et al., 2007). Another added benefit of the aeroponics system is the stability and control of keeping RZTs both in the day and in the night. While heat stress events are often compounded by drought, irradiance and/or salinity conditions in the field, aeroponics can tease out the plant physiological response to heat stress alone, to better elucidate the conferment of heat tolerance.

A total of six commercial cultivars and recombinant in-bred lines (RILs) of *Lactuca sativa* variants were examined in this preliminary study. These include two cultivars: Canasta (*L. sativa*) and Arugula or rocket salad (*Eruca sativa*); the recombinant inbred lines (RIL) of lettuce were obtained from crosses between lettuce cultivars which are thermosensitive (*L. sativa* L. ‘Salinas’)) and thermotolerant *L. serriola* accession UC96US23 (Argyris et al., 2005). *L. sativa* L. ‘Salinas’ (Maternal plant) and *L. serriola* (Paternal plant) were examined alongside thermotolerant RILs 141 and thermosensitive RIL 192 for their response to heat stress in the initial examinations of suitable heat stress indices. The cultivars were chosen both for their commercial importance to the Singapore food market and prior experience in their growth and maintenance in the aeroponic greenhouse. While commercial cultivars provide easy access to seeds and guaranteed productivity and germination success, RILs have the added advantage in that their full genome has been known and described.
Although the benefits of a cooled RZT has been established for the growth of temperate and sub-tropical vegetables in tropical settings of Singapore (He et al., 2001), each plant species has its own specific temperature range for optimal growth (Qin et al., 2007). None of the vegetables explored here has been worked on before in the laboratory and their baseline data with application to aeroponics system growth is largely unknown.

Heat stress on plants could affect their productivity (Goerg, 2003), inactivation of proteins and enzymes (Howarth, 2005) and cellular damage (Schöffl et al., 1999), but workers on the effects of high temperature on plants tend to only report on the existence or absence of heat stress rather than a dose-response relationship (e.g. Haldimann and Feller, 2004). Weibel et al. (1991) and Pörtner (2002) suggested that capacity failures in systemic process, cellular process, structural integrity, and molecular functionality form a hierarchy of stress sensitivity which would correspond to the optimum, pejus (= getting worse) and pessimum (critical) range which defines the stress tolerance of an organism (Shelford, 1931; Frederich and Pörtner, 2000). Pejus tolerance would likely limit species distribution limitations from an ecological point of view.

In this study, temperate vegetables will be used to test the framework of tolerance hierarchy from somatic growth to sub-cellular processes with respect to acute and chronic heat stress. This would also aid in defining the thermal windows of operation for these species (Hofmann and Todgham, 2010).
3.3 Materials and methods

3.3.1 Plant materials

A total of six cultivars and recombinant in-bred lines (RILs) of *Lactuca sativa* variants were used: *L. sativa* (cv. Canasta), *Eruca sativa* (cv. Arugula); two RILs (thermotolerant: 141, thermosensitive: 192) of lettuce population obtained from crosses between lettuce cultivars: “Maternal plant” (*L. sativa* L. ‘Salinas’) which is thermosensitive and “Paternal plant” (*L. serriola* accession UC96US23; (Argyris et al., 2005) which is thermotolerant. Seeds of the two RILs and their parental plants were obtained from the Michelmore lab (Genome Centre, UC Davis, USA) while seeds for the two commercial cultivars Canasta and Arugula were obtained from a commercial seed supplier (Known-You Seed Co, Taiwan).

3.3.2 Growth conditions and experimental treatments within the greenhouse

Seeds were germinated in a 5.5 cm petri dish lined with moist filter paper (Whatman No. 4). After 72 hr of germination, seedlings that sprouted were then inserted into polyurethane cubes (24 x 24 x 24 mm) that were soaked in tap water within trays and left in natural light to acclimate for 4–5 days. Upon root establishment within the sponge block, the seedlings were then transplanted onto Styrofoam boards and placed into the aeroponic system (Day 0). Full-strength Netherlands Standard Composition (Douglas, 1982) nutrient solution (w/v%: K$_2$HPO$_4$ = 0.019; MgSO$_4$ = 0.061; K$_2$SO$_4$ = 0.025; KNO$_3$ = 0.029; Ca(NO$_3$)$_2$$\cdot$4H$_2$O = 0.124; [CH$_2$N(CH$_2$COO)$_2$]$_2$ FeNa = 0.006; trace amount (<0.001 w/v%) of ZnSO$_4$$\cdot$7H$_2$O, CuSO$_4$$\cdot$5H$_2$O, H$_3$BO$_3$, MnSO$_4$$\cdot$H$_2$O, and (NH$_4$)$_6$Mo$_7$O$_{24}$$\cdot$4H$_2$O; conductivity = 2.0±0.2 mS and pH = 6.0 ± 0.5) maintained at target temperature ± 1°C was used (He et al., 2001). Plants were grown in natural light with maximal photosynthetic photon flux density (PPFD) of 600 to 800 photon μmol m$^{-2}$ s$^{-1}$. While root zones temperatures were kept constant (see 3.3.3), environmental temperatures experienced by the shoots ranged between 25–35°C (min-max) while relative humidity ranged between 65–85% (min-max).
Temperatures of the nutrient tank (nutrient spray) and RZTs were recorded using temperature data loggers SL52T (± 0.5°C accuracy, Signatrol, UK) recording at thirty-minute intervals. Nutrient tank temperature was recorded by submerging one data logger into the nutrient tank within a silicon waterproof casing while RZTs were checked with a total of three data loggers deployed along the length of the growing troughs. These RZT data loggers were suspended in mid-air in between the Styrofoam board and the bottom of the trough for a more accurate thermal assessment of RZT where the Styrofoam board would be heated from irradiance while the bottom of the trough would be further cooled by the nutrient spray backflow. Temperatures were downloaded and checked every week for anomalies in the thermal profiles experienced from Day 0 till the final day of harvesting.

Three youngest mature leaves were harvested on day 25 from three different plants per treatment, except for productivity analysis where five whole plants were used per treatment group per cultivar/species. All samples were harvested between 0700hrs ~ 0900hrs.

3.3.3 Chronic and acute heat stress manipulation

For chronic heat stress evaluation, four RZTs of 25, 28, 32 and 36±1°C were maintained after transplanting till parameter assessment. Chronic heat stress plants were examined for their shoot and root productivity, root morphology and development, TRN, $P_{max}$, $A_{sat}$, $g_{s sat}$, transpiration and Chl fluorescence parameters such as ETR, NPQ, qP and $F_v/F_m$ ratio.

For acute heat stress experiments, plants were grown in constant RZT of 25°C from transplantation until harvest. Leaf discs of 1cm diameter were excised from the youngest mature leaf and placed in petri dishes laid with two pieces of filter paper: the bottom layer was lightly infused with a 1 M carbonate/bicarbonate buffer (pH 9) to provide saturating CO$_2$ conditions while the top layer was lightly dampened with deionized water upon which leaf discs were placed. The petri dish was then covered with a transparent plastic film and partially submerged into a water bath pre-heated to the target temperature of 26 (control), 30, 34, 38, 42
and 46°C under PPFD of 500 μmol m⁻²s⁻¹. One hour after heat stress, O₂ evolution, ETR, NPQ, qP and Fv/Fm ratio were examined. For photosynthetic O₂ evolution, an extra set of leaf discs was incubated at 44°C. For Fv/Fm ratio, an extra set of leaf discs was incubated at 48, 49 and 50°C. These parameters were chosen for they would likely respond to a short-term stress.

3.3.4 Chronic and acute heat stress evaluation

3.3.4.1 Productivity of shoot and root

Destructive harvest was carried out and 5 whole plants were used from each RZT treatment. Shoot and roots were separated for FW measurement and the polyurethane cubes removed. The roots of each plant were washed and gently blotted dry before weighing.

3.3.4.2 Root morphology and development

After the roots were detached from their shoots and their FW recorded, they were floated in a transparent tray half-filled with water to maintain the viability of the roots and spatially separate the root structures for the scan. Using the WinRHIZO scanner unit (Québec, Canada), an image of the root was scanned. The root image was later analysed using a WinRHIZO V2012b program (Régent Instruments, Canada). The parameters analysed were total root length (TRL), average root diameter (RSA), number of root tips (RT), and root volume (RV).

3.3.4.3 Measurement of TRN

TRN was assessed via the Kjeldahl method. Briefly, fresh leaf samples were dried in an over at 80°C for 3 days after which 0.05g of dry sample were digested in 5ml H₂SO₄ with a Kjeldahl tablet and 5 ml of concentrated sulphuric acid. The mixture was then heated to 420°C for 1 hour in a digester until complete digestion (Allen, 1989). After the digested content had cooled to room temperature (overnight), TRN was measured using Kjeltec auto 1030 analyser (Model 1030, Kjeltec, Hoganas, Sweden) and calculated from the results based on mg/ g dry weight (DW).
3.3.4.4 Measurement of $P_{\text{max}}$

Leaves harvested for $P_{\text{max}}$ analysis were kept in a covered petri dish on top of a moist filter paper and left under a PPFD of 1000 $\mu$mol m$^{-2}$ s$^{-1}$ prior to measurements. $P_{\text{max}}$ was determined with a leaf disc O$_2$ electrode (Hansatech, King’s Lynn, UK) under a PPFD of 1000 $\mu$mol m$^{-2}$s$^{-1}$ at 25°C at saturating CO$_2$ conditions (1% CO$_2$ from a 1 M carbonate/bicarbonate buffer, pH 9) as described by (He et al., 2001).

3.3.4.5 Measurements of transpiration, $A_{\text{sat}}$ and $g_s_{\text{sat}}$

Readings of transpiration, $A_{\text{sat}}$ and $g_s_{\text{sat}}$ were taken between 0900h to 1200h in the greenhouse with an open infrared gas analysis system with a 6cm$^2$ chamber (LI-6400, Biosciences, U.S.). LED light source which supplied 1000 mol m$^{-2}$ s$^{-1}$ of PPFD were used. The light source emitted in the wavelength ranged between 420 to 510 nm and 610 nm to 730 nm. The spectral output of the light source has one peak centred at about 465 nm and second peak centred at about 670 nm. Average ambient CO$_2$ and relative humidity in the chamber were 400±3.5 $\mu$mol mol$^{-1}$ and 50% respectively. Measurements were recorded when both $A_{\text{sat}}$ and $g_s_{\text{sat}}$ were stable.

3.3.4.6. Measurement of photochemical light use efficiency

The youngest mature leaf was harvested at 0800 h for Chl fluorescence analysis, where NPQ, qP and ETR of young mature lettuce leaves were measured at 25°C in the laboratory, using the Imaging-PAM ChlFluorometer (Walz, Effeltrich, Germany). Leaf samples were predarkened under a piece of black cloth for at least 15 min prior to measurements. Images of fluorescence emission were digitized within the Imaging-PAM Chl Fluorometer camera and transferred to a computer for storage and analysis via a Firewire interface (400 megabits s$^{-1}$) (Firewire-1394, Austin, TX, USA). Light pulses were applied at about low 1 Hz frequencies for the initial, $F_o$, Chl fluorescence image measurements in partially dark state. Light pulse frequencies were then automatically increased to about 10 Hz during actinic illumination and
saturation pulses. Meanwhile, actual fluorescence intensity, F, is continuously measured (van Kooten and Snel, 1990). The dark-level fluorescence yield, $F_o$, is determined in the absence of actinic exposures while the maximum fluorescence yield, $F_m$, is determined after a 0.8 s maximal saturation pulse at PPFD 2400 μmol m$^{-2}$ s$^{-1}$. From these, the Chl fluorescence $F_v/F_m$ ratio was derived. Thereafter, a series of 10 s actinic light exposures with increasing irradiance from PPFD 1 to 1600 μmol m$^{-2}$ s$^{-1}$ was applied to obtain the rapid light curve measurements (Schreiber et al., 1997). A further 0.8 s duration of maximal saturation pulse of PPFD 2400 μmol m$^{-2}$ s$^{-1}$ was applied after each actinic light exposure to obtain maximal fluorescence yield, $F_m$. Thus, the actual fluorescence intensity, $F = F_s$ ($F_s =$ fluorescence in steady state), and the maximum fluorescence ($F_m$) at the steady state were determined and the effective photosystem II quantum yield, $\Delta F/F_m \cdot [(F_m - F)/F_m]$ and ETR (PPFD × $\Delta F/F_m \cdot 0.5 \times 0.84$) could be derived – the 0.5 constant presupposes that there is equal distribution in the excitations between photosystems II and I, while the 0.84 correction factor accounts for the fraction of incident light that is really absorbed by photosynthesis (Rascher et al., 2000). NPQ was defined as: NPQ = $(F_m - F_m)/F_m$, while qP was defined as $1 - (F_m - F_o)/(F_m - F_o)$. ETR, qP and NPQ were all measured across a light spectrum of PPFD 1 to 1600 μmol m$^{-2}$ s$^{-1}$, and also analysed at 835 PPFD for this represented the maximum possible irradiance in the greenhouse. At least 4 measurements from 4 different plants per genotype were used.

3.3.4.7 Measurement of $F_v/F_m$ ratio

$F_v/F_m$ ratio is the ratio of variable fluorescence $F_v$ to maximal fluorescence $F_m$, where variable fluorescence is calculated as maximal fluorescence $F_m$ – minimal fluorescence $F_0$. $F_m$ is the fluorescence level when all antenna pigment complexes associated with the photosystem are assumed to be closed (high intensity flash applied) while $F_0$ is fluorescence level when all PSII traps are assumed to be open (dark adapted). $F_v/F_m$ ratio was read off using a Plant Efficiency Analyser, PEA (Hansatech Instruments Ltd, England) after 15 minutes of dark
adaptation. Three readings were taken for each treatment group, each using the youngest mature leaf of an individual plant.

3.3.4.8 Recovery rate post heat stress and photosynthetic pigment analysis

Leaf discs used in determination of F_v/F_m ratio in response to acute heat stress were further manipulated to evaluate recovery rate post exposure to heat stress greater than 38°C. After 1 h exposure to 38, 42, 44, 46, 48 and 50°C under PPFD of 500 μmol m^{-2}s^{-1} the leaf discs were returned to room temperature (25°C) under high light (500 μmol m^{-2}s^{-1}), low light (150 μmol m^{-2}s^{-1}), and total darkness for 3 hours. F_v/F_m ratio was determined every hour. At the end of 3 hours these leaf discs were analysed for their chlorophyll fluorescence parameters F_v/F_m ratio, ETR, NPQ and qP and photosynthetic pigments content.

Leaf discs were soaked in 1.5 ml N,N- dimethylformamide for 48 h in the dark, at 4°C. The absorption of 3 replicates was read at 480 nm, 647 nm and 664 nm, using a spectrophotometer (UV-2550, Shimadzu, Japan). Concentrations for chlorophyll (Chl) a, Chl b, and carotenoids (Car) were calculated (Wellburn, 1994). The formula for Chl and Car calculations are:-

\[
\text{Chl a, } C_a = (11.65 \times A_{664} - 2.69 \times A_{647}) \times \left(\frac{1.5}{\pi r^2}\right) \mu g/cm^2
\]

\[
\text{Chl b, } C_b = (20.81 \times A_{647} - 4.53 \times A_{664}) \times \left(\frac{1.5}{\pi r^2}\right) \mu g/cm^2
\]

Total Chl = C_a + C_b

\[
\text{Car} = \left(\frac{1000 \times A_{480} - 0.89 \times C_a - 52.02 \times C_b}{245}\right) \times \left(\frac{1.5}{\pi r^2}\right) \mu g/cm^2
\]

3.3.4.9 Statistical analysis and LT_{50}

For each physiological index obtained, each species was analysed separately with one-way analysis of variance and posthoc SNK tests. Parameters in ratio (e.g. F_v/F_m ratio was arcsine transformed before statistical analysis). LT_{50}, or temperature at which index performance changed by 50% compared to that of control plants at 26°C was calculated by
probit analysis. Significant difference in LT₅₀ for each parameter of the six vegetables was scored if there was no overlap in the 95% confidence intervals.

3.3.4.10 Critical (Pessimum) temperature determination and quantum yield (φO₂)

To determine if LT₅₀ of Fᵥ/Fₘ ratio approaches the critical temperature (pessimum) of the plant, the cultivars Arugula and Canasta and the two RILs 141 and 192 were further examined. After exposing excised leaf discs (1 cm diameter) at their respective Fᵥ/Fₘ ratio LT₅₀ temperatures for 1 h under PPFD of 500 µmol m⁻²s⁻¹ the leaf discs were returned to room temperature (25°C). Photosynthetic light response curve and quantum yield (φO₂) were then analyzed together with leaf discs treated at 25°C under PPFD of 500 µmol m⁻²s⁻¹.

The photosynthetic light response curve was obtained by examining the photosynthetic O₂ evolution against varying illumination of total darkness (0 µmol m⁻² s⁻¹) to full light saturation (1850 µmol m⁻²s⁻¹) at 25°C in saturating CO₂ conditions. Quantum yield (φO₂: the rate of oxygen evolution per unit PPFD) was obtained from the light response curve by linear regression of O₂ evolution at low light (PPFD < 150 µmol m⁻²s⁻¹).
3.4 Results

3.4.1 Chronic heat stress responses

There were significant differences in shoot FW (Figure 3.1A) and root FW (Figure 3.1B) across all six species with increasing heat stress at the RZ. A drastic drop in shoot FW was observed as early as 28°C for Arugula (F_{(3,19)} = 45.0, p<0.001), Canasta (F_{(3,19)} = 24.4, p<0.001), RIL 141 (F_{(3,19)} = 24.6, p<0.001), RIL 192 (F_{(3,19)} = 59.9, p<0.001), *Lactuca serriola* accession UC96US23 henceforth known as “Paternal plant” (F_{(3,19)} = 72.3, p<0.001) and *L. sativa* L. ‘Salinas’ henceforth known as “Maternal plant” (F_{(3,19)} = 80.9, p<0.001). A similar trend was observed in root FW of Canasta (F_{(3,19)} = 12.2, p<0.001), RIL 141 (F_{(3,19)} = 16.1, p<0.001), RIL 192 (F_{(3,19)} = 30.1, p<0.001), “Paternal plant” (F_{(3,19)} = 20.5, p<0.001) and “Maternal plant” (F_{(3,19)} = 36.2, p<0.001) where significant difference was detected at 28°C, but for Arugula (F_{(3,19)} = 19.4, p<0.001) this was only observed at 32°C.

Root morphology and development, especially the lettuce species, were drastically affected by higher RZTs (see Plate 3-1). Root length for all six vegetables species decreased significantly as RZT increased (Figure 3-2 A: Arugula, F_{(3,11)} = 144.7, p<0.001; Canasta, F_{(3,11)} = 2042.5, p<0.001, RIL 141, F_{(3,11)} = 596.4, p<0.001, RIL 192, F_{(3,11)} = 1186.8, p<0.001; Paternal plant, F_{(3,11)} = 1613.0, p<0.001; Maternal plant, F_{(3,11)} = 2857.9, p<0.001).

Conversely, average root diameter all increased with RZT (Figure 3-2 B: Arugula, F_{(3,11)} = 7.667, p = 0.010; Canasta, F_{(3,11)} = 8.202, p = 0.001, RIL 141, F_{(3,11)} = 596.4, p<0.001, RIL 192, F_{(3,11)} = 1186.8, p<0.001; Paternal plant, F_{(3,11)} = 1613.0, p<0.001; Maternal plant, F_{(3,11)} = 2857.9, p<0.001).

While the number of root tips for Arugula (F_{(3,11)} = 1.2, p = 0.362) was similar for all RZTs, Canasta (F_{(3,11)} = 92.9, p<0.001), RIL 141 (F_{(3,11)} = 13.7, p = 0.002), RIL 192 (F_{(3,11)} = 102.0, p<0.001), Paternal plant (F_{(3,11)} = 128.5, p<0.001) and Maternal plant (F_{(3,11)} = 30.5, p<0.001) all had significantly decreased root tips with increasing RZTs (Figure 3-2 C).
Figure 3-1. Shoot FW (A), root (B) FW, and shoot FW percentage reduction in comparison to that in 25°C-RZT (C) of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Plate 3-1. Root Scan images of (Top to bottom, left to right) Arugula, Canasta, RIL 141, RIL 192, Paternal plant and Maternal plant grown in RZT 25°C and 35°C. Images are arranged to size of the polyurethane cubes.
Figure 3-2. Root morphology analysis of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C: (A) Total root length; (B) Average root diameter; (C) Number of root tips and (D) Root Volume. Each bar is mean ± SE (n=5). No significant difference in root volume was detected in Canasta, RIL 141, RIL 192, Paternal plant and Maternal plant. Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
There was significant reduction of root volume for Arugula with increasing RZT \( (F_{(3,11)} = 7.0, p = 0.013) \) but not for the other 5 vegetables species (Figure 3-2 D).

There was also a trend of decreasing TRN with increasing RZT (Figure 3-3) for most of the examined vegetables. RIL 141 was the exception where no significant difference was observed for all heat treatments \( (F_{(3,11)} = 2.61, p=0.153) \). Canasta \( (F_{(3,11)} = 3.31, p=0.027) \), RIL 192 \( (F_{(3,11)} = 5.901, p=0.038) \) and “Maternal plant” \( (F_{(3,11)} = 232, p=0.017) \) had the most distinct dose response TRN decrease with heat. Conversely, although significant drop in TRN was observed in 32°C Arugula \( (F_{(3,11)} = 2.726, p=0.044) \) and “Paternal plant” \( (F_{(3,11)} = 7.88, p=0.047) \) relatively high TRN was still maintained at 36°C compared to 25°C plants.

Arugula \( (F_{(3,11)} = 9.74, p=0.013) \), “Paternal plant” \( (F_{(3,11)} = 18, p=0.003) \) and “Maternal plant” \( (F_{(3,11)} = 1.59, p=0.028) \) all showed significant decrease in \( P_{\text{max}} \) at 28°C RZT while Canasta \( (F_{(3,11)} = 3.57, p=0.035) \), RIL 141 \( (F_{(3,11)} = 5.3, p=0.047) \) and RIL 192 \( (F_{(3,11)} = 3.455, p=0.01) \) only showed significant decrease of \( P_{\text{max}} \) at 32°C RZT (Figure 3-4).

There was a decrease in \( A_{\text{sat}} \) with response to increasing RZTs (Figure 3-5A). For Canasta \( (F_{(3,11)} = 771.04, p<0.001) \), RIL 141 \( (F_{(3,11)} = 46.3, p<0.001) \), RIL 192 \( (F_{(3,11)} = 229.9, p<0.001) \), “Paternal plant” \( (F_{(3,11)} = 22.8, p<0.001) \) and “Maternal plant” \( (F_{(3,11)} = 10.9, p<0.001) \) a significant decrease was observed in 28°C RZT plants, but for Arugula \( (F_{(3,11)} = 2.61, p<0.001) \), significant decrease in \( A_{\text{sat}} \) was only observed at 32°C.
Figure 3-3. TRN of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 3-4. $P_{\text{max}}$ of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 3-5. Photosynthetic parameters of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C: (A) $A_{sat}$; (B) $g_{sat}$ and (C) Transpiration rate. Each bar is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
Similarly, $g_s$ sat generally decreased with increasing RZTs (Figure 3-5B), but the decrease was only statistically significant for RIL 141 ($F_{(3,11)}= 3.55$, $p=0.047$), RIL 192 ($F_{(3,11)}= 9.01$, $p=0.006$) and “Paternal plant” ($F_{(3,11)}= 2.76$, $p=0.012$). Transpiration rate also generally decreased with increasing RZTs (Figure 3-5 C). The decrease was, however, only statistically significant for Arugula ($F_{(3,11)}= 6.09$, $p=0.018$), Canasta ($F_{(3,11)}= 4.13$, $p=0.048$), RIL 141 ($F_{(3,11)}= 6.00$ $p=0.019$), RIL 192 ($F_{(3,11)}= 5.82$, $p=0.021$), but not for the parental plants “Paternal plant” ($F_{(3,11)}= 1.18$, $p=0.37$) and “Maternal plant” ($F_{(3,11)}= 3.8$, $p=0.057$).

All six vegetables showed a general decrease in ETR (Figure 3-6) with increasing heat stress except for “Paternal plant”. A similar trend was observed in qP (Figure 3-7) and NPQ (Figure 3-8) where a general decrease in photochemical and non-photochemical quenching capabilities decreased with increasing RZT except for “Paternal plant” which generally retained the same physiological functionality for all RZTs. Data for ETR, NPQ and qP at PPFD of 835 $\mu$mol m$^{-2}$ s$^{-1}$ was extracted and analysed. For chronic heat stressed plants, no significant difference was detected in ETR of “Paternal plant” for all treatment groups ($F_{(3,59)}= 2.084$, $p=0.113$) and qP of “Maternal plant” for all treatment groups ($F_{(3,59)}= 2.026$, $p=0.121$). All other parameters for all treatment groups showed significant decrease in ETR (Figure 3-9 A), NPQ (Figure 3-9 B) and qP (Figure 3-9 C). No significant difference was detected for all chronic heat stress treatment groups across the six species for $F_v/F_m$ ratio (Figure 3-10).
Figure 3-6. Light response curve of ETR per plant in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5).
Figure 3-7. Light response curve of ETR in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5).
Figure 3-8. Light response curve of NPQ in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5).
Figure 3-9. (A) ETR; (B) qP; (C) NPQ at 835 PPFD of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
Figure 3-10. $F_v/F_m$ ratio of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=3). No significant difference was detected for all six species grown in different RZTs.
3.4.2 Acute heat stress responses

For acute heat stressed plants, Canasta and the parental plants “Paternal plant” and “Maternal plant” all showed a significant decrease in $P_{max}$ compared to control plants (26°C) at 34°C (Figure 3-11: Canasta: $F_{(6,20)}= 87.8$, p<0.001; “Paternal plant”: $F_{(6,20)}= 24.9$, p<0.001; “Maternal plant”: $F_{(6,20)}= 112.86$, p<0.001). $P_{max}$ dropped significantly for RIL 192 at 38°C ($F_{(6,20)}= 25.7$, p<0.001) while Arugula had a significant decrease of O$_2$ evolution compared to control plants (26°C) at 42°C ($F_{(6,20)}= 17.4$, p<0.001). Photosynthetic O$_2$ production dropped significantly at the high temperature of 44°C for RIL 141 compared to the other five vegetables ($F_{(6,20)}= 60.1$, p<0.001) while showing no significant differences in O$_2$ production from 26°C to 42°C.

All six vegetables showed a general decrease in ETR (Figure 3-12) with increasing heat stress. A similar trend was observed in qP (Figure 3-13) and NPQ (Figure 3-14), although the decrease in qP was subtler for the 26-42°C plants. Interestingly, for Arugula (Figure 3-13 A) and Canasta (Figure 3-13 B) at 46°C, qP was markedly decreased, with qP being not detected at all in Arugula in spite of increasing light intensity. Conversely, qP of Canasta exposed to 46°C heat stress approached zero only at light intensity above 1200 µmol m-2s-1. For RIL 141 and RIL 192, treatment groups at 46°C had the lowest ETR and qP with increasing light intensity, but performance of both parameters was not significantly different from the 42°C treatment groups for the two respective vegetables. NPQ for both vegetables, conversely, was significantly lower at 46°C compared to the other treatment groups. There was almost no significant difference in ETR, qP and NPQ of “Paternal plant” among all the treatment groups (Figure 3-12 E, 3-13 E and 3-14 E). ETR, qP and NPQ generally all decreased with increased heat stress for “Maternal plant”, and a flat lining of NPQ (Figure 3-14F) for vegetables exposed to 46°C heat stress was prominent.
Figure 3-11. $P_{\text{max}}$ of the six vegetables exposed to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 3-12. Light response curve of ETR in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” exposed to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=5).
Figure 3-13. Light response curve of qP in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” exposed to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=5).
Figure 3-14. Light response curve of NPQ in (A) Arugula; (B) Canasta; (C) RIL 141; (D) RIL 192; (E) “Paternal plant”; (F) “Maternal plant” exposed to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=5).
Data for ETR, NPQ and qP at PPFD of 835 μmol m⁻² s⁻¹ was extracted and analysed. For acute heat stressed plants, except for “Paternal plant”, ETR, qP and NPQ decreased with increasing heat stress for the other five vegetables (Figure 3-15 A, 3-15 B and 3-15 C respectively). There were no significant differences between “Paternal plant” treatment groups for ETR (F(5,89)= 1.8, p = 0.129) and qP (F(5,89)= 2.5, p=0.36), but a significant drop in NPQ was observed for the 46°C treatment group compared to control plants (F(5,89)= 3.2, p=0.01).

For acute stressed plants, F_v/F_m ratio for all six vegetables at 46°C were all significantly lower compared to values obtained for control plants (Figure 3-16, Arugula: F(5,26)= 453.9, p<0.001; Canasta: F(5,26)= 424.9, p<0.001; RIL 141: F(5,26)= 1037.6, p<0.001; RIL 192: F(5,26)= 475.6, p<0.001; “Paternal plant”: F(5,26)= 1365.1, p<0.001; “Maternal plant”: F(5,26)= 617.6, p<0.001). Although there was also a significant drop in F_v/F_m ratio for RIL 141 at 42°C compared to the control plants, the value obtained were still above the optimal value of 0.8.

3.4.3 Recovery rate post heat stress and photosynthetic pigment analysis

Plants heat shocked at 38°C had similar F_v/F_m ratio (≈ 0.80) over time for all three light treatment (Figures. 3-17 A, 3-17 E, 3-17 I, 3-17 M). For leaf discs heat shocked at 42°C, F_v/F_m ratio significantly decreased under high light for all the species except Arugula (Figures. 3-17 B, 3-17 F, 3-17 J, 3-17 N) after 3 hours at 25°C. For leaf discs heat shocked at 44°C, all four species showed significant decrease in F_v/F_m ratio under high light treatment compared to those treated under low light and darkness at 25°C from 2 hours onwards (Figures. 3-17 C, 3-17 G, 3-17 K, 3-17 O). Leaf discs of all species except for Arugula heat shocked at 46°C, showed significant performance drop under high light treatments (Figures. 3-17 D, 3-17 H, 3-17 L, 3-17 P). No recovery of F_v/F_m ratio was detected in all genotypes in all treatment groups.
Figure 3-15. (A) ETR; (B) qP; (C) NPQ of the six vegetables at 835 PPFD after exposure to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 3-16. $F_{v}/F_{m}$ ratio of the six vegetables after exposure to acute heat stress of 26, 30, 34, 38, 44, 46°C for 1 h. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
Figure 3.17. $F_v/F_m$ ratio of the four vegetable species exposed to 1h heat shock at 38°C (A, E, I, M), 42°C (B, F, J, N), 44°C (C, G, K, O), and 46°C (D, H, L, P). The leaf discs were then returned to 25°C for 3h under high light (500 μmol m$^{-2}$ s$^{-1}$), low light (150 μmol m$^{-2}$ s$^{-1}$), and darkness. Each point is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
ETR, qP and NPQ of the leaf discs were analysed after 3 hrs. Generally, ETR (Figure 3-18 A, 3-18 D, 3-18 G and 3-18 J), qP (Figure 3-18 B, 3-18 E, 3-18 H, 3-18 K) and NPQ (Figures 3-18 C, 3-18 F, 3-18 I, 3-18 L) all decreased with increasing heat stress and increasing light exposure.

The same leaf discs that were used for the measurements of Fv/Fm ratios and other Chl parameters were used to analyse the photosynthetic pigments. They all had similar values of total Chl (Figures. 3-19 A, 3-19 E, 3-19 I, 3-19 M), Chl a/b ratio (Figures. 3-19 B, 3-19 F, 3-19 J, 3-19 N), total Car (Figures. 3-19 C, 3-19 F, 3-19 K, 3-19 O), Chl/Car ratio (Figures. 3-19 D, 3-19 H, 3-19 L, 3-19 P) after heat shock at different temperatures for 1 h and followed by 3 h recovery at room temperature (25°C) under high light (500 μmol m⁻² s⁻¹), low light (150 μmol m⁻² s⁻¹), and darkness. No close correlations were obtained between total Chl content and Fv/Fm ratio of the four different vegetable species after different temperature treatment under different irradiances. (Figure. 3-20).

3.4.4 LT₅₀ of chronic and acute heat stress parameters

For chronic heat stressed plants, shoot FW was significantly lower compared to all the other parameters (Figure 3-21 A). LT₅₀ of TRN for RIL 141 could not be resolved as there was no significant difference between the four treatment groups. Transpiration rates and TRN (except RIL 141) were significantly higher compared to the other parameters. In comparing Pₘₐₓ and chlorophyll fluorescence, chronic stressed plants tend to have lower LT₅₀ values compared to acute stressed plants for the same temperature stress (Figure 3-21 B). For acute heat stressed plants, while Arugula and RIL 192 had similar LT₅₀ values for all four parameters, Canasta, RIL 141 and the two parental plant “Paternal plant” and “Maternal plant” all had significantly higher photosynthetic LT₅₀ values compared to their respective O₂ evolution LT₅₀ values (Figure 3-21 C).
Figure 3-18. ETR (A, D, G, J), qP (B, E, H, K) and NPQ (C, F, I, L) of the four vegetables at 835 PPFD. The leaf discs underwent acute heat shock of 38, 42, 44, 36, 48 and 50°C and were returned to room temperature under high light (500 μmol m⁻² s⁻¹), low light (150 μmol m⁻² s⁻¹), and darkness conditions for 3 h. Each point is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
Figure 3-19. Chl pigment analysis of total Chl (A, E, I, M), Chl a/b ratio (B, F, J, N), total Car (C, F, K, O) and Chl/Car ratio (D, H, L, P) for the four vegetables. The leaf discs underwent acute heat shock of 38, 42, 44, 36, 48 and 50°C and were returned to room temperature under high light (500 µmol m⁻² s⁻¹), low light (150 µmol m⁻² s⁻¹), and darkness conditions for 3 h. Each point is mean ± SE (n=3). Different letter groups denote significant differences between treatment groups. Each species was analyzed separately.
Figure 3-20. Correlations between $F_v/F_m$ ratio and total Chl of the four different vegetables. The leaf discs underwent acute heat shock of 38, 42, 44, 36, 48 and 50°C and were returned to room temperature under high light (500 $\mu$mol m$^{-2}$ s$^{-1}$), low light (150 $\mu$mol m$^{-2}$ s$^{-1}$), and darkness conditions for 3 h.
Figure 3-21. LT$_{50}$ of physiological parameters of the six vegetables with response to (A) Chronic heat stress; (B) Chronic heat stress; (C) acute heat stress. Each bar is mean ± 95% confidence interval. LT$_{50}$ of each parameter is significantly different if the 95% confidence intervals do not overlap.
3.4.5 Quantum yield (φO2)

Leaf discs from Arugula, Canasta, RIL 141 and RIL 192 were heated at their respective Fv/Fm ratio LT50 for 1 h (See Table 3-1) under PPFD of 500 μmol m⁻² s⁻¹. Generally, the untreated leaves (Figure 3-22A) had higher O₂ evolution than the heat shocked leaves (Figure 3-22B). Quantum yield of control plants was also significantly higher than the heat treated plants (Figure 3-22C).

Table 3-1 Mean ± 95% confidence interval of LT50 of Fv/Fm ratio for the six vegetables. Values were obtained by probit analysis.

<table>
<thead>
<tr>
<th>Vegetable Species</th>
<th>LT50 of Fv/Fm ratio (°C)</th>
<th>Upper 95% confidence limit (°C)</th>
<th>Lower 95% confidence limit (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arugula</td>
<td>47.03</td>
<td>52.41</td>
<td>41.63</td>
</tr>
<tr>
<td>Canasta</td>
<td>47.49</td>
<td>52.47</td>
<td>42.29</td>
</tr>
<tr>
<td>RIL 141</td>
<td>48.23</td>
<td>53.63</td>
<td>42.83</td>
</tr>
<tr>
<td>RIL 192</td>
<td>47.34</td>
<td>52.89</td>
<td>41.84</td>
</tr>
<tr>
<td>Paternal plant</td>
<td>48.17</td>
<td>52.77</td>
<td>42.37</td>
</tr>
<tr>
<td>Maternal plant</td>
<td>48.07</td>
<td>52.75</td>
<td>42.47</td>
</tr>
</tbody>
</table>
Figure 3-22. Photosynthetic parameters plots of four vegetable species. Photosynthetic O₂ evolution measured at low PPFD (<150 μmol m⁻² s⁻¹) of (A) control plants; (B) heat treated plants and (C) quantum yield (φO₂) of control and heat treated plants against Fv/Fm ratio. Heat treated plants were subjected to 1 h of acute heat stress at their respective Pₚₐₓ LT₅₀ temperature. Control plants were maintained at 25°C for 1 h before starting the analysis.
3.5 Discussion

This study reported the effect of high temperature on the physiological performances of different vegetables. With the exception of \(\text{T}_\text{v}/\text{T}_\text{m}\) ratios (Figure 3-10), increasing heat stress decreased performance of all observed parameters.

3.5.1 Chronic heat stress responses

There was an inverse relationship between shoot and root FW and RZT temperature (Figure 3-1). This trend was also observed by He et al. (2009 and He et al. (2013) in previous studies. Decrease in \(A_{\text{sat}}\) and in \(g_{\text{s, sat}}\) (Figure 3-5) were likely related to the decrease in shoot and root productivity. He (2009) and He et al. (2011) previously reported that stomatal limitation of photosynthesis indicated by decrease in \(g_{\text{s, sat}}\) resulted in \(\text{CO}_2\) decrease at the chloroplast level (Devireddy et al., 2018) and photosynthesis is thus inhibited, ultimately resulting in reduced plant growth. Higher RZT also induced a lower level of TRN in plants (except RIL 141, Figure 3-3). He et al. (2009) attributed this low TRN in high RZT due to the poor development of roots (e.g. less root tips, larger root diameter) which in turn affected the efficiency of mineral and nutrient uptake. Looking at root morphology (Figure 3-2C), even 25°C RZT RIL 141 had lower number of root tips compared to the other five vegetables and difference in root tips in RIL 141 of differing RZTs were less pronounced compared to the other five vegetables. This could be the reason why TRN for RIL 141 was not significantly different (He et al., 2009).

While not as drastic in Arugula, root morphology and development were very distinctly affected by increased RZTs for the lettuce (Figure 3-2). All the lettuce grown in 35°C RZT had very attenuated roots compared to those grown at 25°C-RZT (Plate 3-1). Increased root length and number of root tips are important in acquires resources like water and nutrients (Vries et al., 2016; Pirnajmedin et al., 2017) and the attenuation of root systems could result in the reduced shoot productivity for plants grown in higher RZTs. Similarly, increased root diameter
(root thickening) has been reported as a morphology of poor root development (Tan et al., 2002; Qin et al., 2007) and would likely contribute to the low productivity in plants grown in high RZTs.

3.5.2 Chronic stress responses versus acute stress responses

As acute heat stress was designed to last 1 h and the longest post-heat shock observation was 24 h, shoot and root FW and TRN were not analysed for acute heat stress experiments. As excised leaf discs were used in acute heat experiments, $A_{sat}$ and $g_{s sat}$ were not assessed as well for the leaf discs were too small for analysis. Only $P_{max}$, $F_v/F_m$ ratio, ETR, qP and NPQ were recorded for acute heat stress experiments and the results compared to those in chronic heat stress experiment.

Except for $F_v/F_m$ ratio (Figures 3-10 and 3-16), $P_{max}$ (Figures 3-4 and 3-11), ETR, qP and NPQ (Figures 3-9 and 3-15) in chronic stress plants all showed a corresponding decrease in performance efficiency at a lower temperature compared to acute heat stress. For $F_v/F_m$ ratio, acute heat stress plants showed a significant drop in $F_v/F_m$ ratio at 46°C. Logistically it was very difficult to maintain constant RZT above 36°C in the greenhouse, but it might be expected that plants grown in constant RZTs would start showing a decrease of $F_v/F_m$ ratio above 36°C but lower than 46°C. This agrees with the concept put forth by Tattersall et al. (2007) and Cramer et al. (2011) that chronic stresses are time integrated responses, and tolerance build up would be far more complex than acute stress responses in that energy budget must be strategized for physiological and cellular maintenance processed on daily basis whereas priority in energy expenditure for acute heat stress are often on organismal survival (Pinheiro and Chaves, 2011). Results from this study coincides with previous findings that high RZT inhibits photosynthesis and carbohydrate production (Xu and Huang, 2000a; 2000b; He et al., 2009). ETR was significantly lower for plants with RZT higher than 25°C in chronic stressed
plants (except Paternal plant, Figure 3-6 and Figure 3-9) but this occurred at 38°C for acute stressed plants Arugula and RIL 192 (Figure 3-12).

Apart from the fundamental energy budgeting strategy differences, this difference in chronic stressed plants and acute stressed plants could stem from the compounding effects of root morphology alterations in chronic heat stress plants (He and Lee, 1998) which likely decreased root hydraulic conductivity (Dodd et al., 2000), which in turn lowered transpiration of leaves in the middle of the day, and induced stomatal closure and decreased $A_{\text{sat}}$ and $g_{s\text{ sat}}$ (He et al., 2001). However, a decrease in $P_{\text{max}}$ with heat stress also suggested the presence of non-stomatal limitation of photosynthesis: poor root development leads to nutrient deficiencies, represented here by the significant decrease in TRN with higher RZT (Figure 3-3). He et al. (2001) demonstrated a close relationship between leaf N content and $P_{\text{max}}$ which would explain the greater sensitivity to heat stress in chronic plants compared to acute stress plants. For acute stressed plants, given that photochemical functionality of PSII was relatively stable even at 46°C ($F_v/F_m$ ratio, Figure 3-16) and that chlorophyll pigments do not break down in acute heat stress experiments (Figure 3-19) nor with $F_v/F_m$ ratio drop (Figure 3-20), $P_{\text{max}}$ decrease would be due to some other mechanism failure not elucidated yet in this study.

Tikkanen and Aro (2014) reported the protective effect NPQ has on PSII, but the decreasing NPQ with increasing RZT (Figure 3-8, chronic stressed plants) and increasing heat stress temperature (Figure 3-14, acute heat stressed plants) suggests a capacity or systemic failure when temperatures exceed the optimal range for functioning. The similarity between ETR (Figure 3-6E) and qP (Figure 3-7E) in chronic heat stress and in acute ETR (Figures 3-12E), NPQ (3-13E) and qP (3-14E) is reflective of Paternal plant’s nature in being drought (stress) tolerant (Iba, 2002) although compromises on other plant functions could occur (Heckathorn et al., 1998; Heckathorn, 2002). This is observed when TRN (Figure 3-3, chronic heat stress), ETR, NPQ and qP (Figure 3-15, acute heat stress) all suggested that these
physiological performances are not temperature sensitive, ultimately O\textsubscript{2} evolution and shoot FW were all significantly affected with higher RZTs (chronic) or incubation temperatures (acute).

\(F_v/F_m\) ratio for chronic heat stressed plants (Figure 3-10) and for acute heat stressed plants below 42°C (Figure 3-16) all remained above 0.8, suggesting absence or very mild effects of dynamic PSII photoinhibition (He et al., 2011). This could be due to the relatively lower irradiation in the green house for this experimental period (maximum PPFD circa 500\(\mu\)mol m\(^{-2}\) s\(^{-1}\)) for chronic stressed plants or that the application of the acute heat stress was not intense or long enough to induce PSII photoinhibition for temperatures lower than 42°C. This finding differs from that reported by He et al. (2001) where even on cloudy days when there was no dynamic high PPFD-induced photoinhibition, plants grown in A-RZT still showed lower \(F_v/F_m\) ratio and decreased chlorophyll levels. This could be due to the difference in greenhouse temperatures experienced by plants: He et al. (2000) recorded 38°C on clear hot days, while our greenhouse records a relatively benign 33°C on clear hot days.

Traditionally \(F_v/F_m\) ratio has been the standard indicator for plant stress (Yamada et al., 1996). In this study, \(F_v/F_m\) ratio did not show a significant drop in acute heat stressed plants till 42°C and capacity failure (LT\textsubscript{50}) of \(F_v/F_m\) ratio was relatively high (~47°C). Conversely, while \(F_v/F_m\) ratio may not be sensitive to initial stress events, it could be a good indicator for critical damage to plants. When leaf discs were exposed to heat stress and returned to 25°C, high light stress (a PPFD of 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) did not significantly affect leaf discs heated at 38°C but maintained a \(F_v/F_m\) ratio around 0.8 (Figure 3-17). Conversely, leaf discs with compromised PS II (\(F_v/F_m\) ratio < 0.80) were likely further damaged by the light stress despite being kept in a benign 25°C. Additionally, no recovery of \(F_v/F_m\) ratio back to above 0.80 was observed in all treatment groups even if left in the dark for 3 hours. Thermal stress at the point of \(F_v/F_m\) ratio capacity failure could thus approach the physiological death (critical temperature) of plants.
such that if the prevailing abiotic stress persists, tolerance still exists but becomes progressively
time limited (Pörtner, 2001). This also explains why capacity failures of acute heat stressed
plants were all so much higher than the chronic heat stress observations (Pörtner, 2002).

The fact that $F_v/F_m$ ratio capacity failure approaches physiological death of plants was
further proven by their Chl fluorescence performances post heat shock (Figure 3-18). Nowadays, Chl fluorescence has been used as an indicator of the level of stress. The advantage of this technique over many other techniques is that it provides rapid and non-destructive
measures (Eisvand et al., 2018). In this study, while NPQ remained detectable even for leaf
discs heated at 50°C, ETR and qP generally were not detected beyond 48°C for all four
vegetables.

Effective quantum yield of PSII photochemistry ($\Phi_{PSII}$) and NPQ are both affected by
high temperature (Hassan, 2006). In chronic heat stressed plants, heat stress reduces the amount
of Chl a, Chl b and total Chl levels (Hanif and Wahid, 2018). However, the capacity failure of
$F_v/F_m$ ratio observed here did not correspond strongly with chlorophyll pigment loss (Figure 3-
19 and Figure 3-20), showing that for acute heat stress events, Chl a and Chl b pigments were
not main factors for photosynthetic performance failure.

3.5.3 Hierarchy of stress indices

Thermal tolerance is defined by capacity failure at the highest level of biological
functions or organization complexity (Pörtner, 2002). Thus, more complex integrations like
sexual production or somatic growth would be compromised by increased stress (Shelford,
1931), before physiological functions like aerobic respiration, cellular functions and molecular
process or metabolic complexes will be affected (Pörtner, 2001). In face of extreme
temperatures, organismal survival becomes time dependent as long as organismal energy
reserve could support molecular functions by chaperone molecules and anti-oxidative
molecules as a last line of defence (Pörtner et al., 2005). While the symmorphosis concept has
been widely explored in the animal kingdom with oxygen limitation being the central dogma for tolerance definition (Taylor and Weibel, 1981), capacity failure ($LT_{50}$) could be explored as a way to define tolerance in plants.

Looking at $LT_{50}$ values as a whole (Figure 3-21), $LT_{50}$ in chronic heat stress generally occurred at lower temperatures compared to acute heat stress. Among the parameters explored, shoot and root FW would form the highest level of biological complexity, followed by $A_{sat}$, $g_{s}$, $P_{max}$. $LT_{50}$ of ETR, NPQ and qP generally fall in the intermediate range, followed by $F_v/F_m$ ratio and finally a hypothetical bleaching of Chl where photosynthesis would be damaged beyond repair (Kasa et al., 2015). This is evidential from the fact that total Chl did not correlate with $F_v/F_m$ ratio in the acute heat stress experiment. The biological hierarchy of stress parameters put forth here agrees with the heat tolerance principle (Pörtner et al., 2005) where highly complex organization or process would have the lowest thermal tolerance and capacity failure. While it has always been established that choosing the right stress indicator for plant health assessment is essential (e.g. Haldimann and Feller, 2004; Strasser et al., 2004), defining the thermal biology and optimal, pejus, and pessimum thermal range of a species could shed more light on strategies of energy budgeting and heat shock responses.

3.6 Conclusions

It is a fundamental paradigm within thermal physiology that ectotherms have an optimal physiological temperature range (Pörtner and Farrell, 2008). In the context of global warming, knowing the pejus thermal threshold of an organism is ecologically more relevant than critical temperatures, for the pejus thermal threshold temperature demarcates the boundary between optimal physiological functioning and a compromise of Darwinian fitness. Arguably, survival beyond the point of $CT_{max}$ is a temporal function of the individual organism’s energy store and physiological status prior to heat stress (Iba, 2004), illustrated here in the drop of $F_v/F_m$ ratio signifying a total capability failure in photosynthesis and cessation of energy uptake. Leaf discs
heated at their $F_v/F_m$ ratio LT$_{50}$ temperatures all have very diminished quantum yield $\varphi_{O_2}$ (Figure 3-22). By defining the optimal, pejus and pessimum thermal threshold of the vegetable crops studied here, it is hoped that sub-lethal (hardening) heat shock regimes could be better designed to prime these vegetables to better tolerate heat stress with the imminent threat of a global climate change.
CHAPTER 4

Heat shock response and plant proteins
4.1 Abstract

Plants, being sessile organisms, have several adaptation mechanisms to cope with abiotic stress experienced daily. Of the various chaperone molecules induced under stress, hsp70 is the most highly conserved and is found in all levels of biological organisms. The objectives of this study were threefold: 1) to determine the hsp70 synthesis status in various stages of the day for chronic heat stressed plants, 2) to determine the temperature at which acute heat stress (1 h) would (a) induce hsp70 synthesis, (b) result in peak magnitude of hsp70 synthesis and (c) induce decay of hsp synthesis, and finally 3) determine the temporal effects of heat stress and recovery on hsp70 synthesis, peak and decay. For all plants exposed to chronic heat stress of RZTs above 25°C, shoot and root productivity, total soluble protein and constitutive hsc70 protein levels all decreased with increasing RZT, but hsp70 in the shoot analysed at noon time increased with RZT. Rubisco protein level also decreased with increasing RZT, but when Rubisco levels were expressed as a function of total soluble protein there was no significant differences between plants grown in different RZTs. For acute heat stressed plants, peak hsp was observed at 38°C for Canasta and RIL 192. Conversely, peak hsp70 for Arugula and RIL 141 were only observed in 42°C. Hsp70 synthesis was generally attenuated at temperatures at or above 46°C for all four vegetables. For temporal kinetics of hsp70 synthesis, 30 mins heating at 38°C was found to induce hsp70 synthesis in all four vegetables. Except for RIL192 which showed peak hsp70 synthesis immediately after 60 mins of heat shock at 38°C, Arugula, Canasta and RIL 142 all had peak hsp70 synthesis after 15 mins of recovery post heat shock. While hsp70 decay was observed after 120 mins of recovery for Arugula and RIL 141, Canasta and RIL 192 still had relatively high hsp70 levels at 240 mins post heat shock. Through the kinetics of hsp70 synthesis, it could be concluded that Arugula and RIL 141 are more tolerant to heat shock compared to Canasta and RIL 192.
4.2 Introduction

Heat stress is a main environmental stressor that seriously threatens plant growth and development, causing great economic loss by affecting crop yield world-wide (Xue et al., 2015). To adapt to heat stress, sessile plants have evolved strategies to acquire thermotolerance through heat shock response (Linquist and Craig, 1988). In heat shock response, chaperone molecules like heat shock proteins (hsp) are induced and confer thermotolerance by repairing proteins damaged by heat stress, therein maintaining cellular functions and homeostasis (Vierling, 1991; Sung et al., 2003). Plant hsps can be categorised into five groups based on their molecular mass: small hsps, hsp60, hsp70, hsp90 and hsp100 (Wang et al., 2004). Among these hsps, hsp70 (70 K Dalton heat shock cognate, hsc70) is highly conserved, occurs most broadly among organisms from bacteria to human beings, and is the most abundant protein induced in a heat shock event (Renner and Waters, 2007). They are crucial housekeeping chaperone molecules essential for normal plant functions and growth under normal environmental interactions (Sung et al., 2001).

Works to identify and characterize hsp70 began in as early as the 1960s (Ritossa, 1996). Highly conserved (Wang et al., 2004) and ATP-dependent, these chaperone molecules possess two major domains: a conserved ~44-kD N-terminal ATPase domain (nucleotide binding domain; NBD) and a ~18-kD substrate binding domain (SBD) with a ~10-kD variable C-terminal “lid” (Sakar et al., 2013). NBD is regulatory functionally while SBD binds momentarily to damaged protein via attachment to exposed amino acid residues or surfaces which are hydrophobic and typically found in non-native states (Lin et al., 2001; Dragovic et al., 2006). The constant binding and release of substrate protein are both ATP-dependent and require activation of cohort system and co-chaperones such as the DnaJ-type molecular chaperones (Wang et al., 2004).
Since its discovery and description, a lot of research has been performed on the hsp70 molecular chaperones (Mayer and Bukau, 2005). Hsp70 proteins are found in different cellular locations. While the bacteria Escherichia coli possesses three members, higher organisms have been shown to possess a lot more hsp70 members: 14 in Saccharomyces cerevisiae (Walsh, et al., 2004), 18 in Arabidopsis (Lin et al., 2001), at least 12 in spinach (Guy and Li, 1998), 32 in Oryza sativa (Sakar et al., 2013), 20 in Populus trichocarpa (Zhang et al., 2015) and 61 in Glycine max (Zhang et al., 2015). Plant hsp70 can be broadly differentiated by their sub-cellular locality: cytosol, endoplasmic reticulum, mitochondria and plasmids. Each category consists of its own unique and highly conserved motifs at the C-terminus: EEVD, HDEL, PEAELYEEAKK and PEGVIDADFTSDK, respectively (Guy and Li, 1998). Each category of hsp70 has a unique role closely related to their subcellular location (Lin et al., 2001; Sung et al., 2001).

Hsp70 synthesis are essential for plant development and for adapting to extreme temperatures in: Arabidopsis (Su and Li, 2008; Jungkunz et al., 2011), hybrid poplar (Zhang et al., 2015), rice (Qi et al., 2011) and Sorghum bicolor (Mulaudzi-Masuku et al., 2015). Induction of hsp70s is initiated by heat shock transcription factors (Hsfs), although hsp70 proteins has been reported to inhibit Hsf activity (Sung et al., 2001), probably as a synthesis regulating feedback mechanism due to high energy expenditure in inducing hsp70.

Recent efforts exploring conferment of thermostolerance in vegetables mostly involve genetic engineering (e.g. transformation of tomato with FAC transcription factor gene family and ATAF1 gene, Awais et al., 2018). Arguably, plant responses towards abiotic stress do not rely on a single factor but rather a suite of stress response mechanisms that works synergistically or in a cascade (e.g. Ashraf, 2010). However, a lot of these stress response molecules are not sufficiently identified. For example, while past research has revealed that hsp20s are ATP-independent molecular chaperones and abundantly synthesized at high
temperature in many higher plants (Charng et al., 2006), they exhibit extensive sequence variability and evolutionary divergence (Basha et al., 2012).

For this chapter, the kinetics of hsp70 in heat stressed vegetables will be examined. Hsp70 was chosen for it is considered the most highly conserved hsp (Wang et al., 2004) and as it is an ATP-dependent chaperone molecule (Sarkar et al., 2013) could provide an integrated glimpse into the energy expenditure dynamics and strategy of heat stressed plants. The objectives of this chapter were threefold: 1) to evaluate hsr in various stages of the day for chronic heat stressed plants via hsp70 quantification, 2) to determine the trigger temperature (1h heating) to (a) induce hsp70 synthesis, (b) induce peak magnitude of hsp70 synthesis and (c) induce decay of hsp synthesis, and finally 3) evaluate the temporal effects of heat stress and recovery on hsp70 synthesis, peak and decay. This hsp dynamic comparison has been extensively utilized in species comparison within the animal kingdom: The level of hsp70 response in two genetically diversified populations of killer shrimps *Kikerogammarus villosus* showed a clear difference in hsp70 expression resulting in the more thermal sensitive eastern population of the killer shrimps taking a different invasion route compared to the more thermotolerant population (Hupalo et al., 2018). It has also been found that the hsp mRNA and hsp70 induction temperature were different between salmons acclimated in the Arctic and Atlantic oceans, thus explaining their difference in thermal preference (Lewis, et al., 2016). While such an endeavor has not been attempted in plant physiology, it would be interesting to see if hsp70 dynamics of the examined vegetables would shed light on their physiological performance under heat stress as explored in Chapter 3.
4.3 Materials and methods

4.3.1 Plant materials

Plant materials from Chapter 2 were used in this experiment: *Lactuca sativa* (cv. Canasta), *Eruca sativa* (cv. Arugula); two RILs (thermotolerant: 141, thermosensitive: 192) of lettuce population obtained from crosses between lettuce cultivars *L. sativa* L. ‘Salinas’ (Maternal plant) which is thermosensitive and *L. serriola* (Paternal plant) accession UC96US23 (Argyris et al., 2005) which is thermotolerant. While Paternal plant and Maternal plant were included in the chronic heat stress experiment, they were later excluded from the acute heat stress experiment for the seeds from the same batch had run out and new seeds from UC Davis did not arrive in time for inclusion in the experiment.

Please refer to “Chapter 2 Materials and Methods” for seed germination and growth maintenance.

4.3.2 Chronic heat stress experiments

For chronic heat stress evaluation, four RZTs of 25, 28, 32 and 36±1°C were maintained after transplanting till 30 days after transplantation (DAT). Destructive harvest was carried out and 5 whole plants were used from each RZT treatment. 5 plants were harvested each once in the morning 7am, once during noon time 12pm and once at 7pm. About 0.5 g FW of shoot and root per plant were very quickly weighed and flash frozen in liquid nitrogen for protein analysis. Shoot and roots were separated for fresh weight (FW) measurement and the polyurethane cubes removed. The roots of each plant were washed and gently blotted dry before weighing.

4.3.3 Acute heat stress experiments

For acute heat stress experiments, plants were grown in constant RZT of 25°C from transplantation until 30 DAT when destructive harvest was carried out. Leaf discs of 1cm diameter were excised from the youngest mature leaf and placed in petri dishes laid with two
pieces of filter paper: the bottom layer was lightly infused with a 1M carbonate/bicarbonate buffer (pH 9) to provide saturating CO₂ conditions while the top layer was lightly dampened with deionized water upon which leaf discs were placed. The petri dish was then covered with a transparent plastic film and partially submerged into a water bath pre-heated to the target temperature. For the acute heat stress experiments there were two different heat treatments of the leaf discs.

For the hsp magnitude experiment, the leaf discs were heated at target temperatures of 25 (control), 30, 34, 38, 42, 46, 50, 52 and 54°C under PPFD of 500 µmol m⁻² s⁻¹ for 1 h and then flash frozen for protein analysis. This was meant to determine the magnitude of hsp70 that would be synthesized at each target temperature.

For the hsp kinetic experiment, the leaf discs were heated at target temperature of 38°C for 15 mins, 30 mins, and 1h. For leaf discs heated for 1 h, they were returned to room temperature of 25°C and flash frozen 0, 15, 30, 60, 120, 180 and 240 mins after the heat shock. This was meant to determine the temporal kinetics of hsp kinetics and decay over time since a peak magnitude of hsp70 was observed at 38°C from the hsp magnitude experiment.

4.3.4 Total soluble proteins and Rubisco analysis

The protein in leaves or roots was extracted according to Zhang and Klessig (1997). Briefly, 0.5 g of the frozen leaf or root tissue was ground in liquid nitrogen in 0.6 ml extraction buffer [100 mM HEPES, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM DTT, 10 mM Na₃VO₄, 10 mM NaF, 50 mM b-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 mg ml⁻¹ antipain, 5 mg ml⁻¹ aprotinin, 5 mg ml⁻¹ leupeptin, 10% glycerol and 7.5% polyvinylpolypyrrolidone (PVP)]. After centrifugation at 13 000 g for 20 min, the supernatants were transferred into tubes, quickly frozen in liquid nitrogen, and stored at −80°C for further analysis.
The concentration of protein in the extracts was determined using the Bio-Rad protein assay kit (Bio-Rad Protein Assay Kit II, #5000002) using bovine serum albumin (BSA) as the standard. Very briefly, BSA protein standards from 8.0 µg/ml to 80 µg/ml were prepared with enough volume for duplicate assays. 40 µl of each standard and sample solution was loaded into separate microtiter plate wells (96 well plates were used). Dye reagent concentrate was diluted 1:4 with deionized water and 160 µl of the diluted dye reagent was added to each well. The microplate was then mixed thoroughly using a microplate shaker and incubated for 5 minutes. Absorbance was read using a microplate reader (SpectraMax Plus 293 Microplate Reader, Molecular Devices USA) at absorbance 595 nm.

For Rubisco analysis, 10 µl of protein extract was diluted with 10µl of sample buffer (Bio-Rad, 2X Laemmli Sample Buffer #1610737) and denatured at 95°C for 5 minutes. 1-D SDS-PAGE electrophoresis was performed using Bio-Rad Mini-PROTEIN Tetra Cell and Mini-PROTEIN AnyKD Ready Gel Tris-HCL Precast Gels (8.6 X 6.8 cm) and electrophoresis performed under constant voltage of 200V for 30 minutes (192mM glycine, 3.47 mM SDS, 25mM Tris-HCL pH8.6 running buffer). Separated proteins were then stained for 3 hours in coomassie brilliant blue (0.2% coomassie brilliant blue in 10% acetic acid, 50% methanol) and destained in 7% acetic acid and 25% ethanol. Resultant bands were analyzed using Fluor Chem 8800 gel imaging system. Large and small subunits of the Rubisco protein were combined for densitometric analysis.

4.3.4 Heat shock protein quantification

Denatured protein extracts were then separated using 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), and the proteins were transferred to a PVDF membrane (Trans-Blot® Turbo Mini PVDF Transfer Packs) by semidry electro blotting (Trans-Blot® Turbo, Bio-Rad). The membrane was blocked for 2 h in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.1 mM Na3VO4) with 5% BSA at room
temperature and then incubated for 1 h in TBS buffer (with BSA) containing the Rabbit polyclonal antibody HSP70 (ADI-SPA-811-F, EnzoLifeScience, USA, 1:1000 dilution). After incubation with HRP (horseradish peroxidase)-linked antibody (Cell Signalling Technology, Beverly, MA, USA), the complexes on the blot were visualized using the Clarity Western ECL Substrate (HRP conjugate, Bio-Rad) following the manufacturer’s instructions. For hsc70 examination the primary antibody used was hsc70 (plant) monoclonal antibody 1D9 (ADI-SPA-818-F, EnzoLifeScience, USA, 1:1000 dilution). Protein visualization and quantification was performed using ChemiDoc MP Imaging System (Bio-Rad, 12003154).

For subsequent hsp70 analysis (acute heat stress experiments), the ChemiDoc MP imaging system become unavailable. Remaining protein extracts from the chronic heat stress experiments were re-analyzed using the EnzoLifeScience hsp70 ELISA kits (ADI-EKS-700B, EnzoLifeScience, USA) with comparable results to densitometry analysis from western blot analysis. As such, all hsp70 acute heat stress experiments were analyzed using ELISA kits from EnzoLifeScience. Very briefly, the protein extracts and chemicals of the ELISA kit were brought to room temperature. Hsp70 protein standards were prepared as per the manufacturer’s instructions and 100 µl of prepared standards and sample were added in duplicates to the 96 well microplate and incubated at room temperature for 2 hrs. The wells were then washed 4 times with 1X Wash buffer and 100 µl of hsp70 antibody added to each well. The well was again incubated for 1 h and then washed 4 times with 1X Wash buffer. 100 µl of hsp70 conjugate was then added to each cell. The well was then incubated for 1 h and then washed 4 times with 1X Wash buffer. 100µl of TMB Substrate was added to each cell and incubated at room temperature for 30 mins. Finally, 100µl of Stop Solution 2 was added to each well and absorbance was read off using a microplate reader at 450nm absorbance with a correction at 540 nm.
4.3.5 Statistical Analysis

For each physiological index obtained, each species was analysed separately with one-way analysis of variance and posthoc SNK tests.

4.4 Results

4.4.1 Chronic heat stress responses

There were significant differences in shoot FW (Figure 4-1A) and root FW (Figure 4-1B) across all six species with increasing RZT. A drastic drop in shoot FW was observed as early as 28°C for Arugula (F(3,11)= 844.9, p<0.001), Canasta (F(3,11)= 273.0, p<0.001), RIL 141 (F(3,11)= 184.3, p<0.001), RIL 192 (F(3,11)= 721.7, p<0.001), Lactuca serriola accession UC96US23 henceforth known as “Paternal plant” (F(3,11)= 1369.9, p<0.001) and L. sativa L. ‘Salinas’ henceforth known as “Maternal plant” (F(3,11)= 469.3, p<0.001). A similar trend was observed in root FW of Arugula (F(3,11)= 190.8, p<0.001), Canasta (F(3,11)= 240.6, p<0.001), RIL 141 (F(3,11)= 656.7, p<0.001), RIL 192 (F(3,11)= 352.0, p<0.001), Paternal plant (F(3,11)= 199.9, p<0.001) and Maternal plant (F(3,11)= 500.1, p<0.001) where significant difference was detected at 28°C.
Figure 4-1. Shoot (A) and root (B) fresh weight of the four vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Correspondingly, total soluble protein also decreased significantly with higher RZT (Figure 4-2, Arugula: $F_{(3,11)} = 312.5, p < 0.001$; Canasta: $F_{(3,11)} = 119.1, p < 0.001$; RIL 141: $F_{(3,11)} = 337.9, p < 0.001$; RIL 192: $F_{(3,11)} = 99.7, p < 0.001$; Paternal plant: $F_{(3,11)} = 1.722, p < 0.001$; Maternal plant: $F_{(3,11)} = 281.5, p < 0.001$). For Rubisco, there was no significant differences between treatment groups for Paternal plant (Figure 4-3, $F_{(3,11)} = 1.722, p = 0.239$), but Rubisco synthesis decreased significantly with increased RZT for the other plants (Arugula: $F_{(3,11)} = 312.5, p < 0.001$; Canasta: $F_{(3,11)} = 119.1, p < 0.001$; RIL 141: $F_{(3,11)} = 337.9, p < 0.001$; RIL 192: $F_{(3,11)} = 99.7, p < 0.001$; Maternal plant: $F_{(3,11)} = 281.5, p < 0.001$). If, however, Rubisco synthesis was expressed as a function of total soluble protein, there was no significant differences between the plant species and between treatment groups (Figure 4-4).
Figure 4-2. Total soluble protein of the six vegetables grown in constant RZT of 25, 28 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 4-3. Rubisco amount of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 4-4. Ratio of Rubisco over total soluble protein of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C. Each bar is mean ± SE (n=5). There was no significant difference between the treatment groups for all the vegetables.
Figure 4-5. Hsp70 levels of the six vegetables grown in constant RZT of 25, 28, 32 and 36°C analysed at (A) 7 am in the morning in shoot tissue; (B) at noon time in shoot tissue, (C) at 7pm in the evening in shoot tissue and (D) at noon time in the root tissue. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
There was no hsp70 synthesis differences in the shoot between the treatment groups for all 6 vegetables in the morning 7am (Figure 4-5A) and 7pm (Figure 4-5C) but in analysis during noon time where ambient light and heat were both high in the greenhouse, all six plant species showed higher level of hsp70 synthesis with response to higher RZTs (Figure 4-5B, Arugula (F(3,11)= 496.9, p<0.001), Canasta (F(3,11)= 701.9, p<0.001), RIL 141 (F(3,11)= 300.3, p<0.001), RIL 192 (F(3,11)= 772.7, p<0.001), Paternal plant (F(3,11)= 131.0, p<0.001) and Maternal plant (F(3,11)= 1636.5, p<0.001).

Although hsp70 synthesis from the root showed similar trends to that of the shoot analysed at noon, there was too much variance in the results and no significant differences between the treatment groups were detected (Figure 4-5D).

With the exception of Paternal plant which showed no difference between treatment groups for its constitutive heat shock protein (Hsc70) synthesis (Fig 4-6, Paternal plant (F(3,11)=1.743, p = 0.236), all other 5 plants showed a similar trend to that of shoot and root productivity where hsc70 decreased significantly with higher RZT (Figure 4-6, Arugula (F(3,11)= 314.3, p<0.001), Canasta (F(3,11)= 119.0, p<0.001), RIL 141 (F(3,11)= 339.8, p<0.001), RIL 192 (F(3,11)= 98.6, p<0.001), and Maternal plant (F(3,11)= 280.3, p<0.001)

4.4.2 Acute heat stress responses

There were positive heat shock responses for all 4 vegetables where increased heat shock temperature generally induced higher levels of hsp70. Hsp70 was detected Figure (4-7). Hsp70 was detected at the beginning of the experiment which was significantly elevated at 30°C heating for all 4 vegetables (Arugula (F(8,26)= 3780.4, p<0.001), Canasta (F(8,26)= 2993.1, p<0.001), RIL 141 (F(8,26)= 2281.1, p<0.001), RIL 192 (F(8,26)= 20.1, p<0.001).
Figure 4-6. Hsc70 levels of the six vegetables grown in constant RZT of 25, 28 32 and 36°C. Each bar is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 4-7. Hsp70 levels of the four vegetables exposed to acute heat stress at 25, 30, 34, 38, 42, 46, 50, 52 and 54°C for 1 h. Each bar is mean ± SE (n=5). Significantly elevated hsp was detected at 30°C for all four vegetables. Asterisk marks the peak hsp70 detected in each of the four vegetables.
Figure 4-8. Hsp70 levels of the four vegetables exposed to acute heat stress at 38°C for 15, 30 and 60 mins. After heating, leaf discs were returned to room temperature and analysed for hsp70 levels after 0, 15, 30, 60, 120, 180 and 240 mins at 25°C. Each bar is mean ± SE (n=5).
For the temporal experiment, hsp70 was detected in leaf discs after 15 mins of heat shock at 38°C (Figure 4-8). Peak hsp synthesis was observed for RIL 141 ($F_{(8,26)}= 1366.3$, $p<0.001$) and RIL 192 ($F_{(8,26)}= 2578.5$, $p<0.001$) after 60 min exposure to 38°C, but was only observed after 15 mins of recovery time in room temperature post heat shock for Arugula ($F_{(8,26)}= 2267.2$, $p<0.001$) and Canasta ($F_{(8,26)}= 952.1$, $p<0.001$). Upon returning the leaf discs back to room temperature (25°C), Arugula and RIL 141 both showed significantly decreased hsp levels after 30 mins (15 mins after hsp peak manifestation, while Canasta and RIL 192 still had comparable (relatively high) hsp levels 240 mins after heat stress removal.

4.5 Discussion

This chapter reports the kinetics of hsp70 synthesis in temperate vegetables when subjected to chronic and acute heat stress. This research is unprecedented in plant physiology research in two aspects: firstly, for chronically heat stressed plants the heating was in the RZT instead of the whole plant and secondly, the acute heat stress dynamics of hsp70 synthesis has not been extensively performed in plant species. Generally, there was a significant heat shock response in the vegetables with response to the degree of heat stress: similar to the results obtained in Chapter 2, productivity of the shoot and root FW generally decreased with increasing RZT (Figure 4-1).

4.5.1 Total soluble protein and Rubisco analysis

The response of the shoot and root productivity corresponded with the trend of total soluble protein with increasing RZT (Figure 4-2). While carbon and nitrogen are essential for carbohydrate manufacturing in plants, proteins (amino acids) and lipids are essential for plant biomass (Deans et al., 2018). Thus, it can be inferred that increasing RZTs above 25°C directly limited the productivity and somatic growth of the four temperate vegetables through limitation of total soluble protein available for growth. Interestingly, Rubisco protein levels was also similarly affected by increasing RZT (Figure 4-3). Given that Rubisco proteins are the main
rate-limiting factor for photosynthesis (Yasumoto et al., 2018) the productivity limitation with increasing RZT could be seen as a cascading effect of RZT affecting the total soluble protein availability, which in turn affected the available pool of Rubisco protein, which in turn affected the rate of photosynthesis and somatic growth of plants in higher RZTs. While considering the Rubisco protein level as a function of the total soluble protein (Figure 4-4) it could be seen that Rubisco production was maintained at a fixed percentage of total soluble protein available as there was no significant difference in the Rubisco: Total soluble protein ratio for all plant species and across all treatment groups. This corresponds to the large investment of Rubisco, dedicating approximately 15-30% of total leaf nitrogen in this single enzyme for C3 plants (Evans 1989; Makino et al., 1992; Fukayama et al., 2018). Moreover, overproduction of Rubisco did not necessarily improve photosynthesis (Suganami et al., 2018) and that N allocation to Rubisco could be regulated through preferential production of Calvin-Benson cycle enzymes (Suganami et al., 2018).

4.5.2 Hsp70 expression in chronic heat stress experiment

Interestingly, although there was constant heat stress in the root zone for treatment groups, there was almost no significant difference in heat shock response of the four vegetable between the treatment groups in the morning 7 am analysis (Figure 4-5A). There could be two possible explanations for this: Firstly, given that hsp70 is an ATP-dependent chaperone molecule (requires energy expenditure for synthesis and for binding to damaged protein for sequestering or re-folding, Goloubunoff et al., 2018), chronically heat stressed plants would have acclimated to not synthesise hsp70 continuously. Moreover, one of the triggers for hsp70 synthesis is denatured or damaged proteins (Oliverio et al., 2018): as it is the shoot (youngest mature leaf) that is excised for analysis at 7 am in the morning, there could be minimal trigger for hsp70 synthesis in the shoot tissue. Secondly, heat shock response could be
compartmentalised for plants given that while the roots are being heat shocked, the shoots are experiencing cool early morning ambient temperatures.

By contrast, shoot hsp70 analysis at noon time (Figure 4-5B) showed an increasing heat shock response with increasing RZT. Given that the shoots would be experiencing similar light and heat stress from the ambient greenhouse conditions during noon time analysis, the differential hsp70 synthesis levels among the treatment groups suggests a compounding effect of high RZT on heat stress experienced by the shoot from noon time heating of the shoots. Taking into consideration that high RZT plants have lower total soluble protein pool (He et al., 2001; He et al., 2009) and that hsp70 synthesis is highly ATP demanding (Sakar et al., 2013), this further explains the corresponding lower shoot and root productivity of high RZT plants.

There was a drop in hsp70 levels in the shoot tissue towards the evening in the 7 pm analysis (Figure 4-5C). Given the high energy expenditure of hsp70, we would expect a fast recovery (return to native state) in heat tolerant plants once the heat shock event is over. Interestingly, although there was no significant difference in hsp70 levels among treatment groups within each species, we see that Canasta, RIL 192 and Maternal plant all had significantly higher hsp70 levels both compared to the 7 am cohort and to the other species. This delayed decay in hsp70 levels suggested that Canasta, RIL 192 and Maternal plant are not as thermotolerant as the other 3 vegetables. The results here partially agree with the work by Ali et al. (2018) where rice was heated to 42°C followed by recovery at 28°C. Ali et al. (2018) suggested that prompt induction in hsp70 expression and slow decay during recovery for long-term memory is the desirable trait for thermotolerance in rice. Arguably, in this experimental set up there is no recovery period for the plants, for although shoot experienced temperature would drop to around 25~28°C at night, RZT was kept constant. Moreover, given that hsp70 synthesis and mode of action are all very ATP-dependent, having consistent and strong
expression of hsp70 during recovery would suggest both a greater extent of thermal damage over the same heat stress and a lower efficiency in cellular repair (Goloubunoff et al., 2018).

For hsp70 in the roots, there was no significant difference between analysis at 7 am, noon and 7 pm and thus the data was pooled and presented as hsp70 at noon time (Figure 4-5D). Although there seems to be a trend of increasing root hsp70 with increasing RZT, the variance for each treatment group was too big and hsp70 among the treatment groups were statistically not significant. There could be due to two reasons: Firstly, as the roots were chronically heated at constant target ± 1 °C one might expect that the roots were acclimated to the constant temperature and might have mixed signals for hsp70 synthesis. Secondly, the huge variance might be due to the sampling method: to make up for the 0.5g FW some of the root tissue might not be root tip tissues, especially for higher RZT plants where number of root tips and biomass of root decreased. This might have resulted in inaccuracy of the hsp70 quantification.

Antibody targeting the constitutively synthesized hsc70 ADI-SPA-818-F was used to examine the constitutive hsc70 protein. Unlike the stress induced hsp70 proteins, hsc70 is constitutively secreted at low levels in all organisms (Wang et al., 2018) for housekeeping functions. Interestingly, unlike hsp70, hsc70 levels in the chronic heat stressed plants decreased with increasing RZT much like the behaviour of total soluble protein and Rubisco protein (Figure 5-6). This suggests that hsc70 was limited by the total soluble protein as well and thus decreased with high RZT due to the decreased total soluble protein in high RZT plants.

4.5.3 Hsp70 expression in acute heat stress experiment

The acute heat stress experiment here was modelled after the works of Tomanek and Somero (2000) on Tegula snails. The significance of heat shock responses in situ is indicated by the observations that synthesis of hsp70 is often near the upper thermal limits that organisms will experience in their habitat (Hofmann and Somero, 1995). However, till date, especially in
plant physiology studies, several features of the heat shock response are not well defined. For example, the time course and dose-response relationship of hsp70 production has received very little study. Working with congeners (e.g. *Mytilus*: Roberts et al., 1997; *Tegula*: Tomanek and Somero, 1999), kinetics (time course) and magnitude of hsp70 induction was strongly related to the thermal niche and adaptation/acclimation potentials of intertidal organisms. Thermotolerant organisms tend to be prompt in activating its heat shock response and completing production of hsp70 while thermosensitive organisms would have higher magnitude of hsp70 synthesis (related to greater severity of cellular damages due to heat stress) and prolonged periods of hsp70 synthesis (Lewis et al., 2016; Hupalo et al., 2018).

In the hsp70 magnitude experiment (Figure 4-7), Canasta and RIL 192 both peaked earlier at 38°C while Arugula and RIL 141 had peak hsp70 synthesis at a higher temperature of 42°C, suggesting that Arugula and RIL 141 are more thermotolerant than Canasta and RIL 192. This is also suggested by Ali et al. (2018) in that heat tolerant plants should have fast induction of hsp70 in heat stress events. For temperatures higher than 42°C we see a distinct attenuation in hsp70 synthesis in Canasta, RIL 141 and RIL 192 compared to Arugula, suggesting that among the four vegetables, Arugula is the most thermotolerant plant. In the time course experiment of hsp70 synthesis, we see a peak in hsp70 for all four vegetables at 15 mins post heat shock (38°C for 1 h). However, while hsp70 for Arugula and RIL 141 quickly decreased with time (30 minutes post heat shock), we see hsp70 levels of Canasta and RIL 141 remaining constant post heat shock for at least 60 minutes before declining. This suggests that Canasta and RIL 141 were both more severely affected by the 1h of 38°C heat shock and that firstly, greater energy expenditure was prioritised in heat shock remediation, and secondly, it took a longer time for protein repair work to be completed for these two plants species (Tomanek and Somero, 1999).

4.5.4 Conclusion
The chronic heat stress experiments showed that even though shoot tissue might be experiencing temperatures independent of RZT, the latter has a compounding effect on shoot hsp70. This might have affected the overall fitness of the whole plant in terms of resource allocation and priority strategizing. Through the magnitude and time course experiments of hsp70 synthesis experiments, it has also been shown that Arugula and RIL 141 are more thermotolerant than Canasta and RIL 192. Lastly, based on the hsp70 synthesis relationship with absolute temperature, 38°C would be a good target temperature for sub-lethal priming experiments with regards to the four temperate vegetables.
CHAPTER 5

Effects of sub-lethal heat shock regimes on thermotolerance conferment in temperate vegetables and post-harvest qualities
5.1 Abstract

Having established the thermal limits and critical temperatures at which photosynthetic parameters and physiological performances of selected vegetables species would be compromised, sub-lethal heat shock regimes were employed to prime Arugula, Canasta, RIL 141 and RIL 192. This research aimed to evaluate: 1) if heat priming could enhance crop performance under harsher heat stress environment such that heat primed plants would have comparable productivity with control plants, and 2) if heat priming could enhance the post-harvest quality of the vegetable crop even if productivity was compromised for heat stressed plants. Post-harvest qualities here refer to two key attributes: nutritional value of the crop and longer shelf-life of the crop to resist browning during the process of harvest, storage and transport to the point of sales. The four vegetables were first grown in RZT 25°C. The treatment plants were initially kept at 25°C RZT from first day of transplantation (0 DAT) till 10 DAT, and subsequently subjected to a daily 38°C RZT for 4 hours (1000 hrs to 1400 hrs) to simulate a noon-time heating from 11 DAT to 22 DAT. Root morphology analysis was performed every 3 days to examine if the sub-lethal heat shock would affect root growth and morphology of the treatment plants. From 21 DAT to 30 DAT the plants were divided into four groups: (i) CC: plants were kept constantly at 25°C RZT from transplantation to harvest; (ii) CH: plants kept in 25°C RZT from 0 DAT to 22 DAT and then subjected to a daily heat stress of 45°C for 6 hours (1000 hrs ~ 1600 hrs) 23~30 DAT; (iii) HC: plants subjected to 38°C RZT sub-lethal heat shock 11 DAT to 22 DAT and kept in 25°C RZT 23~30 DAT and (iv) HH: plants subjected to 38°C RZT sub-lethal heat shock 11 DAT to 22 DAT and then subjected to a daily heat stress of 45°C for 6 hours (1000 hrs ~ 1600 hrs) 23~30 DAT. Plants were harvested 30 DAT and examined for their productivity, O₂ evolution, Fᵥ/Fm ratio, chlorophyll fluorescence, total reduced nitrogen (TRN) and total sugar content. Root morphology was examined every three days after heat priming started, but no significant difference between control and heat primed
plants were detected, signifying that the heat priming did not affect root development in heat primed plants. CH plants initially shows a significantly lower Fv/Fm ratio compared to the other treatment groups (26 DAT), but by 30 DAT there was no significant difference in the vegetables except in RIL 192. CH treatment group for all four vegetables were also significantly lowest for shoot fresh weight and Pmax. Heat primed vegetables all had significantly higher phenolic compounds and ascorbic acid while CC and CH plants have higher PPO, POD and PAL browning enzymes towards day 15 of storage. This study suggested that: 1) if root zone chilling is applied during the root formative period (0 DAT~ 11 DAT) temperature is not a very important environmental stressor for later growth of these temperate vegetables as long as water in the form of nutrient solution is constantly available; 2) Heat primed vegetables could have shoot productivity comparable to plants kept in constantly cool RZT throughout the growth period; 3) heat primed vegetables have higher nutritional values like phenolic compound and ascorbic acid levels; 4) heat primed vegetables have lower levels of browning enzymes and thus might have a longer shelf-life.
5.2 Introduction

It is predicted that the imminent global warming and accompanying heat stress will greatly affect global food security and cause severe drop in crop productivity by affecting plant growth rate (Fahad et al., 2017; Deligios et al., 2019). Increased average temperate, extreme hot weather events (especially in the tropics) compounded with loss of good farm areas due to salt and heavy metal contamination of soil are all expected to affect crop yield drastically (Long et al., 2015). Heat stress is the greatest threat to food security as it affects crops at molecular, biochemical and physiological levels, limiting physiological performance and affecting crop yield (Asada, 2006).

To improve food crop survival and boost productivity, different approaches have been adopted to enhance resistance to stress in plants. Traditional cross breeding relies on genetic variation and induced mutations to produce plants with improved stress tolerance, but results have been wanting and few new plants were developed under this method (Lin et al., 2013). Inherently, this approach was flawed due to the inefficiency in selection techniques and complexity of stress tolerance traits (Lin et al., 2010). Current efforts to induce stress tolerance in plants rely on engineering the regulatory or stress signaling processes in plants (Seki et al., 2003, Ohama et al., 2017), triggering genes to produce stress proteins and chaperone molecules (Jacob et al., 2017; Ma et al., 2018), or induction of enzymes for functional metabolite production (Li et al., 2018; Liu et al., 2018). This approach enhances stress tolerance by targeting the endogenous systems at different levels of response: stress signaling elements, gene transcription of stress proteins and antioxidant enzymes (Reguera et al., 2012). Alternatively, plants could also acclimate to environmental stresses through priming.

Priming also boosts plant tolerance to abiotic stresses by triggering defense pathways already present in plants, without need of genetic engineering. Priming primarily works by inducing production of stress proteins (Jacob et al., 2017), antioxidant enzymes (Chiang et al.,
2015) and various metabolites (Li et al., 2018) through expression of regulatory and functional genes (Savvides et al., 2016). Arguably the downstream events of priming are identical to the engineering approach, but genetic engineering techniques are not involved (Ohama et al., 2017).

Environmental stress in plants could lead to generation of secondary metabolites which may not be essential for survival but could play important and primary roles in adaption mechanisms. One such component would be phenolic compounds in vegetables. Due to their antioxidant properties, plant phenolic compounds are very beneficial supplements to the human diet (Balasundram et al., 2006). The other component to be examined in this study is antioxidant ascorbate or Vitamin C. About 90% of our dietary vitamin C are from plant sources (Lee & Kader, 2000) and thus ascorbate content in vegetables would be an invaluable source for dietary enhancement.

Browning in vegetables and fruits is the major problem in the handling and sales of fresh produce. Browning affects the physical appearance and taste of the product for browning reactions produce undesirable flavours (e.g. bitterness) in the food crop. PPO, POD and PAL are enzymes associated with the browning process: PPO causes browning by producing quinones which results in brown pigments (Tomas-Barberan and Espin, 2001). Thus, the PPO activity levels has a direct relationship with browning potential. POD is also an enzyme that causes browning (Chen et al., 2010) while PAL produces the abovementioned undesirable flavours through the synthesis of phenolic compounds via the phenylpropanoid pathway (Tomas-Barberan and Espin, 2001).

Having established the critical temperatures for physiological performance in Chapter 3 and heat shock protein kinetics in Chapter 4, this chapter aimed to use sub-lethal heat stress as a primer to confer thermotolerance to the temperate vegetable species worked on in the previous chapters. The aim of the experiment was two-fold: 1) to investigate if heat priming would enhance crop performance under harsher heat stress environment such that heat primed
plants would have comparable productivity with control plants, and 2) if heat priming could enhance the post-harvest quality of the vegetable crop even if productivity was compromised for heat stressed plants. Post-harvest qualities here refer to two key attributes: nutritional value of the crop and longer shelf-life of the crop to resist browning during the process of harvest, storage and transport to the point of sales.

5.3 Materials and methods

5.3.1 Plant materials

*Lactuca sativa* (cv. Canasta), *Eruca sativa* (cv. Arugula) and two RILs (thermotolerant: 141, thermosensitive: 192) of lettuce population were used in this experiment. Please refer to Chapters 3 and 4 for details on plant materials, germination and plant growth condition maintenance.

5.3.2 Heat priming experiments

The four vegetables were maintained at RZT of 25±1°C from 0 days of transplantation (DAT) till 11 DAT. Upon 11 DAT, treatment group plants (25°C-RZT→38°C-RZT) were subjected to a RZT 38°C heat priming regime that lasted for 4 hrs (10 am to 2 pm) daily and then RZT was cooled to 25°C. Control plants (25°C RZT) were kept in constant RZT of 25°C throughout for the priming period. Heat priming experiment plants were harvested every three days after the heat priming started and examined on their root morphology and development.

5.3.3 Heat shock experiments

The heat priming regime of 38°C for 4 h daily was applied to the treatment group on 11 DAT and continued till 20 DAT. On 21 DAT (corresponding to after 10 days of heat priming), both the control plants (25°C RZT) and heat primed plants (25°C-RZT→38°C-RZT) were subjected to RZT 46°C heat shock for 6 h daily from 10 am to 4 pm for 5 days and then plants from both groups were harvested 26 DAT for shoot and root productivity, chlorophyll fluorescence parameters such as ETR, NPQ, qP, P_max and F_v/F_m ratio.
Plate 5-1. Schematic diagram of the heat shock experiments. Phase I (0 DAT to 11 DAT) all plants were maintained at 25°C-RZT. Phase II (heat priming) starts on 11 DAT and half the plants were left in constant 25°C-RZT while half the plants were subjected to a heat priming of 38°C-RZT daily for 4 hours after which RZT was returned to 25°C. Phase III (heat shock) starts after 10 days of heat priming at 22 DAT. Plants kept in 25°C-RZT throughout were divided into two groups CH and CC while heat primed plants were divided into two groups HC and HH. CC and HC plants were subjected to 25°C-RZT from 22 DAT to harvest time. HH and CH plants were subjected to a heat shock regime of 46°C-RZT daily for 6 hours, after which the plants were left in ambient RZT from 22 DAT to harvest time.

5.3.4 Post-harvest quality experiments

For the post-harvest quality experiments, plants were grown in constant RZT of 25°C till 11 DAT. Half of the plants were then subjected to a 38°C heat priming for 4 h a day (10 am to 2pm) daily till 21 DAT (10 days of priming). Plants kept in 25°C RZT till 21 DAT were then divided into two groups: CC plants were kept in 25°C RZT till 30 DAT while CH plants were given a RZT-46°C heat shock for 6 h daily (10 am till 4 pm) from 21 DAT till 30 DAT. CH plants were left in ambient RZT when not under heat shock. Plants that were heat primed at 38°C were also divided into two groups: HC plants were returned to constant RZT-25°C
while HH plants were left in ambient RZT and given a RZT-46°C heat shock daily for 6 h (10 am to 4 pm). Plants were harvested on 30 DAT and examined for their shoot and root productivity, total reduced nitrogen (TRN), soluble and insoluble sugar, total ascorbic acid level, total phenolic compound levels, and browning agent PPO, POD an PAL activity levels. The remaining plants were individually packed in ziplock bags with their shoot and roots intact in 4°C darkness and analysed every 5 days for their TRN, soluble and insoluble sugar, total ascorbic acid level, total phenolic compound levels, and browning agent PPO, POD and PAL activity levels till day 15 post-harvest. This schematic diagram of this process is shown in Plate 5.1.

5.3.5 Plant stress indicator and post-harvest quality evaluation

5.3.5.1 Productivity of shoot and root

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.2 Root morphology and development

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.3 Measurement of TRN

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.4 Measurements of P_{max}

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.5 Measurement of photochemical light use efficiency

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.6 Measurements of F_{v}/F_{m} ratio

Please refer to Chapter 3 Materials and methods for methodology details.

5.3.5.7 Soluble and Insoluble Sugar

The procedure for the quantification of free sugar is outlined by Buysse and Merckx (1993). Isolation of soluble and insoluble sugars was carried out in two parts.
Dried plant tissue of 50 mg was added to hot 80% ethanol (10 ml, heated to 65°C), and agitated for 15 min. The homogenate was centrifuged at 4000 rpm for 10 min, and the supernatant collected. The pellet was resuspended in an additional 10 ml of hot 80% ethanol, and the process repeated twice more. The final volume was made to 50 ml and used in evaluation of soluble sugar.

The remaining pellet was allowed to dry at room temperature for approximately 24 hours, prior to incubation in an oven (65°C) overnight to ensure the removal of all water. The pellet was stored for later quantification of starch content. Starch was hydrolyzed by incubation with 5 ml of 3% HCl in a water bath at 100°C for 3 h. The pellet was resuspended every 30 minutes during incubation. After 3 hours the solution was centrifuged at 1700 g, the supernatant collected, and the pellet re-extracted twice more using the method for soluble sugar extraction. The supernatants were pooled and the final volume was made to 50 ml with 80% ethanol and used in evaluation of insoluble sugar.

The concentration of sugar in the prepared extracts was determined colourimetrically (Dubois et al., 1956). 0.5 ml of 5% phenol and 2.5 ml of H₂SO₄ was added to 0.5 ml of extraction solution (either from soluble sugar or insoluble sugar). The mixture was allowed to stand for 10 min before gently agitating. They were then allowed to sit for another 10 min during which time the colour of the solution stabilized. Once the colour was stable, a small aliquot was poured into a 1 ml plastic cuvette and absorbance (OD) was measured at 490 nm. The amount of free sugar present was determined from a standard assay.

5.3.5.8 Total ascorbic acid

The concentrations of ascorbic (Asc, reduced form) and dehydroascorbic (DHAsc, oxidized form) acids were spectrophotometrically assayed by the reduction of 2,6-dichlorophenolindophenol (DCPIP) (Leipner et al., 1997). Leaves (0.5 g FW) were homogenized in liquid nitrogen in presence of 1g NaCl and extracted in 5 mL ice-cold 2%
(w/v) metaphosphoric acid. The homogenates were filtered (or centrifuged at 4°C during 15 min at 13,000 x g). An aliquot of 0.3 mL was mixed with 0.2 mL 45% (w/v) K₂HPO₄ and 0.1 mL 0.1% (w/v) homocysteine to reduce DHAsc to Asc in the determination of the total Asc pool (Asc + DHAsc). For the determination of Asc, the homocysteine solution was replaced by the same volume of water. After 15 min of incubation at 25°C, 1 mL of 2 M citrate-phosphate buffer (pH 2.3) and 1 mL 0.003% (w/v) DCPIP were added. The absorbance at 524 nm was immediately measured using a spectrophotometer (UV-2550 Shimadzu, Japan). The content of Asc was calculated by reference to a standard curve. The amount of DHAsc resulted from the difference between the total Asc (vitamin C) content and Asc (DHAsc = Total Asc – Asc). Results were expressed as mg of ascorbic acid per g of FW of leaves.

5.3.5.9 Total phenolic compound

The concentration of total phenolic compounds was determined in methanol extracts using a colorimetric method (Kan and Saltveit 2002, Ragee et al., 2006). 0.5 g of fresh shoot tissues was ground with liquid nitrogen and 5 ml of 80% methanol. The extracts were shaken for 30 min at 2000 rpm and centrifuged for 20 min at 3500 rpm. The supernatants were transferred to clean tubes. 0.5 ml of extract was diluted with 0.5 ml of diluted Folin-Ciocalteau reagent and 1 ml of 7.5% Na₂CO₃ solution. After 20 min, the absorbances were measured at 765 nm using UV-2550 spectrophotometer (Shimadzu, Japan). Total phenolic compounds of the samples were expressed as gallic acid equivalents in micrograms per gram of FW.

5.3.5.6 Browning enzymes PPO, POD and PAL levels

PPO activity was assayed with some modifications as described by Rico et al. (2006). Extraction of crude enzymes related to browning was performed at 4°C. Ten grams of polyvinyl polypyrrolidone (PVPP) was added to 10 g of the vegetable FW and homogenised in 90 ml of 0.2 mol/L sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000g for 5 min and the supernatant was used as a crude enzyme solution. The reaction
mixture consisted of 0.8 ml of the crude enzyme solution and 2.4 ml of 0.02 mol/L catechol dissolved in 0.05 mol/L sodium phosphate buffer (pH 7.0). Enzyme activity was measured by the increase in absorbance of 420 nm. One unit of enzyme activity was defined as an increase in absorbance at 0.01/min. The enzyme activity was expressed as units of enzyme/ g / min.

POD activity was assayed with some modification as described by Yang et al. (2009). Ten grams of PVPP was added to 10 g of the vegetable FW and homogenised in 90 ml of 0.2 mol/L sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000g for 5 min and the supernatant was used as a crude enzyme solution. The reaction mixture consisted of 0.2 ml of the crude enzyme solution, 2.8ml of 25mmol/L guaiacol, and 25 mmol/L hydrogen peroxide dissolved in 0.05 mol/L sodium phosphate buffer (pH 7.0). Enzyme activity was measured by the increase in absorbance at 470 nm. One unit of enzyme activity was defined as an increase in absorbance of 0.001/min. The enzyme activity was expressed as units of enzyme/ g / min.

PAL activity was assayed with some modification as described by Ke and Saltveit (1989). The crude enzyme PAL was extracted by homogenising 4 g of vegetable FW with 16 ml of 50 mmol/L borate buffer (pH 8.8) containing 5 mmol/L β-mercaptoethanol and PVPP (25g/L). Centrifugation was conducted at 4°C and 15,000g for 30 min and the supernatant was used as the crude enzyme. Reaction mixtures contained or non-contained with 0.55 ml of 50 mmol/L L-phenylalanine group were reacted for 2 h at 40°C. The reaction was stopped using 0.1 ml of 6 N HCl, and the absorbance was measured at 290 nm. The enzymatic activity was expressed as μg of cinnamic acid produced ml⁻¹ h⁻¹ fresh weight (FW).

5.3.6 Statistical analysis

For each physiological index obtained, each species was analysed separately either using one-way ANOVA or two-way ANOVA where appropriate using treatment groups and temporal as factors. Posthoc analysis was performed with SNK tests using one-way ANOVA
where two-way ANOVA factor interactions were not significant. Parameters in ratio (e.g. $F_v/F_m$ ratio was arcsine transformed before statistical analysis).

5.4 Results

5.4.1 Heat priming experiments

Upon 11 days after transplantation (DAT), treatment group plants were subjected to a RZT 38°C heat priming regime that lasted for 4 h (10 am to 2 pm) daily and then RZT was cooled to 25°C. Control plants were kept in constant RZT of 25°C throughout for the priming period. Both the control and treatment plants were harvested every three days after the heat priming started and root morphology was analysed for the two treatment groups. For Arugula, there was a significant increase in root length (Figure 5-1A, $F_{(2, 18)} = 27.7, p < 0.001$), root surface area (Figure 5-1E, $F_{(2, 18)} = 15.9, p = 0.001$) and number of root tips over time (Figure 5-1J, $F_{(2, 18)} = 22.8, p < 0.001$), but there was no significant increase in root diameter over time (Figure 5-1M, $F_{(2, 18)} = 0.617, p = 0.556$). There was no significant difference in the root length ($F_{(1, 18)} = 5.5, p = 0.057$), root surface area ($F_{(1, 18)} = 0.594, p = 0.456$), number of root tips ($F_{(1, 18)} = 3.109, p = 0.103$) and root diameter ($F_{(1, 18)} = 4.3, p = 0.062$) between control and heat primed Arugula.

Root morphology of Canasta during the heat priming period followed a similar trend as that of Arugula plant. There was significant increase in total root length (Figure 5-1B, $F_{(2, 18)} = 13.6, p = 0.001$), total surface area (Figure 5-1F, $F_{(2, 18)} = 19.4, p = 0.002$) and number of root tips (Figure 5-1J, $F_{(2, 18)} = 31.7, p < 0.001$) over time for both treatment and control plants. There was no significant increase of root diameter over time for both heat primed and control Canasta (Figure 5-1N, $F_{(2, 18)} = 4.9, p = 0.053$). There was no significant difference between the heat primed plant and control plants in terms of root length ($F_{(1, 18)} = 5.67, p = 0.053$), number of root tips ($F_{(1, 18)} = 0.4, p = 0.554$), and root diameter ($F_{(1, 18)} = 0.2, p = 0.684$). Although there was significant difference between treatment groups for root surface area of
Canasta \( (F_{1, 18} = 15.5, \ p = 0.002) \), this was between samples of control plant Day 3 and treatment plant of Day 6 post heat priming. By Day 9 both treatment and control group had similar root surface area by post hoc analysis.
Figure 5-1. Root morphology analysis of the Arugula, Canasta, RIL 141 and RIL 192 of total root length (A, B, C, D), total root surface area (E, F, G, H), number of root tips and (I, J, K, L), average root diameter (M, N, O, P). Control plants were grown in constant 25°C C-RZT while treatment plants were grown in 25°C C-RZT till 11 DAT and then heat primed for 4 h daily from 1000 – 1400 h at 38°C C-RZT 38°C. Each point is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
For RIL 141 there was significant increase in root length (Figure 5-1C, $F_{(2, 18)} = 38.2, p < 0.001$) over time for both control and treatment group, but no discernable increase over time was observed for root surface area (Figure 5-1G, $F_{(2, 18)} = 3.9, p = 0.05$), number of root tips (Figure 5-1K, $F_{(2, 18)} = 3.5, p = 0.063$) and root diameter (Figure 5-1O, $F_{(2, 18)} = 16.4, p = 0.05$). Correspondingly, there was no significant difference between treatment and control groups in terms of root length ($F_{(1, 18)} = 5.67, p = 0.053$), root surface area ($F_{(1, 18)} = 5.67, p = 0.053$), number of root tips ($F_{(1, 18)} = 5.67, p = 0.053$) and root diameter ($F_{(1, 18)} = 5.67, p = 0.053$).

For RIL 192, there was significant increase in root surface area (Figure 5-1H, $F_{(2, 18)} = 5.6, p = 0.019$) and number of root tips (Figure 5-1L, $F_{(2, 18)} = 6.9, p = 0.01$) over time, but root length (Figure 5-1D, $F_{(2, 18)} = 2.7, p = 0.11$) and root diameter (Figure 5-1P, $F_{(2, 18)} = 0.56, p = 0.584$) were similar throughout the survey period. There was significant difference between treatment and control plants for root length ($F_{(1, 18)} = 8.02, p = 0.015$) and root surface area ($F_{(1, 18)} = 19.97, p = 0.001$), but number of root tips ($F_{(1, 18)} = 0.052, p = 0.823$) and root diameters ($F_{(1, 18)} = 2.67, p = 0.128$) were similar for both treatment groups. For both root length and root surface area, the differences were only in Day 3 sampling of treatment and control groups and by Day 9 both groups have similar root length.

### 5.4.2 Heat shock experiments

The heat priming regime of 38°C for 4 h daily was applied to the treatment group on 11 DAT and continued till 20 DAT. On 21 DAT (corresponding to after 10 days of heat priming), both the control plants (25°C RZT) and heat primed plants (25°C-RZT → 38°C-RZT) were subjected to 46°C heat shock for 6 h daily from 10 am to 4 pm. Shoot and root FW was analyzed for both groups on 14, 17, 20 and 26 DAT which corresponds to after 3, 6, 9 days of heat priming (38°C) for the treatment group and 5 days of heat shock (46°C for 6 h daily) for both plant groups.
Generally, for all four plant species, shoot FW significantly increased over time (Arugula: \( F_{(3, 24)} = 395.9, p < 0.001 \); Canasta: \( F_{(3, 24)} = 40.2, p < 0.001 \); RIL 141: \( F_{(3, 24)} = 182.5, p < 0.001 \); RIL 192: \( F_{(3, 24)} = 16.7, p < 0.001 \)). Except for RIL 192 (\( F_{(1, 24)} = 2.9, p = 0.106 \)), the other 3 vegetables all had significantly higher shoot FW over the control plants after being subjected to 5 days of heat shock (Arugula: Figure 5-2A, \( F_{(1, 24)} = 109.4, p < 0.001 \); Canasta: Figure 5-2B, \( F_{(1, 24)} = 2.6, p = 0.025 \); RIL 141: Figure 5-2C, \( F_{(1, 24)} = 21.86, p < 0.001 \)).

Root morphology development of the four vegetables generally followed a similar pattern as the shoot with statistically significant growth over time except for Arugula control plants (Arugula: \( F_{(3, 24)} = 30.0, p < 0.001 \); Canasta: \( F_{(3, 24)} = 17.3, p < 0.001 \); RIL 141: \( F_{(3, 24)} = 94.5, p < 0.001 \); RIL 192: \( F_{(3, 24)} = 18.0, p < 0.001 \)). While heat primed Arugula (Figure 5-2E, \( F_{(1, 24)} = 17.0, p = 0.001 \)) and RIL 141 (Figure 5-2G, \( F_{(1, 24)} = 17.2, p = 0.001 \)) both had significantly larger root FW compared to control plants, Canasta (Figure 5-2F, \( F_{(1, 24)} = 1.6, p = 0.227 \)) and RIL 192 (Figure 5-2H, \( F_{(1, 24)} = 0.44, p = 0.516 \)) showed no difference in root FW for heat primed and control plants. Arugula (Figure 5-2I), Canasta (Figure 5-2J), and RIL 141 (Figure 5-2K) all showed significantly higher shoot/root ratio in heat primed plants over control plants 26 DAT (5 days post heat shock) while RIL 192 had similar shoot/root ratio for both treatment groups at 26 DAT (5 days post heat shock).

Light response curves generally showed a higher ETR and qP for the control plants over the heat primed plants (Figure 5-3). While Arugula and Canasta had similar NPQ for both treatment groups, RIL 141 showed higher NPQ for control plants while RIL 192 showed higher NPQ for heat primed plants.
Figure 5-2. Productivity of Arugula, Canasta, RIL 141 and RIL 192, showing shoot FW (A, B, C, D) root FW (E, F, G, H) and shoot/root FW ratio (I, J, K, L). Control plants were grown in constant 25°C-RZT while treatment plants were grown in 25°C-RZT till 11 DAT and then heat primed for 4 h daily from 1000 – 1400 h at 38°C-RZT (25°C-RZT → 38°C-RZT). From 20 DAT both treatment groups were heat shocked daily for 4 h from 1000 – 1400 h at 46°C-RZT. Each point is mean ± SE (n=5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-3. Light response curves of Arugula, Canasta, RIL 141 and RIL 192 showing ETR (A, B, C, D), qP (E, F, G, H) and NPQ (I, J, K, L). Control plants were grown in constant 25°C C-RZT while treatment plants were grown in 25°C C-RZT till 11 DAT and then heat primed for 4 h daily from 1000 – 1400 h at 38°C C-RZT (25°C C-RZT → 38°C C-RZT). The plants were analysed after 10 days of heat priming treatment, at 20 DAT. Each point is mean ± SE (n=5).
While heat primed Arugula showed significantly higher $P_{\text{max}}$ compared to control plants (Figure 5-4, $F_{(1,5)} = 15.36, p = 0.017$), RIL 192 had significantly high $P_{\text{max}}$ in control plants compared to heat primed plants ($F_{(1,5)} = 198.4, p < 0.001$). Canasta ($F_{(1,5)} = 0.692, p = 0.452$) and RIL 141 ($F_{(1,5)} = 0.692, p = 0.452$) showed no significant difference in $P_{\text{max}}$ for both control groups.

$F_{v}/F_{m}$ ratio was measured every morning 7am after heat shock at RZT 46°C began (21 DAT). $F_{v}/F_{m}$ ratio was consistently significantly higher in heat primed plants for all four vegetables (Fig 5-5A, Arugula: $F_{(1,18)} = 59.9, p < 0.001$; Fig 5-5B, Canasta: $F_{(1,18)} = 33.0, p < 0.001$; Fig 5-5C, RIL 141: $F_{(1,18)} = 56.2, p < 0.001$; Fig 5-5D, RIL 192: $F_{(1,18)} = 217.6, p < 0.001$). Interestingly, while $F_{v}/F_{m}$ ratio was still significantly higher in heat primed plants for RIL 192 on 26 DAT, the difference was not statistically significant for Arugula, Canasta and RIL 141 between the two treatment groups by 26 DAT.

### 5.4.3 Post-harvest quality experiments

For the post-harvest quality experiments, plants were grown in constant RZT of 25°C till 11 DAT. Half of the plants were then subjected to a 38°C heat priming for 4 h a day (10 am to 2pm) daily till 21 DAT (10 days of priming). Plants kept in 25°C RZT till 21 DAT were then divided into two groups: CC plants were kept in 25°C RZT till 30 DAT while CH plants were given a RZT-46°C heat shock for 6 h daily (10 am till 4 pm) from 21 DAT till 30 DAT. CH plants were left in ambient RZT when not under heat shock. Plants that were heat primed at 38°C were also divided into two groups: HC plants were returned to constant RZT-25°C while HH plants were left in ambient RZT and given a RZT-46°C heat shock daily for 6 h (10 am to 4 pm). Plants were harvested on 30 DAT and examined for their post-harvest qualities.

Shoot FW on 30 DAT was significantly different among treatment groups for all four vegetables (Fig 5-6A, Arugula: $F_{(3,11)} = 159.7, p < 0.001$; Canasta: $F_{(3,11)} = 457.5, p < 0.001$; RIL 141: $F_{(3,11)} = 185.0, p < 0.001$; Fig 5-5D, RIL 192: $F_{(3,11)} = 362.8, p < 0.001$) while CH
plants had significantly lower shoot FW compared to the other treatment groups. The pattern was more varied for root FW on 30 DAT (Figure 5-6B). Arugula ($F_{(3,11)} = 301.2, p < 0.001$), Canasta ($F_{(3,11)} = 8.9, p = 0.006$) and RIL 141 ($F_{(3,11)} = 35.7, p < 0.001$) all had significantly different root FW but there was no significant difference in root FW of RIL 192 ($F_{(3,11)} = 2.2, p = 0.163$).

![Graph](image)

Figure 5-4. $P_{\text{max}}$ of Arugula, Canasta, RIL 141 and RIL 192. Control plants were grown in constant 25°C-RZT while treatment plants were heat primed at 38°C-RZT for 4 h daily from 1000 – 1400 h (25°C RZT $\rightarrow$ 38°C RZT), from 11 DAT. The plants were analysed after 10 days of heat priming treatment, on 20 DAT. Each bar is mean $\pm$ SE (n=5). Each species was analysed separately. Asterisks denote significant differences between treatment groups.
Figure 5-5. $F_v/F_m$ ratios of Arugula, Canasta, RIL 141 and RIL 192. Control plants were grown in constant 25°C-RZT while treatment plants were grown in 25°C-RZT and then heat primed at 38°C-RZT for 4 h daily from 1000 – 1400 h (25°C-RZT → 38°C-RZT), from 11 DAT. From 20 DAT, both treatment groups were heat-shocked at 46°C-RZT for 6 h daily between 1000 – 1600 h. $F_v/F_m$ ratios was measured at 0700 h the day after the start of the heat shock regime. Each bar is mean ± SE (n=5). Asterisk marks denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-6. Productivity of (A) Shoot FW and (B) Root FW. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 -22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
The vegetables were then individually stored in 4°C in darkness with the roots intact and vegetables were analyzed every five days for their post-harvest qualities. Arugula showed significantly different TRN between the four treatment groups upon immediate harvest (Day 0 of storage, Figure 5-7A, \( F_{(3,48)} = 152.8, p < 0.001 \)) but TRN remained stable during the storage days and did not show any significant change over time (\( F_{(3,48)} = 1.5, p = 0.234 \)). The other three vegetables neither showed significant differences between treatment groups (Canasta: \( F_{(3,48)} = 0.648, p = 0.234; \) RIL 141: \( F_{(3,48)} = 1.1, p = 0.362; \) RIL 192: \( F_{(3,48)} = 9.064, p = 0.052 \)) nor over storage time (Canasta: \( F_{(3,48)} = 1.8, p = 0.167; \) RIL 141: \( F_{(3,48)} = 1.5, p = 0.081; \) RIL 192: \( F_{(3,48)} = 0.793, p = 0.507 \)).

Arugula showed significant difference between treatment group for soluble (Figure 5-8A, \( F_{(3,48)} = 10.2, p < 0.001 \)) and insoluble sugar (Figure 5-8B, \( F_{(3,48)} = 72.8, p < 0.001 \)), and also a significant drop over time for both soluble (\( F_{(3,48)} = 5.7, p = 0.003 \)) and insoluble sugar (\( F_{(3,48)} = 24.5, p < 0.001 \)). Conversely, Canasta showed no significant difference between treatment group (Figure 5-8C, \( F_{(3,48)} = 1154.1, p = 0.555 \)) and between harvest time (\( F_{(3,48)} = 0.003, p = 1 \)) for soluble sugar, but showed significant difference both between treatment group (Figure 5-8D, \( F_{(3,48)} = 39.5, p < 0.001 \)) and between harvest time (\( F_{(3,48)} = 5.1, p = 0.005 \)) for insoluble sugar. RIL 141 was similar to Canasta and showed no significant difference between treatment group (Figure 5-8E, \( F_{(3,48)} = 4.64, p = 0.08 \)) and between harvest time (\( F_{(3,48)} = 1.3, p = 0.282 \)) for soluble sugar, but showed significant difference both between treatment group (Figure 5-8F, \( F_{(3,48)} = 73.2, p < 0.001 \)) and between harvest time (\( F_{(3,48)} = 24.3, p < 0.001 \)) for insoluble sugar. RIL 192 showed significant difference between treatment groups for both soluble (Figure 5-8G, \( F_{(3,48)} = 36.1, p = 0.029 \)) and insoluble sugar (Figure 5-8H, \( F_{(3,48)} = 75.1, p < 0.001 \)). Both soluble sugar (\( F_{(3,48)} = 3.42, p = 0.092 \)) and insoluble sugar (\( F_{(3,48)} = 1.78, p = 0.171 \)) did not change significantly over time for RIL 192.
Figure 5-7. TRN of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 -22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of TRN. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-8. Soluble and insoluble sugar content of (A) Arugula, (C) Canasta, (E) RIL 141, (G) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of soluble and insoluble sugar content. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
There was significant difference in total ascorbic acid content between the treatment groups, with HH plants significantly higher than CC plants for all four vegetables species (Figure 5-9, Arugula: $F_{(3,48)} = 237.4, p < 0.001$; Canasta: $F_{(3,48)} = 136.5, p < 0.001$; RIL 141: $F_{(3,48)} = 136.5, p < 0.001$ and RIL 192: $F_{(3,48)} = 136.5, p < 0.001$). Ascorbic acid level significantly decreased with time in storage with Day 15 significantly lower than Day 0 for all four vegetable species (Arugula: $F_{(3,48)} = 18.5, p < 0.001$; Canasta: $F_{(3,48)} = 21.5, p < 0.001$; RIL 141: $F_{(3,48)} = 21.5, p < 0.001$ and RIL 192: $F_{(3,48)} = 21.5, p < 0.001$).

There was significant difference in phenolic compound levels between the treatment groups, with HH plants significantly higher than CC plants for all four vegetables species (Figure 5-10, Arugula: $F_{(3,48)} = 279.9, p < 0.001$; Canasta: $F_{(3,48)} = 177.8, p < 0.001$; RIL 141: $F_{(3,48)} = 177.8, p < 0.001$ and RIL 192: $F_{(3,48)} = 177.8, p < 0.001$). Phenolic compound levels remained relatively stable with time in storage with no significant difference between Day 15 and Day 0 for all four vegetable species (Arugula: $F_{(3,48)} = 0.545, p = 0.655$; Canasta: $F_{(3,48)} = 0.142, p = 0.934$; RIL 141: $F_{(3,48)} = 0.142, p = 0.934$ and RIL 192: $F_{(3,48)} = 0.142, p = 0.934$).

There was significant differences in PPO levels between the treatment groups, with CC plants significantly higher than HH plants on day 20 of storage for all four vegetables species (Figure 5-11, Arugula: $F_{(3,48)} = 96.1, p < 0.001$; Canasta: $F_{(3,48)} = 187.4, p < 0.001$; RIL 141: $F_{(3,48)} = 187.4, p < 0.001$ and RIL 192: $F_{(3,48)} = 187.4, p < 0.001$). PPO levels increased significantly with time in storage such that levels for Day 15 plants were significantly higher than levels for Day 0 plants for all four vegetable species (Arugula: $F_{(3,48)} = 252.103, p < 0.001$; Canasta: $F_{(3,48)} = 218.0, p < 0.001$; RIL 141: $F_{(3,48)} = 218.0, p < 0.001$ and RIL 192: $F_{(3,48)} = 218.0, p < 0.001$).
Figure 5-9. Ascorbic acid content of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of ascorbate content. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-10. Total phenolic content of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of phenolic content. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-11. PPO activity levels of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of PPO activity. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Arugula showed no significant difference in POD levels between treatment groups (Figure 5-12, $F_{(3,48)} = 3.23, p = 0.053$) and no significant difference over time ($F_{(3,48)} = 23.3, p = 0.088$). For the other three vegetable species, there was significant difference in POD levels between the treatment groups, with CC plants significantly higher than HH plants for Canasta ($F_{(3,48)} = 461.8, p < 0.001$), RIL 141 ($F_{(3,48)} = 461.8, p < 0.001$) and RIL 192 ($F_{(3,48)} = 624.6, p < 0.001$). POD levels increased significantly with time in storage such that levels for Day 15 plants were significantly higher than levels for Day 0 plants for Canasta ($F_{(3,48)} = 758.7, p < 0.001$), RIL 141 ($F_{(3,48)} = 758.7, p < 0.001$) and RIL 192 ($F_{(3,48)} = 690.1, p < 0.001$).

There was significant differences in PAL levels between the treatment groups, with CC plants significantly higher than HH plants on day 20 of storage for all four vegetables species (Figure 5-13, Arugula: $F_{(3,48)} = 14.8, p < 0.001$; Canasta: $F_{(3,48)} = 240.9, p < 0.001$; RIL 141: $F_{(3,48)} = 240.9, p < 0.001$ and RIL 192: $F_{(3,48)} = 240.9, p < 0.001$). PPO levels increased significantly with time in storage such that levels for Day 15 plants were significantly higher than levels for Day 0 plants for all four vegetable species (Arugula: $F_{(3,48)} = 38.9, p < 0.001$; Canasta: $F_{(3,48)} = 175.4, p < 0.001$; RIL 141: $F_{(3,48)} = 175.4, p < 0.001$ and RIL 192: $F_{(3,48)} = 175.4, p < 0.001$).
Figure 5-12. POD activity levels of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of POD activity. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
Figure 5-13. PAL activity levels of (A) Arugula, (B) Canasta, (C) RIL 141 and (D) RIL 192. All plants were grown in 25°C-RZT for 11 DAT, prior to the start of treatment. CC plants were left in constant 25°C-RZT throughout the experiment. CH plants continued to grow in constant 25°C-RZT till 22 DAT, and then moved to ambient-RZT, inclusive of a daily heat shock treatment at 46°C-RZT from 1000 – 1600 h till 30 DAT. Both HC and HH plants were heat primed, 12 - 22 DAT, at 38°C-RZT for 4 hours daily between 1000 – 1400 h. HC plants resumed their growth in 25°C-RZT after heat priming, till harvest. HH plants were further exposed to heat shock treatment at 46°C-RZT daily from 1000 – 1600 h, 23 DAT, and left to grow in ambient-RZT till harvest. Whole plants were harvested 30 DAT and kept in the dark at 4 °C prior to the measurement of PAL activity. Each bar is mean ± SE (n = 5). Different letter groups denote significant differences between treatment groups. Each species was analysed separately.
5.5 Discussion

This chapter reported the effects of heat priming on productivity of temperate vegetables when exposed to high heat stress and heat priming effects on post-harvest qualities of vegetables. Generally, heat primed plants had comparable productivity as control plants and higher nutritional values in terms of ascorbic acid and phenolic compounds.

5.5.1 Heat priming experiments

In the initial heat priming phase, root morphology and development of heat primed plants (25°C-RZT → 38°C-RZT) were compared to control plant (25°C-RZT) every 3 days (Fig 5-1). Arugula, Canasta and RIL 141 all had longer root length, larger root surface area and increasing number of root tips as time progressed but there was no significant growth in root length for RIL 192. However, as this was observed in both the control and heat primed plants this was probably a species trait of RIL 192 to have a slightly slower root length growth period (beyond 20 DAT) for there was also no significant increase in root tips over time for RIL 192. The increase in number of root tips translates to active growing roots (He and Lee, 1998; He, 2009) so based on Figure 5-1 both control and heat primed plants are healthily growing. The fact that there was no significant difference between treatment groups for nearly all of the parameters (especially on Day 9 after heat priming began) showed that the priming temperature did not affect healthy growth of roots of the heat primed plants. Considering that RZT-36°C produced very attenuated roots and significantly reduced number of root tips and root length for Chapter 2 (reduced root FW with high RZTs, He et al., 2009; He et al., 2013), it is both interesting and significant that the 38°C priming here did not affect root morphology.

There could be a number of reasons for the difference in root morphology observed here: 1) the priming did not begin under 11 DAT and it could be that the inhibitory effects of high RZT on root morphology reported by He et al. (2009) or He (2010) is less significant once root development is established (post 10 DAT), 2) the priming only occurs for 4 hours and
RZT was returned to 25°C throughout the night which facilitated recovery and active root growth (Head 1965; Mahmud et al., 2018), whereas the RZT-36°C plants discussed in Chapter 2 were kept constantly at RZT-36°C. More importantly, Figure 5-1 showed that the heat priming did not have a detrimental effect on morphology of all four vegetables species.

5.5.2 Heat shock experiments

Both heat primed and control plants had similar shoot and root FW during the heat priming period (Figure 5-2). However, when both groups of plants were subjected to 46°C heat shock, heat primed plants of Arugula, Canasta and RIL 141 all performed better than the control plants. RIL 192 did not have any difference in shoot and root FW between the two treatment groups but this could be linked to the slower development of root systems of this vegetable compared to the other 3 species.

For Arugula, Canasta and RIL 141 this finding mirrored the reports that priming improved heat tolerance in Salvia cultivars (Natarajan and Kuchny, 2008) and creeping bentgrass (Xu and Huang, 2000). Purportedly the better performance of heat primed plants could be due to the production of hsp70 to maintain cellular function and photosynthetic processes during events of heat stress. Prior priming at 38°C has attuned the plants to be more sensitive to heat stress while control plants subjected to 46°C suddenly found their heat shock responses deactivated by the high temperature. This corresponded to the hsp kinetics observed in Chapter 4 where higher temperature attenuated hsp70 production.

Light response curves in Figure 5-3 showed higher ETR in control plants than heat primed plants but almost similar qP and NPQ between treatment groups. The lower ETR in heat primed plants could be a protective mechanism against photoinhibition (Karim et al., 2002; Guo et al., 2006; Mal noe, 2018). Interestingly, RIL 141 which is thermotolerant has a lower NPQ in heat primed plants while RIL 192 which is thermosensitive had higher NPQ in heat primed plants. This difference could be why heat primed RIL 141 had higher productivity.
post heat shock compared to control plants while productivity of RIL 192 was comparable between control and heat primed plants.

Similarly, $P_{\text{max}}$ in Figure 5-4 was significantly lower in heat primed 192 but was significantly lower in control Arugula, suggesting that RIL 192 did not respond well to the heat priming while heat primed Arugula could adapt better when exposed to 46°C heat shock. Compared to the other three vegetables, both control and heat primed RIL.192 had no significant increase in root length and number of root tips in the initial phase of growth (Figure 5-1). Assuming that the heat sensitive period in root development in RIL192 extends beyond 10 DAT, heat priming might have affected root development ultimately, which might have affected subsequent root hydraulic conductivity (Dodd et al., 2000), which in turn resulted in inefficient transpiration (water stress) of leaves in the middle of the day (He et al., 2009), which resulted overall lower photosynthesis and growth of heat shocked plants (He et al., 2001). Canasta and RIL 141 had similar $P_{\text{max}}$ between treatment groups but heat primed plants all had higher productivity in the end, suggesting that control plants of Canasta and RIL 141 had to divert some of the energy into acclimating to the 46°C heat shock and sacrificed somatic growth (especially synthesis of ATP-dependent hsp70: Goloubinoff et al., 2018) whereas heat primed Canasta and RIL 141 could adapt to the 46°C heat shock better than the control group (Sanyal et al., 2018).

$F_v/F_m$ ratio in Figure 5-5 showed a very interesting trend: except for RIL 192, the control plants all had similar $F_v/F_m$ ratio compared to the heat primed plants by day 5 of heat shock at 46°C. This implied that even though the control plants of Arugula, Canasta and RIL 141 were not primed before exposure to 46°C, this heat shock event was a priming experience for the control plants: unless the high temperature resulted in irreversible damage or mortality in the plants, the plant could gradually adapt to the high temperature eventually. Although control plants would have paid a much higher physiological price to adapt to the 46°C heat shock, as
evidential from the lower shoot FW, a dynamic equilibrium would be established sooner or later, hence the recovery of Fv/Fm ratio. This observation draws a parallel comparison with a study of chronic heat stressed vegetables by Lai and He (2016) where plants subjected to constant RZTs of 25, 28, 32 and 36°C all had comparable Fv/Fm ratio eventually.

5.5.3 Post-harvest quality experiments

As seen in Figure 5-6, CH plants basically were affected productivity wise and had much less shoot FW. Interestingly, root development or biomass was not affected by the heat shock event with or without priming. This is probably due to the fact that the 46°C heat shock was only administered 21 DAT, which further supports the hypothesis that high RZT will only affect crop performances in the initial root development period of 0 DAT to 10 DAT. This in turn has huge commercial implications in that a lot of electricity could be saved for cool RZT would only need to be maintained for 10 days in the future instead of 20–30 days for a crop cycle. For RIL 192, again, CH and HH plants both had lower shoot FW, further proving that RIL 192 is thermosensitive and heat priming, or at least this heat priming design, is not optimal for RIL 192 yet.

For post-harvest quality, crops were examined to determine if heat primed plants have higher nutritional values at the point of harvest, and if these nutritional values differ in retention rate between treatment group. The vegetables would also be examined if heat priming will affect the browning potential in storage.

For TRN (Figure 5-7), only Arugula showed significant differences between treatment groups with HC and HH plants having higher TRN compared to CH and CC plants. TRN values did not change with storage time.

Vegetables continue to respire even in storage after harvesting by utilizing stored food reserves. Hence, respiration rate is inversely proportional to the shelf-life duration and quality of fruits and vegetables during post-harvest storage (Hu et al., 2012). Depletion in soluble sugar
over time would indicate an increase in respiration intensity. For insoluble sugar, CC and HC plants tend to have higher insoluble sugar compared to CH and HH plants, probably due to the lack of 46°C heat shock to deplete the energy store. For all 4 vegetables, insoluble sugar mostly decreased with storage time, and were probably converted to soluble sugar to sustain respiration. Canasta and RIL 141 did not have significant difference between treatment groups nor change in soluble sugar content over time, but Arugula (CH) and RIL 192 (HH) plants both showed a distinct soluble sugar degradation over time. Overall, from Figure 5-8, the plants were not drastically affected by insoluble or soluble sugar depletion across the treatment groups. This could be a result of leaving the roots intact with the plants.

In Figure 5-9, HC and HH plants had higher total ascorbic acid in all four vegetables as compared to their CC and CH counterparts, showing that the heat priming affects accumulation and synthesis of this antioxidant. A similar trend was observed in phenolic compound analysis (Figure 5-10) where HH and HC plants tend to have higher phenolic compound compared to CC and CH plants. This agrees with the recent work with rocket salad by He et al. (2016) where higher RZT plants had higher amount of phenolic compounds.

For Figures 5-11, 5-12 and 5-13, with the exception of PAL in Arugula, there is a gradual increase in the PPO, POD and PAL levels over the storage time, with higher levels in CC and CH plants compared to HC and HH plants. This difference might be due to the higher content of ascorbic acids in HC and HH plants which curbs synthesis of the browning enzymes. Phenolic compound did not increase or decrease over the storage time, so PAL activity was probably also kept in check.

5.5.4 Conclusion

This chapter set out to evaluate: 1) if heat priming will enhance crop performance under harsher heat stress environment such that heat primed plants would have comparable productivity with control plants, and 2) if heat priming could enhance the post-harvest quality
of the vegetable crop even if productivity was compromised for heat stressed plants. Post-harvest qualities here refer to two key attributes: nutritional value of the crop and longer shelf-life of the crop to resist browning during the process of harvest, storage and transport to the point of sales. With the exception of RIL 192, it has been shown with Arugula, Canasta and RIL 141 that heat primed vegetables could have comparable, even greater, productivity compared to constantly RZT-cooled plants, but that heat primed vegetables would have higher post-harvest qualities in terms of ascorbic acid content and phenolic compounds. The results also suggested that RZT-cooling would be crucial for the initial phase of root development but RZT may not affect root and shoot development post 11 DAT.
CHAPTER 6

General discussion and conclusion
6.1 Stress indices and heat stress

It has been shown by various studies that high RZT negatively affects shoot and root productivity of temperate vegetables grown in tropical aeroponic greenhouse (*Lactuca sativa*: Choong et al., 2016; Arugula: He et al., 2016; *Brassica* spp: Srikanth et al., 2016) such that high/ambient RZT usually resulted in shorter roots, smaller root surface area, fewer root tips and thicker root diameters (Pardales et al., 1991; Tan et al., 2002; He et al., 2009). In the present study, both shoot and root productivity of vegetable grown in 25°C RZT were significantly higher than those grown in 28, 32, and 36°C RZT (Figure 3-1 and 4-1). This study differs from previous reports in that in addition to showing the detrimental effects of high RZT on plant productivity, a dose-response relationship has been established where productivity decreased proportionally with increasing RZT (Figure 3-1, Figure 4-1).

In comparing dose-response of physiological or photosynthetic performance with regard to heat stress, chronic stressed plants were typically affected at much lower temperatures than acute stressed plants (e.g. Pmax: compare Figures 3-4 and 3-11; ETR, qP and NPQ: compare Figures 3-9 and 3-15). This is most likely because chronic stress effects are often time integrated and daily maintenance of physiological processes and energy budgets are prioritized for heat stress mediation, with greater complexities than acute stress responses (Tattersall et al., 2007; Pörtner and Farrell, 2008; Cramer et al., 2011; Pinheiro and Chaves, 2011).

According to this point of view, the physiological performances of chronically stressed plants probably followed a cascading effect based on biological hierarchy of the measured parameters. Looking at Fv/Fm ratio of chronic stressed plants (Figure 3-10), one would assume that the different RZTs had no effect on the plants. These results indicated that dynamic PSII photoinhibition was rather mild or moderate for all the plants regardless of RZT (He et al., 2001; He and Lee, 2001; Eivand et al., 2018;). However, lower productivity in shoot and root tissue for plants in higher RZTs suggested a different story (Figure 3-1): a dose-dependent
decrease in photosynthetic O₂ evolution under saturated light conditions (P₂max: Figure 3-4) indicated a decrease in overall photosynthetic capacity of plants with increasing RZT which directly resulted in lower shoot and root growth (Figure 3-1). A$_{sat}$ and g$_{s, sat}$ both decreased significantly with increasing RZT (Figure 3-5), indicating stomatal closure as a result of high RZT (He, 2009; He et al., 2016). A$_{sat}$ decreased as a direct result of intercellular CO₂ depletion when stomata closure limited leaf gaseous exchange (Wong et al., 1985) and causing stomatal limitation of photosynthesis (He, 2009; He et al., 2016). Results observed in this study (Figure 3-5) concurs with the works of Gosselin and Trudel (1984) who showed that, in tomato plants grown in RZTs of 12, 18, 24, 30 and 36°C, that photosynthetic CO₂ uptake was optimal at 24°C-RZT but severely limited at 36°C-RZT. Although non-stomatal limitations of photosynthesis due to decreases of Fv/’Fm ratio and Chl content have been reported in butterhead lettuce (Lactuca sativa L. cv. Palma) by He et al. (2001), results of this study were more similar to those of He et al. (2016) working with salad rocket (Eruca sativa): Fv/’Fm ratio of plants grown in different RZTs were similar and close to 0.8 (Figure 3-10), and Car variation among the treatment plants was not statistically significant (Figure 3-19).

Shoot productivity (Figure 3-1) was the result of overall photosynthetic performances measured by P₂max of leaf discs under light- and CO₂-saturated conditions (Figure 3-4). It was also affected by A$_{sat}$ and g$_{s, sat}$ (Figure 3-5), measured only under light-saturated condition of intact plants in the greenhouse. Hence, the decrease in photosynthetic performance was probably deeply rooted in the development of the roots (He, 2016). Various studies have confirmed that poor root development is the cause of poor shoot growth when temperate vegetables were grown in tropical greenhouse setting (Tan et al., 2002; He et al., 2009; He, 2010). Reduction of root length (Figure 3-2A), increase in root diameter (Figure 3-2B) and reduction in number of root tip (Figure 3-2C) and root volume/surface area (Figure 3-2D) correspondingly reduces the efficiency of water and mineral uptake from the root, thus
affecting the plant’s ability to cool down leaf temperature and nutrient uptake from the root for plant growth (Vries et al., 2016; Pirnajmedin et al., 2017). The root thickening may be due to the synthesis of stress chemical signals such as ethylene (Arshad and Frakenberger, 2002; Qin et al., 2007), recently confirmed by our team (Choong et al., 2016). The cascading effect of physiological effects ultimately adversely affected shoot productivity and overall growth of the plant.

6.2 Stress indices and thermotolerance determination

Pörtner (2002) advocates that organismal thermal sensitivity is described by capacity failure in the most complex and integrated biological process, meaning that processes like sexual reproduction would be disrupted or ceased under stress (pejus=getting worse thresholds, Shelford, 1931) before physiological pathways, metabolic and enzymatic reactions and structural integrity become compromised (Pörtner, 2001). At critical temperatures, energy budget of the organism would be strategized to maintain the bare minimal cellular function to ensure survival, and with cessation of complex behaviours like foraging for energy intake and assimilation. Thus, the inherent available energy store directly affects survival time to outlast the stress event (Pörtner et al., 2005). Although frequently demonstrated in the animal kingdom (Taylor and Weibel, 1981), this study is a novel attempt to apply symmorphosis on plant species.

Interestingly, in acute heat stress experiments, significant decreases in photosynthetic light use efficiency measured by ETR, qP and NPQ generally manifested at a lower range of temperatures from 28 to 38°C, (Figure 3-15) compared to P_{max} at a higher range of 34–44°C (Figure 3-11). However, LT_{50} values of ETR, qP and NPQ were either similar to that of P_{max} or significantly higher for both chronic and acute heat stressed plants (except Paternal plant in acute heat stress, Figure 3-21). This implies that among parameters of chlorophyll fluorescence, ETR, qP and NPQ might be more sensitive in detecting heat stress compared to
Fv/Fm ratio (compare Figures 3-9 and 3-10 for chronic stress and Figures 3-15 and 3-16 for acute heat stress) although capacity failure would at first sight appear to be similar for these parameters (except Paternal plant in acute heat stress, Figure 3-21). However, it is to be noted that ETR, qP and NPQ were all measured under actinic illumination, and were, therefore, subject to control by processes downstream of PSII. By contrast, Fv/Fm is a parameter measured in the dark-relaxed state and is thus a less complex parameter.

Instead of higher NPQ, lower NPQ was observed in all the vegetables grown under 36°C-RZT (Figure 3-9). High levels of NPQ were typically associated with higher level of Car content (Camejo et al., 2005; Usman et al., 2014), so it is likely that similar to the results reported by He et al. (2016) Chl and Car composition were not major factors in different manifestation of heat stress in this study (Figure 3-19).

In this study, capacity failure of the physiological parameters agreed with the heat tolerance principle (Pörtner et al., 2005): capacity failure occurred at the lowest temperature for shoot and root FW, followed by Pmax, Asat and gs sat while thermal failures of Fv/Fm ratio, ETR, NPQ and qP were among the highest. Chl pigment breakdown was not observed in this experiment even at extreme temperatures, but Chl breakdown generally signifies permanent irreparable damage to photosynthesis (Kasa et al., 2015). This is a novel approach to assessing thermotolerance or thermosensitivity of plant where in addition to choosing the right physiological index in assessing stress (e.g. Haldimann and Feller, 2004; Strasser et al., 2004), it is proposed here that rather than just monitoring stress effect manifestations, looking at capacity failures of each stress index might shed more light on strategies of plant energy budgeting and inherent thermobiology.

It should be pointed out that in this experiment, while chronic heat stressed plants were exposed to high RZTs, acute heat stressed plants were mainly assessed using excised leaf discs where heat (and light stress) was applied to shoot tissue directly. As plant responses to
environmental stresses are largely systemic, future experiments should be designed where whole plants are placed in heat chambers to further elucidate the effects of acute heat stress on physiological and photosynthetic performances.

6.3 Hsp70 and thermotolerance

Heat shock responses are imperative in acquiring thermotolerance (Usman et al., 2014). Stress proteins and chaperone molecules are important in acclimating to temperature stress. A large portion of the stress proteins are water soluble, and in this way, contribute to stress tolerance by means of hydrating cellular structures (Hanif and Wahid, 2018). Although heat shock proteins (hsp) are exclusively involved in heat-stress responses, other proteins are also additionally included. However, for ease of analysis, only the highly conserved hsp70 was analyzed in this study (Sung et al., 2001; Renner and Waters, 2007). In this study, it is shown that even though plants were grown in constant RZT of 25, 28, 32 and 36°C a heat shock response could still be detected in the shoot tissue at noon time (Figure 4-5B). Although there are some studies looking into promoting hsp70 synthesis in plants (e.g. Ghanem et al., 2011), very few papers examine hsp70 dose-response relationships with heat stress. Based on the hsp70 synthesis relationship with absolute temperature and temporal kinetics experiment (Figures 4-7 and 4-8), 38°C would be a good target temperature for sub-lethal priming experiments with regards to the four temperate vegetables.

It should be noted that acquired thermotolerance is a systemic resistance that involves the expression of several pathways like ROS-scavenging antioxidants, abscisic acid and salicylic acid (Larkindale and Huang, 2004; Charng et al., 2006). Even among the hsps over-expressed in heat shock cells, hsp70 is but only one of the several chaperone molecules that work in synergy (Hupalo et al., 2018). Preferentially, a 2-D gel electrophoresis would be preferred to capture the overall regulation of protein expression in a heat-stress event, especially if the mechanisms of thermotolerance conferment is to be evaluated. This study is a
starting point to first explore if organismal survival and physiological/photosynthetic performances of heat primed plant could be observed in extreme heat shock events and hence only hsp70 was used to first evaluate the presence of hsr. Further work would be necessary to both elucidate the mechanisms of heat tolerance in heat primed plants and to fine tune heat priming manipulations. Moreover, genetically engineered plants could be designed to determine if thermostolerance could be directly derived via intrinsic overexpression of hsp70.

6.4 Heat prime experiment designing and post-harvest qualities

The heat priming experiment was designed based on the findings in Chapter 2 and Chapter 3: 1) LT50 of Fv/Fm ratio (46°C, Figure 3-21) approaches the critical temperature of the vegetables where photochemistry performance of PSII is reduced by 50% and 2) a heat shock response could be observed in vegetables heated at 38°C (Figure 4-7 and 4-8). Thus 38°C was used as a heat priming temperature while 46°C was used as a critical heat shock temperature.

For the priming experiment, it was determined that the 38°C priming temperature had no detrimental effects on roots (Figure 5-1). This is a huge departure from reports that report hot ambient RZT resulted in poor root development (He and Lee, 1998; Tan et al., 2002; He, 2009; He et al., 2009) and from the observations of this study that 36°C-RZT significantly attenuated root development of lettuce species (Plate 3-1, Figure 3-1). This is likely due to the two different treatments here compared to the constant RZT in chronic heat stress experiments: 1) the priming temperature was not applied until 11 days after transplantation (DAT) and 2) the 38°C priming temperature was only applied for 6 h and RZT was returned back to 25°C outside of the priming timing. Choong (2018) has recently shown that aeroponically grown plants are only heat sensitive in the initial phase of transplantation and root development. After root establishment in the aeroponics system the plants are less susceptible to ambient RZT affecting photosynthesis. It has also been reported that root growth is more active at night (Head, 1965; Mahmud et al., 2018), and the cooled RZT at night could allow the heat-primed
plants to recover and actively develop their roots. Extensive root growth in turn confers thermotolerance via increasing transpiration and cooling of leaf temperature during heat stress events (He et al., 2001; Tan et al., 2002; He et al., 2009). In the chronic heat stress experiments (Chapter 3), although RZTs were all lower than the 38°C priming temperature (e.g. Figure 3-1), they were kept constant 24 h and throughout the growing period from 0 DAT to eventual harvest. Hence, plants subjected to the 36°C constant RZT suffered the most from the heat stress, starting with poorly developed roots (Figure 3-2), compromised photosynthetic processes (ETR, qP, NPQ: Figure 3-9, A_sat and gs_sat: Figure 3-5 and P_max: Figure 3-4) and yet have the highest energy expenditure in terms of hsp70 synthesis (Figure 4-5), thus resulting in the smallest somatic growth and productivity (Figure 3-1). Conversely, heat-primed plants were offered a recovery from the heat stress, and with a heat primed heat shock response, would have comparable and even better root development (Figure 5-1) compared to control plants constantly kept at 25°C RZT (Natarajan and Kuehnev, 2008; Mahmud et al., 2018).

Similarly, acquired thermotolerance has been found in both woody and herbaceous plant species. It was reported that heat hardening in redbud (Cercis canadensis L.) seedlings increased leaf thickness and plant growth (Griffin et al., 2004). Seedling survival and recovery growth also increased after priming seedlings of sunflower (Helianthus annuus) (Senthil-Kumar et al., 2003). However, in this study RIL 192 did not respond as well to the priming effect compared to the other three vegetables (Figure 5-6). This coincides with the study showing that plant responses to hardening vary with the extent of temperature increase, its duration and the type of plant (Wahid et al., 2007).

Originally, it was intended that hsp70 levels of the plants would be assessed at various stages of development (e.g. during priming, during heat shock, during recovery at night). Unfortunately, when growing plants for the heat priming and heat shock experiments, the vegetables were attacked by squirrels invading the greenhouse, despite multiple growing
attempts and interference measures like putting up rodent/squirrel repellents (ultra-sonic waves). As the foraging effects of the squirrels would likely induce a stress response, hsp synthesis analysis was later dropped.

Looking at post-harvest qualities of the heat primed vegetables: 1) they had comparable shoot productivity compared to control plants (Figure 5-6); 2) they generally had higher levels of ascorbic acid (Figure 5-9) and phenolic compounds (Figure 5-10) compared to control plants; 3) they generally had lower browning enzyme activities (Figures 5-11 to 5-13) which all translates to commercial values in biomass in harvesting, higher nutritional values, and longer shelf life for storage and transport without browning effects. A greater abundance of phenolic compounds has also been reported in heat primed rocket salad recently (He et al., 2016).

Plant phenolic compounds and ascorbic acid are essential for human diet and are of considerable interest due to their antioxidant properties (Balasundram et al., 2006). It has also been reported that low growth temperature decreased the content of some phenolic compounds in pea (Pisum sativum L.) seedlings (Rudikovskaya et al., 2008). Heat and light stress lead to the production of reactive oxygen species. Anti-oxidants such as ascorbic acid and phenolics compounds are produced by plants in order to resist oxidative stress (Bita and Gerats, 2013).

6.5 Overall conclusion and future works

In assessing a plant’s response to any environmental stress, it would be necessary to use a suite of stress indices which spans across different biological hierarchy. As the strategy and prioritizing energy budget for the multitudes of plant processes would be very complex, it would not be easy to determine if a plant is thermotolerant or thermosensitive. That said, looking at the capacity failure temperatures of the various physiological and photosynthetic performances together with heat shock response would be a comprehensive approach in
determining thermobiology, optimal thermal window of operation and critical temperatures of a plant species.

Arising from this study, heat priming could be applied to some of the temperate vegetables to enhance productivity and nutritional values of the crop. In face of global warming, this could boost crop productivity without resorting to genetic engineering or use of harmful chemicals in chemical priming. Although heat priming by itself may not speed up or greatly enhance productivity compared to plants with cool RZT, heat primed plants would be able to withstand stressors like excess light and heat, and additional LED growth lights could be employed to enhance plant growth as opposed to relying on natural insolation to reduce the growth period while augmenting shoot productivity. Taking the plant growth in the heat shock experiments for this study (Figure 5-2), despite heat priming and the huge difference between shoot productivity of control and heat-primed plants upon subject to heat shock, shoot FW was still less than 10 g on 26 DAT when normal harvest cycle would have ended by 28–35 DAT. This was due to a long period of cloudy and rainy rains depriving the plants of insolation for accelerated growth. While growth light might alleviate this problem, on sunny days with full insolation (>600 PPFD) this might prove stressful for plants. Heat priming would then be a perfect solution in turning this stressful event into a highly productive growth period.

Moreover, by demonstrating that plants may not be so heat-sensitive upon completion of root development this could decrease the energy expenditure of greenhouse and aeroponics systems by increasing the RZT set points. Although it may be argued that with the application of heat source for heat priming electricity would still be utilized in lieu of the cooling, it should be noted that the normal regime of aeroponic spray of 1 min spray and 5 min rest would be sufficient to generate enough heat for the root zone chamber to reach ~37°C even at night. For experimental purposes (and precision in temperature control) an external heater was employed
for this study, but logistically it would be possible to maintain a heat source for heat priming with just the regular operation of the aeroponic system.

With finer exploration of heat priming regime (magnitude, period of application and duration) productivity and post-harvest qualities of temperate vegetables could be better enhanced. However, to better design appropriate heat priming regimes, basic thermal biology and heat shock responses of vegetables should be explored diligently for all potential temperate crops to be developed for Singapore aeroponic cultivation.


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