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Influence of Environmental Stress toward Carotenogenesis Regulatory Mechanism through *In Vitro* Model System

Rashidi Othman, Norazian Mohd Hassan and
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Abstract

Carotenoid biosynthesis is influenced by some aspects and is liable to geometric isomerisation with the existence of oxygen, light, and heat, which affect color degradation and oxidation. The major problems related to carotenoid accumulation inherently originate from pigment instability. This chapter discusses an overview on the influence of stringent control of genetic, developmental, and environmental factors toward carotenoid biogenesis in potato minitubers through the potential model system for rapid initiation, extraction, and analysis of carotenoids. The outcome of this experimental system is a discovery of variables regulating carotenoid accumulation as a result of the environmental change assessment through manipulation of drought stress, light intensity, and nutrient strength on carotenoid accumulation.

Keywords: carotenogenesis, environmental stress, *in vitro*, model system, elicitors

1. Introduction

Considerable research interest has recently focused on the improvement of both transgenic and conventional propagation techniques to enrich total and individual carotenoid composition in potatoes [1–4]. Unfortunately, little information is available on the influence of the environment on the carotenoid content in potatoes, especially growing seasons and locations. Genotype and environment interactions have been reported to account for alteration in free amino acids, protein, and sugar composition [5–13]. In addition, the total glycoalkaloid content of potato tubers was found greatly affected as a result of environmental changes during

the growing season [14], even though there are also strong genetic effects [15, 16]. Seasonal differences, growing conditions, locations, genotypes, and postharvest storage conditions are among the factors that can be significantly affecting the quality and nutritional value of potatoes [17–20]. The bioavailability of carotenoids is characterized as an intricate issue and influenced by various factors [21]. In our study of interseasonal and genotype interactions, the data revealed that variations in total carotenoid content and the concentration of individual carotenoid pigments are due to the strong relationship between genotype and growing seasons. This assumption is supported by Chloupek and Hrstkova [22] in their observations of 26 crops over a 43-year period growing seasons; where yield adaptability over time was controlled largely by weather and small variations from year to year in agronomical practices. In other words, major factors influencing yield are location, year, and their interactions. They also observed that yield variation of the 26 crops, including potato, in the Europe was greater than in the USA by nearly two times. In another case, the level of polyphenols in potatoes has been reported to have significant difference with environmental conditions and genetics [22]. A strong relationship and association between growing location of potato, the yellow color intensity in tuber flesh, and its total carotenoid content have also been reported. Report [1] demonstrated that environmental factors may affect on the yellow intensity of tuber flesh. The correlation between genotypes and environment can be indicative of the particular potato cultivar for best adapted to certain locations. For example, in 2004/2005 growing season in New Zealand, Agria was found to have a substantially higher carotenoid content relative to other cultivars with mostly lutein and no zeaxanthin, whereas in 2006/2007 Agria contained all five carotenoids with relatively high concentration of zeaxanthin [23]. A notable difference between the two seasons was the accumulation of zeaxanthin in 2006/2007 and the absence of zeaxanthin in 2004/2005.

pH can affect epoxidation and de-epoxidation reactions in the xanthophyll cycle [24]. Hydroxylation can convert α- and β-carotene to lutein and zeaxanthin, respectively. Violaxanthin is formed from zeaxanthin due to epoxidation and de-epoxidation that can transform violaxanthin back to zeaxanthin. This reaction sequence is reversible and mediated by pH [25, 26]. Epoxidation will occur in darkness or under low light condition and activity is optimal near pH 7.5 [27, 28], whereas de-epoxidation activity is active at pH below 6.5 and optimal at approximately pH 5.2 [24].

Zeaxanthin happens only in trace amounts under physiological conditions *in vivo* or without stress condition [29–31]. Nevertheless, zeaxanthin occurs upon de-epoxidation through the reversible xanthophyll cycle operation due to exposure under irradiance stress or high light condition [32, 33]. Although zeaxanthin accumulates during irradiance stress, that association is normally only transient. Upon recovery under low light or in darkness, zeaxanthin will disappear [33]. In addition, it was revealed from recent *in vitro* studies [34, 35], upon analysis of zeaxanthin accumulating *Arabidopsis thaliana* mutants [36–38] and from the green alga *Scenedesmus obliquus* [39], that zeaxanthin could replace lutein and violaxanthin under irradiance stress.

There are two possibilities to explain the accumulation of zeaxanthin in 2006/2007 season and not previous season:

(i) The conversion of individual carotenoids such as violaxanthin, neoxanthin to zeaxanthin is due to irradiance stress condition from high light exposure. This will promote the conversion of other carotenoids to zeaxanthin from the β-carotene and α-carotene branch point. As a result, zeaxanthin concentration will increase. Consequently, the precursor supply for ABA biosynthesis and the plant responds will inhibit as the carotenogenic metabolic flux increases to compensate for this restriction [29, 40]. Previous study [41] also reported that lutein and violaxanthin can transform to zeaxanthin successfully under irradiance stress condition.

(ii) The presence and absence of zeaxanthin are in response to alterations in pH. Acidity will trigger the de-epoxidation reaction by the conversion of violaxanthin and other precursors of ABA to zeaxanthin, whereas alkaline conditions will induce lutein or the supply of precursors for ABA biosynthesis, which will lead to the conversion of zeaxanthin to violaxanthin, neoxanthin or other precursors for ABA biosynthesis through epoxidation reaction [42].

Overall, this study clearly demonstrated that the total and the individual pigment content of carotenoids in potato tubers were depending on the growing season, subsequently, affect their quality and nutritional content. Thus, along with genotypic factors, environmental factors also take on an important part in regulating the accumulation of individual carotenoids in potato tubers, especially in Agria and Desiree. Between seasons, lutein has been transformed into zeaxanthin in Agria, whereas neoxanthin has been transformed into zeaxanthin in Desiree. These findings evidently suggest that selection of high or low carotenoid tuber levels cannot be established on the basis of a single year's results. Still, valid comparisons can be established between data from different years if the material is stored and developed under similar environmental conditions. This study suggests that environmental factors such as seasonal climatic variation may influence the accumulation of potato tuber carotenoids content and composition. Apparently, further research using potato plant materials produced under different environmental conditions are needed to support this theory.

2. Experimental design

2.1. Tissue culture and minituber initiation

Virus free *in vitro* plants of cultivars Agria and Desiree were supplied by the New Zealand Institute for Crop & Food Research Ltd. These were cultured in a incubation room at 24°C day and night temperature, with a 16-h photoperiod under cool white fluorescent light at 80–85 µmol m^{-2} s^{-1}. Every 4 weeks, the *in vitro* plants were subcultured as nodal cuttings on potato multiplication medium (PMM) composed of Murashige and Skoog (MS) salts and vitamins [43] added with 30 g/L sucrose, 40 mg/L ascorbic acid, 500 mg/L casein hydrolysate, and 10 g/L agar in accordance with the procedure of Conner et al. [44]. Media was adjusted to pH 5.7 and sterilized by autoclaving (15 min, 121°C) and 50 ml aliquots poured into presterilized 290 ml plastic bottles (80 mm diameter × 60 mm high; Vertex Plastics, Hamilton, New

Zealand). For minituber initiation, individual shoots of 3–4 nodes from vigorously growing 4-week-old cultures were transferred into 40 ml of liquid tuber initiation medium (TIM) in 250 ml polycarbonate culture vessels (7 cm diameter × 8 cm high). The TIM contained the same constituents as PMM, except with the addition of 80 g/L sucrose, 5 mg/L benzyladenine, 2.5 mg/L ancymidol, and no agar. Nine shoots were placed upright into each culture vessel and were incubated in darkness at 25°C. Minitubers were classified as such when their diameter exceeded 2 mm and normally grew up to more than 5 mm diameter within 4 weeks.

2.2. Effect of environmental factors on carotenoid biosynthesis

In three independent experiments, the influence of light, water stress, and nutrient availability on carotenoid biosynthesis were tested in both Agria and Desiree. Minitubers harvested after 4 weeks from two culture vessels were pooled for each of three replicates established under the following conditions:

1. Light versus darkness by incubation under cool white fluorescent light (80–85 $\mu mol\ m^{-2}\ s^{-1}$, 16 h photoperiod) with dark condition imposed by carefully wrapping the culture vessels in aluminium foil.

2. Incubation in darkness with and without 50 mM PEG 4000 to impose water stress.

3. Incubation in darkness at three concentrations of MS salts (one tenth, half, and full strength).

2.3. Minituber extraction and analysis of carotenoids

Minitubers were harvested and pooled for each replicated treatment, cut in half, and freeze-dried as combined skin and flesh samples for 7 days. The samples were then ground into fine powder and kept at −80°C until further analysis.

The extraction procedure followed the methods described in several reports [45–47]. 0.1 g of each powdered sample was rehydrated with distilled water and extracted with a mixture of acetone and methanol (7:3) at room temperature until colorless. The crude extracted was then centrifuged for 5 min at 10,000 g and stored in darkness at 4°C until analysis. The same volume of hexane and distilled water was added to the combined supernatants to extract carotenoids. The mixture was set aside until separation occurred and the upper layer holding the carotenoids was collected. The upper hexane layer was then removed using a gentle stream of oxygen-free nitrogen until the collected carotenoid was dried completely.

The carotenoids HPLC analysis was performed on an Agilent model 1100 series equipped with a binary pump, autosampler injector, micro vacuum degassers, thermostatted column compartment, and a diode array detector [45]. The column used was a Luna C18 end capped 5 μm, 250 × 4.6 mm reverse phase column (Phenomenex Auckland, New Zealand). The solvents used were (A) acetonitrile: water (9:1, v/v) and (B) ethyl acetate. The gradient of solvent used was developed as follows: 0–40% solvent B (0–20 min), 40–60% solvent B (20–25 min), 60–100% solvent B (25–25.1 min), 100% solvent B (25.1–35 min), and 100–0% solvent B (35–35.1 min) at

1.0 ml min^{-1} flow rate. The column temperature was maintained at 20°C and was allowed to reequilibrate in 100% solvent A for 10 min prior to the next injection. The volume of an injection was 10 μL. Carotenoid standards β-carotene, violaxanthin, lutein, and neoxanthin were isolated from *Eruca sativa* (roquette or rocket salad) by open column chromatography [48], whereas zeaxanthin was obtained commercially from Sigma-Aldrich (Auckland, New Zealand).

3. Results

3.1. Effect of light on carotenoid accumulation in potato minitubers

Statistical analysis demonstrated that there was a highly significant difference ($P < 0.0001$) in carotenoid content in Agria minitubers developing in the dark and light. Agra minitubers accumulated four individual carotenoid compounds (violaxanthin, zeaxanthin, lutein, and β-carotene) when developing in both dark and light. The two predominant carotenoids were violaxanthin and zeaxanthin. Neoxanthin was not detectable in either dark or light treatments. However, development of Agria minitubers in light resulted in an approximate doubling of the total carotenoid content compared minitubers developing in darkness (**Figure 1**). The amount of each individual carotenoid also approximately doubled upon development in light, especially for violaxanthin and zeaxanthin. Analysis of variance comparing Desiree minitubers grown in the dark and light also exhibits highly significant differences ($P < 0.0001$) in carotenoid content. As shown in **Figure 1**, five individual carotenoids (neoxanthin, violaxanthin, zeaxanthin, lutein, and β-carotene) were found in Desiree minitubers grown in darkness, but upon development in light only four (neoxanthin, violaxanthin, lutein, and β-carotene) were detected, with an absence of zeaxanthin. After development in light, total carotenoid content approximately doubled and reflected an increase in neoxanthin and violaxanthin.

## 3.2. Effect of PEG on carotenoid accumulation in potato minitubers

Analysis of variance revealed that there was a highly significant difference ($P < 0.0001$) in carotenoid content in response to the water stress treatment during development of Agria minitubers. Agria minitubers developing in the presence of PEG (**Figure 2**) exhibit an increased total carotenoid content. This increase reflected a substantially higher amount of violaxanthin and occurred despite the total absence of zeaxanthin in the presence of PEG. Analysis of variance also indicates highly significant differences ($P < 0.0001$) in carotenoid content for Desiree minitubers developing in the presence of water stress. As shown in **Figure 2**, total carotenoid content increased in minitubers developing in the PEG treatment. This reflected an increase in both neoxanthin and violaxanthin, with traces of lutein being observed in both treatments.

3.3. Effect of nutrient stress on carotenoid accumulation in potato minitubers

Nutrient stress during Agria minituber development resulted in a highly significant difference ($P < 0.0001$) in carotenoid content. When MS salt strength increased from 0.1× to 0.5×, total carotenoid, violaxanthin, and β-carotene content decreased, accompanied by a slight increase in lutein

concentration. However, when MS salt strength increased from 0.5× to 1.0×, total carotenoid, violaxanthin, and β-carotene increased, whereas lutein concentration decreased (**Figure 3**).

Analysis of variance also establishes highly significant differences ($P < 0.0001$) in carotenoid content in Desiree minitubers developing in varying MS salt strengths. As shown in **Figure 3**, when MS salt strength increased from 0.1× to 0.5×, total carotenoid content slightly increased
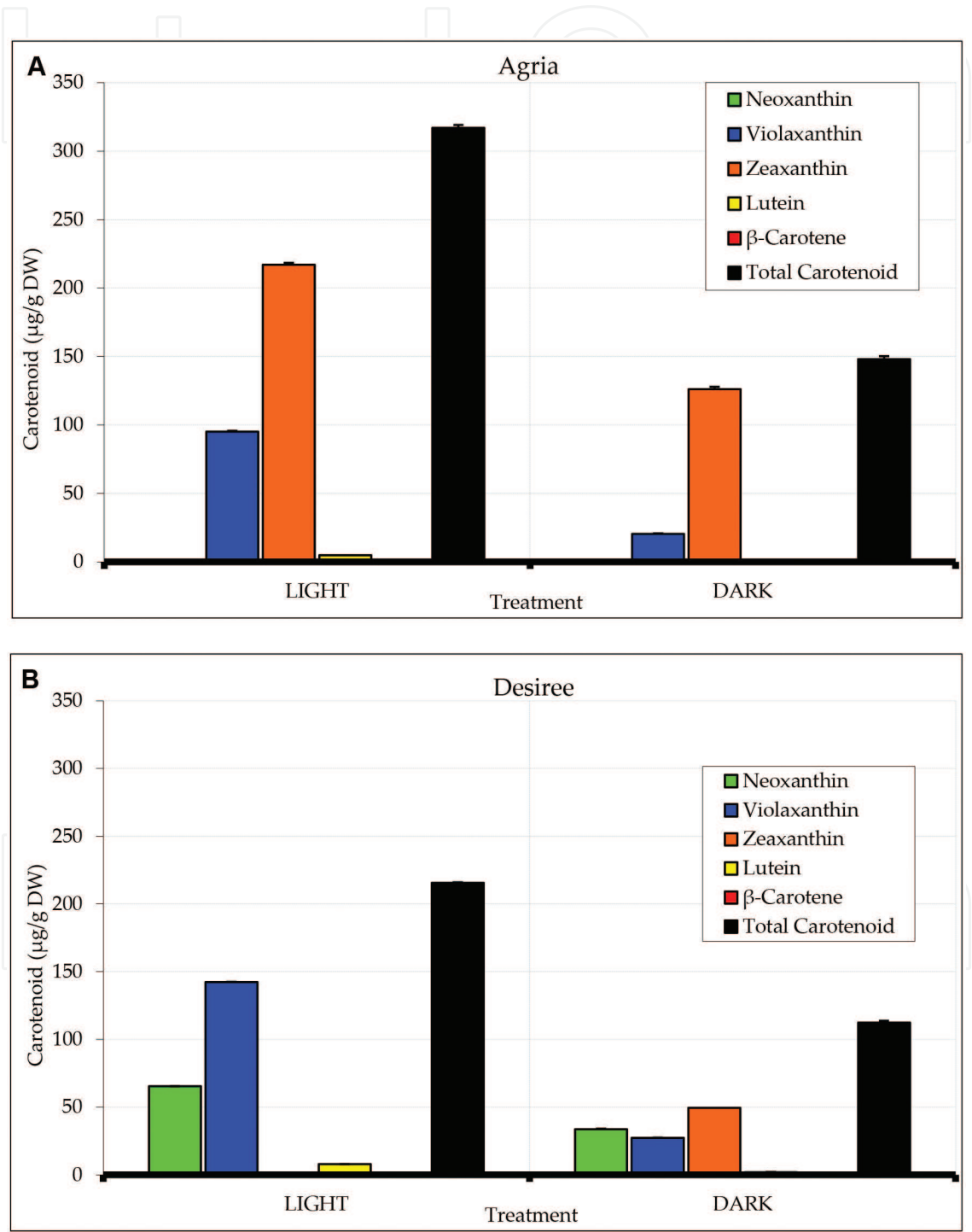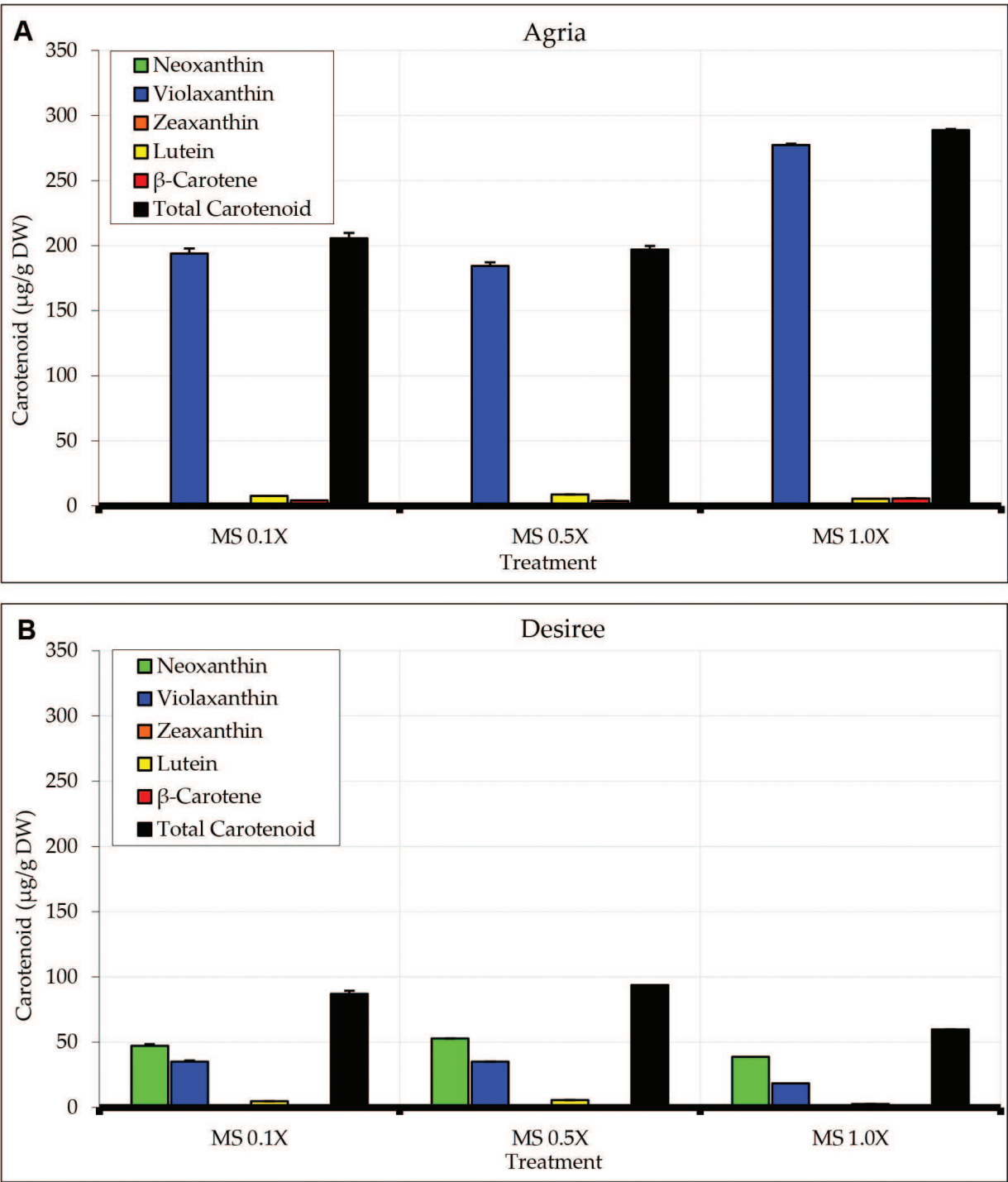

Figure 1. Analysis of carotenoid content (μg/g DW) of Agria and Desiree minitubers in response to light; (A) individual and total carotenoid content (μg/g DW) of Agria minitubers developing in light and dark; (B) individual and total carotenoid content (μg/g DW) of Desiree minitubers developing in light and dark; error bars represent ± SE.

due to minor changes in neoxanthin and lutein. In contrast, upon further increases in MS salt strength, 0.5–1.0×, total carotenoid content and individual carotenoids, especially neoxanthin, violaxanthin, and lutein, decreased. No changes were observed in β-carotene when MS salt strength increased from 0.1× to 0.5× for the development of Desiree minitubers.
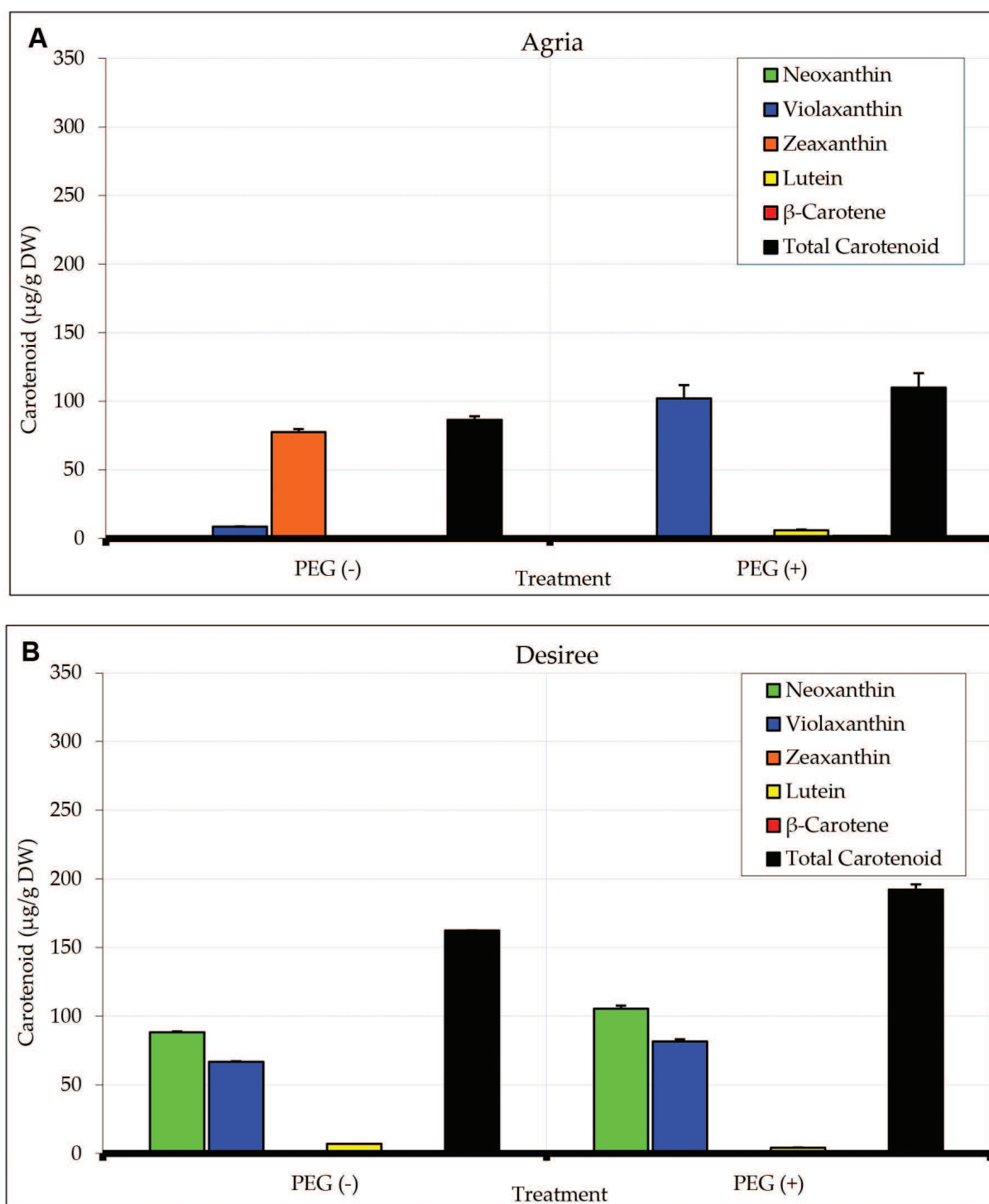
Figure 2. Analysis of carotenoid content (μg/g DW) of Agria and Desiree minitubers in response to water stress; (A) individual and total carotenoid content (μg/g DW) of Agria upon development with and without PEG treatment; (B) individual and total carotenoid content (μg/g DW) of Desiree upon development with and without PEG treatment; error bars represent ± SE.

Figure 3. Analysis of carotenoid content (µg/g DW) of Agria and Desiree minitubers in response to nutrient levels; (A) individual and total carotenoids content (µg/g DW) of Agria upon 0.1×, 0.5× and 1.0× MS salt stress; (B) individual and total carotenoids content (µg/g DW) of Desiree upon 0.1×, 0.5× and 1.0× MS salt stress; error bars represent ± SE.

4. Discussion

The development of potato minitubers through *in vitro* system has proved to be an effective experimental system for investigating the environmental factors involved in regulating the

carotenoid biosynthesis. This potential model system has been used because of several advantages compared to the field cultivated tubers:

(i) Rapid initiation of minitubers within four weeks from establishing the experiment rather than whole growing season in the field;

(ii) The environmental conditions are easy to control because of the small size of the plantlets;

(iii) Potato minitubers were easily exposed to different types of environmental treatments effect;

(iv) Variation between tubers was minimized; and

(v) Extraction and analysis of carotenoids can be done by using potato minitubers.

Environmental stress is described as external conditions that adversely affect growth, development, or productivity [49]. Plants respond to stress by various means such as transformed gene expression, trigger cellular metabolism, and variations in growth rates and crop yields. There are two categories of stress:

(i) Biotic – caused by other organisms; and

(ii) Abiotic – resulted from the physical or chemical environment excess or deficiency.

Abiotic or physical and chemical environmental conditions can trigger stress and regulate the carotenoid biosynthesis and of this light, water stress, and nutrient are among the important factors. Species, genotype, and development age are factors influencing the resistance or sensitivity of plants to stress. Three stress resistance mechanisms are as follows:

(i) Avoidance mechanisms – prevents exposure to stress;

(ii) Tolerance mechanisms – permit the plant to withstand stress; and

(iii) Acclimation – modify their physiology in response stress.

Plants cultivated in full sunlight exposure usually receive and absorb more light for photosynthesis process. Carotenoids have a significant role to protect photosynthetic organisms against excessive light [50, 51] and these functions have been proven *in vitro* in photosystem II complexes [52]. Light is a major stress factor in plants causing photoinhibition and photooxidation in photosynthetic tissues and inhibit productivity. Light is also one of the main factors regulating carotenoid biosynthesis [53]. Current researches have reported a clear relationship between the dissipation of excess excitation energy and the conversion of zeaxanthin from violaxanthin in the light-harvesting complexes of plants [54–56]. Under these situations, violaxanthin is reversibly de-epoxidized by violaxanthin de-epoxidase to zeaxanthin [57–59]. To put it simply, violaxanthin becomes an efficient accessory pigment in weak light and zeaxanthin becomes an efficient photoprotector in strong light [60]. This association also has been relevant for a varied range of environmental conditions, for example, water stress and high temperature and not just under strong light exposure [61].

This chapter demonstrates that light exposure to Agria and Desiree minitubers leads to the similarity that both total and individual carotenoids were elevated up to two-fold higher on a µg/g DW basis than the total and individual carotenoids produced by dark treatment except for violaxanthin. The findings were consistent with the result reported in the previous studies [62, 63], where high violaxanthin was detected in sun-grown crop plants. However, they are not in agreement with Havaux and Niyogi [60] who found high violaxanthin in the dark and high zeaxanthin in strong light. Lutein and total carotenoid content also high in accordance with their observations and others [64–66]. In this study, lutein and total carotenoid in Agria and Desiree minitubers also increased with light. The changes were due to the stoichiometric and cyclical transformations among violaxanthin, antheraxanthin, and zeaxanthin [67]. Light encourages the de-epoxidase reaction and requires acidity for de-epoxidase activity, which can be caused by ATP hydrolysis or supplied by buffer [24, 68, 69]. The de-epoxidase is stereospecific for xanthophylls and as a consequence of that the carotenoid polyene chain must be all-trans. Otherwise, neoxanthin, which is 9-cis, is a passive substrate but becomes active when isomerized to the all-trans form [70].

The phytohormone abscisic acid (ABA) shows a regulatory role in most physiological processes in plants [71]. Various stress conditions, for instances, water, drought, cold, light, and temperature caused an increased amount of ABA. The action of ABA includes alteration of gene expression and analysis of responsive promoters discovered several potential *cis-* and *trans*-acting regulatory elements. In some of the controls in Agria and Desiree minituber experiments, zeaxanthin was detected. The occurrence of zeaxanthin might be in response to the brief exposure of samples to light. Every week, all minituber samples were checked and observed for contamination and size of minitubers. This brief exposure to light might trigger the accumulation of zeaxanthin in some of the minituber control samples.

The presence of zeaxanthin in Agria and Desiree minitubers developing on dark-grown plants could be justified by modification of gene expression in response to stress. Stress recognition may activate signal transduction pathways that transmit information inside the individual cell and through the plant. This may induce gene expression changes that influence growth and development as well as regulate the carotenoid biosynthesis. A stress will trigger and alter cellular metabolism, and as a result zeaxanthin accumulated as a precursor to ABA biosynthesis. Furthermore, plant resistance or sensitivity against stress can be determined by the species, genotype, and development age. In addition, there was a study revealing that different developmental age will accumulate different carotenoid [45]. In their study, 28 day stolons similar to our 4-week minitubers were detected to have highest total carotenoid compared to 80-day developing tubers and 9-month mature tubers. In both cases, zeaxanthin was also detected. Morris et al. [45] also demonstrated comparable results whereby the orange flesh tubers of DB375/1 were detected with high zeaxanthin, while pale yellow Desiree was detected with high violaxanthin. Besides, yellow flesh cultivars were observed to have the capability and ability to generate more carotenoids compared to the white flesh cultivars. Yellow flesh cultivars with high carotenoid content are able to tolerate stress, mainly light with tolerance mechanism [49]. As a result, Desiree minitubers accumulated violaxanthin and neoxanthin when stored in light, while Agria minitubers accumulated zeaxanthin and violaxanthin. In the experiment involving nutrient stress, the higher nutrient concentration given,

the higher content of total and individual carotenoids of Agria found. On the other hand, in Desiree, total and individual carotenoids initially increased with increasing nutrient level, but then decreased at higher nutrient levels. The result showed that Desiree minitubers accumulated violaxanthin and neoxanthin, while Agria minitubers accumulated only violaxanthin.

Water deficiency is another significant environmental stress, which could influence plant growth and development as it is an essential element to meet basic needs. Drought, hypersaline environments, low temperatures, and transient loss of turgor at midday are among environmental conditions that can cause water deficit [49]. In a water stress condition, ABA will be synthesized by the roots and carried it into the shoots, with ABA being an important mediator to trigger plant responses, especially carotenoid biosynthesis to adverse environmental stimuli [72]. A major increase in the ABA content, particularly, in crops such as winter wheat, potatoes, and alfalfa was detected during hardening and cold acclimation [73–76]. However, the extent of the ABA response influenced by several differences [76], for instance, in winter wheat, a freeze resistant variety of wheat had a greater ABA level than a less resistant variety. Likewise, an increase in the ABA content was also observed in *Solanum commersonii*, but not in *S. tuberosum*, which failed to acclimate at −3°C. Besides, the total and individual carotenoid concentrations in both cultivars increased slightly during the PEG treatments, where the drought stress was simulated (**Figure 2**).

Oxidative cleavage is the first committed reaction for ABA biosynthesis in plant and it has commonly been assumed to be the key regulatory step. Various types of stress could encourage the ABA synthesis; therefore, ABA may be thought as a plant stress hormone [71]. ABA was known as one of the crucial plant growth and development regulator. A significant role for ABA in modulation at the gene level of adaptive responses of plants in adverse environmental conditions was also reported in several previous researches [77–80]. In some other physiological processes, ABA is also involved, for example, in leaf senescence [72], stomatal closure, embryo morphogenesis, development of seeds, and synthesis of storage proteins and lipids [81], germination [82], and defense against pathogens [83]. Nevertheless, ABA plays a role as a mediator in regulating adaptive plant responses to environmental stresses [84]. In certain cases, it has been involved in signal transduction at the single cell level [85]. Therefore, the findings of this study clearly demonstrated that environmental conditions for plant growth and development, such as light, dark, water stress, and nutrient concentration were significantly affecting and stimulating the carotenoid biosynthesis. At the same time, like other environmental stress response, disease or pathogen infection can also lead to oxidative stress responses, which implicate stress response genes [86], as well as storage period, which can cause physical changes such as sprouting and dehydration [87].

The results also suggested that ABA could facilitate a regulatory step for the carotenoid biosynthetic pathway versus environmental stress and during the first committed step in ABA biosynthesis, the epoxidation of zeaxanthin to violaxanthin by ZEP has happened. Zeaxanthin acts as a key element and indicator for the occurrence of environmental stress. In response to environmental stress conditions, violaxanthin and neoxanthin merely accumulated toward producing xanthoxin or precursors of ABA biosynthesis pathway. Predictably, the potato genotypes response to that environmental condition seemed to be highly genotype dependent and

time duration exposed to stress. The activity of functional enzymes and candidate enzymes is another factor, which regulates carotenoid biosynthesis that determines the individual carotenoids type and quantity. Since the environmental conditions can influence carotenoid biosynthesis, undoubtedly, the carotenoid type and concentration that accumulates in potato tubers can be induced.

In a nutshell, the differences in carotenoid profile and tuber flesh color from different growing seasons, locations, and cultivars can be explained by the genes regulation, particularly ZEP and VDE, the existence of structure sequestering carotenoids, and the presence of the environmental stress. White flesh cultivars, which have a limited capacity to tolerate excessive light, exhibited an increased susceptibility to photooxidative damage [60]. In contrast, yellow flesh cultivars whose carotenoid content is much higher can specifically tolerate excessive light and also many environmental stress conditions by regulating ZEP and VDE. On top of better yield production, potatoes nutritional value and quality can be enhanced by selecting the appropriate potato cultivars that meet suitable environmental conditions for applicable agronomic practices. In simple words, to increase the accumulation of specific individual carotenoid pigments, implementing the most appropriate environmental factors is required. This invention could be more effective than selecting potato genotypes with higher carotenoid content as parents in a breeding program for the new potato cultivars development with enriched nutrients.

Acknowledgements

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