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Effect of abiotic stress on carotenoids accumulation in orange sweet potato callus under light and dark conditions

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Abstract

Abiotic stress factors are the main limitation to plant growth and yield in agriculture. Orange sweet potatoes may become major sources of carotenoids in the diet, but the extent of environmental and genetic influences on plant carotenoid biosynthesis are poorly understood. Carotenoid biosynthesis is regulated by several factors such as water, light, pathogen, salinity, nutrients and is susceptible to geometric isomerisation in the presence of oxygen, light and heat which causes colour loss and oxidation. The main problems associated with carotenoid accumulation arise from the inherent instability of pigments. In this study carotenoid biogenesis is investigated in sweet potato callus culture as a potential model system for carotenogenesis by analysing the effects of environmental stress agents such as NaCl (for salt tolerance), PEG (for drought tolerance), salicylic acid (for pathogen stress or disease resistance) and nutrient strength towards carotenoid content and composition. Results of this study revealed that the bioactive compounds detected in orange sweet potato callus were α-carotene, β-carotene, lutein and zeaxanthin. Not surprisingly, the response of sweet potato callus culture to such environments appeared to be highly light dependent. Another factor is the activity of functional enzymes and candidate enzymes that regulate carotenoid biosynthesis, which will determine type and quantity of individual carotenoids. By understanding the environmental factors that affected carotenoid biosynthesis, it should be possible to enhance the amount and type of carotenoid that accumulates in sweet potato tubers. In conclusion, *in vitro* callus culture is suggested as a successful new alternative approaches to enhance or enrich certain carotenoids through controlled environment.

Introduction

Abiotic stress is defined as environmental conditions that reduce growth and yield below optimum levels. Plant responses to abiotic stresses are dynamic and complex (Cramer, 2010; Skirycz, 2010). The natural environment for plants is composed of a complex set of abiotic and biotic stresses. Plant responses to these stresses are equally complex (Grant, 2011). The production and productivity of several crops continues to be adversely affected due to various biotic and abiotic stresses. Damages caused by these stresses are responsible for enormous economic losses worldwide. Traditional breeding technologies and proper management strategies continue to play a vital role in crop improvement. The conventional breeding programmes are being employed to integrate favorable genes of interest from inter-crossing genera and species into the crops to induce stress tolerance. However, conventional breeding methods have little success and have failed to provide desirable results (Purohit *et al*., 1998). Therefore, we need to deploy (diffuse or circulate) the biotechnological tools for addressing the critical problems of crop improvement for sustainable agriculture. Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemical (Akula, 2011). Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. Environmental factors such as temperature, humidity, light intensity, the supply of water, minerals, and CO_2 influence the growth of a plant and secondary metabolite production. Drought, high salinity, and freezing temperatures are environmental conditions that cause adverse effects on the growth of plants and the productivity of crops. Plant cell culture

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technologies have been effective tools for both studying and producing plant secondary metabolites under *in vitro* conditions and for plant improvement (Akula, 2011).

Carotenoids are accumulated in most plant organs and they are lipophilic secondary metabolites, derived from the isoprenoid pathway. According to Othman (2009), in plants, carotenoids are synthesised and located in the plastids. Brightly coloured chromoplasts contain high levels of carotenoids and provide the colour to many flowers, fruits and vegetables. Gerontoplasts represent a degrading, but still functional, stage in the plastid life cycle found in senescent tissues. Plastids may differentiate into several forms, which normally consist of chloroplasts, chromoplasts, gerontoplasts and leucoplasts (Wise, 2007). Chloroplasts store carotenoids in thylakoid membranes while chromoplasts store high levels of carotenoids in membranes, oil bodies, or other crystalline structures within the stroma (Howitt and Pogson, 2006). Even though the individual biosynthetic enzymes are known, there is still a gap in the fundamental understanding of complexes and protein interactions involved in mediating the carotenoid biosynthesis (Quinlan *et al*., 2012). In previous studies, many researchers documented that the pathway of carotenoid biosynthesis was likely to function as a multi-enzyme complexes to facilitate the metabolite channeling (Al- Babili *et al*., 1996; Bonk *et al*., 1996; Lopez *et al*., 2008). This indicates that the carotenoid biosynthesis process is a complex study and each process requires a multidiscipline research to investigate them in a holistic manner.

According to Raja *et al*. (2011), *in vitro* culture of plant cells, tissues or organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance, which includes the use of some selective agents that permit the preferential survival and growth of desired phenotypes (Purohit *et al*., 1998). The selecting agents usually employed for *in vitro* selection include sodium chloride or NaCl for salt-tolerance, polyethylene glycol or PEG for drought-tolerance and salicylic acid for pathogen stress or disease resistance (Raja *et al*., 2011). In this study, carotenoid biogenesis was investigated in sweet potato callus culture as a potential model system for carotenoids biogenesis by analysing the effects of environmental stress agents such as NaCl, PEG, salicylic acid and nutrient strength towards carotenoid content and composition.

Materials and methods

Tissue culture and callus initiation

Virus-free *in vitro* plantlets of orange sweet potato were obtained from the Herbarium Research Lab, International Islamic University Malaysia. These plantlets were incubated in a growth room at 24ºC day and night temperature, with a 16-h photoperiod at 80-85 µmol m^{-2} s^{-1} under cool white fluorescent light. Every 4 weeks the *in vitro* plantlets were subcultured as nodal cuttings on Murashige and Skoog medium (MS) composed of Murashige and Skoog (1962) salts and vitamins supplemented with 30 g/L sucrose and 3.5 g/L gelrite following the method of Othman (2009). Media were adjusted to pH 5.7 and sterilized by autoclaving (15 min, 121ºC) and 50 ml aliquots poured into pre-sterilised 290 ml plastic container (80 mm diameter x 60 mm high). For callus initiation, individual shoots of 3-4 nodes from vigorously growing four-week-old cultures were excised (1.0 cm^2) and five explants were transferred into each of 40 ml of callus initiation medium in 250 ml polycarbonate culture vessels (7 cm diameter x 8 cm high). The callus initiation medium contained the same constituents as MS, except with the addition of 1.5 mg/L benzyladenine (BAP).

Effect of abiotic stress on carotenoid biosynthesis in orange sweet potato calluses

In four independent experiments the influence of light, water-stress, disease and salt stress as well as nutrient availability on carotenoid biosynthesis were tested in orange sweet potato callus. Callus harvested after 4 weeks from culture vessels were pooled for each of three replicates established under the following conditions:

1. Light versus darkness by incubation under cool-white, fluorescent lamps (80-85 µmol m^{-2} s^{-1}; 16 h photoperiod) with dark condition imposed by carefully wrapping the culture vessels with aluminium foil.
2. Incubation in darkness condition with and without 5.0 g/L PEG 4000.
3. Incubation in darkness condition with and without 5.0 g/L NaCl.
4. Incubation in darkness condition with and without 0.1 g/L salicylic acid.
5. Incubation in darkness condition at three concentrations of MS salts (half, double and full-strength).

Extraction of carotenoids

The extraction procedure essentially follows the methods described by Othman (2009) with some

modifications: 0.1 g of each powdered freeze-dried sweet potato callus was weighed and rehydrated with 3 ml of distilled water and extracted in 5 ml of acetone and methanol mixture (7:3 v/v) containing calcium carbonate in a centrifuge tube. Samples were mixed well and the mixture was vortexed and allowed to stand overnight in darkness at room temperature. To extract carotenoids an equal volume of hexane and distilled water was added to the combined supernatants. The solution was then allowed to separate and the upper ether layer containing the carotenoids was collected. The combined upper phase was then dried to completion under a gentle stream of oxygen-free nitrogen.

HPLC analysis

The HPLC analysis of carotenoids extracted from sweet potato callus was performed on an Agilent model 1200 series which comprises a binary pump with auto-sampler injector, micro vacuum degassers, thermostat column compartment and a diode array detector in accordance to Othman (2009). The column used was a ZORBAX Eclipse Plus C_{18} end capped 5µm, 4.6 x 250 mm reverse phase column (Agilent Technologies, USA). The solvents used were (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The solvent gradient used is 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 a flow rate of 1.0 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min prior to the next injection. The temperature of the column was maintained at 20°C and the injection volume was 10 µL. Carotenoid standards such as α-carotene, β-carotene, lutein and zeaxanthin were obtained from Sigma-Aldrich. Detection of individual carotenoids was confirmed by their spectral characteristics, absorption maximum and retention time. Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peak was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas. The individual carotenoid concentration was expressed in terms of microgram per 1.0 g dry weight of freeze-dried matter (µg/g DW).

Results

Effect of nutrient stress on carotenoid accumulation in orange sweet potato callus

Nutrient stress during orange sweet potato callus development resulted in a highly significance difference ($p < 0.0001$) in carotenoid content (Figure 1). When MS salt strength increased from 0.5x to 1.0x in light, zeaxanthin, lutein, β-carotene and

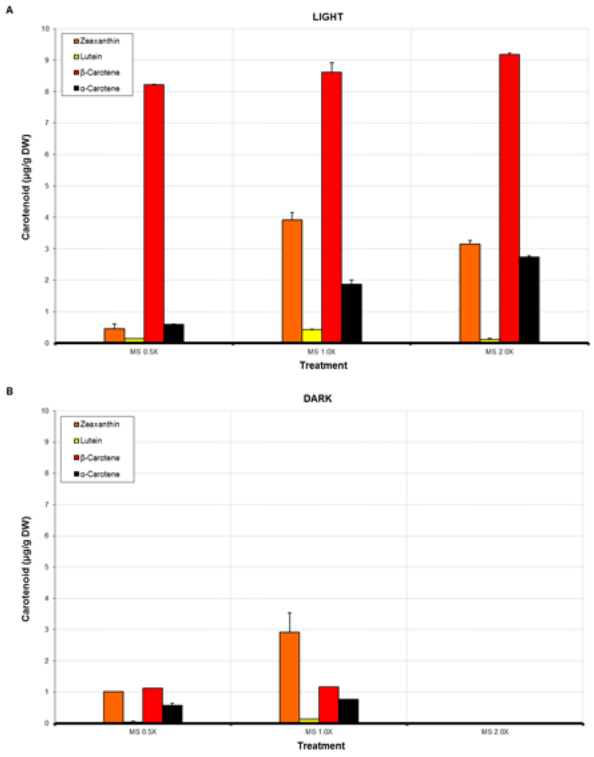

Figure 1. Analysis of carotenoid content (µg/g DW) of orange sweet potato callus in response to nutrient levels.
A - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in light upon 0.5x, 1.0 x and 2.0x MS salt stress.
B - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in dark upon 0.5x, 1.0 x and 2.0x MS salt stress.
Error bars represent ± SE.

α-carotene content increased. However, when MS salt strength increased from 1.0x to 2.0x, β-carotene and α-carotene increased, whereas zeaxanthin and lutein concentration slightly decreased. Analysis of variance also established highly significant differences ($p < 0.0001$) in carotenoid content in orange sweet potato callus developing in varying MS salt strengths in dark. As shown in Figure 1, when MS salt strength increased from 0.5x to 1.0x, carotenoid content of zeaxanthin, lutein, β-carotene and α-carotene slightly increased. In contrast, upon further increases in MS salt strength, 1.0x to 2.0x, all individual carotenoids were not detected. Orange sweet potato callus accumulated four individual carotenoids compounds (zeaxanthin, lutein, β-carotene and α-carotene) when developing in both dark and light condition. The predominant carotenoids were β-carotene, zeaxanthin and followed by α-carotene. However, development of orange sweet potato callus in light condition resulted in an approximate doubling of the all four individual carotenoids content compared in darkness except for β-carotene. β-carotene compound showed its highest concentration (9.18+0.051 ug/g DW) in the MS salt

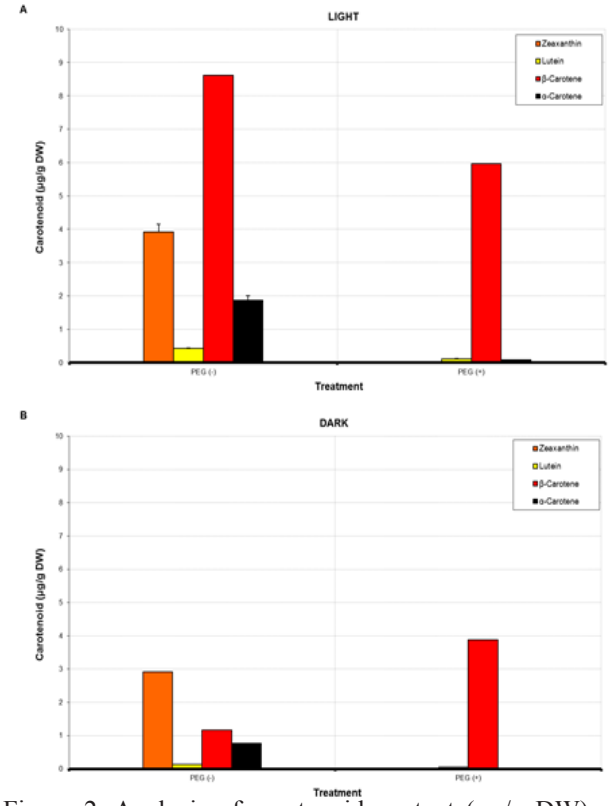

Figure 2. Analysis of carotenoid content (µg/g DW) of orange sweet potato callus in response to water stress.
A - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in light with and without PEG.
B - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in dark with and without PEG.
Error bars represent ± SE.

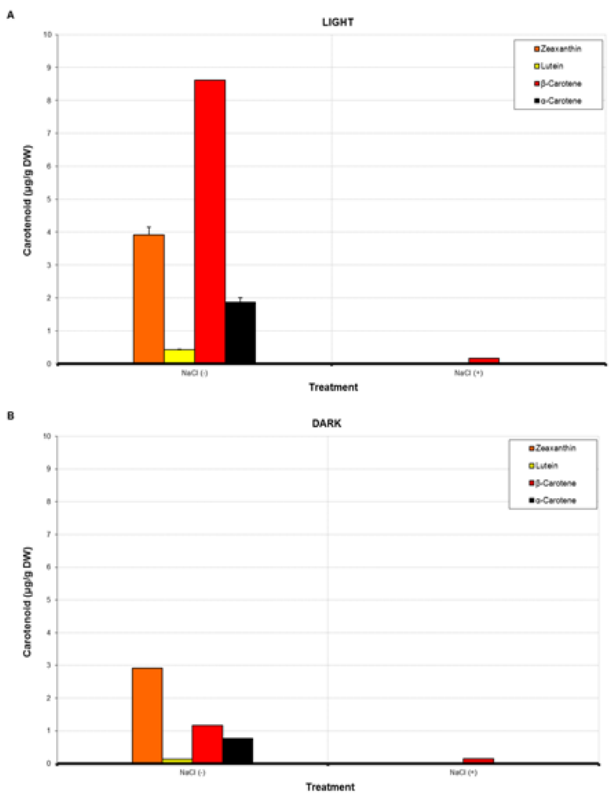

Figure 3. Analysis of carotenoid content (µg/g DW) of orange sweet potato callus in response to salt stress.
A - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in light with and without NaCl.
B - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in dark with and without NaCl.
Error bars represent ± SE.

2.0x strength in light condition in comparison with dark condition for both low and moderate MS salt strength, β-carotene was detected less eight times than its quantity in the light condition. Surprisingly all four individual carotenoids compounds (zeaxanthin, lutein, β-carotene and α-carotene) were disappeared at MS salt 2.0x strength in dark condition.

Effect of water stress on carotenoid accumulation in orange sweet potato callus

Analysis of variance showed that there was a highly significance difference ($p< 0.0001$) in carotenoid content in response to the water stress treatment during development of sweet potato callus (Figure 2). Sweet potato callus developing in the presence of PEG and light exhibited three main carotenoids which are zeaxanthin, lutein and α-carotene being suppressed to almost disappeared whereas β-carotene was slightly decreased. Similar response was observed in callus developing in the presence of PEG in dark condition. Interestingly β-carotene was found increased despite the total absence of zeaxanthin, lutein and α-carotene.

Effect of salt stress on carotenoid accumulation in orange sweet potato callus

Statistical analysis showed that there was highly significance difference ($p < 0.0001$) in carotenoid content in sweet potato callus developing in the dark and light conditions (Figure 3) in the presence of NaCl. Sweet potato callus accumulated four individual carotenoids compounds (zeaxanthin, lutein, β-carotene and α-carotene) when developing in both dark and light conditions without the presence of NaCl. However, development of sweet potato callus in the presence of NaCl with both light and dark conditions resulted only trace of β-carotene was detected whereas other types of carotenoid were totally suppressed and disappeared.

Effect of pathogen stress on carotenoid accumulation in orange sweet potato callus

Analysis of variance comparing sweet potato callus grown in the dark and light conditions in response to pathogen stress also established highly significant differences ($p < 0.0001$) in carotenoid content. As shown in Figure 4, four individual carotenoids compounds (zeaxanthin, lutein, β-carotene and α-carotene) were found in sweet

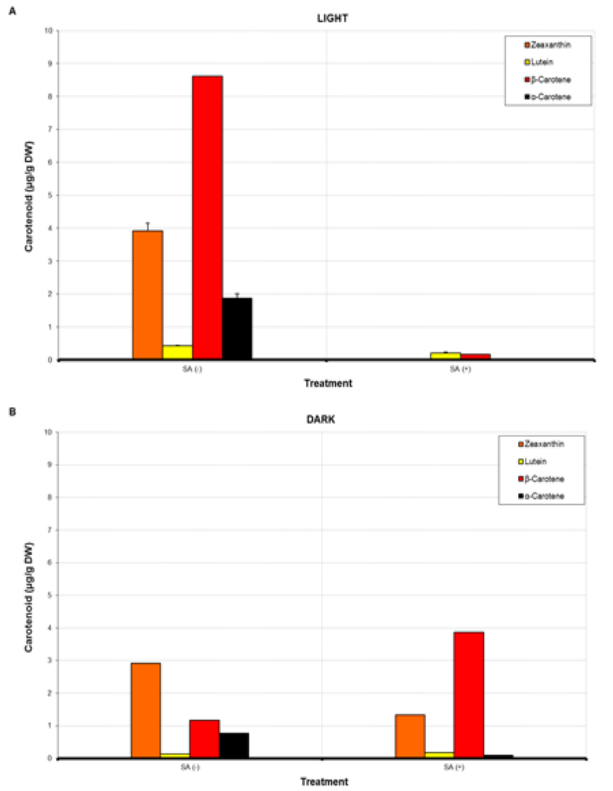

Figure 4. Analysis of carotenoid content (µg/g DW) of orange sweet potato callus in response to pathogen stress.
A - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in light with and without salicylic acid.
B - Individual carotenoid content (µg/g DW) of orange sweet potato callus developing in dark with and without salicylic acid.
Error bars represent ± SE.

potato callus grown in both conditions without the presence of salicylic acid, but upon development in light in the presence of salicylic acid only two (lutein and β-carotene) were detected in trace amount, with an absence of zeaxanthin and α-carotene. After development in dark condition in the presence of salicylic acid, β-carotene content approximately doubled, and reflected in decrease of zeaxanthin and α-carotene.

Discussion

The development of sweet potato callus through *in vitro* model system has proved to be an effective experimental system for investigating the environmental factors involved in regulating carotenoid biosynthesis. This potential model system has been used because of several advantages over the use of field grown tubers:

i. rapid initiation of callus within four weeks from establishing the experiment rather than whole growing season in the field;

ii. the environmental conditions are easy to control;

iii. callus cells were easily exposed to different types of environmental stress agent with minimal variation between treatment

iv. extraction and analysis of carotenoids can be done by using callus

Environmental stress can be defined as external conditions that adversely affect growth, development, or productivity (Buchanan *et al.*, 2000). Plant responses to stress by many ways such as altered gene expression, trigger cellular metabolism and changes in growth rates and crop yields. There are two types of stress:

i. biotic - imposed by other organisms; and

ii. abiotic - arising from an excess or deficit in the physical or chemical environment.

Abiotic or physical and chemical environmental conditions can cause stress and influence carotenoid biosynthesis and of this light, water stress and nutrient are among the important factors. Resistance or sensitivity of plants to stress depends on the species, genotype and development age. There are three stress resistance mechanisms:

i. avoidance mechanisms - prevents exposure to stress;

ii. tolerance mechanisms - permit the plant to withstand stress;

iii. acclimation - alter their physiology in response stress.

In this study light exposure to sweet potato callus in the presence of different nutrient levels leads to the similarity that both zeaxanthin and α-carotene were elevated up to more than 2-fold higher on a µg/g DW basis than the total individual carotenoids produced by dark treatment except for MS salt 2.0x concentration where all carotenoids totally absence. The results were consistent with Demmig-Adams and Adams (1992b), in which observed high violaxanthin in sun-grown crop plants. No previous study reported α-carotene on carotenoid in callus, but Breitenbach *et al.* (2014) analysed the rice callus and found that accumulated β–carotene and phytoene were in similar amounts, as well as trace amounts of α–carotene, lutein, violaxanthin and zeaxanthin. However Havaux and Niyogi (1999) found high violaxanthin in the dark condition and high zeaxanthin in light condition. Lutein and total carotenoid content also were increased in accordance with their observations and others (Thayer and Bjorkman, 1990; Demmig-Adams and Adams, 1992a; Johnson *et al.*, 1993). In this study β–carotene was the key indicator when exposed to light in the presence of different environmental response. Light and increment of MS salt concentration will trigger β–carotene, zeaxanthin and α-carotene accumulation, PEG in light condition will only accumulate β–carotene whereas NaCl and

salicylic acid will silence the gene expression of β–carotene and others carotenoid. Yamamoto *et al.* (1962) demonstrated that the changes were due to the stoichiometric and cyclical conversions among violaxanthin, antheraxanthin and zeaxanthin. Light induces the de-epoxidase reaction and required acidity for de-epoxidase activity, which can be generated by ATP hydrolysis or supplied by buffer (Hager, 1969; Yamamoto *et al*., 1972; Rockholm and Yamamoto, 1996). The de-epoxidase is stereospecific for xanthophylls and because of that the polyene chain of the carotenoid must be all-trans. Otherwise, neoxanthin, which is 9-cis, is an inactive substrate and becomes active when isomerized to the all-trans form (Yamamoto and Higashi, 1978). Swamy and Smith (1999) reported that the phytohormone abscisic acid (ABA) plays a regulatory role in many physiological processes in plants. Different stress conditions such as water, drought, cold, light, and temperature resulted in increase amounts of ABA. The action of ABA involves modification of gene expression and analysis of responsive promoters revealed several potential cis- and trans-acting regulatory elements. Abiotic stress can alter gene expression and trigger cellular metabolism in plants (Buchanan *et al*., 2000). Stress recognition may activate signal transduction pathways that transmit information within the individual cell and throughout the plant. This may induce changes in gene expression that modify growth and development and even influence the carotenoid biosynthesis. A stress will trigger and alter cellular metabolism, and as a result β–carotene accumulated as a precursor to ABA biosynthesis. Furthermore, resistance or sensitivity of plants to stress depends on the species, genotype and development age. The results from this model system suggest that a regulatory step for the carotenoid biosynthetic pathway versus environmental stress is mediated by ABA and involves the up-regulation or inhibition of β-carotene. β-carotene appears to be a key factor and indicator for the presence of environmental stress.

Conclusion

The aim of this research is to study the carotenoids biosynthesis mechanism in sweet potato callus culture through biogenesis manipulation in orange sweet potato calluses using plant elicitors under light and dark conditions. The manipulation of carotenoid content in orange sweet potato calluses consisted of inhibiting or enhancing effects. Under abiotic stress, orange sweet potato calluses were forced to adapt to the stress by altering the gene expression and by triggering the cellular metabolism in them. Through this adaptation, the secondary metabolite including carotenoid was also affected. The effects would be either positive or negative. To sum up, it can be concluded that abiotic stress can affect the accumulation of carotenoids in orange sweet potato calluses under light and dark conditions. On top of that carotenoid compounds can be manipulated and produced through the enhancing and inhibiting effects using orange sweet potato calluses with plant elicitors' treatments under light and dark conditions. The *in vitro* system used in this study gives many advantages such as a controlled environment system to manipulate the carotenoid biosynthesis pathway. However, for better understanding, further studies should be conducted to investigate the mechanism involved in the biogenesis manipulation in this study more thoroughly.

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