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Characterization of environmental stress-regulated anthocyanin production and growth of cranberry callus

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Abstract

Cranberry callus was successfully induced from cranberry (*Vaccinium macrocarpon* Ait, Ericaceae) leaves by using Gamborg's B5 medium containing phytohormones at 25°C in the dark. Anthocyanin-producing cranberry callus was obtained only under conditions of continuous light exposure. Red light and UV light exposure of the callus enhanced anthocyanin content by 41.3 and 29.3%, respectively. The light-dependent anthocyanin production in the callus was regulated by temperature. Anthocyanin content in the callus decreased 81.1% at 42°C, 58.9% at 37°C, 47.0% at 30°C, and increased 10.4% at 4°C, compared to the callus maintained at 25°C after 48 hours of incubation at the given temperature. A temperature decrease of 10°C from 25 to 15°C resulted in a critical increase of the anthocyanin production in the callus, irrespective of differences in pH of culture medium. The growth of the callus cultured in medium at pH 7.0 was 6.2-fold higher than in the same medium at standard pH of 5.8.

Key words: Anthocyanin, biosynthesis, callus, cranberry, Ericaceae, growth, light, pH, temperature, *Vaccinium macrocarpon* Ait

Introduction

American cranberry (*Vaccinium macrocarpon* Ait, Ericaceae) is a non-deciduous, perennial, woody plant. It is grown as a fruit crop in bogs with acidic sandy soil primarily in temperate northern regions of the United States, such as Massachusetts and Wisconsin. The red color of cranberry fruit is due to the presence of anthocyanins, the largest subclass of flavonoids (Harborne and Grayer, 1988), and is considered to be the determining factor of the fruit's quality (Craker, 1971). Anthocyanins have a high potential as natural food colorants, and they have been found to possess important therapeutic properties, including anti-tumor (Kamei *et al.,* 1995; Koide *et al.,* 1996), anti-ulcer (Cristoni and Magistretti, 1987), antioxidant and anti-inflammatory traits (Yan *et al.,* 2002; Wang *et al.,* 1999).

Anthocyanin biosynthesis in plants is regulated by various environmental factors such as light and temperature (Chalker-Scott, 1999). Cranberry plants grown in bogs receive several stresses, which affect the growth and development of the cranberry plant and its fruit. These stresses include biotic stresses (attacks by insects, mites and fungi) and abiotic stresses. Among the abiotic stresses, both physical stresses (light, temperature change and wounding) and chemical stresses (nutrients, water supply, and secretions that are produced by fungi and other microorganisms) occur. The color content of cranberry fruit is also affected by physical (light and temperature) and chemical factors (Craker,1971; Eck, 1972; Farag *et al.,* 1992; Sapers *et al.,* 1986). Effects of decreased light interception by defoliation on carbohydrate and anthocyanin levels were also reported by Onayemi *et al.* (2006). However, very little is understood about the physiological and biochemical basis of these factors.

Part of the reason for the lack of physiological and biochemical information lies in the conditions of growth and development of the cranberry plant and fruit. These limit the adequate availability of samples for detailed analysis. Moreover, manipulation of environmental or nutritional conditions in natural bogs is difficult to control. Plant cell culture technology has been developed to overcome geographical and seasonal restriction of the plants, and it has also been applied to study factors that affect production of useful secondary metabolites. An alternative approach is to grow a cranberry callus which can be exposed to various environmental or nutritional conditions for analyzing responses to environmental stresses.

Cranberry callus has been demonstrated to produce anthocyanins (Madhavi *et al.,* 1995). Cell cultures of other species of *Vaccinium* have also been studied (Madhavi *et al.,* 1998; Smith *et al.,* 1997; Fang *et al.,* 1998; Nawa *et al.,* 1993; Meyer *et al.,* 2002). However, effects of light, temperature, and pH on anthocyanin production have been virtually ignored in previous *Vaccinium* plant cell culture studies. Herein we report studies on how these factors affect anthocyanin production.

Material and methods

Plant materials: 'Early Black' cultivar of cranberry (*Vaccinium macrocarpon* Ait, Ericaceae) was obtained from the State Bog at the University of Massachusetts Cranberry Experiment Station in East Wareham, MA.

Induction of callus: Plants were washed under tap water for one hour and surface-sterilized in 70% ethanol for 10 min then disinfected in 6% chlorine bleach for 5 min. Under sterile conditions, plants were rinsed five times in sterilized water and cut into one-centimeter sections. Each section was placed in a petridish containing Gamborg's B5 medium (Gamborg *et al.,* 1968) containing 5 µM 1-naphthaleneacetic acid (NAA), 5 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 2.5 µM kinetin. The petridishes were cultured at 25°C in the dark.

Examination of optimal culture medium: The growth of

callus was examined in basal B5, MS (Murashige and Skoog, 1962), and WP (Lloyd and McCown, 1980) media containing 0.3 mg L^{-1} NAA, 0.3 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin, 0.1 mg L^{-1} benzylaminopurine, 100 mg L^{-1} $FeNa_2EDTA$ (ethyenediamine-tetraacetic acid ferric-sodium salt), 100 mg L^{-1} PVP (polyvinyl-pyrrolidone), 100 mg L^{-1} myoinositol, 50 mg L^{-1} VC (ascorbic acid), and 10% coconut water, respectively. The above reagents were purchased from Sigma (St. Louis, MO). Growth was evaluated quantitatively in terms of biomass, fresh weight (FW) in g $flask^{-1}$. Callus was separated from the medium, and FW was immediately recorded.

Anthocyanin production: The light yellow-colored callus was cultured in 125 ml-flasks containing the modified WP solid medium under a continuous photosynthetic photon flux density of 25 $\mu M\ m^{-2}s^{-1}$ provided by cool white fluorescent lights (F40CW-RS, General Electric Company, Nela Park, Cleveland, OH) to induce anthocyanin production.

Physical environmental stresses

Light treatment: Anthocyanin-producing callus cultured as above were randomly divided into three groups and subjected to various light treatments (red light treatment, UV light treatment, and darkness) for continuous 48 hours. The red light, at a photon fluence rate of 12 $\mu M\ m^{-2}s^{-1}$, was produced by six 40-w fluorescent tubes (F48T12/R-660/HO, Red, General Electric Company, Nela Park, Cleveland, OH) filtered through a red plastic sheet filter (Roscolux color filter # 27, ROSCO Laboratories, Port Chester, NY). The UV light was produced by a UV-C lamp (TUV 15W/G15T8, Philips, Holland). Light measurements were made with a Model IL1400A Radiometer/Photometer (International Light, Inc., Newburyport, MA).

Temperature treatments

Temperature change treatment between 25°C and 15°C: Callus grown at 25°C under continuous cool white fluorescent light as above was randomly divided into four groups after being transferred to a fresh medium. Groups I and II were cultured at 25°C, and groups III and IV were cultured at 15°C under continuous cool white fluorescent light, for 3 weeks. Group I was then transferred to 15°C, and group III was transferred to 25°C and kept under continuous cool white fluorescent light for 1 week. Groups II and IV remained under their respective culture conditions for 1 week.

High temperature treatment: The anthocyanin-producing callus was cultured in flasks containing the modified WP solid medium at 25°C under continuous cool white fluorescent light for 3 weeks. The callus was then transferred to 15°C and kept cultured under continuous cool white fluorescent light for one additional week. The callus was then randomly divided into six groups. One group remained at 15°C under the same light condition, another group was transferred to 25°C under the same light condition. The other groups were transferred to temperatures at 42°C, 37°C, 30°C, or 25°C and kept in the dark. All were incubated for 48 hours.

Low temperature treatment: The anthocyanin-producing callus cultured in flasks containing the modified WP solid medium at 25°C under continuous cool white fluorescent lights for 4 weeks was randomly divided into two groups. One group remained at 25°C, and the other group was transferred to 4°C for the next 48 hours.

Chemical environmental stresses

pH Treatment: The anthocyanin-producing callus was cultured in flasks containing the modified WP solid medium in different pH conditions of 5.8, 6.5, 7.0, 7.5, or 8.0, at 25°C for 3 weeks then either transferred to 15°C for 1 week, or kept at 25°C for 4 weeks. The growth of callus cultured in the modified WP solid medium in different pH conditions at 25°C for 4 weeks was evaluated quantitatively in terms of biomass, FW (g $flask^{-1}$). The callus was separated from the medium, and the FW was immediately recorded. The pH of all the media was adjusted before autoclaving (at 121°C and 1.05 kg cm^{-2} pressure for 20 min).

Quantitative analysis

Sample preparation: To extract anthocyanins, the callus was removed from the medium, mixed well, and 0.250 g of callus was mixed with 1.25 ml of ethanol-1.5 N HCl (85:15) in a 1.5 ml centrifuge tube and incubated at 4°C overnight.

Anthocyanin analysis: Absorbance of anthocyanin at 535 nm was measured using a Jasco V550 UV/VIS Spectrophotometer (Jasco Corporation, Japan). The total anthocyanin content was calculated in absolute quantities using an extinction coefficient ($\varepsilon^{1\%}_{1\ cm}$) of 982 at 535 nm (Francis, 1982; Zhou and Singh, 2002; 2004).

Results

Induction of cranberry callus and anthocyanin-producing callus: Light yellow callus on the solid medium was visible after 6 weeks of incubation. The callus was separated from the mother tissue after two subculture cycles, and the cell culture was maintained by renewing modified WP medium every 4 weeks at 25°C in the dark. The anthocyanin-producing callus was induced only under the continuous cool white fluorescent light.

Optimal culture medium: Among the three culture media tested, cranberry callus grew best in the modified WP medium (Fig. 1). The growth of the callus was 19.6-fold higher in the modified WP medium than in the modified B5 medium, and 4.2-fold higher than in the modified MS medium. The modified WP medium was used in all further experiments.

Effect of light on anthocyanin production: Forty-eight-hour-treatment with red light and UV light increased the anthocyanin content of callus by 41.3 and 29.3%, respectively, as compared with the anthocyanin production of the callus kept in the dark for 48 hours (Fig. 2).

Effect of temperature on anthocyanin production: Results for callus grown at 25°C for 3 weeks then 15°C for 1 week showed that a temperature change increased anthocyanin content by 3.3-fold in comparison with callus grown at 25°C for 4 weeks without temperature change (Fig. 3). The callus initially cultured at 15°C for 3 or 4 weeks did not show any growth, even after transferring the callus to 25°C for 1 week.

In another set of experiments, the anthocyanin content in callus showed a dramatic change with different temperature treatments for 48 hours. Incubation at temperatures above 25°C decreased the anthocyanin content by 81.1% at 42°C, by 58.9% at 37°C, and by 47.0% at 30°C, in comparison with the anthocyanin content of the callus kept at 25°C (Fig. 4). On the other hand, incubation of the callus at a low temperature (4°C) increased the anthocyanin

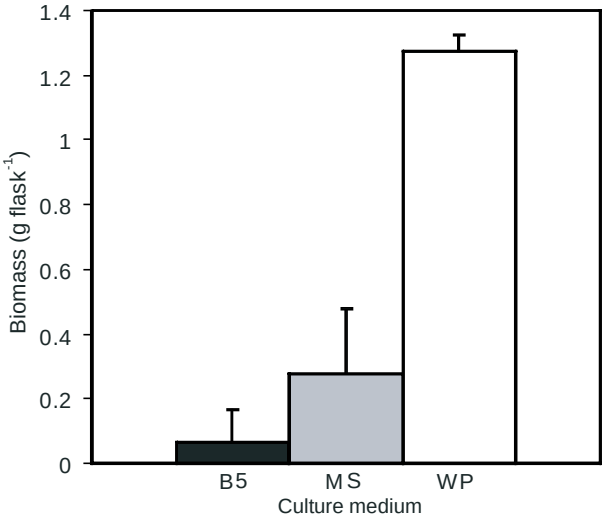

Fig. 1. Growth of cranberry callus in different culture media.The callus was cultured for 4 weeks. Values are mean from six replicates with standard error bars.

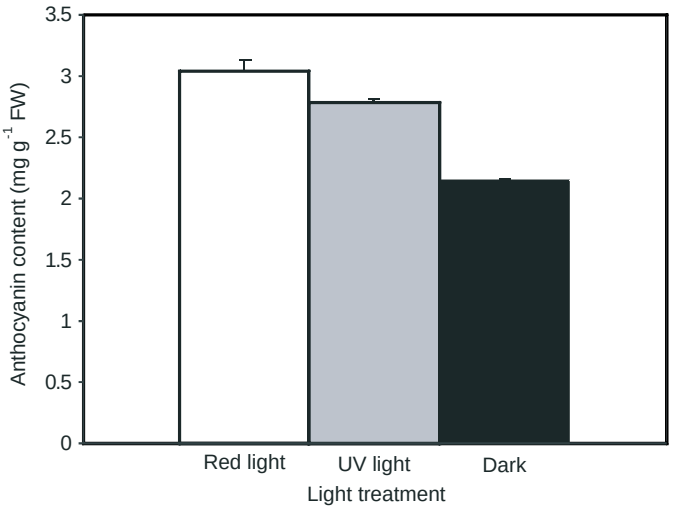

Fig. 2. Effect of light on anthocyanin production in cranberry callus. Anthocyanin-producing cranberry callus was cultured at 25°C for 4 weeks under continuous cool white fluorescent light. Anthocyanin content was analyzed after 48 hours of red light or UV light treatments and darkness. Values are mean from six replicates with standard error bars.

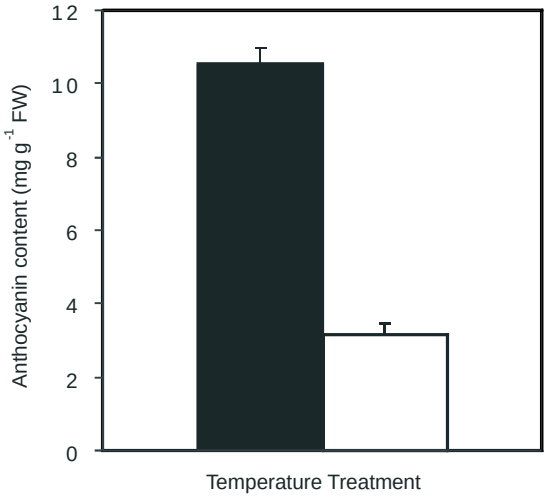

Fig. 3. Effect of temperature change from 25 to 15°C on anthocyanin production in cranberry callus. Anthocyanin-producing cranberry callus was cultured at 25°C for 3 weeks then 15°C for 1 week (black column), or cultured at 25°C for 4 weeks without temperature change (white column). Values are mean from six replicates with standard error bars.

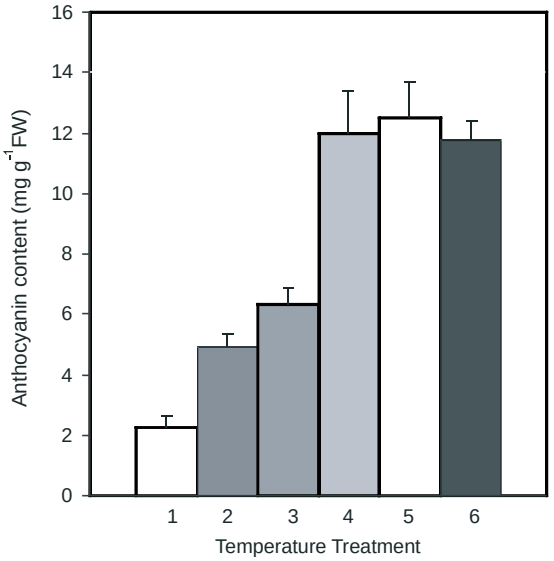

Fig. 4. Effect of high temperature on anthocyanin production in cranberry callus. Anthocyanin-producing cranberry callus was cultured at 25°C for 3 weeks, then 15°C for 1 week. Anthocyanin content was analyzed after 48 hours of incubation at different temperatures. Values are mean from six replicates with standard error bars. 1: 42°C, Dark, 2: 37°C, Dark 3: 30°C, Dark, 4: 25°C, Dark, 5: 25°C, Light, 6: 15°C, Light.

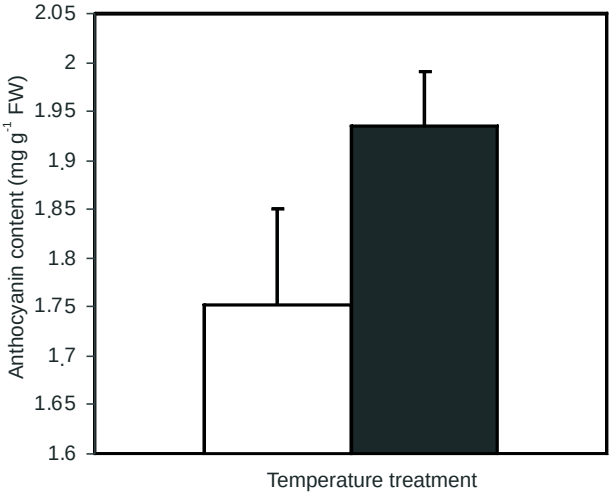

Fig. 5. Effect of low temperature on anthocyanin production in cranberry callus. Anthocyanin-producing cranberry callus was cultured at 25°C for 4 weeks, and anthocyanin content was analyzed after 48 hours of incubation at 4°C (black column), or cultured at 25°C without temperature change (white column). Values are mean from six replicates with standard error bars.

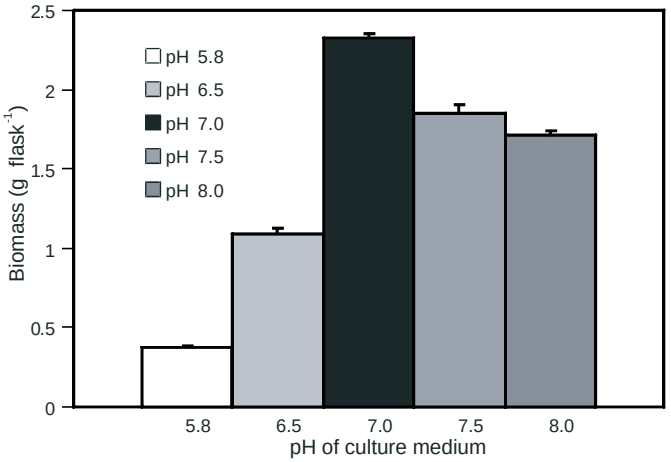

Fig. 6. Growth of the cranberry callus cultured in different pH media. Callus cultured at 25°C for 4 weeks was separated from medium and FW was immediately recorded.

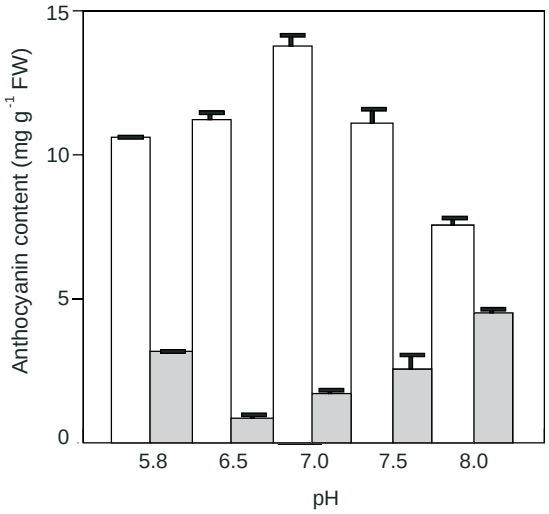

Fig. 7. Effect of culture medium pH on anthocyanin production in the cranberry callus. Anthocyanin-producing cranberry callus was cultured in the modified WP solid medium at pH conditions of 5.8, 6.5, 7.0, 7.5, and 8.0, at 25°C for 3 weeks then 15°C for 1 week (white column), or cultured at 25°C for 4 weeks without temperature change (black column). Values are mean from six replicates with standard error bars.

content by 10.4%, compared to the anthocyanin content of the callus at 25°C (Fig. 5).

Effects of culture medium pH on growth of cranberry callus and anthocyanin production: The growth of callus cultured in different pH media is shown in Fig. 6. Growth at pH 7.0 was higher (6.2-fold) than in the medium with the standard plant cell culture pH of 5.8.

Callus produced more anthocyanins after exposure to a temperature change from 25 to 15°C than with no temperature change, regardless different pH of the medium. Anthocyanin increased 3-fold when temperature changed at pH 5.8, 10-fold at pH 6.5, 6-fold at pH 7.0, 3-fold at pH 7.5, and 1-fold at pH 8.0 (Fig. 7).

Discussion

Different plants and their cell or tissue cultures require different nutrient constituents. The highest growth of the cranberry callus occurred in the modified WP medium (Fig. 1). Although the callus was induced using Gamborg's B5 medium containing phytohormones, the growth of the callus was 19.6-fold higher in the modified WP medium (Fig. 1). Therefore, the modified WP medium was used in all experiments in this study.

Anthocyanins are synthesized due to the activity of key enzymes phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) within the phenylpropanoid/flavonoid pathway; these enzymes can be induced by light (Dixon and Paiva, 1995). Our results show that anthocyanin production in the cranberry callus is light-dependent. The anthocyanin-producing callus itself is obtained only under conditions of continuous light exposure. Anthocyanin was not produced in callus kept in the dark, but red light and UV light exposure increased anthocyanin content by 41.3 and 29.3%, respectively (Fig. 2). This result is consistent with our previous study on the effect of light on cranberry fruit: anthocyanin biosynthesis in cranberry fruit is affected by light quality, and red light selectively promotes higher anthocyanin biosynthesis than UV light (Zhou and Singh, 2002).

Wang and Stretch (2001) reported that storage temperature of cranberries influences anthocyanin content, and the highest anthocyanin content occurred at 15°C storage. We examined the effect of a 15°C environment on anthocyanin production in the cranberry callus, and it was found that a temperature drop from 25 to 15°C resulted in a significant increase of the anthocyanin production in the callus (Fig. 3), which was independent of pH (Fig. 7), and chemicals (glutathione or chlorophyllin, coconut water, metal ions, data not shown) in the medium. Low temperature has been shown to induce anthocyanin synthesis in *Arabidopsis* (Leyva *et al.*, 1995) where low temperature (4°C) induced more PAL and CHS mRNAs accumulation than 20°C conditions in the light after 4 days treatment. Hall and Stark (1972) have reported that early in the fall, cranberry leaves and fruit developed more color at lower temperatures (7.2 -7.8°C in the dark, 12.8-23.9°C in the light). In this study, the cranberry callus also produced more anthocyanin at lower temperature (4°C), compared to the callus maintained at 25°C (Fig. 5).

High temperature (32°C) degrades anthocyanins (Romero and Bakker, 2000). Our high temperature treatments revealed that the higher the temperature, the lower anthocyanin content in callus (decrease of 81.1% at 42°C, 58.9% at 37°C, 47.0% at 30°C, Fig. 4), suggesting that temperature response of cranberry callus is similar to cranberry plant and fruit.

Cranberry bog soils are acidic, with a pH of 4.4 (range 3.3 to 5.5) (Chandler and DeMoranville, 1961) or 4.6 (range 3.9 to 5.9) (Davenport and DeMoranville, 1993). It was interesting to note that the pH of the culture medium not only affected the growth of callus (Fig. 6) but also affected the anthocyanin production (Fig. 7). The standard pH in plant cell culture media, such as B5, MS, WP, and NN (Nitsch and Nitsch, 1969), ranges from 5.5 to 5.8. The growth of the cranberry callus cultured in a medium at pH 7.0 was 6.2-fold higher than in the medium at pH 5.8 (Fig. 6). The anthocyanin content was 27% higher in the callus maintained at pH 7.0 than at pH 5.8 after a temperature drop from 25 to 15°C (Fig. 7). The optimum pH of cranberry culture medium was found to be 7.0.

A limited extent of color development of cranberry promoted by light has been observed (Craker, 1971). The temperature change from 25 to 15°C plays a critical role in increasing anthocyanin production in cranberry callus. This study revealed that a combination of light treatment (to induce anthocyanin production), decreasing temperature from 25 to 15°C (to increase anthocyanin content), and raising pH (to promote growth) would be useful in cranberry culture.

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