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Evaluation of Anthocyanin Production in White and Purple Maize (*Zea mays* L.) Using Methyl Jasmonate, Phosphorus Deficiency and High Concentration of Sucrose

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Anthocyanins are plants metabolites that are recognized by its red/purple coloration produced in flowers, seeds and leaves. These molecules are potentially important to the industry for its antioxidant capacity, disease prevention and as a natural dye. Currently, the production of anthocyanins is carried out using *in vitro* culture of *Vitis vinifera* and its yield is increased by using elicitors or stress factors. *Zea mays* is relevant due to its high content of cyanidin-3-β-glucoside anthocyanin. In the present study the production of cyanidin-3-β-glucoside was evaluated with different mechanisms of elicitation using *in vivo* and *in vitro* culture of purple and white maize varieties. The highest callus induction (85%) for white maize was obtained in MS medium supplemented with 2 mg/L of 2,4-Dichlorophenoxyacetic acid, while for purple maize (93%) was obtained in N6 medium with 2 mg/L of 2,4-Dichlorophenoxyacetic acid, using germinated seed as explant for both varieties. Methyl jasmonate was evaluated as an elicitation tool, however no cyanidin-3-β-glucoside was found to be accumulated or produced *in vitro*. In contrast, using germinated seeds and radicle tissue, elicitation using phosphorus deficiency treatment produced the highest cyanidin-3-β-glucoside accumulation (0.06 mg g^{-1}) in white maize. No elicitation and further production of anthocyanins was found when purple maize were used using this method. Therefore, *in vivo* elicitation in white maize is a potential method to produce a stable anthocyanin that could be optimized for future applications.

Keywords: purple maize, callus induction, anthocyanin elicitation, cyanidin-3-β-glucoside

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Introduction

Anthocyanins are phenolic compounds that belong to the flavonoid group (Loreti et al. 2008). They are secondary metabolites of plants that produce the red/purple coloration of flowers, leaves and seeds, and play an important role in the plant protection, pollination and signaling pathways (Guo et al. 2008). In addition to the various functions in plants, anthocyanins have a wide range of benefits in human health (Carvalho et al. 2015). In various industries, anthocyanins have been used as natural dyes to give red, orange and blue color to different products (Gould et al. 2008). They are employed in food industry, since they are easily incorporated into aqueous systems, and they do not generate adverse health concerns with synthetic dyes (Loreti et al. 2008; Silva et al. 2017). Due to these major advantages, there is a worldwide tendency to expand the use and to improve the production of anthocyanin (Silva et al. 2017).

Diverse species of plants have been utilized to produce anthocyanins both *in vivo* and *in vitro* systems. However, currently, the production of anthocyanins is preferably carried out *in vitro* using cells or plant organs (Ananga et al. 2013). *In vitro* culture works under controlled conditions, which facilities standardization of large scale production, also avoid losses by microbial contaminations or phytopathogens (Silva et al. 2017). On the other hand, the yield of production is limited, which is why several mechanisms have been studied such as variations of culture conditions, addition of precursors and use of growth regulators, stresses factors and elicitors (Silva et al. 2017; Ananga et al. 2013).

Different stress factors were evaluated for anthocyanins production such as application of high concentration of sugar and deficiency of some nutrients in the culture medium (Ananga et al. 2013). The accumulation of anthocyanins after application of high sugar concentrations has been demonstrated to be an efficient method in different plant species like *Arabidopsis thaliana* and *Ceratonia siliqua* (Gould et al. 2008; Loreti et al. 2008). The elicitors are molecules which induce the synthesis and accumulation of substances with antimicrobial and anti-stress effects. The use of inducers related to stress responses and activation of defense mechanisms such as jasmonic acid and salicylic acid have been tested to produce anthocyanins in soybean seeds, *Arabidopsis* seeds, tulip bulbs, peach roots and callus of *Daucus carota* (Gould et al. 2008; Ananga et al. 2013; Deroles 2009).

The most common plant species select for large scale anthocyanin production is *Vitis vinifera*. Several studies demonstrated the use of cell culture is very useful and simple system for industrial and research purposes (Deroles 2009). Elicitation leading the production of anthocyanins was evaluated using phytohormones, nutrients, physical conditions and elicitors (Deroles 2009). In contrast, other studies demonstrated that the modification of abiotic and biotic factors in the different tissues such as fruits and flowers can accumulate anthocyanins therefore leading the tissue for extraction of this compound with different methods (Silva et al. 2017).

Enocianine is the most abundant anthocyanin in *V. vinifera*, however, this compound presents a great limitation for downstream processing and storage (Cuevas et al. 2008). As an alternative, maize (*Zea mays* L.) has aroused great interest as a source of production

of another type of anthocyanin: cyanidin-3-β-glucoside, with greater stability for downstream processing and human health (Cuevas et al. 2008; Silva et al. 2017). Given the properties of cyanidin-3-β-glucoside derived from maize, the aim of the study was to evaluate the production of cyanidin-3-β-glucoside *in vitro* and *in vivo* in two varieties of maize (purple and white) using three elicitation mechanisms: phosphorus deficiency, excess of sucrose and application of methyl jasmonate (MeJA).

Materials and Methods

Plant material

White maize seeds (*Zea mays* L. variety *mishka*) were obtained from a crop in Nayón, Quito-Ecuador; while purple maize seeds (*Zea mays* L. variety *indurata*) were obtained from a collection of seeds grown in El Quinche and Yaruquí, Quito-Ecuador (Fig. S1*).

Callus induction

Seeds from both varieties were disinfected according to Sauer and Burroughs (1986) with some modifications. Seeds were placed in a desiccator along with a beaker that contained a solution of 3 mL of 37% hydrochloric acid and 100 mL of 5% sodium hypochlorite. The seeds were left in the closed compartment full of produced gas for 24 hours.

After disinfection, seeds were placed aseptically in 250 mL glass flask filled with 30 mL of MS medium (Murashige and Skoog 1962), 3% (w/v) of sucrose and 15 g/L Bacto-agar (BA) and pH were adjusted to 5,8 before autoclaving at 121 °C for 45 min.

Two sets consisting of 10 flasks each were incubated at room temperature with a 12/12 photoperiod. After one week, germinated seeds with 10–15 mm long radicle were obtained from the first set of flasks. The second set of flasks were incubated for 4 weeks, after which tip meristems were isolated from seedlings.

For callus induction, germinated seeds and tip meristems were tested in two basal media, MS and N6 (Sauer and Burroughs 1986) both with 3% (w/v) of sucrose. The media were supplemented with 0, 2, 3 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D), in solidified medium previously described. Four explants were placed per 250 mL glass flask filled with 30 mL of medium for 24 h continuous light followed by darkness for 3 weeks, at this point callus was used to test the anthocyanin production.

Elicitation of anthocyanins in vitro

The callus obtained from both varieties of maize were washed with 1 mL of different concentrations of MeJA (0, 30, 100 μM) (Loreti et al. 2008; López et al. 2011) and incubated at room temperature with a 12/12 photoperiod. After 7 days, the concentration of anthocyanins was determined by pH differential method (Puertas et al. 2013).

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Elicitation of anthocyanins in vivo

Prior to the seed germination, seeds were washed with a commercial liquid detergent solution for 5 min, rinsed with tap water to remove traces of soap and then placed in a solution of Benomyl 4 g/L + Captan 4 g/L for 20 min. Finally, they were washed 5 times with distilled water (López et al. 2011).

After washing, 5 seeds per container were placed in 250 ml glass flasks with filter paper and sterile distilled water until the seed was submerged. They were incubated at 20–25 °C in dark until they reached 30 mm long root. At this time, three elicitation mechanisms were tested according to López et al. (2011) and Ulrychová and Sosnavá (1970) with modifications: excess sucrose (MS solution supplemented with 60 g/L sucrose), phosphorus deficiency (MS solution without addition of phosphorus) and MeJA (3.3, 10, 30 and 100 μM MeJA solution). MeJA was diluted first in 70% ethanol as described by Loreti et al. (2008). All solutions were adjusted to pH 5.8 and no solidifying agent was added. Complementary, two solutions were used as control: basal MS without sucrose and sterile distilled water with the corresponding concentration of ethanol used to dilute MeJA.

To each flask, 1 mL of the different solutions were added directly to the germinated seeds. The flasks were incubated at room temperature with 12 h light. After 7 days, the concentration of cyanidin-3-β-glucoside was determined.

Determination of the concentration of cyanidin-3-β-glucoside by differential pH method

To quantify cyanidin-3-β-glucoside concentration from callus (*in vitro*) and germinated seed (*in vivo*), 0.3 grams of the radicle was taken and macerated in a mortar with liquid nitrogen until powder was obtained, then 3 mL of acidified methanol (proportion 1:99 with 37% HCl: 98% methanol) was added and homogenized. The mixture was kept in the dark for 24 hours at 4 °C. Two aliquots of 1 mL were taken and 4 mL of potassium chloride buffer pH 1 (0.025 M) were added to the first aliquot, and 4 mL of sodium acetate buffer pH 4.5 (0.4 M) was added to the second aliquot. The absorbance of each aliquot was measured at 530 nm and 700 nm using distilled water as a blank (Puertas et al. 2013).

Experimental design and statistical analysis

For callus induction, a Completely Randomized Design with a factorial arrangement 2×2×3 was used for the two maize varieties with three repetitions per treatment; giving a total of 36 experimental units. The factors used were: explant type (germinated seed and meristem), culture media (N6 and MS), and 2,4-D hormone concentration (0 mg/L, 2 mg/L and 3 mg/L). The percentage of callus induced per vessel was evaluated.

For anthocyanin elicitation *in vitro*, 6 treatments were obtained in a randomized experimental design with three replicates per treatment; giving a total of 18 experimental units. For *in vivo* experiments, 16 treatments were obtained arranged in a randomized

experimental design with three replicates per treatment; giving a total of 48 experimental units. The concentration of cyanidin-3-β-glucoside (mg/g fresh weight) was quantified by pH differential method.

The data were analyzed using Analysis of Variance (ANOVA) and the differences between treatments were evaluated by the Tukey test at 5% using R studio (RStudio Team 2015).

Results

Callus induction of maize

Callus induction was successful for both maize varieties, however, the addition of 2,4-D had the most significant effect on callus development regardless explant type or medium (Fig. 1). The percentage of callus induction in white maize ranged from 8 to 83%. Germinated seeds in MS medium supplemented with 2 mg/L of 2,4-D was the treatment with the greatest percentage of callus development (83%). In the purple maize, callus induction ranged from 8 to 93%, yet the best treatment (93%) was when used germinated seed as explants in N6 medium, supplemented with 2 mg/L of 2,4-D (Fig. 1). Callus obtained from the best treatments from each variety was used for further experiments.

Elicitation of anthocyanins in maize using callus cells (in vitro)

After obtaining callus from each maize variety, (MeJA) was used to trigger anthocyanins production. After 7 days of MeJA application at two different concentrations (30 μM, 100 μM) was not possible to perceive any red/purple coloration in the callus (Fig. S2),

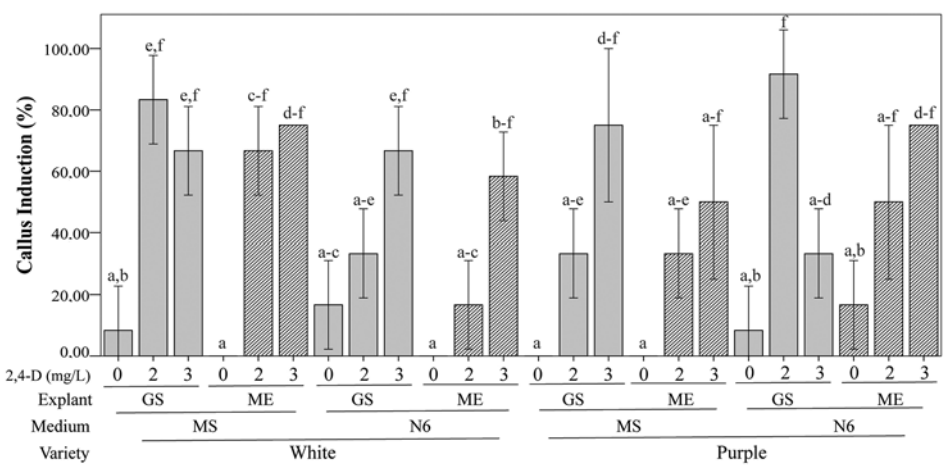

Figure 1. Callus development using white and purple maize varieties under a range of 2,4-D concentrations (0, 2, 3 mg/L), explant type (GS: germinated seed, ME: tip meristem) and culture media (MS: Murashige and Skoog, N6). Means followed by the same letters do not differ from each other by ANOVA with 5% probability Tukey test. Error bars show standard deviation

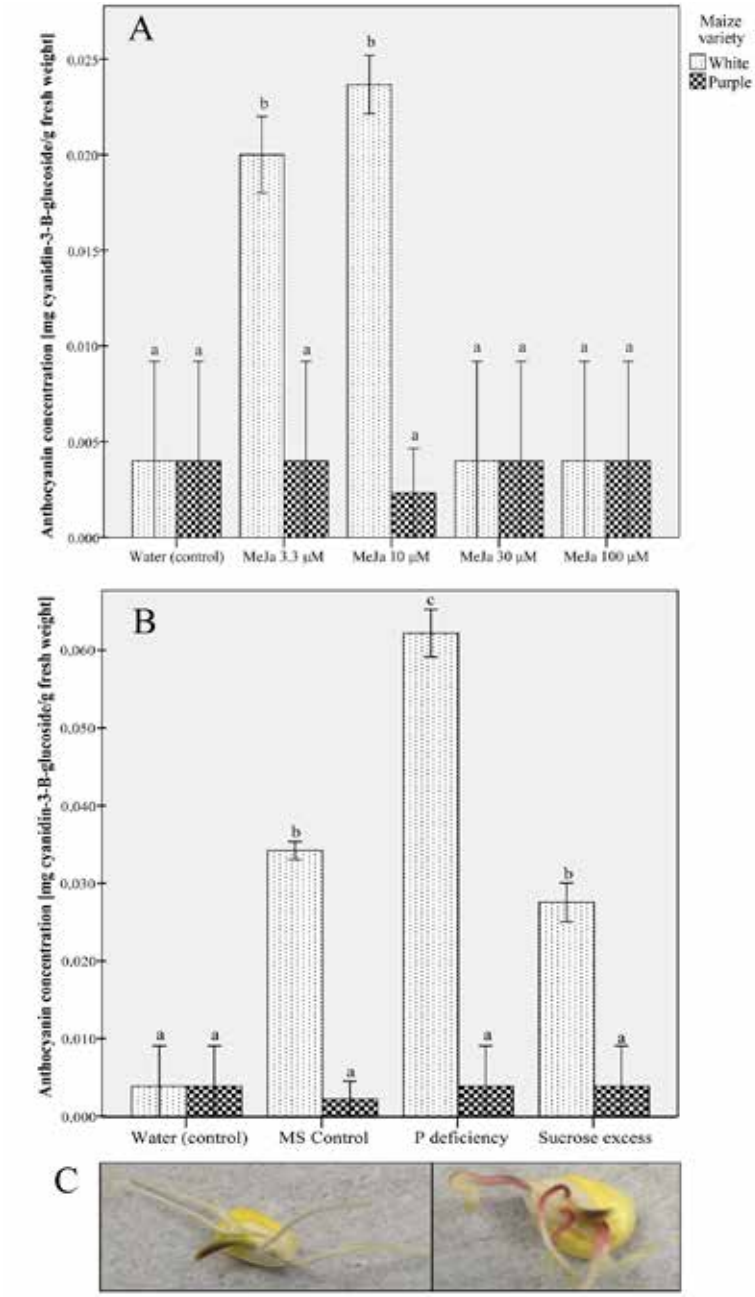

Figure 2. *In vivo* elicitation of cyanidin-3-β-glucoside in white maize and purple maize. A – Different methyl jasmonate (MeJA) concentrations. B – MS medium, excess of sucrose and phosphorus deficiency. Means followed by the same letters do not differ from each other by ANOVA with 5% probability Tukey test. Error bars show standard deviation. C – Left panel: MS medium control, right panel: MS treatment with phosphorus deficiency. Radicle showed changes in coloration

which is the first indication of anthocyanin accumulation (Deroles 2009). To confirm this observation, the concentration of cyanidin-3-β-glucoside was quantified by pH differential method, yet, the production of this anthocyanin could not be detected (Table S1).

Elicitation of anthocyanins using germinated seed (in vivo)

Since no elicitation of anthocyanins was generated *in* callus cells during *in vitro* culture, the effect of elicitation was evaluated using intact tissue (*in vivo*). Here, germinated seeds from purple and white maize were tested with MeJA, excess of sucrose and phosphorus deficiency as a general elicitor of anthocyanins.

As a result, using MeJA as a general inductor there was a statistically significant difference in the production of cyanidin-3-β-glucoside between the two varieties versus elicitation treatment (Fig. 2A). First, the purple variety had almost no production of cyanidin-3-β-glucoside (below 0.005 mg/g fresh weight) for all treatments tested. In contrast, the white variety showed up to 0.025 mg cyanidin-3-β-glucoside /g fresh weight produced after 10 μM of MeJA compared to basal concentration of water control that reached 0.005 mg/g of fresh weight (Fig. 2A).

In contrast, using excess of sucrose and phosphorus deficiency as a specific elicitor the purple variety had almost no production of cyanidin-3-β-glucoside (below 0.005 mg/g fresh weight) for all treatments tested. In contrast, the white variety showed up to 0.065 mg cyanidin-3-β-glucoside /g fresh weight and 0.027 mg/g of fresh weight produced after phosphorus deficiency and excess of sucrose, respectively, compared to MS control that reached 0.035 mg/g of fresh weight (Fig. 2B). The highest accumulation reached using phosphorus deficiency in the white maize was also evidenced visually, due to the red/purple color change in roots and shoots when germinated seed were used (Fig. 2C).

Moreover, in white maize, all elicitation treatments tested were effective and produce more anthocyanins when MS was used as basal medium compared to water control (Fig. 2). Further in white maize, MeJA treatments showed a significant increase of anthocyanins production at 3.3 and 10 μM treatment and had no effect when tested at 30 μM in comparison with water control. As mentioned before, no production of anthocyanins was detected in purple maize.

Discussion

Callus induction

In both varieties of maize, the highest percentage of callus was obtained using germinated seed (mature embryos) in media supplemented with 2 mg/L of 2,4-D. These results agree that embryos regenerates callus more efficiently than other types of explants (Çabuk and Özgen 2016).

The maize varieties showed differences in response to callus formation using different medium, since white maize obtained 83% of callus supplemented with MS medium + 2 mg/L 2,4-D, and 93% of callus in the purple maize cultivated in N6 medium + 2 mg/L

2,4-D (Fig. 1). The concentration of 2,4-D used for callus induction are comparable with previous reports where the optimum concentration was determined using MS medium + 2 mg/L of 2,4-D (Çabuk and Özgen 2016).

In other study demonstrated similar callogenesis development, since the frequency of callus induction ranged from 26.1 to 84% in MS medium + 1 mg/L 2,4-D and using N6 medium + 2 mg/L 2,4-D ranged from 38.1 to 85% (Gorji et al. 2011). The concentration of 2,4-D is an important factor since higher concentration than 4 mg/L callus induction can be inhibited (Gorji et al. 2011).

In the two varieties of maize was observed that when 2,4-D was not applied, a low percentage of callus formation (6.25%) was obtained. This can be explained due to the presence of cytokinin and auxin at the endogenous level in the meristems or in the germinated seed that allow cell de-differentiation and proliferation (Matos and Sánchez 2011).

The variation observed between the two varieties can be explained due genotypic differences since it is an important factor for callus formation and plant regeneration (Gorji et al. 2011; Torres et al. 2012). Thus, the response of callus induction is largely based on concentrations of 2,4-D and their interaction with the maize variety (Shohael et al. 2003). These are the reasons why a difference could be observed in obtaining callus in the two types of maize used for this research, each of the varieties have their optimal conditions (medium and hormone dose) for callus regeneration.

Anthocyanin elicitation

Different elicitation mechanism was evaluated for cyanidin-3-β-glucoside production in two varieties of maize using *in vitro* and *in vivo* systems. The use of 30 µM and 100 µM MeJa *in vitro* did not produced cyanidin-3-β-glucoside (Fig. S2). The absence of anthocyanins production in these treatments could be dependent by antagonisms and tissue dependent. Previously, it has been reported antagonistic effects auxins (2,4-D) on the biosynthesis and inhibition of anthocyanin production in *Daucus carota* and in callus of *Haplopappus gracilis* (Ozeki and Komamime 1986; Stickland and Sunderland 1971). Since in our assays 2,4-D remained in the basal medium for callus maintains, this auxin mediated inhibition of MeJA induce anthocyanins production could be occurring. Also, the lack of anthocyanin accumulation could be attributed to the callus state (Fehér et al. 2003) and MeJA concentrations evaluated could cause an inhibition in anthocyanin production (Fang et al. 1999).

It has been demonstrated that the accumulation of anthocyanin could be triggered by biotic and abiotic factors in flowers and fruits (Silva et al. 2017), however there are not enough information about elicitation of anthocyanin *in vivo* in *Zea mays* L. (Gould et al. 2008). Additionally, exists a great interest to develop methods with high extraction yields of anthocyanin *in vivo* (Silva et al. 2017). In the present study, it was demonstrated that the accumulation of anthocyanin *in vivo* using different elicitation mechanisms showed the production of anthocyanins and accumulation of red/purple color in germinated white maize seeds (Fig. 2).

White maize accumulates more anthocyanin than purple maize under all treatments. The purple maize variety presented accumulation of anthocyanin in the pericarp of mature seeds (Torres et al. 2012) but in the present study, the accumulation of cyanidin-3-β-glucoside was evaluated in the radicle (germinated seed), so anthocyanin production could not be detected. Thus, it demonstrates that depending on the maize variety, plant tissue and elicitation mechanism could lead to different results, being an interesting field of research (Loreti et al. 2008).

The accumulation of cyanidin-3-β-glucoside differed according to the elicitation mechanism (Fig. 2), the treatment of phosphorus deficiency in white maize seeds showed the highest concentration (0.062 mg cyanidin-3-β-glucoside/g fresh weight) in comparison to the MS control and the other treatments. In a study conducted in *Lens culinaris* under conditions of phosphorus deficiency, it was possible to obtain 0.3 mg of phenolic compounds/g fresh weight (Sarker and Karmoker 2011). Therefore, absence of phosphorus in the culture media influenced the phenolic content in the tissue.

On the other hand, in the treatment with the excess of sucrose with a concentration of 0.027 mg cyanidin-3-β-glucoside/fresh weight was obtained, which was not statistically different in relation to the MS control which obtained 0.035 mg/g fresh weight (Fig. 2B). There are reports in *V. vinifera* that such accumulation of anthocyanins can be explained as a function of the osmotic stress that can be generated (Miñaño et al. 2004). Therefore, the stimulation of anthocyanin production presented a proportional increase in relation to the osmolarity present by the medium.

In Figure 2, it is shown that there was no significant difference between the excess sucrose treatment and MS control. MS control contains nitrates and ammonia at 60 mM, this concentration could be considered high and therefore the influence of salts can be determinant for the elicitation of anthocyanins in plants (Guo et al. 2008).

In the case of the different concentrations of MeJA (Figure 2A), a maximum accumulation of cyanidin-3-β-glucoside/g fresh weight was found in the treatments of 3.3 and 10 μM and in higher concentrations produce an inhibitory effect. In a study of maize performed by Kim et al. (2006), it has been shown that at low concentrations of MeJA (3.3 μM and 10 μM) has an induction of 51.8% of anthocyanins than in higher concentrations of MeJA higher than 10 μM. However, in the study of Shimizu et al. (2010) with *Gynura bicolor,* there is a greater accumulation at concentrations between 25 μM to 50 μM of MeJA. The response after methyl jasmonate application that further induces the production of cyanidin-3-β-glucoside may be due to the difference of the genetic background among plant species.

In summary, it is demonstrated that the elicitation of anthocyanins in germinated seeds (radicle) of the white variety produces a greater accumulation of cyanidin-3-β-glucoside in comparison with the purple variety. Additionally, the best elicitation mechanism was the treatment with phosphorus deficiency reaching a concentration of cyanidin-3-β-glucoside of 0.062 mg/g fresh weight. Furthermore, the addition of the MS medium generated an additional elicitation (0.038 mg cyanidin-3-β-glucoside/g fresh weight) without the need of stress. It is important to investigate the combination of these mechanisms *in vivo* to achieve better production and further implementation for industries purposes.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Anthocyanin elicitation *in vitro* in maize callus using different concentrations of methyl jasmonate

Electronic Supplementary *Figure S1.* Purple (left) and white (right) maize seeds

Electronic Supplementary *Figure S2*. Maize callus treated with different concentrations of methyl jasmonate (*in vitro* test). *a* – purple maize callus in 30 μM; *b* – purple maize callus in 100 μM; *c* – white maize callus in 30 μM; *d* – white maize callus in 100 μM