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Betalains and expression of antioxidant enzymes during development and abiotic stress in *Rivina humilis* L. berries

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Abstract: This work investigated the betalains profile and associated molecular changes during *Rivina humilis* L. berry development and 1 month after inducing stress through elicitors such as salicylic acid (SA, 0.05–0.5 mM) and chitosan (CH, 0.1%–1.0%). The results showed that, during berry development, diphenol oxidase activity of tyrosinase increased, which was reflected in an exponential increase in betalains accumulation. Expression levels of a putative *Rivina humilis* betanidin-5-*O*-glucosyltransferase 2 (*RhBGT2*) remained unchanged, while that of antioxidant enzymes superoxide dismutase (*SOD*) and catalase (*CAT*) decreased. Treatment with SA (0.1 mM) and CH (0.5%) enhanced betalains accumulation. The pattern of betalains accumulation during berry development and after elicitor treatment suggested that, probably, both betacyanins and betaxanthins are synthesized from the same pool of betalamic acid. There was no significant change in expression levels of *RhBGT2*, *SOD*, and *CAT* following elicitor stress. However, treatment with CH (1.0%) produced morphological changes (shrinking) in the berries and expression of SOD was significantly suppressed. This is the first report that shows enhancement of betalains accumulation in intact berries through elicitor treatment.

Key words: Salicylic acid, chitosan, glucosyltransferase, superoxide dismutase, catalase

1. Introduction

Rivina humilis L. (Phytolaccaceae) berries are consumed by numerous passerine birds and the berries are likely to be a safe (Khan et al., 2011) dietary source of bioactive betalains (Khan et al., 2012). Betalains are plant pigments that replace anthocyanins in some families of the order Caryophyllales, excluding Caryophyllaceae and Molluginaceae (Clement and Mabry, 1996). These pigments have enormous potential in the food industry as water-soluble colorants specifically due to their stability at $4 \leq pH \geq 6$ (Khan and Giridhar, 2014). Two subgroups of betalains are betacyanins and betaxanthins. Betalains exhibit diverse patterns of coloration ranging from yellow to deep violet through various shades of orange and red depending upon the proportion of betacyanins and betaxanthins. Tyrosinase and DOPA dioxygenase are key enzymes that produce betalamic acid, the betalainic chromophore. *cyclo*-DOPA condenses with betalamic acid to form betanidin, which is glucosylated in the presence of betanidin-5-*O*-glucosyltransferase to betanin, a betacyanin. Betalamic acid conjugates with amino acids or amines to form betaxanthins such as indicaxanthin and dopaxanthin (Gandía-Herrero and García-Carmona, 2013). Advances in betalains research have been reviewed (Gandía-Herrero and García-Carmona, 2013; Khan and Giridhar 2015). However, some pertinent questions still remain unanswered. For example, it is established that betalamic acid is the chromophore of betalains, but it is not understood if betacyanins and betaxanthins are synthesized from the same pool of betalamic acid. About the physiological roles of betalains in planta, it has been proposed that one of their roles is as antioxidants; however, there seems to be a lack of scientific evidence for this.

Enhancement of secondary metabolite accumulation in plants could be achieved through various ecophysiological factors such as biotic and abiotic stress (Georgiev et al., 2008). Few studies on enhancement of betalains in halophyte *Suaeda salsa* (Wang et al., 2007) and *Amaranthus mangostanus* (Cao et al., 2012) seedlings have been reported. During stress, various biosynthetic enzymes are differentially expressed, indicating the molecular mechanism of the enhancement. Salicylic acid and chitosan are well-known elicitors of plant secondary metabolites through abiotic stress (Vasconsuelo and Boland, 2007; Kuzel et al., 2009). However, elicitation of pigment content in matured ex situ plant parts will be very important for commercial production of pigments. The aim of this study was to analyze the biochemical

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changes during development and elicitation of pigment accumulation in intact *R. humilis* berries.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol (MeOH), acetone, isopropanol, ascorbic acid, L-(3,4-dihydroxyphenyl)-alanine (L-DOPA), ammonium sulfate, bovine serum albumin (BSA), and salicylic acid (SA; $C_7H_6O_3$) were obtained from Sisco Research Laboratory (Mumbai, India). 3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), ammonium persulfate, dimethyl formamide (DMFA), and chitosan (from shrimp; ≥75% deacetylated) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade. For HPLC analysis, degassed and 0.22-µm nylon membrane filtered triple-distilled water was used.

2.2. Studies during ontogeny

Berries from *R. humilis* (red variety) were collected (Khan et al., 2012) at green (12–13 days after anthesis), pink (16–17 days after anthesis), and red (35–40 days after anthesis) stages representing early, intermediate, and mature stages, respectively, of ontogeny (Figure 1). Fresh berries (1 g) were crushed using a mortar and pestle and betalains were extracted with 5 mL of 0.25% ascorbic acid in water (w/v, pH adjusted to 5 with 1 N NaOH and 0.5 N HCl). The extract was spun at 8000 × *g* for 15 min at 4 °C. The supernatant was collected and the residue was washed with 5 mL of extraction solvent and spun. The supernatants were pooled and the betalains content in it was quantified using a spectrophotometer, and then HPLC and LC-MS analyses were carried out (Khan et al., 2012). Total proteins were extracted, partially purified using $(NH_4)_2SO_4$ at 80% saturation, and desalted by passing through a 10-kDa cut-off value membrane, and diphenol/DOPA oxidase (DO) activity was assayed (Steiner et al., 1999). Briefly, in 485 µL of 100 mM KPi buffer (pH 6.8) containing 4% DMFA (v/v), 175 µL of 28.6 mM MBTH was added (to inhibit tyrosine hydroxylase activity), followed by 300 µL of 10 mM DOPA as substrate. After 1 min, the oxidation reaction was initiated by adding 40 µL of partially purified protein extract. Yellow color development was monitored at 505 nm every 15 s up to 60 s against a blank solution devoid of enzyme preparation. The difference in the absorbance (15–45 s) was considered for expressing the units of specific activity as a 0.1 increase in absorbance/min/mg protein. Autoxidation of DOPA was 0.002–0.003 absorbance units/min. Total proteins content was quantified using Bradford reagent (Bradford, 1976).

SDS-PAGE of the protein extract was carried out by loading on 7.5% acrylamide gel and resolved on 12.5% acrylamide gel under 100 V. The gel was stained overnight with Coomassie brilliant blue solution. The protein bands were visualized after destaining for 3–4 h in destaining solution. Protein molecular weight marker containing seven purified proteins of molecular weight ranging from 14.4 kDa to 116 kDa (Cat. No. SM0431, Fermentas Life Sciences, GmbH, Germany) was used to calculate the tentative molecular weight of the bands by plotting a graph of Rf value against molecular weight.

2.3. Elicitor treatment, berry harvest, and analyses

Rivina seeds were soaked in 4% urea water overnight, sown in a pot containing vermicompost and soil at a 1:2 (w/w) ratio, and kept in a polyhouse receiving direct sunlight and tap water in April 2011. During the period of seed germination the minimum and maximum temperatures were 18 °C and 30 °C and humidity was in the range of 55%–60%. After 1 month, seedlings were subcultured and flowers of 4-month-old plants were treated with elicitors (salicylic acid- SA, and chitosan- CH) by floral dip method in August 2011. Groups were as follows: control, deionized water; treatment 1, SA (0.05 mM); treatment 2, SA (0.1

Figure 1. Green (A), pink (B), and red (C) *R. humilis* berries representing different stages of ontogeny (bar = 10 cm).

mM); treatment 3, SA (0.5 mM); treatment 4, 0.1% CH (w/v); treatment 5, 0.5% CH (w/v); treatment 6, 1% CH (w/v).

On the third day, the flowers were treated for the second time. One month after the first treatment, the berries were harvested and betalain pigments were extracted and quantified as described above. Total RNA was extracted with a plant total RNA kit (Spectrum, Sigma-Aldrich) and cDNA was prepared using RevertAid reverse transcriptase obtained from Fermentas Life Sciences. The genes were amplified using the primers listed in Table 1. The standard PCR reaction was performed with 1 µL of cDNA in a 10-µL reaction volume containing 1X PCR buffer (Sigma-Aldrich), 200 mM dNTPs, 0.5 units of Taq DNA polymerase, and 400 nM forward and reverse primers of 18S rRNA. The volume was made up to 10 µL with autoclaved triple-distilled H_2O. Final concentration of the primers was 10 mM. Similarly, superoxide dismutase (SOD), catalase (CAT), and *Rivina humilis* betanidin-5-*O*-glucosyltransferase 2 (RhBGT2) were also amplified using 2, 2.5, and 1 µL of cDNA in the reaction mix containing corresponding primers at final concentrations of 4, 3.3, and 10 mM, respectively.

PCR conditions were standardized as follows: for 18S rRNA amplification, initial denaturation was set at 95 °C for 3 min followed by 30 amplification cycles comprising denaturation (95 °C, 30 s), annealing (55 °C, 45 s), and extension (72 °C, 30 s) to get a sufficient quantity of amplified product. Final extension was achieved at 72 °C for 8 min. For RhBGT2, the PCR conditions were same as that of 18S rRNA except that the number of cycles was increased to 35. In addition to the PCR conditions of 18S rRNA, for SOD and CAT the respective annealing temperatures listed in Table 1 were used and the number of amplification cycles was raised to 40 and 45, respectively. The PCR product was resolved on 1.2% agarose gel in horizontal electrophoresis using 1X TAE buffer for 1 h at 100 V. The DNA was stained with 5 µg/mL ethidium bromide in water, visualized under UV light, and documented using a gel documentation system consisting of a camera (E.A.S.Y. 442 K, Herolab, GmbH, Germany) mounted on a UV transilluminator (UVT–20 S/M, Herolab). The band intensity was quantified using the E.A.S.Y Win32 imaging software (Herolab). The PCR product separated on gel was eluted using GenElute (Sigma Aldrich) and ligated into the pTZ57R/T cloning vector following the manufacturer's protocol (MBI, Fermentas). The ligated construct was cloned in the *E. coli* DH5α strain, and plasmid was isolated using Plasmid MiniPrep (Sigma Aldrich) and sequenced (Bioserve Biotechnologies India Pvt. Ltd., Hyderabad, India). Expression levels of the genes were determined by normalizing the band intensity with that of 18S rRNA.

2.4. Statistical analysis

Three independent experiments were carried out for all the analyses and results are presented as mean ± SD. Two-way ANOVA testing was carried out by using Microsoft Excel with Windows 7 software. $P < 0.05$ was considered significant. A multiple range test was carried out manually using the standard deviations of the means.

Table 1. Details of the primers used in this study.

Gene	Primer name	Sequence	Annealing temperature (°C)	Fragment size (bp)	Accession number[a]
18S rRNA	Forward	5′ GTCGTGATGGGGATAGAT 3′	55	207	KC466434
	Reverse	5′ AGGTTCAGTGGACTTCTCG 3′			
SOD	Forward	5′ TGGTCCTCATTTCAATCCTG 3′	54	186	HF572788
	Reverse	5′ TCAGCGTGGACAACAACAG 3′			
CAT	Forward	5′ CATGAATTTCATGCACAGGG 3′	54	165	HF572789
	Reverse	5′ TGAAATTGTTCTCCTTCTCA 3′			
RhBGT2	Forward	5′ GGTGGGACCCTCTCTTATGAT 3′	55	286	KC466436
	Reverse	5′ CGAGTTCCACCCACAATG 3′			

[a]: The eluted PCR products were ligated into the pTZ57R/T vector, which was moved into *E. coli* (DH5α strain) (Sambrook and Russell, 2001). The clones were used for plasmid preparation (GenElute Plasmid Miniprep kit, Sigma-Aldrich Co., MO, USA) and the plasmid was sequenced (Bioserve Biotechnologies India Pvt. Ltd, Hyderabad, India). The gene sequences were submitted to the EBI or NCBI.

3. Results and discussion

3.1. Biochemical profile of *Rivina* berries during ontogeny

During the early stage, only betacyanin (i.e. betanin) was present (Table 2). The major pigment was previously identified as betanin on the basis of the absorbance and mass spectral characteristics presented (Khan et al., 2012). In the intermediate stage, betacyanins level was 3-fold that of betaxanthins, but in matured berries betaxanthins content was 1.6-fold that of betacyanins. This suggests that the same pool of betalamic acid contributed actively to betacyanins biosynthesis in the initial stages, but at the later stage betaxanthins biosynthesis was favored. Similar observations have been documented in *Myrtillocactus geometrizans* (Herrera-Hernández et al., 2011), *Opuntia* sp. (Cayupán et al., 2011), and *Opuntia stricta* (Castellar et al., 2012).

Tyrosinase was shown to catalyze tyrosine hydroxylation (EC 1.14.18.1) as well as oxidation of diphenols (DO, EC 1.10.3.1) and it was assumed that both the active sites were present in a single polypeptide (Steiner et al., 1999). Since tyrosinase is one of the key enzymes required for betalains biosynthesis, its expression profile was found to increase proportionally with pigment content during ontogeny of *Phytolacca americana* berries (Joy et al., 1995). It was also observed that DO activity (a part of tyrosinase) was higher in high betalain-accumulating parts of red beet plants (Steiner et al., 1999). Accordingly, during ontogeny of *Rivina* berries, DO activity increased significantly and the activity was the highest in matured berries, which had the highest betalains content (Table 2). The SDS-PAGE profile of the partially purified protein extract from *Rivina* berries showed the presence of a band corresponding to MW 53.4 kDa (Figure 2). A similar MW of tyrosinase was reported by earlier researchers as well (Steiner et al., 1999).

Since there was less sample available for enzyme assay and due to other practical problems, gene expression study was considered suitable for elicitor-treated samples. Among the betalains' biosynthetic enzymes, only gene amplification of BGT was successful. Four ESTs of a glucosyltransferase were cloned and sequenced (RhBGT1, 2, 3, and 4). Phylogenetic analysis (Figure 3) of the four ESTs revealed that *RhBGT2* was closely related to *P. americana* glucosyltransferase 2 (*PaGT2*), which was previously shown to have betanidin-5-*O*-glucosyltransferase activity (Noguchi et al., 2009). ClustalW2 alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2) of *RhBGT2* produced a score of 81.6% and of 79%

Table 2. Changes in betalains, relative expression levels of putative *RhBGT2*, and antioxidant enzyme genes in *R. humilis* berries from in situ plants during different stages of ontogeny.

Parameter	Green	Pink	Red
Pigment content			
Betacyanins (mg/100 g)	2.8 ± 0.2	26.2 ± 1.3 [c]	156.1 ± 10.6 [d]
Betaxanthins (mg/100 g)	0.51 ± 0.1	8.5 ± 0.2 [c]	247.8 ± 15.0 [d]
Total betalains (mg/100 g)	3.3 ± 0.3	34.7 ± 1.5 [c]	403.9 ± 25.6 [d]
Enzyme expression level			
DO specific activity [a]	1.4 ± 0.3a	2.8 ± 0.5b [e]	4.0 ± 0.2c [f]
RhBGT2 [b]	0.94 ± 0.04a	1.2 ± 0.01a	1.1 ± 0.03a
SOD [b]	2.6 ± 0.04c	1.4 ± 0.39b [g]	0.66 ± 0.02a [h]
CAT [b]	3.0 ± 0.07c	1.2 ± 0.32b [g]	0.92 ± 0.11b [i]

DO– diphenol oxidase enzyme; RhBGT2– putative *R. humilis* betanidin-5-*O*-glucosyltransferase 2 gene; SOD– superoxide dismutase gene; CAT– catalase gene.

[a]: One unit of DO activity was expressed as increase in absorbance unit (0.1)/min/mg protein. Different letters in the same row indicate significant difference at $P < 0.05$ in increasing order. Green, pink, and red are berries from during ontogeny represent early, intermediate, and matured stages. Mean ± SD

[b]: Gene expression level was determined semiquantitatively using RT-PCR of total RNA and normalized with that of 18S rRNA.

[c]: $P < 0.0001$ compared to green, [d]: $P < 0.0001$ compared to pink.

[e]: $P < 0.001$ compared to green, [f]: $P < 0.01$ compared to pink.

[g]: $P < 0.01$ compared to green, [h]: $P < 0.001$ compared to pink.

[i]: $P < 0.001$ compared to green.

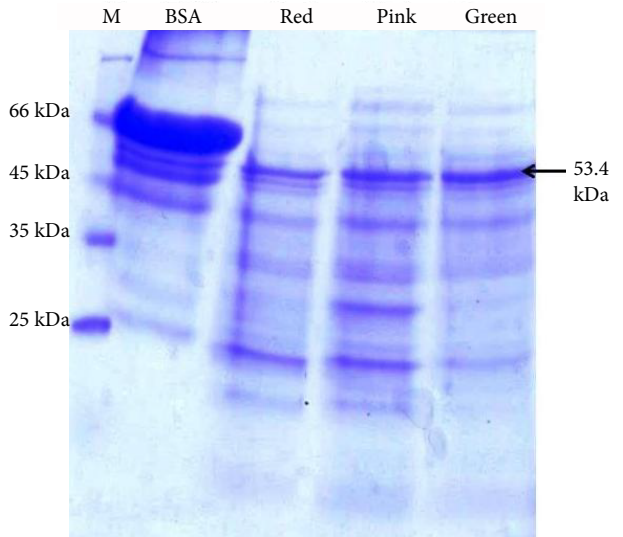

Figure 2. SDS-PAGE of partially purified protein extracts from *R. humilis* berries during ontogeny. The putative tyrosinase band of 53.4 kDa was determined from a graph constructed using MW vs. Rf value of protein molecular weight marker. M– marker, BSA– bovine serum albumin.

and 70% with PaGT3 and PaGT2, respectively. PaGT2 and PaGT3 were shown to glucosylate flavonoids and betanidin (Noguchi et al., 2009). Expression levels of *RhBGT2* during *Rivina* berry ontogeny revealed that there was no significant increase in the enzyme expression among green, pink, and red berries (Table 2), although there was exponential increase in betacyanins content. The reason for this may be that the variant amplified in this study is not actively involved in glucosylation of betanidin/isobetanidin to produce betanin/isobetanin. Attempts to amplify *RhBGT1*, -3, and -4 were not successful.

The role of betalains in combatting ROS in plants has been hypothesized. Supporting this, expression of *SOD* and *CAT* genes decreased during *Rivina* berry ontogeny (Table 2). The lowest level was observed in matured berries that contained the highest level of betalains. This indicated that nonenzymic antioxidant betalains accumulation might have resulted in reduction of antioxidant enzymes. Further studies in this regard should focus on radical scavenging capacity, levels of thiols, etc. in plants. During progression from immature to mature stages, SOD and CAT decreased in strawberry fruits (Luo et al., 2011), which accumulate anthocyanins (functional analogs of betalains).

3.2. Elicitor-mediated betalains enrichment

During *Rivina* berry ontogeny, increased betalains accumulation was concomitant with decreased expression of antioxidant enzymes (discussed in the previous section). On the other hand, externally applied elicitors induce stress in plants causing increase in antioxidant enzymes' expression levels. To verify if similar changes take place in the case of elicitor-mediated enhancement of betalains accumulation in matured berries, expression levels of *RhBGT2*, *SOD*, and *CAT* were determined. Application of SA (0.1 mM) or chitosan (0.5%, w/v) to flowers of *Rivina* increased the betalains content significantly ($P < 0.05$); however, expressions of *RhBGT2*, *SOD*, and *CAT* genes were not affected (Table 3). Foliar application of SA (0.01–1 mM) to intact plants (purple cornflower) elicited phenolics content (Kuzel et al., 2009). SA (0.1 mM) has been reported to increase flavonoids, vitamin C, and glucosinolates in broccoli sprouts (Pérez-Balibrea et al., 2011). However, in *Amaranthus* seedlings, SA (0.1–1 mM) did not affect betalain content over a period of 4 days (Cao et al., 2012). Concentrations of SA ranging from 0.05 to 0.2 mM have been shown to elicit phenolic compound accumulation as well as increased activities of antioxidant enzymes including SOD and CAT in *Salvia miltiorrhiza* cell culture (Dong et al., 2010). These reports indicated that different plant systems may respond variably to different SA concentrations, probably owing to the different basal levels of endogenous SA. Although treatment with SA (0.1 mM) has been implicated in expression of glucosyltranferases in tobacco (Fraissinet-Tachet et al., 1998) after 3 h of treatment, in this study, *RhBGT2* was not affected by SA

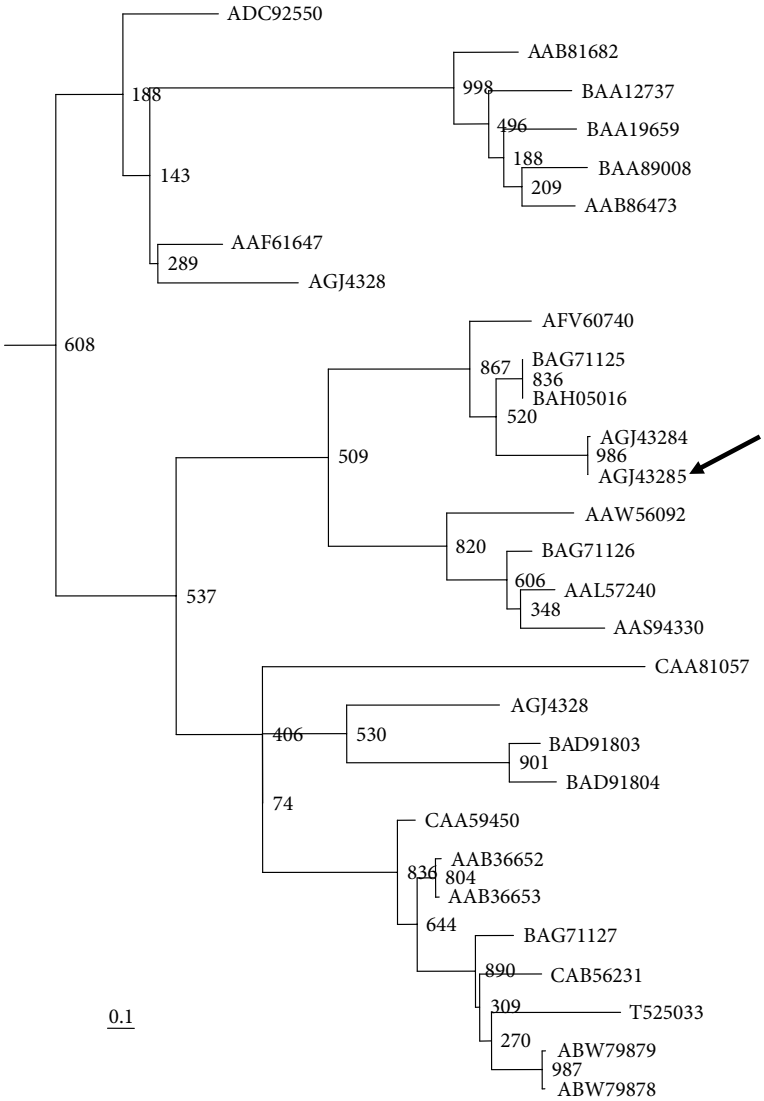

Figure 3. Maximum likelihood phylogeny tree reconstructed in PhyML 3.0 (Guindon et al., 2010) by using amino acid sequences and viewed by using TREEVIEW (Page, 1996). The best fit model (LG+G) was considered after running the program Prot Test 2.4 (Abascal et al., 2005). The EST of *R. humilis* betanidin-5-*O*-glucosyltransferase 2 (RhBGT2) (AGJ43285, indicated by arrow) was used for differential expression studies in this report. GenBank nucleotide entries of the amino acid sequences of glucosyltransferases (GT) in this tree are as follows: RhBGT (AFV60740), RhBGT1 (AGJ43284), RhBGT3 (AGJ43286), RhBGT4 (AGJ43287), *Nicotiana tabacum* UDP-glucose:salicylic acid GT (AAF61647, AAB36653), *Gentiana triflora* UDP-glucose:flavonoid-3-*O*-GT (BAA12737), *Hordeum vulgare* subsp. *vulgare* GT (ADC92550), *Vitis vinifera* UDP glucose:flavonoid-3-*O*-GT (AAB81682), *Perilla frutescens* flavonoid-3-*O*-GT (BAA19659), *Petunia* × *hybrida* anthocyanin-5-*O*-GT (BAA89009), *Petunia* × *hybrida* anthocyanidin-3-glucoside-*O*-rhamnosyltransferase (CAA81057), *Ipomoea purpurea* flavonoid-3-*O*-GT (AAB86473), *Phytolacca americana* glucosyltransferase (PaGT1) (BAG71126), PaGT2 (BAG71125, BAH05016), PaGT3 (BAG71127), *Medicago truncatula* triterpene GT UGT71G1 (AAW56092), *Cleretum bellidiforme* betanidin-6-*O*-glucosyltransferase (CbB6GT) (AAL57240), CbB5GT (CAB56231), *Beta vulgaris* flavonoid-*O*-GT (AAS94330), betanidin-5-*O*-GT (T525033), *Mirabilis jalapa cyclo*-DOPA-5-*O*-GT (BAD91803), *Celosia cristata cyclo*-DOPA-5-*O*-GT (BAD91804), *Solanum lycopersicum* GT (CAA59450), *Opuntia ficus-indica* betanidin-5-*O*-GT (ABW79879, ABW79878).

Table 3. Betalains content (mg/100 g FW) and relative expression levels of *RhBGT2, SOD,* and *CAT* in *R. humilis* berries after 30 days of elicitor application to flowers of ex situ plants.

Treatment	Control	SA (0.05 mM)	SA (0.1 mM)	SA (0.5 mM)	CH (0.1%)	CH (0.5%)	CH (1%)
Pigment content							
Betaxanthins	90.0 ± 2.0	101.9 ± 14.8	157.9 ± 23.6 [a]	62.0 ± 5.9 [a]	91.6 ± 22.7	125.4 ± 11.3 [a]	133.3 ± 40.3
Betacyanins	107.0 ± 3.7	113.7 ± 3.9	136.6 ± 0.51 [a]	118.0 ± 10.7	95.0 ± 15.6	138.4 ± 3.1 [a]	95.4 ± 12.9
Total betalains	197.0 ± 5.6	215.6 ± 14.4	294.5 ± 23.1 [a]	180.0 ± 16.6	186.6 ± 38.3	263.8 ± 14.3 [a]	228.7 ± 53.2
Gene expression level							
RhBGT2	0.58 ± 0.04	0.67 ± 0.2	0.5 ± 0.17	0.45 ± 0.14	0.51 ± 0.26	0.57 ± 0.24	0.45 ± 0.2
SOD	0.7 ± 0.02	0.68 ± 0.13	0.71 ± 0.07	0.68 ± 0.02	0.59 ± 0.02	0.6 ± 0.02	0.3 ± 0.02 [b]
CAT	0.46 ± 0.02	0.39 ± 0.06	0.48 ± 0.002	0.57 ± 0.01	0.52 ± 0.01	0.6 ± 0.02	0.44 ± 0.09

RhBGT2– *R. humilis* betanidin-5-*O*-glucosyltransferase gene; *SOD*– superoxide dismutase gene; *CAT*– catalase gene; SA– salicylic acid; CH– chitosan. Pigment content is on fresh weight basis and values are mean ± SEM (n = 3). Gene expression level was determined semi-quantitatively using RT-PCR of total RNA and normalized with that of 18S rRNA, and values are mean ± SD (n = 3). Data analysis was carried out using two-way ANOVA. [a]: $P < 0.05$ compared to control. [b]: $P < 0.001$ compared to control.

treatment (Table 3). *SOD* and *CAT* expression levels were also not affected by SA treatments (Table 3), probably because there was no significant formation of superoxide anions and hydrogen peroxide.

Chitosan (0.12%, w/v) was shown to elicit isoflavones content in hydroponically grown *Lupinus luteus* (Kneer et al., 1999), and phenolics in wheat seedlings were also elicited by chitosan (>0.4%, w/v) (Reddy et al., 1999). However, application of a lower concentration of chitosan (0.1%, w/v) to soybean sprouts failed to elicit isoflavones content and there was no correlation between isoflavone concentrations and expression levels of enzymes involved in the phenylpropanoid pathway as well as that of isoflavone synthase (Chen et al., 2009). On the other hand, a still lower concentration (0.5 mg/L) of chitosan was effective in eliciting betacyanins in *P. americana* suspension cultures (Bhuiyan and Adachi, 2003). Chitosan-mediated synthesis of plant secondary metabolites involves a complex signaling network comprising the PLC/PKC pathway, PI3K-mediated activation of PKC leading to MAPK cascade, intracellular calcium mobilization, and G-proteins (Vasconsuelo and Boland, 2007).

In this study, even if there was significantly increased betalains accumulation (in the case of SA at 0.1 mM and CH at 0.5%), SA or CH treatment did not produce corresponding increases in the expression of the putative betalain biosynthetic enzyme, RhBGT2, and antioxidant enzymes such as CAT and SOD (Table 3). This could be attributed to the 1-month gap between treatment and harvest of berries for analysis, during which the induced genes, if there were any such changes at all, might have returned to the normal range owing to the absence of an elicitor signal. In general, elicitor signals are short-lived (Vasconsuelo and Boland, 2007; Georgiev et al., 2008). On the other hand, betalains are secondary metabolites and once synthesized they are stored safely in vacuoles, because of which the increased level was detectable even though there was a 1-month gap between elicitor treatment and berry harvest. Interestingly, compared to the control (Figure 4A), treatment with chitosan (0.1%, w/v) induced a delayed ripening effect (Figure 4B), while treatment with chitosan (1%, w/v) suppressed *SOD* expression (Table 3). This could be owing to the enhanced ripening/senescence (Figure 4C). The physiological changes in *Rivina* berries in response to chitosan treatment need further studies.

In conclusion, the pattern of betalains increase during ontogeny as well as abiotic stress indicated that, probably,

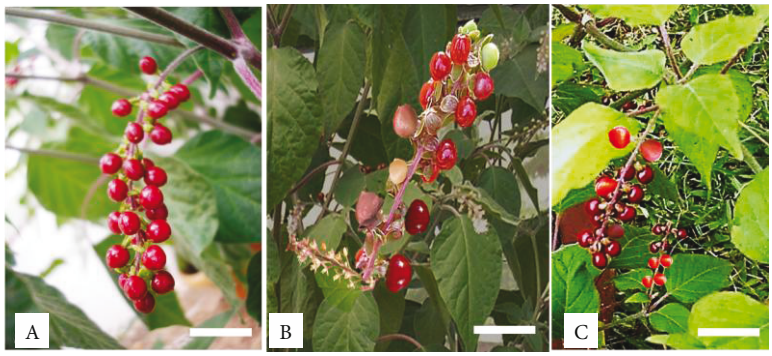

Figure 4. *R. humilis* berries after 30 days of elicitor treatment to ex situ plants by floral dip method. Control (A), delayed ripening of berries in chitosan (0.1%, w/v)-treated plants (B), enhanced ripening and morphological changes of berries in chitosan (1%, w/v)-treated plants (C). Bar = 15 cm.

both betacyanins and betaxanthins are synthesized from the same pool of betalamic acid. Betalains accumulation is accompanied by decreased expression of antioxidant enzymes *SOD* and *CAT*, indicating its possible role in combating ROS in plants. SA/CH-induced stress led to about a 1.3-fold increase in betalains accumulation in intact *Rivina* berries.

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