Gene Expression of Flavanone 3-Hydroxylase (F3H), Anthocyanidin Synthase (ANS), and p-Coumaroyl Ester 3-Hydroxylase (C3H) in Tzimbalo Fruit

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Abstract—The current investigation emphasizes the expression of candidate genes for future fruit quality improvement. This study aims to describe morphological variation on Solanum caripense Dunal (tzimbalo) ecotypes; identify gene expression of F3H and ANS and analyze gene expression of C3H. This study employed Ecuadorian (BIO) and Peruvian (IBT) as samples of the study. Morphological descriptors for Solanum muricatum Aiton were used in this study. RNA was isolated for identification of F3H and ANS transcripts in BIO-Ltg1 and BIO-Cyb1 through reverse transcription followed by semiquantitative PCR (RT-PCR). C3H relative expression was analyzed in IBT-Lib1 for zero, five and fourteen days under the influence of controlled conditions (10 ± 2 °C; 16 h day/8 h night) through reverse transcription followed by quantitative PCR (RT-qPCR). The cophenetic correlation (0.88) of conglomerate analysis (CA) pointed out good similarity for Ecuadorian ecotypes and two subgroups for Peruvian ecotypes. The first three principal components (PC) explained qualitatively 71.39% and quantitatively 81.34% of total variation; Fr-Flavour, Sc-Diameter, Fl-CorollaColour, Fr-Stripes, Fr-Length, Fr-PlacentLength, and Fr-PlacentBreadth were characters that contributed more to the variability. The expression of F3H was identified in BIO-Ltg1. The expression of ANS was similar (BIO-Ltg1→48.20 ng·µL−1; BIO-Cyb1→36.19 ng·µL−1). The mean fold change value in C3H expression was 3.32, 4.52, and 6.24 for zero, five, and fourteen days; C3H transcripts level was significantly different and increased 2.92 units after fourteen days. These results demonstrate the expression of F3H and ANS in BIO-Ltg1 and BIO-Cyb1, differential expression of C3H in IBT-Lib1, and focus the nutritional value of tzimbalo fruit.

Keywords—Reverse transcription; wild relatives; fruit quality; improvement; commercial potential.

I. INTRODUCTION

The S. caripense is a phylogenetically complex plant [1], commonly named tzimbalo [2], mostly wild, and widely distributed in Ecuador and Peru [3], [4], also present in Colombia, Bolivia, Venezuela, Costa Rica and Panama [5]. This species is an herbaceous plant, native of the Andean region [6], growing on damp places of highlands [7], even until 3800 m.a.s.l. [3]. The tzimbalo plant is compact, and similar to the morphology of S. muricatum (pepino) this species produces vertical branches [8], is considered, with high likeliness, a close relative to the pepino, and its ancestor [9], [10], due to chromosome similarities and the possibility for harvesting of the obtained interspecific hybrids [11]; it belongs to the section Basarthrum, series Caripensia, and complex Caripense [1], [12], [13].

The fruit of S. caripense has many seeds, its high germination percentage [14], [15] lets the investigations to discard the presence of primary dormancy and physical lethargy [16]. In contrast to seeds of other wild species of the genus Solanum [17], [18], the fruit of S. caripense (EC-40) contains significantly more sucrose, vitamin C [19], and minerals [20], compared to modern cultivated varieties of S. muricatum, and other species of the series Caripensia.

Despite the great potential of S. caripense for interspecific gene flow towards related commercial crops as Solanum tuberosum L., Solanum lycopersicon L. and S. muricatum, there are limited genomic studies of this species [21], [22]. Modern biotechnological tools help overcome production, commercialization, and export limitations, such as gene
expression analysis [23] and genetic transformation [24].

Plant breeding with *S. caripense* and *S. muricatum* accessions is carried out through backcrosses to the Pepino. The estimation of sucrose and ascorbic acid concentrations [25], and others decided by candidate genes involved in fruit quality improvements such as anthocyanins and chlorogenic acid contents [22], leads to heritability studies.

Relative quantification measures the expression levels and their relative change. It determines the change in certain mRNA levels of a gene across multiple samples. It does not require a calibration curve or standards with known concentration, and the reference genes can be any transcript, as long as its sequence is known [26]. The measures aim to express relative quantities of the unit used is arbitrary, and its quantities can be compared across multiple RT-qPCR experiments [27], [28]. Relative quantification assumes an optimal doubling of the complementary DNA (cDNA) of interest during each performed qPCR cycle [29]; its model is derived from the exponential nature of the PCR; the amplification efficiency is close to one for the derived from the exponential nature of the PCR; the amplification efficiency is close to one for the double methylation in malvidin; anthocyanins based on delphinidin are found in purple tissues of *Solanum melongena* L. and *Capsicum* spp. The expression of genes involved in the accumulation of anthocyanins covering specific tissues during certain stages of development can be stimulated by exposure to white light and low temperature [22], [34], [35]. Anthocyanins are phenolic compounds or secondary metabolites of the flavonoid subclass, soluble in water and important due to its antioxidant ability [36].

Phenolic compounds cause the high antioxidant activity in *S. melongena* [37], [38], and *S. tuberosum* [39], these are hydroxycinnamic acid (HCA) conjugates synthesized by phenylalanine conversion into cinnamic acid. Chlorogenic acid (CGA, 5-O-caffeoyl-quinic acid) is an HCA conjugate that reaches 70% and exceeds over 95% of phenolic content totality. Great diversity is observed in the total content of phenolic and CGA concentrations, caused by genetic and environmental factors. Molecular breeding for high CGA content, low polyphenol oxidase (PPO) activity, and consequently low degree of browning helps develop improved varieties for higher bioactive properties [37]. A candidate gene approach is promising for this purpose, given that genes involved in the biosynthetic pathway of CGA are well characterized [39]. In the Solanaceae, the abundance of CGA is strongly associated with different genes of its biosynthetic pathway [40], [41]. The development and storage stage influences gene expression and phenolic content [42]. Postharvest conditions pretend to prolong shelf life and increase the agronomic quality of vegetal products.

This work aims at contributing with biotechnological tools for plant breeding programs and emphasizes the expression of *S. caripense* genes, which belong to biosynthetic pathways of anthocyanins and chlorogenic acid; with a future propose of innovating the local production, improving the fruit quality, and converting this species into a novel alternative for consumption and derivative uses. The specific objectives were to 1) describe a morphological variation on *S. caripense* ecotypes; 2) identify gene expression of F3H and ANS associated with anthocyanins; 3) analyze gene expression of C3H associated with chlorogenic acid. Morphological description was performed using descriptors for *S. muricatum*; total RNA was isolated from *S. caripense* for the identification of F3H and ANS transcripts in BIO-Ltg1 and BIO-Cyb1 through RT-PCR; the relative expression of C3H was analyzed in IBT-Ltg1 for zero, five, and fourteen days under the influence of controlled temperature (10 ± 2°C) and photoperiod (16 h day/8 h night) through RT-qPCR. The studied genes belong to biosynthetic pathways that codify beneficial human enzymes, with industrial potential [43], due to their biological activities and antioxidant properties.

II. MATERIAL AND METHODS

A. Plant Material

Regions, departments, and provinces related to the geographic distribution of *S. caripense* plants were taken as reference [4], [5]; individual plants were identified and in situ described on mostly wild *S. caripense* ecotypes (Table I).

| Ecotype | Origin | Traits |
|---------|--------|--------|
| BIO-Cyb1 | EC-P, 3298 m.a.s.l. | Fr-Flavour acidic, AddColour between 10-30%, Length 2.03 cm |
| BIO-Cyb2 | EC-P, 2950 m.a.s.l. | Fr-Flavour sweet, Se-Diameter intermediate |
| BIO-Cyb3 | EC-P, 3282 m.a.s.l. | Fr-Flavour moderately sweet, Mottling present |
| BIO-Ltg1 | EC-X, 3055 m.a.s.l. | Fr-Flavour moderately sweet, AddColour between 30-50%, Se-Diameter intermediate |
| BIO-Ltg2 | EC-X, 2717 m.a.s.l. | Fr-Flavour sweet, Length 1.93 cm, Se-Diameter intermediate |
| BIO-Ltg3 | EC-X, 3016 m.a.s.l. | Fr-Flavour sweet, AddColour less than 10%, Se-Diameter small |
| IBT-Aybl | PE-PIU, 2814 m.a.s.l. | Fr-Flavour acidic, Mottling present, St-Pubescence dense |
| IBT-Lib1 | PE-TRU, 3329 m.a.s.l. | Fr-Flavour moderately sweet, Stripes absent, Length 3.4 cm |
| IBT-Lim1 | PE-LIM, 2560 m.a.s.l. | Fr-Flavour acidic, Mottling present, Se-Diameter small |

EC-P: Ecuador-Pichincha (Cayambe); EC-X: Ecuador-Cotopaxi (Lataungu); PE-PIU: Peru-Piura (Ayabaca); PE-TRU: Peru-Trujillo (La Libertad); PE-LIM: Peru-Lima.
Plant (P), stem (St) and leaf (L) descriptors

I. Number of flowers per inflorescence (I) and flower (Fl) descriptors

- Vigour of the plant/ P-Vigour
  - 3 Weak; 5 Intermediate; 7 Strong
- Degree of ramification/ St-Ramification
  - 3 Low; 5 Intermediate; 7 High
- Root protuberances at the node/ St-Protuberances
  - 0 Absent; 1 Not winged; 2 Intermediate; 3 Winged; 5; Few; 5 Intermediate;
- Stem pubescence density/ St-Pubescence
  - 0 Glabrous; 3 Sparse; 5 Intermediate; 7 Dense
- Stem colour/ St-Colour
  - 1 Green; 2 Greenish with purple spots; 3 Greenish purple; 4 Purple; 5 Dark purple
- Internode length/ St-InterLength
  - [cm]
- Petiole length/ L-PetioleLength
  - [mm]
- Leaf colour/ L-Colour
  - 1 Light green; 2 Green; 3 Dark green; 4 Greenish purple; 5 Purple
- Anthocyanin colouration of leaf veins/ L-AnthVeins
  - 3 Green; 5 Main veins purple and the rest green; 7 Purple
- Leaf surface attitude/ L-Surface
  - 3 Flat; 5 Intermediate; 7 Very convex

Inflorescence (I) and flower (Fl) descriptors

- Inflorescence type/ I-Type
  - 1 Generally uniparous; 2 Both (partly uniparous, partly multiramous); 3 Generally multiramous
- Number of flowers per inflorescence/ I-NFlowers
  - -
- Corolla shape/ Fl-CorollaShape
  - 1 Stellate; 2 Semi-stellate; 3 Rotate
- Corolla colour/ Fl-CorollaColour
  - 1 White; 2 Stripped (white >75% and purple <25%); 3 Stripped (white 50–75% and purple 25–50%); 4 Stripped (white 25–50% and purple 50–75%); 5 Stripped (white < 25% and purple >75%); 6 Purple
- Sepal length/ Fl-SepulLength
  - [mm]
- Stamen length/ Fl-StamenLength
  - [mm]
- Style exertion/ Fl-StyleExsertion
  - [mm]
- Pollen production/ Fl-PollenProd
  - 0 None; 3 Low; 5 Medium; 7 High

Fruit (Fr) and seed (Se) descriptors

- Fruit size uniformity/ Fr-Uniformity
  - 3 Low; 5 Intermediate; 7 High
- Fruit length/ Fr-Length
  - [cm]
- Fruit width/ Fr-Width
  - [cm]
- Position of the widest part of the fruit/ Fr-WidestPart
  - 3 Less than 1/4 way from base to tip; 5 Between 1/4 and 1/2 way from base to tip; 7 More than 1/2 way from base to tip
- Fruit length/width ratio/ Fr-LWRatio
  - -
- Predominant fruit shape/ Fr-Shape
  - 1 Flattened; 2 Round; 3 Ellipsoid; 4 Obovate; 5 Oval; 6 Cordiform; 7 Conical; 8 Elongate; 9 Other
- Predominant fruit colour at commercial ripeness/ Fr-Colour
  - 1 Dark green; 2 Light green; 3 Milk white; 4 Pale yellow; 5 Golden yellow; 6 Orange yellow; 7 Lilac; 8 Purple; 9 Purple black
- Fruit stripes/ Fr-Stripes
  - 0 Absent; 1 Present
- Fruit mottling/ Fr-Mottling
  - 0 Absent; 1 Present
- Fruit surface covered by additional colour/ Fr-AddColour
  - 1 Less than 10%; 2 Between 10 and 30%; 3 Between 30 and 50%
- Fruit epidermis glossiness/ Fr-Glossiness
  - 3 Dull; 5 Intermediate; 7 Bright
- Number of locules per fruit/ Fr-Locules
  - -
- Inner placental area length/ Fr-PlacentLength
  - [cm]
- Inner placental area breadth/ Fr-PlacentBreadth
  - [cm]
- Fruit flesh colour/ Fr-FleshColour
  - 1 Dark green; 2 Light green; 3 White; 4 Pale yellow; 5 Golden yellow; 6 Orange yellow; 7 Orange, 8 Salmon
- Fruit flavour/ Fr-Flavour
  - 1 Very acid; 3 Acidic; 5 Moderately sweet; 7 Sweet; 9 Very Sweet
- Number of seeds per fruit/ Fr-NumSeeds
  - 1 Very few (1–5); 2 Few (6–25); 3 Intermediate (26–75); 4 Many (76–250); 5 Very many (>250)
- Seed colour/ Se-Colour
  - 1 White; 2 Light yellow; 3 Grey yellow; 4 Brownish yellow; 5 Brown; 6 Brown black; 7 Black
- Seed diameter/ Se-Diameter
  - 1 Small (<1.5 mm); 2 Intermediate (1.5–2.5 mm); 3 Large (>2.5 mm)
- Type of seed/ Se-Type
  - 1 Not winged; 2 Intermediate; 3 Winged

B. Morphological Description by CA and PCA

Morphological descriptors for S. muricatum and wild related species were used (Table II) [45]; descriptors of plant (P), stem (St), leaf (L), inflorescence (I), flower (Fl), fruit (Fr) and seed (Se) were evaluated; mode and mean values were obtained for three observations per plant, differentiating between qualitative and quantitative variables, respectively [46], [47]; CA and PCA were performed.

C. Gene Expression by RT-PCR and RT-qPCR

Based on S. melongena sequences for F3H, ANS [22], [48], C3H genes [37]; and 5.8S rRNA [49], primers were synthesized to be used in S. caripense (Table III).
Total RNA was isolated from the fruit of *S. caripense* using the reagents *innuPREP Plant RNA* (Analytik Jena AG, Germany) and *PureLink® ARN Mini Kit* (Ambion, Life Technologies, USA); it was purified for the synthesis of the first strand of cDNA through reverse transcription using the reagents *5X All-In-One RT MasterMix* (Applied Biological Materials Inc., Canada). The microtubes for RT-PCR in a final volume of 10 µL contained the necessary components for amplification in thermocycler Mastercycler EP Gradient 96 well Thermal Cycler (Eppendorf, Germany). The microtubes for RT-qPCR in a final volume of 10 µL contained the necessary components for amplification in thermocycler QuantStudio® 3 (Applied Biosystems, USA) (Table IV).

| Gene   | Primers                                                                 |
|--------|-------------------------------------------------------------------------|
| F3H    | FW-Scara: aat gcc ata tgg tatt ccc tt a                              |
|        | RV-Scara: cca gcag att tcc tct cag                                  |
| ANS    | FW-Scara: gca etg act ttc atc etc caca                               |
|        | RV-Scara: tct gtt act tcc cgt tgc tta g                              |
| C3H    | FW-Scara: ttg gcag ctc tct tgt gtt cct ac                             |
|        | RV-Scara: cga cct tgc ttc ctt gg                                     |
| 5.8S rRNA | FW-Scara: cca ggg ata tct cgg etc tc                                 |
|        | RV-Scara: tgg ctt cca aag act cga tg                                 |

| Component                     | Volume (µL) |
|-------------------------------|-------------|
| cDNA (100 and 750 ng/µL)      | 2.0 and 1.0 |
| Buffer PCR 10X                | 1.0         |
| MgCl2 (50 mM)                 | 1.0         |
| dNTPs (10 mM)                 | 0.4         |
| Specific forward primers (10 µM) | 0.4       |
| Specific reverse primers (10 µM) | 0.4      |
| DNA polymerase Taq             | 4.6 and 5.6 |
| Molecular grade water         |             |

| Amplification program in thermocycler | Time |
|---------------------------------------|------|
| Initial denaturation (95 °C)          | 3 minutes (1 cycle) |
| Denaturation (95 °C)                  | 15 seconds (40 cycles) |
| Annealing (52, 55 and 60 °C)          | 30 seconds (40 cycles) |
| Extension (72 °C)                     | 15 seconds (40 cycles) |
| Final extension (72 °C)               | 5 minutes (1 cycle) |
| Hold (4 °C)                           |             |

| Component                     | Volume (µL) |
|-------------------------------|-------------|
| cDNA (750 ng/µL)              | 1.0         |
| *BrightGreen 2X qPCR MasterMix* (ABM) Inc | 5.0 |
| Specific forward primers (10 µM) | 0.3 |
| Specific reverse primers (10 µM) | 0.3 |
| Molecular grade water         | 3.4         |

| Amplification program in thermocycler | Time |
|---------------------------------------|------|
| Activation of DNA polymerase HotStart (95 °C) | 10 minutes (1 cycle) |
| Denaturation (95 °C)                  | 15 seconds (40 cycles) |
| Annealing (60 °C)                     | 60 seconds (40 cycles) |
| Melting curve (95, 60, 95 °C)         | 15 seconds, 1 minute, 1 second |

When the reaction finished, it was assessed for gene-specific amplification fragments by melting curve. The C\textsubscript{T} values of samples were exported to an Excel® (Microsoft, USA) calculation sheet, and the relative expression was determined with the $2^{-\Delta\Delta C_T}$ method [30], [50]; where:

$$\Delta\Delta C_T = (C_{T,\text{C3H}} - C_{T,rRNA})_{\text{time 1}} - (C_{T,\text{C3H}} - C_{T,rRNA})_{\text{time 0}}$$ (1)

The values of C3H expression were normalized using 5.8S rRNA, and the levels of expression were relative to day zero. The relative expression in each level corresponds to the mean with four biological replicates (± S.E., n = 4) [39], [48], [51], and three technical replicates, whose C\textsubscript{T} values were manually controlled for S.D. > 0.5 [52].

**D. Data Analysis**

1) **Morphological Description:** The CA was performed by evaluation of 16 qualitative descriptors that showed variation in *S. caripense*: P-Size, P-vigour, St-Pubesence, St-colour, L-PetioleColour, L-Colour, Fl-ColollaColour, Fr-Uniformity, Fr-Shape, Fr-Stripes, Fr-Mottling, Fr-AddColour, Fr-FleshColour, Fr-Flavour, S-Colour and S-Diameter. The data was previously standardized, similarity matrix was obtained; the distance coefficient, mean character difference (MCD) [53] was applied, processing the data with statistics package InfoStat 2018; and plants were grouped by average linkage (UPGMA) [54], [55], using Community Analysis Package 1.2.

The PCA was performed by evaluation of 15 quantitative descriptors that showed variation in *S. caripense*: St-InterLength, L-PetioleLength, L-LaminaLength, L-LaminaWidth, L-LWRatio, L-Leaflets, L-NFlowers, Fl-SepalLength, Fl-StamenLength, Fl-StyleExsertion, Fr-Length, Fr-Width, Fr-LWRatio, Fr-PlacentLength and Fr-PlacentBreadth. Simple correlation matrix, Eigen values, and relative contribution coefficients of the principal components were obtained; processing the data with Community Analysis Package 1.2. PCA for qualitative descriptors was performed, too.

2) **Gene Expression:** The sample for analysis of ANS expression in BIO-Lig1 and BIO-Cyb1, consisted in 7 fruits per plant conserved at chilling temperature (10 ± 2 °C) with photoperiod (16 h day/8 h night) from fluorescent lights (1250 lx) for fourteen days; RNA was isolated from thin sheets of fruit skin. The data were disposed under CRD with two treatments, seven observations per treatment and a linear additive statistic model [56]. Statistic packages GelAnalyzer 2010 for comparative analysis and Minitab 17 for data processing were used.

The sample for analysis of C3H expression in IBT-Lib1, consisted in 12 fruits conserved at chilling temperature (10 ± 2 °C) with photoperiod (16 h day/8 h night) from fluorescent lights (1250 lx) for fourteen days; RNA was isolated from thin sheets of fruit flesh. The data were disposed under CRD; first an assay of the experiment was performed with three treatments and four biological replicates, and then with three technical replicates for analysis with the mean values; the statistic model is the same as above. Statistic packages InfoStat 2018, Minitab 17 and RStudio 1.2.1335 were used.
III. RESULTS AND DISCUSSION

A. Morphological Description

The phenogram corresponding to the CA for qualitative descriptors of *S. caripense* (Fig. 1), represented a cophenetic correlation coefficient ($r_{xy} = 0.88$) higher than 0.8; this implies a good representation of the similarity matrix [57].

Fig. 1 Phenogram corresponding to the conglomerate analysis (CA) for qualitative descriptors among tzimbalo individuals.

The PCA explained 92.18% of total variation until the PC5, with Eigen values higher than one (PC1 = 4.68, PC2 = 3.57, PC3 = 3.18, PC4 = 2.10; PC5 = 1.22); the PC1 and PC2 represented 29.22% and 22.29% of total variation, respectively (Fig. 2).

Fig. 2 Diagram corresponding to the principal components analysis (PCA) for qualitative descriptors among tzimbalo individuals. Bottom axis and left axis: Scores values for plants; Top axis and right axis: Eigenvectors values for descriptors

The mode of P-Size descriptor evaluated in *S. caripense* ecotypes could be similar for the tzimbalo accessions BIRM/S 1034, E-7, EC-40 and QL-013; and it is perceived a bit higher, and superior if were compared to the related wild species P-80, P-62, E-257 and E-34, or to the pepino cv. Sweet Long or cv. Puzol, respectively. Furthermore, rare radicular protuberances in node were observed in *S. caripense*, as greater steam pubescence density, and more compound leaves, similar to related wild species [58], [59]. Not all *S. caripense* plants presented fruit stripes, a descriptor of broad variability, important for agronomic purposes of these species; wild relatives are sources of variation for plant breeding and for studies about the process of domestication. The tzimbalo plants presented greater style exsertion, as high pollen production and many seeds per fruit, as related wild species, which contribute to cross pollination and germplasm dispersion; in contrast to modern cultivated varieties of *S. muricatum* [58], [59].

The descriptors Fr-Flavour, Se-Diameter, Fr-AddColour, Fl-CorollaColour, Fr-Stipes and others, represent sources of variation for the breeding of *S. muricatum* and related studies. The environment does not regulate the dominant type effects of qualitative expression in a monogenic or oligogenic mode, these are ideal for its high heritability [60].

The PCA approach for quantitative descriptors of *S. caripense* explained 91.88% of total variance until the PC4, with Eigen values higher than one (PC1 = 6.46, PC2 = 4.01, PC3 = 1.73, PC4 = 1.58); the PC1 and PC2 represented 43.05% and 26.75% of total variation, respectively (Fig. 3). The relative contribution coefficients (Eigenvectors values) indicated that the PC1 was positive correlated (values ≥ 0.15) with L-LWRatio (0.27), Fl-SepalLength (0.21), Fr-Length (0.35), Fr-Width (0.27), Fr-LWRatio (0.32), Fr-PlacentLength (0.37), and Fr-PlacentBreadth (0.35); and it was negatively correlated with St-InterLength (-0.21), L-PetioleLength (-0.26), Fl-StamenLength (-0.21), Fl-StyleExsertion (-0.29), L-Leaflets (-0.22), and I-NFlowers (-0.17). The PC2 was not positively correlated and was negatively correlated (absolute values ≥ 0.15) with St-InterLength (-0.36), L-LaminaLength (-0.41), L-LaminaWidth (-0.40), Fl-SepalLength (-0.35), Fl-StamenLength (-0.31), Fr-Length (-0.23), Fruit-Width (-0.28), and I-NFlowers (-0.35).

Fig. 3 Diagram corresponding to the principal components analysis (PCA) for quantitative descriptors among tzimbalo individuals. Bottom axis and left axis: Scores values for plants

On the other hand, the PC1 (43.05%) of the PCA for quantitative descriptors of *S. caripense* was mostly correlated with Fr-Length, Fr-PlacentLength, Fl-SepalLength, and others. These descriptors have additive type effects, are regulated by the environment in a polygenic mode. Therefore, it is optimal to evaluate them by variance decomposition in...
Nevertheless, considering an increase of F3H transcripts in B. pinnatisectum primers alignment at 52 °C (Fig. 4). The expression of F3H in pathway, the enzymatic action can follow or redirect it. It bands of known concentration. The ANOVA did not return to the species of interest [60]; plant breeding with plant reproduction systems, breeding methods are related genotype, environmental, and interactions effects. According to plant reproduction systems, breeding methods are related to the species of interest [60]; plant breeding with S. caripense accessions is carried out through backcrossing to S. muricatum [22].

B. Gene Expression

The expression of F3H was identified in the skin of BIO-Ltg1 fruit with 2 μL of cDNA (100 ng·µL⁻¹) per reaction and primers alignment at 52 °C (Fig. 4). The expression of F3H in BIO-Ltg1 seems to increase slightly after five days of postharvest conditions; after fourteen days well defined and intense F3H transcripts were observed.

The expression of F3H (212 bp) in BIO-Ltg1 induced by controlled temperature and photoperiod. Similarly, S. pinnatisectum tubers [34] showed through RT-PCR that F3H expression increases gradually in controlled conditions. Nevertheless, considering an increase of F3H transcripts in BIO-Ltg1, it is mentioned that early expression of the structural gen F3H is positively correlated with the increase of anthocyanins content in S. tuberosum tuber. It is different from the fruit of S. melongena, S. lycopersicon and Capsicum spp. [32]. This suggests that reached F3H in its biosynthetic pathway, the enzymatic action can follow or redirect it. It takes another way apart of that for anthocyanins accumulation, such as flavonols (kaempferol) formation in the presence of flavonol synthases [61].

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The expression of ANS was identified with 1 μL of cDNA (750 ng·µL⁻¹) per reaction and primers alignment at 55 °C; the transcripts of ANS in BIO-Ltg1 (145.20 ng·µL⁻¹; n=6) and BIO-Cyb1 (36.19 ng·µL⁻¹; n=5) fruit were quantified by comparative analysis of bands intensity on the agarose gel. They were taken as reference molecular weight marker (WM) bands of known concentration. The ANOVA did not return to the species of interest [60]; plant breeding with plant reproduction systems, breeding methods are related genotype, environmental, and interactions effects. According to plant reproduction systems, breeding methods are related to the species of interest [60]; plant breeding with S. caripense accessions is carried out through backcrossing to S. muricatum [22].

The ANOVA applied to the mean fold change of C3H expression returned significant differences, p-value = 0.0085 (Table VI).

### Table V

| FRUIT | Mean Fold Change of C3H Gene Expression in Tzimbalo IBT-Lib1 | 2⁻∆𝑇∆𝑇 | Log2 Mean | Log2 S.E. | Log2 S.D. | C.V. (%) |
|-------|-------------------------------------------------------------|---------|-----------|-----------|-----------|----------|
| IB 1  | 0.89                                                        | 1.30    | 0.33      | 0.66      | 19.96     |
| 2     | 1.08                                                        | 3.32    | 0.33      | 0.66      | 15.01     |
| 14    | 10.84                                                       | 6.24    | 0.73      | 1.46      | 23.47     |

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### Table VI

| Source of Variation | D.F. | M.S. | p-value |
|---------------------|------|------|---------|
| T and P(days)       | 2    | 8.60 | 0.0085  |
| Experimental Error  | 9    | 1.01 |         |
| Total               | 11   |      |         |
The mean fold change of C3H expression in IBT-Lib1 for day fourteen (6.24 ± 0.73) was significantly different from that calculated for day zero (3.32 ± 0.33) and similar for day five (4.52 ± 0.34) (Fig. 6); transcripts level of C3H expression increased in 2.92 units after fourteen days in postharvest conditions.

Fig. 6 Gene expression of C3H (mean ± S.E., n = 4) in tzimbalo IBT-Lib1 fruit exposed to controlled temperature (10 ± 2 °C) and photoperiod (16 h day/8 h night). Log2-transformed values, relative quantification based on the $2^{-\Delta\Delta C_T}$ method; different letters indicate significant differences according to Tukey test (p-value < 0.05)

It is mentioned that the CGA content in S. melongena fruit cv. Lucia increments after two weeks of storage at 10 °C [38]. Furthermore, C3H transcripts levels in Andean varieties of S. tuberosum, increments drastically after storage at 10 °C in darkness, preceded by exposure to drought stress during tuberization; the increase of C3H expression in cv. Huata Colorado coincides with the CGA content caused by drought [41]. Also, the expression level of C3H in S. tuberosum increments in 2.4 units by induction of CGA biosynthesis with sucrose 120 mM [39].

Previous investigations about pepino [65], eggplant [38], and potato [39], [41], support the obtained results with tzimbalo [15], [44], for the future development of improved varieties and the enhancement of the commercial potential of these species [10], [66]. Additionally, the concentrations for phenolic compounds of S. caripense fruit are greater than phenolic contents of melon and cucumber, and these are useful for the development of the new varieties of S. muricatum, focused on the improvement of nutritional and bioactive values of the fruit [20].

IV. CONCLUSIONS

The morphological description of tzimbalo ecotypes indicates that Fr-Flavour, Se-Diameter, Fl-CorollaColour, Fr-Stripes, Fr-Length, Fr-PlacentLength and Fr-PlacentBreadth were characters that contribute more to the variability, and these are agronomical distinctive to be utilized in breeding programs. The expression of F3H and ANS identified through RT-PCR in BIO-Ltg1 and BIO-Cyb1, and the expression of C3H in IBT-Lib1 fruit, constitutes an analysis applied to the exploration of candidate genes, for subsequently transcript quantification in real time.

The expression levels of C3H in the flesh of IBT-Lib1 fruit influenced by postharvest conditions were significantly different; opening the possibility of selecting genotypes that demonstrate good performance in front of different crop conditions. The approach of candidate genes and their expression represents a promising tool for introducing tzimbalo into plant breeding programs, focused on the conservation and utilization of Andean resources (Fig. 7).

Fig. 7 Fruits of tzimbalo (EC-Scl-pl.2) and its surface covered by anthocyanin pigments associated to gene expression of ANS.

NOMENCLATURE

ANOVA Analysis of variance
ANS Anthocyanidin synthase
Bp Base pair
C3H p-Coumaroyl ester 3-hydroxilase
CGA Chlorogenic acid (5-O-caffeoyl-quinic acid)
cDNA Complementary DNA
CRD Completely randomized design
CA Conglomerate analysis
DNA Deoxynribonucleic acid
F3H Flavanone 3-hydroxylase
HCA Hydroxycinnamic acid
MCD Mean character difference
mRNA Messenger RNA
m.a.s.l. Meters above sea level
NTC No template control
PCR Polymerase chain reaction
PPO Polyphenol oxidase
PCA Principal component analysis
RT Reverse transcriptase, reverse transcription
RT-PCR Reverse transcription followed by semiquantitative PCR
RT-qPCR Reverse transcription followed by quantitative PCR
RNA Ribonucleic acid
tRNA Ribosomal RNA
UPGMA Unweighted pair group method with arithmetic mean
WM Weight marker

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RESUMEN

El pepino (Solanum muricatum Ait., Solanaceae), una de las frutas nativas de la montaña, ha sido objeto de diversas investigaciones debido a su amplio uso culinario y su alta diversidad genética. En la actualidad, el pepino se utiliza principalmente como un ingrediente en diversas recetas, sin embargo, su producción es limitada debido a problemas de germinación. En este sentido, el objetivo de este estudio fue evaluar el efecto de diferentes tratamientos de inmersión en hipoclorito de sodio sobre la germinación de semillas de pepino.

MATERIAL Y MÉTODOS

Se usaron semillas de pepino recolectadas de diferentes localidades en el sur del Ecuador. Las semillas fueron inmersas en diferentes soluciones de hipoclorito de sodio a diferentes concentraciones y tiempos de inmersión. Las semillas inmersiones se incubaron a 25°C y se registró la germinación después de 24 horas.

RESULTADOS

Los resultados mostraron que el tratamiento de inmersión en hipoclorito de sodio a 1% durante 24 horas fue el más efectivo para mejorar la germinación de las semillas de pepino. Se observó un aumento del 30% en la germinación en comparación con el control sin tratamiento.

CONCLUSIÓN

El tratamiento de inmersión en hipoclorito de sodio es efectivo para mejorar la germinación de semillas de pepino, lo que podría tener un impacto positivo en la producción del pepino en el sur del Ecuador. Estos resultados pueden ser útiles para los agricultores y entidades de investigación que estén interesados en mejorar la germinación de semillas de pepino.

Palabras clave: Bipolaris solani, inmersión en hipoclorito de sodio, pepino, germinación.
