International Journal on Advanced Science Engineering Information Technology

Vol.11 (2021) No. 2
ISSN: 2088-5334

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

Juan M. Morales[a,*], Raúl H. Blas[a], Viviana P. Chiluisa-Utreras[b], Joel N. Flores[a], Geovanny D. Ortega[c]

[a]*Faculty of Agronomy, Plant Breeding Program, Laboratory of Genomics and Bioinformatics of the Biotechnology Institute, La Molina National Agrarian University, La Molina Ave. w/n, Lima, 15024, Peru*
[b]*Biotechnological Engineering, Laboratory of Molecular Biology, Salesian Polytechnic University, Quito, 170517, Ecuador*
[c]*Industrial Engineering, Quality and Productivity, San Francisco de Quito University, Diego de Robles Ave. w/n, Cumbaya, 170901, Ecuador*
*Corresponding author: *j7juanmos@hotmail.com*

***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, Se-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**.

*Manuscript received 9 Oct. 2020; revised 12 Jan. 2021; accepted 7 Feb. 2021. Date of publication 30 Apr. 2021.*


## 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 likelihood, 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 $2^{-\Delta\Delta C_T}$ method, the target gene quantity is normalized using reference reactions, and it is relative to calibrator reactions [30].

The anthocyanin biosynthetic pathway is already characterized and comprises a well-conserved mechanism in many plants [31]. It is an extension of the general pathway for flavonoid synthesis [32]. The primary anthocyanidin delphinidin shows violet/blue hues [33]. Single methylation of delphinidin results in petunidin and 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-Lib1 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).

TABLE I
PLANT MATERIAL USED FOR THE STUDY OF MORPHOLOGICAL DESCRIPTORS AND GENE EXPRESSION OF F3H, ANS, AND C3H IN TZIMBALO ECOTYPES

| Ecotype | | Origin | Traits |
|---|---|---|---|
| BIO-Cyb1 |  | EC-P, 3298 m.a.s.l. N 0°01′01′′ W 78°05′38′′ | Fr-Flavour acidic, AddColour between 10-30%, Length 2.03 cm |
| BIO-Cyb2 |  | EC-P, 2950 m.a.s.l. N 0°02′38′′ W 78°07′28′′ | Fr-Flavour sweet; Se-Diameter intermediate |
| BIO-Cyb3 |  | EC-P, 3282 m.a.s.l. N 0°01′12′′ W 78°05′51′′ | Fr-Flavour moderately sweet, Mottling present |
| BIO-Ltg1 |  | EC-X, 3055 m.a.s.l. S 0°47′22′′ W 78°33′59′′ | Fr-Flavour moderately sweet, AddColour between 30-50% |
| BIO-Ltg2 |  | EC-X, 2717 m.a.s.l. S 0°58'19′′ W 78°36'52′′ | Fr-Flavour sweet, Length 1.93 cm; Se-Diameter intermediate |
| BIO-Ltg3 |  | EC-X, 3016 m.a.s.l. S 0°45′28′′ W 78°45′28′′ | Fr-Flavour sweet, AddColour less than 10%; Se-Diameter small |
| IBT-Ayb1 |  | PE-PIU, 2814 m.a.s.l. S 4°38'03′′ W 79°43'05′′ | Fr-Flavour acidic, Mottling present; St-Pubescence dense |
| IBT-Lib1 |  | PE-TRU, 3329 m.a.s.l. S 8°00'49′′ W 78°24'38′′ | Fr-Flavour moderately sweet, Stripes absent, Length 3.4 cm |
| IBT-Lim1 |  | PE-LIM, 2560 m.a.s.l. S 11°56'29′′ W 76°29'44′′ | Fr-Flavour acidic, Mottling present; Se-Diameter small |

EC-P: Ecuador-Pichincha (Cayambe); EC-X: Ecuador-Cotopaxi (Latacunga); PE-PIU: Peru-Piura (Ayabaca); PE-TRU: Peru-Trujillo (La Libertad); PE-LIM: Peru-Lima.

TABLE II
LIST OF MORPHOLOGICAL DESCRIPTORS USED FOR TZIMBALO ECOTYPES

| Descriptor/Code | Range (scale)/units |
|---|---|
| **Plant (P), stem (St) and leaf (L) descriptors** | |
| Plant size/ P-Size | 3 Small; 5 Intermediate (e.g., cv. 'Sweet Long'); 7 Large (e.g., cv. 'Puzol') |
| 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; 3 Few; 5 Intermediate; 7 Many |
| 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] |
| Petiole colour/ L-PetioleColour | 1 Green; 2 Greenish with purple spots; 3 Greenish purple; 4 Purple; 5 Dark purple |
| Foliage density/ L-Density | 3 Sparse; 5 Intermediate; 7 Dense |
| Leaf attitude/ L-Attitude | 1 Semi-erect; 2 Horizontal; 3 Dropping |
| Leaf lamina length/ L-LaminaLength | [cm] |
| Leaf lamina width/ L-LaminaWidth | [cm] |
| Position of the widest part of the leaf blade/ L-WidestPart | 1 Base; 3 Bottom 1/3; 5 Middle; 7 Top 1/3 |
| Leaf blade length/width ratio/ L-LWRatio | - |
| Type of leaves/ L-Type | 1 Simple; 2 Compound |
| Number of leaflets/ L-Leaflets | - |
| Leaf colour/ L-Colour | 1 Light green; 2 Green; 3 Dark green; 4 Greenish purple; 5 Purple |
| Anthocyanin coloration 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 multiparous); 3 Generally multiparous |
| 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-SepalLength | [mm] |
| Stamen length/ Fl-EstamenLength | [mm] |
| Style exsertion/ Fl-StyleExsertion | [mm] |
| Pollen production/ Fl-PollenProd | 0 None; 3 Low; 5 Medium; 7 High |

TABLE III *(CONTINUED)*

| **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 Rounded; 3 Ellipsoid; 4 Obovate; 5 Ovate; 6 Cordiform; 7 Conical; 8 Elongate; 9 Other |
| Fruit predominant 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 acidic; 3 Acidic; 5 Moderately sweet; 7 Sweet; 9 Very Sweet |
| Number of seeds per fruit/Se-SeedsFruit | 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 synthetized to be used in *S. caripense* (Table III).

TABLE III
LIST OF PRIMER SEQUENCES USED FOR THE STUDY OF F3H, ANS, AND C3H GENE EXPRESSION IN TZIMBALO ECOTYPES

| Gene | Primers |
|---|---|
| F3H | FW-Sca: aat gcg ata gtg tat ccg tta a<br>RV-Sca: caa gca aga att tcc tca atg |
| ANS | FW-Sca: gca ctg act ttc atc ctc cac<br>RV-Sca: tct tgt act ttc cgt tgc tta g |
| C3H | FW-Sca: tga aga cac tct cat tgg ctt ac<br>RV-Sca: cag cct tag tgc ttc ctt gg |
| 5.8S rRNA | FW-Sca: caa cgg ata tct cgg ctc tc<br>RV-Sca: ttg cgt tca aag act cga tg |

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* (*with AccuRT Genomic DNA Removal Kit*) (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).

TABLE IV
LIST OF PCR COMPONENTS AND AMPLIFICATION PROGRAMS USED FOR THE STUDY OF F3H, ANS, AND C3H GENE EXPRESSION IN TZIMBALO ECOTYPES

| **RT-PCR** | |
|---|---|
| **Component** | **Volume (µL)** |
| cDNA (100 and 750 ng/µL) | 2.0 and 1.0 |
| Buffer PCR 10X | 1.0 |
| $MgCl_2$ (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* | 0.2 |
| Molecular grade water | 4.6 and 5.6 |
| **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) | - |
| **RT-qPCR** | |
| **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 $C_T$ 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:

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

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 $C_T$ 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-Pubescence, St-colour, L-PetioleColour, L-Colour, Fl-CorollaColour, Fr-Uniformity, Fr-Shape, Fr-Stripes, Fr-Mottling, Fr-AddColour, Fr-FleshColour, Fr-Flavour, Se-Colour and Se-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, I-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-Ltg1 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 fine 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 GelAnalizer 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].

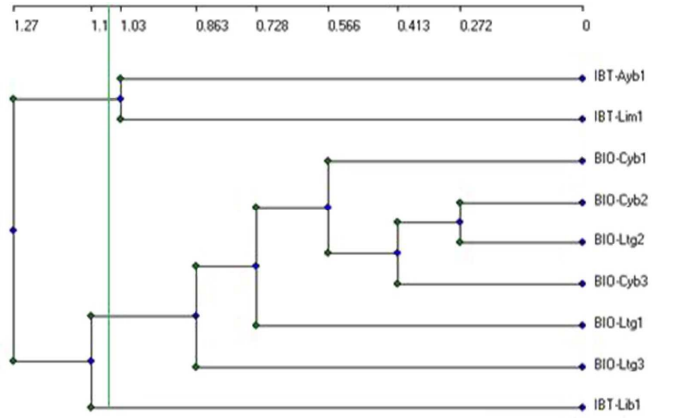


Fig. 1 Phenogram corresponding to the conglomerate analysis (CA) for qualitative descriptors among tzimbalo individuals.

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).

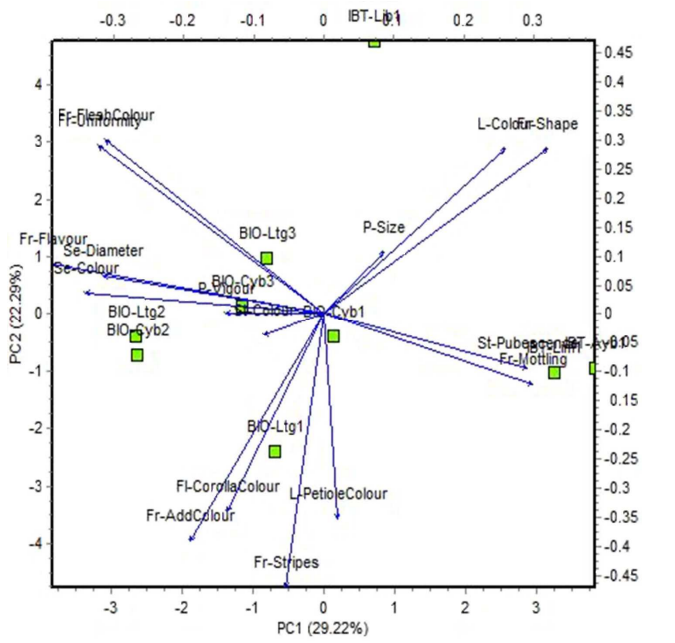


Fig. 2 Diagram corresponding to the principal components analysis (PCA) for qualitative descriptors among tzimbalo individuals. Bottom axis and left axis: Scores values for plants; Top axis and right axis: Eigenvectors values for descriptors

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-Stripes 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).

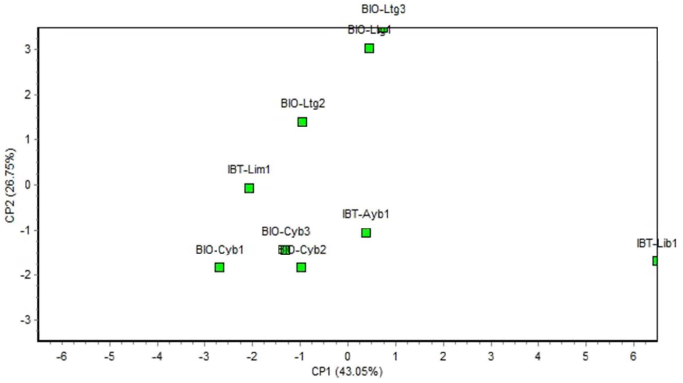


Fig. 3 Diagram corresponding to the principal components analysis (PCA) for quantitative descriptors among tzimbalo individuals. Bottom axis and left axis: Scores values for plants

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

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.

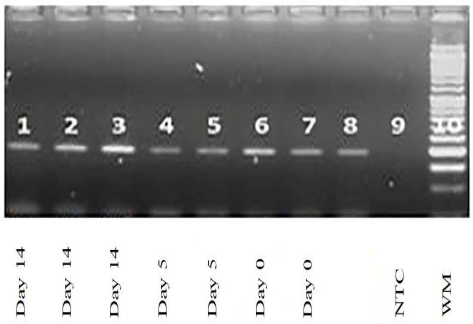


Fig. 4 Gene expression of F3H (212 bp) in tzimbalo BIO-Ltg1 fruit exposed to controlled temperature (10 ± 2 °C) and photoperiod (16 h day/8 h night). Lane 1-7: 100 ng·µL$^{-1}$ cDNA; lane 8: 10 ng·µL$^{-1}$ cDNA at day 14; lane 9: NTC; lane 10: WM

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].

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 (48.20 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 significant differences for the expression of ANS; *p*-value = 0.206, D.F. = 1 according to Tukey test (*p*-value < 0.05).

In relation to the expression of ANS (145 bp) in BIO-Ltg1 and BIO-Cyb1 on agarose gel (Fig. 5), for short amplicons < 150 bp, sometimes is observed very weak and fuzzy bands which migrate ahead of the major-specific bands. A Super-structured or single-stranded version of the specific transcripts in an equilibrium state should be considered specific [62].

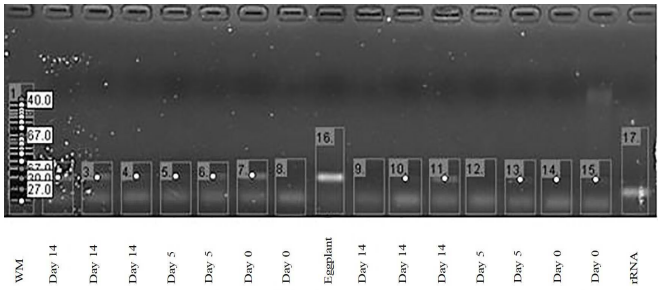


Fig. 5 Gene expression of ANS (145 bp) in tzimbalo fruit exposed to controlled temperature (10 ± 2 °C) and photoperiod (16 h day/8 h night). Lane 1: WM (ng·µL$^{-1}$); lane 2-8 and 9-15: 750 ng·µL$^{-1}$ BIO-Ltg1 and BIO-Cyb1 cDNA; lane 16: 100 ng·µL$^{-1}$ eggplant cDNA at day 0; lane 17: rRNA (100 bp)

The gel composition sometimes interferes with band definition. Therefore, polyacrylamide gels can provide a higher resolution. It could be aberrant reactions that influence the identification, quantification, and analysis. Nevertheless, RT-qPCR provides higher specificity. The plants BIO-Ltg1 and BIO-Cyb1 are phenotypically those that presented greater fruit surface covered by additional color and fruit stripes corresponding to the expression of anthocyanin pigments associated with genes (ANS) identified in *S. caripense* through RT-PCR. The expression of the structural gene ANS is positively correlated with the increase of anthocyanins concentration in *S. tuberosum* tuber, also this occurs in the fruit of *S. lycopersicon*, *S. melongena* and *Capsicum* spp. [32]. Moreover, ANS is required for the production of characteristic pigments of anthocyanins [52].

The expression of C3H in IBT-Lib1 fruit was identified through RT-PCR. It was induced by exposure to postharvest conditions and monitored through RT-qPCR. The term evaluated the expression of the reference gene 5.8S rRNA $2^{-\Delta C'T}$, were:

$$\Delta C'_T = (C_{T,\text{time x}} - C_{T,\text{time 0}}) \quad (2)$$

The corresponding *p*-value = 0.1649 indicates that it was not significantly different during the days of controlled conditions. The log2-transformed values (Table V) represent asymmetric logarithmic scale [63], [64].

TABLE V
MEAN FOLD CHANGE OF C3H GENE EXPRESSION IN TZIMBALO IBT-LIB1 FRUIT

| T and P (days) | $2^{-\Delta\Delta C_T}$ Mean | Log2 Mean | Log2 S.E. | Log2 S.D. | C.V. (%) |
|---|---|---|---|---|---|
| 0 | 1.08 | 3.32 | 0.33 | 0.66 | 19.96 |
| 5 | 2.49 | 4.52 | 0.34 | 0.68 | 15.01 |
| 14 | 10.84 | 6.24 | 0.73 | 1.46 | 23.47 |

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

TABLE VI
ANOVA OF C3H GENE EXPRESSION IN TZIMBALO IBT-LIB1 FRUIT

| 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.

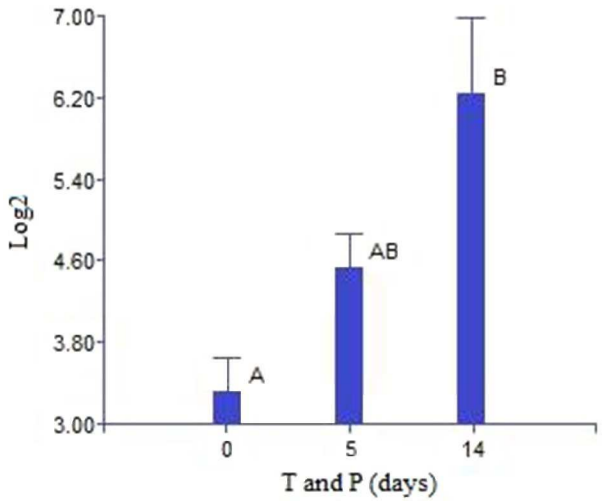


Fig. 6 Gene expression of C3H (mean ± S.E., n = 4) in tzimbalo IBT-Lib1 fruit exposed to controlled temperature (10 ± 2 °C) and photoperiod (16 h day/8 h night). Log2-transformed values, relative quantification based on the $2^{-\Delta\Delta C_T}$ method; different letters indicate significant differences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

It is mentioned that the CGA content in *S. melongena* fruit cv. Lucía 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 Colorada 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 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-Sc1-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 análisis |
| DNA | Deoxyribonucleic 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 |
| rRNA | Ribosomal RNA |
| UPGMA | Unweighted pair group method with arithmetic mean |
| WM | Weight marker |

## Acknowledgment

We are grateful to the Ministry of Higher Education, Science, Technology, and Innovation (SENESCYT),

Government of Ecuador, for the partial funding of this work through the National Program of Scholarships and Educational Loan (PRONABEC), Grant BAPE 2016, Government of Peru. Special thanks to fellows and farmers Mg.Sc. Alberto Salas, Ph.D. Marco Cerna, Eng. Felipe Vinueza, Stanley Vilca, Javier Muñoz and Emerita, whose collaboration and courtesy correspond to plant material.

## REFERENCES


[1] E. Zuriaga, "Análisis de la variabilidad en poblaciones naturales de *Solanum*, secciones *Lycopersicon* y *Basarthrum*," Doctoral thesis, Univ. Politécnica de Valencia, Spain, 2009. https://riunet.upv.es/handle/10251/6362

[2] X. Palomeque, A. Verdugo, S. Criollo, and D. Peña, "Diversidad de frutales nativos comestibles Caricaceae - Solanaceae, fenología, usos y recolección de germoplasma en el Sur del Ecuador," INIAP (Instituto Nacional de Investigaciones Agropecuarias), Estación Experimental Chuquipata, Granja Experimental Bullcay, 2003. https://repositorio.iniap.gob.ec/handle/41000/2368

[3] D. S. Correll, *Flora of Perú*, Volume VIII, Parte V-B, Number 2., Field Museum of Natural History, 1967.

[4] P. M. Jørgensen, and S. León-Yánez, *Catalogue of the Vascular Plants of Ecuador*, Saint Louis: Missouri Botanical Garden Press., 1999.

[5] T. Särkinen, M. Baden, P. Gonzáles, M. Cueva, L. L. Giacomin, D. M. Spooner, R. Simon, H. Juárez, P. Nina, J. Molina, and S. Knapp, "Listado anotado de *Solanum* L. (Solanaceae) en el Perú," *Rev. Peru Biol.* vol. 22, pp. 003-062, Apr. 2015. http://www.scielo.org.pe/scielo.php?script=sci_arttext&pid=S1727-99332015000100001

[6] L. de la Torre, H. Navarrete, P. Muriel, M. Macía, and H. Balslev, Eds., *Enciclopedia de las Plantas Útiles del Ecuador*, Quito & Aarhus: Herbario QCA de la Escuela de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador & Herbario AAU del Departamento de Ciencias Biológicas de la Universidad de Aarhus, 2008.

[7] V. Van den Eynden, E. Cueva, and O. Cabrera, *Plantas Silvestres Comestibles del Sur del Ecuador*, Quito, Ecuador: Univ. Politécnica Salesiana/Abya - Yala,1998.

[8] C. B. Heiser, "*Solanum caripense* y el origen de *Solanum muricatum*," *Revista Politécnica* vol. 1, no. 3, pp. 5-11, 1969.

[9] B. C. Murray, K. R. Hammett, and F. D. Grigg, "Seed set and breeding system in the pepino *Solanum muricatum*, Ait., Solanaceae," *Sci. Hortic.* vol. 49, issues 1-2, pp. 83-92, Jan. 1992. https://agris.fao.org/agris-search/search.do?recordID=NL19920041933

[10] A. Rodríguez-Burruezo, J. Prohens, and A. Fita, "Breeding strategies for improving the performance and fruit quality of the pepino (*Solanum muricatum*): A model for the enhancement of underutilized exotic fruits," *Food Res. Int.* vol. 44, pp. 1927–1935, Aug. 2011.

[11] C. B. Heiser, "Origin and variability of the pepino (*Solanum muricatum*): A preliminary report," *Baileya* vol. 12, pp. 151-158, 1964.

[12] G. J. Anderson, and L. M. Bernardello, "The relationships of *Solanum cochoae* (Solanaceae), a new species from Peru". *Novon* vol. 1, pp. 127-133, Autumn 1991.

[13] G. J. Anderson, C. T. Martine, J. Prohens, and F. Nuez, "*Solanum perlongistylum* and *S. catilliflorum*, new endemic peruvian species of *Solanum*, section *Basarthrum*, are close relatives of the domesticated pepino, *S. muricatum*," *Novon* vol. 16, pp. 161-167, Jun. 2006.

[14] J. Prohens, S. Soler, and F. Nuez, "The effects of thermotherapy and sodium hypochlorite treatments on pepino seed germination, a crucial step in breeding programmes," *Ann. Appl. Biol.* vol. 134, pp. 299-305, May. 1999.

[15] J. Morales, and I. Vaca, "Propagación *in vitro* de tzímbalo (*Solanum caripense* Dunal)," *RTE* vol. 29, pp. 89-104, Dec. 2016. http://www.rte.espol.edu.ec/index.php/tecnologica/article/view/547

[16] S. L. Bithell, B. A. Mckenzie, G. W. Bourdot, and G. D. Hill, "Germination requirements of laboratory stored seeds of *Solanum nigrum* and *Solanum physalifolium*," *N. Z. Plant Prot.* vol. 55, pp. 222-227, Aug. 2002.

[17] M. Ibrahim, M. Munira, M. Kabir, A. Islam, and M. Miah, "Seed germination and graft compatibility of wild *Solanum* as rootstock of tomato," *Online J. Biol. Sci.* vol. 1, no. 8, pp. 701-703, Aug. 2001.

[18] A. Taab, "Seed dormancy and germination in *Solanum nigrum* and *S. physalifolium* as influenced by temperature conditions," Doctoral thesis, Swedish Univ. of Agricultural Sciences, Upsala Sweden., 2009. https://pub.epsilon.slu.se/2064/

[19] J. Prohens, A. Rodríguez-Burruezo, M. Cámara, E. Torija, and F. Nuez, "Morphological and physico-chemical characteristics of fruits of pepino (*Solanum muricatum*), wild relatives (*S. caripense* and *S. tabanoense*) and interspecific hybrids: Implications in pepino breeding," *Eur. J. Hortic. Sci.* vol. 70, pp. 224-230, Oct. 2005.

[20] J. Prohens, F. Herraiz, M. Raigón, S. Vilanova, M. García-Martínez, P. Gramazio, M. Plazas, and A. Rodríguez-Burruezo, "Fruit composition diversity in land races and modern pepino (*Solanum muricatum*) varieties and wild related species," *Food Chem.* vol. 15, pp. 49-58, Jul. 2016.

[21] F. J. Herraiz, S. Vilanova, I. Andújar, D. Torrent, M. Plazas, P. Gramazio, and J. Prohens, "Morphological and molecular characterization of local varieties, modern cultivars and wild relatives of an emerging vegetable crop, the pepino (*Solanum muricatum*), provides insight into its diversity, relationships and breeding history," *Euphytica* vol. 206, pp. 301-318, Apr. 2015.

[22] F. J. Herraiz, J. Blanca, P. Ziarsolo, P. Gramazio, M. Plazas, G. Anderson, J. Prohens, and S. Vilanova, "The first *de novo* transcriptome of pepino (*Solanum muricatum*): assembly, comprehensive analysis and comparison with the closely related species *S. caripense*, potato and tomato," *BMC Genomics* vol. 321, pp. 1-17, May. 2016.

[23] A. Lucca, "Búsqueda de genes candidatos que controlen QTLs involucrados en la resistencia al estrés hídrico mediante el análisis de perfiles transcripcionales en especies silvestres de *Solanum*," Doctoral thesis, Univ. de Buenos Aires, Agentina., 2011. https://repositorio.inta.gob.ar/xmlui/handle/20.500.12123/6192

[24] M. B. Mantilla, "Transformación genética de la naranjilla, *Solanum quitoense*, mediante *Agrobacterium tumefaciens*," Graduate thesis, Univ. San Francisco de Quito, Cumbayá, Ecuador, 2008. http://repositorio.usfq.edu.ec/handle/23000/930

[25] A. Rodríguez-Burruezo, J. Prohens, and F. Nuez, "Wild relatives can contribute to the improvement of fruit quality in pepino (*Solanum muricatum*)," *Euphytica* vol. 129, pp. 311–318, Feb. 2003.

[26] S. Bustin, "Quantification of mRNA using real-time RT-PCR: Trends and problems," *J. Mol. Endocrinol.* vol. 29, pp. 23–39, Sep. 2002.

[27] C. Orlando, P. Pinzani, and M. Pazzagli, "Developments in quantitative PCR," *Clin. Chem. Lab. Med.* vol. 36, pp. 255-269, May. 1998.

[28] J. Vandesompele, K. De-Preter, F. Pattyn, B. Poppe, N. Van-Roy, A. De-Paepe, and F. Speleman, "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biol.* vol. 3, pp. 0034.1–0034.11, Jun. 2002.

[29] P. Fernández, "Cuantificación mediante la técnica de PCR en tiempo real. Usos y aplicaciones," Instituto de Biotecnología, CICVyA, INTA-Castelar, 2011.

[30] K. Livak, and T, "Schmittgen. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method," Methods vol. 25, pp. 402-408, Dec. 2001.

[31] T. Holton, and E. Cornish, "Genetics and biochemistry of anthocyanin biosynthesis," *The Plant Cell* vol. 7, pp. 1071-1083, Jul. 1995

[32] Y. Liu, Y. Tikunov, R. Schouten, L. Marcelis, R. Visser, and A. Bovy, "Anthocyanin biosynthesis and degradation mechanisms in Solanaceous vegetables: a review," *Front. Chem.* vol. 6, pp. 1-17, Mar. 2018.

[33] Y. Tanaka, and A. Ohmiya, "Seeing is believing engineering anthocyanin and carotenoid biosynthetic pathways," *Curr. Opin. Biotechnol.* vol. 19, pp. 190–197, Apr. 2008.

[34] L. Qi-Neng, and Y. Quing, "cDNA cloning and expression of anthocyanin biosynthetic genes in wild potato (*Solanun pinnatisectum*)," *Afr. J. Biotechnol.* vol. 5, pp. 811-818, May. 2006.

[35] M. Jiang, Y. Liu, L. Ren, H. Lian, and H. Chen, "Molecular cloning and characterization of anthocyanin biosynthesis genes in eggplant (*Solanum melongena* L.)," *Acta Physiol.* Plant. vol. 38, Jun. 2016.

[36] N. Martínez-Cruz, K. Arévalo-Niño, M. Verde-Star, C. Rivas-Morales, A. Oranday-Cárdenas, A. Núñez-González, and M. Morales-Rubio, "Antocianinas y actividad anti radicales libres de *Rubus adenotrichus* Schltdl (zarzamora)," *Rev. Mex. Cienc. Farm.* vol. 42, pp. 66-71, Aug. 2011.

[37] P. Gramazio, J. Prohens, M. Plazas, I. Andújar, F. Herraiz, E. Castillo, S. Knapp, R. Meyer, and S. Vilanova, "Location of chlorogenic acid biosynthesis pathway and polyphenol oxidase genes in a new interspecific anchored linkage map of eggplant," *BMC Plant Biol.* vol. 14, pp. 1-15, Dec. 2014.

[38] M. Zaro, A. Vicente, A. Chaves, and A. Concellón, "Cambios en los antioxidantes fenólicos de berenjena violeta durante el desarrollo y almacenamiento refrigerado," *Rev. Iber. Tecnología Postcosecha* vol. 17, pp. 86-92, 2016.

[39] R. Payyavula, R. Shakya, V. Sengoda, J. Munyaneza, P. Swamy, and D. Navarre, "Synthesis and regulation of chlorogenic acid in potato: Rerouting phenylpropanoid flux in HQT-silenced lines," *Plant Biotechnol. J.* vol. 13, pp. 551–564, 2015.

[40] R. Niggeweg, A. Michael, and C. Martin, "Engineering plants with increased levels of the antioxidant chlorogenic acid," *Nat. Biotechnol.* vol. 22, pp. 746–754, Jun. 2004.

[41] C. André, R. Schafleitner, S. Legay, I. Lefèvre, C. Alvarado, G. Nomberto, L. Hoffmann, J. Hausman, Y. Larondelle, and D. Evers, "Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress," *Phytochemistry* vol. 70, pp. 1107–1116, Jun. 2009.

[42] R. Robbins, "Phenolic acids in foods: An overview of analytical methodology," *J. Agric. Food Chem.* vol. 51, pp. 2886–2887, May. 2003.

[43] P. Glorio, *Técnicas Modernas de Investigación en Ciencia de Alimentos (Aplicaciones de Biología Molecular y Uso de Lector de Micropozos)*, Univ. Nacional Agraria La Molina, Lima, Perú, 2010.

[44] J. Morales, R. Blas, V. Chiluisa-Utreras, J. Flores, and G. Ortega, "Gene expression of flavanone 3-hydroxylase (F3H), anthocyanidin synthase (ANS), and p-coumaroyl ester 3-hydroxilase (C3H) in tzimbalo fruit," in *Proc. CIT 2020 - ESPE*, 2020, paper 211, p. 10. https://cit2020.org/life-sciences/

[45] IPGRI (International Plant Genetic Resources Institute)/COMAV (Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana), "Descriptors for pepino (*Solanum muricatum*)," IPGRI, Rome, Italy, 2004. https://agris.fao.org/agris-search/search.do?recordID=QJ2005000012

[46] I. Madroñero, J. Rosero, L. Rodríguez, J. Navia, and C. Benavides, "Caracterización morfoagronómica de genotipos promisorios de papa criolla (*Solanum tuberosum* L. Grupo Andigenum) en Nariño," *Temas Agrarios* vol. 18, pp. 50-66, Jul. - Dec. 2013.

[47] M. Morales, G. Espinosa, R. Morales, B. Sánchez, A. Jiménez, and Y. Milián, "Caracterización morfológica y evaluación de resistencia a *Fusarium oxysporum* en especies silvestres del género *Solanum* sección *Lycopersicon*," *Rev. Colomb. Biotecnol.* vol. 16, pp. 62-73, Jul. 2014.

[48] Y. Zhang, Z. Hu, G. Chu, C. Huang, S. Tian, Z. Zhao, and G. Chen, "Anthocyanin accumulation and molecular analysis of anthocyanin biosynthesis-associated genes in eggplant (*Solanum melongena* L.)," *J. Agric. Food Chem.* vol. 62, pp. 2906−2912, Apr. 2014.

[49] H. Feng, X. Huang, Q. Zhang, G. Wei, X. Wang, and Z. Kang, "Selection of suitable inner reference genes for relative quantification expression of microRNA in wheat," *Plant physiology and biochemistry: PPB/SFPV* vol. 51, pp. 116–122, Feb. 2012.

[50] F. Rivas, "Análisis de la expresión del gen PR-1, mediante la técnica de PCR en tiempo real (RT-PCR), en tomate (*Solanum lycopersicum*) infectado con *Phytophtora infestans*," Graduate thesis, Univ. de las Fuerzas Armadas, Sangolquí, Ecuador, 2010. http://repositorio.espe.edu.ec/xmlui/handle/21000/2640

[51] S. Ballou, K. Yun, C. Cheng, and B. de los Reyes, "Cold sensitivity gradient in tuber-bearing *Solanum* based on physiological and transcript profiles," *Crop Sci.* vol. 47, pp. 2027-2035, 2007.

[52] J. Stommel, G. Lightbourn, B. Winkel, and R. Griesbach, "Transcription factor families regulate the anthocyanin biosynthetic pathway in *Capsicum annuum*," *J. Amer. Soc. Hort. Sci.* vol. 134, pp. 244-251, Mar. 2009.

[53] J. Crisci, and M. López, "*Introducción a la Teoría y Práctica de la Taxonomía Numérica*," Washington, DC, USA: Secretaría General de la Organización de los Estados Americanos. Programa Regional de Desarrollo Científico y Tecnológico, 1983.

[54] R. Sokal, and C. Michener, "A statistical method for evaluating systematic relationships," Univ. Kansas Sci. Bull. vol. 38, pp. 1409-1438, 1958.

[55] C. Quispe, R. Mansilla, A. Chacón, and R. Blas, "Análisis de la variabilidad morfológica del "añu" *Tropaeolum tuberosum* Ruiz & Pavón procedente de nueve distritos de la región Cusco," *Ecol. Apl.* vol. 14, pp. 211-222, Jul. – Dec. 2015.

[56] D. Montgomery, *Diseño y Análisis de Experimentos*, Univ. Estatal de Arizona, Editorial Limusa Wiley, 2004.

[57] R. Sokal, and F. Rohlf, "The comparison on dendrograms by objective methods," *Taxon* vol. 11, pp. 33-40, Feb. 1962.

[58] D. Torrent, "Caracterización morfológica y molecular en pepino dulce (*Solanum muricatum*) y especies silvestres relacionadas," Graduate thesis, Univ. Politécnica, Valencia, Spain, 2014. https://riunet.upv.es/handle/10251/46141?show=full

[59] F. J. Herráiz, "Desarrollo de herramientas morfológicas y genómicas para el estudio del pepino dulce (*Solanum muricatum*) y especies relacionadas. Caracterización de su valor nutracéutico," Doctoral thesis. Univ. Politécnica, Valencia, Spain, 2015. https://riunet.upv.es/handle/10251/61962

[60] F. Camarena, J. Chura, and R. Blas, *Mejoramiento Genético y Biotecnológico de Plantas*, Lima, Perú: Univ. Nacional Agraria La Molina/AGROBANCO, 2014. https://www.agrobanco.com.pe/agrosaber/linea-editorial/

[61] Y. Tanaka, N. Sasaki, and A. Ohmiya, "Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids," *Plant J.* vol. 54, pp. 733–749, May. 2008.

[62] L. Cseke, A. Kirakosyan, P. Kaufman, and M. Westfall, *Handbook of Molecular and CellularMethods in Biology and Medicine*, Boca Ratón, USA: CRC Press, 2011.

[63] T. Little, and F. Hills, *Agricultural Experimentation: Design and Analysis*, Wiley, 1978.

[64] J. Hellemans, and J. Vandesompele, "qPCR data analysis – unlocking the secret to successful results". In *PCR Ttroubleshooting and Optimization: The Essential Guide*, S. Kennedy, and N. Oswald, Eds., U. K.: Caister Academic Press, 2011.

[65] E. España, "Respuesta del cultivo de pepino dulce (*Solanum muricatum* Ait) a la fertilización química mediante el sistema de parcelas de omisión en el cantón Ibarra, provincia de Imbabura," Graduate thesis, Univ. Técnica de Babahoyo, Carchi, Ecuador, 2015. http://dspace.utb.edu.ec/handle/49000/1053

[66] F. J. Herraiz, D. Villaño, M. Plazas, S. Vilanova, F. Ferreres, J. Prohens, and D. Moreno, "Phenolic profile and biological activities of the pepino (*Solanum muricatum*) fruit and its wild relative *S. caripense*," *Int. J. Mol. Sci.* vol. 17, pp. 1-15, Mar. 2016.