RESEARCH NOTE

Decrease of flavonol synthase enzymatic activity in *Ugni molinae* Turcz due to the domestication process

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Abstract

M. Chacón-Fuentes, A. Mutis, L. Bardehle, I. Seguel, A. Urzúa, and A. Quiroz. 2019. Decrease of flavonol synthase enzymatic activity in *Ugni molinae* Turcz due to the domestication process. Cien. Inv. Agr. 46(1): 30-39. Flavonoid biosynthesis may be affected by plant domestication, with flavonoid production being reduced in proportion to the degree of domestication. In this context, kaempferol (3,4´,5,7-tetrahydroxyflavone) has been identified in the leaves of wild and cultivated *Ugni molinae*, a berry endemic to Chile. The biosynthetic pathway of kaempferol production begins with naringenin (4´,5,7-trihydroxyflavanone), which is converted to dihydrokaempferol (3,4´,5,7-tetrahydroxyflavanone), catalyzed by flavanone 3ß-hydroxylase (FHT) and then converted to kaempferol by a bifunctional enzyme called flavonol synthase (FLS). Therefore, our study aims to evaluate how FLS activity is affected in murtilla plants that are subjected to the domestication process. Kaempferol was quantified from methanolic extracts of leaf samples collected from both cultivated and wild *U. molinae* plants using high-performance liquid chromatography, and enzyme extraction was performed to determine FLS activity. The results showed that kaempferol concentration in wild plants from the Soloyo (0.14 μg g^{-1}), Mehuín (0.18 μg g^{-1}) and Queule (0.25 μg g^{-1}) sampling areas was higher than in their cultivated counterparts. Our data are consistent with the FLS activity detected in samples obtained from Manzanal Alto (134.79 pKatal, Soloyo (96.48 pKatal), and Mehuín (119.97 pKatal). These samples also exhibited higher enzymatic activity than their cultivated counterparts. Together, these data suggest that FLS activity is negatively affected by the domestication process.

Key words: Cultivated, enzyme activity, Kaempferol, naringenin, wild.

Introduction

The flavonoid family consists of over 8,000 secondary plant metabolites. Flavonoids are characterized by their C6-C3-C6 skeleton (Nguyen *et al.,* 2016) and can be classified into the following eight subgroups according to their oxidation state and substitution pattern of the C-ring structure: flavanones, dihydroflavonols, flavones, flavonols, flavan-3,4-diols, flavan-3-ols, anthocyanidins, and proanthocyanidins (Xu *et al.,* 2012). Additionally, flavonoids can be conjugated to sugar molecules naturally occurring in plant tissues. These compounds have been associated with a broad range of applications, including antioxidants, insect repellent, and even cancer prevention (Ruiz *et al.,* 2010, Vazhappilly *et al.,* 2017). Flavonols are the most abundant group of flavonoids, and several biological properties have been attributed to these compounds, such as regulation of auxin transport, modulation of flower color, protection from UV radiation, prevention against microorganisms and pest invasions, and signaling interactions with insects and microbes (Nenaah 2013, Dixit *et al.,* 2017, León-Chan *et al.,* 2017, Vazhappilly *et al.,* 2017). Flavonols are produced by the desaturation of dihydroflavonols. These compounds are formed from dihydroflavonols (Figure 1) by the introduction of a double bond between C-2 and C-3, which is catalyzed by flavonol synthase (FLS) (Xu *et al.,* 2012). The B-ring of dihydrokaempferol (3,4′,5,7-tetrahydroxyflavanone) can be hydroxylated at the 3′ position by flavonoid 3′-hydroxylase (F3′H) or at the 3′ and 5′ positions by flavonoid 3′5′-hydroxylase (F3′5′H) to produce dihydroquercetin and dihydromyricetin, respectively. The oxidation reaction introducing the C-2/C-3 double bond is considered to be specific for dihydroflavonol substrates (Preub *et al.,* 2009, Lukacin *et al.,* 2000, Li *et al.,* 2013). Furthermore, FLS has been reported as a bifunctional enzyme capable of transforming not only dihydrokaempferol (3,4′,5,7-tetrahydroxyflavone) but also naringenin (4′,5,7-trihydroxyflavanone) into kaempferol (Lukacin *et al.,* 2003). Regarding

pest management, various studies have focused on the deterrent and anti-feeding properties of these compounds. Onyilagha *et al.* (2012) found that kaempferol, quercetin, and isorhamnetin deterred feeding of the flea beetle, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae), on *Camelina sativa* leaves. Additionally, Selin-Rani *et al.* (2016) reported that quercetin isolated from *Euphorbia hirta* L. was toxic to *Spodoptera litura* Fab. (Lepidoptera: Noctuidae) larvae and showed that 6 ppm caused a 94.6% mortality rate. Furthermore, larval weight was reduced to 100 mg at the same dose. Finally, Nenaah (2003) reported that a leaf methanolic extract from *Calotropis procera* that contained kaempferol showed considerable toxicity to *Sitophilus oryzae* (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae) and that concentrations of 5 mL cm^{-2} caused a mortality rate of 86% in *S. oryzae* with applications of 2 mL of extract.

During the last 20 years, a highly polymorphic perennial and wild native shrub from Chile, denominated "Murtilla", *Ugni molinae* (Hoffmann 2005; Valdebenito *et al.,* 2003), has been domesticated and studied by the Institute of Agricultural Research (Instituto de Investigaciones Agropecuarias, INIA), Carillanca, in the Araucanía region of Chile (Chacón-Fuentes *et al.,* 2016). One hundred sites were originally selected in southern Chile for the collection of wild *U. molinae* plants, and cuttings were grown for the first 10 years under shading and then transplanted to the field. There is strong economic interest in the production of *U. molinae* fruit, both globally and regionally, due to its high antioxidant content that is mainly provided by flavonol compounds (Simirgiotis *et al.,* 2009). The main phenolic compounds reported in murtilla plants are myricetin, quercetin, kaempferol and their glycosides compounds (Peña-Cerda *et al.,* 2017). A recent study by Chacón-Fuentes *et al.* (2015) reported differences in the flavonol concentration present in wild and cultivated plants of *U. molinae*, with domestication decreasing flavonol production in cultivated plants. Nonetheless, information about
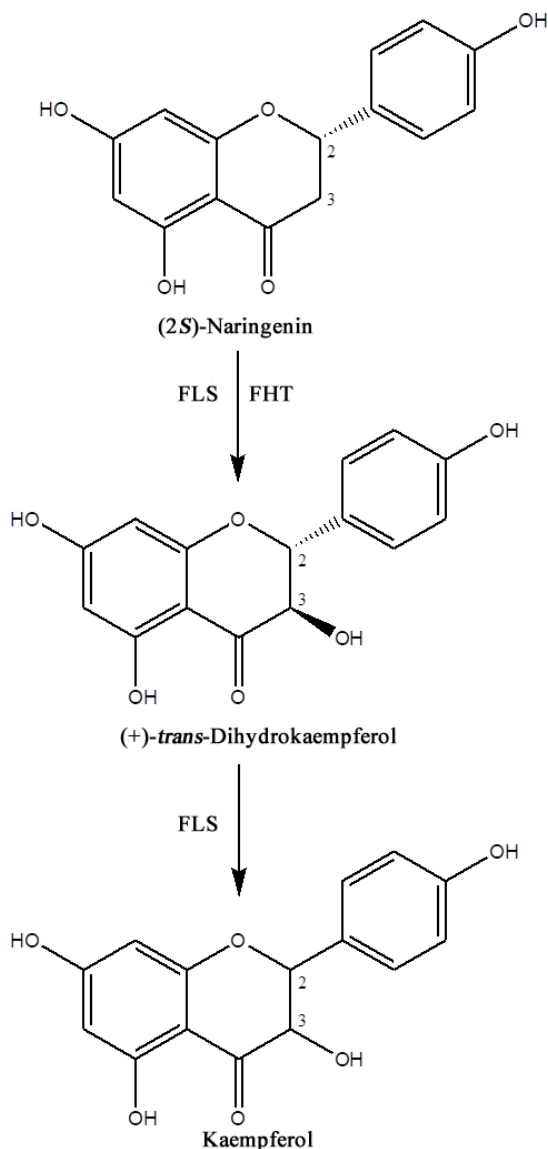
Figure 1. Schematic of the kaempferol biosynthetic pathway from naringenin to kaempferol using flavonol synthase enzyme activity. FLS: flavonol synthase. FHT: flavanone 3ß-hydroxilase.

FLS activity in berries is still limited (Flores *et al.,* 2014) and currently there is no information about flavonol synthase in *U. molinae*. We hypothesize that there is lesser FLS activity and kaempferol production in leaves of cultivated *U. molinae* plants than in wild *U. molinae* plants. To test this hypothesis, kaempferol concentration in leaves of both wild and cultivated plants was analyzed using naringenin as a substrate for the FLS enzyme.

Material and methods

Cultivated material collection

Seven different ecotypes of cultivated plants (Ecotypes: 08-1, 12-1, 14-4, 18-1, 19-1, 22-1, and 23-2) of *U. molinae* were used to obtain cuttings at the Experimental Station-Tranapuente of the Institute of Agricultural Research (Instituto de Investigaciones Agropecuarias, INIA),

Carillanca, in La Araucanía region of Chile (38° 45`S, 73° 21`W). A common garden for wild and cultivated plants was established and cuttings were grown under shading (ambient temperature and humidity) in 8-L pots at INIA Tranapuente, a locality near Puerto Saavedra, a coastal zone close to the Pacific Ocean with a moderate oceanic climate (Scheuermann *et al.,* 2008). After 1 year of acclimation in the common garden, leaves were sampled from the four cardinal directions at the same height and stored at -20 ºC until the kaempferol analysis and enzyme assay were carried out.

Wild material collection

Wild cuttings were sampled from the original geographical area where their cultivated counterparts were collected approximately 20 years ago. The following areas were used for the cutting collections: Caburgua (39º11`S, 71º49`W), Pucón (39º17`S, 71º55`W), Manzanal Alto (38º03`S, 73º10`W), Soloyo (38º35`S, 72º34`W), Porma (39º08`S, 73º16`W) from La Araucanía, Chile and Mehuín (39º26`S, 73º12`W), and Queule (39º23`S, 73º12`W) from Los Ríos, Chile. Plants were established in a common garden for 1 year until their use in chemical and enzymatic analyses. Cultivated plants were paired with their respective wild plant counterparts as follows: Eco 08-1/Caburgua; Eco 12-1/Pucón; Eco 14-4/Manzanal Alto; Eco 18-1/Soloyo; Eco 19-1/Porma; Eco 22-1/Mehuín; and Eco 23-2/Queule. Cultivated and wild murtilla plants were placed in pots with a substrate composed of sand (25%) and soil obtained from the same experimental field. Annually, fertilizer consisting of 80 g of nitrogen (CAN 27), 44 g of P_2O_5 (triple superphosphate), and 43 g of K_2O

(muriate of potash) was applied to each ecotype and wild plant according to the soil analysis. The fertilizer was applied over 4 periods, coinciding with the budding, flowering, ripening and post-harvest plant phenologies (Table 1).

Kaempferol extraction

Leaves of both cultivated and wild plants were collected from plants located in the common garden. Samples were rapidly frozen in liquid nitrogen for 5 s (Mikulic-Petkovsek *et al.,* 2012) and then milled in a grinder (1 mm diameter). Milled samples (5 g) were placed in a flask and 25 mL of chromatographic grade methanol (Sigma-Aldrich, St. Louis, MO) was added (50% v/v in water, solvent-to-solid ratio of 5:1). Flasks were placed on a magnetic stirrer set at 170 rpm for 20 min at 30 °C in the dark. Samples were then filtered through a Whatman nº1 filter paper (Whatman International Ltd., Maidstone, U.K.) and the filtrate was lyophilized (Biobase Biodustry BK-FD1CP, Shandong, China). Finally, each sample was suspended in 10 mL of chromatographic grade methanol and left for 5 min in a Branson 3510 sonicator according to Chacón-Fuentes *et al.* (2015). Samples were stored at -20 °C in amber flasks (25 mL) until High Performance Liquid Chromatography (HPLC) analyses.

Enzyme extraction

The *U. molinae* leaves were ground to a fine powder in a mill. The powder (1.5 g), composed of 0.25 g of quartz sand, 0.25 g of Polyclar AT, and 4 mL of 0.1 M Tris/HCl (containing 0.4% Na-

Table 1. Source of fertilizer (g plant[-1]) applied to murtilla plants during the year according to the plant phenology.

Source	Annual doses	Application (g plant[-1])			
		Postharvest	Budding	Flowering	Ripening
N	80	20	20	20	20
P_2O_5	44	11	11	11	11
K_2O	42	8	8	8	18

ascorbate, pH 7.25) was completely homogenized (Halbwirth *et al.* 2009) with a pestle and mortar. The resulting homogenate was then centrifuged (Hitachi, Himac CT15RE) in polypropylene tubes (2 mL) at 5,000 g for 10 min at 4 ºC. Finally, the supernatant was collected to perform FLS assays. Preparation was done in triplicate for statistical analysis.

Enzymatic activity

Flavonol synthase activities in the enzyme extract were measured according to Halbwirth *et al.* (2009), with slight modifications. Briefly, 20 µL of 50 mM naringenin, 50 µL of the enzyme extract obtained above, 5 µL of 3.48 mM 2-oxoglutarate, 5 µL of 2.01 mM $FeSO_4$-$7H_2O$, and 60 µL of buffer (0.1 M Tris/HCl + 0.4% Na-ascorbate, pH 7.25) were mixed. The mixture was incubated (Major Science MD-01N) for 60 min at 30 °C. The assay was completed by adding 140 µL of ethyl acetate, 10 µL of acetic acid, and 10 µL of 0.1 mM ethylenediaminetetraacetic acid (EDTA). The resulting organic phase was used to perform the HPLC analysis detailed below. Reactions processed without substrates were used as blanks for comparison. Enzyme activity was expressed as pKatal, where one katal is defined as the enzyme activity transforming 1 mol of compound per s (Flores *et al.,* 2014).

HPLC DAD analysis

The methanolic extracts obtained from the leaves and enzyme preparations were filtered through a 0.22 µm membrane and analyzed by HPLC-DAD. Each sample (20 µL) was injected into a Shimadzu autosampler HPLC (SIL-20AHT) equipped with a C-18 column (250 x 4.6 mm I.D.; 5 mm particle size) maintained at 40 °C in a column oven (CTO-20A). The analysis was performed using a linear solvent gradient consisting of 1% formic acid (A) and acetonitrile (B) as follows: 0-5 min,

5% A/95% B; 5-10 min, 30% A/70% B; 10-20 min, 55% A/45% B; 20-30 min, 5% A/95% B at a flow rate of 1 mL min-1 (Simirgiotis et al., 2009). Kaempferol was monitored at 280 nm. Kaempferol identification was based on comparing the peak retention time in samples to the peak retention time of the kaempferol standard. To construct calibration curves for flavonoids, naringenin and kaempferol standards were dissolved in chromatographic grade methanol (Sigma-Aldrich, St. Louis, MO) at 1,000 mg L^{-1}. The stock solutions of each standard were used to prepare a serial concentration between 0.05 and 500 mg L^{-1} (Kumar *et al.,* 2009).

Statistical analysis

The statistical software Statistix 10 (Tallahassee, Florida, USA) was used to analyze the kaempferol concentration and FLS enzyme activity data. The paired sample *t*-test was used to compare wild and cultivated plants. Data were natural-log transformed to meet the assumptions of normality and homogeneity of variance. Values of $p \leq 0.05$ were considered to be significant. The results are expressed as the means with corresponding standard errors.

Results

Kaempferol content

Kaempferol concentration was evaluated for each particular ecotype and compared with the respective wild counterpart. Differences between the ecotypes and their wild counterparts are presented in Figure 2. Significant differences in kaempferol concentrations between cultivated and respective wild counterparts, such as Eco 18-1/Soloyo (0.04 µg g^{-1} and 0.14 µg g^{-1}), ($F_{1,5}$=61.40; *p*=0.0159); Eco 22-1/Mehuín (0.03 µg g^{-1} and 0.18 µg g^{-1}), ($F_{1,5}$=40.97; *p*=0.0235) and Eco 23-2/Queule (0.08 µg g^{-1}, and 0.25 µg g^{-1}) ($F_{1,5}$=84.28; *p*=0.0117) were found using the *t*-tests (*p*<0.05).
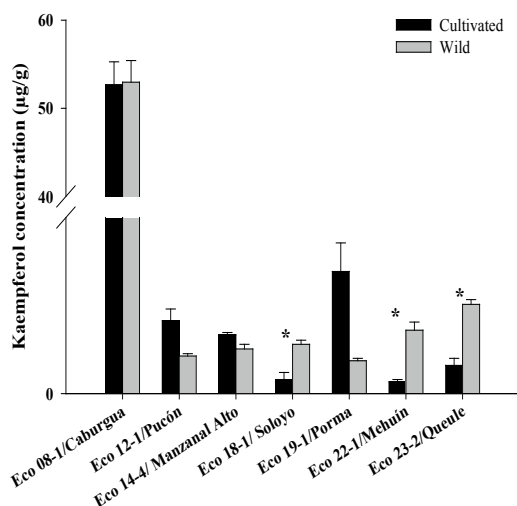
Figure 2. Kaempferol concentration in murtilla plant leaves obtained from all seven cultivated ecotypes and their respective wild counterparts. Bars indicate standard error, and * indicates significant differences between cultivated and wild plants ($p \leq 0.05$).

Enzymatic activity

Kinetic studies were carried out to select the best incubation time for the FLS enzymatic assays. Figure 3 shows how the amounts (in nmol) of naringenin consumed, as well as the amount of kaempferol produced, varied over time (30, 60, 120, and 240 min) at 30 ºC in all cases. The highest level of kaempferol was detected at 60 min (1.13×10^6 nmol) and coincided with a low level of naringenin (2.79×10^4 nmol). With regards to

the enzymatic activity, significant differences were observed in FLS activity between Eco 14-4 and Manzanal Alto, with 134 pKatal measured in the wild plants and 73 pKatal measured in the cultivated plants ($F_{1,5}=7.11$; $p=0.0500$) (Figure 4). The highest pKatal values were observed in wild samples collected in Soloyo ($F_{1,5}=12.45$; $p=0.0243$) and Mehuín ($F_{1,5}=11.81$; $p=0.0264$), at 96 pKatal and 119 pKatal, respectively. Their cultivated counterparts, Eco 18-1 and Eco 22-1, exhibited significantly lower pKatal values of 66 pKatal and 38 pKatal, respectively (Figure 4). There were no significant differences between cultivated and wild plants ($p>0.05$) in the other comparisons. Finally, HPLC analysis showed that enzymatic activity only produced kaempferol when the naringenin substrate from murtilla leaves was available; this enzymatic activity was not detected in the blanks.

Discussion

Plant domestication can affect flavonol biosynthesis, expressed as a decrease in flavonol content, but can increase other productive traits, as part of the so-called domestication syndrome (Meyer *et al.,*
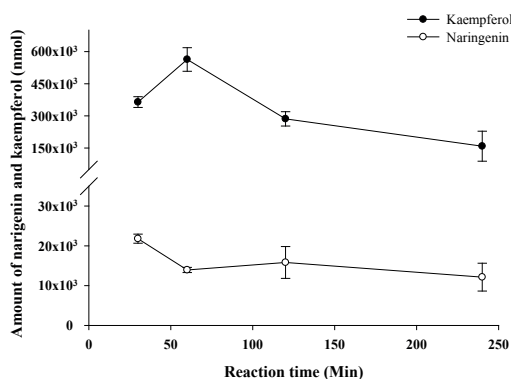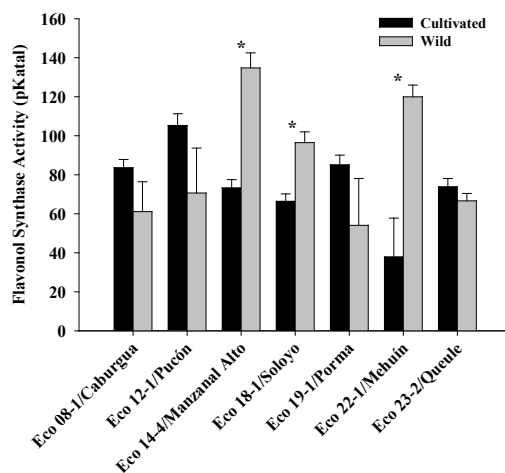

Figure 3. Correlation between the amount of naringenin consumed and kaempferol produced (nmol) during a 240 min reaction time. Data are expressed as the means with corresponding standard errors.

Figure 4. Flavonol synthase activity in murtilla plant leaves from cultivated ecotypes and respective wild counterparts. Bars indicate standard error and * indicates significant differences between cultivated and wild plants ($p \leq 0.05$).

2012). However, chemical defenses in plants, such as flavonols, can be affected in unpredictable ways. For example, the compromise between performance and production may be modified at the expense of flavonoid production. Modifications in flavonoid production can be important since flavonoids are responsible for the chemical defense from insect feeding behaviors in plants. Hence, kaempferol could have undergone concentration changes, due to changes in the enzyme flavonol synthase (FLS) pathway (i.e., transforming dihydroflavonol into flavonol or dihydrokaempferol into kaempferol) during the domestication process in murtilla plants, which in turn affected insect feeding behavior. For instance, Fang *et al.* (2013) observed a positive correlation between kaempferol concentration and FLS enzyme activity in grape berries, *Vitis vinifera* (cv. Cabernet sauvignon). Flores *et al.* (2014) also reported a direct correlation between kaempferol concentration and FLS enzymatic activity for strawberry, raspberry, blackberry, redcurrant, and blackcurrant fruits.

Our results showed that changes in kaempferol concentration were directly related to the FLS activity present in murtilla plants. Decreases in kaempferol concentrations of cultivated plants were due to lower enzymatic activity (FLS) compared to wild plants. Specifically, total kaempferol concentration found in plants from wild localities such as Soloyo (La Araucanía region), Mehuín, and Queule (Los Ríos region) were higher than the respective cultivated ecotypes, Eco 18-1, 22-1 and 23-2. These results were consistent with the higher FLS activity shown in Soloyo and Mehuín wild plants than their respective cultivated counterparts (Eco 18-1 and Eco 22-1). Chacón-Fuentes *et al.* (2017) reported that kaempferol content obtained from the leaves of wild murtilla plants from Mehuín and Queule was higher than in ecotypes 22-1 and 23-2, with concentrations of 0.37 to 0.86 µg g^{-1} in wild plants and 0.024 to 0.58 µg g^{-1} in cultivated plants. Moreover, when wild and cultivated murtilla plants were compared for kaempferol content a decrease from 1.01 to 0.87

mg L^{-1} was reported for the cultivated plants by Chacón-Fuentes *et al.* (2015). Therefore, plant domestication induced a loss in the defense traits associated with kaempferol concentrations in *U. molinae* ecotypes Eco 18-1, 22-1 and 23-2. These results suggest that the low FLS activity in cultivated ecotypes can be a consequence of the domestication process. However, information about adaptations under domestication are still scarce (Ross-Ibarra *et al.,* 2007). In our research, we documented changes in FLS activity, which could be caused by environmental (i.e., soil fertilization) or genetic factors (Yang *et al.,* 2008). Murtilla is a novel plant that has been subjected to the domestication process for less than 20 years. The cultivated ecotypes used in this study are clones recently collected from wild locations and thus can be considered in the primary stage of domestication. The significant differences found FLS activity and kaempferol production between wild and cultivated murtilla plants could be based on the strong genetic traits that are retained by plants in wild systems and lost by plants cultivated in agro-ecosystems. Considering that cultivated ecotypes are clones obtained a few years ago from various wild locations (Seguel *et al.,* 2000) it is reasonable to hypothesize that clonal propagation could transfer favorable genotypes, such as FLS activity, to the next generation according to McKey *et al.* (2009). However, data obtained from Soloyo, Mehuín, and Queule localities showed significant differences between wild plants and their cultivated counterparts indicating adaptation, including adaptations to the kaempferol content, in cultivated plants. Nevertheless, there were no significant differences of kaempferol content and FLS activity among wild and cultivated murtilla corresponding to Caburgua, Pucón, Porma and Eco 18-1, 12-1 and 19-1, respectively. These results would indicate domestication is an ongoing process in which there is a transfer of favorable traits from wild to cultivated ecotypes, specifically in the case of FLS activity and kaempferol production. These findings also suggest that the decrease of both

kaempferol content and FLS activity could be associated with adaptation processes or somatic mutations in the clonal propagation system. In this context, it is necessary to evaluate the role of fertilization in murtilla plants undergoing the domestication process, as well as the transfer of genetic traits by clonal propagation compared to domesticated plants obtained through sexual propagation (Bautista *et al.,* 2012). Our results showed that the biosynthesis of kaempferol through flavonol synthase activity in murtilla is affected by the domestication process. Wild plants coming from Soloyo, Mehuín, and Queule had significantly higher kaempferol concentration than their cultivated counterparts. Moreover, flavonol synthase activity was significantly higher in Soloyo and Mehuín plants. These results suggest an effect of domestication on the flavonol synthase production which affects kaempferol concentration. Therefore, the plant domestication process, which causes a decrease in the key enzyme flavonol synthase and thereby reduces kaempferol production, has implications for pest insect feeding.

Acknowledgments

Resumen

M. Chacón-Fuentes, A. Mutis, L. Bardehle, I. Seguel, A. Urzúa, y A. Quiroz. 2019. Disminución de la actividad enzimática de la Flavonol Sintasa debido al proceso de domesticación en *Ugni molinae* Turcz. Cien. Inv. Agr. 46(1): 30-39. La biosíntesis de flavonoides puede verse afectada por la domesticación vegetal, reduciéndose la producción en relación al grado de domesticación. En este contexto, ha sido identificado el kaempferol (3,4´, 5,7-tetrahidroxiflavona) en hojas de *Ugni molinae* silvestre y cultivada. La ruta biosintética del kaempferol comienza con naringenina (4´, 5,7-trihidroxiflavanona), que se convierte en dihidrokaempferol (3,4´, 5,7-tetrahidroxiflavanona), catalizada por la flavanona 3ß-hidroxilasa (FHT). Finalmente, se convierte en kaempferol por una enzima bifuncional llamada flavonol sintasa (FLS). Por lo tanto, nuestro estudio tiene como objetivo evaluar cómo se ve afectada la actividad de FLS en plantas de murtilla que están sujetas a un proceso de domesticación. El kaempferol fue cuantificado a partir de los extractos metanólicos de hojas recogidas en plantas cultivadas y silvestres de *U. molinae* mediante cromatografía líquida de alta resolución. Además, se realizó una extracción de enzimas para determinar la actividad de FLS. Los resultados mostraron que la concentración de kaempferol en las áreas de muestreo silvestre de Soloyo (0.14 µg g^{-1}), Mehuín (0.18 µg g^{-1}) y Queule (0.25 µg g^{-1}) fue mayor que en sus contrapartes cultivadas. Estos datos fueron consistentes con la actividad de FLS determinada en muestras obtenidas de Manzanal Alto (134,79 pKatal), Soloyo (96,48 pKatal) y Mehuín (119,97 pKatal). Estos también exhibieron una mayor actividad enzimática que sus contrapartes cultivadas. Por lo tanto, nuestros datos sugieren que la actividad de FLS se ve reducida por la domesticación vegetal.

Palabras clave: Actividad enzimática, cultivada, Kaempferol, naringenina, silvestre.

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