Centro de Investigación en Alimentación y Desarrollo A. C.

EFECTO DEL FRÍO Y RADIACIÓN UV-B EN LA EXPRESIÓN GÉNICA Y EL METABOLISMO SECUNDARIO DE PLANTAS DE PIMIENTO MORRÓN (*Capsicum annuum* L.)

POR:

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Los miembros del comité designado para la revisión de la tesis de Rubén Gerardo León Chan, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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RESUMEN

La temperatura baja (LT, siglas en inglés) y la radiación ultravioleta-B (UV-B) son factores de estreses abióticos que limitan la producción de los cultivos, debido a sus efectos en los componentes fotosintéticos. Las plantas de origen tropical y subtropical, como *Capsicum annuum*, sufren daños por LT. Además, los niveles mayores de UV-B en el mundo son en regiones tropicales y subtropicales. LT y UV-B pueden dañar las plantas, alterando su expresión génica y dañando componentes fotosintéticos, directa o indirectamente a través de la producción de especies reactivas de oxígeno. Existe evidencia de degradación de clorofila y carotenoides por efecto de LT y UV-B; así como la producción de ácidos fenólicos y flavonoides, entre ellos antocianinas, componentes de defensa frente a estos factores de estrés. Sin embargo, existe escasa información acerca del efecto de interacción de LT y UV-B (LT+UV-B). Por lo tanto, el objetivo de este estudio fue determinar el contenido de clorofila, carotenoides, ácidos fenólicos y flavonoides a través de métodos espectrofotométricos y cromatográficos; así como también, la expresión de genes que participan en la biosíntesis de antocianinas (*MYB, F3H, F3´5´H, DFR* y *ANS*) a diferentes intervalos de tiempo de exposición de plantas de pimiento morrón a LT y UV-B; lo cual se realizó mediante RT-qPCR, optimizando las condiciones de amplificación con la metodología de superficie de respuesta. Los resultados de la investigación mostraron que la LT+UV-B produjo la mayor degradación de clorofila en hojas de pimiento morrón y la mayor acumulación de carotenoides, ácido clorogénico y los flavonoides apigenina-7-glucósido y luteolina-7-glucósido, en comparación de LT o UV-B por separado. Por otra parte, el modelo de superficie de respuesta permitió encontrar de forma rápida y eficiente las mejores condiciones de amplificación para ensayos de expresión de los genes *MYB, F3H, F3´5´H, DFR, ANS, β-TUB* y *UBI-3*. La mayor expresión de los genes *MYB, F3´5´H, DFR* y *ANS* se presentó en los tallos de plantas expuestas a LT; mientras que la expresión de *F3H* fue mayor en plantas expuestas a LT+UV-B. La mayor expresión de *MYB, F3´5´H* y *DFR* se observó a la misma hora del día (16:00 h), lo que sugiere una posible regulación directa de *MYB*

sobre *F3´5´H* y *DFR*. Mientras que, la expresión de *F3H* incrementó durante el día con un punto de expresión máxima en la madrugada (04:00 h), para posteriormente disminuir nuevamente al inicio del día (11:00 h). Por lo que estos resultados sugieren que la radiación tiene un efecto regulador de la expresión de estos genes.

Palabras Clave: Fotosíntesis; carotenoides; flavonoides; especies reactivas de oxígeno (EROs); capacidad antioxidante; estrés abiótico; temperatura baja; radiación UV-B; expresión génica; biosíntesis de antocianinas.

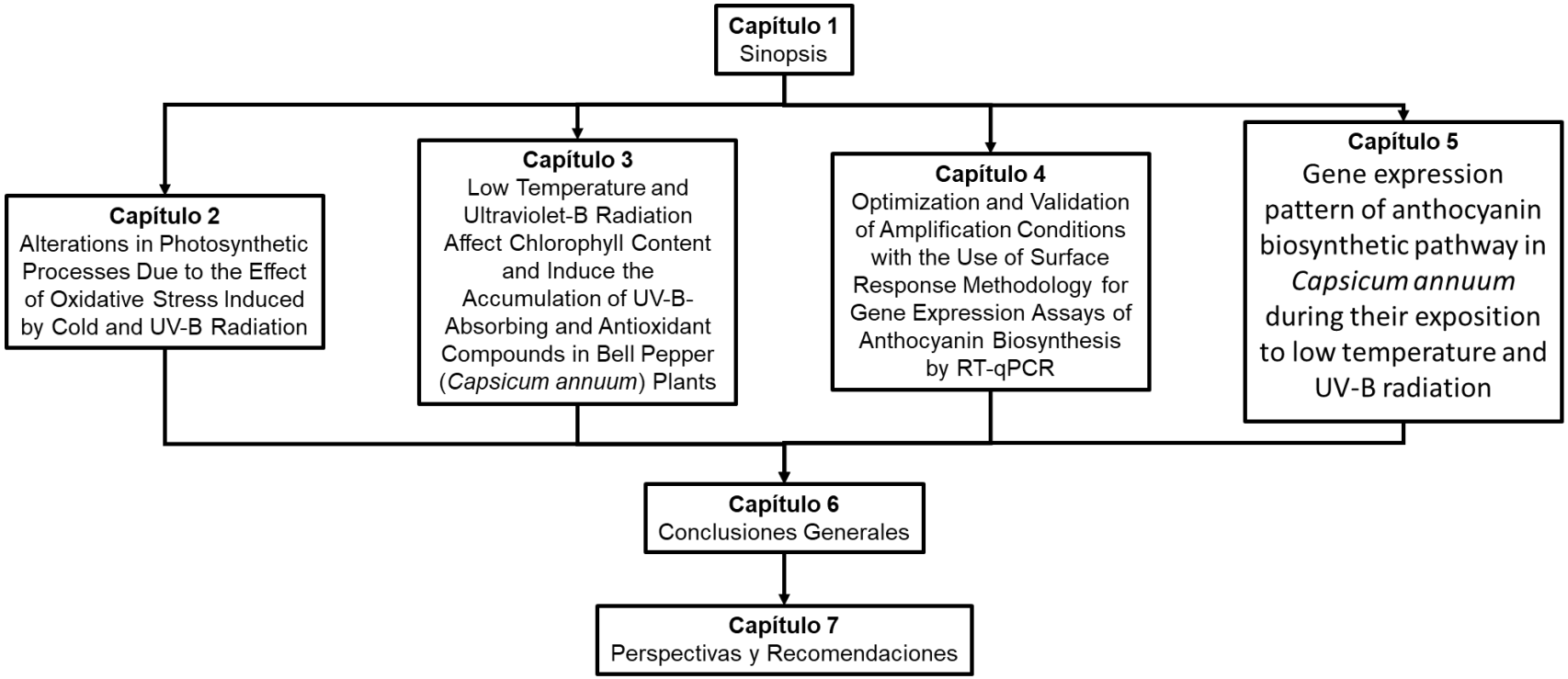
ABSTRACT

Low temperature (LT) and ultraviolet-B (UV-B) radiation are factors of abiotic stress that limit crop production because of their detrimental effects mainly on photosynthetic components. Plants of tropical and subtropical origin, such as *Capsicum annuum* are damaged by LT. Moreover, the higher levels of UV-B radiation in the world are in tropical and subtropical regions. LT and UV-B radiation can damage plants by altering gene expression and affecting photosynthetic components, either directly or through the production of reactive oxygen species. There is evidence of chlorophyll and carotenoid degradation by LT and UV-B radiation; as well as the production of phenolic acids and flavonoids, such as anthocyanins, as defense components against these stress factors. However, there is scarce information about the interactions between LT and UV-B radiation (LT+UV-B). Therefore, the objective of this study was to determine the effect of LT and UV-B radiation, in *Capsicum annuum*, on the chlorophyll, carotenoid, phenolic acids and flavonoid content measured through spectrophotometric and chromatographic methodologies; as well as the expression of genes that participate in the anthocyanin biosynthesis (*MYB, F3H, F3´5´H, DFR* and *ANS*) at different times of bell pepper plant exposition to LT and UV-B; this was done by RT-qPCR, and the amplification conditions were optimized with the response surface methodology. The results of this investigation showed that LT+UV-B condition caused the greatest degradation of chlorophyll in the bell pepper leaves and the highest accumulation of carotenoids, chlorogenic acid, and the flavonoids apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside, compared to the LT and UV-B conditions applied separately. On the other hand, the use of the model of response surface methodology allowed the setting of the best amplification conditions for expression assays of the genes *MYB, F3H, F3´5´H, DFR, ANS, β-TUB* y *UBI-3* in a rapid and efficient way. The highest expression of *MYB, F3´5´H, DFR* and *ANS* were exhibited by stems of plants exposed to LT; meanwhile *F3H* expression was higher in plants exposed to LT+UV-B. The highest expression of *MYB, F3´5´H* and *DFR* were found at the same hour of the day (16:00 h), which suggest

the possible direct regulation of *MYB* over *F3´5´H* and *DFR*. Meanwhile, the expression of *F3H* increased during the day reaching a maximum point at the early morning (04:00) to later decrease again at the beginning of the day (11:00 h). Therefore, the results suggest that radiation has a regulatory effect on the expression of these genes.

Key words: Photosynthesis; carotenoids; flavonoids; reactive oxygen species (ROS); antioxidant capacity; abiotic stress; low temperature; UV-B radiation; gene expression; anthocyanin biosynthesis.

ESTRUCTURA DE LA TESIS



CAPÍTULO 1. SINOPSIS

Los cambios en el ambiente que producen un impacto negativo en el crecimiento y desarrollo de las plantas son considerados como factores de estrés (Bray et al., 2000) y pueden ser clasificados en primarios, cuando el factor de estrés afecta directamente el desarrollo de la planta, y en secundarios, cuando el factor de estrés provoca la generación de especies reactivas de oxígeno (EROs) las cuales afectan negativamente el metabolismo de la planta (Ahmad y Prasad, 2012). Las EROs son producidos principalmente en cloroplastos y mitocondrias donde se lleva a cabo continuamente un intercambio de electrones entre distintos compuestos (reacciones de óxido-reducción). Las EROs pueden participar como microbicidas o como moléculas señalizadoras del sistema de defensa de la planta, sin embargo, su producción desmedida es dañina para diversos componentes de la célula (Perl-Treves y Perl, 2004).

Los factores de estrés pueden ser clasificados de acuerdo con la naturaleza del factor que afecta a la planta en estrés biótico y estrés abiótico (Koyro et al., 2012). El estrés biótico es producido por organismos tales como simbiontes, hongos, bacterias y virus patógenos, herbívoros y plantas parasitas o con las cuales compitan por el alimento. Este tipo de estrés también es conocido como estrés biológico, debido a la naturaleza de los factores que lo causan (Bray et al., 2000). Los factores de estreses abióticos son determinantes en la vida de las plantas, ya que son necesarios en ciertos niveles para su óptimo crecimiento. Cuando los niveles de estos factores se exceden o son deficientes afectan de forma negativa el crecimiento, desarrollo y productividad de las plantas (Koyro et al., 2012).

El estrés abiótico puede ser clasificado de acuerdo con la naturaleza del agente causal en estrés químico, que se presenta por la presencia de metales pesados, toxicidad por sales en exceso, aplicación de herbicidas, contaminantes atmosféricos y/o deficiencia nutrimental y; en estrés físico, el cual es provocado por déficit de agua, temperaturas extremas, radiación solar excesiva, daños mecánicos, entre otros (Koyro et al., 2012).

Cuando en las plantas se presenta cualquier factor de estrés, biótico o abiótico, estas desencadenan un amplio rango de respuestas en resistencia a dicho factor de estrés (Hammerschidt, 2007), mediante reacciones inducidas por diversos compuestos como el etileno, jasmonato, compuestos orgánicos volátiles, entre otros (Lyon, 2007). Las respuestas que se generan en las plantas por efecto del estrés pueden alterar su expresión genética y provocar cambios en el metabolismo celular, lo cual influye en el crecimiento y productividad. La duración, severidad y tipo de estrés al que se exponga la planta, así como las características de la misma, tales como su etapa de desarrollo, tejido afectado y genotipo, influyen en los tipos de respuesta que las plantas generan frente a los efectos deletéreos provocados por el estrés abiótico (Bray et al., 2000; Koyro et al., 2012).

Los mecanismos de resistencia al estrés pueden ser agrupados en dos categorías: 1) mecanismos de evasión, los cuales son un medio de prevención a la exposición del estrés como por ejemplo una reducción de la longitud del tallo, para evitar los efectos de la radiación excesiva o la disminución del área foliar y; 2) mecanismos de tolerancia, los cuales permiten a la planta sobrevivir expuesta al factor de estrés, un ejemplo de ello es la morfología de algunas plantas para sobrevivir a ambientes áridos o bien, la presencia de compuestos del metabolismo secundario, tales como carotenoides y compuestos fenólicos, entre los que se encuentran los flavonoides, relacionados con la protección de la radiación y su capacidad antioxidante para combatir radicales libres (Bray et al., 2000; Salisbury y Ross, 1994).

Los flavonoides son compuestos de 15 carbonos ordenados en dos anillos aromáticos, nombrados A y B, unidos por un puente de tres carbonos que forma un anillo heterocíclico con oxígeno (Figura 1) (Salisbury y Ross, 1994; Ávalos y Pérez-Urria, 2009). Los flavonoides son los compuestos fenólicos más diversos (más de 4000 conocidos) (Croteau, 2000; Manthey et al., 2002); estos compuestos se encuentran extensamente distribuidos en el reino vegetal y están presentes en casi todas las partes de la planta, predominan en la epidermis de las hojas, tallos y en la piel de frutos (Crozier et al., 2006, Molina, 2009; Mazid et al., 2011). Se estima que aproximadamente el 2 % de todo el carbono producido por las plantas es transformado en flavonoides (Barnes, 2010).

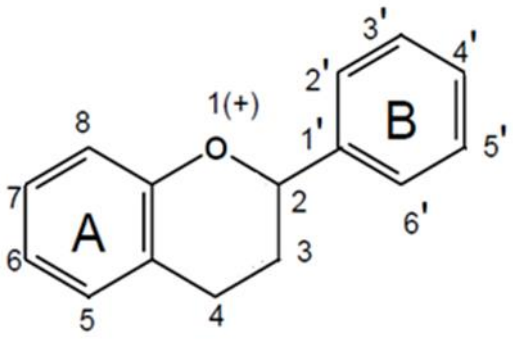


Figura 1. Estructura general de los flavonoides.

La estructura básica de los flavonoides puede presentar aún más enlaces dobles, lo que hace que los compuestos absorban luz visible, dándoles color (Salisbury y Ross, 1994). Algunos flavonoides específicos, como el kaempferol, también pueden proteger a las plantas contra la radiación UV-B (Croteau et al., 2000), por lo que se ha demostrado que la radiación UV-B incrementa la acumulación de flavonoides (Carrasco-Ríos, 2009; Hao et al., 2009).

Los flavonoides se sintetizan a partir de la formación de ácido cinámico por la eliminación de una molécula de amonio del aminoácido fenilalanina, debido a la acción de la enzima fenilalanina amonioliasa (PAL). El ácido cinámico posteriormente se transforma en ácido *p*-cumárico que se une a una molécula de acetil coenzima A (SCo-A) conduciendo a una reacción con tres moléculas de malonil-CoA catalizada por la enzima chalcona sintetasa (CHS), la cual forma una molécula de tetrahidroxichalcona. La molécula de tetrahidroxichalcona se isomerisa por la acción de chalcona isomerasa (CHI) a naringenina, el primer flavonoide de la ruta biosintética, del cual se desprende la formación de los diversos flavonoides (Figura 2) (Croteau et al., 2000; Crozier et al., 2006).

Los flavonoides se clasifican en función del grado de oxidación del puente de tres carbonos, los principales son flavonoles, flavonas, flavan-3-ol, isoflavonas y finalmente las antocianidinas que son la parte principal de la estructura de las antocianinas, además de ser los flavonoides más estudiados por los colores llamativos que proporcionan a los tejidos que los contienen (Crozier et al., 2006; Ávalos y Pérez-Urria, 2009).

La ruta de biosíntesis de antocianinas ha sido bien establecida y dividida en genes tempranos tales como *CHS*, *CHI*, *F3H* (flavanona 3-hidroxilasa), *F3´H* (flavonoide 3´-hidroxilasa) y *F3´5´H* (flavonoide 3´,5´-hidroxilasa); y en genes tardíos de la biosíntesis tales como *DFR* (dihidroflavonol 4-reductasa) y *ANS* (antocianidina sintetasa) (Figura 2) (Lim et al., 2016).

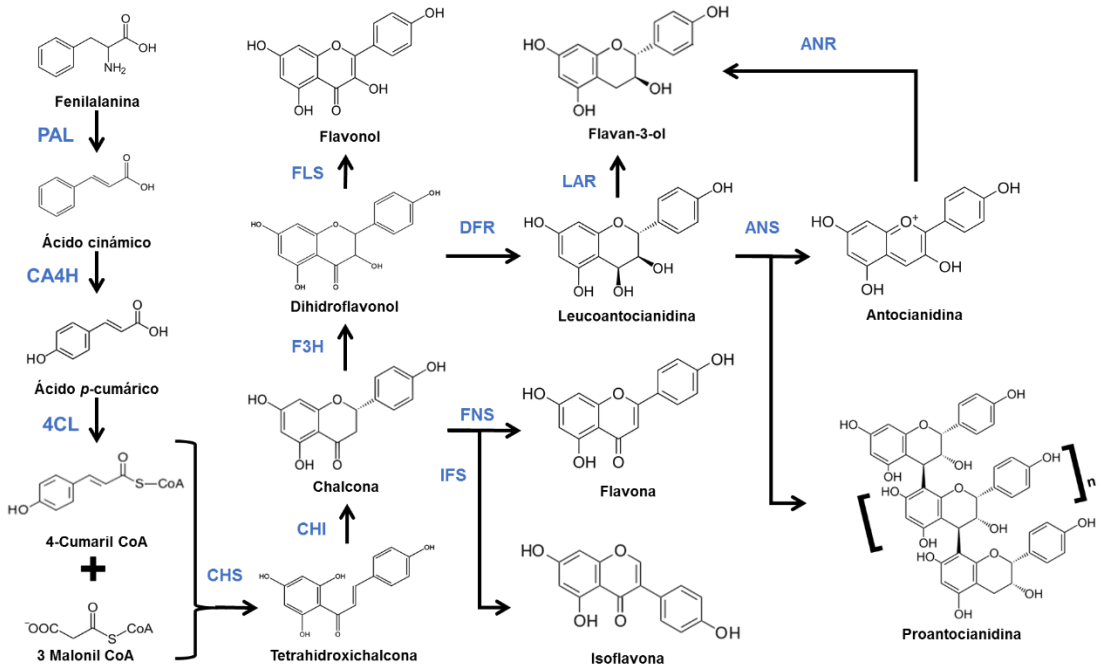


Figura 2. Esquema de la biosíntesis de los principales flavonoides en plantas.

PAL, fenilalanina amonioliasa; CA4H, ácido cinámico 4-hidroxilasa; 4CL, p-cumaril CoA ligasa; CHS, chalcona sintetasa; CHI, chalcona isomerasa; FNS, flavona sintetasa; IFS, isoflavona sintetasa; F3H, flavanona 3-hidroxilasa; FLS, flavonol sintetasa; DFR, dihidroflavonol 4-reductasa; ANS, antocianidina sintetasa; LAR, leucocianidina 4-reductasa; ANR, antocianidina reductasa.

Las hipótesis principales acerca del funcionamiento de las antocianinas en las plantas son: 1) la protección de los cloroplastos de los efectos adversos del exceso de luz. Se ha demostrado que las hojas que contienen antocianinas absorben más luz en los rangos de longitud de onda de los colores verde y amarillo respecto a aquellas hojas que no las contienen; además, se ha demostrado que la energía absorbida en esas longitudes de onda no pasa a los cloroplastos (Oren-Shamir, 2009); 2) protección de la radiación UV, por su capacidad de absorber este tipo de radiación; 3) actividad antioxidante. Se ha demostrado que algunas antocianinas (entre ellas las más comunes en la naturaleza)

tienen hasta cuatro veces más capacidad antioxidante que sus análogos de vitamina E y C; y, IV) moduladores de las cascadas de señales mediante especies reactivas de oxígeno que intervienen en el desarrollo y crecimiento de las plantas, expresión génica y respuestas a estreses bióticos y abióticos (Hatier y Gould, 2009).

Las plantas de origen tropical y subtropical que crecen en regiones meridionales sufren daños por heladas, o incluso por temperaturas por encima del punto de congelación del agua, que, dependiendo del cultivo, pueden ser causados por temperaturas de hasta 15 °C (Knight y Knight, 2012). Además, los niveles mayores de UV-B en el mundo se observan en regiones tropicales y subtropicales (Prado et al., 2012). Kaniuga (2008) reportó que el cultivo de chile (*Capsicum annuum* L.) es una de las plantas más susceptibles al estrés por frío debido a que presenta una mayor degradación de lípidos en las membranas respecto de otros cultivos como tomate, papa, melón, maíz y pepino.

Se ha determinado que el mayor daño causado por frío ocurre en los cloroplastos (Whitaker, 1995). Este daño es causado después de experimentarse el estrés por frío, al subir de nuevo la temperatura ambiental, lo cual ocurre principalmente en presencia de luz. Esto se debe a la producción de radicales libres que también provocan oxidación en las membranas (Kaniuga et al., 2008). Por otra parte, la localización de la planta (altitud, longitud y latitud) influye en la calidad de la luz que llegue a ella; a mayor altitud las plantas están más expuestas a longitudes de onda de las fracciones azul y ultravioleta del espectro de radiación, ya que a menor altitud la luz es parcialmente filtrada por la presencia de nubes, polvo, dióxido de carbono, ozono y vapor de agua, entre otros (Prado et al., 2012).

Los niveles de radiación UV sobre las zonas tropicales son mayores que en las templadas, debido a que en la región ecuatorial es menor la absorción atmosférica de rayos UV, por el ángulo de incidencia y por la capa de ozono, la cual es más delgada en dicha zona (Jaakola y Hohtola, 2010; Prado et al., 2012). En 1987 se estableció el Protocolo de Montreal, en el que se propusieron acciones para llevar a cabo la reducción de “Sustancias que Deterioran la Capa de Ozono”, principalmente los compuestos clorofluorocarbonados (CFCs). Sin embargo, a pesar de que la mayoría de los CFCs se han dejado de utilizar, el proceso de degradación no ha sido completamente revertido

(Tapia, 2010), por lo que el deterioro sigue incrementando hasta en un 0.6 % por año (Prado et al., 2012). En general, cada 1 % de reducción de la capa de ozono provoca un incremento de 1.3 a 1.8 % de la radiación UV-B (Hollósy, 2002).

Existe evidencia de la degradación de clorofila y carotenoides por efecto de LT y UV-B, así como también de la producción de ácidos fenólicos y flavonoides, tales como antocianinas, como componentes de defensa frente a estos factores de estrés (Rodrigues et al., 2006; Mahdavian et al., 2008; Sonoike, 2011; Zlatev et al., 2012). Incluso, la biosíntesis de flavonoles y antocianinas ha sido fuertemente correlacionada con los factores de estrés: temperatura baja y radiación UV-B (Aza-González et al., 2012; Schulz et al., 2015). Sin embargo, existe escasa información acerca del efecto de interacción de temperatura baja y radiación UV-B.

Por todo lo anterior, se hipotetizó que las plantas de pimiento morrón son más susceptibles a los efectos de la combinación de estrés por temperatura baja y radiación UV-B respecto a cada uno de los factores por separado y, por lo tanto, disminuye en mayor medida su contenido de clorofila y carotenoides; a lo cual la planta responde con una mayor síntesis de compuestos de defensa tales como ácidos fenólicos y flavonoides.

Por lo tanto, los objetivos de este estudio fueron determinar el contenido de clorofila, carotenoides, ácidos fenólicos y flavonoides a través de métodos espectrofotométricos y cromatográficos en hojas de plantas de pimiento morrón expuestas a temperatura baja y radiación UV-B. Asimismo, determinar la expresión de genes que participan en la biosíntesis de antocianinas (*MYB, F3H, F3´5´H, DFR* y *ANS*) a diferentes intervalos de tiempo de exposición de las plantas de pimiento morrón a temperatura baja y radiación UV-B, a través de RT-qPCR, optimizando las condiciones de amplificación de los oligonucleótidos con la metodología de superficie de respuesta (Figura 3).

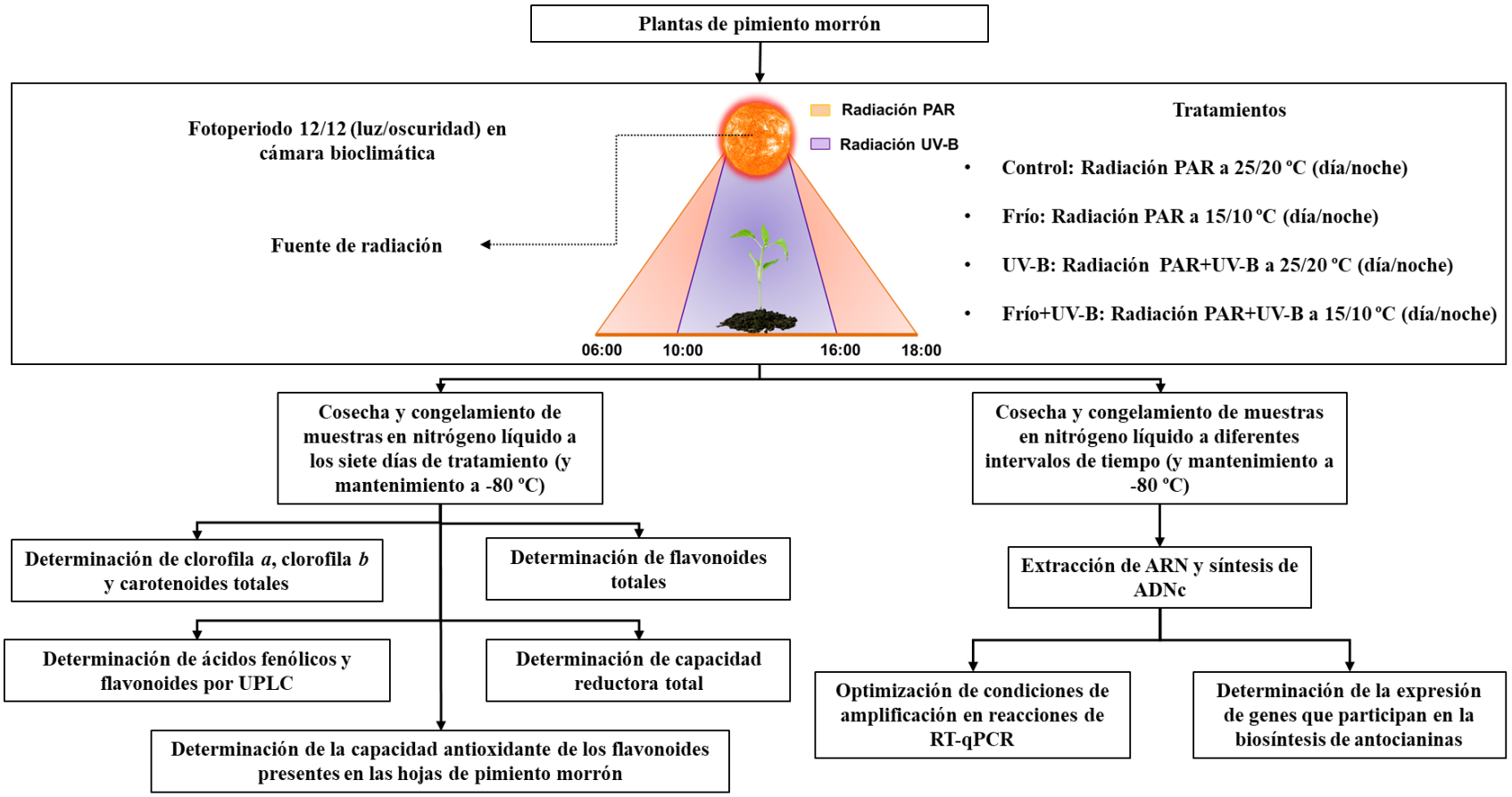


Figura 3. Estrategia metodológica general de la investigación.

*PAR (por sus siglas en inglés, radiación fotosintéticamente activa)

En el Capítulo 2 de la presente tesis se presenta una revisión exhaustiva de las investigaciones más recientes acerca de los efectos de la exposición de plantas a temperaturas bajas y radiación UV-B, y se concluye que los daños causados por temperatura baja y radiación UV-B son principalmente causados mediante la producción de EROs en los cloroplastos, especialmente en los sistemas fotosintéticos; a lo que la planta responde con la síntesis de flavonoides y enzimas con actividad antioxidante. Como resultado de esta revisión se propone un modelo del mecanismo de señalización a través de EROs para la activación de la biosíntesis de flavonoides, tales como las antocianinas, en respuesta al estrés oxidativo inducido por temperatura baja y radiación UV-B.

En el Capítulo 3 se presentan los resultados obtenidos del contenido de clorofila, carotenoides, ácidos fenólicos y flavonoides en hojas de plantas de pimiento morrón expuestas a temperatura baja y radiación UV-B. A partir del análisis de estos resultados concluyó que la combinación de estrés por temperatura baja y radiación UV-B induce una mayor degradación de clorofila y un aumento en el contenido de carotenoides con respecto a los efectos de la exposición de las plantas a cada factor de forma por separado. En estos análisis también se encontró una mayor acumulación de ácido clorogénico y de los flavonoides apigenina-7-glucósido (A-7-G) y luteolina-7-glucósido (L-7-G) por el efecto combinado de temperatura baja y radiación UV-B. Por otra parte, la relación L-7-G/A-7-G fue mayor tanto en las plantas expuestas solo a radiación UV-B como en combinación con temperatura baja, con respecto a plantas expuestas solo a temperatura baja o a condiciones control. Todos estos resultados fueron discutidos en cuanto a la absorción de radiación UV-B y la actividad antioxidante de los compuestos analizados bajo los efectos del estrés por temperatura baja y por radiación UV-B.

En el Capítulo 4 se presenta la optimización de las condiciones óptimas de amplificación para ensayos de qPCR mediante la metodología de superficie de respuesta. Para esto se diseñaron oligonucleótidos para genes que codifican proteínas que participan en la biosíntesis de antocianinas en *Capsicum annuum*. Como resultado se obtuvo que las condiciones óptimas de amplificación para ensayos de RT-qPCR en *C. annuum*, correspondientes a los oligonucleótidos, de genes que participan en la biosíntesis de

antocianinas (*MYB, F3H, F3´5´H, DFR* y *ANS*), diseñados en esta investigación y los genes de referencia *β-TUB* y *UBI-3* se encuentran entre 54.4 y 63.6 °C de temperatura de alineamiento, 233 y 317 nM de concentración de oligonucleótido, y 84 a 116 ng de ADNc. A partir de este trabajo, se propone la utilización de la metodología de superficie de respuesta como una herramienta para facilitar y mejorar la optimización de las condiciones de alineamiento de oligonucleótidos para ensayos de qPCR, reduciendo con ello el tiempo y costo invertidos en este proceso.

En el Capítulo 5 se presenta un análisis de los niveles de expresión de los genes *MYB, F3´5´H, DFR* y *ANS* que participan en la biosíntesis de antocianinas en tallos de plantas de pimiento morrón expuestas a temperatura baja y a radiación UV-B, a diferentes horas del día. En este estudio se determinó que la temperatura baja induce una mayor expresión de los genes *MYB, F3´5´H, DFR* y *ANS* en los tallos de plantas de pimiento morrón con respecto a la radiación UV-B, mientras que el efecto simultáneo de estrés por temperatura baja y radiación UV-B induce una mayor expresión del gen *F3H* en comparación con el estrés inducido por cada uno de los dos factores de por separado. También se encontró que los mayores niveles de expresión de los genes *MYB, F3´5´H* y *DFR* en tallos de plantas de pimiento morrón se presentan a las 16:00 h, mientras que la expresión del gen *F3H* incrementa durante el día con un punto máximo durante la madrugada (04:00 h) y baja al inicio del día siguiente (11:00 h). Finalmente, en el Capítulo 6 se presentan las conclusiones generales de todo el trabajo en conjunto.

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CAPÍTULO 2. ALTERATIONS IN PHOTOSYNTHETIC PROCESSES DUE TO THE EFFECT OF OXIDATIVE STRESS INDUCED BY COLD AND UV-B RADIATION

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Abstract

Plants of tropical and subtropical origin are damaged by temperatures below 15°C; these conditions produce cold stress in plants. The degradation of the ozone layer leads to a greater penetration of ultraviolet-B (UV-B) radiation to the planet's surface, especially in tropical and subtropical latitudes, which interferes with plant development. Cold and UV-B radiation can inhibit photosynthesis by altering gene expression and damaging photosynthetic components, either directly or through the production of reactive oxygen species (ROS). The photosynthetic components that are most affected are photosystems I (PSI) and II (PSII), light-harvesting complex II (LHCII) and the membranes. Through evolutionary processes, plants have developed different defense mechanisms against these types of stress, such as the synthesis of carotenoids and flavonoids, to protect themselves against the incidence of UV-B radiation and the ROS caused by these stressors. Therefore, this review is focused on analyzing the evidence that describes the effects of cold and UV-B radiation on the main components of the photosynthetic apparatus directly and through the production of ROS induced by photoinhibition as well as the mechanisms that plants use to eradicate the excessive production of ROS.

Key words: Photosystems; Defense system; Carotenoids; Flavonoids; ROS

Introduction

In 1987, the Montreal Protocol was established to reduce the emissions of substances that deplete the ozone layer; despite this protocol, the degradation has not been reversed (IPCC, 2014; Abbasi and Abbasi, 2017), and deterioration continues to increase by 0.6% per year (Prado et al., 2012). In general, a 1% reduction of the ozone layer causes a 1.3 to 1.8% increase in ultraviolet-B (UV-B) radiation reaching the earth's surface (Hollósy, 2002). The levels of UV-B radiation at tropical latitudes are higher than in temperate regions due to the thinner ozone layer in the equatorial region, affecting the organisms that inhabit that region to a greater extent (Jaakola and Hohtola, 2010; Prado et al., 2012).

Exposure of plants to UV-B radiation induce a great number of variations in their metabolism, morphology, physiology and molecular levels (Mandi, 2016a; Jenkins 2017). In fact, UV-B radiation can inhibit the process of photosynthesis directly, causing alteration in gene expression and damage to the photosynthetic machinery, mainly to photosystems I and II (PSI and PSII, respectively), as well as to light-harvesting complex II (LHCII). It can also affect photosynthesis indirectly through the generation of reactive oxygen species (ROS) (Smith et al., 2009; Mandi, 2016b). Tropical and subtropical plants are also damaged by temperatures below 15°C (Knight and Knight, 2012). At the cellular level, the greatest damage caused by cold occurs in chloroplasts, since increasing the temperature again increases the activity of galactolipases (enzymes that release fatty acids) and lipoxygenases (LOX, catalysts of the hydroperoxidation of free fatty acids), which occurs mainly in the presence of light due to the production of ROS, all of which ultimately causes oxidation of the membranes (Kaniuga, 2008). In response to cold and UV-B radiation, plants exhibit decreased leaf area, reduced stem elongation, increased leaf thickness, altered stomatal density, increased antioxidant enzyme activity and secondary metabolite synthesis, among other effects (Prado et al., 2012).

Synthesis of secondary metabolites of the phenylpropanoid pathway has been reported as a defense mechanism to counteract the deleterious effects of cold and UV-B radiation on plants; these compounds include phenolic acids, insoluble polyphenols and flavonoids (Theocharis et al., 2012; Zlatev et al., 2012). Flavonoids are found mainly in the epidermis of plant tissues, so they have been associated with the property of absorbing UV-B radiation as their main activity (Barnes et al., 2016; Mandi, 2016a). However, they also have the capacity to counteract the deleterious effects caused by ROS, due to their antioxidant potential (Castañeda-Ovando et al., 2009). The objective of this review is to document the most recent advances in the literature regarding the effects of cold and UV-B radiation on the photosynthesis process, the mechanisms of ROS production during the photoinhibition process and the involvement of defense mechanisms to reverse the effects of stress.

Morphological Changes in Plants Due to the Effects of Cold and UV-B Radiation

Cold and UV-B radiation cause morphological changes in leaf thickness and area, increased axillary branching, cuticle wax production, reduced stem elongation and reduced dry matter content (Kumari et al., 2015; Jenkins, 2017). During cold stress, an increase in stem and leaf flaccidity in pepper plants has been demonstrated (Airaki et al., 2012). In addition, the growth of the leaves is due to an increase in parenchymal cells and an increase in intercellular spaces (Wang et al., 2014). Furthermore, if plants are exposed to temperatures outside optimal growth ranges, the negative effects of UV-B radiation increase (Singh et al., 2014b; Martel and Qaderi, 2016). Physiological changes depend on the plant species exposed to stress. For example, Kakani et al. (2003) reported that out of 40 studies involving 23 plant species exposed to UV-B radiation, only 54% had a biomass reduction, 35% were not affected, and 5% exhibited an increase in biomass, suggesting that factors such as genotype, temperature and the intensity of the

radiation are relevant in the response. Kataria et al. (2013) showed that dicotyledonous plants are more susceptible to UV-B radiation than monocotyledonous plants, which may be related to their morphological differences, since dicotyledons arrange their leaves more horizontally, are more exposed to radiation and are therefore more susceptible to damage to their photosynthetic components, particularly PSII.

Effects of Cold and UV-B Radiation on the Activity of Photosystems I and II (PSI and PSII)

Photosystem II

PSII is the first component of the luminous phase of photosynthesis. It consists of a complex of proteins and pigments that is responsible for taking the energy of the photons of solar radiation and transforming it into chemical energy (Nishiyama and Murata, 2014). The PSII antenna complex consists of the major chlorophyll complex, called LHCII; the minor complex, consisting of the proteins CP29, CP26 and CP24; and the central protein complexes CP43 and CP47 (Pospíšil and Prasad, 2014). The central part of PSII consists of a pair of proteins called D1 and D2, which contain linked chlorophyll and carotenoid pigments as well as cofactors that are responsible for the flow of electrons from water molecules. This protein dimer consists of a manganese complex in which water oxidation occurs, the $P680^{+}$ reaction center, as well as the primary electron acceptor quinone Q_A and the secondary acceptor quinone Q_B, which are bound to the D2 and D1 proteins, respectively (Rodrigues et al., 2006). All the above components are sensitive to UV-B radiation (Dobrikova et al., 2013, Kataria et al.,

2014). Studies of the effects of UV-B radiation have mainly focused on PSII because the D1 and D2 proteins are very sensitive due to chemical transformations in amino acids with double bonds (Table 1) (Zlatev et al., 2012). Amino acids capable of absorbing UV-B radiation are those of the aromatic type, such as phenylalanine, tryptophan and tyrosine, as well as cysteine, cystine and histidine (Hollósy, 2002; Mandi, 2016b). The amino acid histidine is found in protein D1, which contains bound chlorophylls that, during conformational changes by the UV-B radiation effects, are released to facilitate their photo-oxidation (Mahdavian et al., 2008).

Protein D1 is the component of PSII primarily affected by radiation, so plants have developed a complex system of continuous repair for the replacement of damaged D1 proteins, resulting in a process of intense regulation between degradation and D1 synthesis (Nishiyama and Murata, 2014). The repair process begins with the phosphorylation of the PSII containing the inactive D1, which promotes the transport of the PSII from the covered thylakoid membrane to the part of the membrane exposed to the stroma, where the complex is dephosphorylated. Then begins the action of two protease families, called Deg and FtsH (Nath et al., 2013). Deg proteins are endopeptidases of which the DegP 2 isoform is found in the stromal part of the thylakoid membrane associated with D1 dissociation in two 23- and 10-kDa polypeptides (Haußühl et al., 2001). Deg 1 is bound to the part of the thylakoid membrane of the lumen that produces two polypeptides from D1, corresponding to 16 and 5.2 kDa (Kapri-Pardes et al., 2007). However, in mutants of *Arabidopsis thaliana* lacking DegP 2, the same phenotype and D1 change is obtained as in normal plants (Huesgen et al., 2006). So, it can be assumed that the activity of these proteases does not dependent on both to carry out the complete D1 degradation process. On the other hand, the participation of FtsH proteins, also called zinc metalloproteases, has been demonstrated in the degradation of the segments after the action of the endopeptidases (Lindahl et al., 2000). This process is essential for the replacement of D1 proteins and thereby maintenance of PSII (Bailey et al., 2002). In fact, the accumulation of FtsH family has been observed as a recovery system in plants exposed to UV-B radiation (Pascual et al., 2017). Once the inactive D1 is degraded, the assembly of the new D1 pre-protein proceeds; which undergoes some post-translational modifications, and the restored PSII

is subsequently returned to the grana (Nath et al., 2013). Finally, Mlinarić et al. (2017) reported that the completely PSII recovery is totally restored to the initial values during the night.

Cold and hydrogen peroxide (H_2O_2) produced during stress do not have a direct effect on PSII damage; however, these two factors lead to a reduction in the D1 repair system, thereby reducing PSII activity (Allakhverdiev and Murata, 2004). The main point of inhibition of the D1 repair system by ROS is an elongation factor during the translation process, thus also affecting the production of other proteins (Nishiyama et al., 2011). The mechanism consists of the formation of a disulfide bridge due to the oxidation of two cysteine residues, Cys 105 and Cys 242, caused by ROS. These residues are conserved in several plants as well as in prokaryotic organisms (Kojima et al., 2009). Therefore, the reduction in PSII activity due to cold and UV-B radiation is mainly attributed to damage caused by UV-B radiation directly in D1, whereas the excessive production of ROS as a consequence of exposure to both stress factors leads to a reduction in the PSII repair system, which leads to a prolonged recovery time for the plant.

Table 1. Effects of UV-B radiation and cold stress in photosystems

Photosystem	Effects of UV-B radiation	Effects of cold	Reference(s)
PSII	Damages proteins D1 and D2 through conformational changes in aromatic amino acids	Cold has no a direct effect on proteins D1 and D2	Hollósy, 2002; Allakhverdiev and Murata, 2004; Zlatev et al., 2012; Mandi, 2016b
	Conformational changes produced in proteins D1 and D2 release chlorophylls increasing their photo-oxidation	Cellular activity decreases, so the solar radiation becomes excessive producing photo-oxidation of chlorophyll	Rodrigues et al., 2006; Mahdavian et al., 2008; Sonoike, 2011; Zlatev et al., 2012
	Induces the accumulation of proteases which participate in D1 repair system	The protein synthesis is inhibited reducing the D1 repair system	Allakhverdiev and Murata, 2004; Nishiyama et al., 2011; Pascual et al., 2017
PSI	Increases ROS production which can form hydroperoxides from free fatty acids released during cold stress	Induces the activation of galactolipases increasing free fatty acids and therefore, the production of hydroperoxides which damage PSI because is more exposed to stroma than PSII	Zhang and Scheller, 2004; Kaniuga, 2008; Mandi, 2016b
	Reduces the RuBisCO activity which promote PSI photoinhibition by ROS produced	Reduces the RuBisCO activity which promote PSI photoinhibition by ROS produced	Lidon and Ramalho, 2011; Fu et al., 2016; Pascual et al., 2017

Photosystem I

Most studies mention that PSI is less sensitive than PSII to damage by UV-B radiation (Kataria et al., 2014). However, cold stress affects PSI more than PSII (Zhang et al., 2014). During exposure to cold, cellular metabolic activity decreases, so that the solar radiation becomes excessive for the plant, and PSII is damaged (Table 1); however, as mentioned above, PSII has a repair system, and when the plant is again exposed to

higher temperatures, PSII recovers. In contrast, PSI does not have such an efficient repair system. As a result, the electrons from PSII to PSI are not used properly, thus promoting the production of ROS and consequently damaging PSI (Sonoike, 2011). Another process that occurs during cold stress is the activation of galactolipases, which have the function of releasing fatty acids from the membranes. Most fatty acids in chloroplasts are polyunsaturated, making them more susceptible to oxidation by ROS and LOX. Once the temperature increases, LOX activity increases by producing a high amount of fatty acid hydroperoxides that are harmful to membranes and proteins (Kaniuga, 2008). This process coincides with the protein reduction that occurs in PSI during the time after increasing the temperature after cold stress (Zhang and Scheller, 2004). Therefore, the damage to PSI is related to the production of hydroperoxides from the damaged membranes because this photosystem is found mainly in stromal membranes and is therefore more exposed to this type of damage than PSII, which is found mostly in the inner chloroplast membrane.

Cold and UV-B radiation have a negative effect on the Calvin cycle, mainly affecting the activity of the ribulose-1, 5-bisphosphate carboxylase (RuBisCO) enzyme, so the NADH produced in PSI is not used, maintaining the P700 reaction center in a reduced state (Lidon and Ramalho, 2011; Fu et al., 2016; Pascual et al., 2017). This process causes the electrons coming from PSII to promote the production of ROS in PSI and thus its photoinhibition. By blocking the activity of P700, the activity of the electron transport chain is reduced, which induces the reduction of the plastoquinones that causes photoinhibition of PSII. The inactivation of PSI decreases the production of ATP, which is necessary for the production of D1 in PSII repair. Therefore, inactivation of PSI negatively affects PSII. In contrast, inactivation of PSII does not negatively affect PSI activity (Sonoike, 2011). In fact, Zhang et al. (2016) suggest that the reduction of PSII repair or its damage produced by UV-B would be necessary when PSI is damaged by cold stress, because this reduces the production of ROS in PSI and, consequently, allow PSI recovery. However, UV-B radiation is a higher inductor of ROS than cold and other stress factors (Wu et al., 2016), therefore, is necessary to search better ways to improve the PSI recovery.

Because they need oxygen to survive, aerobic organisms are exposed to the negative effects of ROS. In the case of plants, the maximum production of these compounds occurs in chloroplasts (Hasanuzzaman et al., 2012). ROS can be classified into two groups: molecular or non-radical ROS, which are H_2O_2 and singlet oxygen ($^1O_2$) and free radicals, such as the superoxide radical ($O_2•^-$), alkoxyl radicals (RO•) and peroxyls (ROO•), the perhydroxyl radical (HO_2•) and hydroxyl radical (OH•) (Gill and Tuteja, 2010). The last is the radical with the greatest oxidation capacity in plants (Perl-Treves and Perl, 2002). Both cold and UV-B radiation promote a reduction in the activity of the Calvin cycle and thus a lower need for radiation in the photosynthesis process, resulting in an excess of incident radiation in the photosystems, promoting the formation of $^1O_2$ and $O_2•^-$, which alter the redox state and, therefore, change the chloroplast metabolism (Nishiyama and Murata, 2014; Chan et al., 2016). PSII is the highest producer of $^1O_2$, and PSI the greatest producer of $O_2•^-$ (Kataria et al., 2014).

During the exposure of chloroplasts to the energy of solar radiation, the chlorophyll molecules go from being in a basal state to a state of higher energy (^{1}Chl*). The energy is transferred between these molecules until reaching reaction center P680 (in the case of PSII) to continue with the electron transport chain. However, in the case of excess radiation, the chlorophyll remains in an excited state for a prolonged time, whereby the formation of chlorophyll triplets in the excited state (^{3}Chl*) occurs. When ^{3}Chl* is formed, the energy of this molecule can be transmitted to an oxygen molecule and produces $^1O_2$ (Pospíšil and Prasad, 2014). During exposure to UV-B radiation, the D1 protein is damaged, so that the part that blocks the passage of the oxygen molecules produced during the oxidation of water allows access to the P680 reaction center (Nishiyama et al., 2011). During exposure to solar radiation, reaction center P680 also enters an excited state that subsequently also forms an excited triplet (^{3}P680*) and transmits its energy to an oxygen molecule, thereby forming $^1O_2$ (Gill and Tuteja, 2010; Pospíšil and Prasad, 2014).

On the other hand, in PSI, the production of NADPH is accomplished by the reduction of $NADP^+$ and the use of the electrons coming from PSII. When the Calvin cycle undergoes alterations due to cold and/or UV-B radiation, the consumption of NADPH decreases, and $O_2•^-$ is formed in the membrane. This radical is spontaneously transformed into H_2O_2 by the effect of a low pH or by the action of the superoxide dismutase (SOD) enzyme (Perl-Treves and Perl, 2002). Subsequently, H_2O_2 can be reduced to two molecules of water by the action of ascorbate peroxidase (APX) using ascorbic acid as a substrate (Asada, 2006). However, $O_2•^-$ can also be protonated to form the radical $HO_2•$, or, through the presence of metals such as copper and iron, the OH• radical can be formed by Haber-Weiss or Fenton reactions (Gill and Tuteja, 2010). It has also been demonstrated that UV-B radiation impinges on H_2O_2 molecules, causing OH• formation (Czégény et al., 2014). Finally, OH• participates in the oxidation of proteins and lipids in the membranes, the result of which is chain reactions that lead to the formation of RO• and ROO• radicals.

Plant Defense Systems against Oxidative Stress Produced by Cold and UV-B Radiation

Due to the excessive production of ROS caused by cold and UV-B radiation, plants have a number of mechanisms to prevent and combat oxidative stress (Zlatev et al., 2012). According to the location of the defense mechanisms, with respect to the main production sites of ROS in chloroplasts, these organelles have several lines of defense against ROS: first, their content of carotenoids and tocopherols (Pospíšil and Prasad, 2014); second, antioxidant enzymes; and third, phenolic compounds, such as flavonoids.

Carotenoids and Tocopherols

Carotenoids are part of the structure of membranes as well as of photosynthetic complexes, where they have the functions of radiation collectors as well as protectors against ROS (Domonkos et al., 2013). β-Carotene, the most abundant carotene in chloroplasts, is mainly bound to the reaction centers of PSI and PSII (Esteban et al., 2015) and, the xanthophylls are mainly present in the antenna complexes (Ramel et al., 2012). In PSII, most of the β-carotene is in contact with the chlorophyll molecules, thus decreasing the probability of forming ^{3}Chl*, whereas close to reaction center P680, β-carotene is responsible for containing the $^1O_2$ production (Cazzaniga et al., 2012). When β-carotene comes in contact with ^{3}Chl* or with $^1O_2$, it acquires the energy of these molecules, releasing them to their basal state, and β-carotene then releases that energy in the form of heat (Figure 4) (Pospíšil and Prasad, 2014). The products obtained from the exposure of β-carotene to ROS (β-apo-14'-carotenal, β-apo-10'-carotenal, etc.) are more susceptible to oxidation (Ramel et al., 2012), which leads to the rapid oxidation of these compounds during stress due to UV-B radiation (Mahdavian et al., 2008).

It has been reported that the highest amount of $^1O_2$ is produced in PSII (Kataria et al., 2014); however, Cazzaniga et al. (2012) found that the PSI light-harvesting complex (LHCI) produces twice as much $^1O_2$ during stress at 8°C and high irradiance in Arabidopsis mutants lacking β-carotene production. This outcome explains the higher content of β-carotene in PSI of normal plants, which decreases the production of $^1O_2$ and therefore the difficulty detecting $^1O_2$ in this complex.

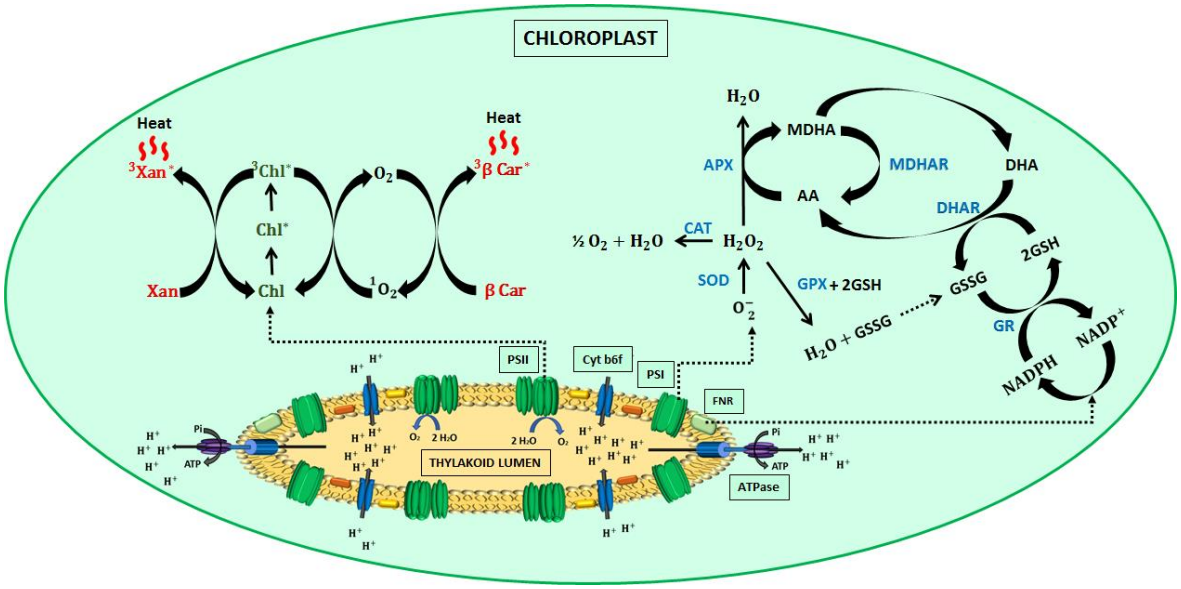

Figure 4. Mechanism to quench triplet excited chlorophylls (^{3}Chl*) and ROS in the chloroplast.

The exceding energy from chlorophyll prodduce ^{3}Chl*, this energy is transferred to oxygen molecules to form the singlet oxygen $^1O_2$. The energy of this molecules, ^{3}Chl* and $^1O_2$, is transferred to xanthophylls (Xan) and β-carotene (β-Car), respectively and is released as heat. The superoxide radical ($O_2^{\bullet-}$) produced in chloroplast is reduced throw the system of reactions of the antioxidant enzymes. PSII, photosystem II; PSI, photosystem I; Cyt b6f, cytochrome complex b6f; FNR, ferredoxin-NADP reductase; ATPase, ATP synthase; ^{3}Xan*, triplet exited xanthophyll; ^{3}β-Car*, triple excited β-carotene; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide, CAT, catalase; APX, ascorbate peroxidase; AA, ascorbic acid; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; DHA, dehydroascorbate; DHAR, DHA reductase; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase.

According to the location of β-carotene in the photosystems and their oxidation products (Ramel et al., 2012), this compound is mainly directed to the neutralization of $^1O_2$. In contrast, due to the increased presence of xanthophylls in light-harvesting complexes (LHCs), these compounds are intended to reduce the production of ^{3}Chl* (Pospíšil and Prasad, 2014). This was verified by Dall'Osto et al. (2013) in Arabidopsis mutants unable to produce xanthophylls but not α- and β-carotene, in which they observed damage in the LHC. In the same work, a significant reduction in PSI activity was described because of a deficiency in the ratio of Psa A/B subunits. This deficiency is attributed to the fact that xanthophylls may play the role of mediators in the interactions between the PSI subunits or with the chaperones that direct these proteins. Alternatively,

xanthophylls also have a role in the post-translational events of these proteins. However, it has also been shown that ROS induce changes in an elongation factor, which causes a decrease in protein production. In addition, the proteins of the photosystems are susceptible to attack from these compounds (Nishiyama et al., 2011), and because the lack of xanthophylls promotes a higher production of ROS, the reduction of PSI may be a consequence of the processes produced by these compounds.

When the carotenoid concentration is insufficient to decrease the production of $^1O_2$, this compound causes oxidation of proteins and thylakoid membranes. In the case of membranes, ROO• radicals are produced that can be neutralized by tocopherol molecules, producing tocopherol radicals that can be reduced by another molecule of tocopherol or react with another ROO• to form the molecule tocopherolquinol (Pospíšil and Prasad, 2014). During cold stress and high radiation levels, α-tocopherol has been shown to reduce membrane lipid damage and has the ability to reduce oxidation of chlorophyll molecules and damage to PSII, assuming that there are α-tocopherol molecules very close to PSII that could be protecting β-carotene and D1 through the neutralization of $^1O_2$ (Havaux et al., 2005). It has recently been proposed that α-tocopherol, due to its ability to neutralize ROS, prevents these compounds from affecting the PSII repair system (Murata et al., 2012). In addition, it has been determined that α-tocopherol and xanthophyll molecules participate in similar actions together, since mutants with α-tocopherol deficiencies exhibit increases in xanthophyll content and vice versa. However, mutants with deficiencies of both compounds present severe damage to membranes, chlorophylls and PSII (Havaux et al., 2005).

Enzymatic Antioxidants

The main components of the antioxidant enzymatic machinery of plant cells are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR),

glutathione peroxidase (GPX) and glutathione reductase (GR) (Figure 4) (Gill and Tuteja, 2010). SOD is the first enzyme to participate during the production of ROS and is responsible for transforming the radical $O_2^{\bullet -}$ into H_2O_2. This process is accomplished by the metal ion contained by SOD (Cu, Mn or Fe). The Fe-SOD and Cu/Zn-SOD isozymes are in the chloroplasts (Asada, 2006). However, H_2O_2 is also a molecule that causes damage to the cell so it presents a further degree of detoxification that is performed by the remaining enzymes together. First, H_2O_2 is reduced to water molecules by the CAT, GPX and/or APX enzymes (Gill and Tuteja, 2010). In the case of CAT, no reduced substrate is required to perform the action, which results in water and oxygen as products (Perl-Treves and Perl, 2002). In contrast, APX and GPX require ascorbic acid (AA) and reduced glutathione (GSH) to obtain water and monodehydroascorbate (MDHA) and oxidized glutathione (GSSG) molecules, respectively. These molecules are transformed to their reduced state by the enzymes MDHAR and GR, respectively. In the case of GR activity, NADPH produced during photosynthesis (Gill and Tuteja, 2010) is used. In chloroplasts, which are the sites of greatest ROS production, APX is the most prevalent H_2O_2-neutralizing antioxidant enzyme. Different chloroplast APX molecules have been classified as tAPX for the molecules located in thylakoids and sAPX for the molecules found in the stroma (Foyer and Noctor, 2009; Murata et al., 2012).

In the majority of cases, both cold stress and UV-B radiation increase the activity of antioxidant enzymes, with some exceptions (Hasanuzzaman et al., 2012), which may depend on the type of plant and even on its nutritional status (Singh et al., 2014a). It has been observed that an increase in SOD activity is greater in the chloroplasts than in the cytoplasm, as is the increase in APX due to the effects of UV-B radiation, whereby the antioxidant activity focuses on metabolizing H_2O_2 and thus $O_2^{\bullet -}$ (Majer et al., 2014), which is mostly produced in PSI (Kataria et al., 2014). However, when cold stress and/or UV-B radiation is very severe, CAT activity (as well as that of APX in chloroplasts) is inhibited and/or insufficient for the amount of H_2O_2 produced (Hasanuzzaman et al., 2012; Liu et al., 2012). A reduction in tocopherols and carotenoids can also be observed (Mahdavian et al., 2008; Zlatev et al., 2012), which increases the concentration of H_2O_2 because it is not neutralized. This outcome is

relevant because H_2O_2 is liposoluble and is able to pass through the membranes to migrate to other cellular compartments and cause greater damage (Mubarakshina et al., 2010).

Flavonoids

Based on the flavonoid structure, they can be classified in: chalcones, flavones, flavonols, dihydroflavonols, flavandiols, anthocyanins, proanthocyanidins, isoflavonoids and aurones (Zhang et al., 2017). Flavonoids are widely distributed in the plant kingdom and in almost all plant organs, though they predominate in the epidermis of the leaves, stems and fruit skin. Due to the absorption capacity of UV-B radiation by compounds such as kaempferol and anthocyanins, the role of photoprotectors is attributed to flavonoids (Croteau, 2000). However, overproduction of flavonoids under the effects of cold and UV-B radiation has also been linked to its antioxidant ability to reverse oxidative stress on the cell (Mazid et al., 2011; Cruceriu et al., 2017; Zhang et al., 2017). Flavonoids containing two hydroxyl groups on their B ring are mostly produced under conditions of exposure to UV-B or high levels of PAR (photosynthetically active radiation) radiation (Guidi et al., 2016) and contain a greater ability to inhibit ROS than those molecules with a single hydroxyl group, which have a greater capacity to absorb UV-B radiation. In addition, dihydroxylated flavonoids have the ability to form complexes with metals such as Al, Cu and Fe, thus also reducing the risk of producing OH• radicals through Fenton reactions with H_2O_2 molecules produced during exposure to cold and UV-B radiation (Castañeda-Ovando et al., 2009; Agati et al., 2013). It has been reported that the content of cinnamic acids is replaced by a higher content of flavonoids, which have a lower UV-B radiation absorption capacity, during exposure to this type of radiation (Agati et al., 2013); therefore, the plant's defenses are mainly focused on reducing the amount of ROS produced by UV-B radiation rather than absorbing the radiation (Hideg et al., 2013).

Quercetin has the ability to neutralize $^1O_2$ in chloroplast membranes (Dobrikova and Apostolova, 2015), and its presence in the nucleus suggests a role in preventing DNA oxidation (Agati et al., 2012). However, after being synthesized at the periphery of the endoplasmic reticulum, most flavonoids are stored in the vacuoles. Under extreme stress conditions, APX activity is inhibited, so that up to 75% of the H_2O_2 produced in the chloroplasts can migrate into the cytoplasm (Mubarakshina et al., 2010), and H_2O_2 can then be transferred into vacuoles transported by TIP aquaporins, where it is neutralized by flavonoids, such as anthocyanins (Agati et al., 2012). The increase in the content of anthocyanins has been tied to the redox changes of the cell caused by ROS such as H_2O_2 that can participate as either an oxidant or a reductant in many reactions (Perl-Treves and Perl, 2002). There are more than 100 members of the MYB transcription factors reported in plants (Wu et al., 2016). Some of this MYB transcription factors participate in the regulation of flavonoid biosynthesis, affecting the capacity to inhibit or increase the production of flavonoids according to their conformation, which is regulated by changes in their structure according to the redox state of the molecules (Figure 5). Therefore, H_2O_2 can activate these transcription factors and induce the synthesis of flavonoids (Heine et al., 2004; Dubos et al., 2008; Queval and Foyer, 2012). Then, H_2O_2 act as a mobile secondary messenger of the chloroplast. In fact, has been mentioned that H_2O_2 produced in chloroplast induces different signals compared to the H_2O_2 produced in other organelles (Chan et al., 2016). Guo and Wang (2010) observed, over time, consecutive increases and decreases in anthocyanin accumulation and expression of the *SlPAL5* gene (phenylalanine ammonia-lyase) in different tissues of tomato plants exposed to UV-A radiation. Therefore, during the exposure of plants to cold stress and/or UV-B radiation, these metabolites may have the ability to regulate their biosynthesis and other metabolic pathways through the neutralization of ROS (Foyer and Shigeoka, 2011).

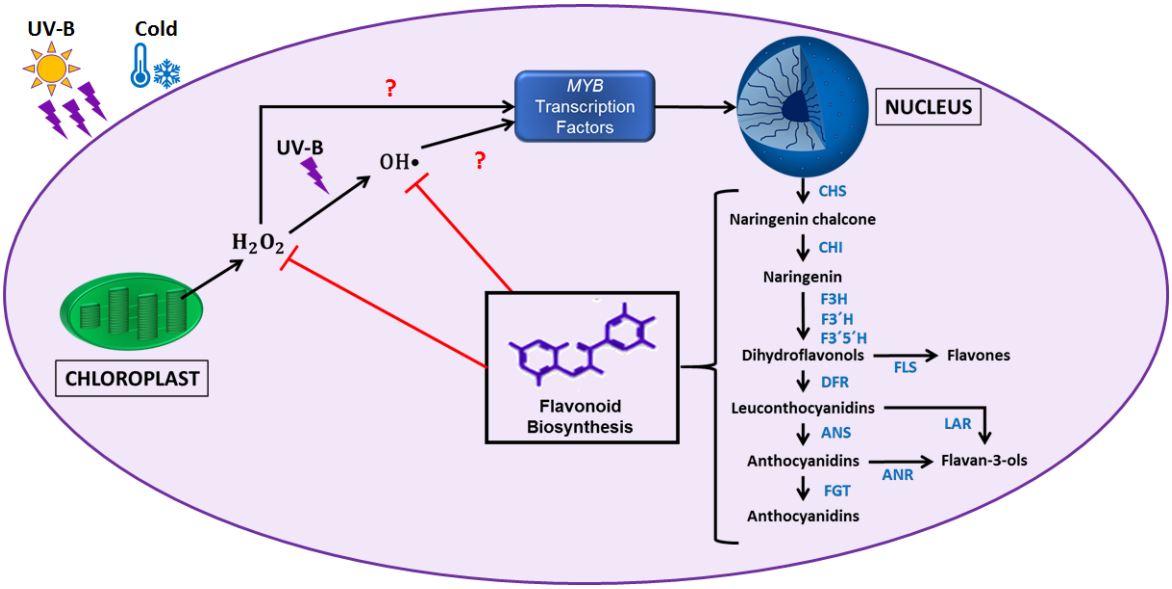

Figure 5. Schematic model of the flavonoid biosynthesis regulation throw the ROS produced during the stress induced by cold and/or UV-B radiation.

The excess of hydroxide peroxide (H_2O_2) produced during the stress is transformed to hydroxyl radical (OH•) by UV-B radiation, Fenton or Harber-Weiss reactions; this ROS can produce redox reactions in transcription factors MYB that active the gene transcription to produce enzymes of the flavonoid biosynthesis. Flavonoids have the capacity to neutralize the ROS produced during the stress and, therefore, regulate their own biosynthesis and other metabolic process affected by this ROS. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3´H; flavonoid 3´-hydroxylase; F3´5´H, flavonoid 3´,5´-hydroxylase; FLS, flavonol synthase; DFR, dihidroflavonoid-4-reductase; LAR, leucocyanidin-4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; FGT, flavonoid glycosyltransferase.

Conclusion

The damages caused by cold and UV-B radiation are the result of the overproduction of ROS in the chloroplasts due to induced damage in the electron transport chain. The functionality of the photosystems is the first component attacked by ROS, especially the protein synthesis system, which is fundamental for the recovery of damaged parts of photosystems, such as protein D1 of PSII. However, the damages in PSI are more critical for the functional recovery of the photosynthetic apparatus. Increase in the production of certain antioxidant and flavonoid enzymes is induced by the increase in ROS, such as H_2O_2, due to the inability of the primary defense system to combat the increase caused by exposure to cold and UV-B radiation.

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CAPÍTULO 3. LOW TEMPERATURE AND ULTRAVIOLET-B RADIATION AFFECT CHLOROPHYLL CONTENT AND INDUCE THE ACCUMULATION OF UV-B-ABSORBING AND ANTIOXIDANT COMPOUNDS IN BELL PEPPER (*Capsicum annuum*) PLANTS

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Highlights

- UV-B induces higher flavonoid synthesis than low temperature (LT) conditions
- LT+UV-B induce higher accumulation of phenolic compounds than each stress alone
- Specific flavonoid concentration is determined by the effect of LT and UV-B stress
- UV-B mainly induces synthesis of flavonoids with greater capacity to quench ROS
- UV-B radiation is more dangerous to plants exposed to LT than to control conditions

Abstract

Low temperature (LT) and ultraviolet-B (UV-B) radiation are abiotic factors that cause plant stress and limit crop production because of their detrimental effects on photosynthetic components. There is evidence of chlorophyll and carotenoid degradation by LT and UV-B radiation, as well as the production of phenolic compounds as defense components against these stress factors; however, there is scarce information about the interactions between LT and UV-B radiation. Therefore, in this study, the contents of chlorophyll, carotenoids, and phenolic compounds were analyzed in response to LT, UV-B radiation and the combination of both (LT+UV-B) in leaves of bell pepper plants. The LT+UV-B condition produced the greater degradation of chlorophyll in the bell pepper leaves and the higher accumulation of carotenoids, chlorogenic acid and the flavonoids apigenin-7-*O*-glucoside (A-7-G) and luteolin-7-*O*-glucoside (L-7-G) compared to the LT and UV-B conditions applied separately. UV-B radiation induced a higher total flavonoid concentration than LT, but the highest flavonoid concentration was observed in the leaves exposed to LT+UV-B. The higher accumulation of chlorogenic acid and A-7-G biosynthesis in leaves exposed to LT than control, indicate a higher resistance of plants to UV-B radiation damage because chlorogenic acid and A-7-G both have high UV-B absorbance capacities. However, a higher concentration of L-7-G, respect to A-7-G, in leaves exposed to UV-B and LT+UV-B indicates a higher necessity to quench ROS, because L-7-G has a higher antioxidant capacity than A-7-G. The increment of carotenoid and L-7-G concentrations in bell pepper plants exposed to LT and UV-B radiation stress displayed a high correlation with the raise of total reducing capacity in their leaves.

Keywords: UV-B radiation, Low temperature, Abiotic stress, Flavonoids, ROS, Antioxidant capacity

Abbreviations: UV-B, ultraviolet-B; LT, low temperature; ROS, reactive oxygen species; PSI, photosystem I; PSII, photosystem II; LHCs, light harvesting complexes; DAS, days after sowing; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; A-7-G, apigenin-7-*O*-glucoside; L-7-G, luteolin-7-*O*-glucoside; TRC, total reducing capacity; PAR, photosynthetically active radiation; CAT, catalase; APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; GR, glutathione reductase; $^1O_2$, singlet oxygen; H_2O_2, hydrogen peroxide

Introduction

Plants are commonly exposed to a great number of factors that can negatively affect their growth and development. Abiotic stress, which includes factors such as salinity, drought, flooding, nutrient imbalances, the wind, extreme temperature and radiation, is the principal cause of reductions in plant growth and worldwide food production, decreasing the average yield of crops by 65 to 87 % (Koyro et al., 2012; Shinozaki et al., 2015). Tropical and subtropical plants suffer damage from temperatures moderately above the water freezing point (0 – 15 °C), defined as chilling or low temperature (LT) stress (Knight and Knight, 2012); in fact, LT is the main reason for limitations in geographic distribution of crops and their yield (Wang et al., 2014b; Król et al., 2015). At the cellular level, the highest damage occurs in the chloroplast because, after the LT exposure, galactolipase is activated, resulting in fatty acid liberation. These free fatty acids are more susceptible to lipoperoxidation by lipoxygenases and reactive oxygen species (ROS), which are highly produced under daylight (Kaniuga 2008). On the other hand, the levels of ultraviolet-B (UV-B) radiation at the tropical and subtropical latitudes are higher than those in the temperate zones because the ozone layer is less thick (Jaakola and Hohtola, 2010). Despite the establishment of the Montreal Protocol, which mandated reductions of the substances that deteriorate the ozone layer, the process of

ozone depletion has not been completely halted (IPCC, 2014); in fact, the ozone layer rate of decline increases continuously at 0.6 % per year. In general, an approximately 1 % ozone layer depletion produces an increase of 1.3 to 1.8 % in the UV-B radiation (Hollósy, 2002; Prado et al., 2012).

LT and UV-B radiation can inhibit photosynthesis through an increase in the production of ROS, which are well known to damage DNA, proteins, and in particular the photosystems (PSI and PSII) and the light harvesting complexes (LHCs) (Allakhverdiev and Murata, 2004; Smith et al., 2009; Nishiyama et al., 2011). The biosynthesis of secondary metabolites, such as phenolic acids and flavonoids, has also been referenced as a defense mechanism of plants against the stress produced by LT and UV-B radiation (Theocharis et al., 2012; Zlatev et al., 2012). In fact, the biosynthesis of secondary metabolites has been reported as the second most representative pathway in the transcriptome analysis of plants exposed to LT, and the total flavonoid content increases in the leaves of some plants such as tomato and *Ginkgo biloba* (Løvdal et al. 2010; Wang et al., 2014a). Flavonoids are predominantly found in the epidermis of plant tissues, so they are mainly related to their capacity to absorb UV-B radiation, and they have also the capacity to quench the ROS produced during stress (Castañeda-Ovando et al., 2009). Each flavonoid has a distinct UV-B absorbance and antioxidant capacity (Peng and Zhou, 2009). Interestingly, significant interactions between the temperature and UV-B radiation have been observed in some growth variables in pea plants, suggesting that LT and UV-B radiation can exhibit synergism in plant stress (Martel and Qaderi, 2016). Although LT and UV-B radiation have both been reported to increase the flavonoid concentration for ROS scavenging and the photoprotection of photosynthesis compounds, there is scarce information about the coordinated effect of LT and UV-B radiation in plants, especially regarding the production and structural changes of defense compounds. Recently, *Capsicum annuum*, a member of the family Solanaceae, has been proposed as a model to understand changes in the secondary metabolism (Dhar et al., 2015). Furthermore, Kaniuga (2008) reported that peppers are very susceptible to LT stress because they present higher lipid degradation than other plants, such as tomato, potato, melon, maize, and cucumber, when they are exposed to LT. We hypothesized that plants, such as *Capsicum annuum*, exposed to LT are more vulnerable to the UV-B

radiation effects than those exposed at optimum growth temperatures. Consequently, plants exposed simultaneously to LT and UV-B radiation show a higher concentration of defense compounds than those exposed only to one of these factors. Therefore, the objective of this study was to determine the combined effect of LT and UV-B radiation on some aspects of the physiology and biochemistry such as photosynthetic pigments, phenolic acids, and flavonoids in bell pepper plants.

Material and Methods

Chemicals and Reagents

Acetone and formic acid were reagent-grade; methanol, water, and acetonitrile were HPLC-grade from J.T. Baker (Edo. De México, México); Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), potassium acetate (KCH_3COO), monobasic (KH_2PO_4) and dibasic (K_2HPO_4) potassium phosphate, fluorescein, AAPH (2,2´-azobis (2-methylpropionamidine) dihydrochloride), Trolox, ABTS (2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate ($K_2S_2O_8$), chlorogenic acid, quercetin, apigenin, apigenin-7-*O*-glucoside (A-7-G), luteolin and luteolin-7-*O*-glucoside (L-7-G) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Plant material and Growth Conditions

Bell pepper seeds Cannon cv (Zeraim Gedera; Israel) were put in a 5 mm depth of germination mix (Lambert Peat Moss, Inc.; Rivière-Ouelle, QC, Canada) and covered with exfoliated vermiculite (Termolita; Santa Catarina, NL, México) in pots of 40 cm^3. The seeds were irrigated periodically with 40 °C water until germination. Bell pepper plants growth in a greenhouse at 26 ºC; and fifteen days after sowing (DAS), they were nourished with 3 $mL \cdot L^{-1}$ water solutions of Ultra NPK, Ultra P, Ultra Ca and HBK (Nubiotek, Bioteksa®; Cd. Jimenez, CHI, México) (Appendix A, Table A1). Twenty-eight DAS bell pepper plants were put in a plant growth chamber (GC-300TLH, JEIO TECH; South Korea) at control conditions, which consisted of a 12 h photoperiod (from 6:00 to 18:00 h) of PAR radiation (972 $\mu mol \cdot m^{-2} \cdot s^{-1}$), temperature of 25/20 °C (day/night) and relative humidity of 65 %. The treatments were applied for seven days (from 31 to 37 DAS) and consisted of the control (described above), low temperature (LT), UV-B radiation (UV-B) and low temperature with UV-B radiation (LT+UV-B). For the LT treatment, the growth chamber was set at 15/10 °C, whereas for the UV-B radiation treatment, the plants were irradiated for 6 h with PAR (from 06:00 to 10:00 and 16:00 to 18:00 h) and for 6 h of UV-B irradiation (72 $kJ \cdot m^2$, from 10:00 to 16:00 h). The UV-B radiation was applied using three Phillips TL 100W/01 lamps (Germany) with a narrow waveband between 305 to 315 nm and peaks at 311 nm; the distance between plant leaves and UV-B lamps was 50 cm, and the UV-B irradiance was measured by a UV A/B light meter (SPER SCIENTIFIC, model 850009; Scottsdale, AZ, USA). The bell pepper leaves were frozen in liquid nitrogen and stored at -80 °C in an ultra-low temperature freezer (Revco UxF40086A, Thermo Scientific; USA) until their analysis.

Extraction and Quantification of Chlorophyll *a*, Chlorophyll *b* and Carotenoids

The chlorophyll and carotenoids were extracted from 2 g of bell pepper leaves with 20 mL of cold acetone (-20 °C). The tissue was homogenized for 40 s with an Ultraturrax Ika T-20 (North Carolina, USA), and then incubated on ice in darkness for 20 min. Subsequently, the solution was filtered with Whatman No. 41 (Maidstone, KE, England) paper, and the final volume was measured. The absorbances at 661.6, 644.8 and 440 nm, which are the wavebands at the maximum absorbance in acetone for chlorophyll *a*, chlorophyll *b* and carotenoids (xanthophylls and carotenes), respectively, were measured with a UV-Vis spectrophotometer (Agilent Cary 60, model G6860A; California, CA, USA). The concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids were determined using the equations proposed by Lichtenthaler and Buschmann (2001),

$$C_a(\frac{\mu g}{mL}) = 11.24\ A_{661.6} - 2.04\ A_{644.8} \quad (1)$$

$$C_b\ (\frac{\mu g}{mL}) = 20.13\ A_{644.8} - 4.19\ A_{661.6} \quad (2)$$

$$C_{(X+C)}\left(\frac{\mu g}{mL}\right) = \left[\frac{1000A_{470} - 1.90\ C_a - 63.14\ C_b}{214}\right] \quad (3)$$

where C_a, C_b and $C_{(X+C)}$ are the chlorophyll *a*, chlorophyll *b* and carotenoid concentrations; and, $A_{661.6}$, $A_{644.8}$ and A_{470} are the maximum absorbances of the sample at 661.6, 644.8 and 470 nm, respectively.

Phenolic Compound Extraction

The phenolic compounds were extracted from 2 g of sample with 10 mL of cold (-20 °C) methanol:water (80:20, v/v) and homogenized for 20 s with an Ultraturrax Ika T-20

(North Carolina, USA). The samples were agitated for two hours with a rotator (HAG, FINEPCR®; Gyeonggi-do, Korea) and centrifuged for 15 min at 4 °C and 10 000 x *g* (Sorvall Legend XTR centrifuge, Thermo Fisher Scientific; Massachusetts, USA), and the supernatant was collected and stored at -20 °C.

Quantification of Total Flavonoid Content

The total flavonoid content was determined according to the modified colorimetric aluminum chloride method described by Ghasemi et al. (2009). Briefly, 15 µL of phenolic extract was mixed with 112 µL distilled water, 60 µL methanol:water (80:20), 4 µL of 10 % aluminum chloride, and 4 µL of 1 M potassium acetate and incubated at room temperature for 30 min in darkness. The absorbance was measured at 415 nm using a microplate reader (Synergy™ HT, BioTek®; Vermont, USA), and the results were expressed as mg quercetin/100 g of fresh weight (FW).

Determination and Quantification of Phenolic Acids and Flavonoids by UPLC

The individual phenolic acids and flavonoids were determined using a Waters Acquity ultra-performance liquid chromatography (UPLC) system equipped with a quaternary pump, photodiode array detector (PDA), autosampler, column heater and BEH C18 column 1.7 µm 2.1 x 100 mm (all from Acquity™, Waters; Massachusetts, USA). Phenolic extracts were passed through disposable Millex® (Merck Millipore; Darmstadt; HE, Germany) syringe filters of 0.45 µm, and the injections consisted of 0.5

µL and at a flow rate of 0.3 mL·min^{-1}. The autosampler was set at 8 °C, and the column was set at 50 and 30 ºC for the phenolic acid and flavonoid analyses, respectively. The elution profile for the phenolic acid determination used two solvent solutions, water:methanol:formic acid (95:2:3, v/v/v) (A) and methanol:water:formic acid (95:2:3) (B): 0-3 min, 10-25 % B in A; 3-5 min, 25-30 % B in A; 5-9 min, 30-40 % B; 9-11 min, 40-50 % B in A; 11-12 min, 50-100 % B in A; 12-13 min, 100 % B in A; 13-15 min, 100-10 % B in A and; 15-16 min, 10 % B in A (Li et al., 2011). The elution profile for the flavonoid analysis used two solvents, 0.05 % aqueous formic acid (A) and acetonitrile (B): 0-5 min, 7 % B in A; 5-20 min, 7-40 % B in A; 20-20.1 min, 40-100 % B in A; 20.1-22 min, 100 % B in A; 22-22.1 min, 100-7 % B in A and; 22.1-24 min, 7 % B in A (Suárez et al., 2008). All solvent solutions were passed three times through a membrane filter of 0.2 µm (Whatman™; Maidstone, KE, England) prior to analysis. The UV spectra were recorded between 210 and 400 nm to obtain the absorbance spectrum, and the phenolic acids and flavonoids were expressed as mg/100 g FW.

Antioxidant Activity of Flavonoids

The antioxidant activities of the standards of apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside, at different concentrations, were determined with the ORAC (oxygen radical antioxidant capacity) and TEAC (Trolox equivalent antioxidant capacity) assays according to Thaipong et al. (2006) with some modifications. Both assays were carried out using a microplate reader (Synergy™ HT, BioTek®; Vermont, USA).

The ORAC assay was conducted in 75 mM phosphate buffer at pH 7.4 and 37 °C. The procedure consisted of dispensing 25 µL of flavonoid solution, 200 µL of 0.96 µM fluorescein and 75 µL of 95.8 µM AAPH (peroxyl radical generator) in a 96-well plate; when the last reagent was added, the reaction started, and the fluorescence was measured

every 70 s for 70 min, with fluorescence excitation at 485 nm and emission at 580 nm. The Trolox standard curve was linear between 25 and 250 µM.

For the TEAC assay, the stock solutions consisted of 7.4 mM ABTS and 2.6 mM potassium persulfate. The ABTS radical ($ABTS^{\cdot+}$) was prepared by mixing the two stock solutions in equal quantities and incubating for 16 h in darkness at room temperature; then, the reaction solution was obtained by mixing 100 µL of $ABTS^{\cdot+}$ with 2900 µL of methanol:water (80:20 v/v). Finally, 150 µL of the flavonoid solution was mixed with 2850 µL of the reaction solution and incubated at room temperature in darkness for 2 h. The absorbance was taken at 734 nm, and the Trolox standard curve was linear between 0 and 1 µM.

For the TEAC and ORAC antioxidant methods, the results were expressed as µM and mM Trolox equivalent (TE) per mg, respectively.

Determination of Total Reducing Capacity

A mix of 15 µL of phenolic extract with 240 µL of distilled water and 15 µL of Folin-Ciocalteu reagent was incubated at 25 ºC for 3 min, and then 30 µL of 4 N sodium carbonate (Na_2CO_3) was added and incubated in darkness at 25 ºC for 2 h. Finally, the absorbance was measured at 725 nm (Swain and Hillis, 1959) using a microplate reader (Synergy™ HT, BioTek®; Vermont, USA), and the results were expressed as mg of chlorogenic acid/100 g FW.

Data Analysis

The quantitative data are shown as the mean values of three experimental replicates, each one measured three times. The analysis of variance was used to test every assay, and Fisher's test was used to determine significant differences at 90 % ($P \leq 0.10$) significance. Pearson´s correlation coefficients were calculated among the data obtained. All statistical analyses were performed with the software Minitab® 17 (Minitab Inc.; Pennsylvania, USA).

Results

Chlorophyll and Carotenoid Concentrations are Inversely Affected in Bell Pepper Leaves Exposed to Low Temperature and UV-B Radiation

The bell pepper leaves showed a higher concentration of chlorophyll *b* than chlorophyll *a*, and the ratio of chlorophyll *a*/*b* was the same in all treatments (Table 2). There was not a significant reduction in the total chlorophyll concentration following exposure to low temperature (LT) (19.92 %) and UV-B radiation (UV-B) (15.90 %), compared to the value in plants grown under control conditions. However, when bell pepper plants were exposed simultaneously to both types of stress (LT+UV-B), the reduction in the total chlorophyll was higher (39.49 %) and significant (Table 2). Thus, the LT+UV-B conditions cause more damage to the bell pepper plants than LT or UV-B radiation alone.

Carotenoids have been associated with protection against chlorophyll oxidation by ROS in the photosystems and light harvesting complexes. The carotenoid concentrations increased when the bell pepper plants were exposed to LT or UV-B, although the difference between the treatments was not significant (Table 2). However, when the bell pepper plants were stressed with LT+UV-B, they had the highest carotenoid concentration in their leaves.

Table 2. Chlorophyll *a*, chlorophyll *b*, and carotenoid concentrations, expressed as mg/100 g FW, in bell pepper leaves under low temperature and UV-B radiation stress.

Treatment	Chl *a*	Chl *b*	Chl *a* + *b*	Chl *a* / *b*	Carotenoids
Control	107.80[a]*	177.16[a]	284.95[a]	0.61[a]	0.02[c]
LT	86.42[ab]	141.78[ab]	228.21[ab]	0.61[a]	1.43[b]
UV-B	91.95[a]	148.20[a]	239.65[a]	0.62[a]	2.18[b]
LT+UV-B	65.10[b]	107.32[b]	172.42[b]	0.61[a]	3.82[a]

*Different letters denote significant difference between treatments in each column ($P \leq 0.10$)

The Combined Effect of Low Temperature and UV-B Radiation Induces a Higher Accumulation of Phenylpropanoid-Derived Compounds in Bell Pepper Leaves in Comparison to Those Under Each Separate Condition

The phenylpropanoids, like phenolic acids and flavonoids, are plant defense compounds that have the capacity to absorb UV-B radiation and exhibit antioxidant properties. The UPLC analysis showed no chlorogenic acid (a phenolic acid) content in the plants of the control treatment but a slight production of this compound when they were affected by LT or UV-B radiation. Interestingly, when the plants were exposed to LT+UV-B, they showed the most significant increase in the concentration of chlorogenic acid (Figure 6).

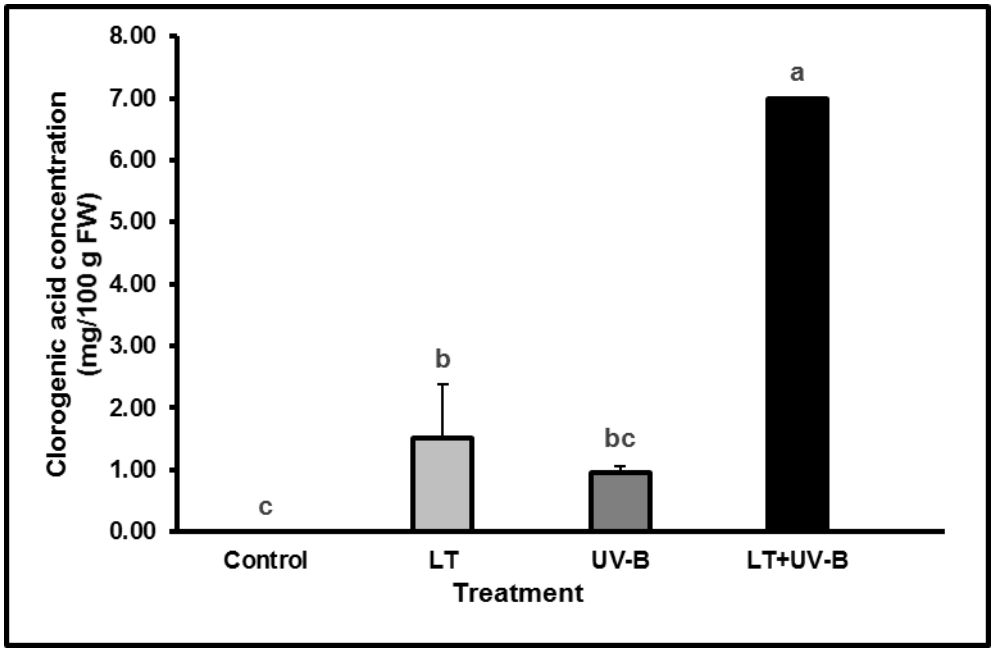

Figure 6. Chlorogenic acid concentration in bell pepper leaves stressed by low temperature and UV-B radiation.
*Different letters denote significant difference between treatments (P≤0.10)

The contents of the individual flavonoid compounds accumulated in the plants were determined in this study by UPLC. These analyses showed four peaks from the bell pepper leaves extracts (Figure 7A). Two of these peaks were identified by comparison to known standard compounds; the first peak corresponded to luteolin-7-*O*-glucoside (L-7-G), a flavonoid that is dihydroxylated in its B ring, and the third peak to apigenin-7-*O*-glucoside (A-7-G), a monohydroxylated flavonoid. Unfortunately, the second and fourth peaks were not identified, but it is possible that these unidentified compounds are derivatives of L-7-G and A-7-G, respectively, since their UV spectra are identical to those of these compounds (Figure 7B). The variation in the retention time could be due to differences in the glycosylation of these molecules.

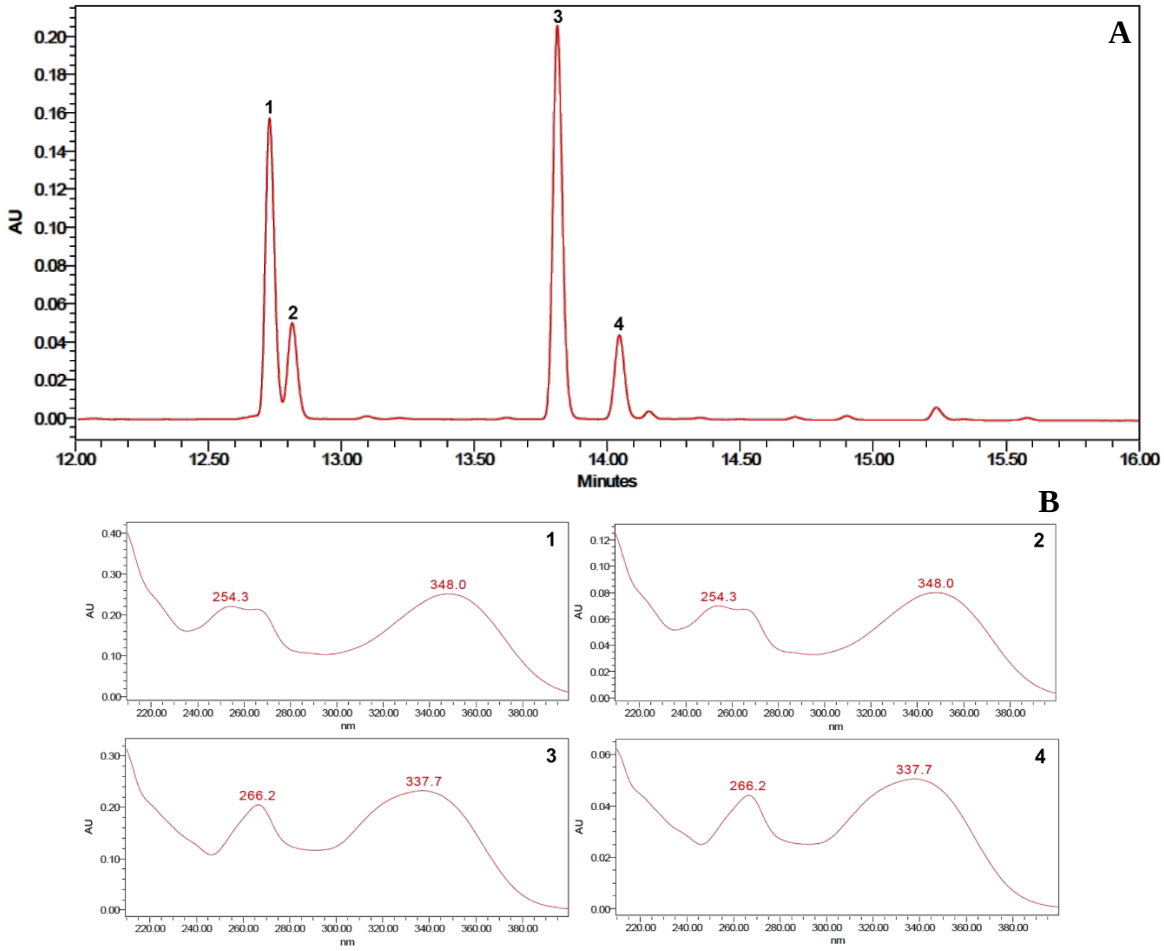

Figure 7. Chromatogram of flavonoid extract showing four defined peaks (A) and UV absorption spectra of the compounds corresponding to each individual peak (B) obtained from bell pepper leaves under low temperature and UV-B radiation stress.
1. Luteolin-7-*O*-glucoside, 2. No identified, 3. Apigenin-7-*O*-glucoside, 4. No identified.

The flavonoids detected in the bell pepper leaves increased their concentration when the plants were exposed to UV-B radiation or LT+UV-B. Meanwhile, only A-7-G concentration increased in plants exposed to LT. Nevertheless, the ratio between L-7-G and A-7-G was different according to the treatment. In the control treatment, the L-7-G:A-7-G ratio was 0.44 (Figure 8). When the bell pepper plants were exposed to low temperature, the A-7-G and L-7-G increased in concentration to approximately twice the control values, and the L-7-G:A-7-G ratio not changed significantly (0.59). On the other hand, in the plants exposed to UV-B radiation, A-7-G showed a similar concentration to

that following the LT treatment, whereas L-7-G showed an almost two-fold higher concentration than that following exposure to LT and five-fold higher in comparison to the control treatment. Furthermore, when the bell pepper plants were exposed to both low temperature and UV-B radiation, they showed the highest concentrations of L-7-G and A-7-G (Figure 8). These results modified significantly the L-7-G:A-7-G ratio of UV-B and LT+UV-B treatments to 1.30 and 1.15 respectively.

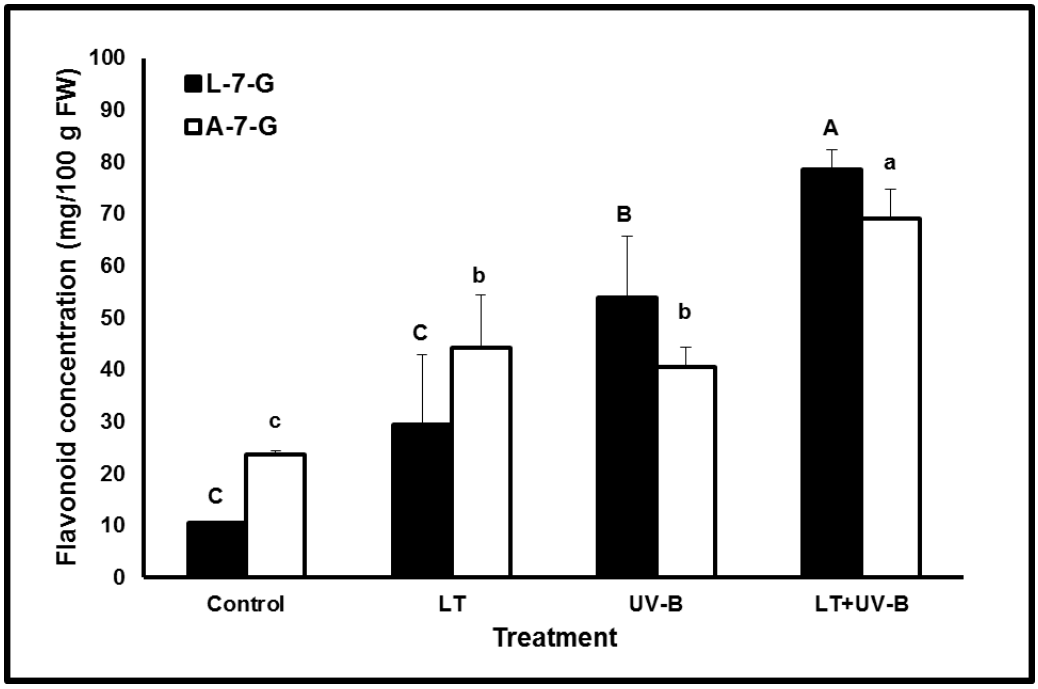

Figure 8. Concentrations of luteolin-7-*O*-glucoside (L-7-G) and apigenin-7-*O*-glucoside (A-7-G) in bell pepper leaves exposed to low temperature and UV-B radiation.
*Different uppercase letters denote significant difference between treatments in L-7-G and lowercase letters in A-7-G ($P \leq 0.10$)

Antioxidant Capacity of A-7-G and L-7-G in Bell Pepper Leaves Exposed to Low Temperature and UV-B Radiation

The antioxidant capacity of L-7-G and A-7-G was measured by two different methods: Trolox equivalent antioxidant capacity (TEAC), which is based on electron transfer, and oxygen radical antioxidant capacity (ORAC), which is based on hydrogen atom transfer. The antioxidant capacity of L-7-G was higher than that of A-7-G as measured by both methods. The antioxidant capacity values obtained for L-7-G were 7.90±0.74 μM TE/mg and 143.89±12.58 mM TE/mg for TEAC and ORAC, respectively, whilst for A-7-G, the values were 1.61±0.59 μM TE/mg and 119.79±9.44 mM TE/mg, respectively. Therefore, L-7-G has a higher antioxidant capacity than A-7-G, as measured by both electron transfer and hydrogen atom transfer.

Using the calculated antioxidant activities of L-7-G and A-7-G, we determined the antioxidant activity provided by these two flavonoids in bell pepper leaves exposed to low temperature and UV-B radiation (Figure 9). The results show that L-7-G contributes importantly to the antioxidant capacity in bell pepper leaves exposed to UV-B radiation, particularly by electron transfer, whilst the changes in the concentration of A-7-G do not represent an increase in this antioxidant capacity (Figure 9A). On the other hand, only the bell pepper leaves exposed to UV-B radiation showed significant differences between A-7-G and L-7-G with respect to the hydrogen atom transfer (Figure 9B).

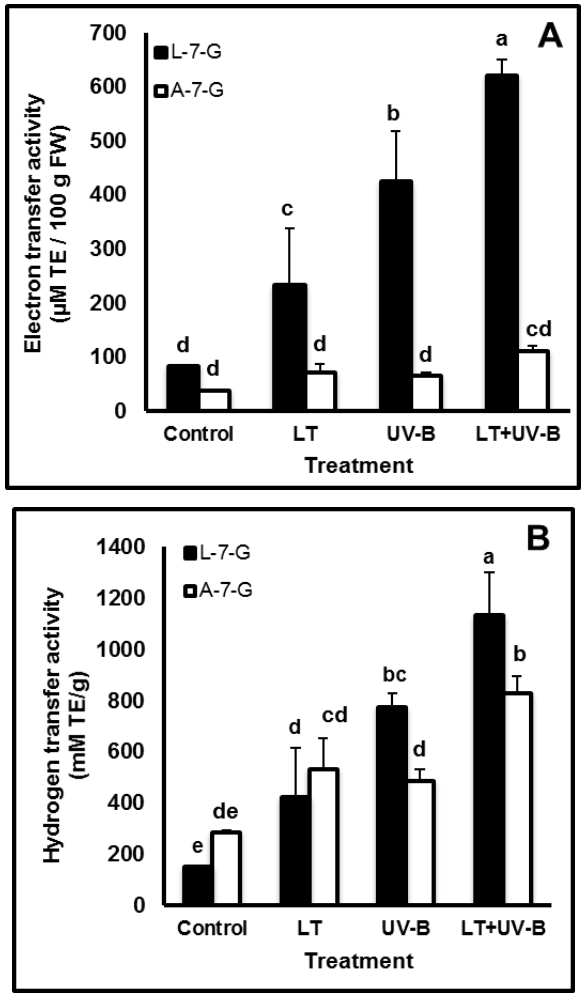

Figure 9. Antioxidant activity of luteolin-7-O-glucoside and apigenin-7-O-glucoside in bell pepper leaves stressed by low temperature and UV-B radiation determined by (A) TEAC (electron transfer) and (B) ORAC (hydrogen transfer) assays.
*Different letters denote significant difference ($P \leq 0.10$)

The Total Soluble Flavonoid Concentration and the Total Reducing Capacity are Predominantly Induced by UV-B Radiation Rather Than by the Low Temperature in the Bell Pepper Leaves

Since it was not possible to identify all the individual flavonoid compounds in the bell pepper leaves, the total soluble flavonoid content was measured by spectrophotometry. The total flavonoid content was not affected when the bell pepper leaves were exposed to LT compared to the control condition (Figure 10); however, the UV-B radiation significantly induced the flavonoid production by more than two-fold with respect to the control treatment. Furthermore, when the plants were exposed to LT+UV-B, the total flavonoid concentration increased to more than three times compared to the control treatment.

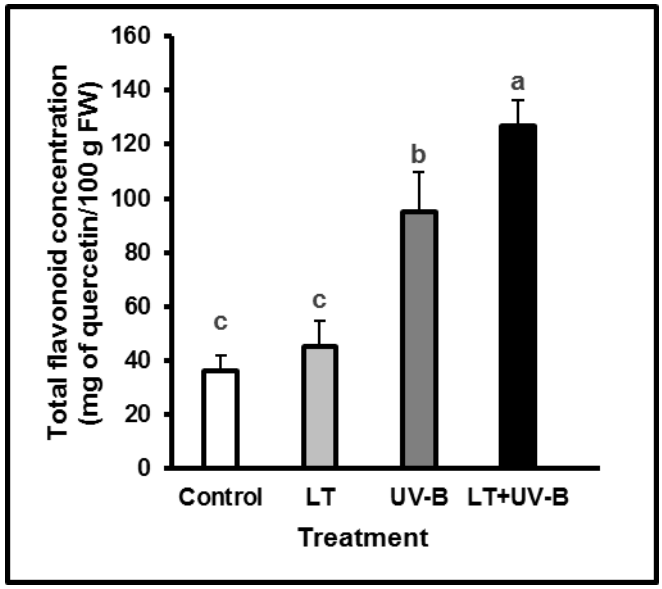

Figure 10. Total flavonoid content in bell pepper leaves exposed to low temperature and UV-B radiation expressed as mg of quercetin per 100 g of FW.
*Different letters denote significant difference between treatments ($P \leq 0.10$)

On the other hand, the bell pepper leaves increased their total reducing capacity (TRC) when they were exposed to LT or UV-B radiation (Figure 11), but there was not significant difference between both treatments. However, plants exposed to LT+UV-B radiation show higher TRC than control and LT.

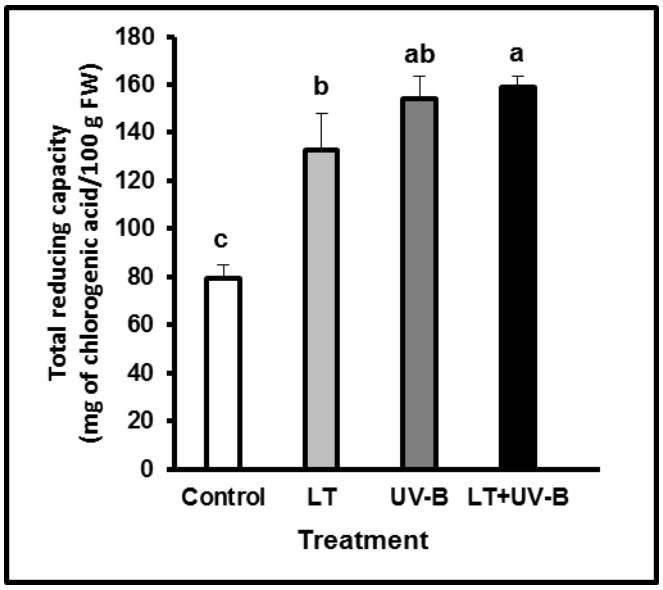

Figure 11. Total reducing capacity of bell pepper leaves exposed to low temperature and UV-B radiation expressed as mg of chlorogenic acid per 100 g of FW.
*Different letters denote significant difference between treatments (P≤0.10)

Correlations Among Chlorophyll, UV-B Absorbing and Antioxidant Compounds During the Exposure of Bell Pepper Plants to Low Temperature and UV-B Radiation

The total chlorophyll concentration had a significant negative correlation with carotenoids ($P<0.01$), chlorogenic acid ($P<0.01$), A-7-G ($P<0.05$) and TRC ($P<0.05$) ranging from -0.601 to -0.764 (Table 3). The correlations obtained among the defense compounds (carotenoids, chlorogenic acid, and flavonoids) were positive, ranging from

0.733 to 0.802 (P<0.01). Finally, the TRC was positively correlated with the carotenoids (P<0.01), total soluble flavonoids (P<0.05), and L-7-G (P<0.05) ranging from 0.649 to 0.733.

Table 3. Pearson´s correlation coefficients of chlorophyll, carotenoids, total flavonoids, chlorogenic acid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, and total reducing capacity of bell pepper leaves exposed to low temperature and UV-B radiation.

Variable[a]	Chl	Car	CLA	A-7-G	L-7-G	Fla
Car	-0.764**					
CLA	-0.714**	0.771**				
A-7-G	-0.615*	0.741**	0.911**			
L-7-G	-0.498^{ns}	0.744**	0.823**	0.898**		
Fla	-0.478^{ns}	0.802**	0.782**	0.824**	0.949**	
TRC	-0.601*	0.733**	0.530^{ns}	0.565^{ns}	0.655*	0.649*

[a]Car = carotenoids, Fla = total flavonoid, CLA = chlorogenic acid, A-7-G = apigenin-7-*O*-glucoside, L-7-G = luteolin-7-*O*-glucoside and TRC = total reducing capacity. ^{ns} = not significant difference, * and ** significant difference at P≤0.05 and P≤0.01, respectively.

Discussion

In the present work, we explored the effect of low temperature and UV-B radiation stresses, both as independent treatments and in combination. Our results showed evidence of a strong relation between the chlorophyll degradation and the consequent production of defense compounds when the bell pepper plants were exposed to these stress conditions.

Chlorophyll Degradation by Low Temperature and UV-B Radiation in Bell Pepper Leaves Presents Simultaneously with Carotenoid Biosynthesis

The photosynthesis and consequently plant yield is directly related to the chlorophyll concentration (Smith et al., 2000). In this work, bell pepper leaves had a higher concentration of chlorophyll *b* than chlorophyll *a*. Chlorophyll *a* predominates in photosystems, while the chlorophyll *b* content is restricted to the light harvesting complexes (LHCs) (Esteban et al. 2015). Therefore, the chlorophyll of the LHCs is predominantly higher with respect to the chlorophyll of the photosystems in the development stage of the bell pepper plants used in this study. Interestingly, the reductions of the chlorophyll induced by LT and UV-B radiation were similar for both chlorophyll *a* and *b*; therefore, the chlorophyll damage occurred equally in the photosystems and the LHCs.

Carotenoids have been linked to the photoprotection of chlorophylls and the neutralization of singlet oxygen ($^1O_2$) produced in the photosystems (Domonkos et al., 2013). In this study, the carotenoid accumulation increased in the bell pepper leaves exposed to LT and UV-B radiation and even more in plants under the LT+UV-B condition. According to Zhang et al. (2014), when the ambient temperature is reduced, the light becomes excessive due to the reduction of the photosystem activity, so there is excessive production of excited chlorophyll (triplet chlorophylls), which generate ROS (Nishiyama and Murata, 2014; Pospíšil and Prasad 2014). On the other hand, UV-B radiation damages proteins of photosystem II (PSII) like D1, which prevents the introduction of oxygen produced during water oxidation to the center of the PSII, where it would be transformed to $^1O_2$ through the energy of the triplet chlorophylls (Nishiyama et al., 2011). Then, it is possible that the increase in the carotenoid concentration by LT+UV-B radiation can neutralize the ROS, like $^1O_2$, that are being produced by the stress. Agati et al. (2013) noted that $^1O_2$ has the capacity to migrate from the thylakoids to the nucleus according to the distance between these organelles. In this work, the carotenoid concentration was negatively correlated with the concentration of

chlorophylls, and then it is possible that $^1O_2$ or some products of the chlorophyll oxidation could participate in the regulation of carotenoid biosynthesis. This finding supports the hypothesis that carotenoids are present in the LHCs and the photosystems with the function of neutralizing the triplet chlorophylls and $^1O_2$, respectively (Cazzaniga et al., 2012; Ramel et al., 2012; Dall´Osto et al., 2013).

The increases in carotenoids like lutein, violaxanthin, zeaxanthin, and antheraxanthin, in comparison to the content of chlorophylls in plants exposed to low temperature and the increase in β-carotene when plants are exposed to light stress, have been recently reported in a meta-analysis (Esteban et al., 2015). Moreover, there are reports of an increase in the carotenoid concentration when plants were exposed to UV-B radiation (Agati et al., 2007; Tapia et al., 2010), and results agree with the data presented in this work.

Low Temperature in Combination with UV-B Induces a Higher Accumulation of Phenolic Compounds in the Bell Pepper in Comparison to Each Separate Condition

The flavonoid content was determined because they are compounds strongly related to the protection of plants from UV-B radiation. However, most studies on these compounds focus only on the total flavonoid content, and there are few studies on the individual flavonoids accumulated in plants exposed to abiotic stress. Four flavonoids were detected in the extracts of bell pepper leaves in this investigation: L-7-G and A-7-G plus two additional unidentified compounds. The UV absorption spectra of the two unidentified compounds were almost identical to those of L-7-G and A-7-G, respectively, but there were not the aglycones of the corresponding flavonoids, according to the standards used in the analysis. Kim et al. (2014) identified the phenolics of three red pepper cultivars, and reported luteolin and apigenin-7-*O*-apiofuranosyl

(1→2) glucopyranoside, which showed peaks of maximum absorbance at 253 and 348 for a luteolin-derived compound and 268 and 340 for an apigenin-derived compound. This profile is very similar to those of the unidentified compounds in this work. Then, it is possible that these compounds are the same as those reported by Kim et al. (2014), and the minor differences in the maximum absorbance between this published information and our results presented in Figure 7 can be due to the different solvents used in the analyses (Harborne, 1958).

In the bell pepper leaves exposed to LT or UV-B radiation, the concentration of A-7-G increased equally in both treatments with respect to the control (Figure 8), although the increase was higher when the plants were exposed to both LT and UV-B radiation in combination. In contrast, there was a significant increase of L-7-G when the plants were exposed to UV-B radiation and LT+UV-B but not in the plants under only LT conditions. The difference between the chemical structures of these two flavonoids is that L-7-G has two hydroxyl groups in its B ring, while A-7-G has only one. Neugart et al. (2014) proposed that UV-B radiation induces the production of compounds with a catechol structure, like the B ring in L-7-G.

A-7-G has a higher capacity to absorb UV-B radiation than L-7-G, but flavonoids with two hydroxyl groups in the B ring, like L-7-G, have a higher antioxidant capacity than flavonoids with only one hydroxyl group (Castañeda-Ovando et al., 2009; Król et al., 2015). On the other hand, the catechol structure of flavonoids can form chelates with metal ions like those of copper, aluminum, and iron (Agati et al., 2012; Sigurdson and Giusti 2014); these metals participate with hydrogen peroxide (H_2O_2) in the Haber-Weiss mechanism or Fenton reactions to produce OH•, the most dangerous ROS (Gill and Tuteja, 2010). In this study, the L-7-G:A-7-G ratio was significantly higher in the bell pepper plants exposed to UV-B and LT+UV-B respect to those exposed to LT and control conditions. Guidi et al. (2016) also found an increase in L-7-G concentrations greater than that of A-7-G in *Ligustrum vulgare* and *Phillyrea latifolia* plants exposed to UV-B radiation, and Markham et al. (1998) found an increase in the luteolin:apigenin ratio in *Marchantia polymorpha*. This suggests that the UV-B irradiation of bell pepper plants preferentially induces the synthesis of flavonoids that have a greater capacity to

quench the ROS formed during the stress and have the ability to inhibit the generation OH•, rather than flavonoids with a higher capacity to absorb UV-B.

On the other hand, the concentration of A-7-G was higher than that of L-7-G in the bell pepper plants of the control and LT treatments, so under that ambient conditions, the chloroplasts produce less ROS than when they are exposed to UV-B radiation. For this reason, the flavonoids synthesized by plants predominantly have a higher capacity to absorb UV-B than to quench ROS to prevent their production. The increase in kaempferol and the decrease in the quercetin concentration in *Ginkgo biloba* leaves when the temperature is reduced support this hypothesis (Wang et al. 2014a).

In this work, chlorogenic acid was the only phenolic acid identified in the bell pepper leaves. This phenolic acid was present in the plants exposed to LT or UV-B radiation but not under the control treatments. Neugart et al. (2012) showed that kale (*Brassica oleracea*) plants present a slight increase in phenolic acids when they are exposed to low levels of UV-B radiation. Løvdal et al. (2010) showed an increase in chlorogenic acid in tomato (*Solanum lycopersicum*) leaves when the plants were exposed to a change in temperature from 30 to 12 °C, especially when they have a nitrogen deficiency, but they did not found a correlation between the temperature and PAR light. In this study, LT+UV-B induced the highest accumulation of chlorogenic acid, showing a synergy in the production of this compound between LT and UV-B radiation. Chlorogenic acid has a higher capacity to absorb UV-B than the flavonoids presented in this work. Agati et al. (2013) noted that the concentration of hydroxycinnamic acids like caffeic acid, a precursor of chlorogenic acid, decreases to produce flavonoids, but in this study, both chlorogenic acid and flavonoids increase their concentrations in bell pepper leaves stressed by LT and UV-B radiation. In fact, the highest concentrations of chlorogenic acid, A-7-G and L-7-G were found in the leaves exposed to LT+UV-B. This result can imply that plants exposed to LT are more susceptible to the direct damage of UV-B radiation, which could be the reason why the plants accumulated more compounds with the capacity to absorb this type of radiation and compounds with a higher antioxidant capacity to reduce the ROS produced in cells by the stress.

Several methodologies to measure the antioxidant capacity of different compounds have been published (Jacobo-Velázquez and Cisneros-Zevallos, 2009), some based on their capacity to donate electrons and others on the capacity to donate protons (Huang et al., 2005). In this work, TEAC and ORAC assays were used to determine the capacities of electron and proton donation, respectively (Zulueta et al., 2009) of A-7-G and L-7-G. When using either method, L-7-G showed a higher antioxidant capacity than A-7-G, but especially in electron donation. Additionally, since the L-7-G concentration was measured to be higher than that of A-7-G, the contribution of L-7-G to the electron donation capacity of the leaves was even higher. Since the capacities to donate protons of the two flavonoids are practically equal, it is suggested that the main antioxidant mechanism against LT and UV-B radiation by the flavonoids is electron donation. In previous experiments with *Capsicum annuum* plants exposed to LT, showed an increase in the activities of several antioxidant enzymes (CAT, MDAR, GR, and APX) that participate in the quenching of H_2O_2 during the first days of exposure, although their activities later decreased even below the values displayed at the start of the stress (Airaki et al., 2012; Ou et al., 2015). Mubarakshina et al. (2010) noted that under severe stress, some antioxidant enzymes are inhibited in the chloroplast, resulting in the increase of H_2O_2 concentration which migrate to the cytoplasm. Therefore, the higher electron donation capacity exhibited by L-7-G can be mainly used to quench H_2O_2 and moreover, as previously mentioned, avoid its transformation to OH• through the Fenton reaction.

UV-B Radiation Induces a Higher Flavonoid Accumulation and Reducing Capacity Than Low-Temperature Conditions in Bell Pepper Leaves

The total flavonoid accumulation increased significantly when the bell pepper plants were exposed to UV-B radiation. This is in agreement with earlier studies in different plants that reported not only an increase in the total flavonoids but also an increase in the expression of the genes of their biosynthetic pathway (Mahdavian et al., 2008; Liu et al.,

2012; Tossi et al., 2012; Choudhary and Agrawal, 2014). It has also been reported that LT induces transcriptomic modifications that lead to the increasing biosynthesis of flavonoids (Crifò et al., 2011; Li et al., 2012). In this work, the total flavonoid concentration did not change significantly when the bell pepper plants were exposed to LT. However, when they were exposed to LT+UV-B, the flavonoid concentration was highest. Therefore, LT interacts with the UV-B radiation to induce the accumulation of flavonoids, but the LT condition by itself has no direct effect on the accumulation of these compounds. These findings are in contrast with the report by Martel and Qaderi (2016), in which no interaction was found between the temperature and UV-B radiation conditions in pea (*Pisum sativum*) plants, however the temperature condition used in that experiment was different than the temperature regime used in this work. The interaction between the temperature and UV-B radiation could occur only at the temperatures in which each plant species is sensitive. It has been reported that during UV-B radiation or LT stress in *Capsicum annuum*, the lipid peroxidation increases due to a rise in ROS production (Mahdavian et al., 2008; Airaki et al., 2012). Additionally, Liu et al. (2012) correlated the production of flavonoids with membrane lipid oxidation and mentioned that chelation of metals like iron and copper by the catechol structures of flavonoids prevents lipid peroxidation.

Phenolic compounds like flavonoids are highly correlated with the antioxidant capacity of different plants during LT and UV-B radiation stress (Pennycooke et al., 2005; Peng and Zhou, 2009). In this experiment, the Folin-Ciocalteu reagent, which has been suggested to be used to measure the antioxidant capacity of plants (Everette et al., 2010; Chen et al., 2016), was used to determine the TRC. The UV-B radiation and LT treatments showed higher measurements of TRC than in the control bell pepper leaves. On the other hand, the LT+UV-B condition induced the highest TRC values. Pennycooke et al. (2005) found a positive correlation between the antioxidant capacity and LT tolerance in the petunia (*Petunia* x *hybrida*). In this work, TRC was correlated positively with carotenoids, L-7-G, and total flavonoids, but not with chlorogenic acid or A-7-G. This support the idea that carotenoids and flavonoids like L-7-G are mainly used by plants to quench ROS and chlorogenic acid and A-7-G to absorb the UV-B radiation.

Conclusions

The present study demonstrates the negative effect of LT and UV-B radiation on the chlorophyll concentrations in bell pepper plants, which were accompanied by increases in several defense compounds such as carotenoids and flavonoids. The stress produced by LT and UV-B radiation in combination induced higher accumulations of chlorogenic acid, A-7-G and L-7-G in the bell pepper leaves in comparison to those under each separate stress condition. Interestingly, A-7-G showed a higher accumulation by LT and L-7-G by UV-B radiation. No difference in the chlorogenic acid accumulation was observed between the LT and UV-B radiation conditions. The results obtained in this work support the idea that the roles of chlorogenic acid and A-7-G are predominantly centered in the absorbance of UV-B radiation to avoid the damage caused by it, while the roles of carotenoids and L-7-G is the quenching of the ROS that is also produced by this stress. On the other hand, our results support the idea that $^1O_2$ and H_2O_2 are the main ROS in stressed bell pepper plants and could be neutralized by carotenoids and L-7-G, respectively. This is based on the observation that L-7-G showed a higher electron donation capacity than proton donation capacity (Figure 9). However, additional experiments are required to determine the specific ROS produced during the LT and UV-B radiation stresses. Finally, the results indicate that the bell pepper plants exposed to LT are more susceptible to UV-B radiation than those under the control condition.

Acknowledgment

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Appendix A

Table A1. Nutrition applied to bell pepper plants during the experiment.

DAS[a]	Product applied	Composition
15	Ultra P	P (33.53 %), K (2.65 %)
18	Ultra NPK	N (10 %), P (10 %), K (10 %)
21	Ultra NPK	N (10 %), P (10 %), K (10 %)
24	HBK	N (10 %), P (5 %), K (10 %)
27	Ultra Ca	N (17 %), Ca (13 %), K (3.5 %)
30	Ultra S	K (30 %), S (30 %)
33	Ultra P	P (33.53 %), K (2.65 %)
36	Ultra NPK	N (10 %), P (10 %), K (10 %)

[a]Days after sowing

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CAPÍTULO 4. OPTIMIZATION AND VALIDATION OF AMPLIFICATION CONDITIONS WITH THE USE OF SURFACE RESPONSE METHODOLOGY FOR GENE EXPRESSION ASSAYS OF ANTHOCYANIN BIOSYNTHESIS BY RT-qPCR

Abstract

Background: Anthocyanins are secondary metabolites that participate in process to reduce the deleterious effects in plant produced by abiotic stress. Consequently, there is a great interest for study the expression of genes, which participate in the anthocyanin biosynthesis during the exposition of plant to abiotic stresses. RT-qPCR is considered as the gold standard for nucleic acids detection and quantification, however, the optimization and evaluation of the protocols used in every assay is required for MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments). In this study, a surface response design is proposed to make the optimization of RT-qPCR assays, therefore the optimized qPCR conditions of primers corresponding to anthocyanin biosynthesis and the reference genes to be used in gene expression analysis in *Capsicum annuum* are reported.

Results: The optimized amplification conditions for the qPCR assays with primers corresponding to anthocyanin biosynthesis genes (*MYB, F3H, F3´5´H, DFR, ANS*) and the reference genes (*β-TUB* and *UBI-3*) were observed between 56.4 and 63.6 ºC, 233 and 317 nM, and 84 to 116 ng for annealing temperature, primer concentration and cDNA quantity, respectively. Finally, the reference genes *β-TUB* and *UBI-3* were confirmed to be used in the gene expression assays of *MYB, F3H, F3´5´H, DFR, ANS* through ΔCq and Pearson correlation analysis.

Conclusions: The surface response design is a good tool which facilitates and improves the process to optimize the qPCR variables like annealing temperature, primer and cDNA concentration with minimal reactions and better results that reduce cost and time of the process.

Keywords: qPCR optimization, Annealing temperature, RNA, cDNA, MIQE, Gene expression

Background

Plants are continuously affected by different abiotic factors such as extreme temperatures, salinity, drought, radiation excess and nutrient imbalances, which can affect negatively their growth and development. In fact, abiotic stress can induce a reduction in the average yield of agricultural crops from 65 to 87 % (Koyro et al., 2012; Shinozaki et al., 2015). Plants have developed defense mechanism to diminish the adverse effects produced for abiotic stress. The mechanism consists in the production of secondary metabolites such as carotenoid, phenolic acids and flavonoids such as anthocyanins. Flavonoids are considered to be able to confer protection against several abiotic stresses (Wang et al., 2016), specially through their antioxidant capacity, which help the plant to reduce the deleterious effect of radical oxygen species (ROS) (Hatier and Gould 2009; Kim et al., 2017; León-Chan et al., 2017), which the main production is through the inhibition of the photosynthesis (Nishiyama and Murata, 2014; Pospíšil and Prasad, 2014; Chan *et al*., 2016). Anthocyanins, which confer a variety of color to the plant tissues, are flavonoids amply distributed in plants and its accumulation is affected by environmental stresses (Aza-Gonzalez et al., 2012; Landi et al., 2015; Wilthshire et al., 2017). The biosynthetic pathway for anthocyanin biosynthesis have

been well established but, its gene expression and regulation through *MYB* transcription factors in response to different environmental stressor continue in discussion (Lightbourn et al., 2007; Li et al., 2012; Kim et al., 2017). Recently *Capsicum* and *Petunia*, which are members of the family Solanaceae, have been proposed as model plants to study, and understand the complex process of anthocyanin biosynthetic pathway regulation through abiotic stress (Dhar et al., 2015). Genome technologies represent an important tool in the study of the mechanism concerning the regulation of metabolic pathways like the anthocyanin biosynthesis, because gene expression profiles under different environmental conditions can provides new insights about its regulation (Wan et al., 2011). The Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) is a widely selected method to determine the gene expression and is considered has the gold standard for nucleic acids detection and quantification, because it can determine low quantities of any transcript with high specificity, sensitivity, reproducibility, high throughput, equipment access and its relative ease performance (Guenin et al., 2009; Wan et al., 2011; Pabinger et al., 2014; Wadle et al., 2016). Because, the high number of published articles and the lack of homogeneity and sufficient experimental details to be the RT-qPCR experiments reproducible, Bustin et al. (2009) showed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), which have a list of the requirements that all RT-qPCR experiment must complete. Optimization of reaction conditions is one of the requirements of MIQE, in which annealing temperature, primer concentration, cDNA quantity and efficiencies of the reactions are the principal variables to be evaluated (Bustin et al., 2009; Raymaekers et al., 2009; Bustin et al., 2010; Taylor 2010). However, until now, the traditional method to optimize the conditions of the PCR reactions has been proof different annealing temperatures making a gradient and sometimes try different primer and cDNA concentrations to select the better condition (Singh et al., 2013; Ahlawat et al., 2014; Cruz et al., 2015; Jacchia et al., 2015). This method can produce a great use of consumables and not evaluate a great number of combinations of the three evaluated variables. In contrast, there are statistical designs to optimize process that can reduce the number of experiments and, therefore, the time and cost of optimization process (Wadle et al., 2016). Therefore, this study reports the

optimized qPCR amplification conditions of primers corresponding to anthocyanin biosynthesis genes and the reference genes *β-TUB* and *UBI-3* to be used in gene expression analysis due to abiotic stress in *Capsicum annuum* using a surface response design which is proposed to include in the process of qPCR optimization assays.

Methods

Plant material and growth conditions

Bell pepper plants grew with 12 h photoperiod consisted of 25/20 ºC (day/night), 972 $\mu mol \cdot m^2 \cdot s^{-1}$ of photosynthetically active radiation (PAR) and 65 % of relative humidity in a plant growth chamber (GC-300TLH, JEIO TECH; South Korea). Stems of bell pepper were collected at 37 days after sowing, frozen immediately in liquid nitrogen and stored at -80 °C until their analysis.

RNA isolation and conversion to cDNA

Stems of bell pepper plants were pulverized with liquid nitrogen and the total RNA was isolated from 50-100 mg of tissue with Trizol reagent (Ambion, life technologies, USA) according to the manufacturer´s instructions with modifications consisted of: repeat the separation with chloroform one more time; in the precipitation phase, we used 0.25 mL

of isopropyl alcohol with 0.25 mL of saline solution prepared with 0.8 M sodium citrate and 1.2 M sodium chloride in DEPC (diethyl pyrocarbonate) treated water and; the RNA wash with 75 % ethyl alcohol was carried out two times. The genomic DNA was removed with the use of Turbo DNA free kit (Invitrogen, life technologies, USA). Quantification and RNA integrity were determined with nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis respectively. The cDNA synthesis was carried out with 2000 ng of RNA and the use of Superscript III kit (Invitrogen, life technologies, USA). Finally, cDNA of several extractions was mixed, quantified with nanodrop 2000c spectrophotometer and stored at -20 ºC until its use.

Primer sequences

We selected the references genes β-tubulin (*β-TUB*) and ubiquitin conjugating protein (*UBI-3*) which have been reported by Wan et al. (2011) with high stability in samples exposed to abiotic stress and in different tissues of *Capsicum annuum* (Table 4). The primers of the transcription factor *MYB* and anthocyanin biosynthesis structural genes (*F3H, F3´5´H, DFR,* and *ANS*) (Table 4) in *Capsicum annuum* were designed using Primer3 software as follow: amplify a product of 165 to 256 bp; primer size of 20 bp; melting temperature near to 60 ºC and GC % between 50 and 60 (Raymaekers et al., 2009; Friedman et al., 2014). The hairpin and dimer formation risk of the primers designed were evaluated *in silico* with OligoAnalyzer 3.1 (D´haene et al., 2010). Finally, the selected primers were manufactured by Sigma Aldrich.

Table 4. Sequences of the primers evaluated

Gene	Gene symbol	Primer sequence (5´- 3´)		Amplicon length (bp)	GenBank accession number
		Forward	Reverse		
R2R3 MYB transcription factor	*MYB*	TACTAAGACCTCGCCCTCGG	ACTGCAGCCACATCTTCCTC	238	AJ608992.1
Flavanone 3-beta-hydroxylase	*F3H*	ATGATGATGTGAAAGCAGCG	TTTCAACTGGTGGCTGCTAC	256	FJ705844.1
Flavonoid-3',5'-hydroxylase	*F3´5´H*	CATGCCACACGTGTCACTTG	GCACCTGCATTAGTTGGACG	165	FJ705845.1
Dihydroflavonol-4-reductase	*DFR*	CGGCTGGATTTATCGGCTCT	CTTCCACGGTCAAGTCTGCT	168	FJ705846.1
Anthocyanidin synthase	*ANS*	CAGACACCGATATCTCCGGC	CGCGGCCTCCAGGATTATAG	207	FJ705847.1
β-tubulin	*β-TUB*	GAGGGTGAGTGAGCAGTTC	CTTCATCGTCATCTGCTGTC	167	EF495259.1
Ubiquitin-conjugating protein	*UBI-3*	TGTCCATCTGCTCTCTGTTG	CACCCCAAGCACAATAAGAC	204	AY486137.1

Optimization of amplification conditions to the quantitative real-time polymerase chain reactions (qPCR)

A composite central design of surface response for three factors was used. The three factors analyzed were: primer concentration, cDNA concentration and annealing temperature (T_a). The design consisted of 20 trials distributed on eight points of the cube, six axial points and six replicates of the central point (Figure 12) (Table 5). The values of coded variables in Table 5 were substituted by the real values of Table 6, in concordance to the evaluated primer. The high and low values (1 and -1, in coded variables respectively) of T_a were selected from the theoretical T_a of the designed primer; meanwhile the high and low values of primer and cDNA concentration were selected from preliminary experiments within the manufacturer recommendations of the

SsoAdvanced™ Universal SYBR™ Green Supermix (Bio-Rad, USA), which was used to the qPCR reactions. The response variable was the Cq value. The best experimental conditions, within the range of values of the three factors (primer concentration, T_a and cDNA concentration) assessed, were found using the response optimal analysis. The goal of the study was decrease the Cq value below 30 preferably to 25 or less to diminish the risk of unspecific products of PCR (Raymaekers et al., 2009).

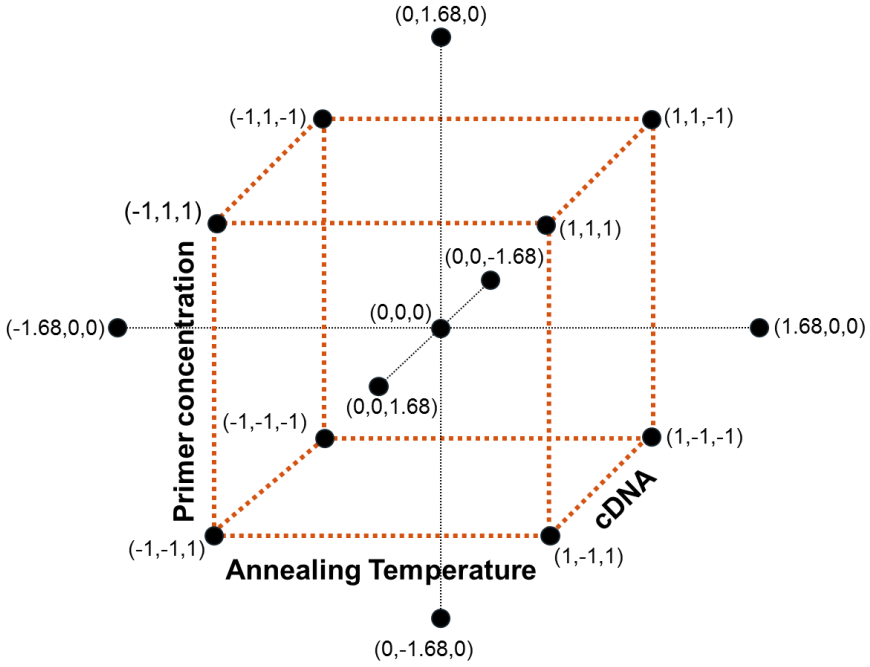

Figure 12. Geometrical view of the composite central design with coded variables.

The qPCR was performed into 0.2 mL PCR tubes with optical ultraclear caps (Bio-Rad, USA) in a CFX96™ Real-Time PCR detection system (Bio-Rad, USA). The reaction mixture consisted of the primer forward and reverse (10 µM) at quantities according to the concentration needed in the final volume, 5 µL of SYBR™ Green Supermix, 1 µL of cDNA at concentration determined by the design of the reaction and water to complete the final volume of 10 µL. The reaction mixture was subjected to the following conditions: 95 ºC for 30 s, followed of 40 cycles of 95 ºC for 10 s and T_a determined by the design during 30 s (Tables 5 and 6).

Table 5. Design of reactions with coded variables to qPCR optimization

Reaction	[a] Ta (ºC)	Primer concentration (mM)	cDNA (ng/µL)
1	-1.00	-1.00	-1.00
2	1.00	-1.00	-1.00
3	-1.00	1.00	-1.00
4	1.00	1.00	-1.00
5	-1.00	-1.00	1.00
6	1.00	-1.00	1.00
7	-1.00	1.00	1.00
8	1.00	1.00	1.00
9	-1.68	0.00	0.00
10	1.68	0.00	0.00
11	0.00	-1.68	0.00
12	0.00	1.68	0.00
13	0.00	0.00	-1.68
14	0.00	0.00	1.68
15	0.00	0.00	0.00
16	0.00	0.00	0.00
17	0.00	0.00	0.00
18	0.00	0.00	0.00
19	0.00	0.00	0.00
20	0.00	0.00	0.00

[a] Annealing temperature

Table 6. Central composite design to optimize the variables primer concentration, cDNA quantity, and annealing temperature in qPCR assays of the genes *MYB, F3H, F3´5´H, DFR, ANS, β-TUB* and *UBI-3*.

Factor	Axial point (-1.68)	Low point (-1)	Central point (0)	High point (1)	Axial point (1.68)
Primer concentration (mM)	232.96	250	275	300	317.05
cDNA (ng/µL)	83.18	90	100	110	116.82
[a]T_a (ºC) of *MYB*	56.64	58	60	62	63.36
T_a (ºC) of *F3H*	56.32	57	58	59	59.68
T_a (ºC) of *F3´5´H*	56.64	58	60	62	63.36
T_a (ºC) of *DFR*	59.32	60	61	62	62.68
T_a (ºC) of *ANS*	60.32	61	62	63	63.68
T_a (ºC) of *β-TUB*	56.32	57	58	59	59.68
T_a (ºC) of *UBI-3*	56.32	57	58	59	59.68

[a] Annealing temperatura

Evaluation of qPCR products

In every qPCR assay, we created melting curves of the products by increase the temperature from T_a – 2 ºC to 95 ºC with increments of 0.5 ºC every 5 s. On the other hand, we make an agarose gel electrophoresis with qPCR products to evaluate that amplicon length of the product corresponded to the design. The qPCR products were sequenced by the Sanger method with an AB3730 DNA analyzer (Applied Biosystems, Japan) and were compared *in silico* with the Basic Local Alignment Search Tool (BLAST) in the NCBI Gen Bank (Table 4) to ensure that the product amplified was correct (Bustin et al., 2010; D´haene et al., 2010; Cruz et al., 2015).

The evaluation of qPCR efficiency was carried out with a cDNA curve of dilutions 1:2 triplicated from 500 to 7.81 ng using volumes ≥ 10 when construct the curve to reduce the possibility of errors (Svec et al., 2015). The efficiency of all primer pairs evaluated were calculated from the slope of the cDNA dilution curves using the formula $E = \left[10^{\left(-\frac{1}{slope}\right)}\right] - 1$ (Friedman et al., 2014). Pearson correlations as well as the ΔCq method and linear regression coefficients, among reference and target genes, were calculated to evaluate if they have similar efficiencies and, therefore, be useful to gene expression analysis (Cui et al., 2015).

Data analysis

The composite central designs of response surface methodology were performed with a confidence level of 95 %. All statistical analysis was performed with the software Minitab® 17 (Minitab Inc.; Pennsylvania, USA).

Results and Discussion

The purity of the RNA analysis with RT-qPCR was important in order to get reliable results. The absorbance ratio A260 / A280 is an indicative of the RNA purity because the presence of DNA or phenol residues alters the values of this ratio (Bustin et al., 2009). In this study, the values of the ratio A260/A280 in the RNA extracted from bell pepper stems were >1.8 which is an acceptable purity (Taylor et al. 2010; De Keyser et

al., 2015). Subsequently, the RNA of several extractions individually was converted to cDNA and finally this was mixed and obtained a final concentration of 1244.3 ng/mL.

The optimization of conditions in a qPCR reaction can help to reduce the dimerization of primers and increase the efficiency and specificity of the amplification process (Raymaekers et al., 2009). In this study, we optimized the conditions to amplify genes that participate in the anthocyanin biosynthesis, with the goal to decrease the Cq values below 30 preferably to 25 or less without the presence of unspecific products, because Cq > 40 are suspect according to the MIQE (Bustin et al., 2009) due to implied low efficiency, therefore, values of Cq lesser are preferable.

The best conditions obtained for qPCR amplification of the primers evaluated, using the response optimal analysis, ranging between 56.4 and 63.6 ºC for T_a, 233 and 317 nM for primer concentration and, between 84 to 116 ng for cDNA (Table 7). However, if exist the necessity to use the same quantity of cDNA to evaluate expression among the genes, we calculate the theoretical Cq using the constant quantity of 100 ng of cDNA, because is approximately the average of the optimized quantity of all the primers evaluated. The results were Cq theoretical values from 17.35 to 25.84 (Table 7) which were confirmed with RT-qPCR assays. These relatively low values of Cq obtained in the optimization can help to utilize this amplifications conditions to evaluate this genes in samples with a wide range of expression. This because the qPCR protocol consists of 40 cycles.

The desirability is a value that represents the approximation of the Cq obtained to the Cq value that is the goal. The maximum value of desirability that can be obtained is 1. In this study, only the primers corresponding to the gene *DFR* did not get the desirability of 1, but get a good approximation to the Cq goal (Table 7).

The products of qPCR reactions of the primers evaluated were validated to confirm their specificity with melting curve analysis, agarose gel electrophoresis and by sequencing (Bustin et al., 2010; D´haene et al., 2010; Cruz et al., 2015). First, the melt curves of all the primer pairs evaluated at the optimizer conditions showed only one pick, suggesting only one product, which have values of melting temperature ranging from 80 to 83.5 ºC (Figure 13); second, the agarose gel electrophoresis also presented only one product for

each gene evaluated at their corresponding size (Figure 14) and; third, the sequences of qPCR products confirmed the primer specificity.

Table 7. Optimized amplification conditions of primers corresponding to anthocyanin biosynthesis genes and *β-TUB* and *UBI-3* reference genes

Gene	Ta[a] (°C)	Primer concentration (nM)	cDNA (ng)	Cq	Cq with 100 ng of cDNA	Desirability
MYB	63.3	280	113	24.51	24.74	1
F3H	58.3	317	106	24.54	24.62	1
F3´5´H	63	303	100	25.00	25.00	1
DFR	59.5	317	110	25.59	25.84	0.8827
ANS	63.6	233	96	24.00	24.27	1
β-TUB	56.5	317	116	18.81	19.20	1
UBI-3	56.4	233	84	17.35	18.30	1

[a] Annealing temperature

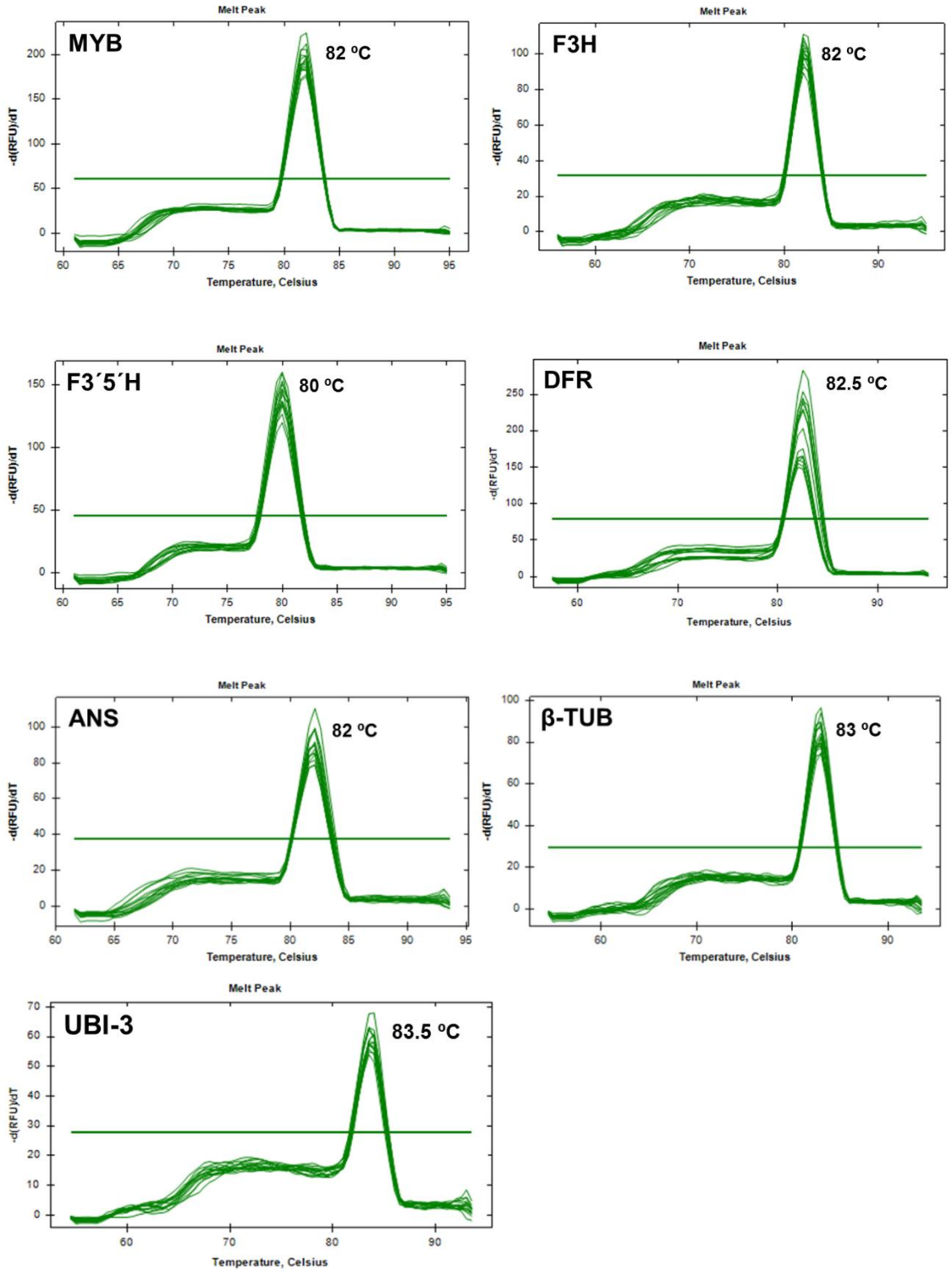

Figure 13. Melt curves of the RT-qPCR reactions of the primers corresponding to the genes *MYB*, *F3H*, *F3´5´H*, *DFR*, *ANS*, *β-TUB* and *UBI-3*.

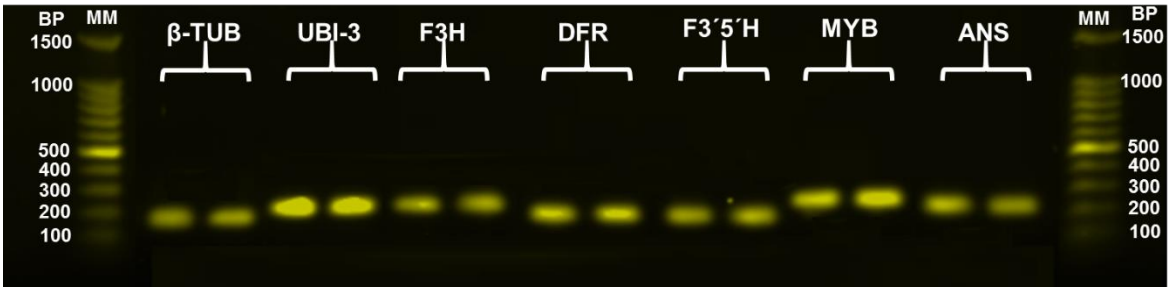

Figure 14. Amplified fragments corresponding to the genes *β-TUB, UBI-3 MYB, F3H, F3´5´H, DFR* and *ANS* shown by agarose gel electrophoresis

We determined the efficiency of the qPCR amplification which is considered one of the major points to be considered when using qPCR to determine relative gene expression (Regier and Frey, 2010). The efficiencies of the qPCR of the primers evaluated ranging between 90.1 to 107.3 % (Table 8). Efficiency values between 90 to 110 % are acceptable (D´haene et al., 2010; Friedman et al., 2014); this because efficiency values lesser to 90 % could be caused by contamination with inhibitors of polymerase, high or suboptimal annealing temperature, poorly designed primers, etc. Meanwhile, efficiency values higher than 110 % could be the result of primer dimers or nonspecific amplicons. Also, both high and low efficiencies could be produced by poorly calibrated pipettes or poor pipetting technique (Taylor et al., 2010).

On the other hand, the R^2 values of the cDNA curve of dilutions are higher than 98 %. The slope values should be between -3.1 and -3.6 (Raymaerkers et al., 2009), in this study slopes ranging from 3.159 to 3.585, which is acceptable. Finally, the intercept values are between 25.291 and 33.510 (Table 8) which are parameters that need to be published according to the MIQE (Bustin et al., 2010).

The relative expression analysis is based on the use of reference genes to determine the fold differences in expression of the target gene (Wan et al., 2011; Pabinger et al., 2014; De Keyser et al., 2015). However, to be valid the use of reference genes, in gene expression analysis, the efficiencies of targets and reference genes must be similar

(Regier and Frey, 2010). The most common method to evaluate this is how ΔCq (Cq target – Cq reference) varies with the curve of template dilution (Livak and Shmittgen 2001). If the amplification efficiencies are approximately equal between both, target and reference genes, the slope of the linear regression between ΔCq and the logarithm of cDNA concentration is close to zero (Regier and Frey, 2010). This based on the assumption that the Cq values of dilutions curves for target and reference genes have a positive correlation (Cui et al., 2015). In this study, the slopes of linear regressions of the ΔCq´s with the logarithm of cDNA concentrations have absolutes values lesser than 0.1 (Table 9). Furthermore, significant positive correlations were presented between target and reference genes (Table 9). These results demonstrated that efficiencies of reference and target genes have similar behavior and, therefore, *β-TUB* and *UBI-3* can be useful for gene expression experiments of *MYB, F3H, F3´5´H, DFR* and *ANS* in *Capsicum annuum*.

Table 8. Data corresponding to the evaluations of dilutions curves corresponding to the genes *MYB, F3H, F3´5´H, DFR, ANS, β-TUB* and *UBI-3*.

Gene	Efficiency (%)	R^2 (%)	Slope	Y-intercept
MYB	90.1	99.6	3.585	32.311
F3H	107.3	98	3.159	29.842
F3´5´H	101.8	98.8	3.279	32.723
DFR	95.6	98.9	3.432	33.510
ANS	106.7	99.2	3.172	30.672
β-TUB	100.5	99.4	3.311	25.291
UBI-3	99.6	99.2	3.332	25.675

Table 9. Slopes of linear regression between ΔCq (Cq target –Cq reference) and logarithm of cDNA concentration and Pearson´s correlation coefficients of references (*β-TUB* and *UBI-3*) with target genes (*MYB, F3H, F3´5´H, DFR* and *ANS*)

Target Genes	**Slopes of linear regression ΔCq vs cDNA with reference genes**		**Pearson´s correlation with reference genes**	
	β-TUB	***UBI-3***	***β-TUB***	***UBI-3***
MYB	-0.0672	-0.0332	0.939*	0.930*
F3H	-0.0365	-0.0025	0.994**	0.989**
F3´5´H	0.0479	0.0567	0.993**	0.992**
DFR	-0.0847	-0.0931	0.992**	0.992**
ANS	-0.0094	0.0707	0.998**	0.995**

* and ** significance at $P \leq 0.005$ and $P \leq 0.001$ respectively

Conclusions

The response surface methodology reduces the number of reactions necessary to assess a great quantity of possible combinations of the values from variables optimized in a RT-qPCR assay. Therefore, the optimization process reduced the analysis costs and evaluation time.

On the other hand, *β-TUB* and *UBI-3* are considered good reference genes to be used in the gene expression assays of *MYB, F3H, F3´5´H, DFR, ANS* in *Capsicum annuum*.

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CAPÍTULO 5. GENE EXPRESSION PATTERN OF ANTHOCYANIN BIOSYNTHETIC PATHWAY IN *Capsicum annuum* DURING THEIR EXPOSITION TO LOW TEMPERATURE AND UV-B RADIATION

Abstract

Tropical and subtropical plants, such as *Capsicum annuum*, are negatively affected by low temperature (LT) stress, defined as temperatures moderately above of the water freezing point (1-15 °C). Moreover, the higher levels of UV-B radiation in the world are in tropical and subtropical regions. Anthocyanin biosynthesis has been strongly correlated with LT and UV-B stress tolerance. Therefore, the objective of this study was to determine the expression pattern of *MYB, F3H, F3´5´H, DFR* and *ANS* in stems of *Capsicum annuum* plants when they are exposed to LT, UV-B radiation and LT+UV-B. The higher expression of *MYB, F3´5´H, DFR* and *ANS* were exhibit in plants exposed to LT; meanwhile *F3H* expression was higher in plants exposed to LT+UV-B. Therefore, anthocyanin biosynthesis in *Capsicum annum* stems is more influenced by LT than UV-B. However, higher expression of *F3H* with LT+UV-B treatment denotes the biosynthesis of other important flavonoids, to protect plants from LT+UV-B. On the other hand, the significant higher increases of *DFR* expression respect to *ANS* against LT stress suggest the possibility of an intensification of proanthocyanidins biosynthesis, which are produced form the leucoanthocyanidins synthetized by *DFR*. Moreover, the higher expression of *MYB, F3´5´H, DFR* at the same hour of the day (16:00 h) indicate the possible direct regulation of *MYB* on *F3´5´H* and *DFR* influenced also by PAR (photosynthetically active radiation).

Key words: UV-B, low temperature, anthocyanin, flavonoid, RT-qPCR

Introduction

Environmental factors such as salinity, drought, flooding, nutrient imbalances, the wind, extreme temperatures and radiation, affect negatively the growth and development of plants; even, 65 to 87 % of the average yield of worldwide food production by crops is reduced by this abiotic stress (Koyro et al., 2012; Shinozaki et al., 2015). Therefore, understand the mechanism of adaptation response, that plants exhibit against abiotic stress is vital to maintain and improve the yield of crops (Li et al., 2015). Tropical and subtropical plants are negatively affected by LT stress, defined as temperatures moderately above of the water freezing point (1-15 °C). Indeed, the main reason for limitations in geographic distribution of crops is LT. On the other hand, the higher levels of UV-B radiation in the world are in tropical and subtropical regions (Jaakola and Hohtola, 2010). Secondary metabolites such as flavonoids have been referenced as defense mechanism of plants against LT and UV-B radiation (Tecocharis et al., 2012; Zlatev et al., 2012). One of the largest groups of secondary metabolites produced by plants, is flavonoids, which are found predominantly in the epidermis of several tissues (Castañeda-Ovando, 2009). The production of these compounds in plants have been related with several stimulus, such as water stress, depletion of nutrients, pathogen attack, low temperature (LT), high light and UV-B radiation (Aza-González et al., 2012; Theocharis et al., 2012; Zlatev et al., 2012; Wiltshire, 2017). Therefore, the roles that scientist propose to these compounds are diverse, but the most common are the antioxidant capacity of this compounds, protection of DNA and photosynthetic elements against radiation, as metal-chelating agents and as a signal transduction regulator (Hatier and Gould, 2009; Landi et al., 2015; Rouholamin et al., 2015). That's a reason because flavonoids have been considered as possible biological markers.

The main flavonoid compounds distributed and studied in plants include flavonols and anthocyanins (Xu et al., 2015). The anthocyanin biosynthetic pathway has been well stablished and it has been divided in the early biosynthetic genes such as: chalcone synthase (*CHS*), chalcone isomerase (*CHI*) and flavanone 3-hydroxylase (*F3H*); and the

late biosynthetic genes such as: dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*) (Lim et al., 2016). On the other hand, transcription factors are proteins that regulates the expression of genes through their interaction with specific DNA cis-regulatory elements, which are usually localized upstream of the transcribed region (Tian et al., 2015). The MYB transcription factors family is one of the largest in plants; moreover, their structures and functions are conserved compared with those in animals and yeast (Li et al., 2015). Overexpression of some MYB transcription factors results in up-regulation of structural genes of anthocyanin biosynthesis, especially the two late biosynthetic genes *DFR* and *ANS* in different plants such as: *Rapahanus sativus, Nicotiana tabacum, Arabidopsis thaliana* and *Capsicum annuum* (Borowsky et al., 2004; Lightbourn et al., 2007; Lim et al., 2016). *Capsicum annuum* has been reported as a very sensitive crop to LT stress and has been considered as a model to the study of anthocyanin biosynthesis (Kaniuga,2008; Dhar et al., 2014)

Flavonol and anthocyanin biosynthesis has been strongly correlated with LT and UV-B stress tolerance (Aza-González et al., 2012; Schulz et al., 2015; León-Chan et al., 2017) and, have been reported that the structural anthocyanin modifications and accumulation in plant tissues, is positively correlated with the expression levels of genes that participate in anthocyanin biosynthetic pathway; suggesting this this as fingerprints of the stress status of the plants (Landi et al., 2015; Rouholamin et al., 2015). Thus, the study of transcriptional regulation of flavonols and anthocyanins in plants can help to elucidate the mechanisms to alleviate the negatively effects of low temperature and UV-B radiation and the possibility of use transcription patterns to identify the specific stress that affect the plants. We hypothesized that the genes of anthocyanin biosynthetic pathway have different expression pattern during the exposition to LT, UV-B radiation and LT+UV-B in *Capsicum annuum*. Therefore, the objective of this study was to determine the expression of *MYB, F3H, F3´5´H, DFR* and *ANS* in plants of *Capsicum annuum* at different times of the day, when they are exposed to LT, UV-B radiation and LT+UV-B.

Plant material and growth conditions

The bell pepper plants were produced such as León-Chan et al., (2017). Twenty-eight DAS (days after sowing) bell pepper plants were put in a plant growth chamber (GC-300TLH, JEIO TECH; South Korea) for three days at the conditions described: 12 h photoperiod (from 6:00 to 18:00 h) of PAR (photosynthetically active radiation) (972 $\mu mol \cdot m^{-2} \cdot s^{-1}$), temperature of 25/20 °C (day/night) and relative humidity of 65 %. Then, the treatments UV-B radiation (UV-B), low temperature (LT) and low temperature with UV-B radiation (LT+UV-B) were applied. For the LT and LT+UV-B treatments, the growth chamber was set at 15/10 °C at the night before to start the sampling (day 30 at 18:00 h). For UV-B and LT+UV-B treatments, the plants were irradiated for 6 h with PAR (from 06:00 to 10:00 and 16:00 to 18:00 h) and for 6 h of UV-B irradiation (72 $kJ \cdot m^2$, from 10:00 to 16:00 h). The UV-B radiation was applied using three Phillips TL 100W/01 lamps (Germany) with a narrow waveband between 305 to 315 nm and peaks at 311 nm; the distance between plant leaves and UV-B lamps was 50 cm, and the UV-B irradiance was measured by a UV A/B light meter (SPER SCIENTIFIC, model 850009; Scottsdale, AZ, USA). The UV-B radiation treatment was started at day 31, the samples were taken at 10:00 h just before starting the radiation with UV-B lamps. Then, samples were taken at 11:00, 16:00 and, finally, at 04:00 and 11:00 of the day 32 (Figure 15). The samples consisted of stems of 10 bell pepper plants. Samples were frozen in liquid nitrogen and stored at -80 °C in an ultra-low temperature freezer (Revco UxF40086A, Thermo Scientific; USA) until their analysis.

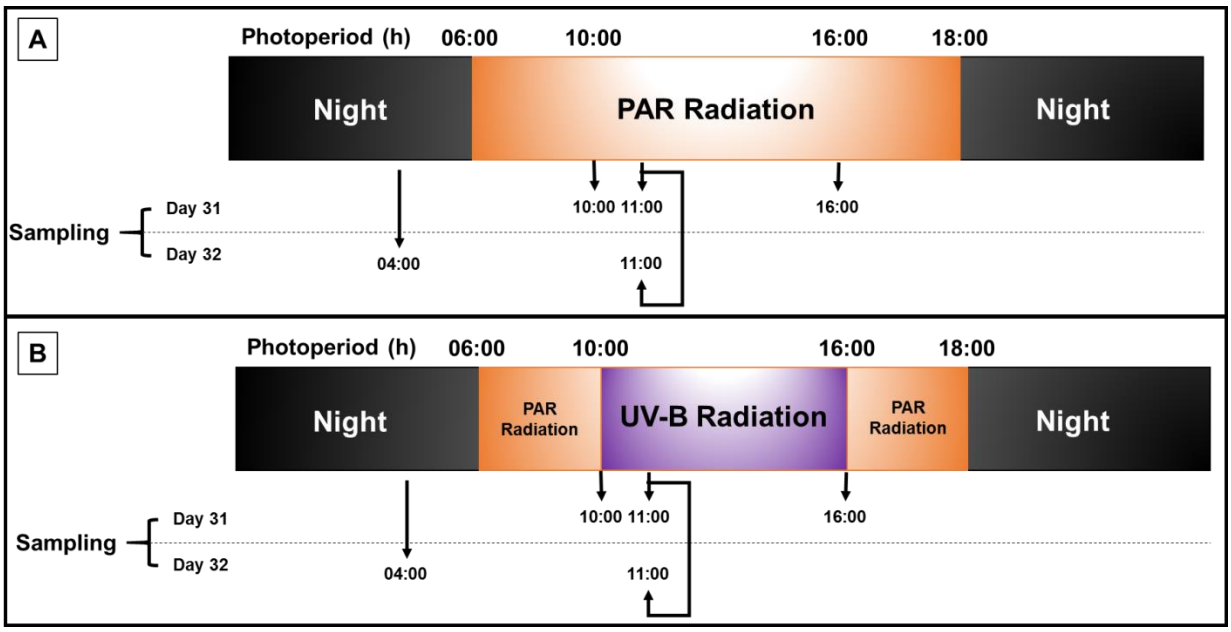

Figure 15. Photoperiod and times of sampling during the treatments (A) low temperature (LT) (15/10 °C, day/night), (B) UV-B radiation (25/20 °C, day/night) and LT+UV-B (15/10 °C, day/night).

PAR (photosynthetically active radiation)
The temperature was reduced, for LT and LT+UV-B radiation treatments, just the night before the sampling day at 18:00 h (day 30)

Primer sequences for relative gene expression analysis

We selected the primers of the reference gene β-tubulin (*β-TUB*) reported by Wan et al. (2011) with high stability in samples exposed to abiotic stress and in different tissues of *Capsicum annuum*. The primers of the transcription factor *MYB* and anthocyanin biosynthesis structural genes (*F3H, F3´5´H*, *DFR*, and *ANS*) (Table 10) in *Capsicum annuum* were designed using Primer3 with the characteristics of amplify 165 to 256 bp, primer size of 20 bp, melting temperature near to 60 ºC and GC % between 50 and 60 (Raymaekers et al., 2009; Friedman et al., 2014). The hairpin and dimer formation risk

of the primers designed were evaluated *in silico* with OligoAnalyzer 3.1 (D´haene et al., 2010). Finally, the selected primers were manufactured by Sigma Aldrich.

Table 10. Sequences of the primers used in the relative gene expression analysis of the anthocyanin biosynthetic pathway and the *MYB* transcription factor

Gene	Gene symbol	Primer sequence (5´- 3´)		Amplicon length (bp)	GenBank accession number
		Forward	Reverse		
R2R3 MYB transcription factor	*MYB*	TACTAAGACCTCGCCCTCGG	ACTGCAGCCACATCTTCCTC	238	AJ608992.1
Flavanone 3-beta-hydroxylase	*F3H*	ATGATGATGTGAAAGCAGCG	TTTCAACTGGTGGCTGCTAC	256	FJ705844.1
Flavonoid-3',5'-hydroxylase	*F3´5´H*	CATGCCACACGTGTCACTTG	GCACCTGCATTAGTTGGACG	165	FJ705845.1
Dihydroflavonol-4-reductase	*DFR*	CGGCTGGATTTATCGGCTCT	CTTCCACGGTCAAGTCTGCT	168	FJ705846.1
Anthocyanidin synthase	*ANS*	CAGACACCGATATCTCCGGC	CGCGGCCTCCAGGATTATAG	207	FJ705847.1
β-tubulin	*β-TUB*	GAGGGTGAGTGAGCAGTTC	CTTCATCGTCATCTGCTGTC	167	EF495259.1

RNA isolation of the bell pepper stems

Stems of bell pepper plants were pulverized with liquid nitrogen and the total RNA was isolated from 50-100 mg of tissue with Trizol reagent (Ambion, life technologies, USA) according to the manufacturer´s instructions with some modifications consisted of: repeat the separation with chloroform one more time; in the precipitation phase, we replaced the use of 0.5 mL of isopropyl alcohol, by 0.25 mL of isopropyl alcohol with 0.25 mL of saline solution prepared with 0.8 M sodium citrate and 1.2 M sodium

chloride in DEPC (diethyl pyrocarbonate) treated water and; the RNA wash with 75 % ethyl alcohol was carried out two times. The genomic DNA was removed with the use of Turbo DNA free kit (Invitrogen, life technologies, USA). Quantification and RNA integrity were determined with nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis respectively.

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) analysis

The cDNA synthesis was carried out with Superscript III kit (Invitrogen, life technologies, USA) using 2000 ng of RNA. The cDNA was quantified with nanodrop 2000c spectrophotometer and stored at -20 ºC until its analysis. To check the primer specificity, melting curves were performed after each qPCR reaction. Furthermore, an agarose gel electrophoresis and an alignment with BLAST (Basic Local Alignment Search Tool) in the NCBI Gen Bank, of the amplicon sequences were performed (Bustin et al., 2010; D´haene et al., 2010; Cruz et al., 2015). The efficiency of each primer set was tested running a dilution series 1:2 of seven points from 500 to 7.81 ng of cDNA. The q-PCR reactions were performed using a CFX96^{TM} Real-Time PCR detection system (Bio-Rad, USA) into 0.2 mL PCR tubes with optical ultraclear caps (Bio-Rad, USA). A total volume of 10 µL reaction mixture containing 1 µL of cDNA (100ng/µL) and 9 µL of master mix was used. The master mix consisted of: 5 µL of SYBR SsoAdvanced^{TM} Universal SYBR^{TM} Green Supermix (Bio-Rad, USA); primers (10 µM), forward and reverse, at the quantities needed according to the concentration required in the final volume (Table 11) and; finally, complete the volume with water. The qPCR reaction conditions consisted of a pre-denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and the annealing temperature (T_a), described in Table 2 to each primer set, during 30 s.

Table 11. Primer concentration and annealing temperature of primers for q-PCR analysis of the anthocyanin biosynthesis genes and the reference *β-TUB* gene.

Gene	Ta[a] (°C)	Primer concentration (nM)
MYB	63.3	280
F3H	58.3	317
F3´5´H	63	303
DFR	59.5	317
ANS	63.6	233
β-TUB	56.5	317

[a] Annealing temperature

Data analysis

The relative gene expression was determined using the software CFX Manager 3.0 (Bio-Rad Laboratories, Inc.). The gene expression levels were normalized against the reference gene *β-TUB*, and the relative amount of amplified product was calculated with the $2^{-\Delta\Delta Cq}$ method using the sample of UV-B radiation treatment at 10:00 h as calibrator. The quantitative data are shown as the mean values of three experimental replicates, each one measured two times. The analysis of variance was used to test every assay, and Fisher's test was used to determine significant differences at 95 % ($P \leq 0.05$) of significance. All statistical analyses were performed with the software Minitab® 17 (Minitab Inc.; Pennsylvania, USA).

The higher expression of *MYB* gene is at 16:00 h independently of LT or UV-B condition

The higher expression of the transcription factor *MYB* was presented in LT and LT+UV-B treatments at 16:00 h showing 18.27- and 17.42-fold higher expression in respect to the calibrator sample (UV-B at 10:00). The expression of the *MYB* transcription factor gene increase slightly with the LT at 11:00 h of the first day of treatment and at 04:00 h of the second day and, with LT+UV-B at 11:00 h of the second day. UV-B radiation at 25/20 ºC (day/night) increases the expression of the *MYB* only at 16:00 h, showing 14.90-fold higher expression (Figure 16).

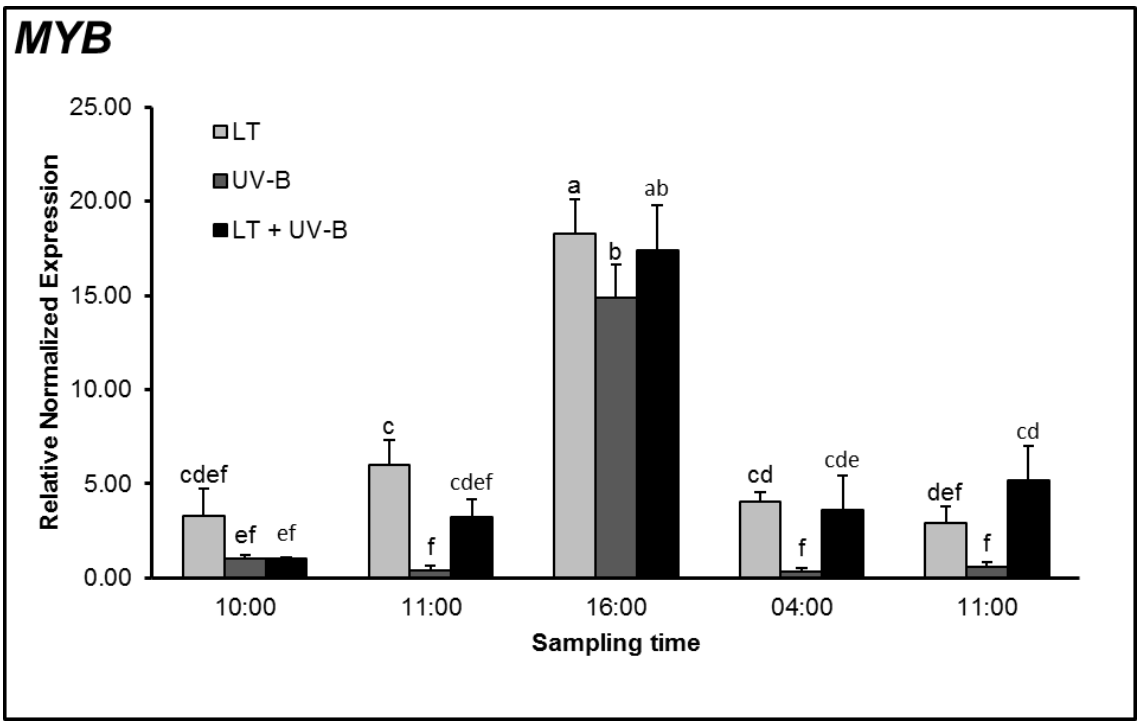

Figure 16. Relative normalized expression of the *MYB* transcription factor gene in *Capsicum annuum* stems exposed to low temperature (LT), UV-B radiation and LT+UV-B for two days. Relative gene expression levels were normalized with *β-TUB* values.

* Statistically significant differences (P≤0.05) of gene expression at different sampling points and between treatments are indicated with different letters.

The combined effect of low temperature (LT) and UV-B radiation induces a higher expression of the *F3H* in stems of *Capsicum annuum* in comparison to those exposed only to LT or UV-B

The expression of the *F3H* gene was higher in stems of *Capsicum annuum* exposed to LT+UV-B followed by stems exposed to LT and finally by UV-B radiation (data not shown). The time of greater expression for *F3H* was at 04:00 h of the second day of

treatment. In stems of the LT+UV-B treatment, the *F3H* gene expression levels increase continuously since the first hour of exposition to UV-B radiation, showing 6-fold higher expression, until the early morning (04:00 h), which showing 11-fold higher expression. Then, the *F3H* gene expression level was reduced at 11:00 of the second day (Figure 17). With LT, the expressions of *F3H* increase from 16:00 to 04:00 h from 3.47- to 6.68-fold higher expression. The UV-B radiation only show difference between 11:00 h (at the first h of UV-B radiation exposure) and 04:00 h (no radiation).

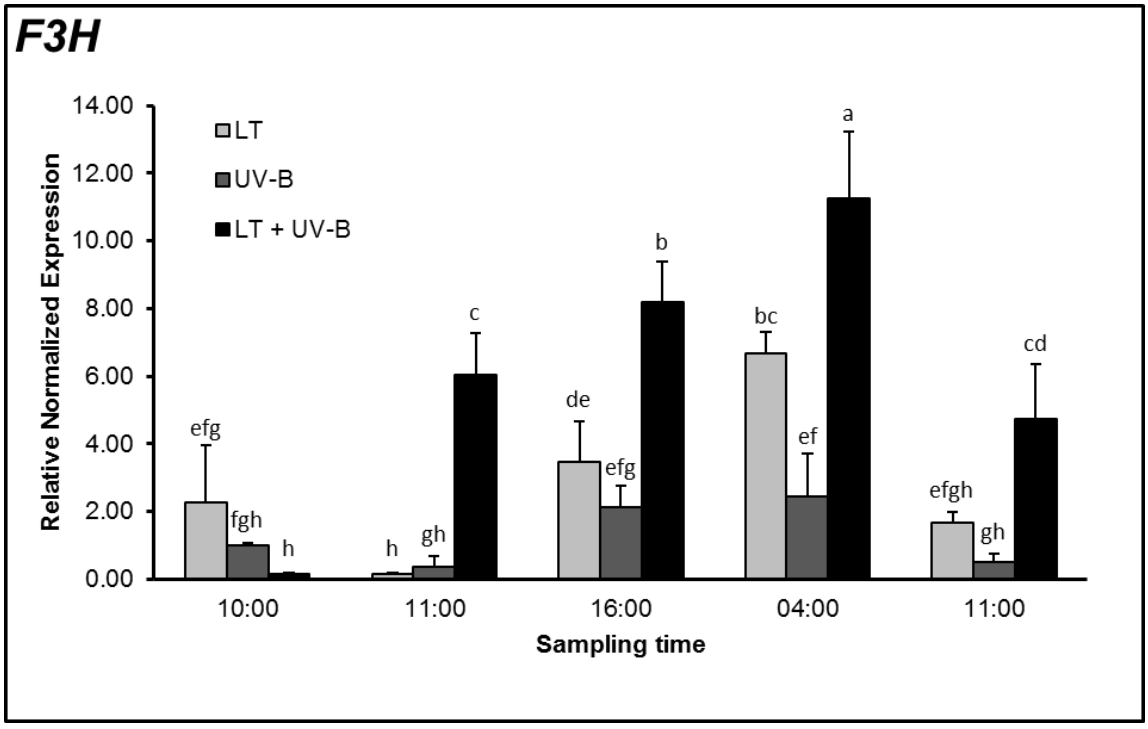

Figure 17. Relative normalized expression of the *F3H* (Flavanone 3-beta-hydroxylase) gene in *Capsicum annuum* stems exposed to low temperature (LT), UV-B radiation and LT+UV-B for two days. Relative gene expression levels were normalized with *β-TUB* values.

* Statistically significant differences (P≤0.05) of gene expression at different sampling points and between treatments are indicated with different letters.

Low temperature (LT) induce higher gene expression of F3´5´H and DFR with similar behavior in stems of *Capsicum annuum* in comparison to those exposed only to LT or UV-B

Stems of *Capsicum annuum* exposed LT show the higher expressions of the *F3´5´H* gene. The expression of *F3´5´H* increases from 11:00 h (14.46-fold higher expression) to 16:00 h (27.23-fold higher expression); then the expression decrease. With LT+UV-B the expression of *F3´5´H* increases at the first hour of exposition to UV-B (11:00 h); then its expression was practically at similar values. Meanwhile, stems exposed only to UV-B radiation did not show significant changes in *F3´5´H* gene expression (Figure 18).

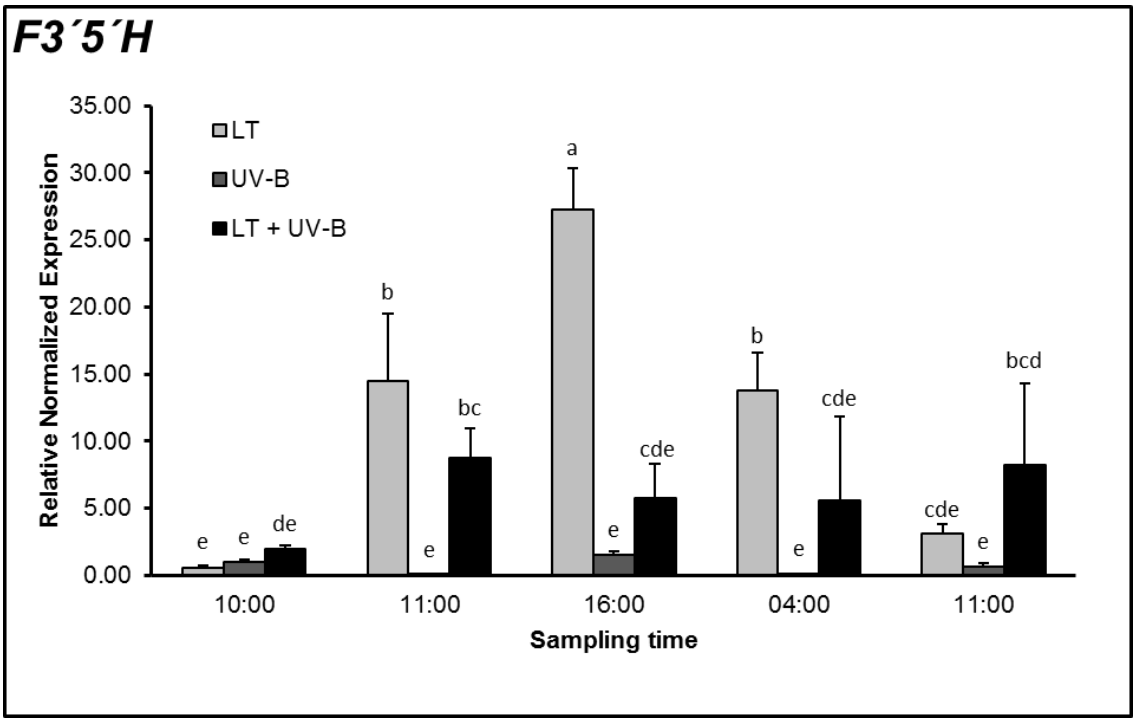

Figure 18. Relative normalized expression of the *F3´5´H* (Flavonoid-3',5'-hydroxylase) gene in *Capsicum annuum* stems exposed to low temperature (LT), UV-B radiation and LT+UV-B for two days. Relative gene expression levels were normalized with *β-TUB* values.

* Statistically significant differences (P≤0.05) of gene expression at different sampling points and between treatments are indicated with different letters.

The expression of the *DFR* only increase significantly in stems exposed to LT. The expression of *DFR* by LT increase from 11:00 to 16:00 h (110.34- and 119.32-fold higher expression), then the expression decrease but keep very higher respect to the 10:00 h of the first day of treatment. Stems exposed to LT+UV-B show a similar behavior than stems exposed to LT but they not present a statistical significance for the expression of this gene (10.64-fold higher expression at 16:00) (Figure 19); however, that increase in gene expression is can be considered biologically significant.

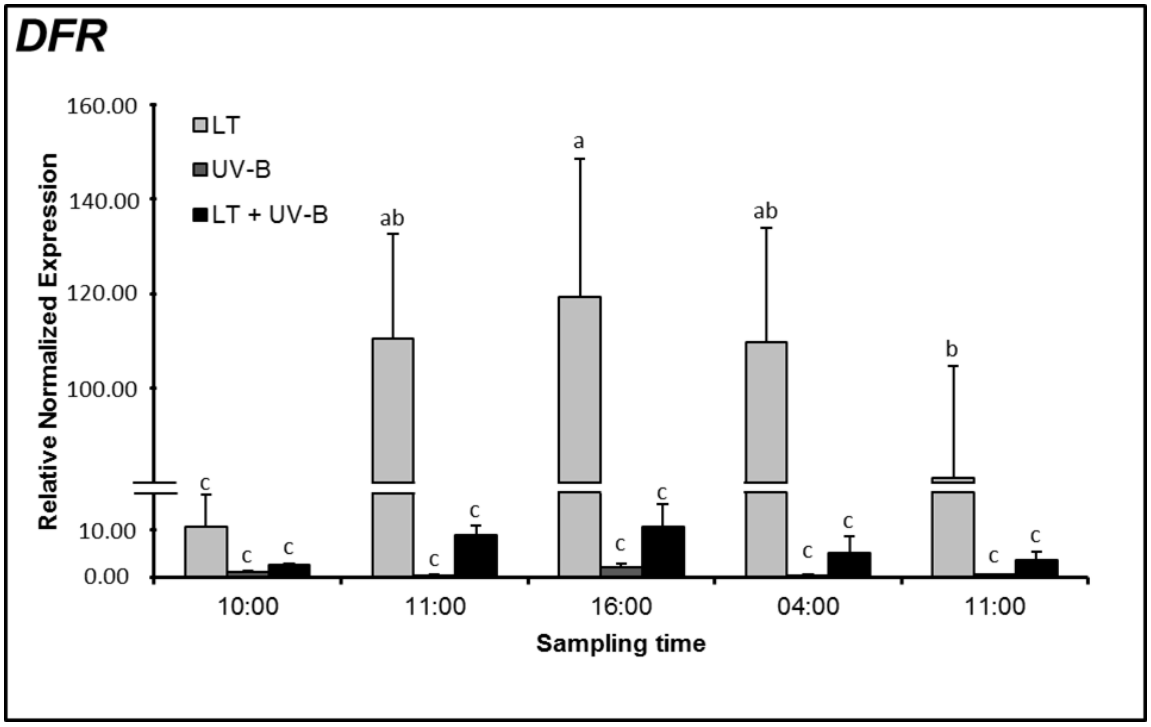

Figure 19. Relative normalized expression of the *DFR* (Dihydroflavonol-4-reductase) gene in *Capsicum annuum* stems exposed to low temperature (LT), UV-B radiation and LT+UV-B for two days. Relative gene expression levels were normalized with *β-TUB* values.

* Statistically significant differences (P≤0.05) of gene expression at different sampling points and between treatments are indicated with different letters.

The expression of ANS increased at less magnitude compared to other anthocyanin biosynthesis related genes

ANS gene show higher expression in stems exposed to LT (1-73-fold higher expression), followed by stems exposed to UV-B radiation (1-33-fold higher expression). The higher expressions of *ANS* gene were present at 11:00 h of both first and second day of treatment. The higher expression of ANS gene was in stems exposed to LT at 11:00 h of the first day of treatment (Figure 20).

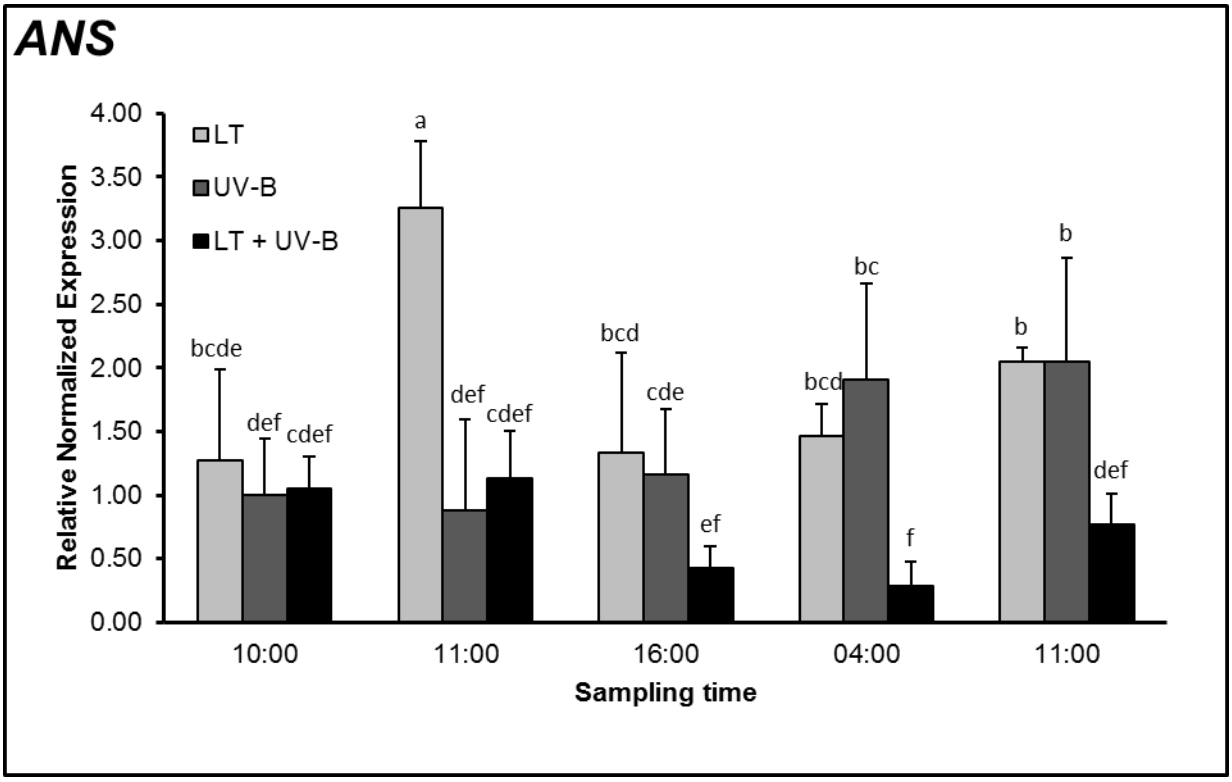

Figure 20. Relative normalized expression of the *ANS* (Anthocyanidin synthase) gene in *Capsicum annuum* stems exposed to low temperature (LT), UV-B radiation and LT+UV-B for two days. Relative gene expression levels were normalized with *β-TUB* values.

* Statistically significant differences (P≤0.05) of gene expression at different sampling points and between treatments are indicated with different letters.

The expression of *F3H* in stems of *Capsicum annuum* is not correlated with the expression of the late biosynthetic genes

The higher expression of *F3H* was exhibit in *Capsicum annuum* plants exposed to LT+UV-B followed by the exposition to LT. UV-B radiation increase the expression levels and enzyme activity of *F3H* in *Reaumuria soongorica* (Liu et al., 2013) and the expression of early biosynthetic genes, like *F3H*, have also been correlated with the anthocyanin accumulation (Lu and Yang, 2006). However, because in this study, the expression of the late genes was higher with LT instead of LT+UV-B, it is probably that the increase of *F3H* expression could be destined to the production of another kind of flavonoids, which help to protect better the stems for the combined effect of LT and UV-B. On the other hand, the higher expression of *F3H* was exhibit at early morning (04:00 h). Therefore, the radiation could be a negatively direct effect on the mRNA of *F3H*.

MYB transcription factor could regulate the expression of *F3´5´H* and *DFR* in stems of *Capsicum annuum* exposed to low temperature

The mechanism that controls the anthocyanin biosynthesis pathway is conserved among several plant species (Lim, 2016). The transcription factors MYB are key regulators of flavonoid biosynthesis in respond to abiotic stress (Wang et al., 2016). In this work, a higher level of *MYB* expression was exhibit at 16:00 h independently of treatment, then

the expression decrease. Higher expression was resulted with LT respect to UV-B. Functions of MYB transcription factors are conserved in plants (Li et al., 2015). Tian et al. (2015) reported that MYB10 positively regulates the transcription of *F3H, F3´H* and *ANS* in ever-red leaf crabapple at low temperature (15°C); but also mentioned that in McMYB10-silenced lines the expression of *F3H, F3´H* and *DFR* is down regulated in apple fruits. In this investigation, the expression of *F3´H* was not measured but *F3´5´H,* which exhibit an expression pattern like *DFR.* In this study, when plants were exposed to LT, the expression of *F3´5´H* and *DFR* increase gradually until their higher point at 16:00 h, matched with the time of higher expression level to *MYB*; then the expression decrease gradually too. Similar results were presented with LT+UV-B but not with UV-B only. The total anthocyanin concentrations have been correlated with the expression levels of *DFR* in different genotypes of *Punica granatum* (Rouholamin et al., 2015). Meanwhile, in *Brassica rapa*, the expression levels of some *DFR* genes have been correlated with the transcription of a *MYB* and cold stress resistance (Ahmed et al., 2014a). Therefore, MYB transcription factor could be the regulator of the expression levels of *F3´5´H* and *DFR* and the anthocyanin accumulation *Capsicum annuum* stems, especially when plants are exposed to low temperature. Xu et al. (2015) mentioned that complex denominated MBW, which include MYB, bHLH and WDR transcription factors, participate in the regulation by abiotic factors, such as light and low temperature, to induce flavonoid accumulation through the activation of late biosynthesis genes such as *ANS* and *DFR* (Li et al., 2012; Zhang et al., 2012; Rouholamin et al., 2015; Xu et al., 2015). Catalá et al. (2011) mentioned that plants required the correct integration of light signals to ensure an appropriate low temperature stress tolerance. In this study UV-B radiation practically did not induce the expression of *F3´5´H* and *DFR*, then, PAR is the radiation necessary to induce the expression of *F3´5´H* and *DFR.* Therefore, could be possible that UV-B blocks partially the expression of *F3´5´H* and *DFR* through the expression or partial inactivation of other transcription factors such as bHLH or WDR, but not MYB.

The higher expression levels of *DFR* in *Capsicum annuum* stems exposed to low temperature could be destined to the biosynthesis of proanthocyanidins

DFR is a pivotal enzyme in the anthocyanin biosynthetic pathway, that use NADPH as cofactor to catalyzes the transformation of dihydroflavonols into leucoanthocyanidins (Wang et al., 2013; Ahmed et al., 2014a) which are subsequently converted to anthocyanidins by ANS (Ahmed et al., 2014b). The higher expression of *ANS* was also in *Capsicum annum* plants exposed to LT. But *ANS* expression pattern was different to *F3´5´H* and *DFR* with a time of higher expression at 11:00 h, then the expression decrease and recovery slightly until the second day. *ANS* expression is strongly associated with cold stress tolerance and the anthocyanin accumulation in *Brassica rapa* and, is regulated by a MYB transcription factor (Ahmed et al., 2014b); however, in this case, *ANS* could be regulated by other molecules or in addition to *MYB*. On the other hand, *Capsicum annum* plants exposed to UV-B radiation have their maximum *ANS* expression until the second day. Therefore, *ANS* in plants exposed to UV-B radiation required more time to respond to this stress respect to LT. The biosynthesis proanthocyanidins are also be produced form the leucoanthocyanidins resulted from DFR activity. This transformation is carried out through the enzyme leucoanthocyanidin reductase (LAR) (Wang et al., 2013). Therefore, because the great differences of expression between *DFR* and *ANS*, a high quantity of DFR products can be destined to the production of proanthocyanidins instead of anthocyanins in *Capsicum annuum* stems exposed to LT. Meanwhile, dihydroxylation of the flavonoids produced during LT and LT+UV-B stress is an important factor to defense system of *Capsicum annum* stems according to the increase of *F3´5´H* expression. About that, León-Chan et al. (2017) reported a higher increase in the concentration and antioxidant activity of the dihydroxylated flavonoid luteolin-7-glucoside respect to the monohydroxylated apigenin-7-glucoside in leaves of *Capsicum annuum*.

Finally, in this study, the expression pattern of *MYB*, *F3H* and *DFR* is the same independently of the treatment. The accumulation of MYBs that regulate anthocyanin

biosynthesis, as well as several mRNAs of structural genes exhibit circadian rhythms in *Arabidopsis* (Harmer et al., 2000). Therefore, *MYB, F3H* and *DFR* have a circadian rhythm in *Capsicum annuum*.

Conclusions

The higher expression of *MYB, F3´5´H, DFR* and *ANS* with low temperature suggests that anthocyanin biosynthesis in *Capsicum annum* stems is more influenced by LT than UV-B. Meanwhile, higher expression of *F3H* with LT+UV-B treatment denotes the possible biosynthesis of other important flavonoids, such as flavonols, to protect plants from LT+UV-B. The significant higher increases of *DFR* expression respect to *ANS* against LT stress suggest the possibility of an intensification of proanthocyanidins biosynthesis, which are produced form the leucoanthocyanidins synthetized by *DFR*. The MYB transcription factor could be the regulator of the expression levels of *F3´5´H* and *DFR* and the anthocyanin accumulation *Capsicum annuum* stems, especially when plants are exposed to low temperature; this because the same time of the day to maxima expression. Moreover, the expression of *MYB, F3´5´H, DFR* is influenced also by PAR (photosynthetically active radiation) because the expression increase with a maxima expression at 16:00 h, the diminish during night; therefore, these genes could be a circadian rhythm.

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CAPÍTULO 6. CONCLUSIONES GENERALES

El efecto combinado de estrés por radiación UV-B y temperatura baja propicia una mayor reducción del contenido de clorofila *a* y *b* el cual se correlaciona con un aumento en el contenido de carotenoides totales respecto del estrés propiciado por cada uno de los dos factores por separado en hojas de plantas de pimiento morrón (*Capsicum annuum* L.).

El estrés producido por el efecto combinado de temperatura baja y radiación UV-B, en hojas de plantas de pimiento morrón, inducen una mayor acumulación de ácido clorogénico y los flavonoides apigenina-7-glucósido (A-7-G) y luteolina-7-glucósido (L-7-G) en comparación con el estrés producido por cada factor por separado.

Las plantas bajo los efectos del estrés por temperatura baja mostraron una mayor acumulación de A-7-G en sus hojas; mientras que, las plantas bajo estrés por UV-B presentaron una mayor acumulación de L-7-G.

Luteolin-7-glucósido les confiere una mayor actividad antioxidante a las plantas de pimiento morrón, a través de donación de electrones (actividad reductora), respecto de apigenina-7-glucósido durante la exposición a temperatura baja y radiación UV-B.

Las condiciones óptimas de amplificación para ensayos de RT-qPCR en *C. annuum*, correspondientes a los oligonucleótidos, de genes que participan en la biosíntesis de antocianinas (*MYB, F3H, F3´5´H, DFR* y *ANS*), diseñados en esta investigación y los genes de referencia *β-TUB* y *UBI-3* se encuentran entre: 54.4 y 63.6 °C de temperatura de alineamiento, 233 y 317 nM de concentración de oligonucleótido y, 84 a 116 ng de ADNc.

Los genes de referencia *β-TUB* y *UBI-3* son adecuados para ensayos de expresión de los genes *MYB, F3H, F3´5´H, DFR* y *ANS* en *C. annuum*.

El diseño de superficie de respuesta es una herramienta que facilita y mejora el proceso obtención de las condiciones óptimas de alineamiento de oligonucleótidos para ensayos de qPCR, reduciendo con ello el tiempo y costo invertidos en este proceso.

La condición de temperatura baja induce una mayor expresión de los genes *MYB, F3´5´H, DFR* y *ANS* en los tallos de plantas de pimiento morrón respecto a la radiación UV-B. Mientras que el efecto simultáneo de estrés por temperatura baja y radiación UV-B induce una mayor expresión del gen *F3H*, comparado con el estrés inducido por cada uno de los dos factores de estrés por separado.

Los mayores niveles de expresión de los genes *MYB, F3´5´H* y *DFR* en tallos de plantas de pimiento morrón se presentan a las 16:00 h, por lo que el factor de transcripción MYB podría ser regulador específico de los genes *F3´5´H* y *DFR*. Mientras que la expresión del gen *F3H* se incrementa durante el día con un punto máximo durante la madrugada (04:00 h) para reducir posteriormente sus niveles de expresión al inicio del día (11:00 h). Por lo que la expresión de estos genes podría estar regulada por el ciclo circadiano.

CAPÍTULO 7. PERSPECTIVAS Y RECOMENDACIONES

Debido a que las hojas de plantas de pimiento morrón sometidas a estrés por frío son más susceptibles al daño por radiación UV-B, es necesario buscar alternativas tecnológicas con las cuales reducir la incidencia de este tipo de radiación en plantas de pimiento morrón, especialmente cuando existe una reducción de la temperatura ambiental.

Se recomienda continuar con investigaciones que conduzcan a inducir la producción de flavonoides dihidroxilados, tales como luteolina-7-glucosido, en las hojas, y antocianinas en los tallos de plantas de pimiento morrón, previo a un descenso de temperatura, lo cual reduciría los efectos deletéreos de la combinación de estrés por frío y radiación.