AMANDA MENDES FERNANDES

De novo SHOOT ORGANOGENESIS AND LEAF DEVELOPMENT IN *Passiflora edulis* SIMS.: A MORPHO-PHYSIOLOGICAL AND MOLECULAR APPROACH

Dissertation submitted to the Universidade Federal de Viçosa, as part of the requirements of the Plant Physiology Graduate Program, to obtain the title of *Magister Scientiae.*

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APPROVED: February 21, 2009.

Diego Ismael Rocha

Diego Silva Batista

Márcia Adriana Carvalho dos Santos

Wagner Campos Otoni
(Advisor)

DEDICATION

To my parents, Eleuza and Waldevi, and my siblings, Bruno e Lara, without whc my career in science wouldnever havebeen possible. To my beloved husbar Fellipe, who has granted me with immeasurable love and support: you are my precious gift!

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. — Marie Curie

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BIOGRAPHY

AMANDA MENDES FERNANDES, daughter of Waldevi Mendes Dias and Eleuza Fernandes da Silva, was born in Montes Claros-MG, on February 06, 1993. She completed her Bachelor's degree in Biological Sciences in 2016, by the State University of Montes Claros. In 2017, she joined the Graduate Program in Plant Physiology of the Federal University of Viçosa, at Master's level, submitting herself to the dissertation defense on February 21, 2019.

SUMMARY

ABSTRACT

FERNANDES, Amanda Mendes, M.Sc., Federal University of Viçosa, February, 2019. ***De novo*** **shoot organogenesis and leaf development in *Passiflora edulis* Sims.: a morpho-physiological and molecular approach.** Advisor: Wagner Campos Otoni. Co-advisors: Lorena Melo Vieira and Priscila Oliveira Silva.

Leaf development is a coordinate process that involves a plethora of events and signaling molecules. *Passiflora edulis* Sims is a tropical herbaceous plant and one of the most economically important species of the family Passifloraceae. *P. edulis* presents a dramatic change in leaf morphology during its development, which distinguishes the juvenile from the adult phase. Although some studies have characterized leaf morphology and some molecular aspects associated with developmental events, there is still a lot to be clarified in the scope of leaf morphogenesis in this species. Therefore, this study aimed to reveal morpho-physiological and molecular mechanisms modulating leaf development and shoot organogenesis in *P. edulis.* The first chapter evaluated the influence of regulators added to the culture media on morphogenesis and the expression of the *miR156/SPL* pathway in *P. edulis* leaves induced *in vitro.* As a result, we found that phytohormones modulate *in vitro* responses of cotyledonary, leaf, apical and nodal explants and that for greenhouse-grown plants, the late physiological condition in leaf explants is a limiting factor for inducing organogenic responses. Also, we produced evidence that the cytokinin:auxin balance up-regulated the expression of *miR172,* suggesting that this gene is regulated by multiple hormones. The second chapter investigated the effect of cytokinin on the expression of miRNAs and *SPL9* and their impact on organogenesis of cotyledonary explants of *P. edulis.* The results achieved in this study point BA as an inhibitor of the expression of *miR319* and *miR164*; thus, we propose a novel link between cytokinin and *miR164* as a key regulatory output of cytokinin in the regulation of SAM function. Lastly, the third chapter evaluated physiological, structural and biochemical properties of *P. edulis* leaves with the aid of hyperspectral and biochemical analyses. As a result, we estimated changes over the course of leaf age and development. Leaf ontogeny affected biochemical, structural and physiological parameters, and some vegetation indexes were shown to be good predictors of pigment levels in leaves of *P. edulis.*

RESUMO

FERNANDES, Amanda Mendes, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. ***De novo* shoot organogenesis and leaf development in *Passiflora edulis* Sims.: a morpho-physiological and molecular approach.** Orientador: Wagner Campos Otoni. Coorientadoras: Lorena Melo Vieira and Priscila Oliveira Silva.

O desenvolvimento de folhas é um processo coordenado que envolve uma grande variedade de eventos e moléculas de sinalização. *Passiflora edulis* Sims é uma herbácea tropical e uma das espécies economicamente mais importantes da família Passifloraceae. *P. edulis* apresenta uma mudança dramática na morfologia das folhas durante o seu desenvolvimento, o que distingue a fase juvenil da fase adulta. Embora alguns estudos já tenham caracterizado a morfologia foliar e alguns aspectos moleculares associados a eventos de desenvolvimento, ainda há muito a ser esclarecido no âmbito da morfogênese foliar nessa espécie. Portanto, objetivo deste trabalho foi revelar os mecanismos morfo-fisiológicos e moleculares que modulam o desenvolvimento foliar e a organogênese das folhas em *P. edulis*. O primeiro capítulo avaliou a influência de reguladores adicionados ao meio de cultura sobre a morfogênese e a expressão da via miR156/SPL em folhas de *P. edulis* induzidas *in vitro*. Como resultado, observamos que os fitormônios modulam respostas *in vitro* em explantes cotiledonares, foliares, apicais e nodais e que, para plantas cultivadas em casa de vegetação, a condição fisiológica tardia em explantes foliares é um fator limitante para induzir respostas organogênicas. Além disso, produzimos evidências de que o balanço citocinina: auxina equilibra a expressão de miR172, sugerindo que esse gene é regulado por múltiplos hormônios. O segundo capítulo investigou o efeito da citocinina na expressão de miRNAs e SPL9, bem como seu impacto na organogênese de explantes cotiledonares de *P. edulis*. Os resultados alcançados neste estudo apontam BA como inibidor da expressão de miR319 e miR164. Assim, propomos uma nova ligação entre a citocinina e o miR164 na regulação da função do meristema apical caulinar. Por fim, o terceiro capítulo avaliou as propriedades fisiológicas, estruturais e bioquímicas das folhas de *P. edulis* com o auxílio de análises hiperespectrais e bioquímicas. Como resultado, verificou-se que a ontogenia foliar afetou parâmetros bioquímicos,

estruturais e fisiológicos, e alguns índices de vegetação mostraram-se bons preditores dos níveis de pigmentos foliares em *P. edulis*.

GENERAL INTRODUCTION

Stem cells have the ability to acquire competence and assume a new developmental fate, giving rise to a completely new organ. This characteristic is fundamental for the establishment of plant regeneration systems and it relies on another important characteristic, pluripotency (Verdeil et al., 2007; Rocha et al., 2015). The establishment of efficient *in vitro* regeneration systems require an explant source that exhibits some developmental plasticity, which might be enhanced by phytorregulators (Rocha et al., 2018). As a result, different morphogenetic pathways might be induced. The most commonly induced pathway in experimental plant regeneration system is *de novo* shoot organogenesis (Davey & Anthony; 2010; Duclercq et al. 2011), which is based on the formation of ectopic shoot or root apical meristems in a monopolar pattern **(**Rocha et al., 2018**)**.

Different explant sources may be cultured in order to induce plant regeneration pathways. Meristematic explants, for instance, apical and nodal segments, are regenerated through the development of preexisting buds, whereas non-meristematic explants, such as leaf and cotyledonary segments, are regenerated through *de novo* organogenesis (Rocha et al., 2018**)**. Also, young juvenile tissues are usually the material of choice for optimum efficiency of plant regeneration, since mature somatic tissues may have a low regenerative capacity (Elhiti & Stasolla, 2011). For both meristematic and non-meristematic explant sources, the acquisition of organogenic competence, organ growth, and development involve phytohormone perception, cell division, and transdifferentiation (De Klerk et al. 1997; Dhaliwal et al., 2003; Fehér et al. 2003; Ikeuchi et al., 2016, 2019). Since the classical breakthrough of Skoog and Miller (1957), much attention has been paid to the effect of phytohormones on regenerative processes, especially the antagonistic effects of auxins and cytokinins (CKs) on apical meristem regeneration.

In general, phytohormones are involved in nearly all, if not all, developmental processes (Leyser, 1998; Wolters & Jurgens, 2009), with highly complex regulatory networks (Santner & Calderon-Villalobos, 2009; Xiong et al., 2009). Within these sophisticated signaling mechanisms, microRNAs (miRNAs) have emerged as critical components in the phytohormone regulation. miRNAs are small non-coding RNA sequences that repress their target expression through post-transcriptional

repression (Voinnet, 2009). These small molecules are important players in a wide range of developmental processes, including phase transitions, organs morphogenesis, and cell division (Voinnet, 2009), as their involvement in the crosstalk with phytohormones modulate developmental processes, affecting plant metabolism, distribution, and perception (Hibara et al., 2003; Guo et al., 2005; Koyama et al., 2010;Curaba et al., 2014). In fact, many studies have demonstrated that a diverse range of miRNAs is affected by plant growth regulators (PGRs)(Achard et al., 2004; Sunkar &Zhu, 2004; Guo et al., 2005; Liu et al., 2009; Marin et al., 2010; Yoon et al., 2010; Chen et al., 2012; Zuo et al., 2012; Srivastava et al., 2013).

It has been demonstrated that the crosstalk between phytohormontes and miRNAs is also of fundamental importance for leaf ontogenesis, especially to a particular processes that describes abrupt changes in leaf morphology marking the transition from the juvenile to the the adult phase - heteroblasty (Tsukaya, 2013; Poethig, 2010; Huijser& Schmid, 2011; Yu et al., 2015, Nguyen et al., 2017). *Passiflora edulis* Sims (yellow passionfruit) is a heteroblastic species, in which juvenile leaves are lanceolate, monolobed and have smooth margins, while the adults are trilobed and have serrated margins (Cutri et al., 2013; Chitwood & Otoni, 2017a, 2017b). Previous work in our laboratory has demonstrated that the expression dynamics of the via *miRNA156/SPL* and *miR172* is altered throughout *P. edulis* leaf development (Silva et al., 2019). However, little is known on the role of auxin:cytokinin in leaf *de novo* formation and development *in vitro* in *P. edulis*.

Although heteroblastic changes are conspicuous and easily detected over leaf development, there has been a huge misunderstanding on the concepts of heteroblastic and ontogenetic changes. As a result, species that undergo only ontogenetic alterations might be classified as heteroblastic (Zotz et al., 2011). In this sense, *P. edulis*is a tropical plant that emerges as a good candidate for new model heteroblastic species due to its well-defined heteroblastic phases (Cutri et al., 2013; Chitwood & Otoni, 2017a, 2017b, Silva et al., 2019). Therefore, when its molecular, physiological, structural and biochemical characteristics are fully characterized, this species might serve as a reference to the classification and validation of other species. In this regard, the development of simple, rapid and

non-destructive methods is extremely necessary, as it will aid the achievement of leaf-related information in a cost-effective manner.

In this scenario, hyperspectral remote sensing arises as a promising technique, as previous studies have successfully used it estimate foliar chemical content, generating unambiguous and accurate estimates (Peñuelas & Filella, 1998; Jacquemoud & Ustin, 2008). This is only possible due to leaf components interaction (absorption, reflection, and transmittance) with radiation, with makes possible that different regions of the spectrum are associated with leaf biochemical or biophysical features (Asner & Martin, 2008; Ustin & Gamon, 2010; Meerdink et al., 2016). Therefore, the advancement of remote sensing technology has created an enabling environment for the development of algorithms and tools for high-quality assessment of plant status through spectral analyses (Xue & Su, 2017), here represented by vegetation indexes, which allow a series of estimations of plant biochemical, structure and morpho-physiological properties (Baek & Cho, 2016; Gitelson et al., 2002; Guo & Trotter, 2006; Ustin et al., 2009; Vandvik & Birks, 2002; Xue & Su, 2017).

The phenomena of leaf development, especially leaf heteroblasty,has stood out in the scope of scientific reports, resulting in a progressive characterization of molecular mechanisms, including the crosstalk between miRNAs and plant growth regulators. However, their implications in leaf development in non-model species, *e.g. P. edulis*, are still largely unexplored. In addition to molecular aspects, physiological, structural, and biochemical properties as affected by leaf development remain an interesting field to be explored.

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Chapter I

Auxin:cytokinin balance, explant type, and miR156/miR172 expression on *in vitrode novo* organogenesis of *Passiflora edulis* Sims.

ABSTRACT

During their lifespan, some plants, such as *Passiflora edulis*, experience a dramatic alteration in leaf morphology, reflecting the transition from the juvenile to the adult phase. This process has been shown to involve a wide number of signaling molecules, *e.g.*phytohormones and miRNAs; thus, raising a great interest into better characterizing the role of these molecules in heteroblasty and leaf developmental processes in a broader perspective. Considering that the role of the auxin:cytokinin balance on juvenile-adult phase transition remains unexplored for non-model species, such as *P.edulis*, we aimed to evaluate the influence of regulators added to the culture media on morphogenesis and the expression of the *miR156* and *miR172* in leaves induced *in vitro.* For this, we evaluated the effect of different combinations of growth regulators on the induction of adult characteristics using cotyledonary, apical and nodal explants obtained from *in vitro*-derivedseedlings and leaf explants from greenhouse-grown plants. Furthermore, we assessed the expression levels of *miR156* and*miR172* in monolobed and trilobed leaves grown induced in cotyledonary explants exposed to different combinations of phytohormes. As a result, we demonstrated that the *in vitro* responses of tissues are influenced differently by phytohormones and that for greenhouse-grown plants, the physiological condition in a major factor regarding the induction of organogenesis. In addition, we demonstrated that the cytokinin:auxin balance up-regulated the expression of *miR172* in trilobed leaves *in vitro*, indicating that this gene is modulated by multiplephytohormones.

Keywords: leaf morphogenesis, heteroblasty, phytohormones.

INTRODUCTION

After germination, two transition stages occur in plants: juvenile to adult and adult to reproductive (Bäurle & Dean, 2006). The transition from juvenile to adult stage is marked by changes in leaf morphology and acquisition of reproductive competence (Poethig, 2010). Throughout their life cycle, plants continue to develop new organs such that the morphological characteristics change according to the morphological stage. As a result, some plant species exhibit significant and dramatic changes in leaf morphology, reflecting the transition from the juvenile to the adult phase during the vegetative period (Tsukaya, 2013; Poethig, 2010; Huijser and Schmid, 2011). This phenomenon is known as heteroblasty and has sparked interest among research groups that have investigated what mechanisms and genes could regulate these changes.

Physiological and genetic studies have shown that both phase transitions in plant cycle are regulated not only by exogenous factors such as day length, light intensity and, temperature, but also by endogenous signals transmitted by age and phytohormones (Yu et al., 2013). Also, it has been demonstrated that the alteration in the production and perception of phytohormones, *e.g*. auxins and cytokinins, modulates leaf development, a highly complex and coordinated process (Shimizu-Sato et al., 2009). Besides their relevant role in regulating plant development, phytohormones also control the maintenance of the shoot meristem and also determine leaf complexity and shape (Capron et al., 2009; Byrne, 2012; Tsukaya, 2013).

In spite of being relatively recent, the characterization of molecular mechanisms in heteroblasty has been progressively successful (Tsukaya, 2013; Yu et al., 2015; Nguyen et al., 2017). In this scenario, microRNAs, small RNAs with a regulatory function on their target genes, play decisive roles in the activity of the shoot meristem, development of leaf primordia, establishment of leaf dorsiventrality and transition of plant vegetative-reproductive phase (Mallory And Vaucheret, 2006; Nogueira et al., 2007; Kidner & Timmermans, 2010; Nonogaki, 2010; Yu et al., 2013). miRNAs negatively regulate specific genes through degradation of target messenger RNAs (Llave et al., 2002; Xie et al., 2003), blocking of translation or regulation of post-transcription (Brodersen et al., 2008).

Despite their relatively recent description in plants, miRNAs are known to play critical roles in development. Many of the miRNA target genes encode proteins and transcription factors that are critical in gene regulation networks (Jones-Rhoades et al., 2006).

The morphological modifications occurring in the leaf along the transition from the juvenile-adult vegetative phase are mediated mainly by *miR156* and *miR172* (Wang et al., 2011). These miRNAs are targeted in the present study, since it has been suggested that *miR156* is an evolutionarily conserved regulator during vegetative phase change in herbaceous and woody plants, whereas *miR172* is a regulator of the reproductive phase (Wu et al., 2009; Chen et al., 2010; Wang et al., 2011; Yu et al., 2013; Levy et al., 2014). By inhibiting transcription factors (TFs) of the family *SQUAMOSA PROMOTER-BINDING LIKE PROTEINS* - SPLs (Schwab et al., 2005; Wu & Poethig, 2006), *miR156* serves as a key component to the maintenance of the juvenile phase, shown to be highly expressed early in the development of *Arabidopsis thaliana* and to have expression levels decreased over the juvenile phase(Wu et al., 2009;Yu et al., 2013; Nguyen et al., 2017). On the other hand, *miR172* is associated with the adult phase, since it has an opposite pattern of expression and has its abundance increased gradually, as *miR156* levels decrease (Wu et al., 2009; Nguyen et al., 2017).

The involvement of phytohormones in heteroblastic changes is well-established, as reported for gibberellic acid, abscisic acid and jasmonates (Robbins, 1957; Rogler & Hackett, 1975). On the other hand, a wide number of studies have shown that the levels of several miRNAs are affected by PGRs, for instance, abscisic acid up-regulates miR393 in *Arabidopsis thaliana* and down-regulates miR167 and *miR319* in *Oryza sativa* (Sunkar & Zhu, 2004; Liu et al., 2009); auxin up-regulates miR390 (Guo et al., 2005; Yoon et al., 2010) and down-regulates *miR156* in *Arabidopsis thaliana* (Marin et al., 2010), and it up-regulates *miR164* in *Brassica juncea* (Srivastava et al., 2013); cytokinin down-regulates *miR172* and *miR319* in *Oryza sativa* (Liu et al., 2009)*;* ethylene down-regulates miR159a in *Medicago truncatula* (Chen et al., 2012) and *miR164*a/b/c, *miR319*, miR390 and miR396ain *Solanum lycopersicum (*Zuo et al., 2012); GA up-regulates miR159 in *A. thaliana*

(ga1-3 mutant) (Achard et al., 2004) and JA up-regulates *miR319* in and *B. juncea* (Srivastava et al., 2013).

In *Passiflora edulis* Sims (yellow passionfruit), phase change is determined by heteroblasty (Chitwood & Otoni, 2017a; 2017b), which in turn is regulated by endogenous factors, such as PGRs, transcription factors (TFs) and miRNAs. Despite the evidence that confirms the role of endogenous factors in the expression profile of *miR156* and *miR172* and their target genes (*SPL*s), little is known on the role of auxin:cytokinin in leaf *de novo* formation and development *in vitro* in *P. edulis*.

Passiflora edulis is an herbaceous climbing plant and one of the most economically important species of the family Passifloraceae due to fruit nutritional value, pharmaceutical properties, and flower ornamental interest (Maluf et al., 1991; Vanderplank, 1991). In addition to the economic relevance, *P. edulis* presents a remarkable heteroblastic pattern which contributed to the selection of the species as an object of study, along with the fact that it results from a consolidated experience of our research group on tissue culture, genetic transformation and gene expression (Otoni et al., 2013, Rocha et al., 2019). Moreover, *P. edulis* shows morphological characters demonstrating a clear juvenile-adult phase change, facilitating the evaluation of adult-characteristic induction and the effect of treatments on plant development. Juvenile leaves are lanceolate, monolobed and have smooth margins, while the adults are trilobed and have serrated margins (Cutri et al., 2013; Chitwood & Otoni, 2017a, b). Furthermore, as the leaf transition becomes more complex, a meristem capable of generating tendril and flower is formed in the leaf axils, and the emergence of the tendril determines the beginning of the adult vegetative phase in *P. edulis* (Nave et al. al., 2010; Cutri et al., 2013; Sousa-Baena et al., 2018).

Although several *in vitro* studies have been established for *Passiflora* species, to our best knowledge, none of them have considered the role of auxin:cytokinin balance on juvenile-adult phase transition, remaining an interesting field to be explored. Therefore, the aim of this study was to evaluate the influence of regulators added to the culture media on morphogenesis and expression of the *miR156*/SPL pathway in *P. edulis* leaf heteroblasty and to provide insights for a

better understanding of the modulatory mechanisms of juvenile-adult phase transition, as well as its physiological, genetic and molecular mechanisms.

MATERIALS AND METHODS

Conduction of in vitro experiments

In vitro experiments were carried out at the Plant Tissue Culture Laboratory, located at the Institute of Biotechnology Applied to Agriculture - BIOAGRO, Federal University of Viçosa (UFV).

Plant material

For induction of *in vitro* organogenesis, plantlets derived from mature seeds of *Passiflora edulis* population FB300, provided by "Viveiros Flora Brasil Ltda." (Araguari, MG, Brazil, http://www.viveiroflorabrasil.com.br), were used as explantsources.Seeds were de-coated using a mini-vice, and further surface-sterilized in a laminar flow chamber using 70% (*v/v*) ethanol for 1 min followed by immersion in commercial sodium hypochlorite (2.5% *v/v*) with two drops of 0.1% (*v/v*) Tween-20 for 15 min. Subsequently, seeds were submitted to a triple rinse in sterile deionized water. Ten seeds were inoculated into each flask (250 mL capacity) containing 60 mL of MS medium (Murashige & Skoog, 1962) with half-strength nutrient solution, MS vitamin complex, 1.5% sucrose (w/v), 0.005% myo-inositol (w/v), and added with 0.25% gelling agent (w/v) (Phytagel®, Sigma Chemical Company, MO, USA), and pH was adjusted to 5.8 prior to autoclaving. Flasks were sealed with a rigid polypropylene lid with two vents (10 mm diameter) covered with a 0.45 μm size pore membrane - 25 μL CO_2 L^{-1} s^{-1} exchange rate (Milliseal®, AVS-045 Air Vent, Japan). The culture media were autoclaved at 120 °C, 1.1 Pa for 20 min. Seeds were kept in the dark at 27 ± 2 ºC, for 15 days. Lastly, flasks were transferred to a growth room, with a photoperiod of 16/8h (light/dark), under irradiance of 50 μmol m^{-2} s^{-1}, provided by two LED lamps with 17W (Vilux®, 27 LLTV03, Brazil) for 15 days.

Effect of different combinations of growth regulators and explant type on the induction of adult characteristics via in vitro *organogenesis*

We evaluated the effect of different combinations of growth regulators on the induction of adult characteristics through the development of *de novo* shoot organogenesis (DNSO), provided by cotyledonary explants, and preexisting

meristems, provided by apical and nodal explants of *P. edulis*. Explants were obtained from 30-d-old seedlings, as previously described.

Explants were inoculated into flasks (250 mL capacity) containing 60 mL of MS medium with full-strength nutrient solution, MS vitamin complex, glucose 44 mM, 0.01% myo-inositol (w/v), and added with 0.25% gelling agent (w/v) (Phytagel®, Sigma Chemical Company, MO, USA). Subsequently, phytohormones were added to the media (Table 1) and pH was adjusted to 5.8 prior to autoclaving. Flasks sealing, media autoclaving and incubation conditions were similar to the aforementioned.

Subsequent to autoclaving, all medium treatments were supplemented with 10 µM silver thiosulfate (STS) in order to prevent ethylene sensitivity in the explants by interacting with the ethylene receptors(Zhao et al., 2002; Reis et al., 2003). Under aseptic conditions, cotyledonary-derived explants (average 6 mm^2) were incubated with the abaxial side facing the medium. The shoot apices and nodal (30 mm) segments were incubated vertically according to the auxin polarity. The explants were incubated in a growth room under irradiance of 50 µmol m^{-2} s^{-1} at 27 ± 2 ºCuntil the end of the experiment.

Table 1: Combination of phytohormonesused in the organogenesis from cotyledonary explants, apical and nodal segments of *Passiflora edulis*.

Treatment	Kinetin (µM)	IAA (µM)	BA (µM)
A1	-	-	-
A2	10	5	20
A3	5	2.5	10
A4	5	5	10
A5	5	2.5	20
A6	10	2.5	10
A7	-	-	4.4

Abbreviations: IAA: indolil-3-acetic acid; BA: benzyladenine).

After 30 days of culture in the media described above, the following variables were evaluated: percentage of responsive explants (PRE) for cotyledonary explants and shoot number per explant (SNE) for apical and nodal explants, percentage of explants presenting monolobed (PML), intermediate (PIL), and trilobed leaves (PTL), leaf area per explant (LAE), and total leaf number per explant (TLE). For leaf area analysis, leaves were detached and fixed individually in white millimetric paper. Digital camera photos were captured and the resulting images were analyzed in the ImageJ software(Schneider et al., 2012).

The experiment using cotyledons as explant sources was conducted in a completely randomized design (CRD) with seven media formulations (A1 - control, A2, A3, A4, A5, A6, A7) and five replicates per treatment, each replicate consisting of a flask containing 10 explants. The experiment using apical and nodal segments wasalso set up in a CRD, in a factorial scheme 2x7 (sources of explant x phytohormone concentration).

Effect of different combinations of growth regulators and physiological condition on the induction of adult characteristics via* in vitro *organogenesis

To obtain the *ex vitro* explants, seeds were germinated in plastic pots (5 L capacity), containing commercial substrate (Plantmax®). Irrigation was done daily in order to keep soil moisture close to field capacity. Plants were periodically fertilized and grown in a greenhouse at the Department of Plant Biology of the UFV.

Twenty plants were selected, from which expanded 3rd (monolobed), 9th (intermediate) and 12th (trilobed) leaves were collected. After collection, leaves were disinfested by immersion in 70% (v/v) ethanol for 1 min, followed by immersion in commercial sodium hypochlorite (1.25% v/v) with 3 drops of0.1% Tween-20 (v/v)for 15 min, followed by a triple rinse in distilled sterile water, immersion in Plant Preservative Mixture (PPM)solution and rinse in distilled sterile water. Leaf explants (10 mm) were obtained by sectioning the midvein of the middle lobule and then, the explants were inoculated into test tubes containing 10 mL of culture medium as described previously. After 60 days of culture, the same variables described previously were evaluated.

The experiment was conducted in a CRD in a factorial scheme 3x4: leaf explants with different ontogenetic ages (monolobed, intermediate and trilobed leaves) x culture media treatments (A1, A4, A6, A7) with 5 replicates containing 10 tubes with one explant each.

Expression profile of miR156 and miR172

Sample collection and preparation

In order to analyze the expression of the *miR156* and *miR172*, total RNA extraction was carried out on samples collected from monolobed and trilobed leaves originated from cotyledonary explants cultivated for 30 days in media A3, A4, A6, and A7. The plant material was collected under RNAse-free condition. The collected samples were immediately frozen in liquid nitrogen and stored at -80 °C until the moment of use.

RNA extraction

Total RNA was isolated using the TRI-Reagent® solution (Sigma-Aldrich, St. Louis, MO, USA) and treated with DNAse I (Thermo Scientific NanoDrop Technologies, Wilmington, DE, USA), according to manufacturer's recommendations. RNA quantification was performed with a NanoDrop™ 2000/2000c spectrophotometer (NanoDrop Technologies) and the integrity of the RNA samples was verified by 1.5% agarose gel electrophoresis (RNAse free). Subsequently, all samples were treated with DNAse I RNAse-Free (Ambion-Life Technologies, CA, USA), according to manufacturer's recommendations, and RNA quantification, as well as integrity, were verified again.

Table 2: Primer sequences used in the PCR reactions.

Primer Name	Sequence
miR156 F	5’CCTGAGTGACAGAAGAGAGTG 3’
miR156 RT	5’GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCTCT 3’
miR172 F	5’CCTGAGAGAATCTTGATGATG 3’

miR172 RT	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACATGCAG 3'
Reverse Universal	5'GTGCAGGGTCCGAGG 3'

cDNA synthesis via '*pulsed-stem loop'* RT-PCR

cDNA synthesis was performed using the *Pulsed stem-loop* RT-qPCR methodology (Varkonyi-Gasic et al., 2007) from 500 ng of total RNA. Then, 1 µL of dNTP (10 mM), 1 µL (1 mM) of the mature miRNA-specific RT primer and 1 µL of oligodT (500ng/µL) were added together with the total RNA treated with DNAse I. The samples were incubated at 70 °C for 10 min for denaturation of the secondary structures and then incubated at 4 °C for 5 min. Then, 4 µL of the 5x RT buffer and 1 µL of the MMLV Reverse Transcriptase enzyme (Ludwig Biotec®, Alvorada, Brazil) were added to the samples. Reactions were incubated at 16 °C for 30 min, followed by reverse transcription of 60 cycles of 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 sec. For inactivation of the enzyme, the reaction was incubated at 85 °C for 5 min. Subsequently, the reactions were stored at -20 °C.

RT-qPCR analysis

The expression profile of the *miR156* and *miR172* genes was analyzed via RT-qPCR on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). All RT-qPCR samples were run using 20 ng of cDNA, 400 nM of each primer, and qPCR SYBR-Green mix/Rox (Ludwig Biotec®, Alvorada, Brazil) and diethylpyrocarbonate-treated water to a final reaction volume of 10 µL. The constitutive gene *actin* was used as a normalizer. The PCR program was as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 16 s at 95 °C and 1 min at 60 °C, and 15 s at 95 °C, 1 min at 60 °C, 30 s at 95 °C and 15 s at 60 °C. Transcription levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primers used are listed in Table 2.

Statistical analyses

Statistical analyses were performed using GENES software (Cruz, 2013). Percentage values were transformed to arcsin $\sqrt{x}$ before analysis. Data were processed by analysis of variance (ANOVA) followed by Tukey's test at a 5% significance level. For the analysis of gene expression via RT-qPCR, three biological and two technical replicates were used and means were compared by Dunnett's test at a 5% significance level.

RESULTS

Effect of different combinations of growth regulators and explant type on the induction of adult characteristics via in vitro organogenesis

In the present study, different leaf developmental stages were commonly observed within the same explant (Fig. 1A). Furthermore, adult traits similar to the ones observed in greenhouse grown*P. edulis* individuals were also observed *in vitro* for leaves induced in cotyledonary explants (Fig. 1A-D). Interestingly, when nodal explants from greenhouse-grown plants were cultured in media supplemented with phytohormones, asynchronous development was also observed, but the adult leaves induced in this type of explant presented more pronounced adult characteristics (Fig. 1E, F).

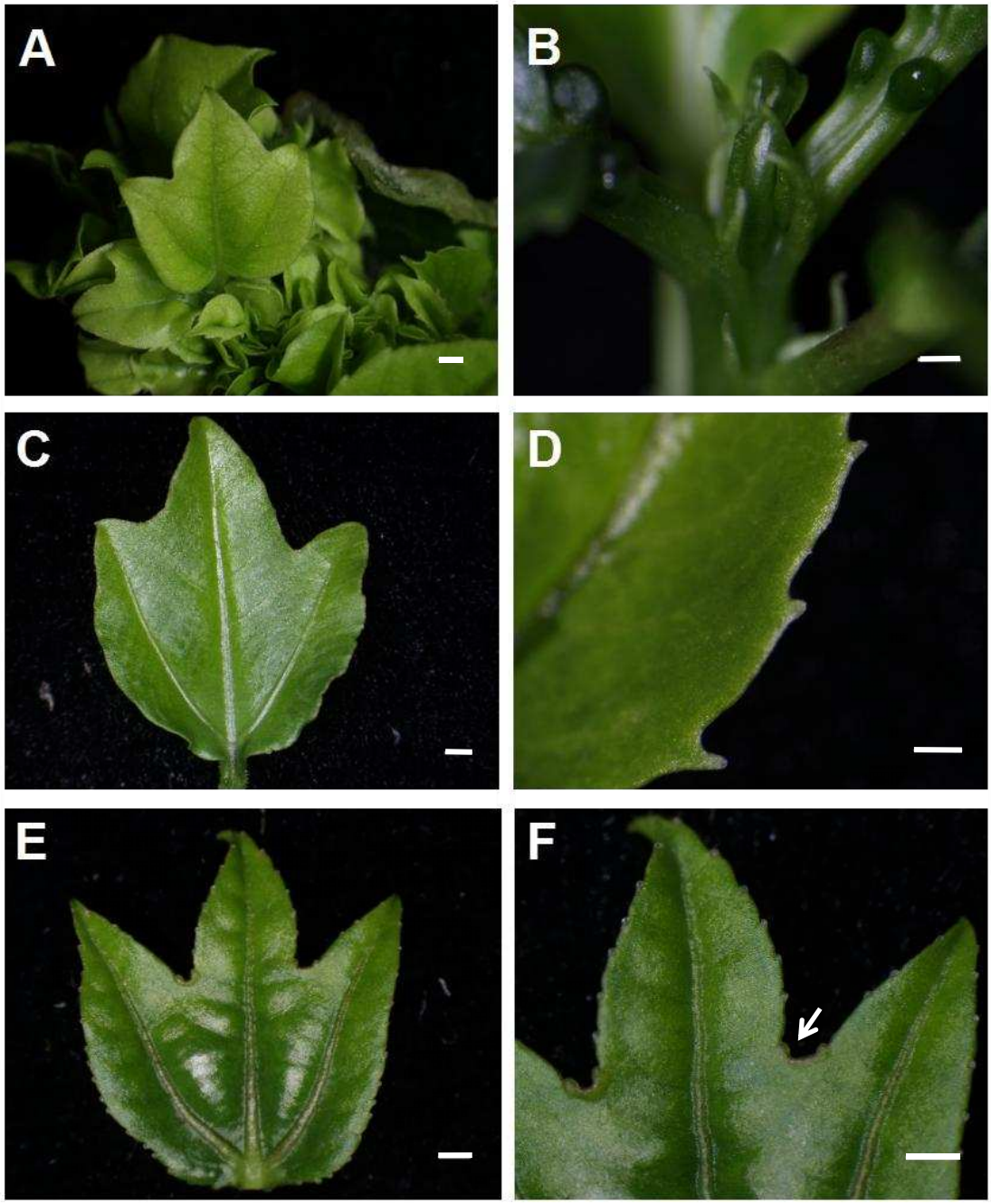

Figure 1 - Leaf morphology and transition markers of *Passiflora edulis* Sims. cultured*in vitro* under different phytohormone treatments. (A-D) Cotyledonary explants from *in vitro* grown seedlings; (E-F) Nodal segments from greenhouse-grown plants. (A) Adult leaf (trilobed); (B) *In vitro* leaves presenting nectaria on the petiole; (C) Isolated *in vitro* grown trilobed leaf; (D) Serration of an *in vitro* grown leaf (an adult trait); (E) Trilobed leaf presenting leaf serration; (F) Nectaria occurring on the flank of trilobed leaves. Bar= 4mm.

The control treatment (A1) - devoid of phytohormones - did not induce any of the organogenic responses evaluated forcotyledonary explants (Fig. 2A-F). The medium A2 promoted low percentage of responsive explants (PRE) and percentage of explants presenting monolobed leaves (PML), 36 and 10%, respectively. Furthermore, it did not induce adult traits (intermediate and trilobed leaves) on the cotyledonary explants, besides presenting decreased total leaf area per explant (TLE) in comparison to all the other media supplemented with phytohormones and decreased leaf area per explant (LAE), when compared to A5 and A7. The treatments A3, A4, A5, A6, and A7 presented the highest PRE, PML, percentage of explants presenting intermediate leaves (PIL) and TLE. Although presenting the same TLE as treatments A3, A4, A6, and A7, the culture medium A5 presented one of the highest leaf area values, indicating that this condition promoted larger leaves. Trilobed leaves were only observed in the explants cultured in media A3, A4, and A6, with the treatment A6 exhibiting the highest values in comparison to all of the other conditions.

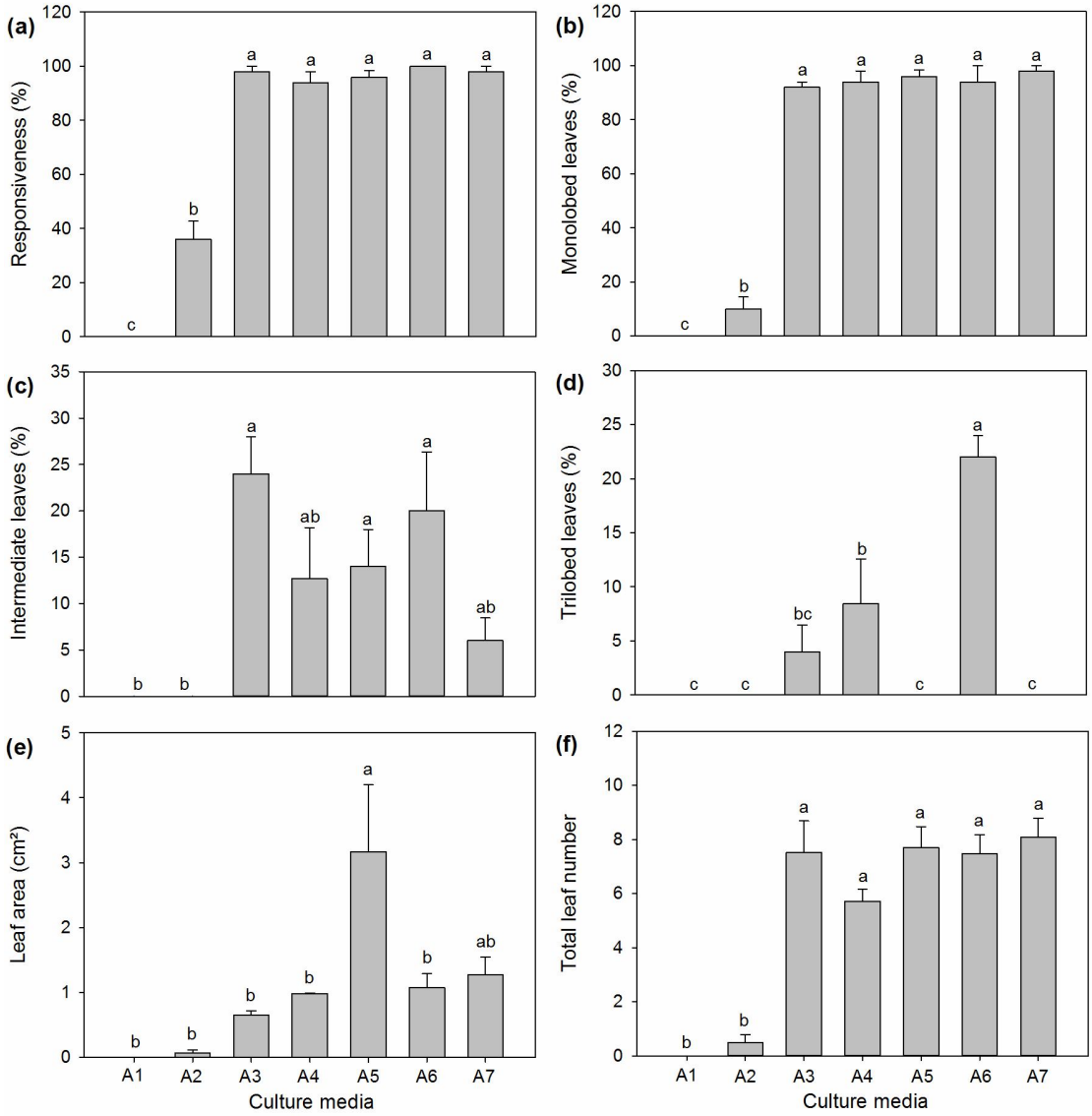

Figure 2 - Effect of different media on organogenic responses of cotyledonary explants of *Passiflora edulis*Sims. (a) percentage of responsive explants, (b) percentage of explants presenting monolobed leaves, (c) intermediate leaves, (d) and trilobed leaves, (e) leaf area per explant and (f) total leaf number per explant. Treatments followed by the same letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Regarding apical and nodal segments, a significant interaction between the factors explant type and culture medium was evident for two of the variables that represent adult characteristics: percentage of explants presenting intermediate and

trilobed leaves (P=0.049; 0.040, respectively). Apical segments presented the highest frequency of intermediate leaves when exposed to media A3 and A7 (Fig. 3C). Although nodal segments did not differ statistically in relation to culture medium composition, it is worth to point out that neither explant types presented intermediate or trilobed leaves when cultured in media A1 and A5 (Fig. 3C, D). As observed for intermediate leaves, apical segments maintained a similar pattern in relation to trilobed leaves, also presenting the highest percentage when exposed to medium A7.

A similar pattern was also found when comparing the explant types, since nodal segments presented a higher frequency of intermediate and trilobed leaves in medium A4, but the opposite was observed for medium A7. On the other hand, apical segments presented higher PIL when exposed to medium A3, but the same was not observed for trilobed leaves.

The variables shoot number, percentage of monolobed leaves, leaf area and total leaf number per explant did not present significant interaction between the factors, but they were all significantly influenced by either explant type, culture media or both. Apical and nodal segments presented opposite organogenic responses when exposed to media A4 and A7. Whereas on medium A4, nodal segments presented increased shoot number per explant (SNE) in comparison to apical segments, on medium A7, the opposite occurred (Fig. 3A). For both explants, the medium treatments that promoted the highest SNE were A3, A4, A6, and A7.

When comparing the explant type, monolobed leaves were induced differently only in the media A6, where apical segments showed higher values. For both explant types, the medium A2 induced lower values (Fig. 3B). In addition, apical segments exhibited lower PML in the medium A3.

Except for medium A4, which induces superior TLE in nodal segments, leaf area and total leaf number did not differ between the explant sources, being verified mostly the effect of the culture media. Interestingly, both A1 (control) and A7, resulted in the highest LAE, for both explant types (Fig. 3E). Medium A7 was also responsible for the highest TLE in apical and nodal segments, not differing from medium A3 and A4 for nodal segments (Fig. 3F). Both apical and nodal segments

rooted in the control media (Fig. 6). On the other hand, rooting was not verified in any other treatment.

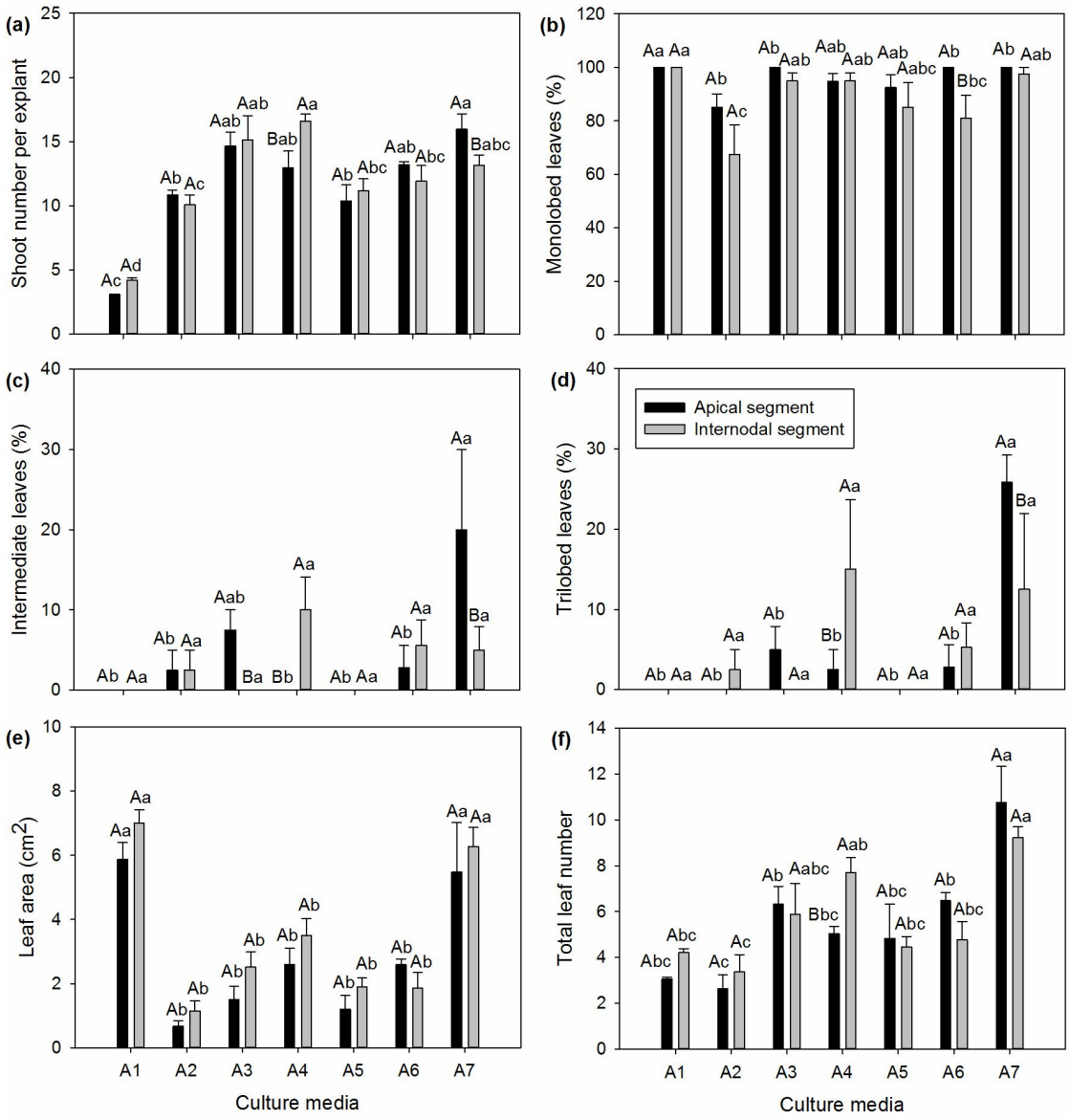

Figure 3 -Effect of different media and explant type (apical and nodal segments) on organogenic responses of *Passiflora edulis*Sims. (a) percentage of responsive explants, (b) percentage of explants presenting monolobed leaves, (c) intermediate leaves, (d) and trilobed leaves, (e) leaf area per explant and (f) total leaf number per explant. Explant sources followed by the same upper-case letters and culture media treatments followed by the same lower-case letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Effect of different combinations of growth regulators and physiological condition on the induction of adult characteristics via* in vitro *organogenesis

A significant interaction was verified for most organogenic responses assessed in this experiment: percentage of responsive explants, monolobed leaves, trilobed leaves, and total leaf number per explant (P= 0.0059, 0.0068, 0.0135, 0.0216, respectively). In general, monolobed and intermediate-type explant induced higher PRE, PML, PIL, and PTL in comparison to the trilobed leaf-type explant source (Fig. 4A-D). Not surprisingly, the control treatment did not induce any of the responses evaluated in this study. As the trilobed leaf explant responded poorly to culture media formulations in general, some medium combinations did not allow responses at all. Medium A4 and A7 did not induce monolobed, intermediate or trilobed leaves in trilobed leaf explants. In addition, medium A7 did not allow production of intermediate and trilobed leaves in intermediate leaf explants and media A6 did not induce trilobed leaves in trilobed leaf explants.

Analyzing the variables percentage of responsiveness and monolobed leaves (Fig.4 A,B), it was verified no significant difference as to the culture media within each type of explant, except for the trilobed leaf explant, which exhibited higher PRE and PML in the medium A6. Medium A6 was also the most beneficial treatment in relation to the induction of intermediate and trilobed leaves in monolobed-type leaf explants. On the other hand, culture media did not affect PIL and PTL in intermediate and trilobed-type leaf explants (Fig. 4C, D).

Crossing data information from the variables leaf area and total leaf per explant, a similar pattern is observed for monolobed and intermediate-type leaf explants, which do not differ statistically, except for TLE of intermediate leaf-type explants being lower than monolobed-type leaf explants. Nevertheless, trilobed-type leaf explants presented the lowest TLE and LAE cultured on treatments A4 and A7, in comparison to the other explant types, but in treatment A6, trilobed-type leaf explants produced the same LAE, indicating that it produced less but larger leaves (Fig. 4E, F). For both variables, there was no difference as to the LAE and TLE among the culture media treatments supplemented with phytohormones, having monolobed leaves as explant source. Intermediate-type leaf explants were similarly

influenced by culture formulations, except for medium A7, which promoted a lower number of leaves per explant.

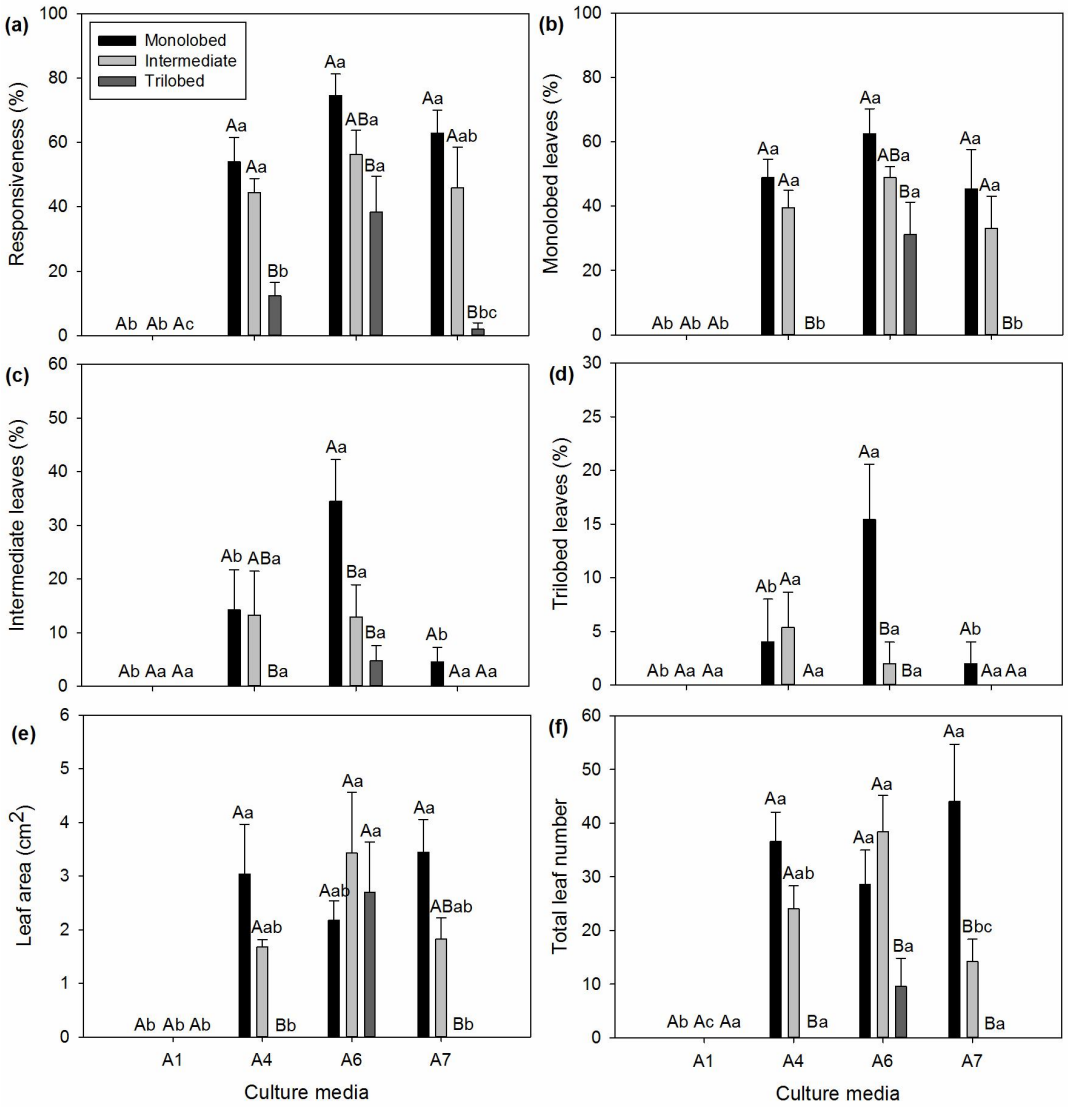

Figure 4 - Effect of different media and physiological condition of the leaf (monolobed, intermediate and trilobed leaf explant) on organogenic responses of *Passiflora edulis*Sims. (a) percentage of responsive explants, (b) percentage of explants presenting monolobed leaves, (c) intermediate leaves, (d) and trilobed leaves, (e) leaf area per explant and (f) total leaf number per explant. Explant sources followed by the same upper-case letters and culture media treatments followed by the same lower-case letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Expression profile of miR156 and miR172

The expression of miRNAs herein investigated was normalized by both monolobed and trilobed leaves in the medium A3, since this medium is used in the literature for the induction of organogenesis in *Passiflora* (Drew, 1991). Therefore, monolobed and trilobed leaves induced by cultivating cotyledonary explant in media A3, A4, A6, and A7 were collected and had their expression profiles for *miR156* and *miR172* evaluated (Fig. 4).

Expression levels of *miR156* in monolobed and trilobed leaves grown *in vitro* were not altered by medium combinations (Fig. 4A. On the other hand, the trilobed leaves collected from cotyledonary explants exposed to media A4 presented significantly increased expression of *miR172* (Fig. 4B).

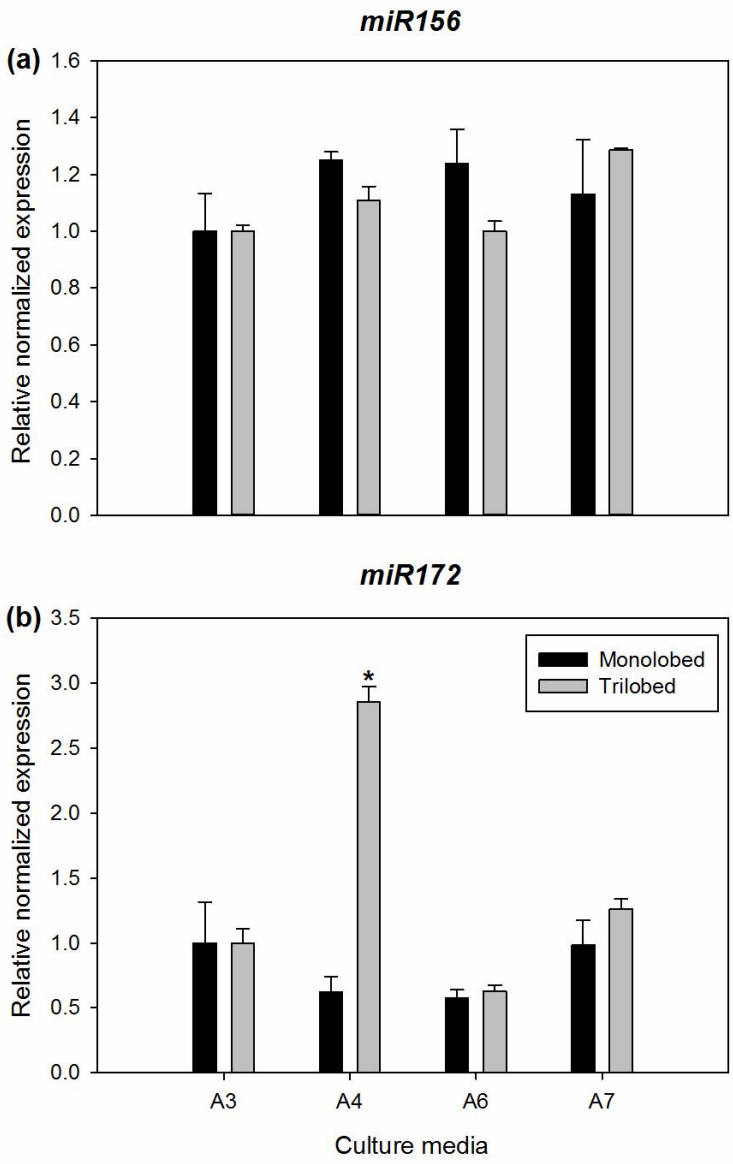
miR156
(a)
Relative normalized expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
miR172
(b)
Relative normalized expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Monolobed
Trilobed
A3
A4
A6
A7
Culture media

Figure 5 - Effect of different media on the relative expression levels of (A) *miR156* and (B) *miR172* in monolobed and trilobed leaves of *Passiflora edulis* Sims. induced *in vitro*. Treatments marked with an asterisk differ statistically to A3 medium by Dunnett's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

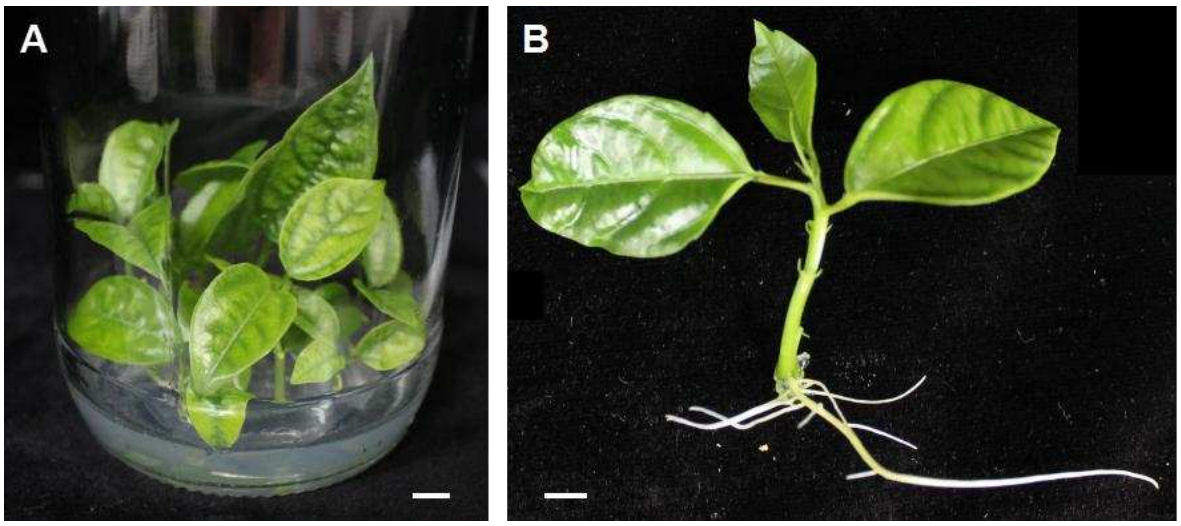

Figure 6 - Effect of the control medium on organogenic responses of apical segments of *Passiflora edulis*Sims. (A) Apical segments growing on control medium. (B) A rooted apical segment with evident large and monolobed leaves. Bar= 5mm.

DISCUSSION

Auxins and cytokinins are known to be the major phytohormes involved in the regulation of plant cell differentiation, and studies have demonstrated that the ratio between them is of utmost importance for the specification of cell identity during early stages of morphogenesis (Jimenez, 2005; Rocha et al., 2015). Furthermore, the use of cytokinins to promote shoot formation in species of *Passiflora* is well established, being the hormonal balance necessary for the differentiation process dependent on the concentration of endogenous auxins in different tissues of the explant (Dornelas & Vieira, 1994; Lombardi et al., 2007).

The ratio between auxin and cytokinin controls a variety of *in vitro* developmental processes (Skoog & Miller, 1957). According to the balance of these phytohormones, some species may even respond with completely different morphogenic responses and pathways (Dornelas et al., 1992; Jeannin et al., 1995; Wang et al., 2008; Zhang et al., 2010; Bouamama et al., 2011; Lu et al., 2013; Rocha et al., 2015).

In this work, shoot formation occurred through the development of preexisting meristems, from apical and nodal segments, and direct organogenesis, from cotyledonary explants. Here, we present the induction of adult traits from cotyledonary, nodal, and apical segments of *P. edulis* cultured in diverse *in vitro* media conditions, modulated mostly by the ration between auxin and cytokinin in the medium. We also describe the expression profile for monolobed and trilobed leaves regenerated from cotyledonary explants cultivated in different media conditions.

Explant source drive the organogenic responses induced by different auxin:cytokinin ratios

Passiflora edulis exhibits heteroblastic behavior: juvenile leaves are lanceolate while adult leaves are trilobed (Cutri et al., 2013), and an intermediate bilobed leaf between the juvenile-adult stage is usually observed. After a gradual transition, a tendril meristem, a small leaf, and a flower are formed in each node (Nave et al., 2010). Herein, different leaf developmental stages were commonly observed within the same explant (Fig. 5). The lack of synchrony in the organogenic process was

also reported in *P. edulis* f. flavicarpa (Dornelas & Vieira 1994; Appezzato-da-Glória et al., 1999; Biasi et al., 2000; Lombardi at al., 2007) and it may be caused by the achievement of competence and determination of different-stage cells (Lombardi et al., 2007).

We found that exposing the explants for 30 days to different combinations of phytohormones on the media induced adult traits *in vitro* (Fig. 5). Therefore, phytohormones can change the developmental program within a species, as demonstrated by Cutri et al. (2013), who after spraying *P. edulis*cv. 'Passion Dream' with commercial cytokinins (5-Phenylcarbamoylamino-1,2,3-thiadiazolea and Forchlorfenuron) increased the formation of flowers flanking a tendril primordium per node, showing that the number of flowers within a specific genotype can be modulated by applying cytokinins.

The highest frequency of adult characteristics was observed in cotyledons after 30 day-culture in medium A3 and A6, whereas apical segments presented better results in medium A7 and nodal segments in media A2, A4, A6 and A7 (Fig. 1C, D). Organogenesis did not occur in the absence of growth regulators in cotyledonary explants, and although it did occur in apical ad nodal segments, adult characteristics were not observed in this condition.

In *Passiflora edulis,* the addition of only one type of cytokinin to the culture medium generally promotes the expected regeneration response of buds (Fernando et al., 2007; Dias et al., 2009; Prammenee et al., 2011; Rocha et al., 2012), probably because the endogenous amount of auxin in the explant is sufficient to establish the necessary balance for differentiation (Schaller et al., 2015; Aremu et al., 2016). Considering the fact that in this study adult traits were not observed in treatments lacking both or only one of the phytohormones tested, we speculate that induction of adult characteristics in *P. edulis* is modulated by a balance between auxin and cytokinin.

In addition, organogenesis was clearly affected by the explant source used in *P. edulis* tissue culture, once different explant sources presented distinct responses. The induction of adventitious roots in the absence of exogenous auxins, as well as numerous and large leaves, may indicate the existence of significant levels of

endogenous auxins and cytokinins in apical and nodal segments from juvenile plants. However, the endogenous concentration of these phytohormones is not sufficient for induction of adult traits, being necessary the addition of exogenous phytohormones in a concentration that probably relates to the varying concentration of these substances in each explant type. Therefore, it might be hypothesized that endogenous phytohormones could have interacted with those present in the culture media, generating diverse and complex responses (Ćosić et al. 2015; Aremu et. al, 2016).

Physiological condition of the explant is limiting for the induction of adult characteristics via* in vitro *organogenesis

Trilobed-leaf explants revealedlower responsiveness, percentage of monolobed an intermediate leaves per explant compared to juvenile explants. Apparently, in this case, phytohormones treatments were not enough to reverse a later physiological state occurring in trilobed-leaf explants, which may be revealing a decrease in the content of auxins associated with loss of morphogenic capacity.

Studying the effect of age and physiological condition of *P. edulis* f. flavicarpa donor plants on *in vitro* morphogenesis of leaf explants, Becerra et al. (2004) verified gradual loss of morphogenic capacity as a function of physiological condition. Adult plants were reinvigorated by pruning and the new emerging leaves, which remained trilobed, were exposed to 4.44 μM benzyladenine (BA) and 2.32 μM kinetin. In agreement with the observations herein presented, Becerra et al (2004) also observed that both age and physiological state of the donor plant affected morphogenic capacity, in a sense that adult reinvigorated plants (trilobed leaves) and older plants presented lower shoot number per explant.

In the current study, the medium supplemented with 10 μM kinetin, 2.5 μM IAA and 10 μM BA (A6) was the only treatment capable of inducing monolobed and intermediate leaves in trilobed-leaf explant and it also induced superior percentage of intermediate and trilobed leaves in monolobed-leaf explants. The response of explants to high levels of cytokinin and low auxin was also reported by Drew (1991) and may have an application with other species where adult tissue is recalcitrant in culture.

Young juvenile tissues are generally desirable explant sources (Peña-Ramírez et al., 2010, Ćosić et al., 2015), since they are composed of cells at the beginning of their developmental path and are potentially totipotent (Dornelas et al., 1992; Elhiti & Stasolla, 2011). Vaz et al. (1993) compared protoplast release in leaves taken from glasshouse-grown plants prior and subsequent to the development of tendrils. It was found that protoplast yield was reduced four-fold, and protoplast-derived cells failed to undergo division in culture.

Auxin:cytokinin balance modulates miR172 levels in cotyledonary explants

Along with an inherent genetic program, plant growth and development require the integration of a variety of environmental and endogenous signals that determine plant morphology. Plant growth regulators play an important role in this process and recent data have suggested that phytohormones promote regeneration through the action of several key transcription factors that respond to PGR signals to determine cell-fate transition (Xu &Huang, 2014; Dubas et al., 2014).

Small RNAs are among the regulators that control signaling pathways of PGRs during plant development (Liu et al., 2009). Even though many reports have associated plant miRNAs with a great variety of developmental processes such as leaf morphogenesis (Palatnik et al., 2003), leaf polarity (Kidner & Martienssen, 2004; Juarez et al., 2004; Mallory et al., 2004b), flowering time (Aukerman & Sakai, 2003; Achard et al., 2004; Chen, 2004), and flower development (Laufs et al., 2004; Mallory et al., 2004a), the relationship between miRNA expression and PGR response remain to be explored.

Vegetative-phase transition and flowering competency are controlled by *miR156* and *miR172* throughout angiosperms (Huijser & Schmid, 2011), having their expression affected by age, temperature, and light, in an opposite manner: *miR156* expression gradually decreases with age in the shoot, whereas *miR172* expression increases (Wu et al., 2009; Jung et al., 2011). At a molecular level, *miR156* down-regulate genes coding for the SQUAMOSA PROMOTER BINDING-LIKE (SPL) and *miR172* act through the downregulation of APPETALA2-like (AP2-like) transcription factor (Wu et al., 2009). Interestingly, *SPL* genes (*e.g. SPL9*), which are targeted by *miR156*, regulate *miR172*, (Wu et al.,

2009; Jung et al., 2011), hence highlighting a molecular connection between both miRNAs. Therefore, we exposed cotyledonary explant to different combinations of phytohormones in order to determine whether there is a connection between *miR156/miR172* and phytohormone signaling pathways in the control of shoot development.

Liu et al. (2009) investigated the expression pattern of miRNAs in response to phytohormone treatments in rice seedlings. In this study, *miR172* was found to be downregulated by BA, while *miR156* remained unaffected byphytohormones. Although it has been reported that *miR156* is downregulated by exposureto auxin in *Arabidopsis* roots (Marin et al., 2010). In this work,*miR156* remained unaffected by the phytohormones tested, which is in agreement with the report made by Liu et al. (2009). However, the isolated effect of auxin was not tested in this experiment; thus, it is possible that the cytokinin present in combination with auxin in the medium circumvents the downregulatory effect of auxin over *miR156*.

The fact that *miR156* was repressed by IAA in *Arabidopsis* roots and *miR172* was inhibited by BA treatment in rice seedlings suggest a probable correlation between the antagonistic function of auxin and cytokinin, required to coordinate the balance between cell division and differentiation, and the apparent opposite role play by *miR156* and *miR172* during vegetative-phase transition (Curaba et al., 2014). Here, *miR172* was upregulated by the combination 5μM kinetin, 5 μM IAA and 10 μM BA. Interestingly, this is the treatment with the lowest citokynin:auxin ratio. Hence, it is likely that a lower concentration of cytokinins and a higher concentration of auxin favored the expression of *miR172*.

Even though medium A4 was capable of inducing adult traits in cotyledonary explants, the elevated expression of *miR172* induced by this medium did not result in remarkable morphological differences, when compared to medium A6, which also allowed for high percentages of intermediate and trilobed leaves but did not present change in *miR156* and *miR172* expression. On the other hand, profiling microRNAs in *Eucalyptus grandis* indicated no mutual relationship between alterations in *miR156* and *miR172* and rooting induction, given that rooting ability was not mutually related to a high *miR156* expression, and loss of rooting ability was not mutually related to high *miR172* expression during development (Levy et

al., 2014). The results achieved here suggest that *PemiR172* is not affected by cytokinin alone, but the conjugation of auxin and cytokinin, as the treatment with BA in isolation did not alter the expression of *miR172*, nor did the treatments with other concentrations of these phytohormones.

CONCLUSION

We demonstrated that the *in vitro* responses of tissues are influenced differently by phytohormones and that for greenhouse-grown plants, the late physiological condition in leaf explants is a limiting factor for inducing organogenic responses. Also, we demonstrated that cytokinin:auxin balance up-regulated the expression of *miR172*, probably due to the regulatory effect of these phytohormones. To the best of our knowledge, this is the first study aiming to investigate the effect of phytohormones on adult traits *in vitro* and on miRNAs expression in *Passiflora eduli*s. The results achieved by this research will develop a better understanding of the roles of cytokinin:auxin balance on tissue competence for organogenic events occurring *in vitro* as well as insights into the mechanisms and endogenous cues implied in the heteroblastic process, using *Passiflora* as a model system.

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Chapter II

Expression profile of miRNAs and *SPL9* during *de novo* shoot organogenesis in cotyledonary explants of *Passiflora edulis* Sims.

ABSTRACT

De novo shoot organogenesis (DNSO) is the most common pathway in *in vitro* culture studies as it allows the development of a new plant in regeneration systems. Noticeably, the acquisition of organogenic competence and shoot development depends on phytohormone perception, cell division, and transdifferentiation. As opposed to plant growth regulations, whose relevance in developmental processes has been known for decades, recently, the important role of miRNAs in leaf organogenesis and cell division has been highlighted in a range of scientific reports. Hence, the aim of this study was to evaluate the interactions between miRNAs/*SPL9* and cytokinin at a molecular and as a consequence elucidate pathways leading shoot apical meristem regeneration. For this, cotyledonary explants were exposed to media with and without BA for 30 days. Over the course of the experiment, at different time points, organogenic responses,and the expression profile of *miR156*, *miR164*, *miR172*, *miR319* and SPL9 were evaluated. The results of the experiment supported the proposal of a link between cytokinin and *miR164* in the modulation of leaf development in *P. edulis*, in which cytokinin down-regulated *miR164* to allow the expression of *CUC* genes. Furthermore, *miR164* and *miR319* presented antagonistic effects, which might be associated with their roles in leaf development.

Keywords: Cytokinin, shoot apical meristem, *CUC*.

INTRODUCTION

Pluripotency is the ability of stem cells to originate a new complete organ, an important characteristic for plant cell fate plasticity (Verdeil et al., 2007). A fundamental pathway that relies on pluripotency of apical cells is*denovo* organogenesis, which refers to the formation of shoot or root meristems from cultured explants (Rocha et al., 2015). For it allows experimental plant regeneration, *de novo* shoot organogenesis is the most common pathway leading to *in vitro* plant regeneration (Davey & Anthony; 2010; Duclercq et al. 2011). The acquisition of organogenic competence, organ growth, and development involves phytohormone perception, cell division, and transdifferentiation (De Klerk et al. 1997; Dhaliwal et al., 2003; Fehér et al. 2003; Sugimoto et al., 2011). Therefore, efforts have focused on the effect of phytohormones on regenerative processes, especially the antagonistic effects of auxins and cytokinins (CKs) on the regeneration of roots and shoots.

The phytohormones are signal molecules produced by plants that regulate almost every developmental process, including embryogenesis, seed germination, vegetative growth, fruit ripening, and leaf development (Wolters & Jurgens, 2009). Virtually all aspects of plant growth and development are under hormonal regulation (Leyser, 1998). This regulation involves amplifying cascades as well as fine-tuning responses modulated by gene expression. Even though the chemical structures of phytohormones are remarkably simple, their action and physiological effects involve complex regulatory networks (Santner & Calderon-Villalobos, 2009). Therefore, one single phytohormone might be involved in a range of developmental processes, while a particular developmental process may be regulated by multiple phytohormones simultaneously (Xiong et al., 2009). Consideration has been given to this matter by plant science, which has recently developed towards characterizing critical components involved in the phytohormone signaling. In this scenario, microRNAs (miRNAs) have emerged as key regulators of phytohormone response pathways as they are clearly involved the crosstalk of developmental processes, affecting plant metabolism, distribution, and perception (Hibara et al., 2003; Guo et al., 2005; Koyama et al., 2010;Curaba et al., 2014;).

miRNAs are small non-coding regulatory RNA molecules, typically 20–21 nucleotide-long, which trigger the post-transcriptional repression of one to several target genes (Voinnet, 2009). A simplified description of miRNAs biogenesis begins with MIR gene(s), from which the transcript (primiRNA) is able to form an imperfect stem-loop structure that is recognized and processed by a protein complex into a small double-stranded RNA molecule containing the miRNA. After being exported outside the nucleus, this small molecule is incorporated into the RNA-induced silencing complex (RISC) to trigger the post-transcriptional regulation of one or several target genes through a base-pairing mechanism (Voinnet, 2009; Curaba et al., 2014).

Important roles have been attributed to miRNAs in a wide range of developmental processes, including phase transitions, organs morphogenesis, cell division, and stress responses (Voinnet, 2009). Noticeably, these small endogenous molecules might as well interplay with hormones in modulating such processes and the expression of many miRNAs is induced by one or various phytohormones simultaneously (Xiong et al., 2009). Similarly to phytohormones, highly conserved miRNAs often control more than one developmental process. In fact, a knockout on *Arabidopsis HYL1* gene, which encodes a nuclear double-stranded RNA-binding protein, produces a pleiotropic mutation that causes developmental abnormalities. Plants carrying this mutation exhibit increased sensitivity to abscisic acid, and reduced sensitivity to auxin and cytokinin, as well as reduced levels of miR159, miR167, and miR171, providing evidence of the functional link between miRNAs and phytohormones(Han *et al.*, 2004). Review articles and experimental studies have concentrated current knowledge on the molecular intersections between miRNAs pathways and phytohormone responses and discuss their implication as potential mediators of hormonal crosstalk in developmental control. (Liu et al., 2009; Liu & Chen, 2009; Xiong et al., 2009; Curraba et al., 2013; 2014)

Plant hormones play crucial roles in regulating organogenesis, and cytokinins, in particular, have been shown to affect cell division, elongation, differentiation, shoots initiation, apical meristem function or senescence, depending on the developmental context. (Müller & Sheen, 2007; Ariel et al., 2012). Lu and Fedoroff (2000) reported miRNAs response to phytohormones for the first time investigating

the*hyl1*mutant, which shows decreased sensitivity to cytokinin. Later, Liu et al. (2009) revealed that a treatment with 6-BA down-regulates *miR172* and *miR319* in *Oryza sativa*. Although the importance of cytokinin has been known for decades, the underlying molecular mechanisms by which CKs induce the regeneration of shoot apical meristems are not fully understood.

miRNAs 156 and 172 participate in a highly conserved regulatory module across angiosperms and their roles in regulating phase changes in the shoot meristem are the best described in the literature (Huijser & Schmid, 2011). In Arabidopsis, rice and maize, *miR156* regulates shoot branching, leaf initiation and juvenile-to-adult phase transition through the down-regulation of several SQUAMOSA PROMOTER BINDING-LIKE (SPL) (Schwab et al., 2005; Xie & Xiong, 2006; Wu & Poethig, 2006; Chuck et al., 2007; 2010; Schwarz et al., 2008; Jiao et al., 2010; Miura et al., 2010). Antagonistically, *miR172* is highly expressed in reproductive shoots and promotes floral initiation and flower development by repressing expression of APPETALA2-like (AP2-like)genes (Aukerman & Sakai, 2003; Chen, 2004; Jung et al; Chuck et al., 2008; Wu et al., 2009; Li & An, 2012). Furthermore, some genes of the *SPL* family are connected with these miRNAs, once *miR156* downregulates the expression of *SPL9* and *10,* which are known to up-regulate *miR172*, which in turn promote the expression of *SPL3, 4* and *5* in Arabidopsis (Wu *et al.*, 2009; Jung *et al.*, 2011). Also, it has been demonstrated that over-expression of *miR156* leads to a decrease in *miR172* abundance in young shoots (Chuck et al., 2007; Wu et al., 2009). Recent studies have shown that this crosstalk between these two miRNAs is responsible for coordinating the timing of vegetative phase changes and competency to flower across all angiosperms (Huijser & Schmid, 2011).

Also very relevant players of shoot morphogenesis are *miR164* and *miR319*. Their mechanisms involve down-regulation of genes coding for NAC (NAM, ATAF1/2, and *CUC2* domain-containing proteins) and *TCP* (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR) transcription factor families, respectively (Palatnik *et al.*, 2003; Mallory *et al.*, 2004; Nag *et al.*, 2009; Koyama *et al.*, 2010). *miR319* is a positive regulator of the AUX signal (Tian, 2002; Kant *et al.*, 2009), once this microRNA represses the expression of

AtmiR164a and inhibitors of the primary AUX responses:*SHY2* and *SAUR39* (Palatnik *et al.*, 2003; Koyama *et al.*, 2010), playing a crucial function as an upstream regulator of leaf morphogenesis. Additionally, the overexpression of *miR319* in *Arabidopsis* and tomato leaves altered leaf cell differentiation through indirect inhibition of GA biosynthesis (Ori *et al.*, 2007;Yanai *et al.*, 2011), providing an indication that the control of leaf organogenesis by *miR319* involves the regulation of at least two phytohormones, AUX and GA. On the other hand, *miR164* has been mentioned in a wider range of developmental processes, especially root development. AUX induction of *miR164* and the subsequent degradation of its target *NAC1* provide a homeostatic mechanism to control lateral root formation (Xie, 2000; Guo *et al.*, 2005).

Although studies on the involvement of miRNAs in phytohormone actions have become hot topics in the last few years, the roles of miRNAs in plant hormone signaling are still largely unexplored. Therefore, the objective of this study was to provide insight into the molecular connections between miRNAs and *SPL9* and BA and discuss their implication in the organogenesis of cotyledonary explants of *Passiflora edulis.*

MATERIALS AND METHODS

Conduction of in vitro *experiments*

In vitro experiments were carried out at the Plant Tissue Culture Laboratory, located at the Institute of Biotechnology Applied to Agriculture - BIOAGRO, Federal University of Viçosa (UFV).

Plant material

For induction of *in vitro* organogenesis, plantlets derived from mature seeds of *Passiflora edulis* population FB300, provided by "Viveiros Flora Brasil Ltda." (Araguari, MG, Brazil, http://www.viveiroflorabrasil.com.br), were used as explantsources.Seeds were de-coated using a mini-vice, and further surface-sterilized in a laminar flow chamber using 70% (*v/v*) ethanol for 1 min followed by immersion in commercial sodium hypochlorite (2.5% *v/v*) with two drops of 0.1% (*v/v*) Tween-20 for 15 min. Subsequently, seeds were submitted to a triple rinse in sterile deionized water. Ten seeds were inoculated into each flask (250 mL capacity) containing 60 mL of MS medium (Murashige & Skoog, 1962) with half-strength nutrient solution, MS vitamin complex, 1.5% sucrose (w/v), 0.005% myo-inositol (w/v), and added with 0.25% gelling agent (w/v) (Phytagel®, Sigma Chemical Company, MO, USA), and pH was adjusted to 5.8 prior to autoclaving. Flasks were sealed with a rigid polypropylene lid with two vents (10 mm diameter) covered with a 0.45 μm size pore membrane - 25 μL CO_2 L^{-1} s^{-1} exchange rate (Milliseal®, AVS-045 Air Vent, Japan). The culture media were autoclaved at 120 °C, 1.1 Pa for 20 min. Seeds were kept in the dark at 27 ± 2 ºC, for 15 days. Lastly, flasks were transferred to a growth room, with a photoperiod of 16/8h (light/dark), under irradiance of 50 μmol m^{-2} s^{-1}, provided by two LED lamps with 17W (Vilux®, 27 LLTV03, Brazil) for 15 days.

Effect of 6-benzylaminopurine (BA) on miRNAs expression profile and organogenesis

We evaluated the effect of 4.4 μM BA on *de novo* shoot organogenesis (DNSO), provided by cotyledonary explants, as well as miRNAs *miR156, miR164,*

miR172, and *miR319* and *SPL9* expression profile of *P. edulis*. Explants were obtained from 30-day-old seedlings, as previously described.

Explants were inoculated into flasks (60 mL capacity) containing 10 mL of MS medium with full-strength nutrient solution, MS vitamin complex, glucose 44 mM, 0.01% myo-inositol (w/v), and added with 0.25% gelling agent (w/v) (Phytagel®, Sigma Chemical Company, MO, USA). Subsequently, BA was added to the medium to a final concentration of 4.4 μM and pH was adjusted to 5.8 prior to autoclaving. Test tubes were sealed with rigid polypropylene, media autoclaving and incubation conditions were similar to the aforementioned.

Subsequent to autoclaving, the culture medium was supplemented with 10 μM silver thiosulfate (STS) in order to inhibit ethylene action by repressing its responses (Zhao et al. 2002; Reis et al., 2003). Under aseptic conditions, cotyledon discs (6 mm^2) were incubated with the abaxial side facing the medium. The explants were incubated in a growth room under irradiance of 50 μmol m^{-2} s^{-1} at a temperature of 27 ± 2 ºCuntil the last collection point.

Upon each collection period, the following variables were evaluated: percentage of responsive explants (PRE), percentage of explants presenting monolobed (PML), intermediate (PIL) and trilobed leaves (PTL), leaf area per explant (LAE) and total leaf number per explant (TLE). For leaf area analysis, leaves were detached and fixed individually in white millimetric paper. Digital camera photos were captured and resulting images were analyzed in the ImageJ software(Schneider et al., 2012).

The experiment was conducted in a completely randomized design (CRD) in a factorial scheme 2x9 (media formulation x time points), five replicates per treatment, each replicate consisting of ten test tubes with one explant each.

Expression profile of miRNAs and SPL9

Sample collection and preparation

In order to analyze the expression of the miRNAs and *SPL9* genes, RNA extraction was carried out on samples collected from the explant edge, where the

organogenic responses are usually observed. The plant material was collected under RNAse-free condition. The collected samples were immediately frozen in liquid nitrogen and stored at -80 °C until the moment of use.

RNA extraction

Total RNA was isolated using the Concert® Plant RNA Reagent solution (Invitrogen, CA, USA) and treated with DNAse I (Thermo Scientific NanoDrop Technologies, Wilmington, DE, USA), according to manufacturer's recommendations. RNA quantification was performed with a NanoDrop™ 2000/2000c spectrophotometer (NanoDrop Technologies) and the integrity of the RNA samples was verified by 1.5% agarose gel electrophoresis (RNAse free). Subsequently, all samples were treated with DNAse I RNAse-Free (Ambion-Life Technologies, CA, USA), according to manufacturer's recommendations, and RNA quantification, as well as integrity, were verified again.

Table 1: Primer sequences used in the PCR reactions.

Primer Name	Sequence
PeSPL9 RT F1	5’ GGT CTA ACC CAA ATC CCG CA 3’
PeSPL9 RT R1	5’ AGA GAC CAG TGT GTG TGA TGA G 3’
miR156 F	5´CCTGAGTGACAGAAGAGAGTG 3´
miR156 RT	5’GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCTCT 3’
miR172 F	5’CCTGAGAGAATCTTGATGATG 3’
miR172 RT	5’GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG 3’
Reverse Universal	5’ GTGCAGGGTCCGAGG 3’
miR164 F	5’ GCGGCGTGGAGAAGCAGGGCA 3’
miR164 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG 3’

miR319 F	5’ GCGGCGTTGGACTGAAGGGA 3’
miR319 RT	5’GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAGGGAGC 3’

cDNA synthesis via ‘*pulsed-stem loop’* RT-PCR

cDNA synthesis was performed using the *Pulsed stem-loop* RT-qPCR methodology (Varkonyi-Gasic et al., 2007) from 500 ng of total RNA. Then, 1 µL of dNTP (10 mM), 1 µL (1 mM) of the mature miRNA-specific RT primer and 1 µL of oligodT (500ng/µL) were added together with the total RNA treated with DNAse I. The samples were incubated at 70 °C for 10 min for denaturation of the secondary structures and then incubated at 4 °C for 5 min. Then, 4 µL of the 5x RT buffer and 1 µL of the MMLV Reverse Transcriptase enzyme (Ludwig Biotec®, Alvorada, Brazil) were added to the samples. Reactions were incubated at 16 °C for 30 min, followed by reverse transcription of 60 cycles of 30 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s. For inactivation of the enzyme, the reaction was incubated at 85 °C for 5 min. Subsequently, the reactions were stored at -20 °C.

RT-qPCR analysis

The expression profile of the *miR156* and *miR172* genes were analyzed via RT-qPCR on a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). All RT-qPCR samples were run using 20 ng of cDNA, 400 nM of each primer, and qPCR SYBR-Green mix/Rox (Ludwig Biotec®, Alvorada, Brazil) and diethylpyrocarbonate-treated water to a final reaction volume of 10 µL. The constitutive gene *actin* was used as a normalizer. The PCR program was as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 16 s at 95 °C and 1 min at 60 °C, and 15 s at 95 °C, 1 min at 60 °C , 30 s at 95 °C and 15 s at 60 °C. Transcription levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primers used are listed in Table 1.

Statistical analysis

Statistical analyses were performed using GENES software (Cruz, 2013). Percentage values were transformed to arcsin $\sqrt{x}$ before analysis. Data were

processed by analysis of variance (ANOVA) followed by Tukey's test at a 5% significance level. For the analysis of gene expression via RT-qPCR, three biological and two technical replicates were used and means were compared by Dunnett's test at a 5% significance level.

RESULTS

Expression profile of miRNAs/SPL9 and organogenesis from cotyledonary explants exposed to exogenous BA

In the present study, the control treatment - devoid of BA - did not induce any of the organogenic responses evaluated forcotyledonary explants throughout the entire experiment evaluation period (Figs. 1 and 2). On the other hand, the medium supplemented with BA promoted responsive explants after 12 days of culture, and by the 18th day onwards (Fig. 1A) the explants achieved the highest response rates. Monolobed leaves were only observed after 18 days of culture (Fig. 1B), and, similarly to leaf area and total leaf number per explant, the highest values were obtained at the 30th day of culture (Fig. 1C and D). Although some leaves presented leaf serration, a characteristic of adult leaves, no intermediate or trilobed leaves were induced by BA-supplemented medium.

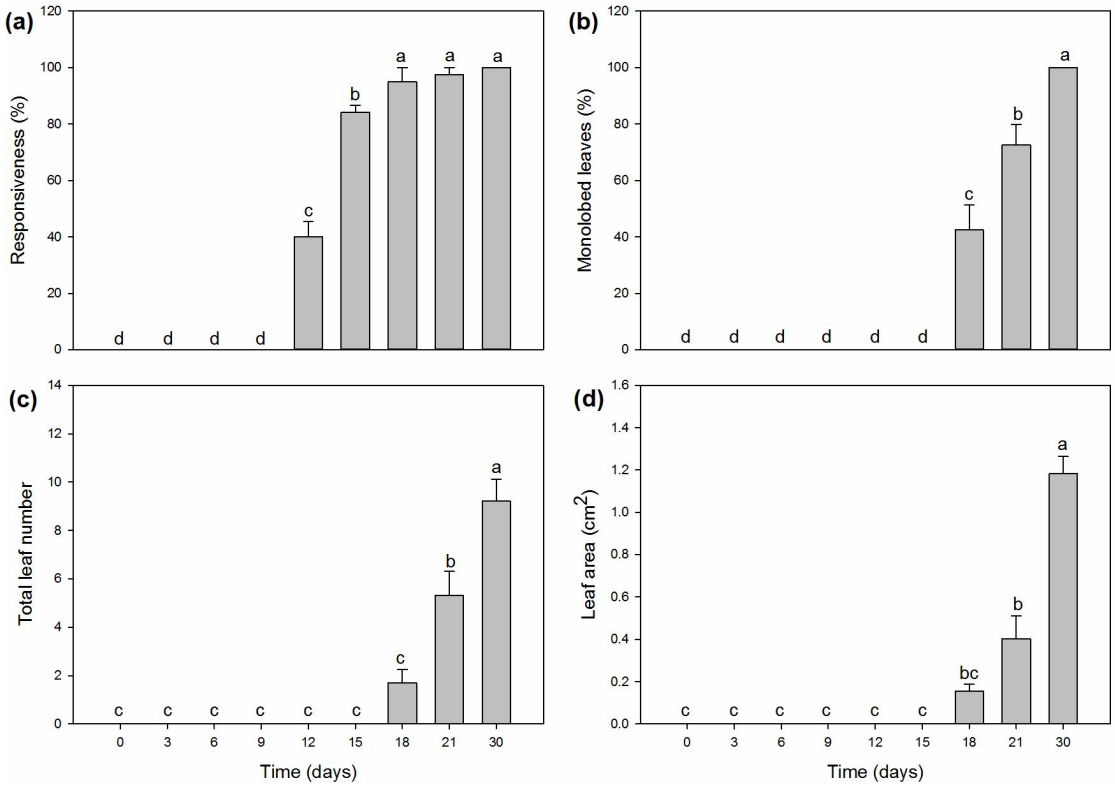

Figure 1 - Effect of BA on organogenic responses of cotyledonary explants of *Passiflora edulis*Sims. (A) Percentage of responsive explants; (B) percentage of explants presenting monolobed leaves: (C) leaf area per explant; and (D) total leaf

number per explant. Treatments followed by the same letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

The expression of miRNAs and *SPL9* investigated in this study was normalized by cotyledons harvested before inoculation into culture media. Expression levels of *miR156, miR172* and *SPL9* were neither altered by time or by medium supplementation with BA (Fig. 3). On the other hand, *miR164* and *miR319*havedifferent expression patterns over the culture period and dependent on the medium treatment. Downregulation of *miR164*started to occur three days after exposing cotyledonary explants to medium supplemented with BA, which persisted until the last day of the experiment. Regarding explants exposed to the control medium, *miR164* levels were decreased only 12 days after culture and *down-regulation* was maintained until the end of the experiment. At days 3, 6, 12, and 30, down-regulation of *miR319* was observed in cotyledonary explants inoculated into BA media. Otherwise, this gene was up-regulated by the control treatment after 6 and 30 days of culture.

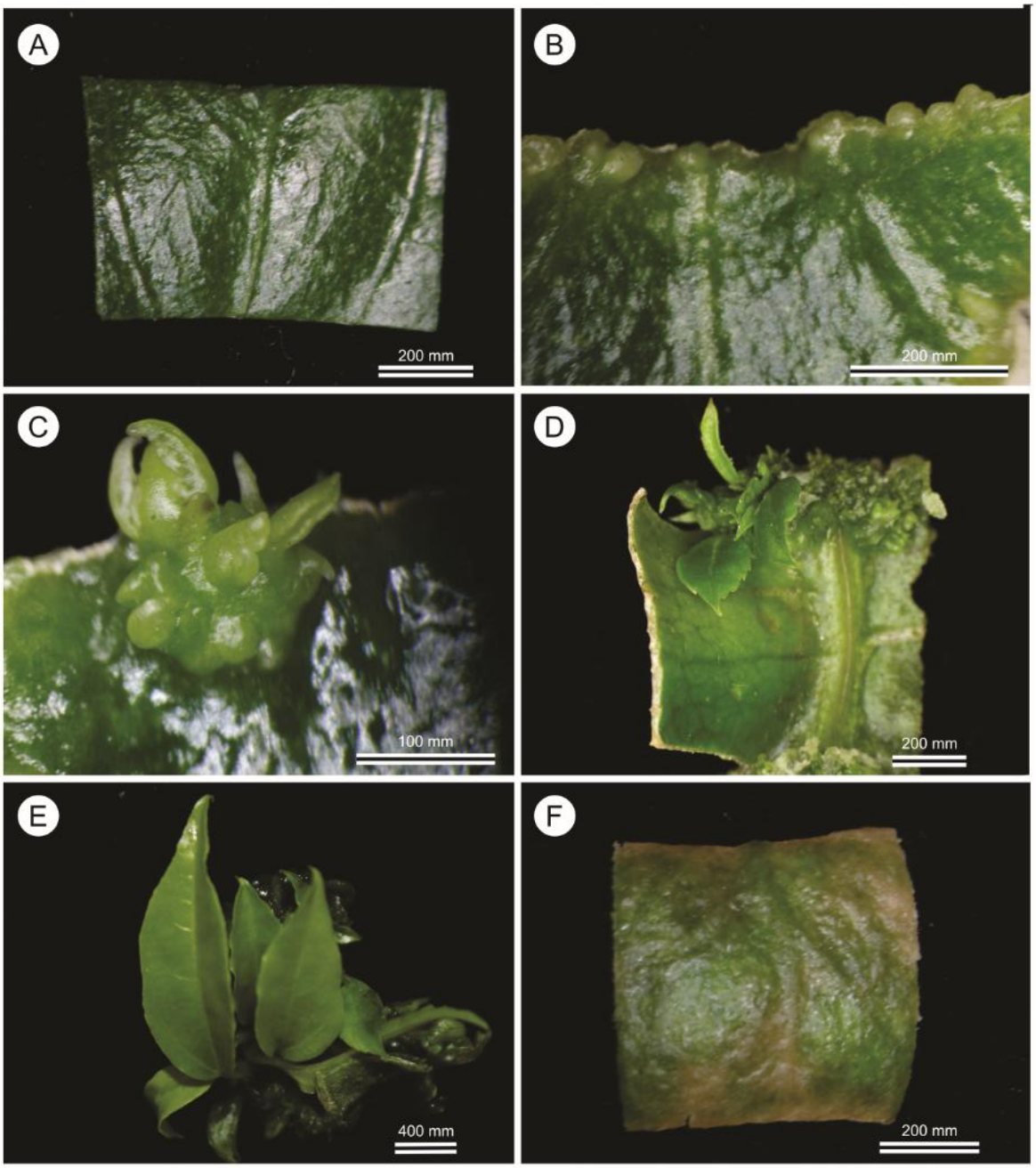

Figure 2 - Effect of BA on organogenic responses of cotyledonary explants of *Passiflora edulis*Sims. (A) Explant prior to culture; (B) explant after 9 days of culture in BA media; (C) explant after 12 days of culture in BA media; (D) explant after 15 days of culture in BA media; (E) explant after 30 days of culture in BA media; and (F) explant after 15 days of culture in the control media.

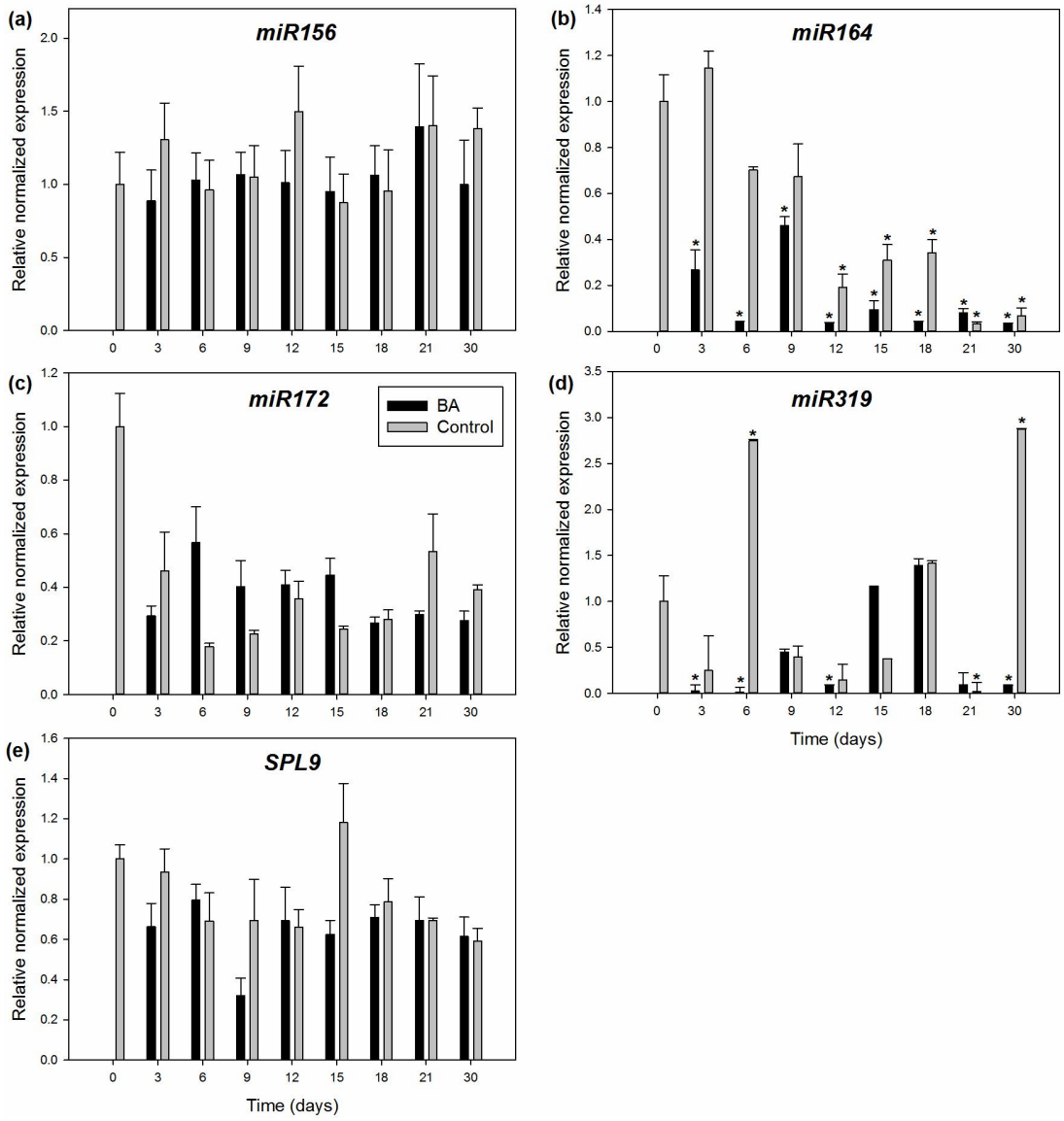

Figure 3 - Effect of BA on gene expression of cotyledons of *Passiflora edulis*. Levels of *miR156*(A),*miR164*(B), *miR172*(C), *miR319* (D)and *SPL9*(E) over 30 days of culture. Treatments marked with an asterisk differ statistically from the time zero by Dunnett's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

DISCUSSION

Regardless of the explant source, the induction of shoots with the use of cytokinins in species of *Passiflora* is quite common and well-established, as it promotes regeneration responses (Fernando et al., 2007; Dias et al., 2009; Prammenee et al., 2011; Rocha et al., 2012). Indeed, the medium MS supplemented with BA has been satisfactory in inducing *de novo* shoot organogenesis (DNSO) in cotyledonary explant of *P. edulis* in our previous studies (Silva et al., 2019). In concert with previous observations, the media supplemented with BA induced only monolobed leaves, strengthening the association of this phytohormone with juvenile characteristics (Fig. 2). Taken together, these results reinforce the hypothesis that the induction of adult traits in organogenesis of *Passiflora edulis* is modulated by a balance between auxin and cytokinin.

Previous work in our research group described the expression profile for monolobed and trilobed leaves regenerated from cotyledonary explants cultivated in different media conditions. It was verified that the exposure of cotyledonary explants of *P. edulis* to cytokinin and auxin simultaneously was able to up-regulate the expression of *miR172* in trilobed leaves, while *miR156* was unaffected in both leaf types. This finding suggests that *miR172* might be under regulation of multiple phytohormones.

In order to set up a deeper investigation whether isolated phytohormones might regulate miRNAs during organogenesis of *P. edulis*, we cultured cotyledonary explants into media supplemented with 4.4 µM BA and assessed organogenic responses and expression profile of *miRNAs 156, 164, 172* and *319* as well as *SPL9* throughout 30 days. We expected that this experiment would provide insight into the isolated action of cytokinin upon genes previously reported to be associated with leaf development.

miR156, 172 and SPL9 expression profilesare not modulated by 6-benzylaminopurine

Cytokinin alone was not able to affect the expression profile of *miR156*, *SPL9,* and *miR172*, in comparison to the control treatment. These results differ from those of Liu et al. (2009), who found *miR172* to be down-regulated by exogenous BA

applied to *Oryza sativa* seedlings*;* however, similarly to our results, *miR156* remained unaffected by this treatment. Moreover, the fact that cytokinin alone did not alter the expression profile of these genes, but the combination of cytokinin and auxin did, provide a strong indication that *miR172* in *P.edulis* is under constraint of multiple phytohormones.

miR164 and miR319 expression profiles are down-regulated by 6-benzylaminopurine

Differently from *miR156*, *SPL9,* and *miR172*, *miR164* was down-regulated by the BA and control treatments. Inhibition of this gene started 3 and 12 days after of culture for explants exposed to cytokinin and MS media without phytohormones, respectively. After down-regulation of *miR164* occurred in both treatments, the gene expression was kept down-regulated, as opposed to *miR319*, whose expression oscillated in the course of the experiment. Regarding the expression of *miR319*, mostly up-regulation and down-regulation were observed for BA and control treatments, respectively, except for day 21, in which the expression of this gene was down-regulated by the control medium.

Recently, *miR319* was reported to target the TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factor genes, already well known for their effects on leaf development (Doebley et al., 1997; Almeida et al., 1997; Luo et al., 1999). By affecting jasmonate levels, TCP transcription factors function throughout leaf development to coordinate the balance between leaf growth, which they negatively regulate, and leaf senescence, which they positively regulate. Thus, *miR319*-controlled TCP transcription factors coordinate two sequential processes in leaf development: leaf growth and senescence (Schommer et al., 2008). Here, comparing organogenic responses of cotyledonary segments (Figs. 1 and 2), it was observed that explants exposed to the control treatment did not produce any organogenic responses and underwent a severe senescence process. On the other hand, explants exposed to BA were highly responsive, producing shoots and leaves, not showing signs of senescence. Interestingly, miRNA319 was upregulated only in the control treatment, which suggests a response towards senescence, which is a commonly observed process in explants exposed to medium without phytohormones. Beyond that, down-regulation of *miR319* was observed at some

time points during culture of explants submitted to BA, which agrees with the result observed by Liu et al. (2009), who reported a decline in the expression of *miR319* in rice seedlings exposed to cytokinin. However, 15, 18 and 21 days after culture, *miR319* exhibited an increased relative expression, when compared to the earlier days, which might be related to the functions of this gene in targeting TCP transcription factors, an inhibitor of leaf growth, and; therefore, promoting leaf growth.

miR319 has also been pointed out to suppress and act antagonistically to *miR164* (Palatnik *et al.*, 2003; Koyama *et al.*, 2010), which agrees with the results presented here, since the down-regulation of *miR164* in the control treatment coincides with the up-regulation of *miR319* in some control treatments. In fact, *miR319* is primarily known as a modulator of leaf morphogenesis by repressing TCP transcription factors and, thus indirectly repressing the expression *AtmiR164a,* ASYMMETRIC LEAVES1 (*AS1*) and INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2(*IAA3/SHY*), which in turn act as negative regulators of *CUC* genes (Palatnik *et al.*, 2003; Koyama *et al.*, 2010). Interestingly, over-expression of *miR319* in creeping bentgrass was shown to reduce the expression of both *miR319* and *miR164* target genes in the root (Zhou *et al.*, 2013).

In addition to the probable repression exerted by *miR319*, the negativeregulation of *miR164* in control treatments might be related to its described decline with the progression of senescence. Throughout leaf aging, *miR164*may function in the age-dependent cell death pathway as an important player against premature overexpression of the NAC transcription factor *ORESARA1* (*ORE1*)and may finely tune the timing of senescence and cell death. *ORE1* positively regulates aging-induced cell death in Arabidopsis leaves, whose expression is up-regulated concurrently with leaf aging by *EIN2*, but is negatively regulated by *miR164*. The expression of *miR164* gradually decreases with aging through negative mediation by *EIN2*, which leads to the elaborate up-regulation of *ORE1* expression (Kim *et al.*, 2009). However, as a strategy to prevent senescence at earlier stages, *ORE1* is negatively regulated by *miR164*, which is only relieved at later stages (Kim *et al.*, 2009). This mechanism is consistent with the results described in this study, since the expression of *miR164* in explants exposed to the control treatment was higher at

the beginning of the experiment and subsequently down-regulated at 12 days after culture, implying an initial effort to prevent senescence through increase in *miR164*, followed by a decline of this gene, the so-called "relief" of its inhibition prior to senescence.

The CUP-SHAPED COTYLEDON (*CUC*) genes encode members of the NAC family, which are involved in shoot apical meristem (SAM) formation, as well as cotyledon and specification of organ boundaries (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). As a matter of fact, cuc1 cuc2 double mutant seedling completely lacks an embryonic SAM and exhibit cup-shaped cotyledon, that is, fused along both edges (Aida et al., 1997; Takada et al., 2001). Hence, it has been suggested that *CUC1* promotes SAM formation during embryogenesis (Takada et al., 2001; Hibara et al., 2003). Interestingly, *CUC* genes regulate SHOOT MERISTEMLESS *(STM*) genes, which are required for SAM formation and maintenance of stem cells on cotyledons (Hibara et al., 2003). In its turn, *CUC* is negatively regulated by TCPs, also important players in the coordination of undifferentiated fates in the SAM and promotion of differentiation of cells in leaves (Nath et al., 2003; Palatnik et al., 2003; Koyama et al., 2007; Ori et al., 2007; Efroni et al., 2008; Schommer et al., 2008). It has been postulated that *TCP3* might trigger a mechanism that indirectly regulates the expression of *CUC* genes (Koyama et al., 2007). Koyama et al. (2010) showed that *TCP3* directly activate target genes, including *miR164, AS1, IAA3, SHY2* and several auxin-inducible genes, that act as negative regulators of the expression of *CUC* genes.

Guo et al. (2005) demonstrated a mechanism of *miR164* induction by auxin in *Arabidopsis* to regulate lateral root formation. Therefore, we speculate that the down-regulation of *miR164* promoted by the BA treatment in the very beginning of the experiment might be related to the antagonistic effect of cytokinin. A recent work proposed that cytokinin signaling promotes CUC expression (Li et al., 2010). Taken our results and these findings together, we propose a signaling pathway involving both *miR164* and*miR319* to regulate *in vitro* leaf development in *P. edulis* (Fig.4), in which cytokinin regulates *miR164* levels. CUC-related lead development is promoted through the direct induction of this gene by cytokin, which also presents an indirect induction through suppression of *miR164,* an inhibitor of CUC

expression. *miR319* is another important player in this process, as it represses the transcription factor TCP, a positive regulator of *miR164*. This mechanism explains the decreased levels of *miR164* in explants exposed to exogenous cytokinin. However, further investigations are required to determine the influence of cytokinin on the levels of CUC genes. This investigation would clarify the mechanism by which cytokinin suppresses *miR164* expression.

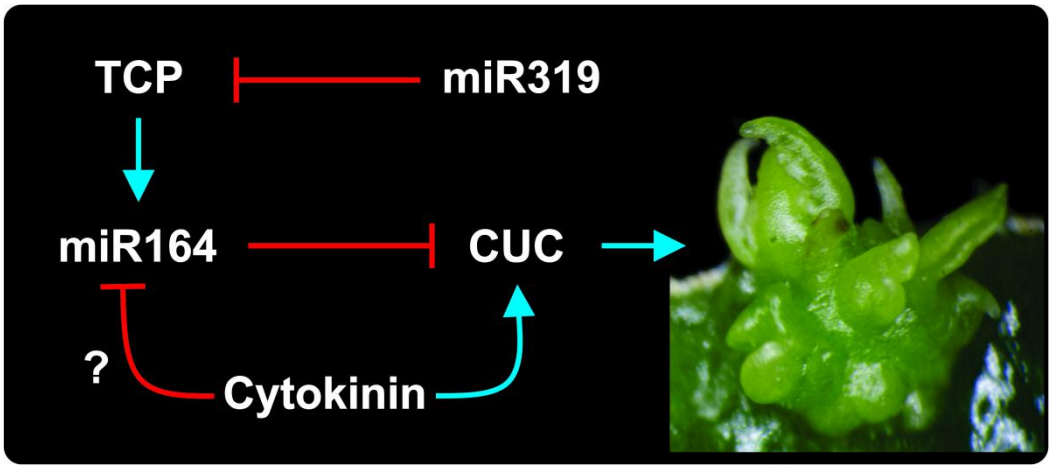

Figure 4 - A proposed mechanism for *in vitro* leaf development of *P. edulis*.*CUC* genes are involved in the promotion of leaf development and are directly induced by cytokinin, which in turn down-regulates the expression of *miR164*, a repressor of *CUC*.*miR319* is another important regulator in this process, since this mRNA inhibits the expression of the transcription factor family TCP, an inducer of *miR164*.

CONCLUSION

As research progresses, more pieces of the puzzle behind leaf development are unraveled, and as a consequence, it is now known that there is a miRNA/hormone network increasingly in complexity that plays a fundamental role to this process. Detailed analysis have demonstrated the role of transcription factors, i.e. TCP family genes, SAM development-related genes, i.e. STM, CUC, and phytohormones.

In the present study, we propose a novel link between cytokinin and *miR164* as a key regulatory output of cytokinin in the regulation of SAM function. Moreover, we demonstrate what appears to be antagonistic interactions between two miRNAs involved in leaf development. Still, further clarification and validation of the connection between cytokinin and the biological role miRNAs in developmental processes will be aided by the spatio-temporal investigation of CUC and other related genes expression levels. Also, Further examination of the regulatory interactions between miRNAs *164* and *319* and their target genes will provide insights into the complex crosstalk between phytohormone signaling and miRNAs in regulating plant development.

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Chapter III

Leaf development stages and ontogeny changes of passionfruit (*Passiflora edulis* Sims.) are detected in narrowband spectral signal

ABSTRACT

Leaves are highly specialized organs whose components (*e.g.* pigments, proteins, cellulose, lignin) interact with light. This feature makes leaves a target for reflectance studies, once different regions of the spectrum can be associated with leaf biochemical, anatomical, structural or biophysical features. As a result, relationships between the spectral curves and the leaf traits might be established, enabling the assessment of these features in a non-destructively and rapidly. As the first report onhow leaf development and ontogeny affect the spectral signature of *Passiflora edulis* leaves and their traits, we assessed physiological, structural and biochemical properties of leaves with the use of leaf hyperspectral and biochemical analyses. We also evaluated leaf pigment contents in order to compare the results for both methods and validate hyperspectral indexes. We performed hyperspectral measures of expanding and fully-expanded leaves, from the 2nd to the 12th phytomer of green-house grown individuals. Moreover, we compared hyperspectral data from 70-d-old plants with biochemical analyses in order to compare and validate pigment-related indexes proposed in the literature. We verified that all of the variables investigated are affected by leaf ontogeny and leaf photosynthetic efficiency might be influenced by heteroblasty. Also, comparing biochemical and hyperspectral analyses of pigments, we conclude that remote sensing as a great potential method for diagnosing phenology and physiological conditions for *P. edulis,* as some indexes were highly correlated with chlorophylls and carotenoids.

Keywords: remote sensing, leaf ontogeny, vegetation indexes.

INTRODUCTION

Over the course of ontogeny, plants naturally experience a certain degree of variation in leaf size, shape and geometry (Chitwood & Otoni, 2017a; 2017b). Thus, in the vast majority of cases, this size-related variation is gentle and gradual. In some cases, however, there are abrupt and dramatic changes in form and function (e.g. in leaf form, leaf size, phyllotaxy, internode length, anthocyanin pigmentation, rooting ability, or wood structure) (Rumball, 1963; Frydman & Wareing, 1973). This phenomenon is known as heteroblastyand has been used to distinguish plants that undergo abrupt and conspicuous alterations from subtle and gradual so-called ontogenetic changes (Zotz et al., 2011).

Over the last years, there has been a huge confusion around the concepts of heteroblastic and ontogenetic changes, leading species that undergo only ontogenetic alterations to be classified as heteroblastic (Zotz et al., 2011). In this context, heteroblastic species from *Passiflora* genus such as *Passiflora edulis* Sims. are targets for studies in heteroblasty (Chitwood & Otoni, 2017a, b; Silva et al., 2019), consisting of a great opportunity to unravel possible functional, physiological, structural and biochemical changes of vegetative organs modulated by this process. Although heteroblasty is not restricted to leaves, these are the organs in the plant where larger measurable variations are observable due to their greater phenotypic plasticity (Zotz et al., 2011).

Leaves are specialized organs and also the main site of photosynthesis. Therefore, considering the leaf optical properties interaction (absorption, reflection, and transmittance) with radiation, different regions of the spectrum can be associated with leaf biochemical or biophysical features. While wavelengths up to 700 nm are typically reflected by pigments, spectral regions beyond the near-infrared (NIR), i.e., the short wave infra-red (SWIR), contain information on biochemical compounds, such as lignin, cellulose and leaf water (Huntjr & Rock, 1989; Martin & Alber, 1997; Martin et al., 1998). Therefore, this interaction of the physical properties reflection, absorption, and transmittance, influenced by the biophysical and biochemical characteristics of the leaves, establishes meaningful relationships between the spectral curves and the leaf traits. As a result, this association allows for the distinction of plants from other targets, classification of

plant communities and species, and contribution for studies in remote sensing (Asner & Martin, 2008; Ustin & Gamon, 2010; Meerdink et al., 2016).

Remote sensing is a widely used technique in geographic and ecological studies to investigate vegetation dynamic, plant/animal diversity distribution and ecological interactions and processes at a local to global scale. More specifically, hyperspectral remote sensing of vegetation has been in widespread and rapid development since the 1990s and the first successful spectral discrimination of native species of tropical environments was reported by Clark et al. (2005). Now that spectral analysis can range from 350 nm–2500 nm, as opposed to a spectral range of up to 1100 nm in the 1980s (Eller & Will, 1977; Tanner & Eller, 1986), high spectral resolution remote sensing techniques show promise to estimate chemical parameters that were not available in the past (Curran, 1989). As a consequence, remotely sensed data are being used to estimate foliar chemical content in response to our increasing ability to understand and measure foliar spectra as well as to generate unambiguous and accurate estimates of foliar chemical content in a rapid, inexpensive and non-destructive method (Peñuelas & Filella, 1998; Jacquemoud & Ustin, 2008).

The progression of remote sensing technology, involving the increasing number and resolution of sensor-captured bands, has created an enabling environment for the development of algorithms and tools for high-quality assessment of plant status through spectral analyses (Xue & Su, 2017). In this sense, vegetation indexes - mathematical formulas that use specific spectral regions defined by laboratory methodologies and/or field experiments (Roberts et al., 2011; Xue & Su, 2017) are being widely implemented in several applications of remote sensing, from quantifying pigments to assessing physiological status of a vegetation (Baek & Cho, 2016; Gitelson et al., 2002; Guo & Trotter, 2006; Ustin et al., 2009; Vandvik & Birks, 2002; Xue & Su, 2017). Furthermore, considering the most hyperspectral systems collect a large volume of data in wavelengths that are highly correlated, hyperspectral indexes are a system by which the wide load of information captured in a spectrum can be transformed in a few, physically meaningful variables (Roberts et al., 2011).

In addition to the appealing advantage of providing instant and cost-effective answers, hyperspectral systems are demonstrated to discriminate plant physiological condition (Pontius et al., 2008) even at early phases of senescence (Campbell et al., 2004). Some previous studies have used hyperspectral remote sensing to detect plant stress due to water deficit (Stimson et al., 2005), insect damage (Pontius et al., 2008; Radeloff et al., 1999), pest outbreaks (Wolter et al., 2008), and pollution (Campbell et al., 2004). Moreover, spectral signatures have been used to measure biochemical components, such as pigment concentration (Sims & Gamon, 2002; Blackburn, 2007), nutrient content (Hellmann et al., 2015), and tannins (Asner et al., 2014; Lehmann et al., 2015); as well as biophysical properties such as cellular leaf structure (Kalacska et al., 2007), leaf water content (Sims & Gamon, 2003); and anatomical traits such as epidermal structures (Da Luz, 2003; Heim et al., 2015).

Even though the number of hyperspectral studies has been increasing in the literature, especially the ones related to plant communities and canopy, investigations onhow leaf development and ontogeny affect the spectral signature of leaves and their traits - especially the heteroblasty process - are not available. Therefore, here we assessed physiological, structural and biochemical properties of the leaves by means of leaf hyperspectral and biochemical analyses of *Passiflora edulis* Sims. We also assessed leaf pigment contents in order to compare the results for both methods and validate hyperspectral indexes.

MATERIALS AND METHODS

Plant material

In order to obtain individual of *P.edulis*, mature seeds of population FB300, provided by Viveiros Flora Brasil Ltda. (Araguari, MG, Brazil, http://www.viveiroflorabrasil.com.br), were germinated in plastic pots (5 L capacity), containing commercial substrate (Plantmax®). Irrigation was done daily in order to keep soil moisture close to field capacity. Plants were periodically fertilized and grown in a greenhouse.

Thirteen plants were selected for hyperspectral analyses, from which the 2nd up to the 12th phytomer were evaluated, after 15 and 30 days of leaf emission. All measurements were performed in the same period of the day (14:00h). At the end of the experiment, the leaves from the 2nd up to the 12th phytomer were collected from ten plants for biochemical analyses, after 70 days of culture. In addition, the same leaves had their hyperspectral signatures collected so the outcome results of biochemical and spectral analyses could be compared. For this, leaves from the 2nd up to the 12th phytomer were packed into a black plastic bag inside a cooler containing ice and taken for hyperspectral analysis followed by biochemical analysis.

Hyperspectral data collection

Leaf samples were selected and analyzed during the experiment. In order to ensure material integrity, plants were carefully transported to the laboratory, where leaf spectral signatures were collected using a spectroradiometer FieldSpec® 4 Hi-Res (Analytical Spectral Devices, Bolder, CO, United States). The equipment consists of a probe (pistol) and a leaf clip, providing orthogonal projection and equal lighting conditions, with little influence from the atmosphere, given the direct contact with the probe. This instrument is capable of recording the reflectance of targets in the range of the optical electromagnetic spectrum from 350-2500 nm, at a spectral resolution of 3 nm for shorter wavelengths (350-700 nm) and 10 nm for long wavelengths, resulting in a total number of 2151 bands (Danner et al., 2015). The analysis was performed on the adaxial side of the leaves, in the middle lobe, excluding the midvein when possible.

Hyperspectral measures were performed at expanding and fully-expanded leaves in order to assess physiological, structural and biochemical features with the use of HVIs. Besides that, at the end of the experiment in order to compare pigment-related indexes with biochemical data, hyperspectral measures from 70-d-old plants were also performed in 10 individuals. In total, 396 spectral data were collected and evaluated from 13 plants, considering leaves from the 2nd to the 12th phytomer. Based on the hyperspectral data collected, vegetation indices were calculated to obtain an estimate of physiological, biochemical and structural aspects of the leaves (Table 1).

Table 1- Vegetation spectral indexes applied in leaf hyperspectral data of *Passiflora edulis* Sims.

Index/equation	Description	Reference
$NDVI_{750} = (p_{750}-p_{705})/(p_{750+}p_{705})$	Green biomass	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$WBI = p_{900}/p_{970}$	Water content	(Peñuelas et al., 1995a)
$ARI1 = (1/p_{550})-(1/p_{700})$	Anthocyanin/chlorophyll	(Gitelson et al., 2002)
$SIPI = (p_{800}-p_{445})/(p_{800}-p_{680})$	Pigments, carotenoids	(Peñuelas et al., 1995b)
$PSRI = (p_{680}-p_{500})/p_{750}$	Pigments, carotenoids	(Merzlyak et al., 1999)
$MSI = p_{1650}/p_{830}$	Water stress	(Rock et al., 1985)
$PRI = (p_{530}-p_{570})/(p_{530}+p_{570})$	Light use efficiency	(Gamon et al., 1997)
$RVSI = (p_{714} +p_{752})/2 - p_{733}$	Vegetation Stress	(Merton & Huntington, 1999)

* *p=wavelength*

Analyses of photosynthetic pigments

Photosynthetic pigments - chlorophyll *a*, *b,* carotenoids, and anthocyanins - were determined by collecting leaves 2 up to 12 from greenhouse-grown plants. In order to compare biochemical and spectral results, pigment-related indexes (Table 2) were compared to biochemical outcomes. For this, leaf samples were collected from the same region considered in the hyperspectral analysis - the middle region of the middle lob, excluding the midvein. Sample collection was followed by maceration in liquid nitrogen with the aid of porcelain grail and pistil. In addition, pigment reading was carried out in a 96-well microplate reader (OptiMax Tunable Microplate Reader).

For chlorophylls *a*, *b,* and carotenoid determination, the protocol described by Wellburn (1994) was used. For that purpose, 500 µL acetone 80% (v/v) were added to a microtube with 100 mg of fresh leaf material. Throughout the analyses period, samples were kept on ice and low light irradiance. Each sample was vigorously agitated and centrifuged at 12000 x *g* for 10 min. Then, the resulting supernatant was collected and again submitted to the aforementioned agitation and centrifuging steps. Reading was performed with 200 µL of the leaf extract/well in three wavelengths, 470, 646 and 663nm. Chlorophylls *a* and *b* contents were determined by the equations below and expressed in mg g^{-1} fresh weight (FW):

$$\text{Ch a} = 12.21 \times Abs_{663} - 2.81 \times Abs_{646}$$

$$\text{Ch } b = 20.13 \times Abs_{646} - 5.03 \times Abs_{663}$$

Carotenoid content was determined by the equations below and expressed in mg g^{-1} fresh weight (FW):

$$Cx + c = (1000 \times Abs_{470} - 3.27 \times \text{Chl a} - 104 \times \text{Chl b})/198$$

Relative anthocyanin levels were determined by incubating 100 mg of leaf fresh material with 300 µL methanol acidified with 1% HCl overnight after vortexing the samples for 20 sec (Neff & Chory, 1998). After the addition of 200 µL distilled water and 500 µL chloroform, samples were centrifuged at 14000 g for 5 min and anthocyanins were separated from chlorophylls. Reading was performed with 200 µL of the leaf extract/well in two wavelengths, 530 and 657nm. Relative

anthocyanin contents were determined by the equations below and expressed in mg g^{-1} fresh weight (FW):

$$ANT = Abs_{530} - Abs_{657}$$

In this study, a set of narrowband vegetation indexes developed in earlier studies were tested to explore their potential for chlorophylls, carotenoids and anthocyanins estimation. These indexes are listed in Table 2.

Table 2- Pigment-related vegetation spectral indexes used in correlation analyses of biochemical assays of *Passiflora edulis* leaves.

Index/equation	Description	Reference
$SR_{680} = p_{800}/p_{680}$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$SR_{705} = p_{750}/p_{705}$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$NDVI_{680}= (p_{800}-p_{680})/(p_{800+}p_{680})$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$NDVI_{750}= (p_{750}-p_{705})/(p_{750+}p_{705})$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$mNDVI_{750}= (p_{750}-p_{705})/(p_{750+}p_{705} -2 \times p_{445})$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)
$mSR_{705}= (p_{750}-p445)/(p_{705+}p_{445})$	Chlorophyll	(Gitelson & Merzlyak, 1994; Sims & Gamon, 2002)

Achl = p_{550}/p_{500}	Chlorophyll	(Pontius et al., 2008)
BNb = p_{800}/p_{550}	Chlorophyll	(Buschman & Nagel, 1993; Pontius et al., 2008)
PRI = $(p_{530}-p_{570})/(p_{530}+p_{570})$	Carotenoid/chlorophyll	Gamon et al.,1997.
PSRI = $(p_{680}-p_{500})/p_{750}$	Carotenoid/chlorophyll	(Merzlyak et al., 1999)
SIPI = $(p_{800}-p_{445})/(p_{800}-p_{680})$	Carotenoid/chlorophyll	(Peñuelas et al., 1995b)
CRI1 = $(1/p_{510})-(1/p_{550})$	Carotenoid/chlorophyll	(Gitelson et al., 2002)
CRI2 = $(1/p_{510})-(1/p_{700})$	Carotenoid/chlorophyll	(Gitelson et al., 2002)
Red/green = p_{red}/p_{green}	Anthocyanin/chlorophyll	(Gamon and Surfus (1999); Sims & Gamon, 2002)
ARI1 = $(1/p_{550})-(1/p_{700})$	Anthocyanin/chlorophyll	(Gitelson et al., 2002)
ARI2 = $p_{800\ x}$ $((1/p_{550})-(1/p_{700}))$	Anthocyanin/chlorophyll	(Gitelson et al., 2002)

** p=wavelength*

Statistical analyses

The experiments were conducted in a completely randomized design (CRD) in a factorial scheme 2x11 (leaf age x leaf developmental stage) and statistical analyses, including Pearson correlation, were performed using GENES software (Cruz, 2013). Data were processed by analysis of variance (ANOVA) followed by Tukey's test at a 5% significance level. Here, age-related treatments consisted of expanding 15-d-old leaves, also referred to as young leaves, and 30-d-old expanded leaves, also referred to as old leaves. Developmental stages were composed of phytomers from the 2nd to the 12th position along the plant axis. Leaves closer to the base were referred to as juvenile and the ones closer to the top were referred to as adult leaves.

RESULTS

Spectral signature of Passiflora edulis *leaves*

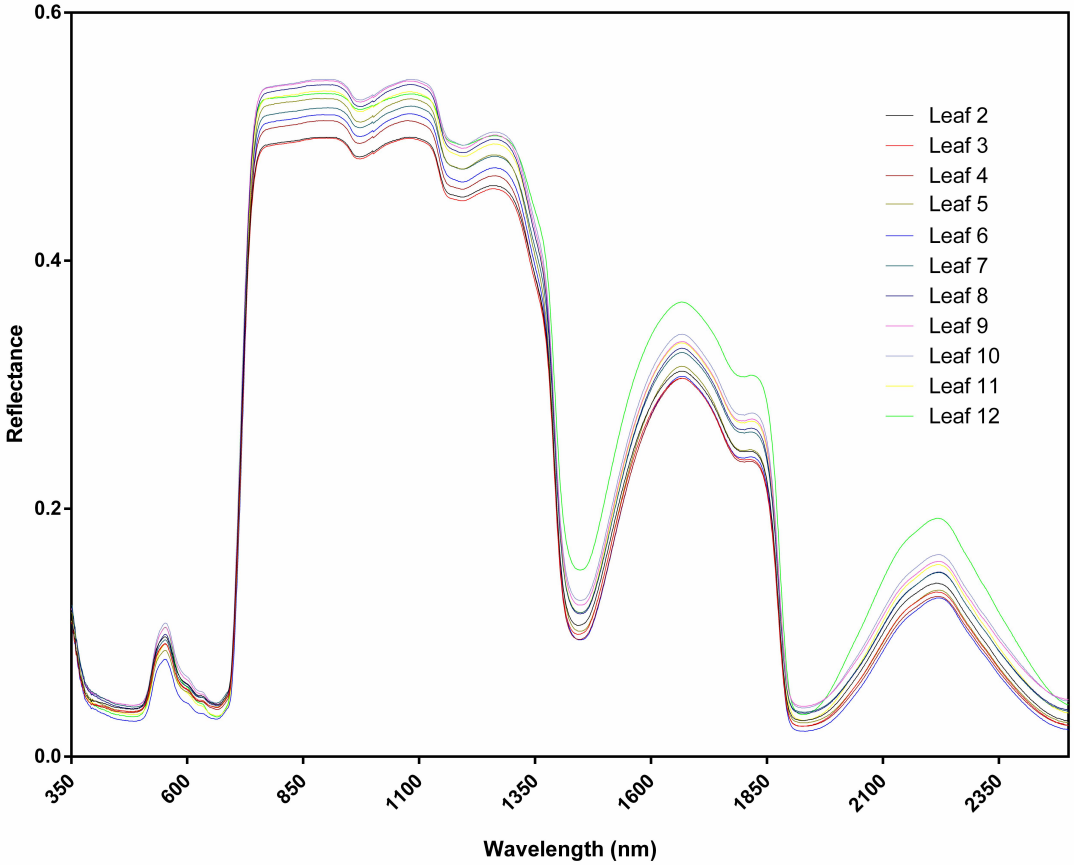

Figure 1 - Reflectance spectra of *Passiflora edulis* leaves.Average spectra representing the signatures of fresh leaves from the 2nd up to the 12th phytomer of *Passiflora edulis* Sims and indications of regions where foliar pigment, biochemical and water absorbing features occur, as well as energy spreading due to leaf structure.

Figure 1 shows the averaged reflectance spectra of fully expanded (30-d-old) fresh state leaves of *Passiflora edulis*. The wavelength regions in which the basic plant components have strong absorption features are also indicated (Kokaly et al., 2003). According to Curran (1989), reflectance spectra of all types of vegetation in the 400-2400 nm spectral region are greatly similar. Because of absorption by chlorophyll, reflectance in the visible region of green plants has a maximum at approximately 550 nm and minimum in the blue (450 nm) and red (680 nm). Beyond visible wavelengths (greater than 700 nm), the spectra of fresh plants show a strong rise in reflectance. The region of high plant reflectance at the short wavelength end of the near-infrared (750–1300 nm) is called the near-infrared

plateau (NIR-plateau). Two absorption features centered near 980 and 1200 nm are evident on the NIR-plateau. At 1400 nm, another water absorption feature reduces the reflectance and an even stronger water absorption occurs at 1900 nm (Kokaly, 2001; Kokaly et al., 2003). Difference in structural and physiological aspects are usually seen in leaves at different stages of development. Here, trilobed leaves (Fig. 1 - leaf 12) were found tohave different spectral signature from leaves at other developmental stages, especially at 1600-1850 nm and 2100-2400 nm. These regions of the reflectance spectrum in vegetation indicate differences in the composition of organic and structural molecules, such as lignin and cellulose Curran (1989).

Biochemical analyses of leaf pigments

Biochemical analyses of leaf pigments present were used as a reference technique to validate and compare the results obtained from hyperspectral vegetation indexes (HVIs). The pigment analyses showed that the contents of chlorophylls *a* and *b,* total chlorophyll, carotenoids, and anthocyanins exhibited similar patterns. For all the pigments evaluated, the second and third leaves, which are juvenile, presented the highest contents (Fig. 2 A-F).

Chlorophylls *a* and *b,* total chlorophyll and carotenoids (Fig. A-E) shared a pattern along leaf development: levels of pigments are increased in the second juvenile leaves and decline gradually until the 9, 10 or 11th leaves, which for chlorophylls is followed by an increase in the 12th leaf - a trilobed leaf that indicates the beginning of the adult phase in this species.

In regard to anthocyanins, these pigments are also more abundant in the second juvenile leaf. In comparison to the second leaf, the 3rd phytomer experience a decline in anthocyanins that is maintained up to the 12th phytomer (Fig. 2F).

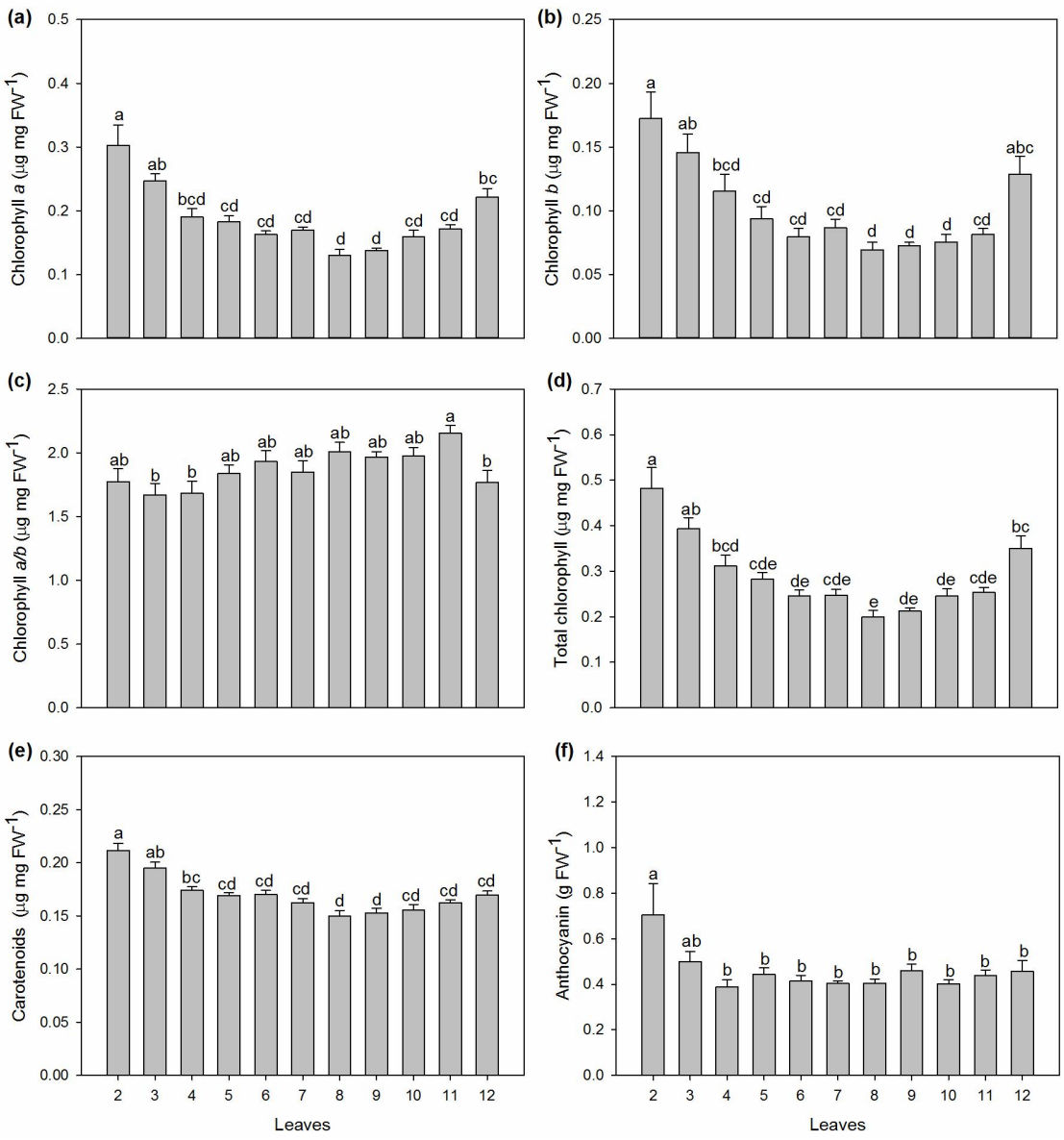

Figure 2 - Pigment concentrations of fresh leaves of *Passiflora edulis* from the 2nd to the 12th phytomer. (A) Chlorophyll *a*, (B) Chlorophyll *b*, (C) Chlorophyll *a/b*, (D) Total chlorophyll, (E) Carotenoids and (F) Anthocyanins. Leaf development treatments followed by the same letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Figure 3 - Heat map visualization of Pearson correlation coefficients between indexes and biochemical measures of pigments in fresh leaves of *Passilora edulis*.x1: PSRI; x2: SIPI; x3: carotenoids; x4: mND705; x5: mSR705; x6: PRI; x7: Red/green; x8:SR680; x9: SR705; x10: ND680; x11: ND705; x12: red/green; x13: BNb; x14: CRI1; x15: CRI2; x16: ARI1; x17: ARI2; x18: Chl *a*; x19: Chl *b*; x20: chl *a*/*b*; x21: total chl; x22: anthocyanins. Pearson correlation coefficients were considered significant at a 5% probability level by the *t*-test.

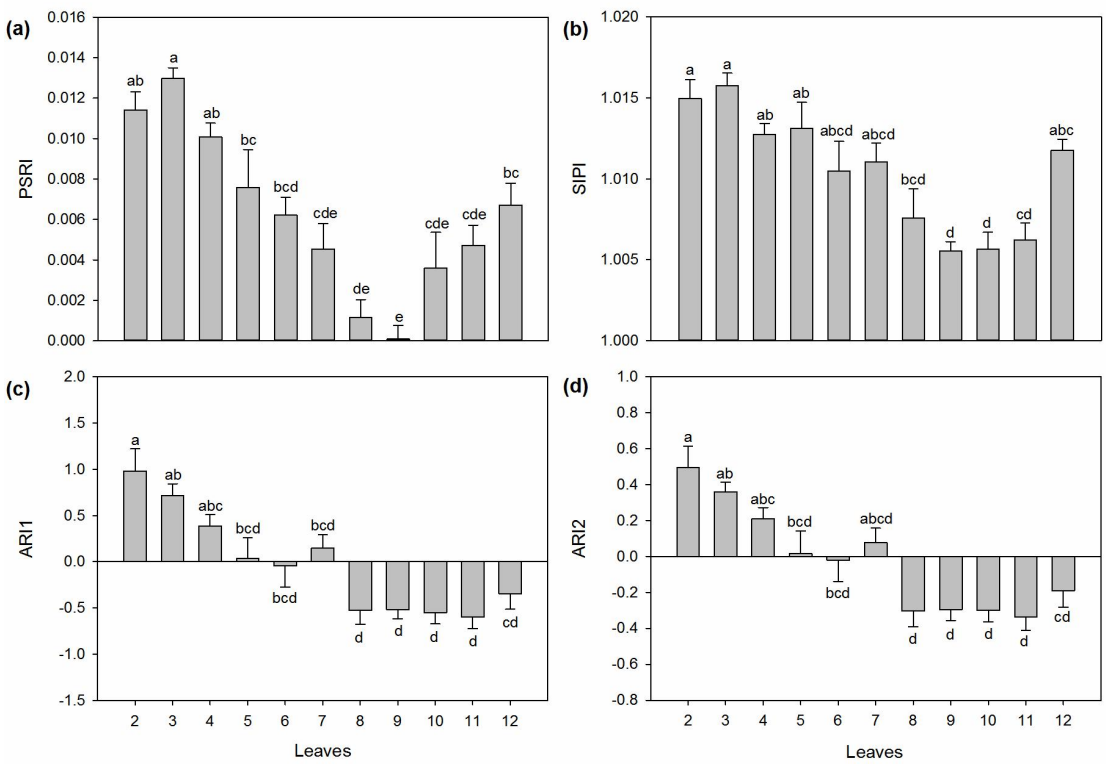

Figure 4 - Biochemical indexes applied to hyperspectral data of fresh leaves of *Passiflora edulis* from the 2nd to the 12th phytomer. (A) PSRI - Plant Senescence Reflectance Index, (B) SIPI - Structure-Insensitive Pigment Index, (C) ARI1 and (D) ARI2 - Anthocyanin Reflectance Indexes 1 and 2. Leaf development treatments followed by the same letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Correlation between pigment content and hyperspectral data

Positive and negative correlation values between indexes and pigment contents were classified as: 0-0.2: no correlation; 0.2-0.4: weak; 0.4-0.6: moderate; 0.6-0.8: strong; and 08-1: very strong. In order to evaluate the ability of indexes to predict pigments in leaves of *Passiflora edulis* over a juvenile gradient, at the end of the experiment, leaves were collected and biochemicals and hyperspectral analyses were performed. The indexes tested are listed in Table 2 and correlation coefficients between indexes and pigments are presented in Figure 3.

The indexes SR680, SR705, ND680, ND705, mND705 and MSR705, red/green, and BNb were selected to test correlation with chlorophylls *a* and *b* and

total chlorophyll, as they were designed for that purpose. Interestingly, no significant correlations were found between any of these indexes and chlorophylls. On the other hand, a very strong correlation was observed between total chlorophyll and other three indexes: PSRI (r=0.856; P=0.008), ARI1 (r=0.817; P=0.021) and ARI2 (r=0.809; P=0.026). Chlorophylls *a* and *b* had a close to perfection correlation (r=0.979; P=0.000), and so did total chlorophyll with chlorophyll *a* (r=0.995; P=0.000) and *b* (r=0.989; P=0.000). It is also worth pointing out that not only did total chlorophyll correlate significantly with the aforementioned indexes and different types of chlorophylls, but it was also correlated with carotenoids (r=0.955; P=0.001) and anthocyanins (r=0.824; P=0.019).

The PSRI, SIPI, and PRI were the indexes originally designed for carotenoids. Both PSRI and SIPI correlated highly with carotenoids: (r=0.8933; P=0.002) and (r=0.832; P=0.016), respectively. On the other hand, PRI did not correlate significantly with PSRI or SIPI, nor did it correlate with carotenoids. Unexpectedly, carotenoids were best correlated with ARI1 (r=0.910; P=0.001) and ARI2 (r=0.902; P=0.018), both designed for detection of anthocyanins, which may be partially explained by the significant correlation between carotenoids and anthocyanins (r=0.822; P=0.020).

While presenting high correlation levels with carotenoids and chlorophylls, anthocyanins measured by biochemical analyses were not significantly correlated to any of the indexes herein evaluated. Instead, indexes referred to estimate levels of anthocyanins in the literature (ARI1 and ARI2) were found to be strongly correlated with carotenoids: r=0.910, P=0.001 and r=0.902 and P=0.002, respectively. Considering that PSRI, SIPI, ARI1, and AR2 provided very strong correlation coefficients for carotenoids and chlorophylls, they were plotted against leaf developmental treatments (Fig. 4) and also used in the analyses of the effect of leaf age and development on pigments of *P. edulis* leaves.

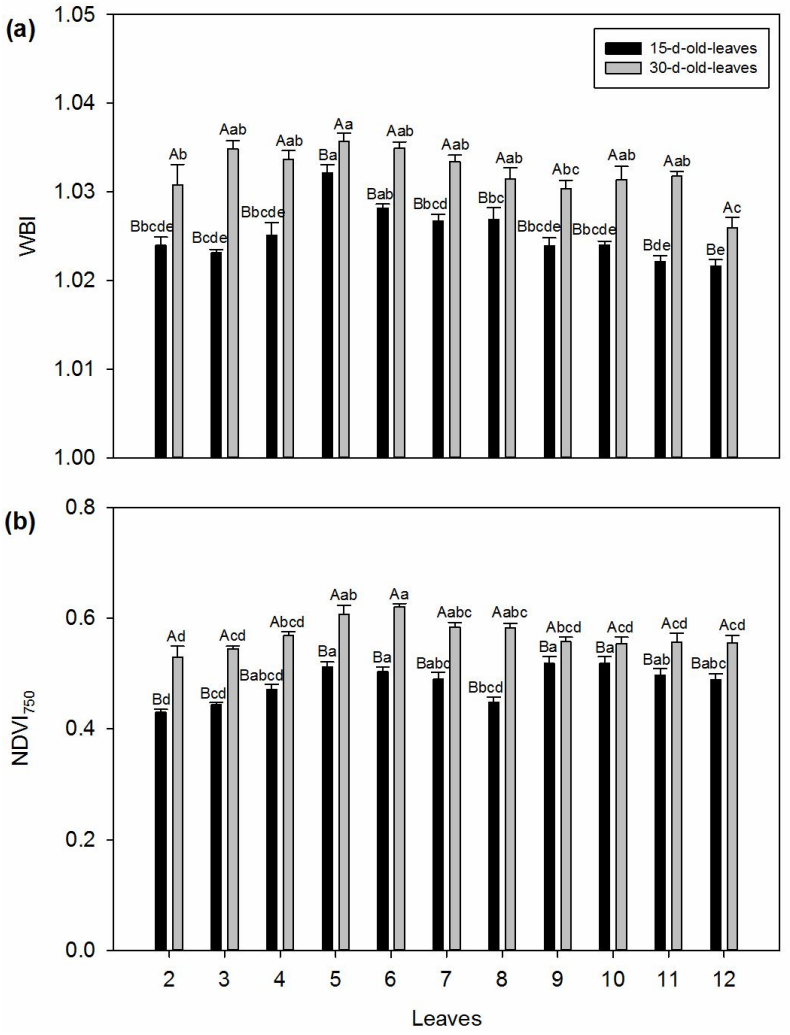

Figure 5 - Structural indexes applied to hyperspectral data of 15 and 30-d-old fresh leaves of *Passiflora edulis* from the 2nd to the 12th phytomer. (A) WBI - Water Band Index, (B) $NDVI_{750}$ - Normalized Difference Vegetation Index. Leaf age treatments followed by the same uppercase letters and leaf development treatments followed by the same lowercase letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

The Water Band Index (WBI) has been proposed to compare the expression of subtle or strong liquid water bands relative to a reference non-absorbing wavelength (Peñuelas et al., 1997). The WBI is calculated as the reflectance ratio of 900 nm to 970 nm and is described (Roberts et al., 2011) to be increased by water content in plants. Here, this variable presented high interaction between the two

factors at investigation: leaf age and leaf development ($P<0.01$). In all developmental stages, 30-d-old leaves exhibited higher water contents than 15-d-old leaves (Fig. 5A). Both ages, 15- and 30-d-old leaves, achieved the lowest WBI values on the latest phytomers. However, 30-d-old leaves experienced a subtler decline in water content throughout leaf development.

The Normalized Difference Vegetation Index (NDVI) is a good predictor of wet and dry green biomass (Tucker, 1979).It is defined as the difference between the reflectances in near-infrared and red regions of the spectrum normalized to the sum of these reflectances. In this study, the two factors under evaluation present strong interaction ($P<0.01$). NDVI was found to be higher in 30-d-old leaves compared to 15-d-old leaves and a non-linear pattern was observed over the developmental phases (Fig. 5B). Noticeably, the NDVI increased in the leaves of the latest developmental phases in expanding leaves.

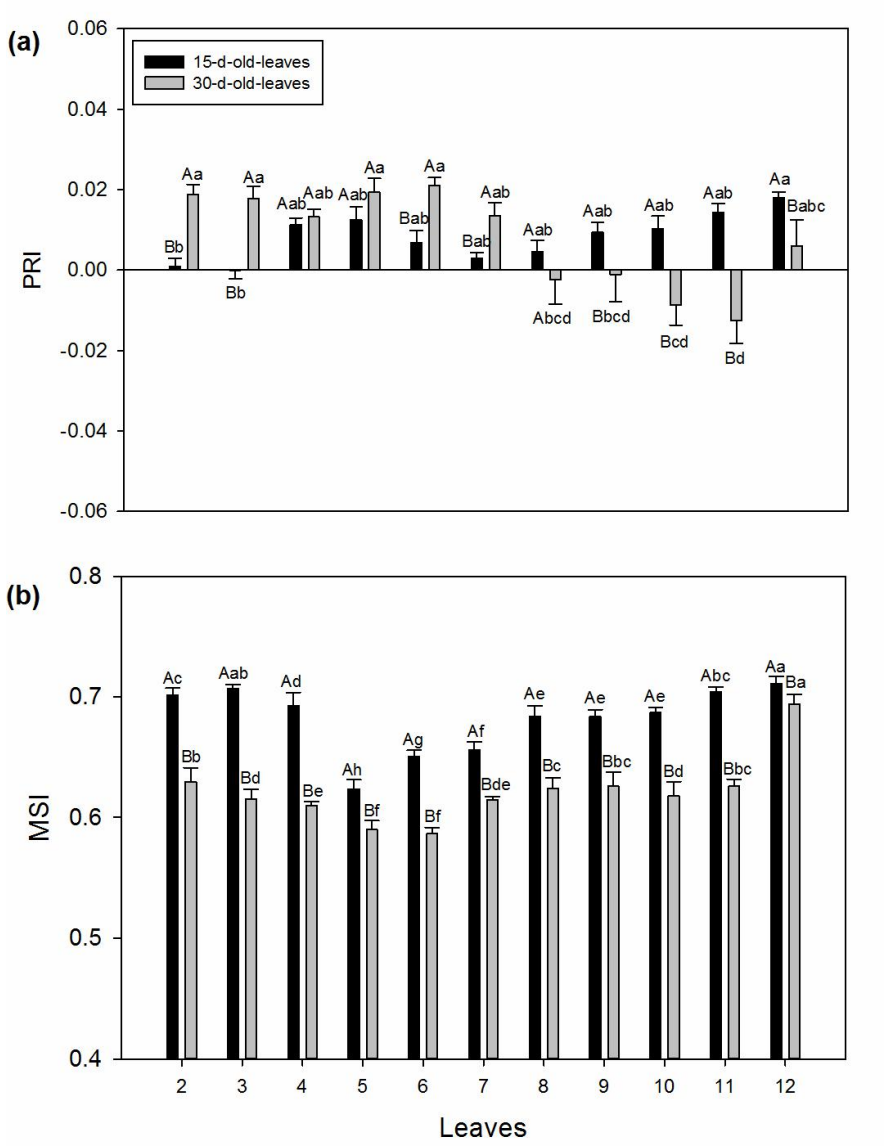

Figure 6 - Physiological indexes applied to hyperspectral data of 15 and 30-d-old fresh leaves of *Passiflora edulis* from the 2nd to the 12th phytomer. (A) PRI - Physiological Reflectance Index, (B) RVSI - Red-edge Vegetation Stress Index, (C) MSI - Moisture Stress Index. Leaf age treatments followed by the same uppercase letters and leaf development treatments followed by the same lowercase letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Effect of leaf ontogeny on physiological features of Passiflora edulis *leaves*

In this study, we observed a significant interaction between leaf age and development (P=0) regarding PRI. Expanding leaves presented lower PRI values in the second phytomer, which increased gradually until the last phytomer, evidencing that expanding leaves in the early developmental stages are under more stressful conditions (Fig. 6A). On the other hand, fully-expanded leaves were less stressed at

the beginning of plant development and become more stressed from the 8th phytomer up to the 11th, exhibiting negative values. On the 12nd expanded leaf, an increase in the PRI value is observed, indicating some stress relief.

Previous work has shown that the ratio of SWIR to NIR band of a spectral curve can discriminate between high and low-damage sites (Rock et al., 1985; Vogelmann and Rock, 1986). This ratio was developed using field data and has been referred to as the moisture stress index (MSI; Rock et al., 1985). Similarly to WBI, the MSI compares reflectance at water absorbing to non-absorbing wavelengths. Therefore, high ratios are associated with high-damage material, and low ratios with low-damage material (Rock et al., 1986). Here, the variable MSI presented highly significant interaction between the two factors at investigation: leaf age and development (P=0.002). In agreement with the results achieved with the WBI index, expanding leaves demonstrated higher MSI than fully expanded leaves, which implicates in more leaf damage (Fig. 6B). Moreover, both 15- and 30-day-old leaves had the highest MSI values at the 12th phytomer, and 30-day-old leaves also presented increased MSI at the 3rd phytomer.

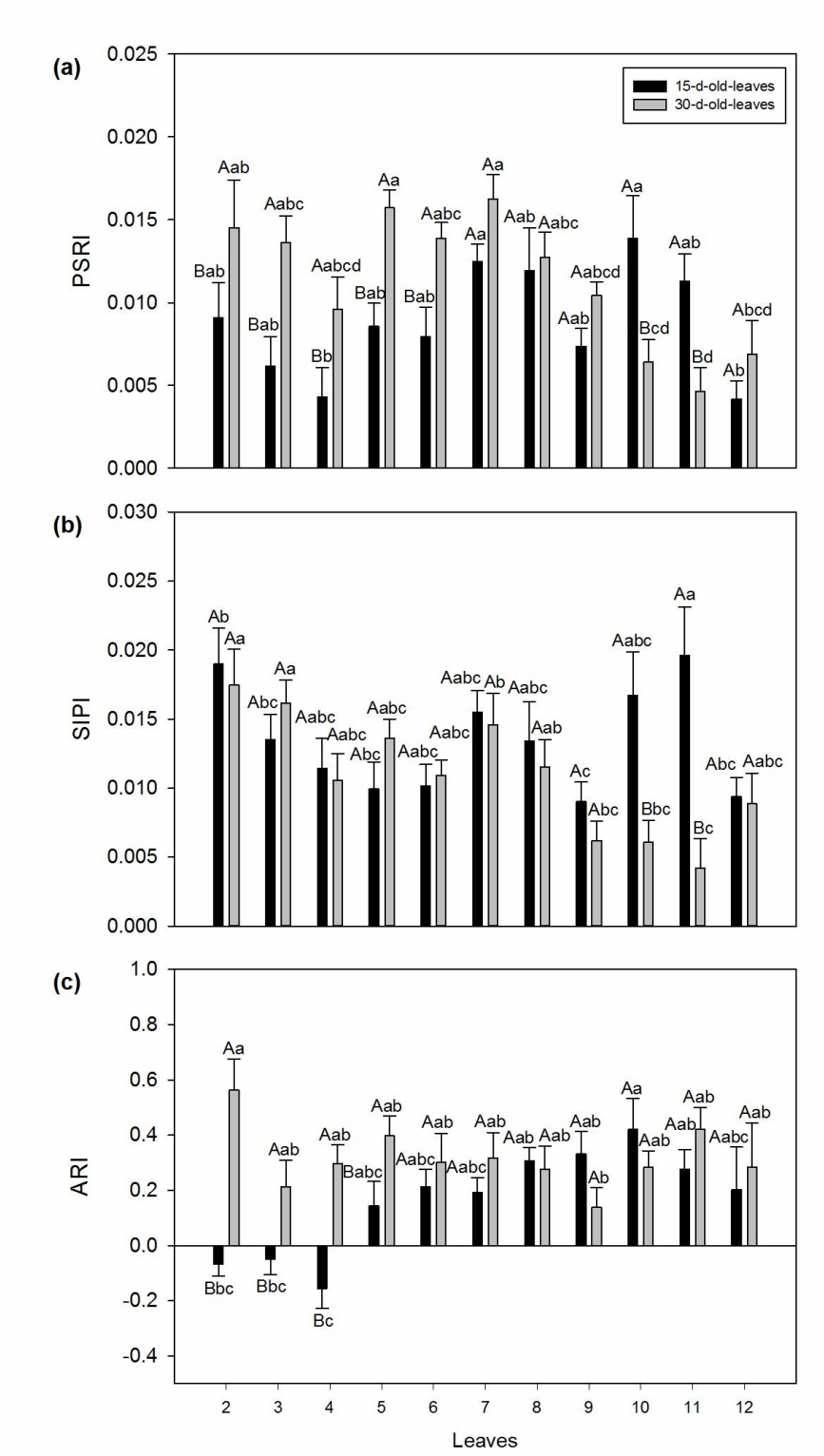

Figure 7 - Biochemical indexes applied to hyperspectral data of 15 and 30-d-old fresh leaves of *Passiflora edulis* from the 2nd to the 12th phytomer. (A) PSRI - Plant Senescence Reflectance Index, (B) SIPI - Structurally Insensitive Pigment Index, (C) ARI-Anthocyanin Reflectance Index. Leaf age treatments followed by the same uppercase letters and leaf phytomers followed by the same lowercase letters do not differ statistically by Tukey's test at 5% probability. Bars and limit lines represent mean and standard error values, respectively.

Effect of leaf ontogeny on biochemical features of* Passiflora edulis *leaves

A reasonable number of indexes has been developed to measure overall pigment concentrations or to quantify specific pigments in plant leaves and canopies (Roberts et al., 2011). Many indexes are formulated either as a simple ratio, such as the Anthocyanin Reflectance Index (ARI: Gitelson et al., 2001) or a normalized ratio, such as the Structurally Insensitive Pigment Index (SIPI: Penuelas et al., 1995b). These indexes respond to either a single pigment or combinations, such as the ratio of carotenoids to chlorophyll *a* (SIPI). Similar to SIPI, the Plant Senescence Reflectance Index (PSRI: Merzlyak et al., 1999) also changes in response to a change in the ratio of carotenoids to chlorophyll as plants senesce (Roberts et al., 2011). On the ground that PSRI, SIPI, and ARI were the indexes that best correlated to pigment data from biochemical analyses, they were chosen to describe pigment dynamics on expanding and fully-expanded leaves of *P. edulis* over plant development.

The PSRI index describes the dynamics of senescence processes in leaves and fruits, estimating the carotenoid/chlorophyll ratio (Merzlyak et al., 1999). In the first six juvenile phytomers, fully-expanded leaves presented higher PSRI than expanding leaves, becoming statistically equal from the 7th phytomer onwards, excepted for phytomers 10 and 11th, which presented expanding leaves with higher PSRI (Fig. 7A). Except for phytomers 10 and 11th, whose expanded leaves had a higher carotenoid/chlorophyll ratio, a similar pattern was observed for both kinds of leaves, presenting oscillating values that did not follow a linear trend throughout leaf development.

Similar to PSRI, the SIPI index responds to alterations in the ratio of carotenoids to chlorophyll (Penuelas et al., 1995b). This ratio was not significantly different in the two expanding periods evaluated in this study, except for the 10 and 11th phytomers, in which SIPI was higher for expanding leaves (Fig. 7B). The non-linear and oscillating values of SIPI in 15- and 30-day-old leaves followed a similar trend of PSRI values.

Anthocyanins are plant pigments that can increase in response to environmental stress and may play a role in minimizing photoinhibition (Gitelson et

al., 2001). Gitelson et al. (2001) proposed an index to evaluate anthocyanins in plants, based on the concept of reciprocal reflectance developed for chlorophyll. Hence, the Anthocyanin Reflectance Index (ARI) is calculated as the difference between reciprocal green reflectance (540-560 nm) and reciprocal red-edge reflectance (690-710 nm). In the biochemical analyses that we carried out, ARI was significantly correlated with chlorophylls and carotenoids, and it was not correlated with anthocyanins. When comparing leaf expansion throughout plant development, only in the early juvenile leaves, 2nd to 5th, ARI values were statistically different, achieving increased numbers in expanded leaves (Fig. 7C). In terms of the effect of the development phase on ARI, it was verified that ARI values increased gradually in 15-day-old leaves, whereas it decreased gradually in 30-day-old leaves until the 9th phytomer.

DISCUSSION

The most promising contribution and important goal in monitoring vegetation with high-sensitivity remote sensing are to detect physical, structural and biochemical changes in plants as affected by diverse factors. For such purpose, the use of spectral signatures is indispensable for determining quantitative and qualitative relationships between optical properties and plant composition (Kim et al., 2015). Generally, these signatures are obtained via spectroscopy, and leaves are usually the organ of choice to be analyzed for spectral changes (Ceccato et al., 2001). Having recently found their way into small-scale and controlled environments, hyperspectral approaches with spectral indexes are of utmost importance to establish a correlation between leaf composition and spectral traits (Inoue et al., 2008;Singh et al., 2013).

Spectral signature of **Passiflora edulis** *leaves*

Plant functional and optical properties are highly regulated by leaf anatomical, structural, biochemical and biophysical characteristics (Heim et al., 2014). Each plant component has its particular light-absorbing characteristics, but jointly all molecules contribute to the plant spectral signature (Gates et al., 1965; Knipling, 1970), providing valuable information not only to distinguish plant species, but also environmental conditions to which they have been submitted. In this scenario, spectroscopy is an important tool to obtain information about a material by associating the interaction of electromagnetic radiation to its chemical and physical traits (Kokaly et al., 2003).

Many recent studies have shown that spectral signatures might be valuable for distinguishing among plant species (Clark et al., 2005; Ghiyamat et al., 2013; Prospere et al., 2014), including cultivars of tea (He et al., 2009), wine (Edelmann et al., 2001) and other commercially-relevant crops (Rao et al., 2007). In this regard, Ustin and Gamon (2010), based on the idea of plant functional types (Duckworth et al., 2000), proposed a new hypothesis that supports the classification of plants as optical types.

As described in the literature and similarly observed in *Passiflora edulis* leaves (Fig. 1), in near-infrared wavelengths at approximately equal distances throughout

the spectrum, there are five major absorption features. These absorption features are the result of electron transitions in chlorophyll (400-700 nm) and of the bending and stretching of the O--H bond in water and other chemicals (970, 1200, 1400, 1940 nm) (Danks et al., 1984; Curran, 1985; Osborne and Fearn, 1986). In dry vegetation, the water absorption features conceal to different absorption characteristics in the NIR region due to organic bonds in plant biochemicals (Kokaly et al., 2003). Organic compounds such as proteins, lignin, and cellulose all contribute to these traits, once their C–H, N–H, and C–O bonds have overtone and combination bands that absorb in the near infrared region of the spectrum (Kokaly, 2001; Peterson & Hubbard, 1992).

Here, we observed high reflectance values in the NIR plateau. According to Gausman et al. (1998), this characterizes healthy leaf tissues. Reflectance in this region results from an increased amount of light scattering at cell wall interfaces due to a change in the index of refraction, the absence of absorption by pigments, and the weakening of absorption by water in leaves at these wavelengths (Rock et al. 1985; Kokaly et al., 2003). Similarly, Rock et al. (1986) suggest that changes in cellular health within leaves are associated with reduced reflectance along the NIR plateau. Interestingly, this information reveals remote sensing techniques as important tools to provide diagnoses of plant health conditions.

Although the effect of water on NIR regions has been discussed, Curran (1989) pointed out that water absorption wavelengths overlap the absorbance of some biochemical compounds, such as cellulose and lignin, making their estimation through hyperspectral data rather compromised or biased. For this reason, it is desirable to perform spectral measurements of fresh and dry leaves. In this study, only fresh leaves were evaluated, but in order to have reliable information regarding cellulose, lignin and other parameters that absorb in high-absorbing water regions, further investigations considering dry leaves must be considered.

This is the first report that provides the spectral signature of *Passiflora edulis* leaves and evaluation of leaf age and development as affected by ontogeny.Based on multiple traits evident in vegetation optical properties, there have been a number of recent successful demonstrations of biodiversity assessment through optical diversity (Carlson et al., 2007; Lucas & Carter, 2008; Asner & Martin, 2009). Some

of the studies argue that it is possible to characterize spectral types based on the underlying unique biochemical signatures, a concept termed 'spectranomics' (Asner & Martin, 2009).

Young and adult leaves are under more water stress

Water concentration assessment might be easily and reliably performed with a standard method in which the plant material is dried in an oven. It is important to highlight that while this method is time-consuming and demanding, the use of indexes, such as WBI, which allow water measurement through the water absorption of electromagnetic infrared radiation, may provide a rapid, easy and reliable method for the same purpose (Peñuelas et al., 1997). In fact, plant water concentration has been successfully estimated with the use of methods relying on high-resolution reflectance on the water absorption spectrum (Carter, 1991; Danson *et al.,* 1992).

In the spectral region from NIR to SWIR, water presents narrow absorption peaks, similar to a pigment. Hence, it is possible to estimate water content based on quantitative methods, such as ratio-base indexes defined by water absorption-related bands and the reference non-absorbing bands. Water Band index (WBI) (Peñuelas et al., 1997) and Moisture Stress Index (MSI) (Rock et al., 1986) are negatively correlated, meaning that the increase of water content causes the high value in WBI, whereas MSI value is reduced with the increase in moisture level.

Given that previous experiments have found a relationship with progressive drying and a decrease in the reflectance of 970nm, the WBI index was suggested considering the reflectance of water at 970 nm and a reference wavelength at 900 nm (Peñuelas et al., 1993, 1996, 1997). Working with a range of Mediterranean species growing in the field, Peñuelas et al. (1997) found that WBI was significantly correlated with plant water content, with the highest water concentrations corresponding to also higher WBI. Furthermore, Roberts et al. (2006) also observed that the water index had a high correlation with the moisture of shrubland vegetation.

Physiology based-methods for detection of water stress constitute a major goal for remote sensing and have been proposed using indexes of Near-Infrared (NIR, 0.7–1.3 μm) and Middle-Infrared (MIR, 1.3–2.5 μm) wavelengths (Jackson, 1982; Hunt et al., 1987). HVIs using red and NIR wavelengths have been successfully reported to infer plant water stress and a consequent reduction of plant productivity (Richardson and Everitt, 1987), being strongly correlated with total leaf water mass (Tucker, 1979). One such method is termed the MSI and it uses NIR and MIR reflectances to determine plant water stress and damage (Rock et al., 1985). The MSI has been successfully used to detect vegetation moisture status in both vascular plant species and mosses (Bryant & Baird, 2003; Ceccato et al., 2001; Vogelmann & Moss, 1993; Hunt & Rock, 1989; Hunt et al., 1987; Rock et al., 1986; Vogelmann & Rock, 1986).

As expected, increases in MSI (Fig. 6B) corresponded to decreases in the WBI (Fig. 5A). While younger leaves presented higher MSI compared to older leaves, the opposite behavior was observed for WBI, indicating that 30-d-old leaves suffered from lower damage and presented higher water contents, whereas the opposite occurred to expanding leaves. Both younger and older leaves presented higher and lower values of MSI and WBI, respectively, at the 12th phytomer, indicating that adult leaves have lower water contents and are under more stressful conditions in comparison to juvenile leaves. Previous studies demonstrated that there is a causal relationship between these variables (Defraeye et al., 2014; Torres-Ruiz et al.,2015; Zhang et al., 2015; Breś et al., 2016; Beyschlag & Zotz, 2017), that is, the latest leaves are under more stressful physiological conditions and the decreased water contents might be a causing agent.

Older leaves exhibit higher green biomass

The normalized difference vegetation index (NDVI) is one of the most widely used and has been extremely helpful to monitor plant biomass and other growth variables across time and space, predominantly in ecological studies. In this type of studies, NDVI has been found to correlate highly with biophysical properties of the vegetation, e.g. green biomass and the leaf area index (LAI), (Lu et al., 2005; Wang et al., 2005). In addition, plant crop investigations have shown that the NDVI has been correlated to many variables such as crop nutrient deficiency, final

yield in small grains, and long-term water stress (Govaerts & Verhulst, 2010). However, rather than reflecting the effect of one parameter only, NDVI must be interpreted as a measurement of combined plant growth, reflecting a set of plant growth factors (Govaerts & Verhulst, 2010).

The results herein presented evidenced that 30-d-old leaves presented higher NDVI when compared to 15-d-old leaves during the whole developmental process of *P. edulis* (Fig. 5B). This finding suggests that the interlude of 15 days, which separate expanding from fully-expanded leaves, is fundamental for biomass gain in yellow passionfruit leaves as well as leaf growth and development. It appears that this period exerts an even higher impact over juvenile leaves, considering the bigger difference between NDVI values of 15 and 30-d-old leaves at the beginning of the development of *P. edulis* leaves. A recent study with durum wheat (*Triticum turgidum* L. var. *durum*) grown in pots showed a strong correlation between NDVI measurements and dry aboveground biomass, total green area, green area without spikes, and aboveground nitrogen content (Cabrera-Bosquet et al., 2011). Moreover, Bégué et al. (2010) reported the usefulness of NDVI to describe biomass and sugar content in the stalk of sugarcane, as significant correlations were achieved between these variables. In combination with our findings, this report points out this index as a reliable and informative predictor of plant growth and development.

Ontogeny plays an important role in photosynthetic efficiency

The Photochemical Reflectance Index (PRI) is a spectral index that has shown promise as an indicator of photosynthetic efficiency using narrow-band reflectance (Gamon et al., 1992; Peñuelas et al., 1995), through the estimation of xanthophyll pigment interconversion — a plant photoprotective mechanism that results in lowered photosynthetic efficiency (Magney et al., 2016). Once PRI measures the relative reflectance on either side of the green reflectance "hump", at 531 and 570, it also compares the reflectance in the blue (chlorophyll and carotenoids absorption) region of the spectrum with the reflectance in the red (chlorophyll absorption only) region (Garbulsky et al., 2011). As a result, this index can evaluate relative chlorophyll:carotenoid levels (Filella et al., 2009; Stylinski et al., 2002).

Plants used in this study were grown in a greenhouse free of stressful conditions, leading us to believe that the negative PRI observed in this study (Fig. 6A) were more certainly related to endogenous factors, such as ontogeny. Here, this effect was apparent in expanding leaves in early phytomers, probably due to lower pigment pool usually observed in these types of leaves. In addition, fully-expanded leaves in later developmental stages presented the lowest PRI values, except for the 12th leaf. Therefore, to the extent that photosynthetic activity correlates with changing chlorophyll/carotenoid ratios in response to stress, ontogeny might play an important role in plants grown under stable conditions. In fact, changes in bulk pigment content and ratios due to leaf development, aging or chronic stress have been reported to play a significant role together with the xanthophyll pigment epoxidation in the PRI signal (Gamon et al., 2001; Peñuelas et al., 1997b; Sims and Gamon, 2002; Stylinski et al., 2002), corroborating with the hypothesis that ontogeny could be a stress agent itself to promote change in the chlorophyll/carotenoid ratio and investment in the photoprotective xanthophyll cycle pigments. Furthermore, an increase in the PRI was observed in the 12th leaf, which might be an indication of heteroblastic change, since this leaf is the first adult leaf in the lifespan of *P. edulis.*

Pigments of *P.edulis* leaves are highly correlated to HVIs

After analyzing pigment accumulation through biochemical analyses, we next compared biochemical data with hyperspectral signatures to evaluate the efficiency of indexes described in the literature for the particular case of *Passiflora edulis* leaves. Thus, we chose NDVI 705, mNDVI 705, NDVI 680, SR 750, SR 680, mSR 705, red/green and BNb for chlorophylls; PRI, SIPI, PRSI, CRI1 and CRI2 for carotenoids; and Ant, ARI1 and ARI2 for anthocyanins.

Unexpectedly, the chlorophyll measurement performed by laboratory assays was not correlated to any of the indexes built for chlorophyll in the literature, which suggests that these indexes are not good predictors of chlorophyll in leaves of greenhouse-grown *P. edulis*. Instead, chlorophyll was significantly correlated to PRI (r=0.644; P=0.031), an index proposed to estimate light use efficiency and carotenoid/chlorophyll ratio. Also, chlorophyll was strongly correlated to PRSI(r=0.856; P=0) and SIPI (r=0.795; P=0.003), indexes proposed to estimate

carotenoid/chlorophyll ratio. In addition, chlorophyll presented a significant correlation with ARI1 and AR12, which have been linked to anthocyanin/chlorophyll estimation. Noticeably, chlorophyll also correlated strongly to chlorophyll *a* (r=0.996; P=0), chlorophyll *b* (r=0.989; P=0) and anthocyanins (r=0.824; P=0.019). The fact that chlorophyll *a* and chlorophyll *b* correlated so well (r=0.980; P=0) might be explained by the homogeneous light conditions in which the plants were grown, with no leaves under shaded spots. While the PRI was not significantly correlated to carotenoids, both SIPI and PSRI were highly correlated with this pigment. Interestingly, PRI correlated only with chlorophyll, being the only index specific to one pigment. It is important to notice that the indexes built for anthocyanins, ARI1 and ARI2, yielded the best correlation coefficient to carotenoids: r=0.91 and r=0.90, respectively. On the other hand, none of the tested indexes correlated significantly with anthocyanins.

In short, chlorophyll was significantly correlated with PRI, PSRI, SIPI, ARI1, and ARI2, being best correlated with PSRI. Carotenoids correlated significantly with PSRI, SIPI, ARI1, and ARI2, presenting its highest correlation coefficient with ARI1. Although PRI was the only index specific for chlorophyll, PSRI and ARI1 outperformed all the indexes tested for chlorophyll and carotenoids, respectively.

Recent studies on PRI indicate that when PRI is applied over longer periods or across species, its variations appear to assess relative composition of chlorophylls and carotenoids (Filella et al., 2004; Sims & Gamon, 2002; Stylinski et al., 2002). In the present work, this index was only correlated to chlorophyll but not to carotenoids. Similarly, after evaluating the relationship between PRI and chlorophyll fluorescence parameters over different life stages of *Solanum melongena* L., Rahimzadeh-Bajgiran et al. (2012) found overall photosynthesis capacity and steady-state PRI to be better correlated with chlorophyll content than xanthophylls.

Estimating carotenoids precisely in leaves using remote sensing is usually challenging for the smaller proportion of carotenoids in relation to chlorophylls in most leaves, and most importantly, the fact that both pigments present overlapping spectral absorption features in the blue region of the spectrum (Sims & Gamon,

2002). Consequently, reflectance indexes have been more useful as predictors of the ratio of carotenoid to chlorophyll than absolute carotenoid content (Merzlyak et al., 1999; Peñuelas et al, 1995). Hence, most indexes for estimation of carotenoid/chlorophyll ratios are built upon the comparison of reflectance in the region of the carotenoid absorption peak (400–500 nm) with reflectance in the red region, which is influenced only by chlorophyll (Sims & Gamon, 2002).

The indexes tested here proved to lack sensitivity to both chlorophyll and carotenoids, except PRI and SIPI, respectively. Besides the lack of sensitivity due to overlap, we faced another challenge due to the high correlation that all pigments revealed between each other, especially chlorophyll and carotenoids. This ambiguity might have masked the efficiency and specificity of some indexes that correlated well to more than one pigment, making it difficult to evaluate if the indexes are indeed unspecific or the correlation to another pigment only happened because of a similar pattern presented by the non-target pigment. Hence, further studies are needed in order to minimize the effects of spectral convolution, enabling the isolation of the index performance.

Even though the indexes with the highest correlation coefficients to the biochemical data lacked some specificity, they presented a satisfactory performance, and perhaps in a scenario with different and unrelated patterns of pigments, these indexes will probably be more specific. Therefore, the results achieved in this study point out remote sensing as a great potential method for diagnosing phenology and physiological conditions for *P. edulis* leaves in a non-destructive way.

Here, indexes designed to estimate carotenoid/chlorophyll values, except PRI, were well correlated to carotenoids, whereas indexes designed for chlorophylls and anthocyanins performed poorly. It is possible that the other indexes achieved a saturation level with increasing chlorophyll and anthocyanin contents, which is usually not a problem for carotenoids due to their lower concentration in plants. Indeed, previous studies have shown that vegetation indexes tend to reach a saturation point with increasing levels of a given pigment (Blackburn, 1998; Fassnacht et al., 2015). According to Kong et al. (2016), PRSI and SIPI are only affected by saturation at high carotenoid levels, which is not the case for *P. edulis*.

Considering that pigment-related indexes were validated through biochemical analyses and PSRI, SIPI and ARI were found to be good predictors of leaf pigments in leaves of *P. edulis,* these indexes were also applied in the evaluation of the effect of age and development on leaf pigments (Fig. 4). It stands out that PRSI and SIPI in expanding leaves presented superior values in comparison to fully-expanded leaves in the phytomers 10 and 11, which are related to the phase transition from juvenile to adult leaves. This behavior is likely to be associated with the fact that intermediate and adult leaves present necataries on the leaf margin, a characteristic that is exclusive to these types of leaves, implicating in higher energy expenditure and production of photoassimilates, which demands an increased pigment level.

Anthocyanin estimation in leaves is also challenging, once its absorption also overlaps with chlorophyll, as occurs to carotenoids (Sims & Gamon, 2002). This might be a minor factor that contributed to the correlation of ARI1 and ARI2 to other non-target pigments. However, unlike carotenoids and chlorophylls, none of the indexes tested in this study were good predictors of anthocyanin levels in *P. edulis* leaves. For this reason, further studies are suggested in order to design a novel and efficient index specifically for estimating anthocyanins levels, which also considers important aspects of leaves, such as saturation point, leaf structure, and pigment overlapping.

CONCLUSION

Leaf growth, development, and aging encompass a plethora of structural, physiological and biochemical changes. In this study, we were able to estimate some of these changes with the aid of vegetation indexes and biochemical analyses. The clear message that emerged from the results obtained here is that all of the variables investigated are affected by leaf ontogeny and we speculate that PRI might be influenced by heteroblastic changes, due to less subtle changes from juvenile to adult leaves.

The results obtained from the comparison of hyperspectral and biochemical data reveal remote sensing as a great potential method for diagnosing phenology and physiological conditions for *P. edulis* leaves nondestructively. Although most indexes were not specific for one pigment, high correlation coefficients were achieved for chlorophylls and carotenoids. Still, further studies are necessary to uncouple the effect of carotenoid to chlorophyll correlation. Moreover, due to the overlapping of water with spectral regions where organic molecules also absorb, it would be necessary to run further analyses with dry leaves in order to access the contents of compounds such as lignin and cellulose.

GENERAL CONCLUSION

We demonstrated that *P. edulis* tissues respond differently to phytohormone stimuli and that the organogenic responses were negatively affected by the late physiological condition in leaf explants from green-house grown plants. Additionally, we verified that *miR172* is up-regulated by the cytokinin:auxin balance, which is likely due to the regulatory effect of these phytohormones.Furthermore, we suggest a crosstalk between cytokinin and miR1*64*, which might be relevant for the modulation of leaf development, as antagonistic interactions between *miR164* and *miR319*is proposed. In addition to a molecular approach, we also investigated ontogenic changes during leaf development in *P. edulis* with the aid of vegetation indexes and biochemical analyses. Therefore, we showed that physiological, biochemical and structural alterations occur over the heteroblastic process in *P. edulis* and might be accounted for with the use of remote sensing.

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