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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E MELHORAMENTO

CRISTIANA TORRES LEITE

Looking for influence of the chromosome number, ploidy level and nuclear 2C value on the in vitro response in *Passiflora* genus

ALEGRE

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Dissertação apresentada à Universidade Federal do Espírito Santo como requisito parcial para obtenção do Título de Mestre pelo Programa de Pós-Graduação em Genética e Melhoramento.

Orientadora: Dra. Milene Miranda Praça Fontes

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06 de setembro de 2016.

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Dedico

Aos meus pais, meus irmãos, minhas avós e minhas tias.

Ofereço

À minha orientadora Milene Miranda Praça Fontes e meu co-orientador Wellington Ronildo Clarindo.

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“A ciência nunca resolve um problema sem criar pelo menos outros dez”

George Bernard Shaw

Lista de abreviaturas

2,4-D – 2,4-dichlorophenoxyacetic acid

APM – Amiprophos-methyl

BAP – Benzylaminopurine

FEC – Friable embryogenic calli

IO – Indirect organogenesis

ISE – Indirect somatic embryogenesis

IZE – Immature zygotic embryos

FC – Friable calli

FCM – Flow cytometry

LEC – *Leafy cotyledon*

MZE – Mature zygotic embryos

PCR – Polymerase chain reaction

SE – Somatic embryos

SERK – *Somatic embryogenesis receptor kinase*

VIES – Herbarium of Universidade Federal do Espírito Santo

WOX – *Wuschel-related homeobox*

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Sumário

Article: Looking for influence of the chromosome number, ploidy level and nuclear 2C value on the in vitro response in *Passiflora* genus

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Resumo

Assim como para outros taxa, diferente respostas morfogênicas in vitro (organogênese direta / indireta ou embriogênese) foram relatadas para as espécies de *Passiflora*, utilizando as mesmas condições ambientais in vitro. O número de cromossomos distintos entre algumas espécies do gênero *Passiflora* tem sido apontado como um possível fator genético relacionado com as diferentes respostas in vitro. Com base nesta hipótese, o presente estudo teve como objetivo avaliar as respostas in vitro de espécies de *Passiflora* que exibem diferentes números cromossômicos, níveis de ploidia e conteúdo de DNA nuclear para responder a seguinte pergunta: estes aspectos genômicos influenciam à resposta in vitro? Para isso, embriões zigóticos maduros (MZE) de cinco espécies, pertencentes a quatro subgêneros de *Passiflora*, foram inoculados em meio MS suplementado com 4.4 µM de benzilaminopurina (BAP) e nove concentrações de 2,4-ácido diclorofenoxiacético (4.53 - 144.96 µM 2,4-D). Corroborando com os estudos anteriores, diferentes respostas morfogênicas foram observadas sob as mesmas condições in vitro. Apenas calos friáveis (FC) foram obtidos a partir MZE de *Passiflora coriacea* Juss (2n = 12 cromossomos, 2C = 1,00 pg), *Passiflora lindeniana* TR & Planch (2n = 24, 2C = 2.42 pg) e *Passiflora contracta* Vitta (2n = 48 cromossomos, 2C = 4.78 pg). Plântulas foram recuperadas a partir de MZE de *Passiflora foetida* L. (2n = 20, 2C = 1.04 pg) e *Passiflora miniata* Vanderpl. (2n = 18, 2C = 3,40 pg) via organogênese indireta e embriogênese, respectivamente. Como em outros estudos, as plântulas foram regeneradas a partir de embriogênese somática indireta somente para as espécies de Passiflora com 2n = 18 cromossomos *(P.*

miniata). Apesar do número de cromossomos e do nível de ploidia relativamente mais baixo do que em outros taxa de *Passiflora*, o tamanho do genoma nuclear das espécies com 2n = 18 é relativamente mais elevado. Assim, as mudanças no cariótipo (poliploidia, hibridização e disploidia) que amplamente ocorrem durante a evolução de *Passiflora*, provavelmente resultaram em número de cópias distintas dos genes relacionados ao processo morfogênico em plantas. Portanto, para o gênero *Passiflora* é importante olhar simultaneamente algumas características genômicas para compreender as respostas in vitro.

Palavras-chave: embriogênese somática indireta, evolução do cariótipo, genes morfogênicos**,** maracujá, organogênese indireta

Abstract

As well as for other taxa, different morphogenetic in vitro responses (direct/indirect organogenesis or embryogenesis) have been reported for the *Passiflora* species, even in the same in vitro environmental conditions. The distinct chromosome number between some *Passiflora* genus species has been appointed as a possible genetic factor related to the differences in in vitro responses. Based on this hypothesis, this study aimed to evaluate the in vitro responses of *Passiflora* species exhibiting distinct chromosome number, ploidy level and nuclear DNA content to answer the following questions: these genomic aspects influence the in vitro response? For this, mature zygotic embryos (MZE) of five species, which belong the four *Passiflora* subgenus, were inoculated in MS medium supplemented with 4.4 µM de benzylaminopurine (BAP) and nine concentrations of 2,4-dichlorophenoxyacetic acid (4.53 – 144.96 µM 2,4-D). Corroborating to the previous studies, different morphogenic responses were observed under the same in vitro conditions. Only friable calli (FC) were obtained from MZE of *Passiflora coriacea* Juss (2n = 12 chromosomes, 2C = 1.00 pg), *Passiflora lindeniana* TR & Planch (2n = 24, 2C = 2.42 pg) and *Passiflora contracta* Vitta (2n = 48 chromosomes, 2C = 4.78 pg). Plantlets were recovered from MZE of *Passiflora foetida* L. (2n = 20, 2C = 1.04 pg) and *Passiflora miniata* Vanderpl. (2n = 18, 2C = 3.40 pg) from indirect organogenesis and embryogenesis, respectively. As in other studies, plantlets were regenerated from indirect somatic embryogenesis only for the *Passiflora* species with 2n = 18 chromosomes (*P. miniata*). In spite of the chromosome number and ploidy level relatively lower than other *Passiflora* taxa, the nuclear

genome size of the species with 2n = 18 is relatively higher. So, the karyotype changes (polyploidy, hybridization and disploidy) that wide occur during evolution in *Passiflora* probably resulted in distinct copy number of the genes related to plant morphogenic process. Therefore, for *Passiflora* genus is important to simultaneously look some genomic characteristics to understand the in vitro responses.

Keywords: indirect somatic embryogenesis, indirect organogenesis, karyotype evolution, morphogenic gene, passion fruit

Introduction

Since the first studies about in vitro plant tissue culture, the totipotency theory has been treated. Haberlandt in 1902 related that the plant cells possessed genetic potential to regenerate an entire organism, i.e., differentiated cells can be dedifferentiated, acquired competence and then become morphogenically determined following a morphogenic route. These ontogenetic changes are influenced by various factors, such as in vitro environmental, and the genetic and epigenetic aspects of the cell plant (Fehér et al. 2015).

Chemical and physical conditions of the in vitro culture are determinant for the all morphogenic route, from the cell dedifferentiation until the plantlet regeneration by organogenesis or embryogenesis (Fehér et al. 2003). Jointly and equally important, genetic (such as chromosome number and nuclear DNA content) and epigenetic (chromatin remodeling by nitrogen basis and histone modifications) features directly determine the in vitro responses. These genomic features vary between species and individuals of the same species, resulting in distinct morphogenic responses even on same in vitro conditions (Fehér et al. 2015).

As a direct consequence of the chromosome number, ploidy level and nuclear genome size divergences, the number of gene copies related to morphogenesis, as the *BABY BOOM1 – BBM1*, *CUP-SHAPED COTYLEDON – CUC, LEAFY COTYLEDON1 – LEC1*, *SOMATIC EMBYOGENESIS RECEPTOR KINASE1 – SERK1, SHOOT MERISTMEM LESS – STM* and *WUSCHEL-RELATED HOMEOBOX4 – SERK1* (Irikova et al. 2012; Chandler et

al. 2008) may also vary between the species. In spite of this, the approaches have been conducted based on the single focus to show the expression and influence of these genes during the in vitro culture. In that sense, the expression of some genes has been quantified, providing the basis for understanding of the in vitro system establishment: *Arabidopsis thaliana* L. (*AtSERK1* gene, Hecht et al. 2001), *Coffea canephora* L. (*LEC1, BBM1* and *WOX4* gene, Nic-Can et al. 2013), *Momordica charantia* L. (McSERK gene, Talapatra et al. 2014) and *Passiflora edulis* Sims. (*PeSERK* gene, Rocha et al. 2015).

Distinct morphogenic responses were observed in the genus *Passiflora*, mostly in the subgenus *Passiflora* (Ozarowski and Thiem 2013; Table 1). For this taxon, the plantlet regeneration has been established from direct and indirect organogenesis (IO) and indirect somatic embryogenesis (ISE) (Ozarowski and Thiem 2013). Besides, some studies reported only the calli formation, without plantlet recovering (Pinto et al. 2011; Ozarowski and Thiem 2013). Most of these works involve *Passiflora cincinnata* Mast. and *Passiflora edulis* Sims. (Ozarowski and Thiem 2013; Table 1). However, for the first time, Rosa et al. (2015) expanded the number of *Passiflora* species in the same in vitro tissue culture study. Mature zygotic embryos (MZE) of five species were inoculated in vitro. Of these, in ISE was established for four, which all possess 2n = 18 chromosomes, *Passiflora alata* Curtis, *Passiflora crenata* Feuillet & Cremers, *P. edulis* and *Passiflora gibertii* N. E. Brown. In the same in vitro conditions, plantlets were recovered from IO only for *Passiflora foetida* with 2n = 20 chromosomes. So, Rosa et al. (2015) suggested that the differences in chromosome number, indicate differences in the regulation of genes involved in morphogenesis, which may influence the in vitro response.

Passiflora genus covers taxa with distinct chromosome number, as the *Decaloba* subgenus with 2n = 12, *Passiflora* subgenus with 2n = 18 or 2n =20 (*Dysosmia* section), *Astrophea* and *Deidamioides* with 2n = 24 (Hansen et al. 2006). Due to this cytogenetic feature, Melo et al. (2001) suggested the ancestor basic chromosome number as x = 6 and the other derived from karyotype changes involving the polyploidy (x = 12) with subsequent disploidy (x = 10 → x = 9). The cytogenetic differences, which are caused by numeric and structural chromosome changes, between *Passiflora* resulted in DNA content divergences, varying from 2C = 0.42 pg for *Passiflora organensis* (*Decaloba subgenus*) to 2C = 4.41 pg for *Passiflora alata* (*Passiflora* subgenus) (Yotoko et al. 2011). Due to differences in in vitro responses related for the congeneric *Passiflora* species and the hypothesis about the correlation with the chromosome number, this genus becomes an important taxa to search the genetic factors that influence the in vitro responses. Thus, the aim of the present work was to answer the questions: What factors promote the different responses observed in the regeneration pathways between species *Passiflora* subgenus? Considering the same in vitro conditions, had the chromosome number, ploidy level and DNA content influence on the morphogenic in vitro response?

Table 1. Different responses in vitro observed for the *Passiflora* genus using zygotic embryos as explants.

Species/ subgenus	2C value (pg) *	Chromosome number (2n)	Explant	PGR	% responsive explants	Regeneration medium	Morphogenic responses	% of plantlets regenerated	Reference
P. lindeniana (Astrophea)	2.42	24	MZE	22.65 µM 2,4-D + 4.5 µM BAP	73%	MS + CA + MI	Calogenesis	0%	Present study
P. coriacea (Decaloba)	1.00	12	MZE	72.48 µM 2,4-D + 4.5 µM BAP	97%	MS + CA + MI	Calogenesis	0%	Present study
P. contracta (Deidamioides)	4.78	48	MZE	27.18 µM 2,4-D + 4.5 µM BAP	46%	MS + CA + MI	Calogenesis	0%	Present study
P. foetida (*Passiflora*)	1.04	20	MZE	9.06 µM 2,4-D + 4.5 µM BAP 13.59 µM 2,4-D + 4.5 µM BA	60% 70-85%	MS + CA + MI MS ½	Organogenesis indiretc	60% 70-85%	Present study Rosa et al. (2015)
P. miniata (*Passiflora*)	3.40	18	MZE IZE	13.59 µM 2,4-D + 4.5 µM BAP 18.1 µM 2,4-D + 4.5 µM BAP	20% 95%	MS ½ MS + CA + MI	Indirect SE	20% 60%	Presente study Ferreira et al. (2015)
P. speciosa (Passiflora)	3.08	18	IZE	0 µM 2,4-D + 4.44 µM BAP	80%	MS + CA + MI	Indirect SE	90%	Ferreira et al. (2015)
P. cincinata (Passiflora)	2.93	18	MZE	18.1 µM 2,4-D + 4.5 µM BA	60%	MS + CA + MI	Indirect SE	60%	Silva et al. (2009)
P. alata (Passiflora)	5.06	18	MZE	18.1 µM 2,4-D + 4.5 µM BA	70 -85%	MS ½	Indirect SE	26 ± 12%	Rosa et al. (2015)
P. crenata (Passiflora)		18	MZE	18.1 µM 2,4-D + 4.5 µM BA	70 -85%	MS ½	Indirect SE	28 ± 10%	Rosa et al. (2015)
P. edulis (Passiflora)	3.39	18	MZE	18.1 µM 2,4-D + 4.5 µM BA 72.4 µM 2,4-D + 4.4 µM BA	70 -85% 70%	MS ½ MS + CA + AIA	Indirect SE	35 ± 15% 0%	Rosa et al. (2015) Pinto et al. (2011)
P. gibertii (Passiflora)		18	MZE	18.1 µM 2,4-D + 4.5 µM BA	70 -85%	MS ½	Indirect SE	35 ± 15%	Rosa et al. (2015)

2,4-D – 2,4-dichlorophenoxyacetic acid; BAP – benzylaminopurine; CA – activated charcoal; IZE – immature zygotic embryos; MI – myo-inositol; MS – Murashige and Skoog 1962; MZE – mature zygotic embryos; PGR - plant grow regulation; SE – somatic embryogenesis.

Materials and methods

Experimental design

Passiflora species were chosen in order to sample the four subgenre proposed by Feuillet and MacDougal (2003): *Astrophea*, *Decaloba*, *Deidamioides* and *Passiflora*. So, different chromosome numbers, ploidy level and nuclear DNA content (2C) values were also sampled. To assess the influence of these variables on morphogenic response, the experiment was planned as the culture medium that regenerated plantlets in vitro (Silva et al. 2009; Rosa et al. 2015; Ferreira et al. 2015).

Plant material

Mature seeds from *Passiflora* species were obtained in different localities. *Passiflora coriacea* Juss (*Decaloba* subgenus) was acquired in established genotypes in the botanical collection of the researcher Mauro Peixoto, located in Mogi das Cruzes, São Paulo (SP) Brazil. *Passiflora miniata* Vanderpl. and *Passiflora foetida* L. (*Passiflora* subgenus) were collected in an Amazon Rainforest fragment situated in city of Carlinda, Mato Grosso (MT), Brazil. For *Astrophea* subgenus, seeds from *Passiflora lindeniana* TR & Planch were kindly provided by Dr. Maurizio Vecchia from your collection located in Rome, Italy. Already the *Deidamioides* subgenus, seeds were sampled by *Passiflora contracta* Vitta in Atlantic Rainforest fragments located in the city of Linhares, Espírito Santo (ES), Brazil. The collected materials are in preparation

process to deposit in the herbarium of the Universidade Federal do Espírito Santo (VIES).

Chromosome number and nuclear 2C value

Seeds of the five species were scarified, disinfected, inoculated on MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar, and cultivated under photoperiod of 16 h light at 25 ± 2°C. Roots of the recovered plantlets were excised and treated for cytogenetic approaches (Praça et al. 2008). Slides were prepared (Carvalho et al. 2007) and the metaphases were captured in 100x objective and a CCD camera (Nikon Evolution™) coupled to a Nikon 80i microscope (Nikon).

Leaves of the same *Passiflora* plantlets were used for nuclear DNA content measurement using as internal standard *Solanum lycopersicum* L 'Stupické' (2C = 2.00 picograms – pg). Nuclei suspensions were prepared (Galbraith et al. 1983; Otto 1990; Praça-Fontes et al. 2011) and analyzed in a flow cytometer Partec PAS® (Partec® GmbH, Munster, Germany) equipped with a laser source (488 nm), which generated histograms that were used to measure the nuclear genome size comparing the G_0/G_1 nuclei peak of each *Passiflora* in relation to internal standard.

In vitro morphogenic responses

Zygotic embryos were manually excised, disinfected in laminar flow chamber upon immersion in 70% ethanol for 1 min, in 2.5% sodium hypochlorite

added to 0.1% Tween 20 for 20 min, then washed four times in sterile dH_2O. The explants were inoculated in semi-solid friable calli induction medium (Ferreira et al. 2015), supplemented with 4.44 µM benzylaminopurine (BAP), and one of the nine different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D): 4.53 µM (M1), 9.06 µM (M2), 13.59 µM (M3), 18.12 µM (M4), 22.63 µM (M5), 27.18 µM (M6), 36.24 µM (M7), 72.48 µM (M8) and 144.96 µM (M9). Five explants were inoculated per Petri dish containing 15 ml of the friable calli induction medium, totalizing 10 dishes (repetitions) for each medium in each species. Petri dishes were maintained in the dark at 25 ± 2°C. Randomly, resulted friable calli (FC) were transferred to the regeneration medium denominated M10 (Silva et al. 2009) and M11 (Rosa and Dornelas 2012). The cultures were submitted to a photoperiod conditions (16 h light) and dark, maintained at a temperature of 25 ± 2 ° C. The regenerated somatic embryos (embryogenesis) and the plantlets (organogenesis) were transferred to growth medium (M12, Ferreira et al. 2015), and maintained under a photoperiod of 16 h light and maintained at 25 ± 2°C (Figure 1).


a
1
2
b
3
4
5

Figure 1. Plant regeneration pathways in vitro in *Passiflora*. a) Plantlet regeneration via IO from MZE *P. foetida*. (1) FC derived from M2 (9.06 µM of 2,4-D) were transferred to the M10 in photoperiod, recovering several seedlings per callus. (2) The plantlets were transferred to tubes containing germination medium, promoting the plantlet development. b) Plantlet recovering through ISE from MZE *P. miniata*. (3) Friable embryogenic callus formed in M3 (13.59 µM of 2,4-D) were transferred to the M11 in photoperiod. From this callus, somatic embryos were found at distinct development stages, such as globular, heart and cotyledonary. (4) Cotyledonary somatic embryo obtained from FC. (5) The somatic embryos formed in M11 were individualized and transferred to tube for germination and plantlet development. Bars = 1cm (1 – 3, 5), and bar = 1 mm (4). Photos: Joaquim Gasparini dos Santos.

Prospection of genes related to the development of plants

Genomic DNA extraction was accomplished from leaves of the *Passiflora* species (Doyle and Doyle 1990). DNA concentration and purity were determined by spectrophotometer (NanoDrop 2000 Thermo Scientifc®), and its integrity was evaluated in 1.2% agarose gel electrophoresis. From previous amplification, three pairs of primers were chosen: *LEC1, SERK1* and *WOX4* (Table 2). PCR products were separated by electrophoresis in agarose gel 1.2%, visualized under UV light and the images were digitally photographed.

Statistical analysis

FC responses were compared considering the factors: species (*P. lindeniana, P. coriacea, P. contracta, P. foetida* and *P. miniata*), 2,4-D concentration (4.53 µM, 9.06 µM, 13.59 µM, 18.12 µM, 22.63 µM, 27.18 µM, 36.24 µM, 72.48 µM, and 144.96 µM) and time (7, 14, 21, 28 and 35 days).

Statistical analysis was performed by variance (ANOVA) followed by regression analysis at 5%.

For organogenic response, the variables considered were: friable callus origin (M1 – M9), regeneration medium (M10 or M11) and the physical environment (dark and photoperiod). This statistical comparison was performed ANOVA followed by Tukey's test at 5%. All analyses were accomplished in software R (Version 3.1.1, 2014-07-10).

Table 2. DNA molecular primers used to prospect the amplification of gene sequences related with in vitro morphogenic responses. The primers, their functions and size of the fragments fond in the literature and in this study.

Genes	Primer sequence (5'-3')	Encoding	Function	Amplicon (pb)*	Amplicon (pb) in this study	Reference
LEC1[2]	F: ATGATGAGAGCAGCAGAGATAAGC R: ATATTTGCCCTCTTCCCCACT	HAP3 subunit of the CCAAT-binding transcription factor (CBF)	To induce embryogenic potential and promote the somatic embryo maturation	477 pb	180 - 390 pb	Nic-Can et al. (2013)
SERK1[1]	F: GAAGAGGATCCTGAAGTTCA R: CCATAACCAAAAACATCAGT	A transmembrane protein containing the intracellular domain conserved in kinases of an extracellular domain containing leucine-rich repeats, associated with protein-protein interactions	Play a key role in embryogenic competence, acting in the signaling the embryo formation process	608 bp	690 - 750 pb	Talapatra et al. (2014)
WOX4[2]	F: GGAGGGACGAGGTGGAATCCA R: TACTAATGGTAGTGGTGGGGTGAC	Transcription factors that have a conserved sequence, *homeobox*, a sequence coding for 60 amino acids, homeodomain.	To promote and maintain procambial vascular and act on the germination of embryos	262 pb	250 - 510 pb	Nic-Can et al. (2013)

* Literature

[1] PCR (15µL): 40ηg DNA; 1X GoTaq DNA polymerase buffer; 1.25U GoTaq DNA polymerase; 0.25µM each of foward and reverse primers; 1.3mM dNTPs; 3mM MgCl2. The thermal amplification parameters for the PCR reaction were as follows: an initial denaturation at 95°C for 5 min followed by 30 cycles of amplification (95°C for 40 s, 55°C for 45 s, 72°C for 1 min 10 s) and a final extension step at 72°C for 5 min.

[2] PCR (15µL): 50ηg DNA; 1X GoTaq DNA polymerase buffer; 0.75U GoTaq DNA polymerase; 0.13µM each of forward and reverse primers; 0.8mM dNTPs; 3mM $MgCl_2$. The thermal amplification parameters for the PCR reaction were as follows: an initial denaturation at 95°C for 5 min followed by 10 cycles of amplification (95°C for 40 s, 55°C for 45 s, 72°C for 1 min 10 s), and in each cycle the temperature was lowered by 1°C, 20 cycles of amplification (95°C for 40 s, 45°C for 45 s, 72°C for 1 min 10 s), and a final extension step at 72°C for 5 min.

Results

Chromosome number and nuclear 2C value

Root meristems, which were subjected to treatment with APM, provided prometaphases and metaphases, exhibiting chromosomes without overlapping, with primary constrictions well defined and different levels of chromatin compaction. So, the chromosome number was determined, differing among the *Passiflora* species: *P. coriacea* presented 2n = 12, *P. miniata* 2n = 18, *P. foetida* 2n = 20, *P. lindeniana* 2n = 24 and *P. contracta* 2n = 48 (Table 1, Figure 2). As a consequence of the distinct chromosome number, each *Passiflora* species showed a particular nuclear 2C value. The mean values were: *P. coriacea* 2C = 1.00 pg, *P. foetida* 2C = 1.04 pg, *P. miniata* 2C = 3.40 pg, *P. lindeniana* 2C = 2.42 pg and *P. contracta* 2C = 4.78 pg (Table 1, Figure 2).

In vitro morphogenic responses

FC were found for all *Passiflora* species in all tissue culture media (M1 – M9). For all species, the calli formation rate gradually increased during the time in calli induction medium (Figure 3). First callogenesis signals were observed after 7 days for *P. coriacea* and *P. foetida* explants, 14 days for *P. contracta*, and 21 days for *P. lindeniana* and *P. miniata*.

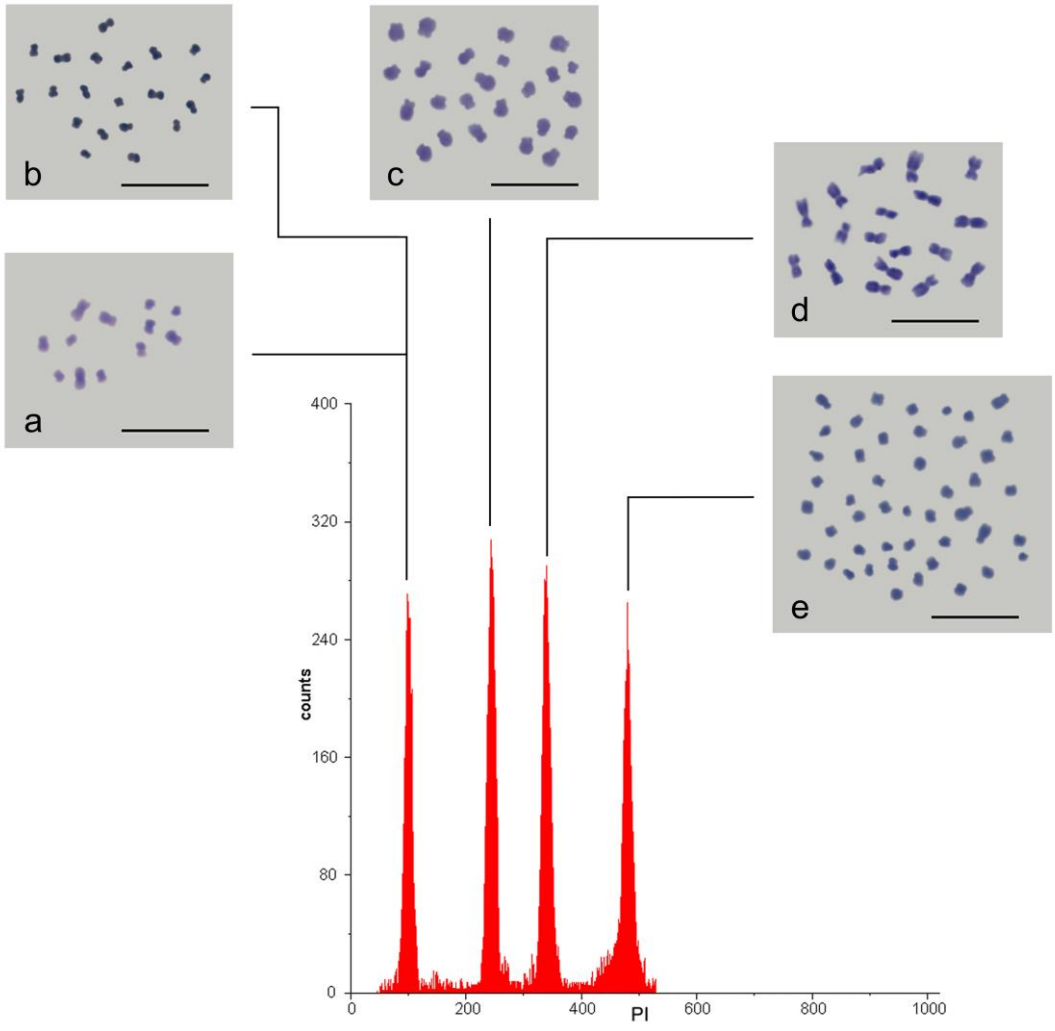

Figure 2. Schematic histogram and karyotype of the *Passiflora* species. FCM was made separately for all species using the internal standard *Solanun lycopersicum* (2C = 2.00 pg, Praça-Fontes et al. 2011). G_0/G_1 peaks nuclei of each *Passiflora* species are represented in the same histogram, being the *P. coriacea* (2C = 1.00 pg) and *P. foetida* (2C = 1.04 pg) G_0/G_1 peak in the channel 100, *P. lindeniana* (2C = 2.42 pg) G_0/G_1 peak in the channel 242, *P. miniata* (3.40 pg) G_0/G_1 peak in the channel 340, and *P. contracta* (2C = 4.78 pg) G_0/G_1 peak in the channel 478. Following the lines from each G_0/G_1 peak has been showed the karyotype of each species: (a) *P. coriacea* with 2n = 12 chromosomes, (b) *P. foetida* with 2n = 20, (c) *P. lindeniana* with 2n = 24, (d) *P. miniata* with 2n=18, and (e) *P. contracta* with 2n=48. Bars = 5 µm.

2,4-D concentration (M1 – M9) also influenced the calli formation rate, being that during all period in the calli induction medium the rate of responsive explants ranged between the species (Figure 3). After 35 days, *P. coriacea* showed 97% of responsive explants in M8 (72.48 µM of 2,4-D), 92% of the *P. foetida* explants in M9 (144.96 µM of 2,4-D), 74% of the *P. miniata* explants in M5 (22.65 µM of 2,4-D), 73% of the *P. lindeniana* in M7 (36.24 µM of 2,4-D) and 46% of the *P. contracta* explants in M6 (27.18 µM of 2,4-D). After 35 days, all FC were transferred to the M10 or M11, resulting in the organogenesis and embryogenesis responses. *P. foetida* FC (Figure 1), originated from M2 – M5, provided plantlets by IO in M10 and M11, being that the higher rate of plantlets was obtained in the photoperiod.

By indirect somatic embryogenesis (ISE), somatic embryos were recovered from *P. miniata* (Figure 1) friable embryos calli (FEC) formed in M1 – M3 and after 120 days in M11 in photoperiod. Somatic embryos rate was statistically equal in M1 – M3. For the other species (*P. Lindeniana, P. coriacea and P. contratca*), plantlet regeneration from organogenesis and embryogenesis did not obtained.

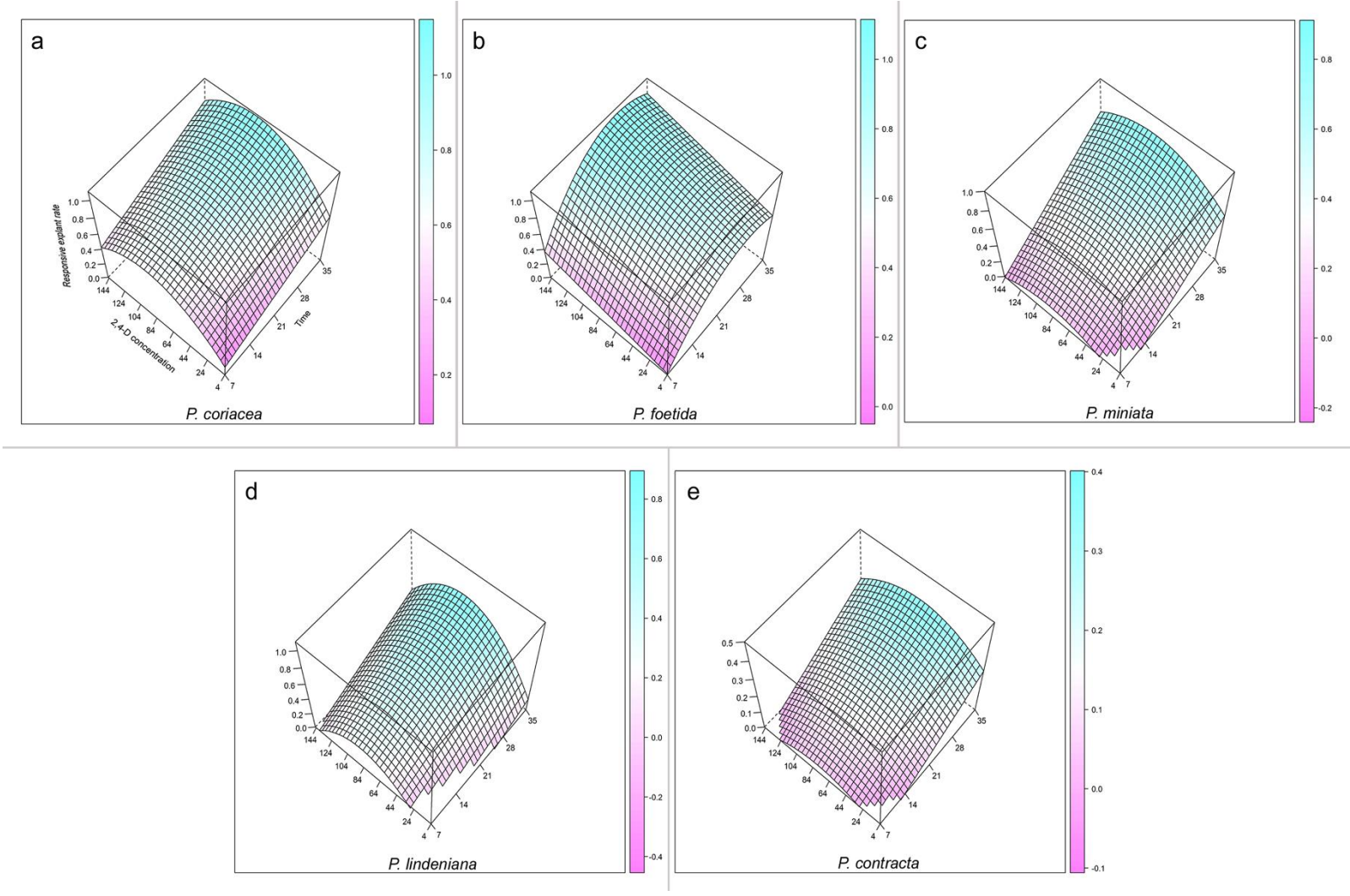
a
Responsive explant rate
2,4-D concentration
Time
P. coriacea
b
P. foetida
c
P. miniata
d
P. lindeniana
e
P. contracta

Figure 3 – Response surface showing the influence of the time in days (*x*) and the 2,4-D µM concentration (*y*) in explant responsive rate (*z*) for the five species. The graphics show that for all species the explant responsive rate (FC formation rate) increased in relation to the time and increasing of the 2,4-D concentration. In concentrations up to 72.48 µM of 2,4-D were observed the highest explant responsive rate, except for *P. foetida* that showed the higher rates in 144.96 µM 2,4-D. Note that for *P. contracta*, which possesses 2n = 48), was observed the lower rate of calli formation. Quadratic models fitted were significant ($p < 0.05$) by regression analysis for all species: a) *P. coriacea* $z = 0.5619 + 21{,}0x + 38{,}7544y + 3271{,}8428y^2$; b) *P. foetida* $z = 0.556 + 21{,}0x + 539{,}0x^2 +, 38{,}7544y$; c) *P. miniata* $z = 0.3066 + 21{,}0x + 387544y + 3271{,}8428y^2$; d) *P. lindeniana* $z = 0.2209 + 21{,}0x + 47{,}8857y + 4191{,}9973y^2$; and e) *P. contracta* ($z = 0.1342 + 21{,}0x + 38{,}7544y + 3271{,}8428y^2$).

Prospection of genes related to the development of plants

The primers *LEC1, SERK1* and *WOX4* provided amplification profiles for all species. The size of the fragments generated by amplification of the primers varied between species. *LEC1* amplification products varied of 180 pb for *P. lindeniana, P. contracta* and *P. foetida*, 300 pb for *P. coriacea* to 390 pb for *P. miniata*. *SERK1* oscillated from 690 pb for *P. foetida*, 700 pb for *P. contracta*, 710 pb for *P. lindeniana*, 720 pb for *P. miniata* to 750 pb for *P. coriacea* (Figure 4). *WOX4* resulted in amplicons of 250 pb for *P. miniata*, 290 pb for *P. contracta*, 320 pb for *P. coriacea*, 490 pb for *P. foetida* to 510 pb for *P. lindeniana*.

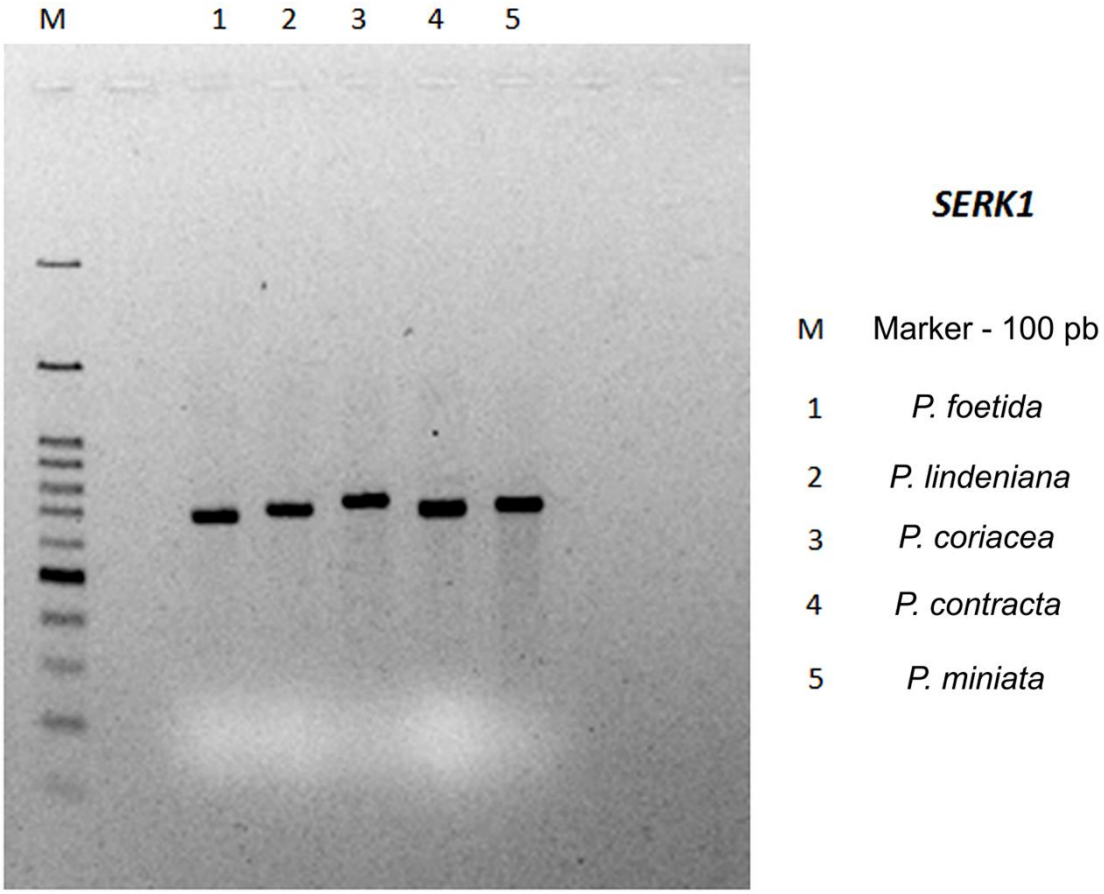

Figure 4. PCR profile showing the amplification from *SERK1* primer in the five *Passiflora* species. This result showed *SERK1* gene occurrence in orthologous species analyzed. The largest fragment was observed in *P. coriacea* with 750 bp and the smallest in *P. foetida*, which showed 690 bp.

Discussion

Differently of the other approaches, which were conducted mainly from *Passiflora* subgenus (Ozarowski and Thiem 2013, Table 1 – present study) and from *Decaloba* subgenus (only *P. suberosa*, Ozarowski and Thiem 2013), here the in vitro procedures were designed involving four subgenres of *Passiflora*

genus: *Astrophea, Decaloba, Deidamioides* and *Passiflora*. This design was performed to look the influence of the chromosome number, ploidy level and nuclear 2C value on morphogenic in vitro responses.

Besides the species chosen, other fundamental aspect was the tissue culture condition, inasmuch as the in vitro morphogenic route is directly influenced by species genotype and in vitro conditions (Fehér et al. 2003). Considering the data summarized in the Table 1, the in vitro tissue culture conditions, chemical (medium composition) and physical (environmental of propagation), were established varying the: (a) for calli induction the 2,4-D concentration; (b) for regeneration the in vitro conditions proposed by Silva et al. (2009) or Rosa and Dornelas (2012) and photoperiod and dark.

The different in vitro morphogenic responses in Passiflora

The time and 2,4-D auxin concentration directly influenced the rate of calli formation and the cell mass proliferation of them in all species (Figure 1 a, b, 3). During the 35 days occurred the increase of the calli formation rate and the progressive proliferation of the cell mass of them. The same responses were observed in relation to the concentrations of 2,4-D up to 72.48 µM in *P. miniata*, *P. coriacea*, *P. contracta* e *P. lindeniana*. Concentrations higher than 72.48 µM 2,4-D also reduced the calli formation rate in *P. cincinnata* (Silva et al. 2009), *P. edulis* (Pinto et al. 2011; Rosa et al. 2015), *P. alata*, *P. crenata, P. gibertii* (Rosa et al. 2015), *P. miniata* and *P. speciosa* (Ferreira et al. 2015).

After transfer of the FC in to plantlet regeneration medium, three different morphogenic responses were obtained from the *Passiflora* MZE explants (Table

1). Independently of the in vitro conditions, plantlets did not regenerate from FC of *P. coriacea, P. lindeniana* and *P. contracta*, which remained in callus stage. Differentiated plant cells, in certain circumstances, revert to its previous state, recovering the totipotency. Subsequently, the cellular fate can be changed under hormonal and environmental influences, driving the formation of new tissue, organ or whole plant (Sugimoto 2010). However, the in vitro conditions did not allow that the undifferentiated cells of the *P. lindeniana, P. coriacea* and *P. contracta* FC to become competent and regenerate plantlets.

Unlike, in vitro conditions allowed the plantlet regeneration by IO from FC of *P. foetida* and ISE from FEC of *P. miniata*. Both species belong to the subgenus *Passiflora*. In spite of the large differences in relation to plantlet regeneration rate (Table 1, Ozarowski and Thiem 2013), ISE has been established only for species this subgenus and organogenesis for species belong to the *Decaloba* (only *Passiflora suberosa*) and *Passiflora* subgenus (Ozarowski and Thiem 2013; Ferreira et al. 2015). The in vitro conditions that proved plantlets here are similar to other authors (Rosa and Dornelas 2012; Ferreira et al. 2015; Rosa et al. 2015).

Considering these data (Table 1), for establishment of the in vitro propagation in *Passiflora* subgenus is crucial the combination of 4.44 µM BAP with until 22.65 µM 2,4-D (M1 – M5). Auxins and cytokinins play a key role in organogenesis (Rosa and Dornelas 2012) and somatic embryogenesis (Fehér et al. 2003), stimulating the gene expression reprogramming leading the plant cell division and dedifferentiation (Fehér et al. 2003). Among these plant growth regulators, the 2,4-D and BAP are widely used mainly in the first phase of the ISE in *Passiflora* subgenus, in which is induced the calli formation. In the

second phase, the medium is devoid of 2,4-D and BAP (Silva et al. 2009) for cell differentiation and, thus, developing of somatic embryos, conversion, maturation and progression to plantlets (Heringer et al. 2015).

The activated charcoal did not essential for the induction of IO in *P. foetida.* The activated charcoal role is related to its ability to adsorb unwanted substances in culture medium, such as secondary metabolites and waste plant growth regulators (Johansson et al. 1982; Pan and Van Staden 1998). Activated charcoal also promotes the inactivation of polyphenol oxidase and peroxidase, increasing organogenesis and the survival rate of the regenerated plantlets (Pan and Van Staden 1998). Differently of the organogenesis in *P. foetida*, the activated charcoal adding to the culture medium caused an inhibition of the somatic embryo formation in *P. miniata*. Although adsorb undesirable substances, the activated charcoal can also adsorb need substances for development of somatic embryos, such as macro and micro nutrients, vitamins and sucrose (Thomas 2008). Thus, activated charcoal can be detrimental to the formation of embryos, as observed by Heringer et al. (2015), where in the culture medium supplemented with 3% produced 5 times fewer somatic embryos than half with 1,5%.

Luminosity environmental affected the regeneration of *P. foetida* and *P. miniata*, seeing that the higher rate of recovered plantlets was obtained in photoperiod condition. Plant development and growth depend on light to accomplish the photosynthesis, photomorphogenesis and phototropism. According to Stefano and Rosario (2003), the light is related primarily to in vitro photomorphogenesis.

Based on data found here and reported since 2007 (Table 1, Ozarowski and Thiem 2013), the tissue culture procedure for regeneration of species belongs to the *Passiflora* subgenus should involve the steps and media summarized in the Figure 1.

Genomic aspects of Passiflora and its outcomes on in vitro responses

Different in vitro responses were observed in this study for *Passiflora* species (only FC, IO or ISE). Morphogenic in vitro responses vary between species, individuals of the same species and explant used due to genetic features (Brown et al. 1995; Ozarowski and Thiem 2013; Rosa et al. 2015). However, the genetic aspects related to the in vitro responses are still poorly understood (Xu and Huang 2014). So, for the *Passiflora* species, the chromosome number, ploidy level and nuclear DNA content were determined to answer: these genomic aspects influence the in vitro response?

Since 40.5 Mya (Eocene, Cenozoic, Muscher et al. 2012), several karyotype changes (polyploidy, hybridization and disploidy, Melo et al. 2001) clearly occurred during *Passiflora* evolution. This variation was showed for all *Passiflora* sampled here, seeing that these species differed in at least one karyotype aspect. n = x = 6 (*P. coriacea* 2n = 12, 2C = 1.00 pg) is considered the primary basic chromosome number of the *Passiflora* genus (Melo et al. (2001). Polyploidy events (auto- or allopolyploidy) yielding the species with n = 2x = 12, as *P. lindeniana* (2n = 24, 2C = 2.42 pg), and n = 4x = 24, as *P. contracta* (2n = 48, 2C = 4.78 pg). In some polyploid species, disploidy events occur forming the species with 2n = 20, as *P. foetida* (2C = 1.04 pg), to 2n = 18,

as *P. miniata* (2C = 3.40 pg). A wide genomic variation in *Passiflora* genus was characterized by an euploid series based on the basic chromosome number x = 6 (*P. coriacea* diploid, *P. lindeniana* tetraploid, and *P. contracta* octaploid), and species with other basic chromosome number generated by disploidy (*P. foetida* and *P. miniata*).

As demonstrated here (*P. miniata*, 2n = 18, 2C = 3.40 pg) and in other studies (Table1), ISE has been established only for species of the *Passiflora* subgenus that possess 2n = 18 chromosomes. Between all in vitro systems (direct/indirect, organogenesis and embryogenesis), ISE depends of the reprogramming and expression *de novo* of a large number of genes (*BBM1*, *CUC, LEC1*, *SERK1, STM* and *WOX4* (Irikova et al. 2012; Chandler et al. 2008) Thus, it is expected the establishment of the ISE from explants of *Passiflora* species with a higher chromosome number.

However, spite of the chromosome number lower than *P. foetida* (2n = 20, 2C = 1.04 pg, *Passiflora* subgenus) and *P. lindeniana* (2n = 24, 2C = 2.42 pg, *Astrophea* subgenus), the species with 2n = 18 show higher nuclear DNA content, oscillating of 2C = 2.93 pg (*P. cincinata*) to 2C = 5.06 pg (*P. alata*) (Table 1). Therefore, the copy number of the genes involved with in vitro responses can be superior in the species with higher DNA content. The disploidy in *Passiflora* subgenus promoted the chromosome number reducing by aneuploidy and chromosome fusion (Melo et al. 2001). Besides, chromosomal rearrangements, mainly duplication, occur, increasing of the DNA content and, consequently, the copy number of the genes related to plant morphogenesis. The establishment only of FC for *P. coriacea* (*Decaloba*), which

exhibits chromosome number and nuclear 2C value lower than the *Passiflora* subgenus species, corroborates to this hypothesis.

Considering this assumption, differently of the observed here, plantlets should be regenerated from FC of *P. contracta*, which presents 2n = 48 chromosomes and 2C = 4.78 pg. However, the karyotype characterization and reproductive viability of this species suggest a true allopolyploid origin. So, *P. contracta* was likely originated from crossing between two *Passifora* species with 2n = 24 chromosomes. Thus, it is expected that the in vitro responses from MZE of the progenitors of *P. contracta* to be similar in relation to *P. lindeniana* (2n = 24). Therefore, for *Passiflora* genus is important to simultaneously look some genomic characteristics to understand the in vitro responses.

The amplification of some sequences related to ISE (*SERK1*, *WOX4* and *LEC1,* Nic-Can et al. 2013; Talapatra et al. 2014) in the five *Passiflora* species indicates the presence of possible orthologous genes (Figure 4). Orthologous genes are genes from different species that have similarity with each other by being derived from a common ancestor. These genes are separated by a speciation event, or when a species diverges to two different species, distinct copies of a gene in the resulting species are referred to the orthologous gene normally retain the same function (Dessimoz et al. 2012). Due to the presence of these genes in all species, possible differences in the number of gene copies can be promoted to different responses observed in this study.

In associating to tissue culture responses and karyotype aspects, the molecular result represents the basis to determine the gene copy number and sequencing of the amplicons. From these data, new aspects will be known, as:

the genetic factors that interfere in the indirect organogenesis in *P. foetida*, the different rate of plantlet regeneration in *Passiflora* subgenus, the evolution of the gene sequences related to plant morphogenesis, and the epigenetic influence in the in vitro responses.

Conclusions

The approaches have been conducted based on the single focus to show the expression and influence of genes during the in vitro culture. In this study, different morphogenetic responses are associated with several genetic factors, being a pioneering study. Genetic changes that occurred in the *Passiflora* genus during their evolution, as chromosome number, ploidy level and DNA content and presence of gene related with morphogenesis, influenced the in vitro morphogenic response in this study. Only in the *Passiflora* subgenus species was regenerated plantlets, due to its recent diversification with higher in karyotypes changes occurring in the group. In *Passiflora*, the chromosome number, DNA content, ploidy level and in vitro conditions did not use as only genetic aspect to understand the in vitro responses. Therefore, understanding the various genetic factors together with the in vitro conditions allow comprehension of the morphogenetic responses and propose specific culture media formulations. Because of different genetic aspects, the *Passiflora* genus becomes an interesting group to provide the understanding of evolutionary aspects that occurred in the group.

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