BA and TDZ in the Morphogenesis *in Vitro* of *Passiflora Setacea*

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Abstract

Passiflora setacea DC (Passifloraceae) is considered an important species in the genetic breeding of passion fruit. However, its use is limited due to low seed germination. This paper aimed to study the effect of cytokinins 6-benzyladenine (BA) and thidiazuron (TDZ) on the *in vitro* morphogenesis of *P. setacea* using three explants: hypocotyl, nodal segment, and root segment. The explants were induced to morphogenesis in MS medium modified and with different concentrations of BA and TDZ. After 55 days, the percentage of calluses and shoots were evaluated, and anatomical characterization was performed. The three explants used in the *in vitro* morphogenesis of *P. setacea* showed callus and shoots formation, but in greater numbers in the nodal segments treated with BA. TDZ isolated affected equal to or less than BA on callus and shoots formation for the three explants. Direct and indirect organogenesis was observed in the three types of explants. From the results obtained for plant regeneration via *in vitro* morphogenesis of *P. setacea*, it is recommended to use a nodal segment in MSM medium and supplemented with $2.22 \mu\text{mol L}^{-1}$ of BA.

Key Message

1. *P. setacea* has disease resistance genes, however, it has low germination via seminiferous and morphogenesis *in vitro* is a technique enabling the multiplication and regeneration of plants *in vitro*.
2. Different concentrations of cytokinins 6-benzyladenine and thidiazuron combined were used to promote the morphogenesis *in vitro* of *P. setacea* for three types of explants.
3. A protocol containing medium supplemented with BA is efficient for the morphogenesis *in vitro* of the nodal segment of *P. setacea*.

Introduction

Passiflora setacea DC. (Passifloraceae) is a wild species known popularly as the passion fruit of sleeping, this species has resistance genes for to the main diseases that are common to sour passion fruit (*P. edulis* Sims), the most commercially used species (Fuhrmann et al., 2014; Viana et al., 2016; Sacoman et al., 2018). This characteristic encourages passion fruit in genetic breeding programs, aiming to obtain interspecific hybrids (Carvalho et al., 2019; Santos et al., 2019). However, it is reported a low percentage of germination via seeds and a low rooting capacity of cuttings (Chaves et al., 2004; Pádua et al., 2011). Thus, *in vitro* cultivation techniques support propagation with efficient multiplication protocols in cases of low germination. For the *Passiflora* genus, these protocols are developed by combining explant types (Antoniazzi et al., 2018; Silva et al., 2020), the growth regulators (Faria et al., 2018), and the culture environment (Vieira et al., 2014).

Thus, this paper aims to present a protocol for *in vitro* multiplication and regeneration of *P. setacea* plants induced to morphogenesis from the use of cytokinins 6-benzyladenine (BA) and thidiazuron (TDZ) for three types of explants, hypocotyl (HP), nodal segment (NS) and root segment (RS).

Material And Methods

Seeds of *P. setacea* (cultivar BRS Pérola do Cerrado, crop from 2015/2016) from the Embrapa Mandioca e Fruticultura germplasm bank were used. The research was carried out in the Horticulture Sector of the Phytotechnics Laboratory of Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF).

The explants HP, NS, and RS were obtained from seedlings 35 days after *in vitro* germination. For the induction of morphogenesis, it was used the MSM medium (Monteiro et al., 2000), plus 30.0 g L⁻¹ of sucrose, 0.10 g L⁻¹ of myo-inositol, vitamins of White (Murashige and Skoog, 1962), pH of medium adjusted to 5.7±0.1 before adding 7.0 g L⁻¹ of agar (Vetec®) and autoclaved for 20 minutes at 121°C and 1.1 atm of pressure.

The experiment was installed in a completely randomized design, in a 4 x 4 factorial scheme, to test for each type of explant (HP, NS, and RS) four concentrations of BA (0.00; 2.22; 4.44; and 8.88 µmol L⁻¹) combined with four concentrations of TDZ (0.00; 2.27; 4.54 and 6.81 µmol L⁻¹) with three replicates, each consisting of a Petri dish (90 mm x 15 mm) with 25 mL of culture medium and three explants. The plates were kept in a growth room for 55 days to check the percentage of callus formation and sprouts in the explants, in addition to visual descriptive evaluation with the aid of a stereoscopic microscope (Tecnival®).

The explants that formed shoots were induced to elongate in MSM medium, supplemented with 2.88 µmol L⁻¹ of gibberellic acid (GA₃), 30.0 g L⁻¹ of sucrose, 0.10 g L⁻¹ of myo-inositol, White's vitamins (Murashige and Skoog, 1962), pH of the medium adjusted to 5.7±0.1 before the addition of 7.0 g L⁻¹ of agar (Vetec®), 40 mL of medium were distributed in flasks (125 mm x 60 mm), then autoclaved for 20 min at 121°C and 1.1 atm. The flasks were kept in a growth room and after 60 days, the shoots in the elongation phase were evaluated and followed by rooting. The explants that formed shoots were induced to elongate in MSM medium, supplemented with 4.92 µmol L⁻¹ of gibberellic acid (GA₃), 30.0 g L⁻¹ of sucrose, 0.10 g L⁻¹ of myo-inositol, White's vitamins (Murashige and Skoog, 1962), pH of the medium adjusted to 5.7±0.1 before the addition of 7.0 g L⁻¹ of agar (Vetec®), 40 mL of medium were distributed in flasks (125 mm x 60 mm), then autoclaved for 20 min at 121°C and 1.1 atm. Afterward, they were kept in flasks with medium for 30 days in a growth room for the rooting process, and then they were taken to acclimatization. The rooted plants were transplanted into disposable cups with a substrate composed of 1:1 (Basaplant®) and sand, at an average temperature of 27 °C, remaining under greenhouse conditions for 60 days.

Preparation of samples for anatomical characterization

For anatomical characterization, samples of the three explants (HP, NS, and RS) were collected after 30 days in the morphogenic induction medium and fixed (Klein et al., 2004). The selected samples were washed with sodium cacodylate buffer and dehydrated in ascending alcoholic series (10 % to 90 %) and included in Histoiresina® Kit (Kraus; Arduin, 1997, adjusted) for the transverse sectioning at an average

thickness of 5 μm with a rotary microtome (SM 2010R, LEICA, Germany). The histological sections were stained with Toluidine Blue O (Johansen, 1940; Sass, 1941; Feder and O'Brien, 1968), mounted on permanent slides, and were visualized under a light microscope (Axioplan, ZEISS, USA) with a digital camera (Moticam Pro 282A, MOTIC®, Asia) for capturing images.

To determine whether the data would meet the assumptions of analysis of variance (ANOVA), they were subjected to preliminary analyses to determine the normality and homogeneity of variances among treatments, for each factor, based on the Lilliefors (1967) and Bartlett, Bobko, and Mosier (1978) tests. Subsequently, the data were subjected to analysis of variance, and the means were compared according to the Tukey's test at 5% probability, utilizing the software SISVAR® (Statistical Analysis System, 5.6 version) (Ferreira, 2011).

Results And Discussion

In callogenesis, it was observed that the explants from the aerial part of the plant (HP and NS), when submitted to the medium without plant growth regulator, did not presently callus formation. On the other hand, there was callus formation in RS in the absence of plant growth regulator (PGR). For explants derived from HP and NS, any isolated concentration of TDZ and BA induces callus formation. The high concentrations of cytokinins BA and TDZ combined favor the proliferation of calluses in HP and NS, however, they are harmful in the formation of calluses derived from RS (Table 1). According to Huetteman and Preece (1993), at low concentrations ($<1 \mu\text{M}$) TDZ can induce greater proliferation of shoots than many other cytokinins, but it can inhibit their elongation. At concentrations above $1 \mu\text{M}$, however, TDZ can stimulate callus formation, pursuant to results presented in this paper.

Table 1

Percentage of callus formation in the explants: hypocotyl (HP) (A), nodal segment (NS) (B) and root segment (RS) (C) of *P. setacea* submitted to different concentrations of BA and TDZ.

(A) Hypocotyl					
TDZ ($\mu\text{mol L}^{-1}$)	BA ($\mu\text{mol L}^{-1}$)				Mean
	0.00	2.22	4.44	8.88	
0.00	0.00 bB	100.00 aA	77.66 aA	83.33 aA	63.66 b
2.27	100.00 aA	100.00 aA	89.00 aA	100.00 aA	97.33 a
4.54	77.66 aA	100.00 aA	89.00 aA	0.00 bB	63.66 b
6.81	100.00 aA	100.00 aA	100.00 aA	22.33 bB	80.66 ab
Mean	69.33 B	100.00 A	89.00 A	48.33 C	76.67
MSD:	0.5802				
(B) Nodal Segment					
0.00	0.00 bB	89.00 aA	77.66 aA	100.00 aA	66.66 b
2.27	100.00 aA	100.00 aA	100.00 aA	100.00 aA	100.00 a
4.54	100.00 aA	100.00 aA	100.00 aA	0.00 bB	75.00 b
6.81	100.00 aA	100.00 aA	100.00 aA	100.00 aA	100.00 a
Mean	75.00 B	97.33 A	94.33 A	75.00 B	85.33
MSD:	0.3571				
(C) Root Segment					
0.00	22.33 aB	77.66 aA	66.66 aAB	100.00 aA	66.66 a
2.27	66.66 aA	77.66 aA	77.66 aA	0.00 bB	55.67 a
4.54	66.66 aA	100.00 aA	77.66 aA	0.00 bB	61.00 a
6.81	66.66 aA	89.00 aA	66.66 aA	0.00 bB	55.67 a
Mean	55.67 B	86.00 A	72.33 AB	25.00 C	59.67
MSD:	0.7318				
Means followed by equal uppercase letters in the line and lowercase in the column do not differ among them ($p \leq 0.05$) according to the Tukey-test.					

The use of explants combined with different concentrations of BA and TDZ contributed to *de novo* organogenesis (Table 2). RS formed calluses and shoots even in the absence of PGR (Table 2C). This

response is reported using this same type of explant in other *Passiflora* species (Lombardi et al., 2007). This is due to the endogenous balance of this type of tissue, as cytokinins are synthesized in the roots (Aloni et al., 2006). Thus, by excising the root, cytokinin activity increases (Van Staden and Smith, 1978), inducing the formation of calluses and shoots on the roots even without the addition of the PGR to the medium. On the other hand, explants extracted from the aerial part (HP and SN) when submitted to the absence of PGR did not show significant *de novo* organogenesis. In this study, direct and indirect organogenesis was observed. The direct was detected in NS and RS (Fig. 1a, b; 2 c, e, f) with a vascular system directly connected to the vascular tissue of the explant. This pattern was not observed in HP derivatives, for the conditions mentioned.

Table 2

Shoot formation percentage in explants from hypocotyl (HP) (A), nodal segment (NS) (B) and root segment (RS) (C) of *P. setacea* submitted to different concentrations of BA and TDZ.

(A) Hypocotyl					
TDZ (μmol L ⁻¹)	BA (μmol L ⁻¹)				Mean
	0.00	2.22	4.44	8.88	
0.00	0.00 bB	100.00 aA	77.66 aA	83.33 aA	63.66 b
2.27	100.00 aA	100.00 aA	89.00 aA	100.00 aA	97.33 a
4.54	77.66 aA	100.00 aA	55.67 aA	0.00 bB	54.67 b
6.81	77.66 aA	100.00 aA	77.66 aA	22.33 bB	69.33 b
Mean	64.00 BC	100.00 A	75.00 AB	48.33 C	71.67
MSD:	0.7522				
(B) Nodal Segment					
TDZ (μmol L ⁻¹)	BA (μmol L ⁻¹)				Mean
	0.00	2.22	4.44	8.88	
0.00	0.00 bB	100.00 aA	77.66 aA	100.00 aA	69.33 b
2.27	100.00 aA	100.00 aA	100.00 aA	100.00 aA	100.00 a
4.54	100.00 aA	100.00 aA	100.00 aA	44.33 bB	86.00 ab
6.81	100.00 aA	100.00 aA	89.00 aA	83.33 abA	94.00 a
Mean	75.00 B	100.00 A	91.67 AB	75.00 B	85.33
MSD:	0.6961				
(C) Root Segment					
TDZ (μmol L ⁻¹)	BA (μmol L ⁻¹)				Mean
	0.00	2.22	4.44	8.88	
0.00	22.33 aB	66.66 aAB	66.66 aAB	89.00 aA	61.00 a
2.27	66.66 aA	77.66 aA	55.67 aA	0.00 bB	50.00 a
4.54	66.66 aA	100.00 aA	66.66 aA	0.00 bB	58.33 a
6.81	66.66 aA	89.00 aA	66.66 aA	0.00 bB	55.67 a

Means followed by equal uppercase letters in the line and lowercase in the column do not differ among them ($p < 0.05$) according to the Tukey test.

(A) Hypocotyl					
Mean	55.67 B	83.33 A	64.00 AB	22.33 C	56.33
MSD:	0.8143				
Means followed by equal uppercase letters in the line and lowercase in the column do not differ among them (p<0.05) according to the Tukey test.					

A diversity of explants can be explored to establish an efficient *in vitro* regeneration protocol in species of the genus *Passiflora* (Garcia et al., 2011; Antoniazzi et al. 2018; Faria et al., 2018). All three types of explants were able to regenerate at least one complete plant from an induction medium supplemented with BA. The explants derived from NS are the most responsive to regeneration.

The treatment with the highest number of shoots had the highest number of regenerated plants. Vieira et al. (2014) state that it is expected that explants more responsive in the morphogenic stage are those presenting a greater number of regenerated plants. In the following steps of elongation and rooting it was found that the treatments, which in the morphogenic step presented TDZ in the medium, were not successful for plant regeneration. TDZ can have a cumulative effect over cycles by inhibiting the elongation and rooting of shoots (Huetteman and Preece, 1993).

The anatomical analyses revealed that the morphogenetic responses observed in HP, NS, and RS began with the intense asymmetric divisions of the explant originated cell clusters with cytological characteristics with the new meristemoids. These cells are small, isodiametric, with reduced volume, dense cytoplasm, and evident nucleus compared to adjacent cells that are already more developed and vacuolated. So, rise meristematic regions proliferating precursor cells with stem cell-like properties with an intense callus formation along with the cutting areas of leaf-derived and HP explants that later differentiate into shoots. Organogenesis was observed both indirectly (Fig. 2a, b) and directly (Fig. 2c-f) as observed in the RS (Fig. 2e, f), in which the vascular system tends to connect to the vascular tissue of the explant, consistent with the pattern of organogenic regeneration. Consequently, organogenic structures appear, giving origin to leaf primordia, stem buds, and other tissue in NS (Fig. 2c) and root apex in RS (Fig. 2f). These structures were observed over the callus (Fig. 2).

All types of explants had elongated shoots derived from at least four different concentrations of BA x TDZ in the morphogenesis phase (Fig. 3a). The largest number of rooted plants was obtained from shoots derived from NS induced in medium supplemented with $2.22 \mu\text{mol L}^{-1}$ BA without TDZ (Fig. 3b). For shoots derived from HP and SR, the rooting of 1 and 2 shoots, respectively, was observed (Fig. 3b). The rooted plants were acclimatized for 60 days in a greenhouse, being obtained 15 acclimatized and well-developed plants. At least one plant was fully regenerated and acclimatized for the three types of explants (HP, NS, and RS), which confirms the development of an efficient protocol for *in vitro* morphogenesis in *P. setacea* (Fig. 4).

Conclusion

In this study, it was possible to obtain the regeneration of *P. setacea* plants after *in vitro* morphogenesis using HP and NS in medium with 2.22 $\mu\text{mol L}^{-1}$ BA and using RS in medium with 8.88 $\mu\text{mol L}^{-1}$ BA. Therefore, we recommend using explants derived from NS and MSM medium supplemented with 2.22 $\mu\text{mol L}^{-1}$ of BA for the *in vitro* morphogenesis of *Passiflora setacea*.

Declarations

CONTRIBUTION OF AUTHORS

LSA conducted the experiments and wrote the manuscript. VSC supervised the research. ALG contributed to conducting and evaluating the experiments. JGSS contributed to statistical analysis and graphing. GC conducted the anatomical analyses. RW contributed to the statistical analyses. MC inspected the anatomical analyses. Finally, all authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with participating humans or animals performed by any of the authors.

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Figures

Figure 1

Direct organogenesis in NS (a) and RS (b), at the respective concentrations of $8.88 \mu\text{mol L}^{-1}$ of BA with $4.54 \mu\text{mol L}^{-1}$ of TDZ and $4.44 \mu\text{mol L}^{-1}$ of BA and $0.00 \mu\text{mol L}^{-1}$ of TDZ, after 30 days of *in vitro* morphogenesis induction (bar = 1 mm).

Figure 2

In vitro development of *P. setacea* after 30 days in induction medium of the morphogenic stage. **(a, c, e)**: Stereoscopy microscope. **(b, d, f)**: Light microscopy. **(a-b)**: HP submitted to the induction medium with $2.22 \mu\text{mol L}^{-1}$ BA e $4.54 \mu\text{mol L}^{-1}$ TDZ. **(c-d)**: NS submitted to the induction medium with $2.22 \mu\text{mol L}^{-1}$ BA e $0.00 \mu\text{mol L}^{-1}$ TDZ. **(e-f)**: RS submitted to the induction medium with $8.88 \mu\text{mol L}^{-1}$ BA e $0.00 \mu\text{mol L}^{-1}$

TDZ. It is possible to observe the presence of meristemoids (Me) in the three types of explants (b, d, f), leaf primordia (LP) (d) and root primordia (RP) (f). Bars: a, c, e: 1 mm. b, d, f: 100 μ m.

Figure 3

Number of elongated (a) and rooted (b) shoots derived from HP, NS and RS of *P. setacea* cultivated in different concentrations of BA and TDZ in μ mol L⁻¹, respectively. A - 0.00 + 0.00; B - 0.00 + 2.27; C - 0.00 + 4.54; D - 0.00 + 6.81; E - 2.22 + 0.00; F - 2.22 + 2.27; G - 2.22 + 4.54; H - 2.22 + 6.81; I - 4.44 + 0.00; J - 4.44 + 2.27; K - 4.44 + 4.54; L - 4.44 + 6.81; M - 8.88 + 0.00; N - 8.88 + 2.27; O - 8.88 + 4.54; P - 8.88 + 6.81.

Figure 4

Plants acclimatized in a greenhouse for 60 days. One plant from HP at a concentration of $2.22 \mu\text{mol L}^{-1}$ BA x $0.00 \mu\text{mol L}^{-1}$ TDZ (a), 12 plants from NS at concentrations of $2.22 \mu\text{mol L}^{-1}$ BA x $0.00 \mu\text{mol L}^{-1}$ TDZ (b), two plants from RS, at concentrations of $8.88 \mu\text{mol L}^{-1}$ BA x $0.00 \mu\text{mol L}^{-1}$ TDZ and $4.44 \mu\text{mol L}^{-1}$ BA x $0.00 \mu\text{mol L}^{-1}$ TDZ, respectively, (c) (bar = 20 cm).