

Influence of type of explant, plant growth regulators, salt composition of basal medium, and light on callogenesis and regeneration in *Passiflora suberosa* L. (Passifloraceae)

Renata Garcia · Georgia Pacheco · Erica Falcão ·
Gabriela Borges · Elisabeth Mansur

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Abstract *Passiflora suberosa* is used in popular medicine, improvement programs, and as an ornamental plant. The goal of this study was to establish efficient protocols for plant regeneration and callus induction from nodal, internodal and leaf segments excised from in vitro-grown plants. The different morphogenetic responses were modulated by the type and concentration of plant growth regulators, according to the basal medium and light conditions. Shoot formation occurred through three pathways: (1) development of preexisting meristems, (2) direct organogenesis, and (3) indirect organogenesis. Development of preexisting meristems was observed from nodal segments (1 shoot/explant) in response to α -naphthaleneacetic acid (NAA), picloram (PIC), and 2,4-dichlorophenoxyacetic acid (2,4-D), using two basal media (MS and MSM). Direct organogenesis in this species was obtained for the first time in this work, through shoot development from internodal segments in the presence of 6-benzyladenine (BA). The highest regeneration rates were achieved on MSM medium, regardless of the BA concentration. Indirect organogenesis was achieved from all explant types on media supplemented with BA, used alone or in combination with NAA. The highest regeneration efficiency was obtained from internodal segments cultured on MSM medium plus 44.4 μ M BA. Compact, friable, or mucilaginous non-morphogenic

calluses were induced by thidiazuron, PIC, 2,4-D, and NAA. High-yielding friable calluses obtained on MSM medium supplemented with 28.9 μ M PIC are being used for the establishment of suspension cultures and further analysis of the production of bioactive compounds.

Keywords Passion fruit · Morphogenesis · Micropropagation · Tissue culture

Introduction

The genus *Passiflora* is the most economically important of the family Passifloraceae, comprising 24 subgenera and 465 species distributed in tropical regions. Brazil is an important center of diversity of this genus, with about 200 native species found mostly in the north-central region (Vanderplank 1996). Several wild species of *Passiflora* are used in breeding programs aimed at the development of high-yielding cultivars, with resistance to diseases that affect the cultivated species, including bacterial spot caused by *Xanthomonas axonopodis* pv. *passiflorae*, fusarium wilt caused by *Fusarium oxysporum* f. sp. *passiflorae*, passion fruit woodiness disease, and anthracnose (Gardner 1989; Vieira and Carneiro 2004; Junqueira et al. 2005).

Passiflora suberosa L. is widespread in the West Indies and in many parts of Mexico and Central and South America (Ulmer and MacDougal 2004). It shows high genetic variability, with intraspecific variation in the diploid chromosome set ($2n = 12, 24, \text{ and } 36$ chromosomes) (Otoni et al. 1996). In Brazil, it is known as “maracujá-mirim” or “maracujazinho-cortiça-preto” due to the color and small size of the fruits. This species is used as an ornamental plant (Vanderplank 1996; Ulmer and MacDougal 2004), and in popular medicine as a sedative and to treat hypertension,

R. Garcia · G. Borges
Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

R. Garcia · G. Pacheco · E. Falcão · G. Borges ·
E. Mansur (✉)
Instituto de Biologia Roberto Alcântara Gomes, Núcleo de
Biotecnologia Vegetal, Universidade do Estado do Rio de
Janeiro, Rua São Francisco Xavier no 524 PHLC sala 505,
Maracanã, Rio de Janeiro 20550-013, Brazil
e-mail: elisabeth.mansur@gmail.com

diabetes, and skin diseases (Miller 1998; Ulmer and MacDougal 2004). Studies on the phytochemical composition of *P. suberosa* have detected several important phyto-constituents, such as the cyanogenic glycosides passisuberosin and epipassisuberosin, as well as eight types of anthocyanins (Spencer and Segler 1987; Kidoy et al. 1997). *P. suberosa* was also reported as a source of resistance to the passion fruit woodiness virus (PWV) (Otoni et al. 1996) and to the fungus *Fusarium oxysporum* f. sp. *passiflorae*, and therefore can be used as rootstock in cultures of the yellow passion fruit (Gardner 1989).

Current tissue culture protocols for *Passiflora* species aim at obtaining disease-free plants, large-scale production, and providing material for breeding programs (Passos and Bernacci 2005). The first study on micropropagation of *Passiflora* was carried out in 1978 and tested different compositions of culture medium for the development of shoots and roots of *P. edulis* f. *flavicarpa* and *P. mollissima*, a wild species (Moran Robles 1978). Subsequently, several protocols were established for different species of the genus, especially for the yellow passion fruit, based on the processes of direct and indirect organogenesis induced by different cytokinins, used alone or in combination with auxins, as a tool for genetic transformation, somatic hybridization, and in vitro conservation (Drew 1991; Dornelas and Vieira 1994; Faria and Segura 1997; Freitas 1997; Becerra et al. 2004; Trevisan and Mendes 2005).

The occurrence of symptoms of mineral deficiency in micropropagation systems of passion fruit, including chlorosis and interrupted or arrested development, has been well documented (Kantharajah and Dodd 1990). To circumvent this problem, Monteiro et al. (2000a) proposed a modification in the mineral formulation of MS medium based on the composition of leaves of field-grown plants (MSM). The positive effect of MSM compared to MS was also significant in the process of shoot bud elongation in cultures of *P. alata* (Pinto et al. 2010). In addition, Faria and Segura (1997) studied the influence of nitrogen on the induction of adventitious buds of passion fruit from hypocotyls and leaves, and observed that the omission of ammonium or nitrate and an imbalance in the MS medium nitrate:ammonium ratio reduced bud-forming rates.

To the best of our knowledge, there are only two reports on tissue culture of *P. suberosa*. The first was based on the use of buds and nodal segments maintained on MS medium supplemented with 6-(γ,γ -dimethylallylamino) purine (2iP) and indole-3-acetic acid (IAA) (Drew 1991). In vitro regeneration of this species was also accomplished from leaf explants cultured on MS medium supplemented with BA, followed by transfer of calluses to MSM medium plus gibberellic acid (GA₃) for shoot production (Monteiro et al. 2000b). Both studies used field-grown plants as sources of explants, and attained low regeneration efficiencies.

The specific requirements of each species, together with problems that affect in vitro passion fruit cultures, including chlorosis and high ethylene production, which may lead to morphological variations and necrosis, justify continued research on the development of new protocols for in vitro regeneration of *Passiflora* species. In addition, in vitro systems may also represent alternative tools for the multiplication, conservation, and availability of wild *Passiflora* germplasm. Therefore, the goal of this work was to evaluate different explant types, plant growth regulators, salt composition of the basal medium, and light conditions for callus induction and plant regeneration of *P. suberosa*.

Materials and methods

Culture media and conditions

The basal media consisted of MS (Murashige and Skoog 1962) or MSM (Monteiro et al. 2000a) salts, supplemented with MS vitamins and 3% sucrose (w/v) and solidified with 7% agar (w/v). MSM medium has reduced concentrations of nitrogen, potassium, zinc, boron and chloride, together with increased concentrations of calcium, magnesium, sulfur, iron, manganese, copper, sodium, and EDTA, and does not contain iodine. The reduction of chloride is accomplished by changing the calcium source from calcium chloride to calcium nitrate. The pH of all media was adjusted to 5.8, and different concentrations of plant growth regulators (BA, NAA, PIC, TDZ, and 2,4-D) were added before autoclaving for 15 min at 121°C.

Cultures were maintained in a growth chamber at $25 \pm 2^\circ\text{C}$ in the dark or under a 16-h light photoperiod, using a total irradiance of $46 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent lamps.

Plant regeneration and callus induction

Seeds of *P. suberosa* were surface-sterilized with 1.0% (w/v) sodium hypochlorite for 20 min under agitation and washed three times with sterile deionized water. Disinfected seeds were distributed between two layers of filter paper soaked with 2.5% GA₃ for 5 days (Ferreira et al. 2005). After this period, the seeds were mechanically scarified with a scalpel and inoculated on $\frac{1}{2}$ MS medium (half-strength of MS salts, MS vitamins, and 15 g/l sucrose), followed by incubation in a germinator with a photoperiod of 8 h and alternate temperatures (30°C for 8 h and 20°C for 16 h), for 60 days (Osipi and Nakagawa 2005). In order to obtain primary cultures, shoot apices and nodal segments were excised from the seedlings and inoculated on $\frac{1}{2}$ MSM medium (half-strength of MSM salts, MS vitamins, and 15 g/l sucrose). This material was incubated under a 16-h photoperiod, originating new plants after 60 days.

Plants of the primary cultures described above were used as sources of explants for experiments on micropropagation and induction of callogenesis. Nodal segments (0.5 cm), internodes (1.0 cm), and leaf segments with the midvein (1.0 cm²) were inoculated on MS or MSM media supplemented with different concentrations of TDZ (4.54, 13.2, 22.7, 31.8, 45.4 μM), 2,4-D (4.5, 13.5, 22.6, 31.6, 45.2 μM), NAA (5.4, 16.2, 26.9, 37.8, 54 μM), picloram (4.14, 12.4, 20.7, 28.9, 41.4 μM), or BA (4.4, 13.2, 22.0, 31.0, 44.4 μM) used alone or in combination with NAA (0, 2.7, 5.4 μM), and maintained in the presence or absence of light. Four glass flasks (8 × 7 cm) each containing three explants and closed with polypropylene caps were cultured per treatment. Subcultures were performed after 30 days of culture. Shoots were transferred to ½ MSM for elongation and rooting after 30 days of culture. Regeneration rates, expressed as the percentage of responsive explants, and the number of shoots per responsive explant, were evaluated for each treatment. The friable calluses obtained in response to PIC were subjected to analysis of biomass accumulation through the evaluation of fresh and dry weights, after 60 days of culture. Dry weights were obtained after drying at 60°C for 24 h.

Acclimatization

In vitro-grown plants were removed from the culture vessels 60 days after transfer to ½ MSM, and washed several times with distilled water to remove traces of medium on root surfaces. Then, the plants were transferred to styrofoam trays filled with a mixture of substrate (Plantmax[®]) and sand (1:1). The trays were placed in glass chambers (80 cm × 40 cm × 40 cm) at 28 ± 2°C under a 12-h photoperiod, for 2 months. In order to reduce the relative humidity inside the chambers, the covers were gradually opened after the second week and completely removed 8 weeks after transplanting. Survival rates of plants were recorded after this period.

Statistical analysis

The experiments were repeated at least twice, using groups of 12 explants. Regeneration rates and the mean number of shoots produced per explant were recorded after 60 days of culture. Data were subjected to analysis of variance (ANOVA), and comparisons of means were carried out with the Tukey–Kramer test at 0.05% significance level, using the software GraphPad Instat.

Results

Different plant regeneration processes and types of non-morphogenic calluses were obtained from internodes, leaf

segments, and nodal segments excised from in vitro plants and cultured on both MS and MSM media supplemented with different plant growth regulators and incubated either under light or in darkness.

Plant regeneration

Because no significant shoot formation occurred from tissues incubated in the dark, only the results obtained in the presence of light were considered.

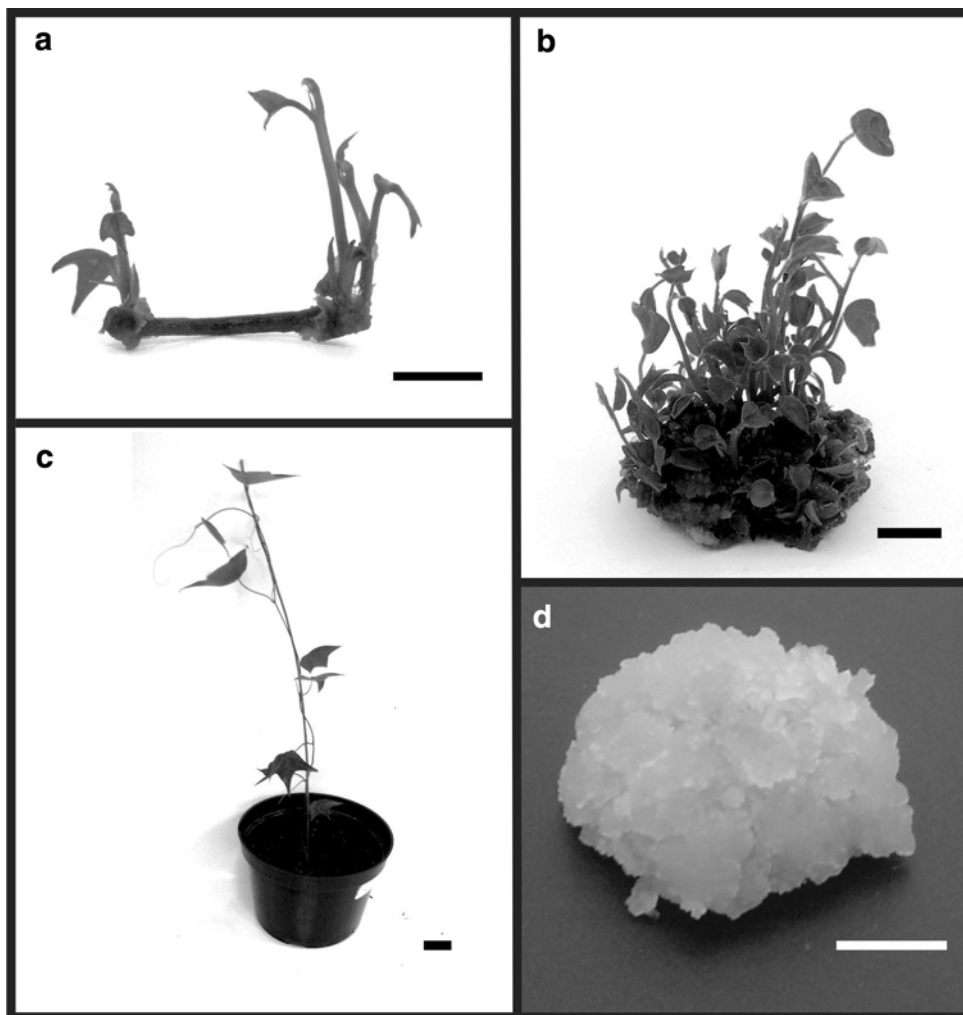
Shoot production via the development of preexisting meristems was observed from nodal segments (1 shoot/explant) cultured on MSM or MS medium in response to all concentrations of 2,4-D, PIC, and NAA, except for explants cultured on MS medium supplemented with 26.9, 37.8, and 54 μM NAA. However, shoots developed on MS medium had a chlorotic appearance, independently of the growth regulator used.

Direct organogenesis was only displayed by internodes cultured on medium supplemented with BA. Shoot formation was observed after 15 days of culture, with a mean number of five shoots per explant, on MSM medium supplemented with all BA concentrations tested (Fig. 1a). A similar response was observed on MS medium, although at significantly lower rates (1.5 ± 0.07 shoots/explant). After this period, the explants also formed compact organogenic calluses (data not shown).

Indirect organogenesis was achieved from all explant types cultured in the presence of BA, except for internodes cultured on MS medium supplemented with BA plus 5.4 μM NAA in darkness, which gave rise to friable non-organogenic calluses. The regeneration frequencies and shoot production were significantly influenced by the basal medium, and higher multiplication rates were observed when using MSM medium. These conditions also resulted in the formation of more vigorous shoots compared to those obtained on MS medium, which also displayed chlorosis. The best results for leaf and nodal segments were obtained on MSM medium supplemented with 22 μM BA, resulting in the production of 9.33 ± 1.4 and 8.37 ± 0.90 shoots/explant, respectively (Table 1). However, the highest shoot production occurred from internodes cultured on MSM medium supplemented with 44.4 μM BA (12.79 ± 0.95 shoots/explant) (Fig. 1b; Table 1). The combination of BA and NAA also resulted in the formation of shoots via indirect organogenesis, although with reduced efficiency. Similarly to cultures with BA alone, the best results were obtained by using MSM as the basal medium (Table 1).

The shoots obtained in these experiments were transferred to ½ MSM to induce root formation, and rooting was achieved at a frequency of 100%. For acclimatization, the plantlets were kept in the acclimatization chambers with a progressive reduction of the relative humidity. After

Fig. 1 In vitro regeneration and callogenesis of *P. suberosa*. **a** Direct organogenesis from internodal segment on MSM medium supplemented with 44.4 μM BA after 15 days of culture in the presence of light; **b** indirect organogenesis obtained from internodal segment on MSM medium supplemented with 44.4 μM BA after 60 days of culture in the presence of light; **c** acclimatized in vitro-grown plant, 60 days after transfer to ex vitro conditions; **d** friable callus formed from leaf segment cultured on MSM medium supplemented with 28.9 μM PIC, after 60 days in darkness. Bars 1 cm



60 days ex vitro, plants showed normal features (Fig. 1c), with a survival rate of 100%.

Non-morphogenic callus formation

Three different non-morphogenic callus types (compact, friable, and mucilaginous) were obtained, according to the growth regulator, basal medium, and light conditions. The three explant types cultured on MSM medium supplemented with TDZ developed compact calluses, regardless of the light condition. In contrast, when cultured on MS medium, leaf segments developed mucilaginous calluses in both light and darkness, whereas nodal segments originated compact calluses in the presence of light and friable calluses in the dark. Internodes formed compact and mucilaginous calluses in the presence and absence of light, respectively. When cultured on media supplemented with different concentrations of 2,4-D, all explant types originated compact or friable calluses on both basal media, according to the light condition (Table 2).

NAA and PIC induced the formation of friable calluses from all explant types on both basal media and light

conditions (Fig. 1d), except for leaf segments cultured on MS medium supplemented with NAA and kept in the presence of light, which formed compact calluses (Table 2). Friable calluses formed on MSM medium supplemented with both plant growth regulators showed greater biomass accumulation in comparison to the material obtained on MS medium. The most callogenic explants were leaf segments, which were then used for further evaluations. The expression of the callogenic capacity of these explants was influenced both by the concentration of the plant growth regulators and by light. When incubated in the presence of light, media supplemented with NAA induced higher biomass accumulation in response to the concentration of 54 μM . On the other hand, cultures incubated in the dark showed better growth on media supplemented with lower concentrations (Fig. 2). On media supplemented with PIC and incubated in the presence of light, the best callus proliferation was obtained in response to the concentration of 28.9 μM . In contrast, similar growth rates were induced by the different concentrations upon incubation in the dark (Fig. 2). Because of their characteristics, calluses formed in

Table 1 Indirect organogenesis from different explants excised from in vitro plants of *P. suberosa* cultured in the presence of light on MS or MSM medium supplemented with different concentrations of plant growth regulators, after 60 days of culture

Plant growth regulators		Leaf segment						Nodal segment						Internodal segment					
		MS			MSM			MS			MSM			MS			MSM		
		Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a	Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a	Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a	Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a	Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a	Regeneration (%)	Shoots/explant ^a	Shoots/explant ^a
4.4	15	3.53 ± 1.05 b	86.4	4.27 ± 0.27 b	50	2.00 ± 0.36 b	59.1	5.30 ± 0.90 b	41.7	1.90 ± 0.21 b	100	7.28 ± 0.71 b							
13.2	25	3.74 ± 0.75 b	95.4	5.84 ± 0.60 b	50	1.13 ± 0.09 b	100	7.30 ± 0.50 b	25	2.77 ± 0.64 b	100	6.78 ± 0.58 b							
22	0	5.60 ± 2.50 a	100	9.33 ± 1.40 a	50	1.69 ± 0.38 b	100	8.37 ± 0.90 a	25	1.92 ± 0.26 b	100	8.50 ± 0.98 b							
31	50	1.50 ± 0.26 bc	75	9.68 ± 1.50 a	50	1.33 ± 0.23 b	81.1	5.75 ± 0.60 b	25	1.87 ± 0.26 b	100	8.84 ± 0.75 b							
44.4	50	3.08 ± 0.48 b	85.7	8.33 ± 1.20 ab	30	3.00 ± 0.84 a	81.1	5.60 ± 0.60 b	41.7	2.15 ± 0.26 b	100	12.79 ± 0.95 a							
4.4	35.7	2.00 ± 0.71 bc	70	4.27 ± 0.40 b	57.5	2.42 ± 0.54 a	68.2	2.88 ± 0.40 c	37.5	1.58 ± 0.34 bc	41.7	1.60 ± 0.0 d							
13.2	65	1.00 ± 0.00 c	90	4.38 ± 0.43 b	40	1.29 ± 0.12 b	68.2	4.12 ± 0.80 bc	8.4	1.80 ± 0.58 bc	37.5	3.24 ± 0.36 c							
22	2.7	3.00 ± 0.00 b	100	5.81 ± 0.58 ab	11.1	1.09 ± 0.09 b	86.4	4.00 ± 0.50 bc	4.2	3.00 ± 0.38 b	41.7	3.20 ± 0.20 c							
31	18.75	4.00 ± 0.60 b	100	4.14 ± 0.53 b	33.3	1.08 ± 0.08 b	86.4	4.00 ± 0.70 bc	12.5	5.22 ± 0.21 a	37.5	5.29 ± 1.27 bc							
44.4	30	3.60 ± 1.10 b	100	4.19 ± 0.40 b	33.3	1.09 ± 0.09 b	72.7	3.40 ± 0.40 bc	12.5	3.36 ± 0.45 b	20.8	4.40 ± 1.75 bc							
4.4	45	2.50 ± 0.44 b	56.4	1.62 ± 0.30 c	20	1.20 ± 1.11 b	36.4	1.22 ± 0.10 d	4.2	1.60 ± 0.25 bc	24.1	1.81 ± 0.15 d							
13.2	20	4.00 ± 0.58 b	60	4.17 ± 0.30 b	11.1	1.00 ± 0.00 b	59.1	3.56 ± 0.40 bc	12.5	2.40 ± 0.81 bc	24.7	4.00 ± 0.0 c							
22	5.4	0	70	4.56 ± 0.30 b	10	1.57 ± 0.20 b	90.1	2.33 ± 0.30 cd	20.8	1.33 ± 0.21 c	28.7	3.17 ± 0.1 c							
31	5	2.67 ± 0.88 b	90	3.76 ± 0.40 b	25	1.14 ± 0.14 b	68.2	2.12 ± 0.40 cd	15	1.40 ± 0.25 c	21.5	3.00 ± 0.0 c							
44.4	10	2.00 ± 0.00 b	100	3.20 ± 0.40 b	10	1.83 ± 0.48 b	36.4	1.87 ± 0.20 cd	15	1.00 ± 0.00 c	21.5	3.00 ± 1.30 c							

Means within each column followed by the same letter are not significantly different at $P \leq 0.05$

^a Mean ± SE

Table 2 Types of non-morphogenic calluses obtained from different explants excised from in vitro plants of *P. suberosa* cultured on MS or MSM supplemented with different plant growth regulators and incubated in the presence or absence of light, after 60 days of culture

Plant growth regulators	Leaf segment				Nodal segment				Internodal segment				
	MS		MSM		MS		MSM		MS		MSM		
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	
TDZ (4.54–45.4 μ M)	Mucilaginous	Mucilaginous	Compact	Compact	Compact	Friable	Compact	Compact	Compact	Mucilaginous	Compact	Compact	Compact
2,4-D (4.5–45.2 μ M)	Compact	Friable	Compact	Compact	Friable	Compact	Friable	Compact	Compact	Compact	Compact	Compact	Compact
NAA (5.4–54 μ M)	Compact	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable
PIC (4.14–41.4 μ M)	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable	Friable

the presence of both NAA and PIC will be used for the establishment of cell suspension cultures and further phytochemical and pharmacological studies.

Discussion

In this work, we evaluated different parameters that modulate in vitro responses of *P. suberosa*, using three explant types excised from in vitro-grown plants. Plant regeneration was obtained through three different mechanisms: (1) development of preexisting meristems, (2) direct organogenesis, and (3) indirect organogenesis. These processes were studied using MS or MSM as basal media. MSM is a formulation proposed by Monteiro et al. (2000a) to circumvent problems of chlorosis, retarded elongation, and low production of shoots in in vitro cultures of different species of *Passiflora* grown on MS medium.

Development of preexisting meristems was accomplished from nodal segments incubated in the presence of light, in response to NAA, PIC, and 2,4-D. This regeneration pathway was previously reported by Drew (1991), who used shoot tips cultured on MS medium supplemented with kinetin in combination with IAA.

Direct organogenesis in *P. suberosa* was observed for the first time in this work. This process only occurred from internodal segments, in response to all BA concentrations tested, at the initial stages of culture. The composition of the basal medium significantly affected the efficiency of this process, since the cultures maintained on MSM medium showed higher regeneration frequencies and number of shoots per explant. Following this morphogenic pathway, indirect organogenesis was also observed from these explants both in response to BA alone and in combination with NAA.

Shoot regeneration through indirect organogenesis was obtained from all explant types in response to BA, regardless of the basal medium. In *Passiflora*, the use of cytokinins to promote shoot formation is well established, and the hormonal balance necessary for the differentiation process depends on the amount of endogenous auxins in different tissues of *Passiflora* (Dornelas and Vieira 1994; Lombardi et al. 2007). Indirect organogenesis in *P. suberosa* was previously reported by Monteiro et al. (2000b), who induced callus formation from leaf discs cultured on MS medium supplemented with BA, and shoot regeneration (maximum of 4 shoots per explant) after transfer to MSM medium supplemented with GA₃. In our study, shoot production from all explants inoculated on MSM medium plus BA occurred at higher rates and in a shorter time compared to this protocol. In addition, the same culture medium was effective for both callus induction and shoot regeneration.

We also evaluated the effect of TDZ, which is a synthetic phenylurea derivative with cytokinin activity, known to

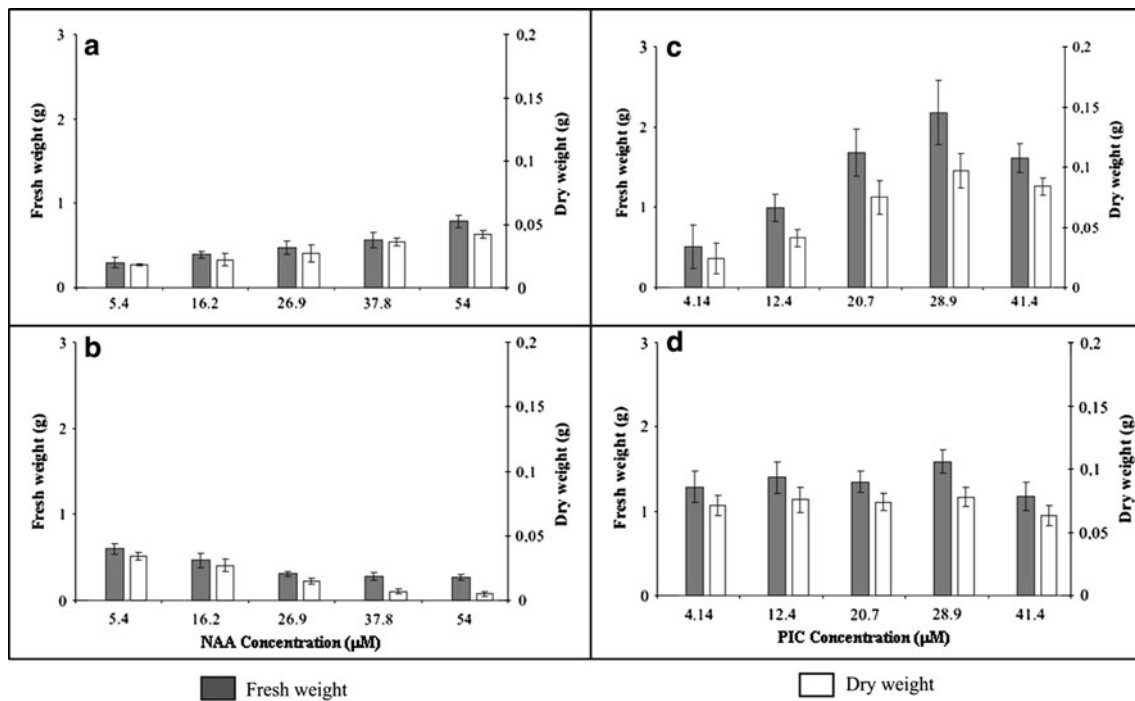


Fig. 2 Biomass accumulation (g) of friable calluses derived from leaf segments inoculated on MSM medium supplemented with different concentrations of NAA or PIC, after 60 days in culture. Cultures

obtained in the presence of NAA: **a** incubation under light, **b** incubation in the dark; Cultures obtained in the presence of PIC: **c** incubation under light, **d** incubation in the dark

show higher activity and to promote better shoot organogenesis in comparison to adenine-type cytokinins such as BA and kinetin (D'Onofrio and Morini 2005; Khurana-Kaul et al. 2010). In contrast, cultures of *P. suberosa* maintained in the presence of TDZ only developed non-morphogenic calluses.

In addition to plant regeneration, we observed the formation of non-morphogenic friable calluses in cultures maintained in the presence of NAA, 2,4-D, and PIC. Treatments with exogenous auxins often give rise to totipotent cells with the ability to proliferate and regenerate somatic embryos. However, in our study these plant growth regulators only caused the acquisition of callogenic capacity by the explants, and cell modification toward an embryogenic pathway was not achieved. Nevertheless, the different systems for induction of non-morphogenic calluses reported here provide a basis to develop studies on the modulation of in vitro synthesis of secondary metabolites by different plant growth regulators and culture conditions.

Conclusion

We demonstrated that the in vitro responses of tissues of *P. suberosa* are influenced differently by plant growth regulators, according to the basal medium and light

condition. Our data confirmed the positive effect of MSM medium on the micropropagation of *Passiflora*, extending its application to different regeneration pathways and callus cultures induced by various plant growth regulators. The plant regeneration protocols described here can be used for mass propagation and to provide plant material for improvement programs of the cultivated species. Those based on the development of preexisting meristems and direct organogenesis are particularly useful for in vitro conservation. The systems established for the induction of friable calluses are currently being used for the establishment of cell suspension cultures and further analyses of the production of bioactive compounds.

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