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In vitro propagation of *Passiflora edulis* through internodal segments as affected by medium composition

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Abstract

The aim of the experiment was to evaluate the effect of the basal medium and the type and concentration of cytokinin on direct regeneration from internodal segments of passion flower (*Passiflora edulis* Sims). Internodal segment explants were cultured on the Murashige and Skoog (MS) and woody plant medium (WPM) without growth regulators or supplemented with 0.5–3.0 mg L^{-1} of 6-benzylaminopurine (BAP), thidiazuron (TDZ) or zeatin (ZEA). The cultivation of the internodal segment explants on media supplemented with cytokinins resulted in direct organogenesis without a callus phase. The shoot formation frequency and shoot number per explant were strongly influenced by the type and concentration of cytokinin. MS medium supplemented with BAP resulted in a shoot formation frequency that was higher in comparison with TDZ but significantly lower as compared to 0.5–2.5 mg L^{-1} ZEA. On the other hand, on WPM supplemented with BAP the shoot formation frequency was significantly lower in comparison with that for analogous ZEA and TDZ concentrations. The shoot formation frequency and shoot number per explant were strongly influenced by interactions of the basal medium and type and concentration of cytokinin. The MS medium supplemented with 2.0 mg L^{-1} ZEA resulted in the highest shoot formation frequency, while the highest shoot number per explant was obtained on WPM supplemented with 1.5 mg L^{-1} ZEA. The highest shoot formation frequency (98.1%) with the highest number of shoots per explant (9.53) was observed on the WPM supplemented with 1.5 mg L^{-1} ZEA. The formed shoots were rooted on MS medium containing ½ macro and micro salts supplemented with 2.0 mg L^{-1} IBA (indole-3-butyric acid); about 92% of them survived and grew normally with true-to-type morphology.

Key words: basal medium, direct organogenesis, growth regulators, passion flower.

Introduction

Passion flower (*Passiflora edulis* Sims) is a very important horticultural plant that is increasingly being grown around the world for several purposes. In many tropical and subtropical climates, *P. edulis* is grown as an alternative to low-nutrition plants making it very important in the food industry. Due to its positive properties for human health, *P. edulis* has spread throughout the world. Various research groups have shown that *P. edulis* is characterized by anticonvulsant, anxiolytic and sedative (Coleta et al., 2006; Barbosa et al., 2008; Sena et al., 2009; Deng et al., 2010; Li et al., 2011), antioxidant (Ferreres et al., 2007; Sunitha, Devaki, 2009; Zeraik et al., 2011), blood pressure attenuating (Zibadi et al., 2007), anti-inflammatory (Zucolotto et al., 2009) and antibacterial (Kannan et al., 2011) activities. The anti-carcinogenic activity of *P. edulis* was studied by Li et al. (2013), who determined that the pulp, seed and peel showed 97, 97 and 89 % inhibition, respectively of HT-29 (human colon adenocarcinoma cells) cancer cell line activity. According to Cazarin et al. (2015), the oral intake of aqueous extract of *P. edulis* leaves, which is a source of vitexin, isovitexin and isoorientin, significantly improved endogenous antioxidant status and decreased lipid peroxidation in the liver, colon and serum. In addition, *P. edulis* is valued as ornamental plant because of the great beauty of the flowers (Abreu et al., 2009).

The traditional propagation of *P. edulis* is very complicated, because this plant is characterized by low seed germination, seedling viability and growth rate. Besides this, *P. edulis* is sensitive to pathogenic microorganisms such as viruses, bacteria and fungi, which can significantly reduce plant productivity (Fischer, Rezende, 2008).

Because of these problems, it is important to find the most efficient way to propagate *P. edulis* through *in vitro* culture for the micropropagation of true-to-type plant regenerants from elite plants. *In vitro* micropropagation of the Passifloraceae family has been reported for some species such as *Passiflora alata* (Pinto et al., 2010), *P. caerulea* (Jafari et al., 2017) and *P. foetida* (Shekhawat

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et al., 2015 a). *In vitro* regeneration of *P. edulis* has been obtained from internodal segments (Biasi et al., 2000), leaf segments (Trevisan, Mendes, 2005), hypocotyl explants (Fernando et al., 2007), apical meristem (Prammanee et al., 2011) and root (da Silva et al., 2011) and nodal (Shekhawat et al., 2015 b) segments. In the study of Biasi et al. (2000), simultaneous callus and adventitious shoot formation from internodal segments with asynchronous development of shoots from explants on MS medium supplemented with BAP was observed. However, indirect shoot formation in many cases may create somaclonal variations; therefore, it cannot be used for the production of a genetically identical plants. To the best of our knowledge, the effect of basal medium and cytokinin interaction on direct organogenesis from internodal segments of *P. edulis* has not been reported before.

Therefore, the aim of the present study was to evaluate the effect of the basal medium and the type and concentration of cytokinin on direct regeneration from internodal segments of passion flower (*Passiflora edulis* Sims).

Materials and methods

Induction of in vitro organogenesis. The experiment was conducted in 2019–2020. Six-month-old donor passion flower (*Passiflora edulis* Sims) plants from Botanical Garden of Klaipėda University were maintained in a growth chamber with a 16/8 h photoperiod, 25/22°C (day/night) temperature and 100 µmol m^{-2} s^{-1} light density. Explants – internodal 20 mm long segments – were washed thoroughly under running tap water for 5 min. The surfaces of explants were disinfected in 70% ethanol for 3 min and then in 5.0% active chlorine solution supplemented with 0.05% Tween 20 (Sigma-Aldrich) for 20 min. They were then washed three times with sterile distilled water. For organogenesis induction, the explants were horizontally placed on Murashige and Skoog (MS) (Murashige, Skoog, 1962) or woody plant medium (WPM) (Lloyd, McCown, 1980) basal media without growth regulators or with 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1} of 6-benzylaminopurine (BAP), thidiazuron (TDZ) or zeatin (ZEA). Basal media were supplemented with 30.0 g L^{-1} sucrose and 8.0 g L^{-1} agar, and the pH was adjusted to 5.7. Culture media (20 mL) were dispensed into 90 mm in diameter Petri dishes and then sealed with parafilm. Explants were cultivated in a growth chamber at 25/22°C (day/night) temperature under a 16/8 h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1}. Shoot formation frequency and adventitious shoot number per explant were recorded after 4 weeks of culture.

Shoot elongation, root formation and plantlet acclimatization. For elongation, regenerated shoots were removed from the explants and transferred to the same medium additionally supplemented with 1.2 mg L^{-1} GA_3 (gibberellic acid) and cultivated in the growth chamber under the same conditions as for organogenesis induction. For rooting, elongated shoots were transferred to MS medium containing ½ macro and micro salts supplemented with 2.0 mg L^{-1} IBA (indole-3-butyric acid) and 10.0 g L^{-1} sucrose. The medium was solidified with 8.0 g L^{-1} agar, and the pH was adjusted to 5.7. Regenerated shoots were cultivated at 25/22°C (day/night) under a 16/8 h photoperiod at a light intensity of 45 µmol m^{-2} s^{-1}. Plantlets with roots were removed from the medium, washed with water to remove the medium from the roots and planted in plastic pots with perlite and vermiculite in a 1:1 ratio. The *P. edulis* plants were initially covered with a plastic bag and maintained in a growth chamber at 25/22°C (day/night) under a 16/8 h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1} for 14 days, and then they were transferred to the greenhouse.

The experiment was arranged in a completely randomized design with three replicates per treatment and 44 explants per replicate. The percentage of shoot formation frequency and the number of shoots per explant was calculated using the following formulas:

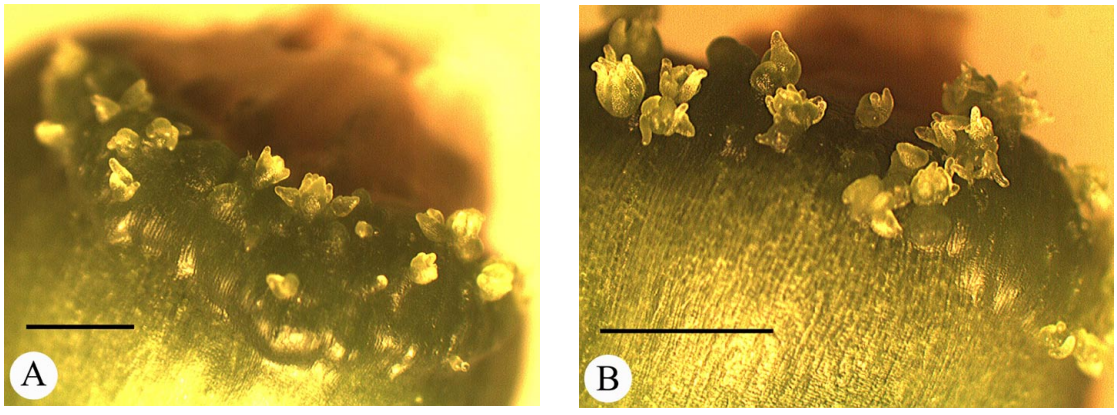
Shoot formation (%) = (number of explants with adventitious shoots / total number of explants) × 100;

Number of shoots = number of adventitious shoots / number of explants forming adventitious shoots.

Statistical analysis of the experimental data was done using the software package *Statistica*, version 10 (TIBCO Software, USA). The mean value of shoot formation frequency and corresponding standard error (SE) for every treatment were computed based on the number of independent replications. Fisher's least significant difference (LSD) test was carried out with a significance level of $p < 0.05$.

Results

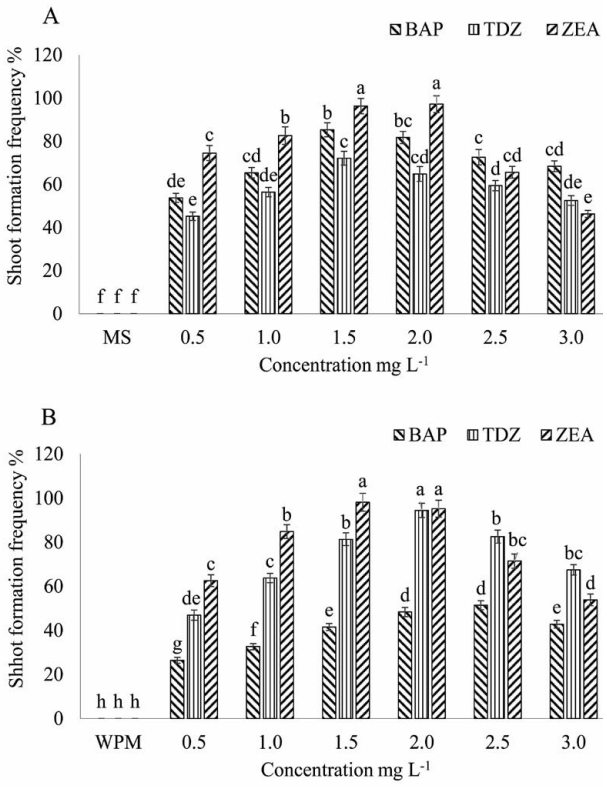
The internodal segments of *Passiflora edulis* that were grown on the medium without growth regulators demonstrated no response. Cultivation of the internodal explants on the medium supplemented with cytokinins resulted in direct organogenesis close to the cutting end (Figure 1).



Bar = 1.0 mm

Figure 1. Initiations of adventitious *Passiflora edulis* shoots from internodal segments cultured on the MS (A) and WPM (B) basal media

The adventitious shoot formation frequency varied from 26.4% to 98.4% depending on the basal medium and the type and concentration of the cytokinin (Figure 2).



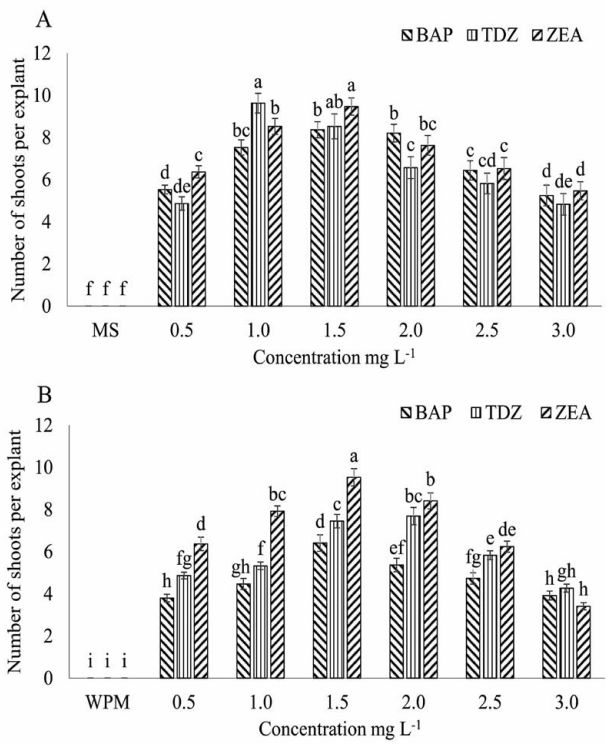
Note. BAP – 6-benzylaminopurine, TDZ – thidiazuron, ZEA – zeatin; values with the same letter do not significantly differ at $p > 0.05$; the bars above the columns denote the standard errors.

Figure 2. Effect of basal medium and cytokinin type and concentration on *Passiflora edulis* shoot formation frequency on the MS (A) and WPM (B)

On the MS medium supplemented with BAP, the shoot formation frequency varied from 53.7% to 85.3% (Figure 2) with in average of 71.18% (Table), while the incorporation of BAP into WPM promoted average shoot formation of 40.5%. The addition of TDZ to both tested basal media resulted in average shoot formation frequency of 58.4% on MS and 72.7% on WPM. On the MS and WPM media supplemented with ZEA, average shoot formation frequencies were 77.08% and 77.63%, respectively.

The shoot formation frequency was strongly influenced by the type and concentration of cytokinin. Polynomial regression analysis showed that the highest shoot formation frequency was observed on MS medium at 2.0 mg L^{-1} ZEA ($y = -8.294x^2 + 71.242x - 52.843$; $R^2 = 0.9134$), while on WPM it was observed at 1.5 mg L^{-1} ZEA ($y = -11.473x^2 + 88.71x - 72.79$; $R^2 = 0.9514$).

The number of shoots per explant varied from 3.41 to 9.63 depending on the basal medium and the type and concentration of the cytokinin (Figure 3).



Note. BAP – 6-benzylaminopurine, TDZ – thidiazuron, ZEA – zeatin; values with the same letter do not significantly differ at $p > 0.05$; the bars above the columns denote the standard errors.

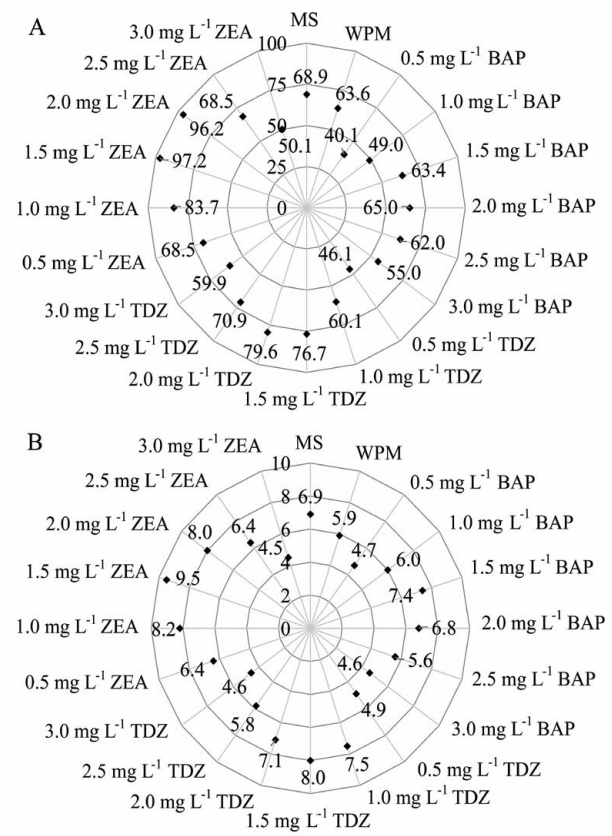
Figure 3. Effect of basal medium and cytokinin type and concentration on the number of *Passiflora edulis* shoots per explant on the MS (A) and WPM (B)

The addition of BAP to the MS medium resulted in an average of 6.89 shoots per explant, while addition of this cytokinin to the WPM resulted in a lower shoot number per explant by an average of 4.78 (Table). In the MS medium, the influence of TDZ on the shoot number per explant was similar to that of BAP, and each explant formed on average 6.71 shoots. On the other hand, on the WPM, TDZ promoted a higher (5.91) average shoot number per explant in comparison with BAP. ZEA was superior to BAP and TDZ in both tested basal media with average shoot numbers per explant of 7.33 on MS and 6.98 on WPM.

Table. Effect of basal medium and cytokinin type on mean values of *Passiflora edulis* shoot formation frequency and number of shoots per explant

Type of cytokinin	MS		WPM	
	shoot formation frequency %	number of shoots per explant	shoot formation frequency %	number of shoots per explant
Control	0.00	0.00	0.00	0.00
BAP	71.18	6.89	40.50	4.78
TDZ	58.40	6.71	72.70	5.91
ZEA	77.08	7.33	77.63	6.98

BAP – 6-benzylaminopurine, TDZ – thidiazuron, ZEA – zeatin; MS – Murashige and Skoog, WPM – woody plant medium



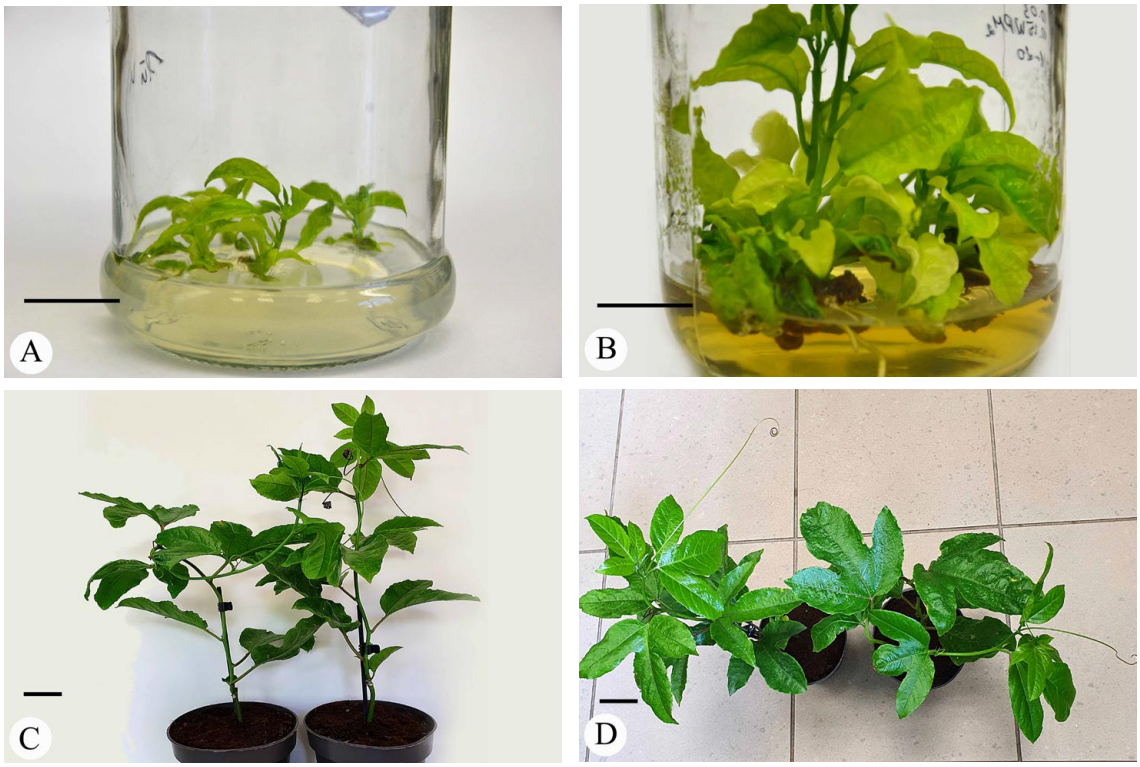
BAP – 6-benzylaminopurine, TDZ – thidiazuron, ZEA – zeatin

Figure 4. Average shoot formation frequency (A) and average number of *Passiflora edulis* shoots per explant (B) depending on the basal medium, type and concentration of cytokinin

Polynomial regression analysis showed that on MS medium the highest shoot number per explant was observed at 1.0 mg L^{-1} TDZ ($y = -0.6973x^2 + 6.0542x - 4.5214$; $R^2 = 0.8115$), while on WPM it was observed at 1.5 mg L^{-1} ZEA ($y = -0.834x^2 + 7.046x - 5.52$; $R^2 = 0.9612$).

The evaluation of separate factors affecting organogenesis response in the tested explants showed that the MS medium was superior to the WPM in terms of both shoot formation frequency and the number of shoots per explant. The average shoot formation frequencies on MS and WPM were 68.9% and 63.6%, respectively (Figure 4A) with average shoot number per explant of 6.9 and 5.9, respectively (Figure 4B). The average shoot formation frequency was strongly dependent on the tested cytokinin concentration. Increased cytokinin concentration up to 2.0 mg L^{-1} BAP and TDZ or up to 1.5 mg L^{-1} ZEA resulted in an increased average percentage of responding explants with subsequent decreasing average shoot formation frequency under the influence of higher concentrations of cytokinins (Figure 4A). All tested cytokinins induced the highest average shoot number per explant at 1.5 mg L^{-1} (Figure 4B).

On the elongation medium, the regenerated shoots were elongated within 21 days (Figure 5A). The formed roots were observed within 4 weeks on the rooting medium, about 98% of shoots developed roots (Figure 5B). *P. edulis* plants with roots were transferred to the greenhouse; about 92% of them survived (Figure 5C) and grew normally with true-to-type morphology (Figure 5D).



Bars: A, B = 2 cm; C, D = 5 cm

Figure 5. Elongated shoots on the elongation medium (A), rooted shoots on the rooting medium (B) and hardened *Passiflora edulis* plants (C, D)

Discussion

The culture medium composition is one of the most important factors affecting somatic tissue response throughout cultivation *in vitro*. WPM has been suggested to be more suitable for the micropropagation of some horticultural plants, for example, *Vaccinium corymbosum*, *V. vitis-idaea* and *V. virgatum* (Meiners et al., 2007; Schuchovski, Biasi, 2019). In our investigation, internodal segments of *Passiflora edulis* cultured on the MS medium showed greater average organogenic response than did explants cultured on the WPM.

A very important factor for organogenesis induction also is the type and concentration of growth regulators in the culture medium. Prammanee et al. (2011) reported that a higher number of shoots from the apical meristem of *P. edulis* was obtained on MS medium supplemented with 1.0 and 1.5 mg L^{-1} BA (benzyladenine). In their study, the medium with 1.5 mg L^{-1} BA generated more numerous but shorter shoots in comparison with the medium supplemented with 1.0 mg L^{-1} BA, where shoots were longer and, therefore, more suitable for sub-cultivation. For shoot formation from thin cell layer explants of *P. edulis*, 1.0 mg L^{-1} BA was suggested in the study by Nhut et al. (2007). Jafari et al. (2017) reported that the combination of 1.5 mg L^{-1} BAP and 0.15 mg L^{-1} IBA promoted the highest regeneration frequency with the highest number of shoots from cotyledonary node and shoot tip explants of *P. caerulea*, while semi-solid MS medium supplemented with 2.0 mg L^{-1} BAP was found to be most appropriate for shoot formation from nodal shoot meristems of *P. foetida* (Shekhawat et al., 2015 a).

According to the research reports, TDZ stimulates direct organogenesis from the somatic tissues of blackberry (Vujović et al., 2010) and strawberry (Cappelletti et al., 2016). The higher number of shoots per explant from leaf discs of *P. edulis* obtained using TDZ as compared to BAP was obtained in studies by Trevisan and Mendes (2005). Kumar et al. (2010) reported that TDZ shows greater influence on shoot regeneration from cotyledonary leaf explants of *Jatropha curcas* as compared to BAP. ZEA was suggested as a more appropriate cytokinin for direct organogenesis in blueberry (Welander et al., 2017; Schuchovski, Biasi, 2019).

In our experiment, MS and WPM supplemented with ZEA resulted in a shoot formation frequency that was higher in comparison with BAP and TDZ. The highest shoot formation frequency on MS medium was obtained at 2.0 mg L^{-1} ZEA, while 1.5 mg L^{-1} ZEA resulted in the highest shoot formation frequency on the WPM. The highest shoot number per explant on MS medium was observed under the influence of 1.0 mg L^{-1} TDZ, and on WPM it was observed with 1.5 mg L^{-1} ZEA.

The results obtained in our experiment clearly demonstrate that WPM supplemented with 1.5 mg L^{-1} ZEA is most appropriate for micropropagation of *P. edulis* from internodal segments.

Conclusions

1. The cultivation of the internodal segment explants of passion flower (*Passiflora edulis* Sims) on the basal media supplemented with cytokinins resulted in direct organogenesis without a callus phase.

2. The shoot formation frequency and shoot number per explant were strongly influenced by the type and concentration of cytokinin. Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) resulted in a shoot formation frequency that was higher in comparison with thidiazuron (TDZ) but significantly lower as compared to zeatin (ZEA) at 0.5–2.5 mg L^{-1} concentration. On the other hand, on woody plant medium (WPM) supplemented with BAP the shoot formation frequency was significantly lower in comparison with that for analogous ZEA and TDZ concentrations.

3. The shoot formation frequency and shoot number per explant were strongly influenced by the interactions of the basal medium and type and concentration of cytokinin. The MS medium supplemented with 2.0 mg L^{-1} ZEA resulted in the highest shoot formation frequency, while the highest shoot number per explant was obtained on WPM supplemented with 1.5 mg L^{-1} ZEA.

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Maitinamosios terpės sudėties poveikis *Passiflora edulis* dauginimui *in vitro* tarpubamblių segmentų kultūroje

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Santrauka

Tyrimo tikslas – nustatyti bazinės maitinamosios terpės, citokinino tipo ir koncentracijos poveikį pasifloros (*Passiflora edulis* Sims) tiesioginei regeneracijai tarpubamblių segmentų kultūroje. Tarpubamblių segmentų eksplantai buvo auginti Murashige ir Skoog (MS) ir sumedėjusių augalų (WPM) terpėse be augimo reguliatorių ir papildytose 0,5–3,0 mg L^{-1} 6-benzilaminopurino (BAP), tidiazurono (TDZ) ir zeatino (ZEA). Citokininų priedas maitinamojoje terpėje skatino tiesioginę organogenezę be tarpinės kaliaus fazės. Ūglių susiformavimo dažnis ir kiekis iš eksplanto kito priklausomai nuo citokinino tipo bei koncentracijos. BAP priedas MS terpėje ūglių susiformavimo dažnį skatino labiau nei TDZ, tačiau esmingai mažiau nei ZEA. Kita vertus, BAP priedas WPM terpėje ūglių susiformavimo dažnį skatino esmingai mažiau nei analogiškos ZEA ir TDZ koncentracijos. Be to, nustatyta priklausomybė tarp bazinės terpės, citokinino tipo, koncentracijos sąveikos ir ūglių susiformavimo dažnio bei ūglių kiekio iš eksplanto. Didžiausias ūglių susiformavimo dažnis (98,1 %) ir didžiausias ūglių kiekis iš eksplanto (9,53) gautas WPM maitinamojoje terpėje, papildytoje 1,5 mg L^{-1} ZEA. Susiformavę ūgliai buvo įšaknydinti ½ MS terpėje, papildytoje 2,0 mg L^{-1} ISR (indolil-3-sviesto rūgšties). Apie 92 % jų išgyveno aklimatizacijos metu ir buvo morfologiškai identiški donoriniams augalams.

Reikšminiai žodžiai: bazinė terpė, tiesioginė organogenezė, augimo reguliatoriai, pasiflora.