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MORPHOGENETIC PATHWAYS FROM *Narcissus* L. 'CARLTON' *IN VITRO* CULTURES OF PC STAGE FLOWER BUD EXPLANTS ACCORDING TO CYTOKININ AND AUXIN RATIOS

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Abstract. The effect of the concentration and proportion of growth regulators in an initial medium on *Narcissus* L. 'Carlton' morphogenesis in cultures of Pc stage flower bud explants was investigated. Ovary and flower stem explants were cultured on solid Murashige and Skoog (MS) media with cytokinin – 6-Benzyladenine (BA) or Zeatin (ZEA) (0.5–50 μM) – in combination with auxin – 4-Amino-3,5,6-trichloropicolinic acid (Picloram), 2,4-Dichlorophenoxyacetic acid (2,4-D) or α-Naphthaleneacetic acid (NAA) (1–50 μM). The ratios of cytokinin and auxin in the initial medium determined the morphogenetic pathways and the level of cytokinin proved to be a critical factor. Somatic embryogenesis was achieved for a cytokinin and auxin ratio of 1:1–10, bulb organogenesis – for a 1:0.4–1 ratio and rhizogenesis – for a 1:20–100 ratio. The minimum concentration of cytokinin in the initial medium for somatic embryo and bulb formation was 5 μM. A lower level of cytokinin (0.5 μM) resulted in rhizogenesis. Considering the effect of auxin, Picloram stimulated the initiation of somatic embryos and the 2,4-D enhanced embryo maturation and conversion.

Key words: callus, growth regulators, organogenesis, somatic embryogenesis

INTRODUCTION

The genus *Narcissus* (Amaryllidaceae family), including the large-cup cultivar 'Carlton', occupies an important position in the worldwide bulbous plant industry. They are grown for their decorative qualities and used as garden flowers, cut flowers as well as pot flowers. Because of the pharmacologically active alkaloids (for the treatment of Alzheimer's disease and anti-cancer therapy) and lectins that narcissus produce, they are

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also a potential material for the pharmaceutical industry [Ooi et al. 1998, El Tahchy et al. 2011].

The narcissus plant regeneration systems that are known are based on two major *in vitro* pathways: organogenesis or somatic embryogenesis**.** Shoot organogenesis was induced on bulb scales, flower stems, leaves and basal plates as well as mature seeds, while cytokinin BA in combination with auxin NAA was most frequently used for the initiation of the culture [Seabrook et al. 1976, Chen 1982, Hussey 1982, Kozak 1991, Bergonon et al. 1996, Selles et al. 1997, Sochacki and Orlikowska 1997]. Somatic embryogenesis was obtained on the bulb scale, leaf, flower stem, ovary, anther explants as well as mature and immature embryos excised from mature seeds [Selles et al. 1999, Sage et al. 2000, Chen et al. 2005, Malik 2008]. Studies carried out so far on narcissus somatic embryogenesis only generally demonstrate that somatic embryos formed in callus cultures obtained under the influence of 2,4-D or Picloram auxins applied alone or in combination with cytokinin BA.

The present study describes for the first time the induction of different alternative morphogenetic pathways in *Narcissus* L. 'Carlton' cultures. It concerns the organogenesis (root and bulb formation) and the somatic embryogenesis, on flower bud explants cultured in the same *in vitro* conditions, depending on different proportions of cytokinin (BA, Zeatin) and auxin (Picloram, 2,4-D, NAA). We also evaluated the effects of different growth regulators and their proportions at the level of somatic embryogenesis initiation on later somatic embryo development and conversion.

MATERIALS AND METHODS

Plant material. Pc stage flower buds (the stage of development of complete flower differentiation, called from the paracorolla, the last formed part of the flower bud) originating from *Narcissus* L. 'Carlton' bulbs (12 cm in circumference) stored after harvest at 20ºC, were sterilised as Malik [2008] described. Flower stems and ovaries isolated in September from buds (approx. 4 cm in length) were cut into 1–2 mm thick slices (primary explants) and, with their basal cut surface, placed on the culture medium.

Culture initiation. Primary explants (5–8 pcs per Petri dish and 5 Petri dishes per treatment) were cultivated on MS [Murashige and Skoog 1962] supplemented with the following auxins: 2,4-D (1–50 μM), Picloram (10–50 μM) or NAA (5 μM), and cytokinins: BA (0.5–50 μM) or zeatin (25–50 μM) (tabs 1, 2). The initiation media were supplemented with 3% sucrose, adjusted to pH 5.5 before autoclaving (121ºC, 0.1 MPa) and gelled with 0.7% agar (Purified Difco Agar). Plastic Petri dishes (90 × 25 mm) were used for media distribution. The cultures were maintained at 20 ±2ºC in the darkness and subcultured every four weeks.

Somatic embryogenesis. The process of somatic embryogenesis was evaluated in two ways (i-ii). Initial cultures were carried out for 12 weeks on the initial media (i) or six weeks on initial media and then during the next six weeks on the regeneration medium supplemented with 5 μM BA + 0.5 μM NAA + 3% sucrose (pH 5.8) (ii). After 12 weeks the percentage of explants forming somatic embryos, the total number of

somatic embryos per primary explant and the number of converted embryos per primary explant were determined.

Organogenesis. After 20 weeks of initial cultures the percentage of explants forming adventitious bulbs, the number of adventitious bulbs per regenerating explant and the number of adventitious roots per explant were determined.

Histological analysis. Histological analysis was carried out using the paraffin method. Staining was done with fast green and safranin. Squash preparations of callus were made in acetocarmine.

Statistical analysis. The results of the observations were evaluated by analysis of variance. The percentage data were transformed by arcsine transformation and then subjected to ANOVA. The means that differed significantly were identified using Tukey's multiple test at a significance level of $P \leq 0.05$ (Statistica version 10, StatSoft).

RESULTS AND DISCUSSION

Culture initiation. In cultures of Narcissus L. 'Carlton' Pc stage flower buds treated with different ratios of cytokinin and auxin, alternative morphogenetic pathways were observed: somatic embryogenesis (callus and somatic embryos formation) and organogenesis (adventitious root or bulb development) (fig. 1).

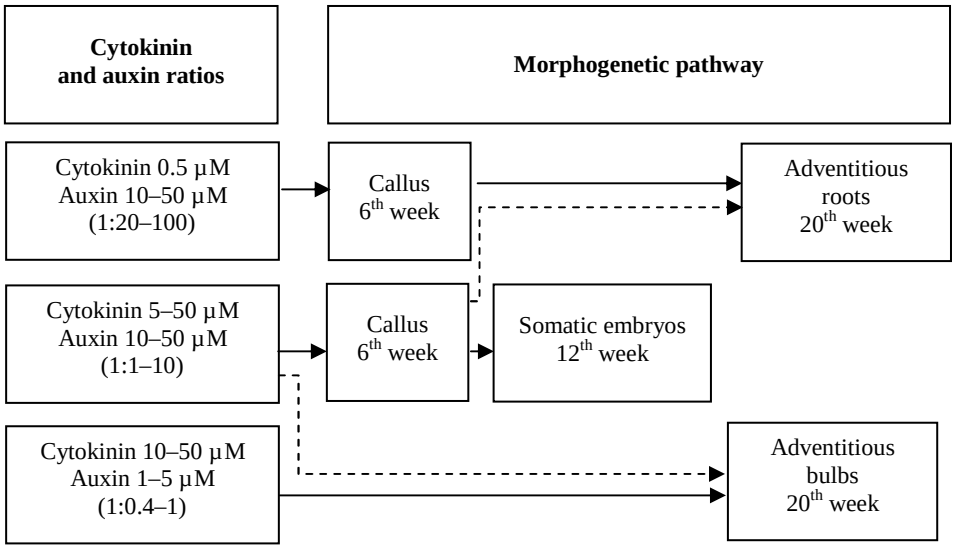

Fig. 1. Concept of morphogenetic pathways in *Narcissus* L. 'Carlton' *in vitro* cultures of Pc stage flower bud explants according to cytokinin and auxin ratio in initial medium (alternative morphogenetic pathways: ⟶ – main pathway; - - ▸ – side pathway)

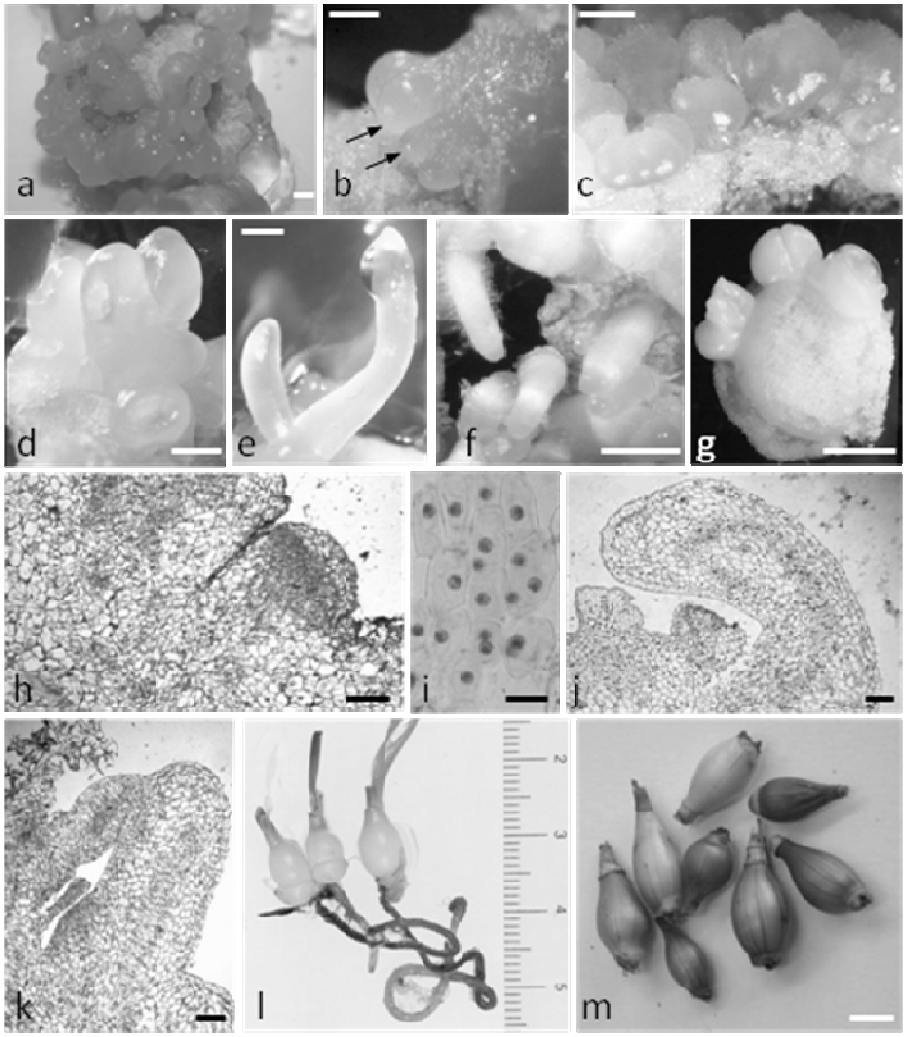

Fig. 2. Somatic embryogenesis and organogenesis in *Narcissus* L. 'Carlton' cultures: (a) Yellow nodular callus obtained on medium with 25 µM Picloram and 5 µM BA. Bar = 2 mm; (b) Embryos formation on a nodule of callus on medium with 25 µM Picloram and 25 µM BA. Bar = 1 mm; (c) Somatic embryos at early stage of development – 25 µM 2,4-D and 5 µM BA. Bar = 1 mm; (d) Mature embryos on medium containing 0.5 µM NAA and 5 µM BA. Bar = 1 mm; (e) Conversion of somatic embryo. Bar = 1 mm; (f) Adventitious roots development in callus cultures derived on medium with 50 µM 2,4-D and 0.5 µM BA. Bar = 1 cm; (g) Adventitious bulb formation under the influence of 5 µM NAA and 25 µM BA. Bar = 3 mm; (h) Cross--section of the shoot and nodular callus formation. Bar = 200 µm; (i) Isolated embryogenic cells of nodular callus. Bar = 40 µm; (j) Transverse section of the somatic embryo. Bar = 125 µm; (k) Longitudinal section of the somatic embryo. Bar = 100 µm; (l) Properly developed plants on medium with 0.5 µM NAA and 5 µM BA; (m) Bulbs derived from acclimatization process. Bar = 1 cm

Somatic embryogenesis. In the fourth week of the culture yellow, nodular callus appeared on explants originated from the flower stem and ovary under the influence of media containing more auxin than cytokinin, and media containing equal concentrations of auxin and cytokinin (fig. 2a).

Table 1. Efficiency of somatic embryogenesis in *Narcissus* L. 'Carlton' *in vitro* cultures of Pc stage flower buds

Growth regulators (μM)	Somatic embryos formation (% of explants)			No. of somatic embryos per explant		
	flower stem	ovary	mean	flower stem	ovary	mean
10 2,4-D + 0.5 BA	0.0 a*	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
25 2,4-D + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
50 2,4-D + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
10 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
25 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
50 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A
10 2,4-D + 5 BA	29.6 de	50.0 f	39.8 D	3.2 a–e	8.1 gh	5.6 DE
25 2,4-D + 5 BA	31.2 de	34.0 de	32.6 C	14.4 i	6.7 e–g	10.6 F
50 2,4-D + 5 BA	8.4 ab	16.0 bc	12.2 B	3.0 a–d	3.6 b–e	3.3 BC
10 Picloram + 5 BA	51.6 f	2.6 a	27.1 C	5.0 c–g	5.0 c–g	5.0 C–E
25 Picloram + 5 BA	74.0 g	60.0 f	67.0 G	22.9 j	17.5 i	20.2 H
50 Picloram + 5 BA	18.2 bc	16.0 bc	17.1 B	1.9 a–c	5.0 c–g	3.5 B–D
10 2,4-D + 10 BA	17.8 bc	40.0 e	28.9 C	4.4 b–f	2.1 a–c	3.3 BC
25 2,4-D + 25 BA	90.0 h	10.0 ab	50.0 E	29.2 k	10.3 h	19.7 H
50 2,4-D + 50 BA	26.0 cd	3.2 a	14.6 B	4.0 b–f	1.0 ab	2.5 B
10 Picloram + 10 BA	31.0 de	24.0 cd	27.7 C	3.3 a–e	2.0 a–c	2.7 B
25 Picloram + 25 BA	84.0 h	52.0 f	68.0 G	21.9 j	6.4 d–g	14.1 G
50 Picloram + 50 BA	90.4 h	26.2 cd	58.3 F	7.2 f–h	4.5 b–f	5.9 E

* – means followed by different letters (a, b, c… or A, B, C…) are significantly different at $P < 0.05$, Tukey's test

The morphogenesis direction of nodular callus obtained under the influence of high concentrations of auxin (10, 25 or 50 μM) was dependent on the concentration of cytokinin. We found that the concentration of cytokinin in the medium inducing callus had a clear effect on the further course of somatic embryogenesis (tab. 1). Somatic embryos occurred on media containing from 5 to 50 μM BA in combination with auxin at a higher or equal concentration (ratios between cytokinin and auxin of 1:1–10). The BA concentration in the initial medium of 0.5 μM proved to be too low to develop embryogenic cultures with a high initial concentration of auxins (ratios between cytokinin and auxin of 1:20–100). It can therefore be hypothesised that the induction of an embryogenic nodular callus in narcissus requires a minimum initial concentration of cytokinin in the medium. Too low a concentration of cytokinin or its lack in the initial medium can prevent the formation of somatic embryos. In cultures of *Eucharis grandiflora* and *Hippeastrum hybridum*, Mujib et al. [2013] did not obtain somatic embryos on initial media containing only auxins (2,4-D or NAA). Narcissus somatic embryos differentiation was observed as early as six weeks into the culture (fig. 2b). The most explants forming embryos (tab. 1, means 67 and 68%) were cultivated on media containing 25 μM of Picloram and 5 or 25 μM of BA. The most embryos per explant (tab. 1) were

obtained under the influence of 25 μM of Picloram and 5 μM of BA as well as 25 μM of 2,4-D and 25 μM of BA. The number of explants forming embryos (up to 84–90.4% for the media: 25 μM Picloram + 25 μM BA, 25 μM 2,4-D + 25 μM BA and 50 μM Picloram + 50 μM BA) and the number of embryos per explant was higher in the flower stem than in the ovary (tab. 1). Most numerous embryos (29.2 embryos per explant) were formed in flower stem cultures on medium with 25 μM 2,4-D and 25 μM BA (tab. 1, fig. 2c).

The maturation and conversion of narcissus 'Carlton' somatic embryos into plants was achieved on regeneration media with a lowered auxin concentration (figs 2d, 2e, 3). Reducing the concentration of auxins in the medium is necessary for the further development and germination of somatic embryos in *Tulipa* and *Nerine* cultures [Lilien-Kipnis et al. 1994, Ptak and Bach 2007].

In the present paper, the appearance of the next stages of somatic embryo development was also observed on media with a high content of initial auxins (fig. 3). This fact can be explained by the gradual usage of auxin during the four-week passage, which, as noted by Zimmerman [1993], removes the blockade against the expression of genes responsible for achieving later stages of development by globular embryos.

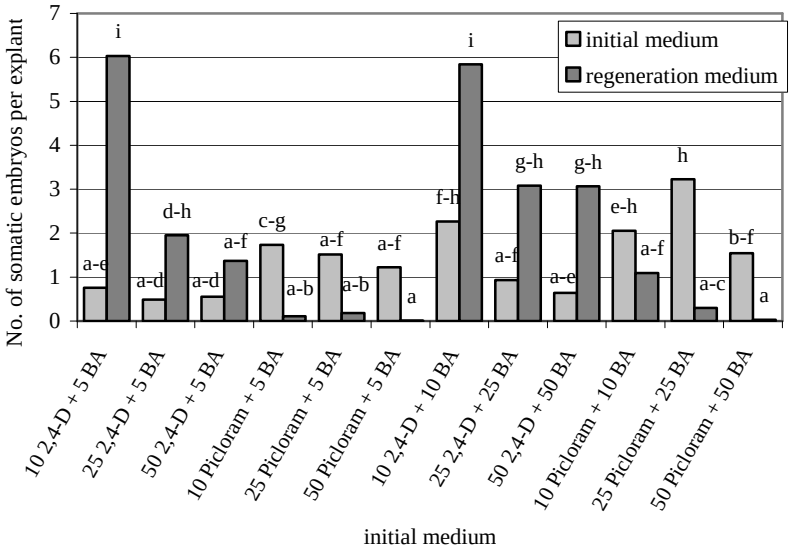

Fig. 3. The effect of growth regulators in initial and regeneration medium on *Narcissus* L. 'Carlton' somatic embryos conversion in cultures of initial explants

The conversion efficiency of embryos in plants was influenced by the type of auxin used for embryogenesis induction. Embryos initiated on media enriched with Picloram more often evolved into plants when the composition of the medium remained unchanged. The regeneration medium containing 5 μM BA and 0.5 μM NAA inhibited the conversion of embryos obtained under Picloram (fig. 3). In turn, embryos under 2,4-D developed better when they were transferred from the initial to the regeneration

medium. The highest conversion was achieved in embryogenic cultures derived on initial explants treated with 10 μM 2,4-D and 5 or 10 μM BA. Similar results were obtained from narcissus shoot culture leaf explants by Sage et al. [2000], who more frequently observed the development of embryos initiated under 2,4-D than under Picloram on a regeneration medium containing 5 μM BA.

Organogenesis. In addition to somatic embryogenesis the formation of adventitious roots and bulbs was observed in narcissus cultures of Pc flower bud explants. Numerous roots were noticed in callus obtained on media with high concentration of auxin (10–50 μM) and 0.5 μM of BA (ratios between cytokinin and auxin of 1:20–100).

The most frequent rhizogenesis was observed under the influence 10 or 25 μM 2,4-D used in combination with 0.5 μM of BA. For media with Picloram, roots were recorded only in the callus cultures initiated with 0.5 μM of BA (tab. 2). Rhizogenesis is most likely related to the level of cytokinin in the initial medium. Its increased concentration blocks this process and triggers the embryogenic potential. Selles et al. [1999] induced and then cultivated narcissus callus only with 2,4-D or Picloram without added cytokinin. Similar to the results of the present study, the growth of callus on this media was accompanied by the formation of roots. Non-embryogenic calli obtained under the influence of 2,4-D in maize, *Haloxylon aphyllum* and rye cultures developed only the roots [Bronsema et al. 2001, Jimenez and Bangerth 2001, Ramirez and Birnbaum 2001]. In *Narcissus tazetta* L. var. chinensis cultures, a medium supplemented with 2,4-D facilitated callus formation but usually promoted root differentiation and inhibited bulblet regeneration [Chen et al. 2005].

Table 2. Efficiency of organogenesis in *Narcissus* L. 'Carlton' *in vitro* cultures of Pc stage flower buds

Growth regulators (μM)	Adventitious bulbs formation (% of explants)			No. of adventitious bulb per explant			No. of adventitious roots
	flower stem	ovary	mean	flower stem	ovary	mean	
10 2,4-D + 0.5 BA	0.0 a*	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	8.7 e
25 2,4-D + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	8.0 e
50 2,4-D + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	5.4 d
10 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	6.4 d
25 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	5.9 d
50 Picloram + 0.5 BA	0.0 a	0.0 a	0.0 A	0.0 a	0.0 a	0.0 A	3.6 c
25 2,4-D + 5 BA	32.2 ef	16.4 a–e	24.3 CD	1.3 ab	1.4 ab	1.3 B	1.9 b
25 Picloram + 5 BA	6.8 a–c	13.6 a–e	10.2 AB	1.0 ab	1.1 ab	1.0 B	0.0 a
25 2,4-D + 25 BA	15.6 a–e	30.8 ef	23.2 CD	1.4 ab	1.5 b	1.4 B	1.9 b
25 Picloram + 25 BA	1.2 ab	9.2 a–d	5.2 AB	0.6 ab	1.0 ab	0.8 AB	0.0 a
1 2,4-D + 10 BA	15.8 a–e	16.8 a–e	16.3 BC	1.5 b	1.1 ab	1.3 B	–[a]
1 2,4-D + 25 BA	14.4 a–e	14.8 a–e	14.6 BC	1.4 ab	1.3 ab	1.3 B	–
5 NAA + 25 BA	0.0 a	28.0 de	14.0 BC	0.0 a	1.8 b	0.9 AB	–
5 NAA + 50 BA	20.0 b–e	50.0 f	35.0 D	2.1 bc	3.5 c	2.8 C	–
5 NAA + 25 ZEA	96.0 g	10.0 a–d	53.0 E	6.8 d	1.3 ab	4.1 D	–
5 NAA + 50 ZEA	0.0 a	24.0 c–e	12.0 A–C	0.0 a	1.6 b	0.8 AB	–

* – means followed by different letters (a, b, c… or A, B, C…) are significantly different at $P < 0.05$, Tukey's test; [a] – data not analyzed

We observed direct organogenesis (adventitious bulb formation) on media containing higher concentrations of auxin than cytokinin, at a level of cytokinin of 5 μM as well as that containing auxin and cytokinin in equal concentrations, as well as with a higher concentration of cytokinin than auxin (tab. 2, fig. 2g). The media containing a very low concentration of cytokinin (0,5 μM) in combination with higher concentrations of auxin did not result in the adventitious bulb formation. Sporadic direct organogenesis next to the formation of callus was also observed by Ziv et al. [1994] on explants isolated from flower stem of nerine cultivated on media containing 10 μM 2,4-D and 10 μM BA. Both, embryogenic and organogenic abilities were also noted in cultures of gladiolus inflorescens [Kumar et al. 1999] and mature zygotic embryos of passion fruit [Rocha et al. 2015].

The greatest number of narcissus adventitious bulbs was recorded in flower stem cultures under 25 μM zeatin and 5 μM NAA. High concentration of BA (33,5 μM) has also resulted in adventitious shoots and bulbs in scales cultures of *Crinum* sp. [Ulrich et al. 1999], while adventitious shoots on the secondary shoot of *Lilium nepalense* explants were obtained as a result of high concentrations of zeatin (5–20 μM) [Wawrosch et al. 2001]. The process of direct organogenesis was more effective when not accompanied by callus formation. A similar pattern was observed by Guohua [1998] in cultures of fragments of cassava somatic embryos.

Histological analysis. As in other studies on the somatic embryogenesis of geophytes [Gude and Dijkema 1997, Ptak and Bach 2007], embryogenic cell formation was localised around vascular bundles (fig. 2h). Vascular bundles contain receptors that are sensitive to the effects of auxins, therefore, embryoid centres form in their immediate vicinity [Yoshida and Komae 1994]. Our histological squash preparation studies showed the inconsistent structure of nodular embryogenic callus tissue. Large, aging parenchymatous cells, highly vacuolated, were located at the centre of the callus tissue. In contrast, the surface portion of the callus mass holds small, isodiametric cells with a dense cytoplasm and a large, centrally located nucleus (fig. 2i). The presence of embryogenic callus of the similar, nodular structure was also observed in cultures of *Tulipa gesneriana* [Ptak and Bach 2007] and *Nerine* × *Mansellii* [Lilien-Kipnis et al. 1994]. The anatomical study of the longitudinal and transverse section of narcissus somatic embryos showed a normal embryogenesis structure (fig. 2j, 2k). Sage et al. [2000] revealed similar structures based on the study of the internal organisation of somatic and zygotic embryos.

The resulting plants were successfully acclimated (fig. 2l, 2m).

CONCLUSIONS

1. The ratios of cytokinin and auxin in the initial medium determined morphogenetic pathways in *Narcissus* L. 'Carlton' cultures of Pc flower bud explants.

2. The level of cytokinin in the initial medium is a critical factor responsible for somatic embryos, adventitious bulbs and adventitious roots formation.

3. Picloram stimulated the initiation of somatic embryos, while 2,4-D enhanced embryos maturation and conversion.

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MORFOGENEZA W KULTURACH *IN VITRO* EKSPLANTATÓW *Narcissus* L. 'CARLTON' IZOLOWANYCH Z PĄKÓW KWIATOWYCH W STADIUM PC W ZALEŻNOŚCI OD POZIOMU CYTOKININY I AUKSYNY

Streszczenie. Zbadano wpływ stężenia i proporcji regulatorów wzrostu w pożywce inicjalnej na morfogenezę narcyza (*Narcissus* L.) 'Carlton' w kulturach eksplantatów izolowanych z pąków kwiatowych w stadium Pc. Fragmenty zalążni i pędów kwiatowych kultywowano na pożywce stałej MS uzupełnionej regulatorami wzrostu: cytokininą – BA lub zeatyna (0.5–50 µM) i auksyną – Picloram, 2,4-D lub NAA (1–50 µM). Zastosowane proporcje regulatorów wzrostu determinowały kierunki morfogenezy, a poziom cytokini-

ny okazał się czynnikiem krytycznym. Somatyczną embriogenezę uzyskano w przypadku proporcji cytokininy do auksyny – 1:1–10, cebule przybyszowe – 1:0,4–1, korzenie przybyszowe – 1:20–100. Minimalne stężenie cytokininy w pożywce do inicjacji somatycznej embriogenezy i formowania cebul przybyszowych wyniosło 5 μM. Niższe stężenie cytokininy (0.5 μM) wywoływało ryzogenezę. Obserwując wpływ auksyny na proces somatycznej embriogenezy, stwierdzono, że Picloram stymulował inicjację zarodków somatycznych narcyza, natomiast 2,4-D przyspieszało ich dojrzewanie i konwersję.

Słowa kluczowe: somatyczna embriogeneza, organogeneza, *in vitro*, kalus

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