ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 16(2) 2017, 107–112
www.acta.media.pl ISSN 1644-0692

ORIGINAL PAPER

Accepted: 24.10.2016

HIGH-YIELDING REPETITIVE SOMATIC EMBRYOGENESIS IN CULTURES OF *Narcissus* L. 'CARLTON'

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ABSTRACT

An innovatory protocol for large-scale production of narcissus 'Carlton' somatic embryos was developed based on the repetitive somatic embryogenesis (RSE). RSE was established as a stepwise process beginning with primary somatic embryogenesis (PSE) on ovary explants followed by secondary somatic embryogenesis (SSE) and continuously repeating cycles of SSE. A highly embryogenic lines of callus were sourced from primary embryogenic tissue, callus and embryos, which were continuously exposed to Amino-3,5,6-trichloropicolinic acid (Picloram) or 2,4-Dichlorophenoxyacetic acid (2,4-D) (both 25 µM) and 6-Benzyladenine (BA) (5 µM). Thus obtained calluses were multiplicated in six-week repetitive cycles and the efficiency of multiplication was 2.1- to 2.3-fold. Using RSE protocol, on regeneration medium containing 5 µM BA and 0.5 µM α-Naphthaleneacetic acid (NAA) it was possible to receive more than 20 embryos per 100 mg of callus. PSE yielded only 3.3–11 embryos. RSE resulted in the production of cream-coloured soft calluses which retained high efficiency of multiplication and differentiation of somatic embryos for over two years.

Key words: primary somatic embryogenesis, secondary somatic embryogenesis, growth regulators, *in vitro*, ovary, callus

INTRODUCTION

Conventional methods of *Narcissus* sp. propagation are very slow [Rees 1969], what makes it difficult to introduce new cultivars or to obtain elite material free from pests and diseases. The use of advanced tissue culture techniques and methods, in particular somatic embryogenesis (SE) process, can significantly increase the number of plants obtained [Selles et al. 1999, Sage et al. 2000, Chen et al. 2005, Malik 2008, Malik and Bach 2016].

The efficiency of primary (PSE) and secondary (SSE) somatic embryogenesis in narcissus had previously been assessed at the stages of initiation and differentiation of somatic embryos [Selles et al. 1999, Sage et al. 2000, Chen et al. 2005, Malik 2008]. Primary somatic embryogenesis is defined as the process in which embryos are formed on initial plant explant or on callus derived from it whereas secondary somatic embryogenesis is defined as the process in which embryos are formed (directly or via callus) from previously formed embryos. Furthermore, new embryos can proliferate continuously by repeated cycles of secondary embryogenesis in repetitive somatic embryogenesis (RSE) [Raemakers et al. 1995]. There is no information on the efficiency of primary, secondary or repetitive SE in the varieties of narcissus derived from *N. pseudonarcissus* L. at the stage

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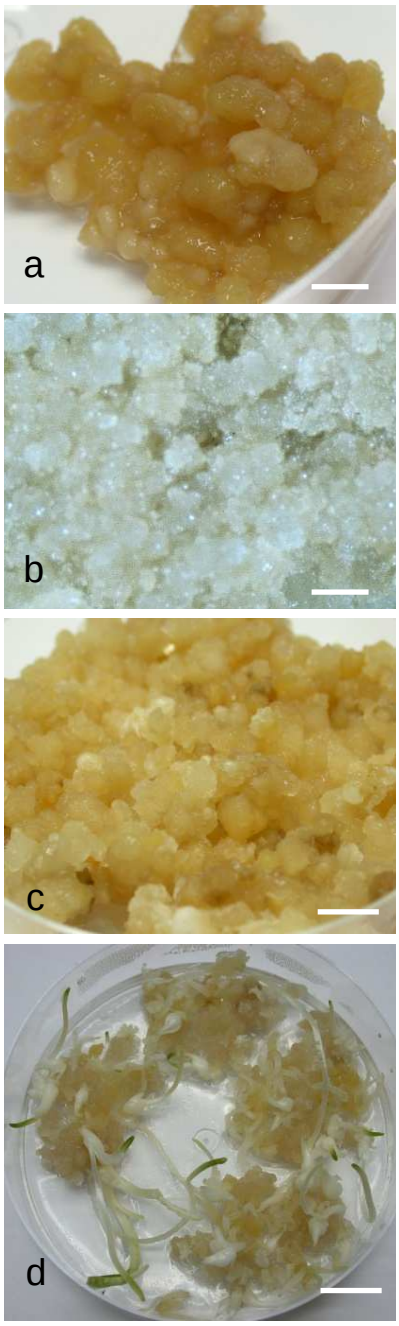

Fig. 1. Different types of *Narcissus* L. 'Carlton' callus: (a) embryogenic – yellow nodular (EYN5), bar = 4 mm; (b) non-embryogenic – white soft (NWS1), bar = 2 mm; (c) embryogenic – cream-coloured soft (ECS2), bar = 10 mm; (d) embryogenic – cream-coloured soft (ECS1) during regeneration of somatic embryos, bar = 15 mm

of embryogenic callus multiplication. According to Raemakers et al. [1995] and Vasic et al. [2001], repetitive somatic embryogenesis presents potentially the greatest opportunity for increasing the scale of plantlets production by continuous multiplication of embryos. The high efficiency of plantlet recovery from the secondary embryos obtained by RSE was already described for some species, e.g. *Camellia sinensis* [Akula et al. 2000], *Petiveria alliacea* [Cantelmo et al. 2013], *Hepatica nobilis* [Szewczyk-Taranek and Pawłowska 2015] but not for narcissus. The aim of this study was to assess, for the first time, the efficiency of multiplication and, consequently, regeneration of the multiplied calli of narcissus 'Carlton' obtained by RSE in comparison to PSE. Here, we present high-yielding repetitive somatic embryogenesis protocol for narcissus 'Carlton'.

MATERIALS AND METHODS

Plant material. Different types of callus tissue obtained on ovary explants isolated from *Narcissus* L. 'Carlton' flower buds originating from bulbs (12 cm in circumference, chilled for 12 weeks at 5°C) were used for the experiment.

Culture initiation. The primary explants, prepared as described by Malik [2008], (5–8 slices per Petri plate and 5 plates per treatment), were cultivated on MS medium [Murashige and Skoog 1962] with growth regulators (auxins – Picloram or 2,4-D, and cytokinin – BA) (tab. 1). Media were selected based on results of Malik and Bach (2016). The initial media were supplemented with 3% sucrose, adjusted to pH 5.5 before autoclaving, gelled with 0.7% agar (Purified Difco Agar) and dispensed into plastic Petri dishes (9 × 2.5 cm). The cultures were maintained at 20 ±2°C in darkness.

Callus multiplication. Calli were separated from the primary explants as different lines (tab. 1) and multiplied (5 clusters of 1 g per Petri plate, and 5 plates per treatment) on media identical to the initial ones. After six weeks, the callus multiplication rate was determined using the formula: (final fresh weight – initial fresh weight)/initial fresh weight.

Table 1. Characteristics of callus lines obtained in *Narcissus* L. 'Carlton' *in vitro* cultures

Callus line	Growth regulators in initial medium (μM)	Callus initiation period	Callus morphology	Callus longevity	Somatic embryogenesis type
EYN1*	25 2,4-D + 25 BA	6 weeks	yellow nodular, E**	< 18 weeks	primary (PSE) ***
EYN2	50 2,4-D + 50 BA	6 weeks	yellow nodular, E	< 18 weeks	primary (PSE)
EYN3	25 Picloram + 25 BA	6 weeks	yellow nodular, E	< 18 weeks	primary (PSE)
EYN4	50 Picloram + 50 BA	6 weeks	yellow nodular, E	< 18 weeks	primary (PSE)
EYN5	25 2,4-D + 5 BA	6 weeks	yellow nodular, E	< 18 weeks	primary (PSE)
EYN6	25 Picloram + 5 BA	6 weeks	yellow nodular, E	< 18 weeks	primary (PSE)
NWS1	1 2,4-D + 25 BA	6 weeks	white soft, NE	> 24 months	non-embryogenic callus
ECS1	25 2,4-D + 5 BA	18 weeks	cream-colored soft, E	> 24 months	repetitive (RSE)
ECS2	25 Picloram + 5 BA	18 weeks	cream-colored soft, E	> 24 months	repetitive (RSE)

* EYN1-6 – lines of embryogenic yellow nodular callus, NWS1 – line of non-embryogenic white soft callus, ECS1-2 – lines of embryogenic cream-coloured soft callus; ** E – embryogenic, NE – non-embryogenic, *** PSE – primary somatic embryogenesis, RSE – repetitive somatic embryogenesis

Somatic embryo regeneration (development and conversion to plant). To evaluate the regeneration of somatic embryos, the calli (separated from primary explants and multiplied for 6 weeks on initial media) were placed on a regeneration medium containing 5 μM BA + 0.5 μM NAA + 3% sucrose (5 tissue clusters of 100 ±5 mg per plate, and 5 plates per treatment). The regeneration medium was adjusted to pH 5.8 before autoclaving. After six weeks, the number of regenerated embryos per 100 mg of callus was estimated.

Statistical analysis. The results of observations were evaluated by analysis of variance. The means that differed significantly were identified using Duncan's multiple test at a significance level of $P \leq 0.05$ (Statistica 10, Stat-Soft, Inc., USA).

RESULTS AND DISCUSSION

In tissue cultures of ovary explants of *Narcissus* L. 'Carlton', three types of callus were obtained – embryogenic yellow nodular (EYN, six lines), non-embryogenic white soft (NWS, one line), and embryogenic cream-coloured soft (ECS, two lines) (tab. 1, fig. 1). The EYN and NWS calluses formed on the primary explants at the initial stage of SE. The EYN callus was induced under the influence of media containing more 2,4-D or Picloram than BA, and media containing equal concentrations of auxin and cytokinin. By contrast, the NWS callus was obtained from a single ovary explant on a medium supplemented with more cytokinin than auxin (25 μM BA + 1 μM 2,4-D). The most efficient type of callus – ECS, was obtained when the primary explants, differentiated nodular callus (EYN5 and EYN6) and embryos, were exposed for an additional 12 weeks to growth regulators (25 μM Picloram + 5 μM BA or 25 μM 2,4-D + 5 μM BA) (tab. 1). This continuous exposure resulted in the induction of ECS calluses from PSE tissues (primary callus and embryos). The ECS callus kept multiplying and differentiated secondary somatic embryos. Secondary embryogenic tissues were capable of developing both embryogenic callus and secondary somatic embryos. Thus, embryogenic cultures were obtained in which new embryos were being formed continuously by repeated cycles of SSE – RSE. The formation of embryogenic callus and somatic embryos during SSE has also been reported for cultures of *Cocos nucifera* [Pérez-Núñez et al. 2006].

The three types of described above callus lines (EYN1-6, NWS1, ECS1-2) showed varying capacity for multiplication and regeneration of somatic embryos (fig. 2). The most intensively multiplying was the NWS callus (multiplication rate of 5.9), in whose

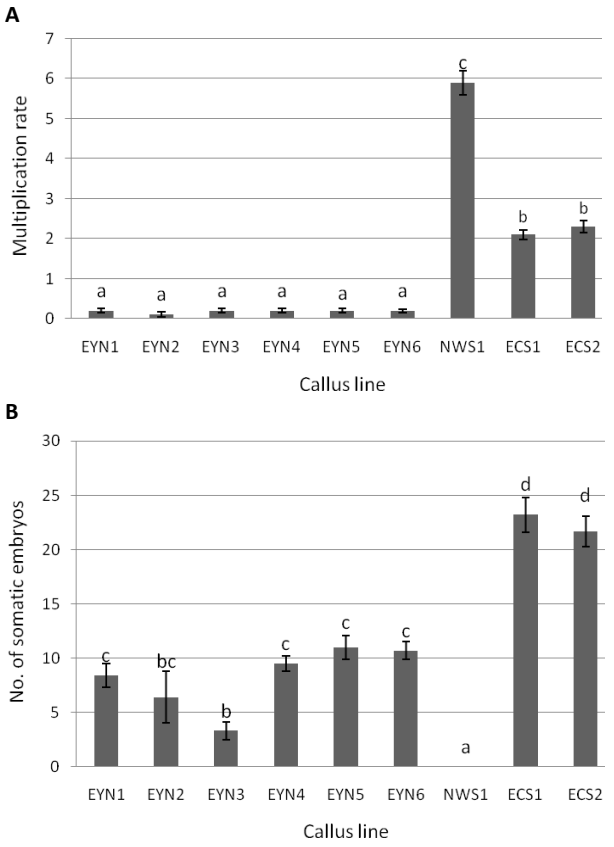

Fig. 2. Efficiency of (A) callus multiplication and (B) somatic embryo regeneration in *Narcissus* L. 'Carlton' *in vitro* cultures (EYN1-6 – lines of embryogenic yellow nodular callus obtained by primary somatic embryogenesis (PSE), NWS1 – line of non-embryogenic white soft callus, ECS1-2 – lines of embryogenic cream-coloured soft callus obtained by secondary somatic embryogenesis (SSE); callus multiplication rate was determined using the formula: (final fresh weight – initial fresh weight)/initial fresh weight; the number of somatic embryos was estimated per 100 mg of callus; mean values ±SD marked with the same letters are not significantly different according to Duncan's test at $P = 0.05$, n = 25)

mass no embryo differentiation was observed, thus proving it to be non-embryogenic. The embryogenic callus lines (EYN and ECS, obtained by PSE and RSE) multiplied at a lower rate than the non-embryogenic line. The multiplication rates for EYN calluses were 0.1–0.2, and for ECS calluses – 2.1–2.3. Jimenez and Bangerth [2001] explained the slow growth of maize embryogenic callus as a consequence of the formation of meristematic centres. In the presented experiment with narcissus, somatic embryos were differentiated in both types of embryogenic callus, which may explain the lower rate of multiplication.

The largest number of somatic embryos appeared in the ECS callus obtained by RSE after transfer on regeneration medium containing 5 µM BA and 0.5 µM NAA (21.7–23.2 embryos per 100 mg of callus). It is about 2-fold greater if compared with that obtained during PSE. However, since multiplication rate of ECS calluses is 2.1–2.3, around 50 embryos can be obtained within 12 weeks. The efficiency of somatic embryogenesis in narcissus reported in earlier research works was not high. Sage et al. [2000] received in primary cultures of *Narcissus pseudonarcissus* 'Golden Harvest' and 'St. Keverne' up to a dozen or so of primary embryos. The efficiency of narcissus 'Carlton' PSE varied from 0.1 to 20.2 of somatic embryos per explant depending on proportion of growth regulators and culture system [Malik 2008, Malik and Bach 2016]. According to Raemakers et al. [1995], the efficiency of primary embryogenesis is, in many species, lower than that of secondary embryogenesis. In narcissus 'Carlton' embryogenic cultures, SSE and RSE open the opportunity to further somatic embryo multiplication independently of primary explant. The experiment demonstrated, for the first time, that RSE is possible in narcissus cultures. Due to the higher efficiency of embryogenic callus proliferation and higher productivity of somatic embryos, RSE allows large scale multiplication of narcissus. The ECS callus obtained by RSE was characterized by retaining (for over 24 months) high efficiency of multiplication and production of somatic embryos of narcissus (tab. 1).

The efficient RSE systems have also been reported in *Camellia sinensis* [Akula et al. 2000], *Petiveria alliacea* [Cantelmo et al. 2013], *Cocos nucifera* [Pérez-Núñez et al. 2006], *Allium cepa* [Saker 1997]. In cultures of these species, RSE was more abundant than PSE. Pérez-Núñez et al. [2006] noted 50 000-fold increase in the yield of secondary somatic embryos compared to the yield of primary embryos.

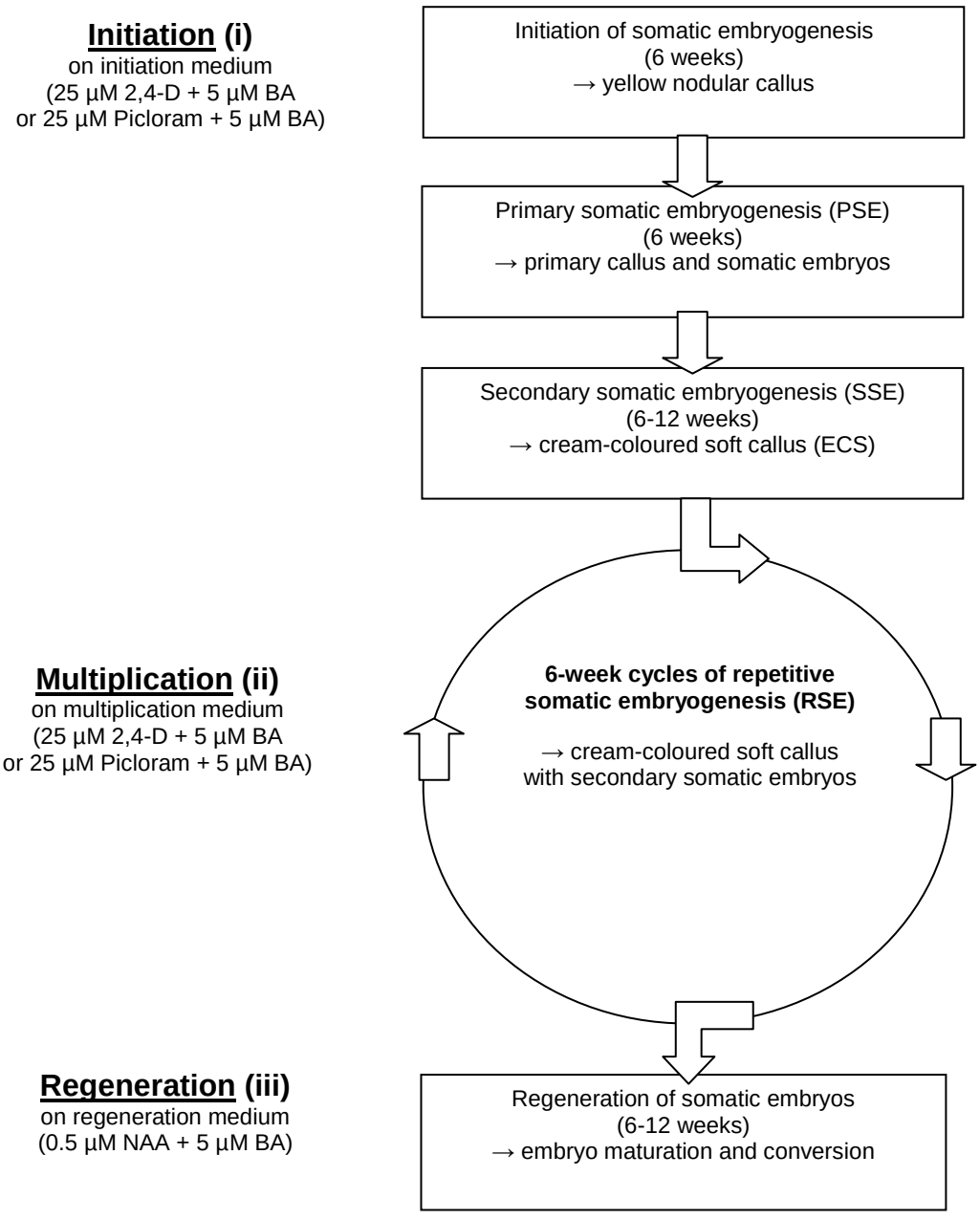

Fig. 3. Scheme of three-step protocol of *Narcissus* L. 'Carlton' embryo production based on the repetitive somatic embryogenesis

Compared to PSE, RSE is a cost-effective method due to the higher productivity, independence of a primary plant explant source (saving the labour, time and medium), maintenance of embryogenic capacity for prolonged periods of time, availability year-round and suitability for automation. However, genetic fidelity of plants obtained *via* developed protocol needs to be confirmed for commercial application.

CONCLUSION

Three-step protocol of narcissus 'Carlton' embryo production (fig. 3) was developed. It includes: (i) initiation of PSE followed by embryogenic cream-coloured soft callus (ECS) development as a result of secondary somatic embryogenesis (SSE), (ii) multiplication of ECS callus by repetitive cycles of secondary somatic embryogenesis and (iii) embryo maturation and conversion to plant. The developed method allows obtaining for about 50 somatic embryos per 100 mg of callus within 12 weeks.

ACKNOWLEDGEMENT

This study was financed by the Polish Ministry of Science and Higher Education (DS 3500/KRO/2014--2015).

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