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SOMATIC EMBRYOGENESIS IN APOMICT *BOECHERA HOLBOELLII*

K. M. TASKIN,[1]* K. TURGUT[2] and R. J. SCOTT[3]

[1] Department of Biology, Canakkale Onsekiz Mart University, 17100 Canakkale, Turkey
[2] Department of Field Crops, Akdeniz University, 07059 Antalya, Turkey
[3] Department of Biology & Biochemistry, University of Bath, Bath BA2 7AY, Avon, England

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In this study we establish an efficient method for the regeneration for *Boechera holboellii* via somatic embryogenesis. Immature cotyledons from siliques of 4–6 month-old plants were cultured on MS medium supplemented with plant growth regulators (BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; TDZ – 1-phelyl-3-(1,2,3-thiadiazol-5-yl) urea: 2,4 D-2,4-dichlorophenoxy-acetic acid). A high frequency of embryogenic callus was produced after two weeks in culture. The somatic embryos were obtained with a frequency of 10% of explants on MS medium supplemented with 1.34 μM NAA + 8.87 μM BA and 2.68 μM NAA + 17.74 μM BA within 3 weeks in culture. The alternative regime of MS medium supplemented with 1.34 μM NAA + 4.44 μM BA produced somatic embryos at a frequency of 38%.

Keywords: Boechera – Brassicaceae – apomixis – somatic embryogenesis – tissue culture

INTRODUCTION

Apomixis, an asexual mode of reproduction, results in embryo formation without fertilization of the egg. Since meiosis is usually absent or modified to provide unreduced female gametes, the progeny of apomictic plants is genetically identical to that of the mother plant. Three developmental components are common in apomixis: generation of a cell capable of forming an embryo without prior meiosis (apomeiosis); the spontaneous, fertilization-independent development of the embryo (parthenogenesis); and the capacity either to produce endosperm autonomously or to use a sexual endosperm derived from fertilization [1].

* Corresponding author; e-mail: kmtaskin@gmail.com

Apomixis occurs naturally among many angiosperm families [3]. The major apomictic pathways have been divided in the following types:

i) Adventitious embryony; the embryo is formed directly from a somatic cell, typically a nucellar, but sometimes an integumentrary cells outside the embryo sac.

ii) Diplospory and Apospory; embryo originates from an egg with unreduced chromosome number [7–9, 15, 19].

Both diplospory and aposory has also known as gametophytic apomixis as they involve modifications in gametophyte development. In diplospory, the megaspore mother cell (MMC) either bypasses or fails to complete meiosis, and divides to form an unreduced embryo sac.

In the genus Boechera there is a variety of reproductive mechanisms [2, 13, 16–18, 21]. *Boechera holboellii* is an attractive model species to study molecular biology of apomixis. It belongs to Brassicaceae and has been reported as a facultative apomict in which both sexual reproduction and apomixis can be seen together at both the diploid and triploid levels [2, 13]. The study of the molecular processes controlling apomixis in *B. holboellii* will be facilitated by the development of a genetic transformation system. The floral dip method [4], commonly used in *Arabidopsis thaliana*, is unlikely to be effective, requiring the development of a method of regenerating plants from tissue culture. Here, we describe an efficient *in vitro* regeneration protocol for *B. holboellii* using immature cotyledon explants from siliques of 4–6 months old plant.

MATERIAL AND METHODS

Seeds were originally collected from North America (Colorado) and obtained from Dr. Roy (University of Oregon, USA). *B. holboellii* plants were grown in a greenhouse under long-day conditions at a regime of 16 h light (20 °C): 8 h dark (18 °C) on peat moss: sand mix (1 : 3). The plants started to flower after 4 to 6 months.

The siliques were surface sterilized in 10% commercial bleach for 10 minutes, and then rinsed with sterile tap water. The surface-sterilized siliques were cut open under a stereo microscope with needles and the ovules were carefully collected into sterile Petri dishes containing liquid 1/2 MS [12] medium to protect from drying. The immature cotyledons were then dissected from the ovules and aseptically implanted in 9 cm Petri dishes containing 30 ml of semi-solid MS medium supplemented with plant growth regulators (BA, Sigma B-9395; NAA, Sigma N-06040; TDZ, Sigma P-6186; 2,4 D, Sigma, D-8407).

Mature seeds were also surface sterilized in 10% (v/v) commercial bleach for 10 min, then rinsed three times with sterile tap water. The surface-sterilised seeds were aseptically implanted in Petri dishes. They were germinated at 25 °C with a 16-h photoperiod under white fluorescent light. After 7 days, different types of explants (hypocotyl, cotyledons and leaves) were excised from seedlings. The cut surface of each explant was embedded in solid MS medium supplemented with PGRs at a density of eight explants per Petri dish. All cultures were maintained at 25 °C in 16-h

photoperiod. MS medium (Sigma M5519) containing 3% (w/v) sucrose was solidified with 0.8% (w/v) agar. The pH was adjusted to 5.7 with 1 M NaOH, and medium was sterilized by autoclaving at 120 °C for 20 min. PGRs were added to medium according to manufacturer's instructions. All the tissue culture works were carried out in a laminar flow hood.

RESULTS AND DISCUSSION

The morphogenic potential of various *B. holboellii* tissues was investigated in order to establish an efficient regeneration system. Hypocotyls, cotyledons, roots and leaves from 7-day-old *in vitro* growing seedlings and immature cotyledons from 6 months old plants were tested. The effect of various concentrations of the PGRs on *in vitro* regeneration of these explants was also investigated. Initial experiments revealed that hypocotyls, cotyledons, leaves and roots were not suitable to induce organogenesis under the studied conditions (Table 1). As the most efficient regeneration systems, somatic embryogenesis was achieved through the use of immature cotyledon explants. The induction of callus and somatic embryos were varied with concentration and type of PGRs. The results were obtained after 2 months in culture (Table 1). MS basal medium supplemented with various concentrations of BA or TDZ alone or in combination with NAA promoted a high frequency of callus formation (up to 100%) within 10 days in cultured hypocotyls, roots and immature cotyledons (Fig. 1a). Calli that appeared at the cut ends of the immature cotyledon explants were compact and dark green, and became embryogenic within 15 days. In contrast, the hypocotyls and root explants gave no regeneration. Subsequently, a high frequency of somatic embryo regeneration was obtained (Table 1). On the other hand, a high level of auxins was found to suppress somatic embryo regeneration.

Table 1
Effect of naphthaleneacetic acid (NAA) and benzylaminopurine (BA) on regeneration of hypocotyl explants of *B. holboellii*

PGRs (µM)	Callus (%)[a]	Somatic embryos (%)[a]	Number of embryos per explant[b,c]
0	0	0	0.00 ± 0.00
26.85 NAA	0	0	0.00 ± 0.00
53.71 NAA	0	0	0.00 ± 0.00
107.41 NAA	0	0	0.00 ± 0.00
1.34 NAA + 4.44 BA	100	38	3.60 ± 0.60
1.34 NAA + 8.87 BA	100	10	0.45 ± 0.20
2.68 NAA + 17.74 BA	100	10	0.27 ± 0.01

[a] Frequency of regeneration (percentage of regenerated explant).
[b] ± Represents standard error of mean.
[c] One-hundred explants per treatment were used.

Regeneration capacity of various B. holboellii *tissues*

The various *B. holboellii* tissues including hypocotyls, cotyledons, roots and leaves from 7-day-old *in vitro* growing seedlings were tested. To determine the regeneration capacity of the explants firstly the cytokinin BA, was used in a range of 8.87, 17.74 and 35.48 µM in combination with 0.54 µM NAA.

The MS medium supplemented with 8.87, 17.74 and 35.48 µM BA alone, or in combination with 0.54 µM NAA produced a high frequency (up to 100%) of callus formation on hypocotyl and root explants. The other tissues, such as leaves and cotyledons did not produce any regeneration. Therefore, we preferred to continue our works with the hypocotyls and root explants.

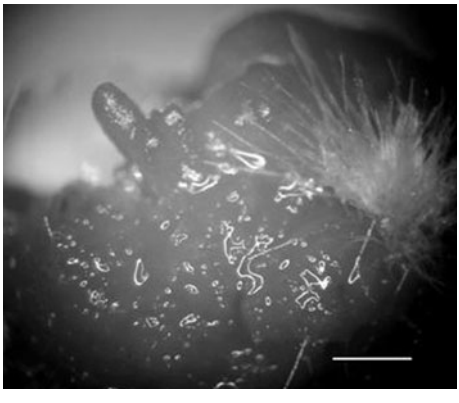

Fig. 1a

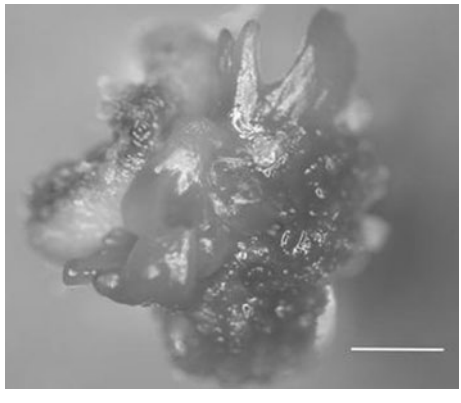

Fig. 1b

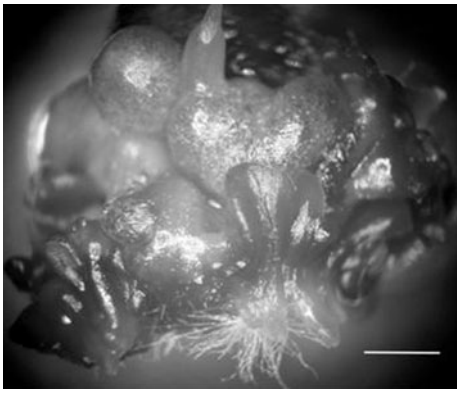

Fig. 1c

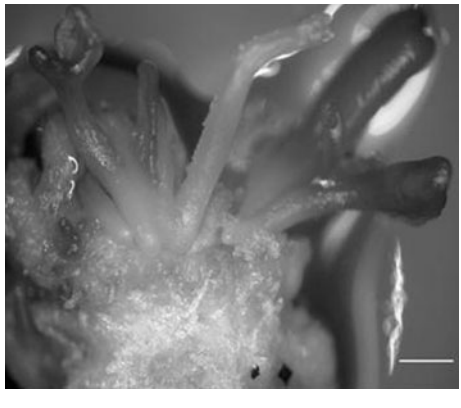

Fig. 1d

Fig. 1a. Callus formation from immature cotyledons in 10 days of culture – *Fig. 1b.* The embryogenic callus formation from immature cotyledons in 15 days of culture – *Fig. 1c.* Somatic embryos on MS medium supplemented with 1.34 µM NAA plus 4.44 µM BA after 3 weeks in culture – *Fig. 1d.* The somatic embryos fused with their roots on each explant. Bar: 1 mm

The cytokinin TDZ used in a range of 2.27–9.08 μM in combination with 0.54 μM NAA was also tested on hypocotyls and root explants. The MS medium supplemented with 2.27, 4.54 and 9.08 μM TDZ in combination with 0.54 μM NAA gave the highest frequency (up to 100%) of callus. However, MS medium supplemented with 2.27, 4.54 and 9.08 μM TDZ alone suppress the *in vitro* response of the explants.

Calli appearing at the cut ends of the explants were compact and dark green and covered surface of the explants rapidly. Although these explants produced a high frequency of callus, their compact structure and green colours disappeared within two months in culture and failed to produce regeneration. MS medium without PGRs also did not induce any organogenesis. These results show that the cytokinins (BA and TDZ) in combination with the auxin (NAA) were able to induce callus formation but failed to produce organogenesis.

Somatic embryogenesis from immature cotyledons

The effects of various concentrations of BAP and NAA on *in vitro* regeneration of the immature cotyledons were also investigated. The immature cotyledons were dissected from the ovules within siliques of 6 months old *B. holboellii* plants that were grown in the greenhouse. We first removed the seed coat with a fine needle without harming the ovules. The immature cotyledons were then dissected from the ovules under a stereo microscope and implanted in MS medium supplemented with PGRs.

The cytokinin BAP was used in the range of 4.44–17.74 μM in combination with NAA and produced a high frequency of somatic embryo regeneration in immature cotyledon explants of *B. holboellii* (Table 1). The immature cotyledons first expanded rapidly in culture then, the callus formation started (after 10 days in culture). MS medium supplemented with 4.44 and 8.87 μM BA plus 1.34 μM NAA and 2.68 μM NAA plus 17.74 μM BA produced high frequency (up to 100%) of callus formation (Fig. 1a). However, MS medium supplemented with 26.85, 53.71 and 107.41 μM NAA did not induce any regeneration. Calli obtained from immature cotyledons were more compact than that obtained from hypocotyls and roots. Within 15 days in culture, the calli showed evidence of embryogenic development in the MS medium supplemented with 1.34 μM NAA + 4.44 μM BAP, 1.34 μM NAA + 8.87 μM BAP and 2.68 μM NAA + 17.74 μM BAP (Fig. 1b). Somatic embryos were obtained at a frequency of 10% of explants on MS medium supplemented with 1.34 μM NAA + 8.87 μM BA and 2.68 μM NAA + 17.74 μM BA within 3 weeks in culture. While, MS medium supplemented with 1.34 μM NAA and 4.44 μM BA produced the somatic embryos with a frequency of 38% (Fig. 1c). However, the number of embryos per explants varied depending on the BA concentrations used. MS medium supplemented with 1.34 μM NAA and 4.44 μM BA gave the highest number of embryos per explants (3.6). Higher BA concentrations such as 8.87 and 17.74 μM reduced the number of shoots per explants to 0.45 and 0.2, respectively (Table 1).

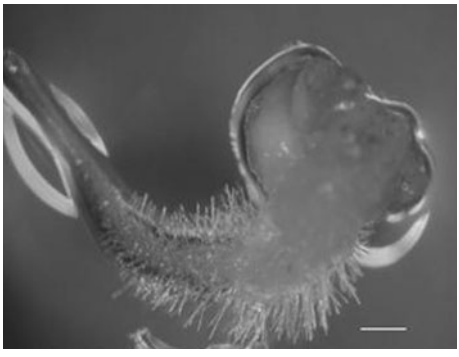

Fig. 2a

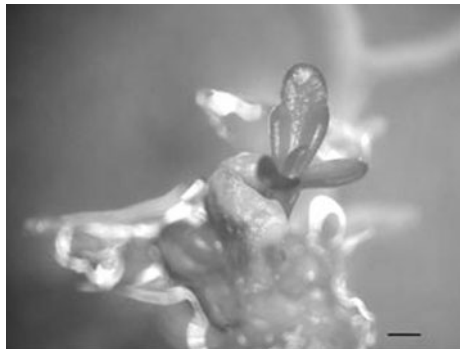

Fig. 2b

Fig. 2a. A germinated embryo on MS medium without PGRs – *Fig. 2b.* Fully developed embryo with cotyledons and roots. Bar: 1 mm

Somatic embryos were appeared to be green, tubular in shape and attached to the explants in groups of 3–5 (Fig. 1d) and, multiple root and shoot meristems were evident (Fig. 1c). When the somatic embryos were separated and transferred to MS medium without PGRs, they developed cotyledons and roots (Fig. 2a–b).

In this research, our results showed that the cytokinin (BA) in combination with the auxin (NAA) were necessary for the highest somatic embryo induction from *B. holboellii*. On the other hand, NAA alone gave no regeneration. Also, higher BA concentrations such as 8.87 and 17.74 μM reduced the frequency of embryos to 10%. The immature cotyledons have been used in other tissue culture systems including *Arabidopsis thaliana* [6, 10, 11, 24], *Brassica napus* [22], *Zea mays* [14], pigeon pea [11], Triticale [23] and watermelon [5] to induce somatic embryogenesis. We previously suggested that the use of low salt MS medium for rooting of *in vitro* regenerated shoots of *Boechera* species (such as *B. gunnisoniana*) was crucial [20]. Therefore, the embryos may also develop into plantlets on half-strength MS basal medium supplemented 1% (v/v) sucrose.

In this work, we obtained fully developed somatic embryos from immature cotyledons on MS medium supplemented with 1.34 μM NAA and 4.44 μM BA. This tissue culture system will be used to optimize a genetic transformation system for *B. holboellii*. Such a system will provide tools such as marker genes to follow inheritance, and will allow gene tagging of the apomixis locus or reverse genetic approaches.

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