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Induction of direct somatic embryogenesis and genetic stability of somatic embryo-derived plants of broccoli

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Abstract: The influence of the developmental stage of zygotic embryos and the composition and pH of the Gamborg induction medium B5 on the initiation and development of somatic embryos was investigated. The optimal medium was B5 medium with a pH value of 5.0 and without plant growth regulator, at which the highest frequency of somatic embryogenesis (56.67%) and the highest average number of somatic embryos per explant (3.35) were achieved. Somatic embryos appeared directly on the hypocotyls of the explants, without the callus stage. On zygotic embryos in the early cotyledonary phase, three times higher regeneration was achieved compared to larger embryos in the cotyledonary phase. The induction of somatic embryogenesis did not occur during the growth of explants on the medium containing 2,4-dichlorophenoxyacetic acid, nor on zygotic embryos in the late cotyledonary phase. Random amplified polymorphic DNA analysis showed the genetic stability of somatic embryo-derived plants, which makes this newly established protocol suitable for the regeneration and propagation of desirable broccoli genotypes.

Keywords: embryos germination; immature zygotic embryos; RAPD analysis

Broccoli (*Brassica oleracea* var. *italica*) is a vegetable of high nutritional value in the family Brassicaceae. It is a source of vitamin A, B and C, iron, fibre, calcium and zinc among others. Micropropagation is a process that enables the rapid production of uniform plants (Vujović et al. 2020). Somatic embryogenesis is the fastest method of plant propagation which not only helps to obtain a large number of plants irrespective of season (Shashi & Bhat 2021), but also can act as a promising biotechnological tool for crop improvement (Quiros-Figueroa et al. 2006). Somatic embryogenesis (SE) is also favoured as a method that allows cryopreservation of somatic embryos and/or whole embryogenic cultures, which in turn makes it possible to establish gene banks (Konieczny et al. 2010). Somatic embryogenesis is a process in which somatic plant cells undergo differentiation to form embryos. The plant-specific phenomenon of somatic embryogenesis is the strongest argument for the totipotency of differentiated plant cells. The inducing conditions result in the dedifferentiation of somatic plant cells followed or paralleled by the reacquisition of developmental totipotency (Feher 2015). Direct somatic embryogenesis has been reported for *Brassica campestris* (Soma & Sikdar 2005), *Brassica napus* (Burbulis & Kupriene 2005; Abdollahi et al. 2012), *Brassica oleracea* (Pavlović et al. 2013), *Brassica juncea* (Kumari et al. 2000; Faisal et al. 2021) and *Arabidopsis thaliana* (Gaj 2001; Kurczyjska et al. 2007). Successful genetic modifications have been

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achieved using embryogenic culture (Von Arnold et al. 2002; Pavlović et al. 2020).

In experiment, immature zygotic embryos from Br-3 line of broccoli were used, which is part of the selection collection held by the Institute for Vegetable Crops (Smederevska Palanka, Serbia), divided into three groups on the basis of their developmental stage: early cotyledonary (EC, 1.5 mm), cotyledonary (C, 1.8 mm) and late cotyledonary (LC, 3.0 mm). Broccoli zygotic embryo-donor plants were grown in the greenhouse and cross-pollination between two flowering plants was carried out by hand. Induction of somatic embryogenesis was performed using a previously developed method for *Brassica* species (Pavlović et al. 2013). The immature zygotic embryos were isolated from the seeds of immature siliques under a stereomicroscope. The excised embryos were grown in 90 mm Petri dishes on solid induction medium, containing B5 salts and vitamins (Gamborg et al. 1968), 20 g/L sucrose and 3 g/L Gelrite (Sigma). The effects of zygotic embryo age, presence of 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/L) in the culture medium, and the pH (5 and 5.8) of the B5 media on somatic embryogenesis were investigated. The pH of the medium was adjusted with 1M NaOH prior to autoclaving at 121 °C for 20 min. Cultures were maintained at 23 ± 2 in 16/8 day length for 4 weeks.

Somatic embryos were visible on the explants hypocotyls after 20–25 days of incubation on B5-0 medium (Figure 1A) and in different stages of development at the same time (Figure 1B). The presence of 2,4-D in the medium (B5-D) caused thickening in the hypocotyl region of explants that did not elongate (Figure 1C). The greater embryogenic potential of immature embryos compared to mature ones has previously been shown in rapeseed (Koh & Loh 2000; Burbulis et al. 2007).

The results of the induction of somatic embryogenesis shown in Table 1 indicate that explant regeneration ability was influenced by both the composition of the substrate (presence of 2,4-D) and the developmental stage of the immature embryos. The highest frequency and highest mean number of SE per explant were obtained on B5-0 medium pH 5.0 using immature zygotic embryos at the EC stage (Table 1). Both parameters of induction decreased in cotyledon immature embryos (C), while induction of somatic embryogenesis was not achieved on zygotic embryos at the late cotyledonary stage. Koh and Loh (2000) reported that in *B. napus* the potential for somatic embryogenesis on plant growth regulator (PGR)-free media was inversely correlated with zygotic embryo developmental stage, while a study in *Arabidopsis* indicated that the high embryogenic potential of immature zygotic embryos was correlated with the stage of development of the explant (Gaj 2001). In contrast in broccoli the best embryo-forming capacity was observed at the EC stage of zygotic embryos with this capacity being lower in LC embryos stages.

In our study, induction of SE was performed on B5-0 medium without exogenously added plant growth regulator (over 55 %), while the presence of 2,4-D in the media caused complete inhibition of the process (Table 1). The obtained results contrast with the results of the induction of somatic embryogenesis of other species, including *Arabidopsis thaliana* (Gogate & Nadgauda 2003; Elhiti et al. 2010) in which the presence of exogenous auxins in the medium, particularly 2,4-D, was necessary for the induction of somatic embryos. The capacity for somatic embryogenesis in the absence of PGRs has also been in several different *Brassica* species (Burbulis & Kupriene 2005; Faisal et al. 2021).

Figure 1. Somatic embryos directly formed on zygotic embryo hypocotyl after 4 weeks in culture on B5-0 medium (bar = 1 mm) (A), somatic embryos at various stages of development (bar = 2 mm) (B), zygotic embryos cultured on B5-D medium (bar = 10 mm) (C)

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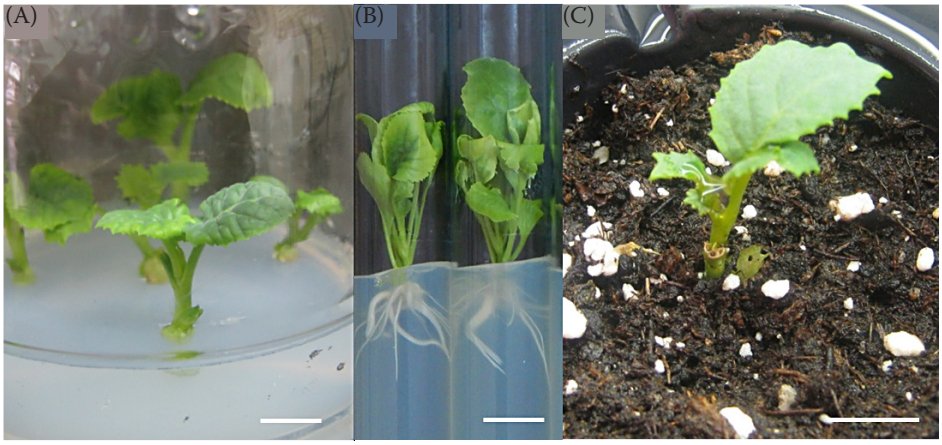

Figure 2. Plantlet obtained by germination of somatic embryo (bar = 10 mm) (A), rooted (bar = 10 mm) (B) and acclimatized plant of broccoli (bar = 5 cm) (C)

The lower pH value of the medium (5.0) was optimal for the induction of SE. At this pH value, the frequency of somatic embryogenesis increased significantly in the EC stage and increased slightly in the C stage, along with the average number of SEs per explant (Table 1). It has previously been shown that the induction of somatic embryogenesis in *B. napus* was favoured by a lower pH of the medium (3.5–5.0) (Koh & Loh 2000; Burbulis et al. 2007). According to our results (Table 1), EC embryos and B5-0 medium pH 5.0 were the optimal combination for induction of somatic embryogenesis in broccoli.

MS medium without PGR (MS-0) containing 2% sucrose and 0.3% Gelrite was used for embryos germination. Plantlets with well-developed leaf and root systems were transferred to pots containing sterile soil substrate and incubated in growth chamber at 21 °C under a 16/8 h photoperiod for 6 weeks, and then transferred and acclimated to greenhouse conditions. On the MS-0 the development of plants with developed leaves and root system from SE occurred spontaneously (Figure 2A, B), and these were subsequently successfully acclimatized to greenhouse conditions (Figure 2C).

Table 1. Frequency of somatic embryogenesis and mean number of somatic embryogenesis (SE) after 4 weeks of culture

Zygotic embryo stage	Treatment			
	induction medium B5 pH	2,4-D (1 mg/L)	frequency of SE induction (%)	mean number of SE/explant
EC	5.0	–	56.67^{a}	3.35 ± 0.41^{a}
EC	5.0	+	0^{d}	0^{c}
EC	5.8	–	36.67^{b}	2.73 ± 0.27^{b}
EC	5.8	+	0^{d}	0^{c}
C	5.0	–	13.00^{c}	3.2 ± 0.10^{a}
C	5.0	+	0^{d}	0^{c}
C	5.8	–	10.00^{c}	2.67 ± 0.12^{b}
C	5.8	+	0^{d}	0^{c}
LC	5.0	+	0^{d}	0^{c}
LC	5.0	–	0^{d}	0^{c}
LC	5.8	–	0^{d}	0^{c}
LC	5.8	+	0^{d}	0^{c}

EC – early cotyledonary; C – cotyledonary; LC – late cotyledonary zygotic embryo stage; 2,4-D – 2,4-dichlorophenoxyacetic acid; values are mean or mean ± standard error, n = 30 (3 replicates); numbers followed by a different letter within each column are significantly different at $P \leq 0.05$ according to the LSD test

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Table 2. The primer sequences used for random amplified polymorphic DNA (RAPD)-PCR

Primer	Sequence 5'→3'	Number of lines	Product size range (bp)
B01	GTTTCGCTCC	3	1 031–3 000
B03	GGGCCACTCA	7	700–3 000
B05	AGGTCGGCGT	6	900–3 000
C01	GTGCCCGATC	5	1 200–2 000
C05	CAGGCCCTTC	3	1 500–3 000

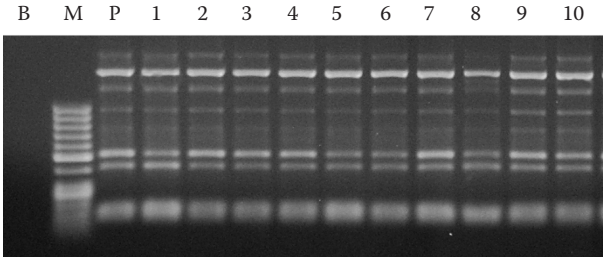

Figure 3. RAPD profile of somatic embryo-derived plants and mother plant of broccoli using B03 primer
M – DNA marker (GeneRuler 100 bp DNA Ladder Plus); P – mother plant; lines 1–10 – somatic embryo-derived plant

For the commercialization of a micropropagation process, it is of utmost importance to evaluate whether the obtained plants are genetically uniform. Random amplified polymorphic DNA (RAPD) markers are considered to be reliable in assessing the genetic stability of *Brassica* species (Qin et al. 2006, 2007). Genomic DNA was extracted from leaves of the mother plant as well as from 10 regenerated plants using the modified cetyl trimethyl ammonium bromide (CTAB) method (Zhou et al. 1994). Genomic DNA was then PCR amplified using RAPD primers under the conditions described by Qin et al. (2007). In the study, 10 primers were screened, of which 5 that gave polymorphic bands were used further in PCRs. Each primer generated an unique set of amplification products ranging in size from 700 to 3 000 bp. The number of bands for each primer varied from 3 in B01 and C05 to 7 in B03 (Table 2). Figure 3 shows the RAPD amplification using primer B03 in broccoli. The absence of genetic variation confirmed by RAPD amplification makes this newly established protocol suitable for the regeneration and propagation of desirable broccoli genotypes.

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