BIOLOGIA PLANTARUM 50 (4): 785-788, 2006

BRIEF COMMUNICATION

Comparison of induction frequency, maturation capacity and germination of *Abies numidica* during secondary somatic embryogenesis

B. VOOKOVÁ* and A. KORMUŤÁK

Institute of Plant Genetics and Biotechnology, Slovak Academy of Science, Akademická 2, P.O. Box 39A, SK-95007 Nitra, Slovak Republic

Abstract

Efficiency of the method for improving repetitive somatic embryogenesis and plant recovery of Algerian fir (*Abies numidica* De Lann.) was investigated by evaluating of induction frequency, maturation capacity and germination. Individual zygotic embryos differed only slightly in induction frequencies (6.8 %) from somatic embryos of the first (5.7 %) and second cycle (5.5 - 9.0 %). The yield of mature embryos differed significantly among the cell lines of the same cycle and among cell lines of the different cycles. Percentage of abnormalities was lowest in the first cycle of somatic embryos, whereas the second and the third cycles of somatic embryos were branded by a higher frequency of abnormalities. The differences in germination of well developed somatic embryos depended on cell lines rather than on cycle of somatic embryos.

Additional key words: abscisic acid, Algerian fir, benzylaminopurine, polyethylene glycol.

Secondary somatic embryogenesis is a phenomenon whereby new somatic embryos are initiated from somatic embryos. As an experimental system it offers some advantage compared to primary somatic embryogenesis – *e.g*. high multiplication rate, independence of an explant source and repeatability. Furthermore, embryogenicity can be maintained for a prolonged period of time by repeated cycles of secondary embryogenesis (Raemarkers *et al*. 1995). The method of secondary somatic embryogenesis was used in our laboratory as a tool for improving the maturation potential of *Abies numidica* lines that exhibited decline during their prolong cultivation.

In our previous work (Vooková *et al.* 2003) the efficient protocol for initiation of secondary somatic embryogenesis in *A. numidica* De Lann. was presented. The objective of the present study was to characterize the process of secondary somatic embryogenesis in more detail. For that reason, the induction frequency, maturation capacity and germination in three cycle of somatic embryogenesis of *A. numidica* were compared.

Mature somatic embryos of *Abies numidica* De Lann. were derived from immature zygotic embryos according to Vooková and Kormuťák (2001). Mature somatic embryos and immature zygotic embryos were subjected to initiation treatment in SH medium (Schenk and Hildebrandt 1972) with 100 mg dm^{-3} *myo*-inositol, 1 mg dm^{-3} benzylaminopurine (BAP) and 20 g dm^{-3} sucrose. Ten embryos were incubated per 9-cm Petri dish with 25 cm^3 medium in the dark at 21 - 23 °C during 10 weeks. Total number of embryos used as explants was recorded.

Proliferation was achieved in induction SH medium supplemented with 500 mg dm^{-3} L-glutamine (GL) and 1000 mg dm^{-3} casein hydrolysate (CH). Embryonal suspensor mass (ESM) was maintained on proliferation medium in the dark and subcultured at a three week intervals.

Maturation medium contained 1/2 strength MS (Murashige and Skoog 1962) macro and micro elements, FeEDTA and modified vitamins (Vooková *et al*. 2003).

Received 14 April 2005, *accepted* 20 August 2005.

Abbreviations: ABA - abscisic acid; BAP - benzylaminopurine; GL - L-glutamine; CH - casein hydrolysate; ESM - embryonal suspensor mass; MS - Murashige and Skoog (1962) medium; PEG-4000 - polyethylene glycol-4000; SH - Schenk and Hildebrandt (1972) medium.

Acknowledgements: The study was financially supported by the Slovak Grant Agency VEGA, project no. 2/3192/25. The authors are grateful to Margita Pavčírová for excellent technical assistance.

* Corresponding author; fax: (+421) 87 73 366 60, e-mail: nrgrvook@savba.sk

The medium was supplemented with 40 g dm^{-3} maltose, 100 g dm^{-3} polyethylene glycol-4000 (PEG-4000), 10 mg dm^{-3} abscisic acid (ABA), CH and GL in concentrations of 500 mg dm^{-3}. Experiments consisted of 10 replicate 60 mm plastic dishes, containing 300 mg of ESM per dish (cultured two weeks on proliferation SH medium). Cultures were maintained in the dark at 21-23 °C for 8 - 10 weeks. Maturation was tested in three independent experiments.

Prior to germination the isolated mature somatic embryos were undertaken to partial desiccation under high humidity in the dark at 21 - 23 °C during 3 weeks as described previously for *Abies* hybrids (Vooková *et al*. 1997/98). Then somatic embryos (at least with three cotyledons) were transferred to germination medium and cultured at 16-h photoperiod with irradiance of 110 μmol m^{-2} s^{-1} and temperature 21 - 23 °C. The standard medium for germination was SH medium containing 1/2 concentration of macro and micro elements, SH vitamins, 100 mg dm^{-3} *myo*-inositol, 10 g dm^{-3} sucrose and 10 g dm^{-3} activated charcoal. Ten embryos were cultivated in 150-cm^3 Erlenmayer flask with 50 cm^3 media, 60 embryos was used per treatment. Germination percentages were evaluated after 40 d of cultivation. The experiment was repeated thrice.

Media for the different stages of somatic embryogenesis were gelled with 3 g dm^{-3} *Phytagel*^{TM}. Comparison of percentage of maturation capacity, abnormality as well as germination were done in cell lines randomly selected from those with maturation potential. Statistical evaluation of the data was carried out using Student's *t*-test.

Both, immature zygotic embryos and mature somatic embryos produced new embryogenic tissue after 5 -10 weeks culture on initiation medium. Embryogenic tissue extruded from somatic embryo hypocotyl similarly as during initiation on mature zygotic embryos of *Abies* spp. (Nørgaard and Krogstrup 1995, Salaj and Salaj 2003/04). The procedure can be repeated several times. The embryogenic cultures contained early somatic embryos and long suspensor cells. Cell lines of embryogenic tissue differed in morphology of the early somatic embryos. Jalonen and von Arnold (1991) categorized the cell lines of *Picea abies* into two main groups: group A – polar/solar tissue type, group B – undeveloped type. Mature somatic embryos of *Picea abies* developed only in embryogenic culture with densely packed cells in the embryonic regions, *i.e*. in group A cell lines (Mo *et al*. 1996). We obtained cell lines with the both types of developing embryos. Zygotic embryos exhibited only slightly higher induction frequencies (6.8 %) than somatic embryos from first (5.7 %) and second cycle (from 5.5 to 9.0 %). The strongest response to induction medium was obtained from somatic embryos of the cell line 12/11 from second cycle (Table 1). Decrease or increase of embryogenic capacity among the explants of the same genotype could be attributed to the differentiation process and is related to the reduction in the number of cells competent to undergo somatic embryogenesis. The results of Wann *et al.* (1987) and Saly *et al.* (2002) suggest that there exists relationship between ethylene biosynthesis of the tissues of conifers and their embryogenic potential. Somatic embryos of larch hybrid with high embryogenic potential release little ethylene and inhibitors of ethylene action improved the development of embryonal masses.

Table 1. Frequency of formation of embryogenic tissue from zygotic (ZE) and somatic (SE) embryos on SH initiation medium with 1 mg dm^{-3} BAP and 20 g dm^{-3} sucrose.

Explant	Line	Number of explants	Frequency [%]
ZE		68	6.8
1st cycle SE	12	100	5.7
2nd cycle SE	12/2	49	8.1
	12/3	90	5.5
	12/7	110	6.3
	12/11	111	9.0

In our experiment, both embryogenic cell lines capable to develop and produce mature embryos and cell lines lacking this ability were obtained. In order to evaluate the maturation capacity, we used cell lines randomly selected from those with maturation potential. Somatic embryos started to develop after 3 weeks on maturation medium and reached the cotyledonary stage in 8 weeks. Well developed somatic embryos were not produced from all the lines. Numerous globule shaped embryos were formed in the cell lines 12/7 and 12/2. Embryogenic tissue of these lines contained many undeveloped embryos (group B). Mature somatic embryos of *Picea abies* developed only in embryogenic culture with densely packed cells in the embryonic regions, i.e. in the group of A cell lines (Mo *et al*. 1996).

The tested lines differed in maturation capacity (Table 2). The maturation capacity of the original line 12 (developed from an immature zygotic embryo) was similar to that of the line 12/11 of the second cycle and to 12/3 line of the third cycle. Our results do not coincide with those obtained with *P. glauca* × *engelmannii* where the parental and secondary lines exhibited similar maturation patterns (Eastman *et al*. 1991). Also the embryogenic line of *Larix* obtained through secondary somatic embryogenesis yielded three times more mature somatic embryos than the original (Lelu *et al.* 1994). In our experiment with Algerian fir, the yield of mature embryos was lower in third and second cycle than in the line of the first cycle. The yield of mature embryos was very significantly different also among the cell lines of the same cycle and among other cycles (Table 2). Some mature cotyledonary embryos exhibited morphological abnormalities (number and size of cotyledons, fused cotyledons, robust hypocotyl) which were common not only for conifer but also for other plant species, *e.g*. pea

Table 2. Number of mature somatic embryos (per g of ESM) and germination of mature well developed embryos after desiccation. Comparison of 1st, 2nd and 3rd cycles of embryogenic tissue and somatic embryos, respectively (means ± SE, a,b,c,d - highly significant difference between cell lines at $P \leq 0.01$, e - significant difference between cell lines at $P \leq 0.05$).

Explants	Line	Number of mature embryos	Abnormal embryos [%]	Germination [%]
1st cycle	12	26.50 ± 1.07ae	25.0	84.0 ± 4.10a
2nd cycle	12/2	13.35 ± 2.66be	70.9	70.7 ± 7.58
	12/3	34.58 ± 0.54ab	33.0	50.0 ± 6.31
	12/7	40.65 ± 2.96ab	45.0	70.5 ± 7.42
	12/11	26.10 ± 7.53	61.8	71.3 ± 7.44
3rd cycle	12/2a	5.80 ± 0.41ac	70.0	56.7 ± 4.94abe
	12/2b	11.80 ± 2.13ac	66.6	71.8 ± 4.09e
	12/2c	4.77 ± 2.42ae	60.0	63.4 ± 13.48
	12/3c	34.98 ± 3.10d	38.8	89.5 ± 4.98b
	12/3d	18.66 ± 3.04d	37.0	76.5 ± 7.55

(Griga 2002). Percentage of abnormalities was lowest in the first cycle of somatic embryos, whereas the second and third cycles of somatic embryos were branded by higher frequency of abnormalities.

The mature somatic embryos could develop into plantlets only if they were subjected to the three weeks desiccation. In our experiment, well developed embryos were chosen for comparison germination in individual cell lines (Table 2). The results described indicated that difference in germination of somatic embryos was depending on cell lines rather than on cycle of somatic embryos.

In our previous work (Kormuťák and Vooková 2000) the genetic stability of embryogenic tissue and emblings of Algerian fir was described. In present study we found significant difference in maturation capacity among cell lines of the same cycle and also among cell lines of other cycles with an increased frequency of abnormalities in each next cycle of embryos. The differences in number of germinating somatic embryos among some cell lines of the same cycle and among cell lines of different cycles were observed as well. It seems that each new embryo is characterized by the different developmental level reached which contributed to their particular nature though it is evident that those distinctions are conditioned physiologically. This is a real feature of the clonal multiplication we should take into account.

References

Eastman, P.A., Webster, F.B., Pitel, J.A., Roberts, D.R.: Evaluation of somaclonal variation during somatic embryogenesis of interior spruce (*Picea glauca engelmannii* complex) using culture morphology and isozyme analysis. - Plant Cell Rep. **10**: 425-430, 1991.

Griga, M.: Morphology and anatomy of *Pisum sativum* somatic embryos. - Biol. Plant. **45**: 173-182, 2002.

Jalonen, P., Von Arnold, S.: Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. - Plant Cell Rep. **10**: 384-387, 1991.

Kormuťák, A., Vooková, B.: Somatic embryogenesis and genetic stability of embling in Algerian fir (*Abies numidica* De Lann.). - In: Iljev, I., Zhelev, P., Tzvetkov, I., Gjuleva, V., Smidt, G. (ed.): IPPS in Bulgaria. Pp. 65-69. Forest University, Sofia 2000.

Lelu, M.K., Klimaszewska, K., Charest, P.J: Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*. - Can. J. Bot. **24**: 100-106, 1994.

Mo, L.H., Egertsdotter, U., Von Arnold, S.: Secretion of specific extracellular proteins by somatic embryos of *Picea abies* is depended on embryo morphology. - Ann. Bot. **77**: 143-152, 1996.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobaco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nørgaard, J.V., Krogstrup, P.: Somatic embryogenesis in *Abies spp*. In: Jain, S.M., Gupta, P.K., Newton, R.J. (ed.): Somatic Embryogenesis in Woody Plants. Vol. 3. Pp. 341- 355. Kluwer Academic Publishers, Dordrecht 1995.

Raemarkers, C.J., Jacobsen, E., Visser, R.G.F.: Secondary somatic embryogenesis and application in plant breeding. - Euphytica **81**: 93-107, 1995.

Salaj, T., Salaj, J.: Somatic embryo formation on mature *Abies alba* × *Abies cephalonica* zygotic embryo explants. - Biol. Plant. **47**: 7-11, 2003/04.

Saly, S., Joseph, C., Corbineau, F., Lelu, M.A., Côme, D.: Induction of secondary somatic embryogenesis in hybrid larch (*Larix* × *leptoeuropaea)* as related to ethylene. - Plant Growth Regul. **37**: 287-294, 2002.

Schenk, R.V., Hildebrandt, A. C.: Medium and techniques for induction and growth of monocotyledonous plant cell cultures. - Can. J. Bot. **50**: 199-204, 1972.

Vooková, B., Gajdošová, A., Matúšová, R.: Somatic embryogenesis in *Abies alba* × *Abies alba* and *Abies alba* × *Abies nordmanniana* hybrids. - Biol. Plant. **40**: 523-531, 1997/98.

Vooková, B., Kormuťák, A.: Effect of sucrose concentration, charcoal, and indole-3-butyric acid on germination of *Abies*

numidica somatic embryos. - Biol. Plant. **44**: 181-184, 2001.
Vooková, B., Matúšová, R., Kormuťák, A.: Secondary somatic embryogenesis in *Abies numidica* De Lann. - Biol. Plant. **46**: 513-517, 2003
Wann, S.R., Johnson, M.A. Noland, T.L., Carlson, J.A.: Biochemical differences between embryogenic and non-embryogenic callus of *Picea abies* (L) Karst. - Plant Cell Rep. **6**: 39-42, 1987.