Efficiency of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. under various *in vitro* culture conditions

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Summary

In *Arabidopsis thaliana*, *in vitro* culture of immature zygotic embryos on medium supplemented with 2,4-D results in formation of somatic embryos via direct embrogenesis (DSE). The analysis of the nature of signals/stimuli involved in determination of embryogenic response in cultured explants can reveal genetic and physiological mechanisms involved in plant embryogenesis. The key factors for DSE induction in *A. thaliana* are the developmental stage of the explant and the presence of 2,4-D in induction medium.

The study was undertaken to analyze DSE efficiency under modified tissue culture conditions. The studied factors included: pH of induction medium, temperature during embryogenesis induction, polyamines and their precursors, genotype and origin of the explants (seed-grown or *in vitro*-regenerated donor plants). The significant increase of the DSE efficiency was indicated on media with higher pH (7.0-8.0) and in culture of the explants obtained from plants regenerated via secondary embryogenesis. Moreover, embryogenic potential of the regenerant-derived explants was also observed on medium lacking of 2, 4–D. Spermidine and precursors of polyamines (ornithine and arginine) included in induction medium as well as any of the tested temperatures (5,28,32°C) did not stimulate the DSE efficiency in comparison to standard conditions. All 16 tested ecotypes displayed the ability for the DSE under standard culture conditions. DSE efficiency varied between the studied ecotypes, however, most of the genotypes (75%) showed high (60-100%) frequency of explants producing somatic embryos.

Key words:

Arabidopsis thaliana, direct somatic embryogenesis, immature embryo culture, stress factors.

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1. Introduction

Recent progress in plant genetics and biotechnology highly depends on the use of *in vitro* cultures. Among different *in vitro* systems applied, somatic embryogenesis (SE) is of special value as it offers opportunities for basic research and commercial applications. Somatic embryogenesis enables production of true-to-type as well as genetically modified plants when genetic transformation, somatic hybridisation and mutagenesis are involved in culture system. Moreover, SE is a useful tool in basic research on totipotency and other fundamental processes of plant morphogenesis.

The broad applications of somatic embryogenesis both in basic and applied research, have stimulated the studies on the determination of *in vitro* conditions for the induction of somatic embryos. As a result, efficient protocols on SE induction and plant regeneration have recently become available for many plant species including *Arabidopsis thaliana* (L.) Heynh. (1). *A. thaliana,* a model plant in molecular genetics, has been proved to be a convenient and powerful model system for the study on plant embryogenesis. The great usefulness of *Arabidopsis* in analysis of plant embryogenesis results from advanced structural and functional genomics of this plant (2,3) and the recent availability of protocols enabling *in vitro* induction of somatic embryogenesis. In *Arabidopsis,* somatic embryos can be induced through two different embryogenic pathways i.e. directly – from immature zygotic embryos (4,5) or indirectly – via embryogenic callus (6-8).

Studies on factors controlling *in vitro* plant morphogenesis are highly desirable not only for the development of improved regeneration systems which are useful for plant biotechnology, but also for the analysis of molecular mechanisms underlying plant embryogenesis. The use of immature zygotic embryos in advanced developmental stage and application of 2,4-D in culture medium were found to be the crucial factors for successful SE induction in *Arabidopsis* (5). Moreover, it was showed that stress treatment imposed by chemical mutagens or application of darkness during induction culture increases SE efficiency in culture of less-responsive pre-cotyledonary stage immature embryos (9).

The aim of the presented work was to characterize the influence of the selected *in vitro* conditions on the efficiency of direct somatic embryogenesis (DSE) in the culture of immature zygotic embryos at different developmental stages. Among *in vitro* conditions studied, there were stress factors (pH of induction medium and temperature imposed during induction of embryogenesis), medium composition (different carbon sources, polyamines and their precursors) as well as genotype and physiological status of explants.

2. Material and methods

2.1. Induction of direct somatic embryogenesis (DSE)

Plants of *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia were used as explant source for *in vitro* culture. Plants were grown in the soil mixed with vermiculite (1:1) at 20-23°C and 16 h of white fluorescent light of 100 μmol $m^{-2}s^{-1}$. Siliques with immature seeds were collected from 8- to 10-weeks old *A. thaliana* plants and were surface sterilised for 20 min in 2% solution of sodium hypochloride with 3 drops of Tween 20 per 100 ml. After rinsing in sterile water, the siliques were opened with very fine needles and immature zygotic embryos were excised under a dissecting microscope.

Immature embryos representing two different stages of development were used to induce DSE. The early stage embryos explants including torpedo shape (100-300 μm in length) were classified as the early cotyledonary stage (EC) of development, while the embryos with developed cotyledons (300-500 μm in length) were considered to represent the late stage (LC) of development.

In control, standard condition for DSE induction was applied (5). Explants, immature zygotic embryos, were cultured in Petri dishes (60 mm in diameter) with agar-solidified (8 g l^{-1} Oxoid agar) or liquid induction medium (E5) consisting of basal B5 medium (10) supplemented with 5 μM 2,4-D (2,4-dichlorphenoxyacetic acid, Sigma) and 20 g l^{-1} sucrose. Ten immature zygotic embryos were cultured in each Petri dish. The cultures were grown at 22°C under a 16 h photoperiod of 55 μmol $m^{-2}s^{-1}$ of white, fluorescent light or in darkness. After 3 weeks of culture on E5 medium, the explants undergoing somatic embryogenesis were counted and the efficiency of DSE was expressed as the percentage of explants producing somatic embryos.

2.2. *In vitro* factors studied

2.2.1. pH of induction medium

DSE efficiency was analysed in culture of EC and LC explants on E5 liquid and solid induction media supplemented with 0.5 g l^{-1} MES and adjusted to different pH values ranging from 5.0 to 9.5.

2.2.2. Temperature

The effect of different temperatures on DSE efficiency was studied in culture of EC and LC explants on E5 and E0 (2,4-D free E5 medium) solid induction media. Cul-

tures were incubated at 5, 22, 28, and 32°C in darkness for 1 or 2 weeks and subsequently transferred to standard DSE-induction conditions (Chapter 2.1).

2.2.3. Induction medium composition

2.2.3.1. Carbon source

Sucrose in E5 standard induction medium was replaced with sugars: galactose or cellobiose and sugar alcohols such as sorbitol, mannitol or xylitol at concentration of 150 mM. Response of LC explants under standard DSE-induction condition was analysed.

2.2.3.2. Polyamine and precursors

Polyamine spermidyne was used in E5 solid medium in two concentrations: 0.5 and 1.0 mM. Precursors of polyamines, arginine and ornithine were added to E5 solid medium in 0.5 mM concentration. DSE efficiency in culture of EC explants was monitored.

2.2.4. Genotype

In addition to the control genotype Columbia, fifteen other ecotypes of *Arabidopsis* were analysed according to their ability for DSE in culture of LC explants in standard conditions. The tested ecotypes included: Mühlen, Landsberg erecta, Nossen, RLD, RLD-1, Aua/Rhon, Cape Verde, Estland, Bensheim, Niederzenz, Wassilewskija, Col-0, Dijon-G, Greenville, Col-PRL. Standard culture conditions were applied to induce DSE.

2.2.5. Origin of explants

The embryogenic capacity of explants excised from seed-grown plants was compared to the DSE efficiency of explants obtained from *in vitro*-derived plants. Two types of *in vitro*-derived plants were used as explant-donor regenerants: (i) R_1DSE^1 plants obtained via DSE process; (ii) R_1DSE^2 plants regenerated through secondary embryogenesis where the primary somatic embryos induced by DSE were used as explants to start a new culture. The primary somatic embryos developed callus on E5 medium and subsequently secondary somatic embryos. The secondary embryos developed into plants (R_1DSE^2) on media used in DSE system (5).

LC explants excised from each tested plant group were cultured on induction media with various concentration of 2, 4-D (0.0, 0.3, 0.6, and 5 μM) and DSE and callus formation were evaluated for combination.

2.3. Statistical analysis

The LSD (least significant difference) test was used to compare treatment means at $P \leq 0.05$. Every treatment mean was based on the analysis of 2-4 experiments. In each experiment, *in vitro* response of 2-5 replicates (a replicate was a culture of 10 explants in one Petri dish) was scored.

3. Results

3.1. pH of induction medium

pH of induction medium was modified within range of 5.0 to 9.5. The analysis of embryo-forming explants indicated an increase of embrygenic potential of cultures on media with pH of 7.0 and 8.0. The significantly higher DSE efficiency on these media was observed in cultures of both types of the explants i.e. EC (fig. 1) and LC

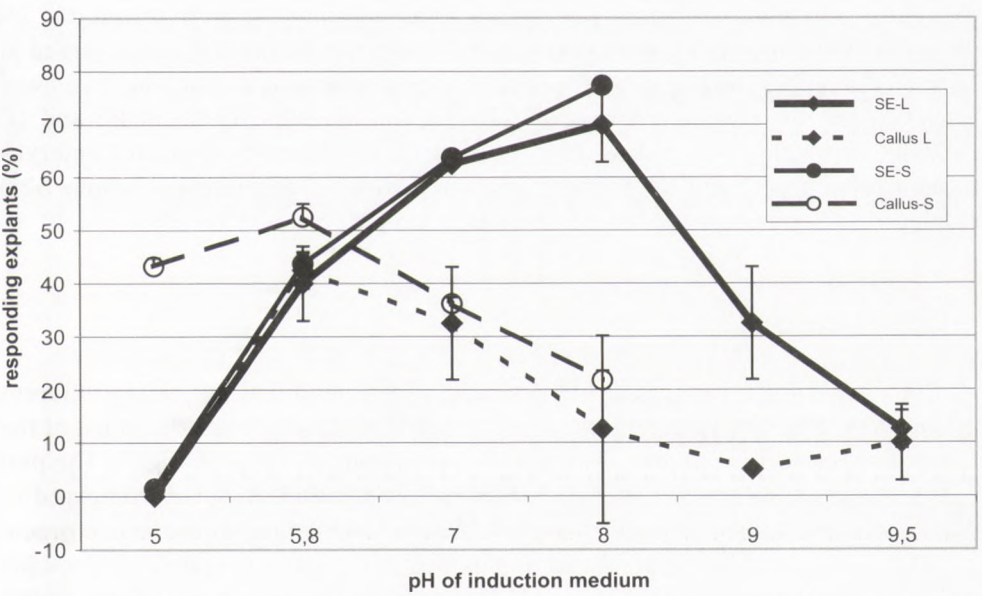

Fig. 1. Efficiency of somatic embryo (SE) and callus formation in culture of EC explants on liquid (L) and solid (S) E5 media of different pH.

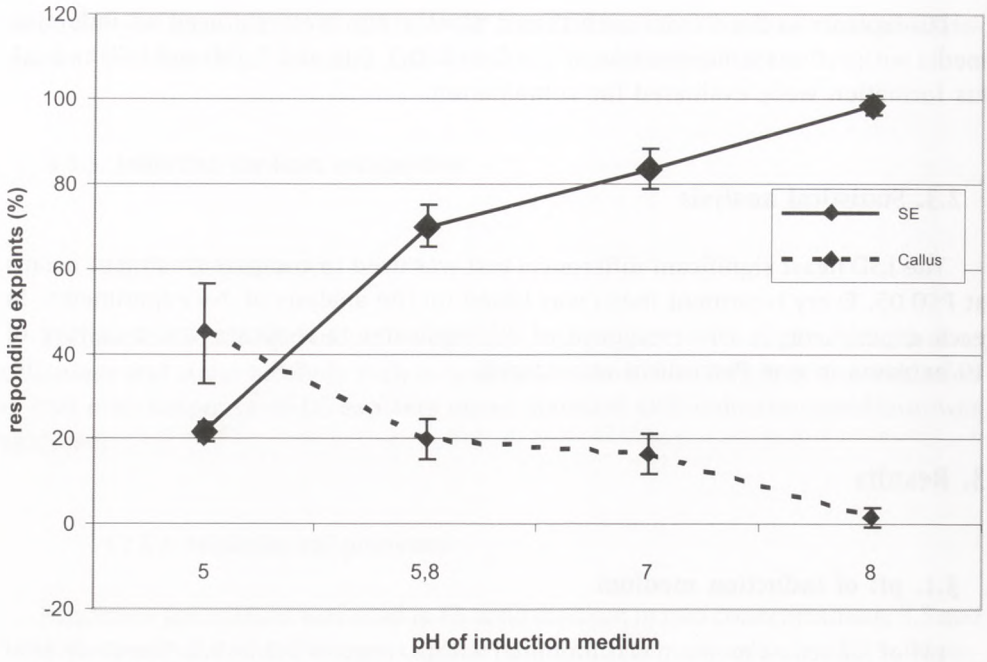

Fig. 2. Efficiency of somatic embryo (SE) and callus formation in culture of LC explants on solid E5 media of different pH.

(fig. 2). In culture of EC explants, when solid and liquid media were tested, the increase of embryogenic response over standard response (at pH 5.8) was observed in both media types. The highest frequency of explants forming somatic embryos was observed for pH 8 where more than 77% (fig. 1) and 90% (fig. 2) of EC and LC explants, respectively, displayed DSE. The explants not developing somatic embryos formed callus tissue and the highest frequency of callus development (above 52%) displayed EC explants cultured on standard medium (pH 5.8).

3.2. Temperature

Standard culture conditions of LC explants were modified by testing different culture temperatures during induction of DSE process. It was found that none of the tested temperatures increased DSE efficiency over control, i.e. 22°C (fig. 3). The percentage of explants forming somatic embryos was significantly lower compared to control culture at low (5°C) as well as high (28°C) temperature. Moreover, detrimental effect of applied temperature treatments was found when longer i.e. 2-week period of treatment was used. While over 80% of explants incubated at 22°C developed embryos, only 63 and 55% of explants displayed DSE at 5°C and 28°C, respectively.

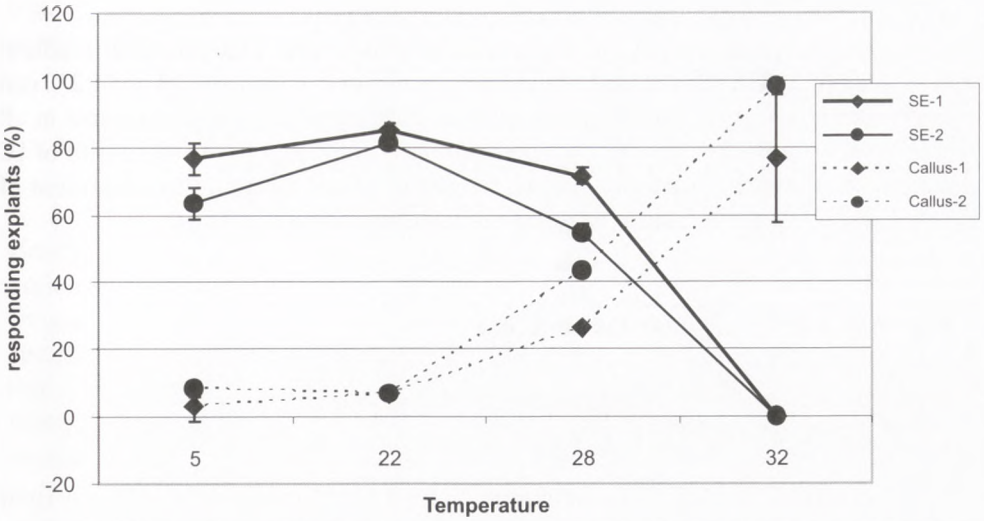

Fig. 3. Efficiency of somatic embryo (SE) and callus formation in culture of LC explants on E5 medium at different temperature conditions. Cultures were incubated at temperatures as indicated for one (1) or two (2) weeks.

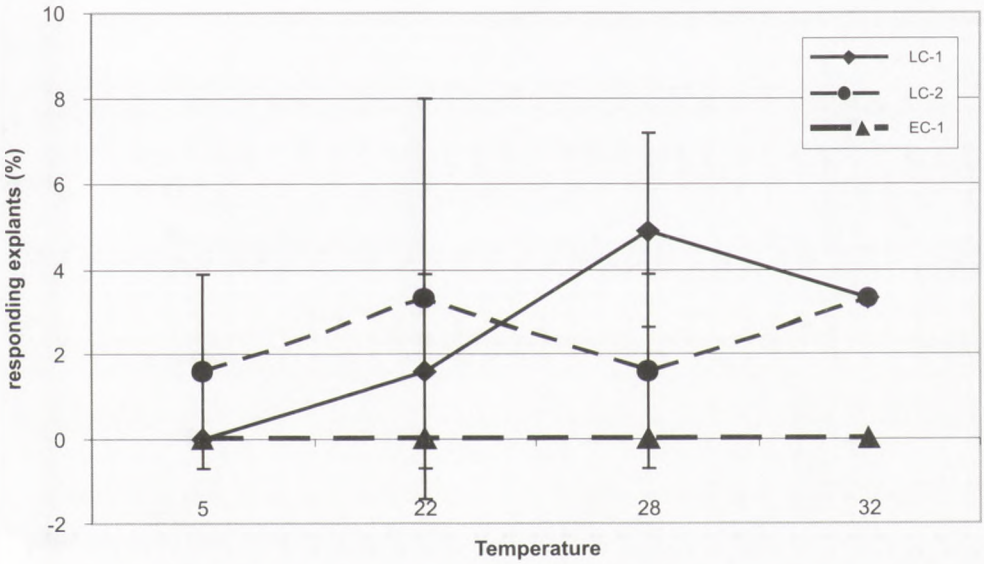

Fig. 4. Efficiency of callus formation in culture of LC and EC explants on hormone-free E0 medium at different temperature conditions. Cultures were incubated at temperatures as indicated for one (1) or two (2) weeks.

The culture of explants in high temperature of 32°C resulted in profound formation of callus tissue with no signs of somatic embryogenesis.

Beside of medium containing 2,4-D (E5), hormone-free (E0) induction medium was applied to test if sub-optimal, stressful temperatures such as 5, 28 and 32°C can stimulate DSE induction. The analysis showed lack of embryogenic response in all temperature regimes tested (fig. 4). The production of callus tissue in culture of LC explants was sporadically observed after 1 and 2 weeks of temperature-treatment of LC explants while EC explants, did not respond and died.

3.3. Composition of induction medium

3.3.1. Source of energy

Various sugars and sugar alcohols were added to E5 medium to evaluate their utilization of as energy source for *in vitro* cultured LC explants. While on sucrose-supplemented standard E5 medium almost 80% of explants developed somatic

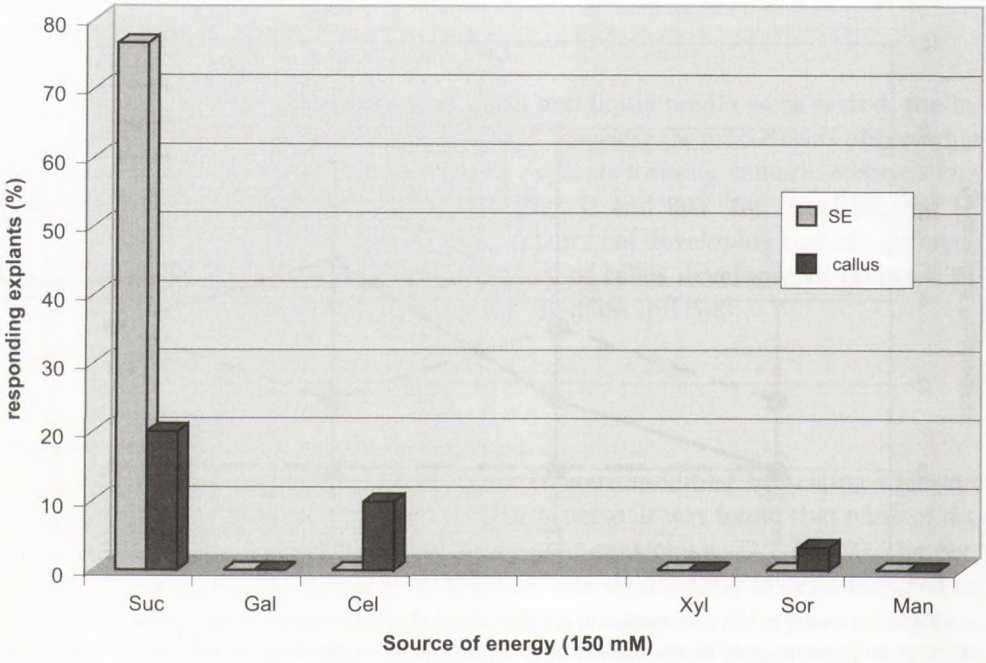

Fig. 5. Efficiency of somatic embryo (SE) and callus formation in culture of LC explants on E5 medium supplemented with different sugars (Suc – sucrose; Gal – galactose; Cel – cellobiose) or alcohols (Xyl – xylitol; Sor – sorbitol; Man – mannitol).

embryos, none of the tested substances was able to induce embryogenic response (fig. 5). Formation of callus (3.3-9.9%) was only sporadically noticed on media supplemented with cellobiose and sorbitol.

3.3.2. Polyamines and their precursors

For evaluation of the effect of exogenously supplied polyamines on DSE efficiency, the induction E5 medium was modified by the addition of spermidine, arginine or ornithine. The analysis of the embryo-forming explants indicated higher frequencies (up to 44.5%) of DSE on media supplemented with arginine and spermidine (fig. 6) as compared to control (38.7%), but the observed increase was not statistically significant. Significant decrease in DSE efficiency was found for ornithine where only 5.8% of explants developed somatic embryos on medium supplemented with this polyamine precursor. On the other hand, ornithine was found to induce profound formation of callus tissue which was observed in 67% of explants cultured on Orn 0.5 medium.

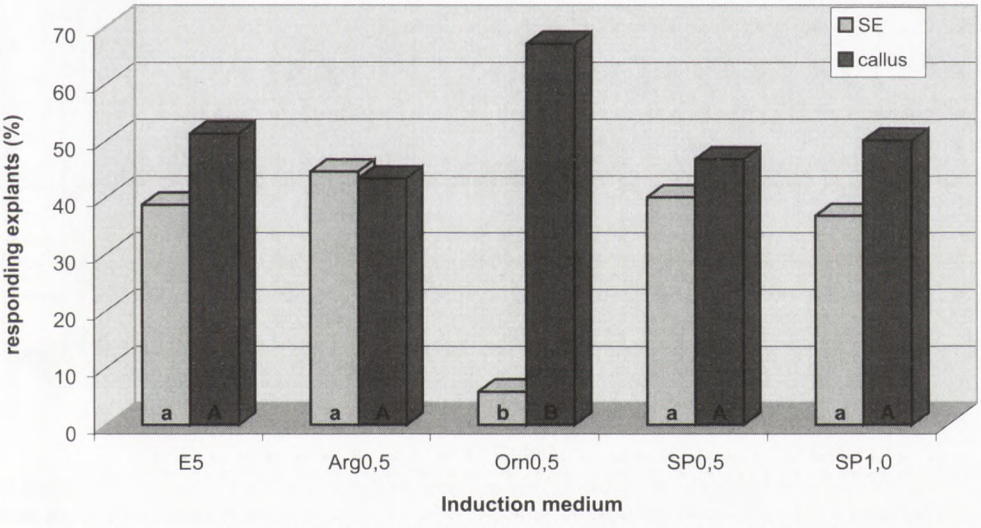

Fig. 6. Efficiency of somatic embryo (SE) and callus formation in culture of EC explants on E5 medium supplemented with precursors of polyamines: arginine (Arg) or ornithine (Orn) and polyamine – spermidine (SP) at concentration of 0.5 or 1.0 mM. Control – E5 standard medium. Values marked by the same letter are not significantly different at $P<0.05$.

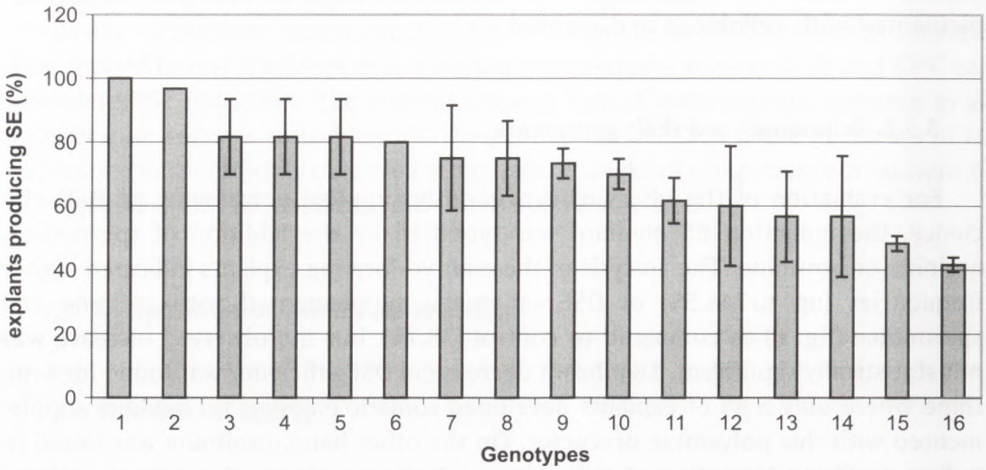

Fig. 7. Frequency of explants producing somatic embryos in culture of different ecotypes: 1 – Nossen; 2 – RLD 1; 3 – Greenville; 4 – Dijon G; 5 – Niederzenz; 6 – Columbia; 7 – Cape Verde; 8 – RLD; 9 – Col-0; 10 – Col-PRL; 11 – Mühlen; 12 – Wassilewskija; 13 – Aua/Rhon; 14 – Landsberg erecta; 15 – Estland; 16 – Bensheim.

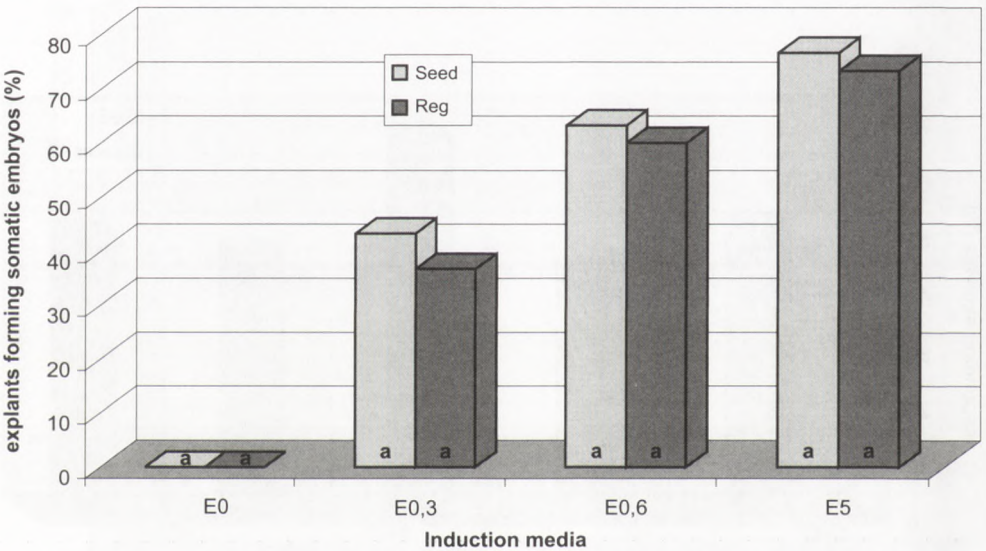

Fig. 8. Efficiency of somatic embryo formation in culture of explants excised from seed- (Seed) and *in vitro*-derived R_1DSE^1 (Reg) plants on medium without 2,4-D (E0) and containing 0.3 (E0.3), 0.6 (E0.6), and 5.0 (E5) μM of 2,4-D. Values marked by the same letter are not significantly different at $P<0.05$.

3.3.3. Genotype

LC explants of sixteen ecotypes were cultured on E5 standard medium to evaluate their ability for somatic embryo production. Variation in DSE efficiency was noticed among the studied genotypes (fig. 7). Ecotypes Nossen and RLD-1 displayed the highest efficiency of DSE with 100.0 and 96.7% frequency of explants formed embryos, respectively. The explants of another two tested ecotypes (Estland and Bensheim) produced somatic embryos with much lower frequency (below 50%), while DSE efficiency for the most (75%) of the genotypes varied between 55-81%.

3.3.4. Origin of explants

The explants excised from seed – and *in vitro* – derived plants were used to analyse the influence of explant origin on DSE efficiency. The explant-donor plants were regenerated through short-term procedure via DSE (R_1DSE^1 regenerants) or through secondary embryogenesis (R_1DSE^2 regenerants).

The comparison of DSE efficiency in cultures originating from seed-derived plants and regenerants obtained via DSE process indicated no significant differences in their embryogenic capacity (fig. 8). Two groups of analysed explants pre-

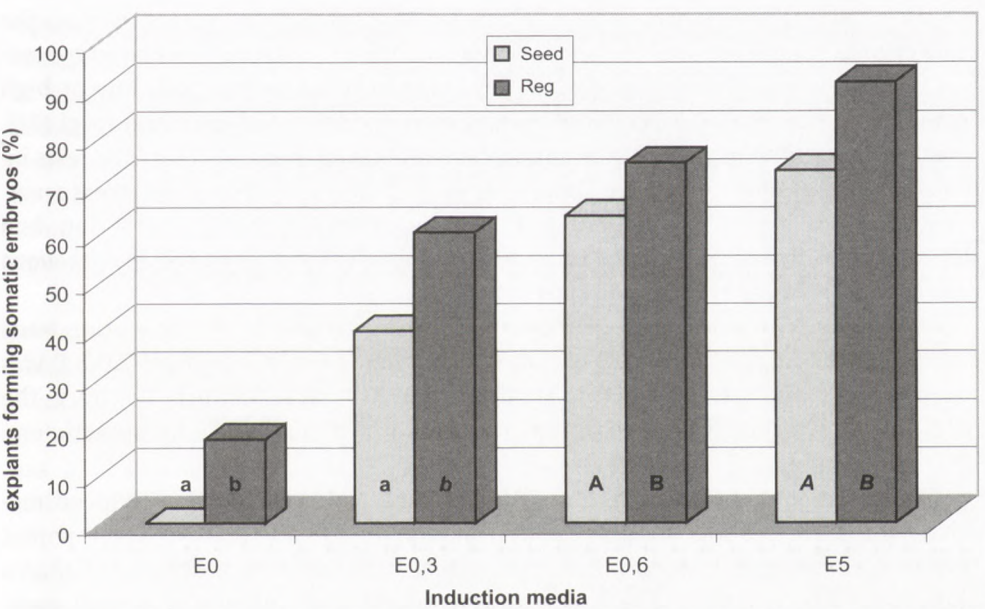

Fig. 9. Efficiency of somatic embryo formation in culture of explants excised from seed- (Seed) and *in vitro*-derived R_1DSE^2 plants (Reg) on medium without 2,4-D (E0) and containing 0.3 (E0.3); 0.6 (E0.6) and 5.0 (E5) μM of 2,4- D. Values marked by the same letters and font type are not significantly different at $P<0.05$.

sented similar mode of response i.e. correlation between concentration of 2,4-D in induction medium and efficiency of DSE. The highest frequency of explants producing somatic embryos was noticed on medium E5 containing standard concentration of 2,4-D i.e. 5 μM (1.1 mg/L) where over 70% of explants, regardless of their origin, formed embryos. DSE efficiency dropped with the decrease of 2, 4 – D concentration and lack of somatic embryo formation was noticed on E0 medium.

Contrary to explants derived from regenerants obtained via DSE, explants excised from plants regenerated via secondary embryogenesis indicated increase of embryogenic potential over control combination i.e. explants originated from seed-derived plants (fig. 9).

Significantly higher DSE efficiency displayed R_1DSE^2-derived explants cultured on all tested media. The highest frequency (over 90%) of somatic embryo formation displayed these explants on E5 medium. Moreover, R_1DSE^2-derived explants were found to be able to produce somatic embryos on hormone-free E0 medium with 17.4% frequency.

4. Discussion

Recently, it has become widely recognized that somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli, generally called stress factors. It is a case of *Daucus carota* where the embryogenic competence of *in vitro* cultured somatic cells can be stimulated by various factors, such as osmotic pressure, chlorides of heavy metals, pH, low or high temperature, starvation, mechanical wounding of explants or high auxin level (11). Stress is also commonly recognized as an essential component of embryogenesis induced in microspore culture, i.e. androgenesis (12). Recently, based on stress treatment used in carrot system, several stresses (osmotic, heavy metal ion and dehydratation stress) were found to be required for the induction of SE in *A. thaliana* explants other than zygotic embryos (13).

In this study, the increase of medium pH over standard 5.8 value stimulated embryogenic competence of the explants. The stimulatory effect of pH 7.0-8.0 was observed in both types of explants applied i.e. in EC and LC culture. Contrary, the pH 5.0 and lower (data not presented) was found to be inhibitory for somatic embryo development.

Similar phenomenon was observed in *D. carota* cultures where a reduced medium pH (below 4.5) was found to be detrimental for somatic embryo development (14). In *Brassica napus,* a low pH (3.5-5.0), however, was more conducive to SE than a higher (6.0-7.0) one (15). The concentration of H^+ ions influences several basic physiological processes related to the cell growth and differentiation. It was recognized that pH affects an auxin response and calcium metabolism (16), ion uptake in cultured tissues (17) or cell elongation and differentiation (18). However, the knowl-

edge of various effects of pH on plant morphogenesis is still incomplete, and thus the optimal pH for certain culture system (including SE induction) has to be established experimentally.

Besides medium pH, the temperature during the SE induction was studied in terms of the DSE efficiency. Temperature is considered as a critical factor determining efficiency of androgenesis induction in microspore culture of plants. Reduced temperatures (4-7°C) are commonly used to enhance embryogenesis in cereals, while incubation at high temperature (32°C) is routinely used to induce the embryogenic response in microspore culture of *Brassica napus* (19). However, the results presented in this paper indicate that neither lower (5°C) nor higher (28 or 32°C) than usually used (22°C) temperature promoted somatic embryo formation in *Arabidopsis* DSE system.

In general, in most species, including *Arabidopsis*, somatic embryo formation occurs on media containing auxins (1). However, in some plant systems, it was shown that stress factors can substitute for auxin and SE can be induced on hormone-free media. Thus, the DSE induction frequency was estimated for explants cultured on the hormone-free (E0) induction medium and treated with sub-optimal, stressful temperatures such as 5, 28 and 32°C. Interestingly, the callus formation in culture of LC explants only was noticed.

Sugars are included in all tissue culture media as an essential source of energy, and they provide the appropriate osmotic conditions for *in vitro* cell growth. The type and concentration of sugar used in media influences SE. The most frequently used sugar to induce SE is sucrose. It is also recommended for indirect and direct development of *Arabidopsis* somatic embryos (6,1). It was indicated that substitution of sucrose for maltose or glucose resulted in reduced efficiency of DSE induction in *Arabidopsis*, and fructose was found to inhibit embryogenesis (20). Similarly, the application of sugars studied in this work, galactose and cellobiose as well as sugar alcohols (xylitol, sorbitol and mannitol), frequently recommended for protoplast culture media, resulted in lack of culture growth.

Recently, several biologically active molecules have been reported to stimulate somatic embryogenesis in different plant species. Among them polyamines, small ubiquitous polycations, have been indicated to enhance embryogenesis in some plant species (21-23). Polyamines are suggested to mediate the action of hormones, and their complex role in various plant developmental and morphogenic processes has been investigated (24). Polyamine spermidine and two precursors of polyamines, arginine and ornithine were employed in the present study to enhance DSE efficiency in culture of less responsive EC explants, but no significant increase in the frequency of embryogenesis was noticed. Moreover, in case of ornithine, a distinct decrease of the SE efficiency and increase of callus formation was observed.

Our earlier studies indicated that DSE system can be applied in culture of several *Arabidopsis* ecotypes commonly used for research (5). The list of genotypes for which the system can be applied was provided in this paper. Most of the analysed geno-

types (75%) showed high DSE efficiency. Particular consideration is desired for the genotypes showing extreme SE capacity. Among them there is Nossen ecotype explants of which developed numerous somatic embryos with the 100% efficiency, and Estland and Benheim genotypes with the reduced (below 50%) DSE efficiency. The genotypes showing distinct differences in the SE efficiency are a valuable experimental material for research on genetic determination of somatic embryogenesis.

The comparison of the DSE efficiency of the explants from seed-grown plants and *in vitro* obtained regenerants (R_1DSE^2) showed significant increase of embryo formation in culture derived from the plants regenerated through secondary embryogenesis. To explain this phenomenon, physiological changes in the explants excised from *in vitro*-derived plants have to be considered. Among such changes, *in vitro* established, different endogenous fitohormone composition in the regenerants' tissues resulting in their higher totipotency can not be excluded. It seems that prolonged *in vitro* culture and callus formation associated with secondary embryogenesis can be especially conducive to physiological (hormonal) changes in tissue of the regenerated plants, as the explants taken from regenerants obtained via rapid and direct process (DSE) did not show an increase in embryogenic potential over seed-originating plants. The increased embryogenic potential of R_1DSE^2-explants was especially marked on hormone-free induction medium, as over 17% of these explants displayed embryogenesis, while no embryo formation was noticed on this medium when explants from seed-derived plants were employed. The increased frequency of somatic embryogenesis in culture of explants from regenerated plants was also described for *Medicago trunculata*, and possible higher number of totipotent cells in these explants was suggested to explain this phenomenon (25).

The presented analysis of the DSE efficiency under modified *in vitro* conditions revealed that the embryogenic potential of *Arabidopsis* immature zygotic embryos can be influenced by externally applied chemical or physical factors. Significant stimulation of the DSE frequency can be achieved by application of higher than standard induction medium pH (8.0) and the use of explants originated from regenerants. Further investigations are needed to elucidate mechanisms by which these factors influence embryogenic potential of the explants.

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