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Somatic embryogenesis and plant regeneration in immature zygotic embryos of *Brassica napus*

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Abstract

The effect of zygotic embryo age and pH of media on somatic embryogenesis induction of spring rapeseed was investigated. Immature zygotic embryos cultured on a growth regulator-free Murashige & Skoog medium produced primary somatic embryos without an intervening callus phase. Secondary somatic embryo induction was also possible on hormone-free medium. Similar to direct embryogenesis, the efficiency of secondary embryogenesis depended on pH of media. Higher pH (5.0) stimulated the proliferation of primary somatic embryos 25 to 26 days after pollination of zygotic embryos, while a lower pH (3.5) significantly increased the number of responding primary somatic embryos developed 20 to 21 days after pollination of zygotic embryos. Our study showed that primary somatic embryogenesis proceeded at a lower rate than secondary, but the highest number of somatic embryos per responding explant was obtained in primary somatic embryogenesis. Upon transfer to B5 medium, cotyledonary embryos developed into plantlets at a frequency of 7.3 - 30 %.

Key words: age of zygotic embryos, *Brassica napus*, pH, somatic embryogenesis.

Introduction

Oilseed rapes (*Brassica napus*, *B. campestris* and *B. juncea*) are now the third most important source of edible vegetable oil in the world (Kott 1998). Over the last decade, researchers have made great efforts into developing biotechnological methods to facilitate rapeseed breeding. All biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation to improve traits of important crops strongly depend on an efficient recovery of plants through *in vitro* systems. Proliferative embryogenic cultures provide suitable and convenient target tissues for genetic transformation, although initiation and maintenance of these cultures are time-consuming and labor-intensive. Induced somatic embryos develop directly on the explant in a few weeks, and then can be targeted for genetic transformation. Direct somatic embryogenesis from the immature zygotic embryos has been reported: *B. napus* (Koh, Loh 2000), *Arabidopsis thaliana* (Luo, Koop 1997), *Pisum sativum* (Tetu et al. 1990), *Solanum tuberosum* (Pretova, Dedicova 1992) and *Arachis hypogea* (Reddy, Reddy 1993).

Secondary somatic embryogenesis is a phenomenon whereby new somatic embryos are initiated from somatic embryos (Vasic et al. 2001). It has, compared to primary somatic

embryogenesis, advantages such as a high multiplication rate, independence of an explant source and repeatability. Furthermore, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis (Raemakers et al. 1995). Secondary somatic embryogenesis has potential application for both plant breeding practice and research.

In the present study, the influence of zygotic embryo age and pH of media on induction of primary and secondary somatic embryogenesis from immature zygotic embryos of spring rapeseed was investigated.

Materials and methods

Plant material

To study the induction of somatic embryogenesis, the double haploid lines NL-302-01, NL-302-02, NL-302-25 were used. Doubled haploids were generated using the Guelph doubled haploid production method (Fletcher et al. 1998). Donor plants were grown in a growing chamber under controlled conditions: temperature 22 ± 2 °C, light intensity – 5000 lx, photoperiod – 16/8 h (day/night).

Isolation of explants and induction of somatic embryogenesis

For the study of somatic embryogenesis, immature zygotic embryos were used. The age of immature zygotic embryos was counted as days after pollination (DAP) – from 14 to 29 days. Explants were sterilized in 70 % ethanol for 2 min, then three times rinsed with sterile distilled water. The embryos under aseptic conditions were transferred to 90 mm Petri dishes containing 25 ml basic Murashige and Skoog (1962) culture medium, supplemented with 2 % sucrose and 8 g l^{-1} Difco-Bacto agar. For each treatment 30 embryos were cultured (10 embryos per Petri plate) and each treatment was done in triplicate. The MS basal medium was adjusted to pH 3.5; 4.0 and 5.0 prior to the addition of agar and autoclaving. Explants were grown under controlled conditions: light intensity 5000 lx, photoperiod 16 h, temperature 25 ± 2 °C. After three days of cultivation, immature zygotic embryos were transferred on the same fresh media and then cultivated for 28 days under the same conditions. The percentage of formed primary somatic embryos and the number of somatic embryos per explant were estimated.

Part of the primary somatic embryos at the cotyledonary stage were carefully separated and transferred to a fresh culture media of the same composition and then cultivated for 28 days under the same conditions. After 28 cultivation days, the percentage of formed secondary somatic embryos and their number per primary embryo were estimated.

Plantlet regeneration

For the regeneration of embryos, a modified B5 (Fletcher et al. 1998) culture medium supplemented with 0.1 mg l^{-1} gibberellic acid (GA_3), 30 g l^{-1} sucrose and 8 g l^{-1} Difco-Bacto agar was used. The percentage of plants regenerated from primary and secondary embryos in B5 medium was estimated.

For statistical analysis, the computer programme STAT 1.55 from "SELEKCIJA" (Tarakanovas 1999) and ANOVA for EXEL, vers. 2.1 were used. Mean values and SE's were calculated based on the number of independent replications

Results

Primary somatic embryogenesis

Immature zygotic embryos cultured on medium with various pH formed primary somatic embryos (SE) without an intervening callus phase. Variable embryogenic responses were expressed by all of the three genotypes tested on the different induction media. Zygotic embryos at the age of 14 to 15 DAP cultivated on pH 3.5 medium did not show any response (Fig. 1A). Higher pH (4.0) improved frequency of embryogenesis of NL-302-01 and NL-302-25 lines (Fig. 1B); however, increasing pH to 5.0 raised the embryogenic potential only in the NL-302-01 line (Fig. 1C). Zygotic embryos at the age of 20 to 21 DAP formed primary somatic embryos on all of the tested media. Explants of NL-302-25 and NL-302-01 lines formed somatic embryos at the highest rate on pH 5.0 medium, whereas pH 4.0 promoted somatic embryogenesis of the NL-302-02 line. Zygotic embryos isolated 25 to 26 days after pollination underwent somatic embryogenesis in all of the media tested, except explants of NL-302-02 line cultivated on pH 3.5 medium. Zygotic embryos of NL-302-02 and NL-302-25 showed the best response on pH 5.0 medium, while a lower pH (4.0) was more suitable for NL-302-01. Immature 28 to 29 DAP zygotic embryos had the highest embryogenic potential on pH 5.0 medium (NL-302-25 and NL-302-02 lines) and pH 4.0 medium (NL-302-01 line).

The number of primary SE produced per responding explant was significantly affected by zygotic embryo age and pH of the medium. Generally, zygotic embryos isolated 20 to 21 days after pollination showed the highest number of primary SE per explant (Fig. 2A-C). Lines NL-302-25 and NL-302-02 formed the highest number of primary SE per responding explant in pH 4.0 media, respectively 18.2 and 7.8. The highest number of primary SE per explant of the NL-302-01 genotype was obtained from zygotic embryos cultivated on pH 5.0 media.

Most primary somatic embryos were formed on the hypocotyls, while some somatic embryos were formed on the cotyledon. Most primary SE had at two cotyledons, but some were multiple with asymmetric cotyledons and had no apical apex. Part of the primary SE after four weeks were transferred to B5 regeneration medium supplemented with 0.1 mg l^{-1} GA_3, and another part were used to induce secondary somatic embryogenesis.

Secondary somatic embryogenesis

Secondary somatic embryos were visible from the root pole of the primary SE within 7 days of culture. The tissue at the root pole of the primary SE proliferated into a small mass of tissue from which several secondary SE emerged. The processes continued in a recurrent manner and by the end of 28 days of culture secondary SE at different developmental stages were observed.

Similar to direct embryogenesis the efficiency of secondary embryogenesis depended on the primary explant age and pH of medium used for inducing the primary embryos. Generally primary SE developed from 14 to 15 DAP zygotic embryos had a very low embryogenic response with some exceptions. Primary SE of NL-302-01 showed the best induced secondary SE on pH 4.0 medium, while secondary SE formation in this line was strongly reduced by pH 5.0 medium and completely inhibited by pH 3.5 medium.

Primary somatic embryos developed from immature zygotic embryos at the age of 20 to 21 DAP exhibited the greatest frequency of secondary embryo formation at pH

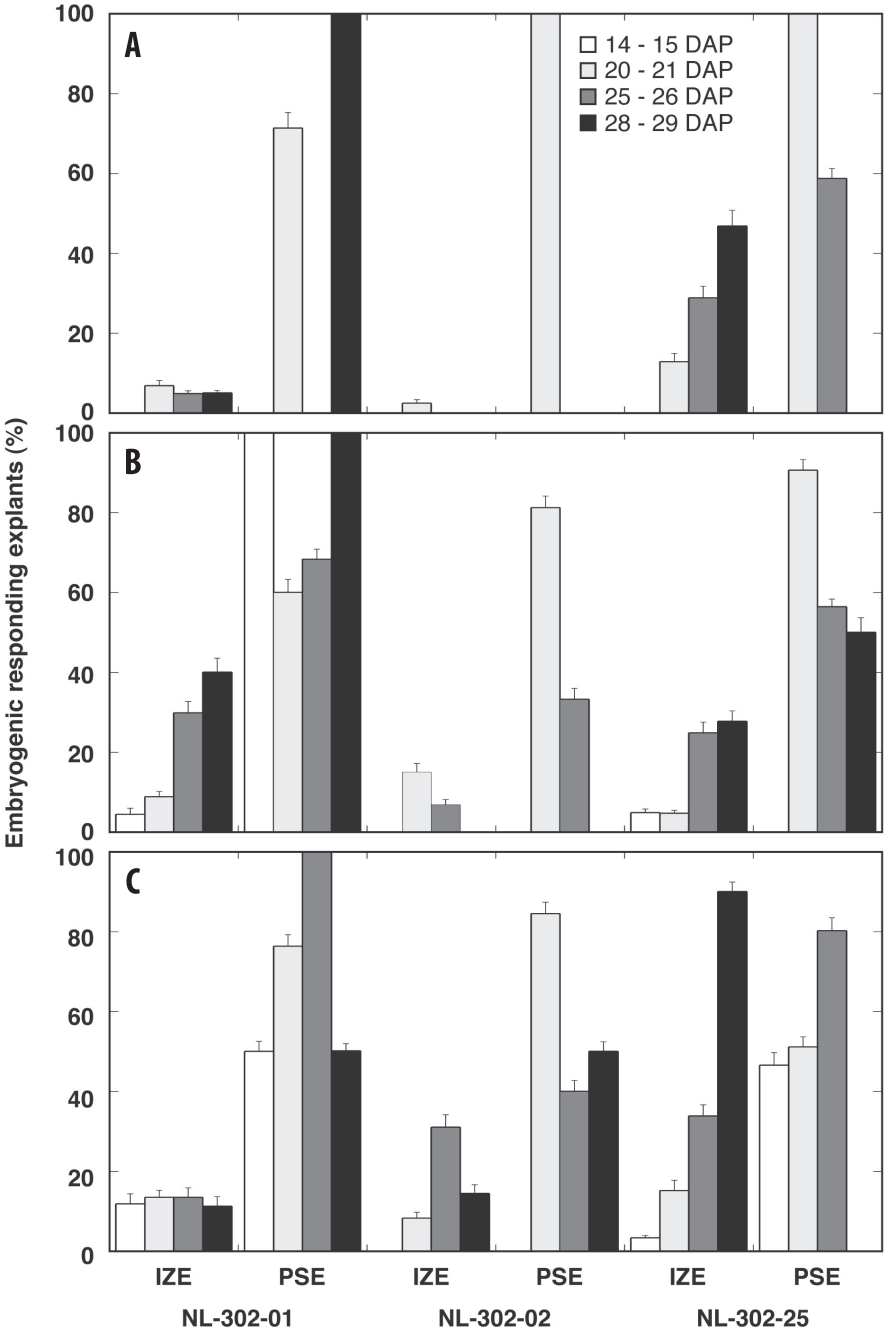

Fig. 1. Effect pH of medium and the age of embryos (days after pollination; DAP) on somatic embryogenesis from immature zygotic embryos (IZE) and primary somatic embryos (PSE) of tested rapeseed lines. A, pH 3.5; B, pH 4.0; C, pH 5.0. White columns – 14 to 15 DAP; light grey columns – 20 to 21 DAP; dark grey columns – 25 to 26 DAP; black columns – 28 to 29 DAP.

3.5 medium (Fig. 1A). In contrast, the higher pH 5.0 of medium promoted secondary embryogenesis of primary SE developed on 25 to 26 DAP zygotic embryos (Fig. 1C).

Cultivating primary SE from 28 to 29 DAP age zygotic embryos, the highest frequency of secondary embryogenesis was observed for the NL-302-01 line in pH 3.5 and 4.0 media, and an increased medium pH up to 5.0 significantly decreased the embryogenetic potential of this line . The efficiency of secondary embryogenesis depended on pH of medium used for the induction of primary embryos, and on the genotype. The highest average number (7.9) of secondary somatic embryos per explant was obtained in pH 4.0 medium from the primary SE of NL-302-01 line, formed from 28 to 29 DAP zygotic embryos.

Plantlet regeneration

Globular somatic embryos developed into cotyledonary embryos after an additional four weeks of culture. Most somatic embryos possessed two cotyledons, some had three or more cotyledons, and a few somatic embryos were fused. Upon transfer to B5 medium, cotyledonary embryos developed into morphologically normal plantlets at a frequency from 57.7 % to 87.3 % (Fig. 3). Experiments did not show significant differences between the capability of primary and secondary somatic embryos to regenerate morphologically normal plants. Somatic embryos that did not develop into plantlets became abnormally enlarged, and frequently formed calluses on their surfaces prior to eventual disorganization. After 28 days, regenerated plantlets bearing three to four leaves were transferred to small cups containing soil, kept for 10 days under a glass cover and finally transferred to a greenhouse.

Discussion

Somatic embryos have shown to be excellent source for secondary embryos. This is associated with loss of integrated group control of cell organization in the somatic embryos. Some cells break away from group control and initiate new somatic embryos. In many species immature zygotic embryos possess and mature zygotic embryos lack the ability to express somatic embryogenesis (Raemakers et al. 1995). In rapeseed it has been reported that immature zygotic embryos had significantly greater embryogenic potential than mature embryos (Koh, Loh 2000; Burbulis, Kupriene 2005). Immature zygotic embryos of various species comprise mitotically arrested pre-embryogenic determined cells, therefore growth regulators in culture medium are required for induction of somatic embryogenesis. It has been reported that exogenous cytokinin is necessary for somatic embryogenesis in immature zygotic embryo cultures of various species, including *Ginkgo biloba* (Laurain et al. 1996) and *Rosa hybrida* (Kim et al. 2003). However, the BAP and other cytokinins have been reported to suppress secondary embryogenesis or cause partial or complete inhibition of embryo development in cell suspension and tissue cultures (Luo, Koop 1997). Other species have been found to require exogenous auxin for embryo induction, while prolonged exposure to auxin in some species inhibits continued development of globular embryos (Merkle et al. 1995). In some species somatic embryos are formed directly from specific explants cultured on medium without growth regulators (Koh, Loh 2000; Zegzouti et al. 2001; Burbulis, Kupriene 2005).

It has been reported that pH of medium or an application of electric field can affect initiation of somatic embryogenesis (Smith, Krikorian, 1990). Exogenous growth regulators

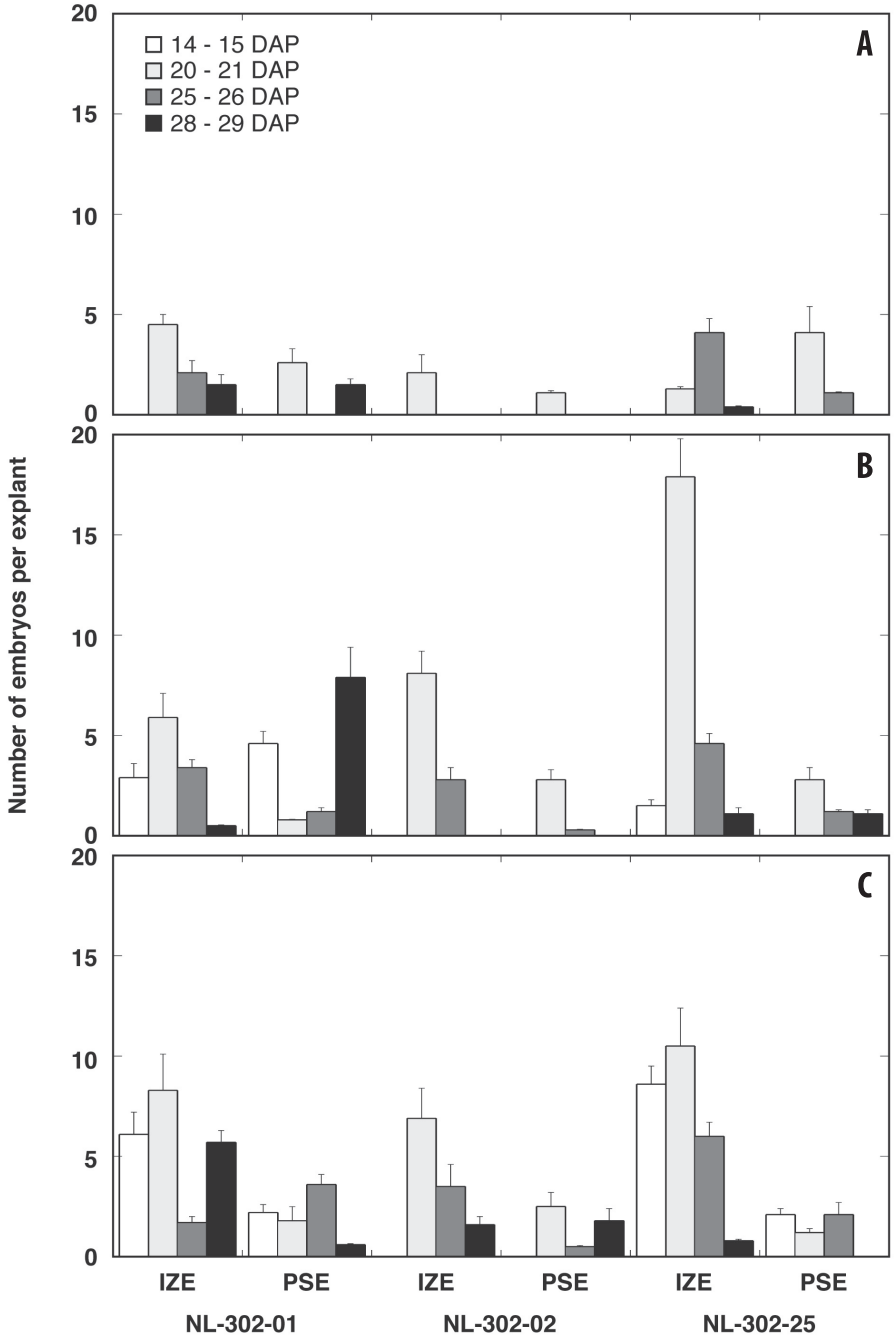

Fig. 2. Effect pH of medium and the age of embryos (days after pollination; DAP) on number somatic embryos per responding immature zygotic embryo (IZE) and primary somatic embryo (PSE) of tested rapeseed lines. A, pH 3.5; B, pH 4.0; C, pH 5.0. White columns – 14 to 15 DAP; light grey columns – 20 to 21 DAP; dark grey columns – 25 to 26 DAP; black columns – 28 to 29 DAP

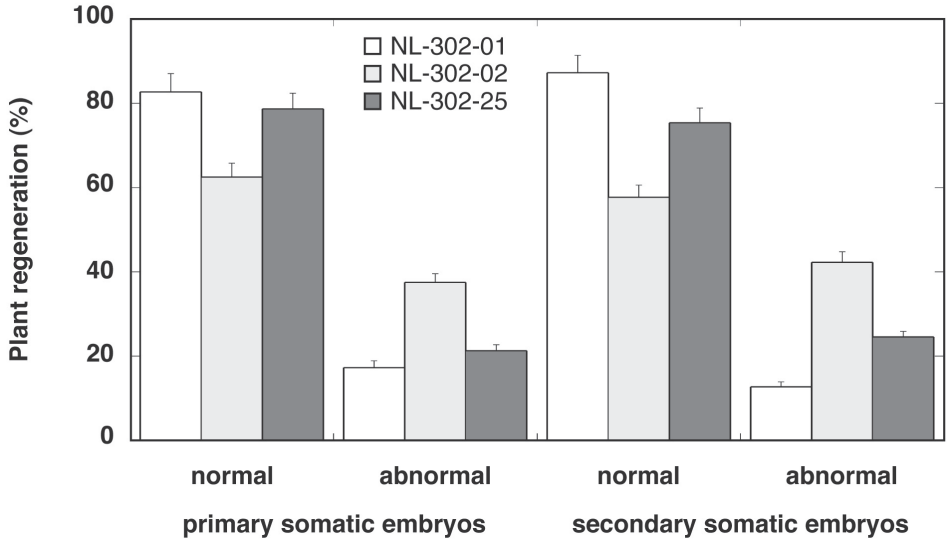

Fig. 3. Plant regeneration from primary and secondary somatic embryos.

probably modify the cell polarity by interfering with the pH gradient or electrical fields around the cells (Dodeman et al. 1997). In soybean it was reported that auxin concentration and pH value influenced somatic embryo production, with optimal levels of 10 mg l^{-1} napthaleneacetic acid and pH 7.0 (Bonacin et al. 2000). However, Hofmann et al. (2004) did not find any significant differences in soybean somatic embryogenesis between 5.7 and 7.0 pH levels.

In the present study somatic embryogenesis of the tested rapeseed lines was induced directly from immature zygotic embryos on hormone-free media. Thus, we suggest that pH of medium can reverse the arrest of pre-embryogenic cells, resulting in somatic embryogenesis. Continued secondary embryogenesis was also possible on hormone-free medium, thereby excluding the complication of exogenous plant grow regulators.

The production of embryos is determined by the number of responding explants and the number of embryos produced per responding explant. It has been documented that in many species the production of embryos in primary embryogenesis is lower than in secondary embryogenesis (Raemakers et al. 1995; Zegzouti et al. 2001). In our study primary somatic embryogenesis proceed at a lower rate than secondary, but a higher amount of somatic embryos per explant was obtained during the primary somatic embryogenesis (Fig. 1, 2).

Detachment of primary SE from the original explant might have stimulated the proliferation of small quantities of the embryogenic tissue attached to its root pole to give a mass of embryogenic tissue or a proembryogenic mass, which ultimately gave rise to further embryos in a cyclic manner. The intensity of secondary embryo formation depended on medium pH used for culturing the explants. Medium pH 5.0 stimulated the proliferation of primary SE from 25 to 26 DAP zygotic embryos, while a lower pH (3.5) significantly increased the number of responding primary SE developed from 20 to 21 DAP zygotic embryos.

In conclusion, a reliable method to induce primary and secondary somatic embryogenesis from new genotypes of rapeseed was established. This system may be highly useful for developing effective transformation systems to improve important economics traits such as oil and protein content and disease resistance.

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Somatiskā embrioģenēze un augu reģenerācija no nenobriedušiem zigotiskajiem embrijiem *Brassica napus* augiem

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Kopsavilkums

Pētīta zigotisko embriju vecuma un vides pH ietekme uz somatiskās embrioģenēzes indukciju vasaras rapsim. Nenobrieduši zigotiskie embriji, kultivēti Murašiges un Skūga vidē bez augšanas regulatoru pievienošanas, veidoja primāros somatiskos embrijus bez kallusa fāzes. Bezhormonu vidē bija iespējama arī sekundāro somatisko embriju indukcija. Līdzīgi tiešajai embrioģenēzei, arī sekundārā embrioģenēze bija atkarīga no vides pH. Augstāks pH (5,0) stimulēja primāro somatisko embriju proliferāciju uz zigotiskajiem embrijiem 25 līdz 26 dienas pēc apputeksnēšanas, bet zemāks pH (3,5) ievērojami paaugstināja primāro somatisko embriju veidošanos no zigotiskajiem embrijiem 20 līdz 21 dienu pēc zigotisko embriju apputeksnēšanas. Mūsu pētījumi parādīja, ka primārā somatiskā embrioģenēze notiek ar zemāku ātrumu, nekā sekundārā, bet lielāku skaitu somatisko embriju uz reaģētspējīgo eksplantu skaitu iegūst pirmās somatiskās embrioģenēzes procesā. Pēc pārvietošanas uz B5 vidi 7,3 līdz 30 % dīgļlapu embriju attīstījās par mikroaugiem.