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In vitro culture of immature embryos of *Cytisus laburnum*

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Abstract

Immature embryos of *Cytisus laburnum* L. were cultivated *in vitro* and four culture media, different techniques of substrate preparation, sucrose concentration and the effect of suspensor removal were tested. The best results were obtained with N6 medium supplemented with 2 mg dm^{-3} glycine and set up using a double-layer culture system, in which the top layer had a higher osmotic potential than the bottom one. These conditions allowed normal embryogenic development in up to 45 % of early globular embryos, that were able to develop until a complete maturity. Osmotic potential and mineral nutrients of the medium demonstrated to be crucial for the successful culture and their effects were dependent on embryo age at the time of excision. The presence of an intact suspensor showed to be beneficial only for early globular embryos while older developmental stage embryos were not significantly affected.

Additional key words: Golden chain, suspensor, zygotic embryogenesis.

Introduction

Zygotic embryogenesis in angiosperms is one of the fundamental steps of the whole plant development. This event is a complex developmental process in which the zygote undergoes various morphological changes to give rise to the mature seed. During the progressive transition from a single cell to an organised mature embryo, some critical events such as polarity formation and cell differentiation occur, determining the different tissues and organs of the future plant (Mayer *et al.* 1993, Goldberg *et al.* 1994, Breton *et al.* 1995, Jurgens 1995). However, few information is available about the mechanisms which allow the establishment of the embryo structures. One obstacle to understanding in detail the events which govern early embryo formation is the location of embryos within the plant and their relative inaccessibility to experimental manipulation particularly at the early stages of plant development.

Several approaches have been employed, notably at the biochemical, genetical and molecular levels, with the aim to provide important insights into the mechanism underlying embryogenesis (Goldberg *et al.* 1989, Meinke 1991). Another approach can be represented by *in vitro* manipulation of proembryos. Such strategy implies that appropriate media and culture conditions are available and allow the normal growth and development of proembryos. Recently this culture procedure has been used to overcome seed inviability and to induce somatic embryogenesis in several plant species (Monnier 1995, García and Molina 2001).

Up to date few publications have described procedure for the culture of the globular embryos which resulted in normal developmental patterns of zygotic embryogenesis and germination (Liu *et al.* 1993, Fisher and Neuhaus 1995, Matthys-Rochon *et al.* 1998).

In this paper we present a work on *in vitro* culture of immature embryos of *Cytisus laburnum*. In this species the presence of a massive suspensor and the relative easiness of proembryo excision at a very immature embryo development provide an excellent system for embryogenesis studies. The main objectives of this investigation were to study the suitability of different culture media, different techniques of substrate preparation, the influence of the sugar concentration and the role of the suspensor.

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Materials and methods

Plants and embryo dissection: Plants of *Cytisus laburnum* L. were grown in the field. Pods were collected daily early in the morning and washed in tap water. The pods were surface sterilised in 1 % sodium hypochlorite solution, with a drop of *Tween-20* as a wetting agent and then rinsed four times in sterile distilled water. The ovules were removed aseptically from the pods under a dissecting microscope. The size of the ovule was used to estimate the stage of the embryo within it. The ovule was held with a pair of fine tweezers and cut longitudinally, taking great care not to damage embryo tissues. A small proembryo with a massive suspensor situated at the micropylar end could be observed under microscope. In some experiments the suspensor was either completely removed or partially damaged with a dissecting needle, carefully avoiding not to injure the embryo proper tissues. Proembryos were then picked up and transferred to the culture medium by using sterile plastic micropipettes. Proembryos were subdivided in three classes, based on their developmental stage at the time of their excision: globular stage (diameter 60 - 100 μm, spherical shape), late globular or transition stage (diameter 100 - 200 μm), and heart shape (diameter over 200 μm, two distinct cotyledon primordia). Embryos length was measured from the top of the suspensor to the extreme tip of the embryo, using a microscope equipped with an eyepiece micrometer.

Culture media and growth conditions: Four different culture media were tested: N6 medium (Chu *et al.* 1975) supplemented with 2 mg dm^{-3} glycine, Gamborg B5 medium (Gamborg *et al.* 1968), MS medium (Murashige and Skoog 1962), and ECM medium (Liu *et al.* 1993). No phytohormones were added to the tested media.

Culture media were set up using two different techniques: single layer and double layer system. The media were dispensed in 24 well multiplates. All the components of the nutrient media were sterilized by autoclaving, except of vitamins and organics that were filter-sterilized (millipore, diameter 0.2 μm). In the single layer system proembryos were carefully placed on the surface of each medium, containing rising sucrose concentrations (3, 6, 9, 12, 15 and 18 %). When a double layer system was used proembryos were posed between two medium layers (each of 0.4 cm^3) in which the top layer contained 16 % sucrose and the bottom layer 5 % sucrose. The bottom layer was overlaid with the top layer medium which was held at 34 °C.

The cultures were incubated in a growth chamber at 24 ± 1 °C in the dark for 4 d and then at 16-h photoperiod (irradiance of 70 μmol m^{-2} s^{-1}, *Philips TL 40W/33CS*) at the same temperature. Embryo growth was recorded at 2-d interval and periodically photographed. After 14 d proembryos were transferred in 6 cm diameter Petri dishes, containing MS medium with 2 % sucrose for germination. Efficiency of each medium was evaluated on embryos percentage that developed until a complete maturity without any morphological abnormalities and that resembled the ones developed *in vivo*. In order to establish optimal culture conditions for the different stages of embryo development other parameters were also tested: sugar concentration, and two different sugar sources. In each experiment 72 proembryos (24 for each developmental stage) were used and the experiments were always made in duplicate.

Results

Single layer system: The percentage of developed embryos in all culture media increased as a consequence of the rising sucrose content (Fig. 1), however, the rate of this increment was strongly dependent on the age of isolated embryos. Globular and post globular proembryos got limited benefit by sucrose increase and in all tested media the percentage of developed embryos never exceeded the value of 15 and 26 %, respectively. The best sucrose concentration was 15 - 18 % in both developmental stages. On the contrary, the growth of heart stage embryos were markedly affected by sucrose content, varying from 2 to 70 % with rising sugar concentrations. Below 6 % sucrose concentration, the percentage of developed embryos in all developmental stages never exceeded the value of 10 %.

Concerning the four culture media tested, no significant differences were detected in the development of post globular and heart stage embryos, while we observed a slight increase in globular embryos survival on N6 and ECM media (from less than 10 to 15 %) in comparison with the other media. Embryos cultured on N6, MS and Gamborg B5 medium always showed a normal growth, with the development of the main axis and two expanded cotyledons (Fig. 2*C*), and were able to grow until maturity within two weeks. Globular embryos developed into transitional stage within 48 - 72 h, and successively into the heart stage with further 2 - 3 d (Fig. 2*A,B*). No morphological differences were observed compared with embryos grown *in vivo* except for embryos grown on ECM where about 20 % of developed embryos showed an abnormal cotyledon development and the occurrence of brownishments on the surface (Fig. 2*D*).

Double layer system: Proembryos in all developmental stages were positively influenced by this culture system, in which the percentage of developed embryos tend to rise steadily (Fig. 3). Globular embryos cultured on ECM and N6 double layer medium were able to develop until maturity in a frequency of 45 %, always showing two normal and expanded cotyledons. On the other hand, globular proembryos cultured on Gamborg B5 medium did not exceed 15 % of frequency, while with the MS medium beneficial effect was not detected in comparison with the single layer culture system.

Late globular embryos were also positively influenced by this culture system. As already observed for globular embryos, best results were obtained with ECM and N6

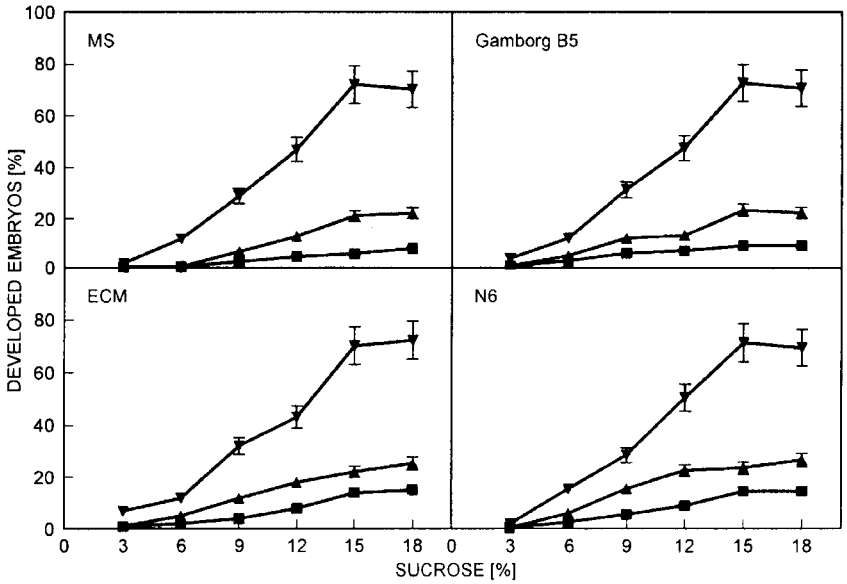

Fig. 1. Percentage of globular, late globular and heart-shaped stage embryos of *Cytisus laburnum* normally developed *in vitro* on MS, Gamborg B5, ECM and N6 media with different sucrose concentration and set up with the single layer technique (*squares* - globular embryo, *triangles* - late globular embryo, *upside down triangles* - heart-shaped embryo.

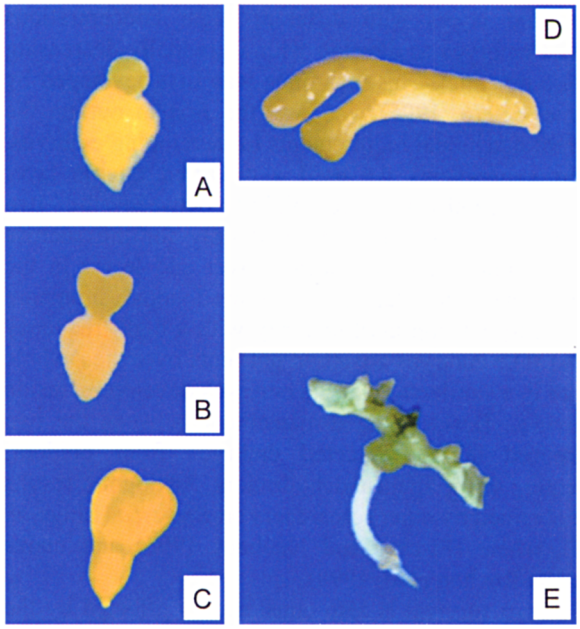

Fig. 2. *In vitro* development of *Cytisus laburnum* proembryos: globular embryo (*A*), heart-shaped embryo (*B*), cotyledon embryo (*C*), and abnormal embryo developed on ECM medium (*D*); embryo germination on MS medium (*E*).

media (≈ 55 % of developed embryos), while with MS and Gamborg B5 culture media 35 and 42 % of embryos developed into mature ones, respectively. With this system up to 85 - 90 % of the heart-shaped stage cultured embryos developed into mature embryo.

Globular proembryos were also cultured on N6 medium with maltose instead of sucrose. Results showed that both sugars could promote a regular embryo development, with no significant difference on the percentage of developed embryos (data not shown).

Embryos germination: Germination of mature embryos occurred when they were transferred into an MS medium containing 2 % of sucrose. The best time to transfer was after two weeks of culture in the initial medium. Delays in transferring caused the occurrence of brownishment on the embryo surface that led quickly to the arrest of the growth.

Using this medium 80 - 90 % of the embryos were able to germinate. However, the formation of the radicle and the first leaves underwent slow development compared to the one observed *in vivo* and often the seedlings showed the occurrence of green callus on the first formed leaves (Fig. 2*E*).

Influence of suspensor removal: The removal of the suspensor had no effect on the development of proembryos after late globular stage. For the globular embryo the removal of suspensor showed a limited effect on the embryo survival frequency with a 10 % reduction of developed embryos (Fig. 4).

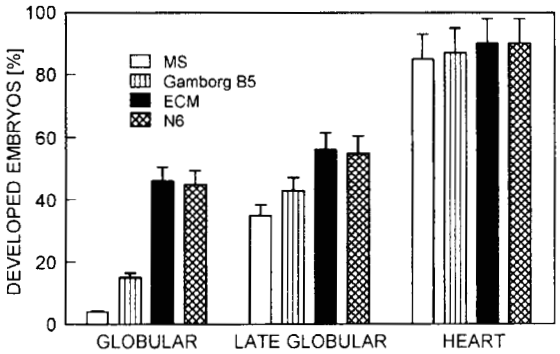

Fig. 3. Percentage of globular, late globular and heart-shaped stage embryos of *Cytisus laburnum* normally developed *in vitro* on MS, Gamborg B5, ECM and N6 medium set up with the double layer technique (5 and 16 % sucrose in the bottom and in the upper layer, respectively).

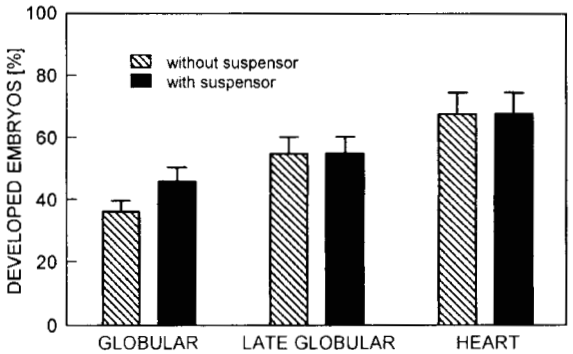

Fig. 4. Influence of the suspensor removal on *in vitro* proembryo development of *Cytisus laburnum*. Proembryos cultured on N6 medium set up with the double layer technique.

Discussion

Much effort has been addressed to the improvement of techniques allowing the culture of zygotes or several-celled proembryos up to a mature embryo. However, despite many years of experimentation, *in vitro* culture of immature embryos is still far to be solved.

The experiments reported in this paper describe an *in vitro* culture system that allows development of *Cytisus laburnum* proembryos to mature embryos. As previously reported by several authors (Monnier 1984, Goldberg *et al.* 1994) the chemical and physical composition of the medium for *in vitro* embryo culture should provide all the requirements for a comparable time schedule of embryo development *in vitro* and *in vivo*. Therefore, the influence of four different media with various concentrations of sucrose (3 - 18 %) and set up using the single layer system was evaluated. As pointed out by Smith (1973) on *Phaseolus,* the osmotic potential of the medium during culture of the embryos plays a critical role. In *Cytisus* we found that the sucrose concentration for freshly excised embryos was optimal between 15 - 18 % in all media. Below this concentration the survival frequency of the embryos was strongly reduced, especially for heart shaped embryos. Matthys-Rochon *et al.* (1998) also outlined that sugar source was crucial for embryo development. In their studies pretransitional and transitional maize embryos were able to develop only when maltose was used instead of sucrose and they hypothesised that cells utilized preferentially glucose as carbohydrate source. However, in our work both sugars were able to promote proembryo development without any significant difference.

In *Cytisus*, the displacement of the proembryos on the top of a single layer medium did not allow to promote with high efficiency the development of most immature embryos, whereas the percentage of developed globular embryos never exceeded the value of 16 %. The utilization of the double layer system demonstrated to be the best way to support osmotic requirements of developing embryos. The slow mixing of the two superposed layers with different sugar concentrations allowed embryos to develop with a gradually decreasing osmotic potential. This technique was first developed by Monnier (1976, 1995) who was the first to outline the different nutritional requirements of developing embryos. In our experiments we used a modified double layer system; proembyos were placed between the two superposed layers which differed only for the sucrose concentration, higher in the top layer and lower in the bottom one. This technique allowed most immature embryos to develop until maturity with a high frequency (45 %). The embryos absorbed nutrients over their whole surface in a similar way of the embryos developing inside the ovules. Besides, the displacement between the two superposed layers protected embryos from outdoor, preventing them against dehydration. Analogous results were obtained by other researchers on *Brassica* (Liu *et al.* 1993), wheat (Fischer and Neuhaus 1995) and maize (Matthys-Rochon *et al.* 1998).

Embryo survival is also considerably affected by the composition of the substrate and not all culture media could be utilised for the culturing of young embryos. We demonstrated that most immature embryos are affected by the mineral solutions of the medium and found best conditions with ECM and N6 media. On the contrary, MS

and Gamborg B5 medium appeared to inhibit the development of youngest embryos that showed a decrease of the survival percentage. With the going on of embryo maturation the choose of the substrate becomes less important, as all tested media were able to allow embryo development. The positive effect of ECM medium on the growth of very immature embryos was already outlined in previous study on *Brassica* (Liu *et al.* 1993). The satisfactory results obtained with this substrate were attributed to various factors such as the presence of a complex sugar mixture and the addition of organic compounds (organic acids, caseine and coconut water). Besides, the higher ratio of nitrate nitrogen to ammonium nitrogen (3.5 times higher than in MS medium) appeared to greatly increase the growth of embryos, reducing the toxic effect of the NH_4^+. In N6 medium, the absence of the complex sugar mixture and of additional organic compounds seemed to indicate inorganic nutrients as mainly responsible for the positive results obtained. As above mentioned for ECM medium, the high content of nitrate nitrogen form present also in N6 medium could explain the positive influence on the survival of early globular embryos.

The occurrence of irregularly conformed cotyledons, green callus and brownishment of the whole surface of some embryos grown on ECM medium was noted. Our hypothesis is that these morphological aberrations could be addressed to the coconut water. Several studies showed that this organic additive is very rich in plant hormones (Letham 1974, Van Staden and Drewes 1975) and its stimulatory effect on *in vitro* embryo development has been clearly outlined (Raghavan 1986). On the contrary, proembryos cultured on N6 medium always showed a regular morphology, resembling the ones developed *in vivo*. Consequently, N6 medium appeared as the best substrate to reproduce *in vitro* the normal development of *Cytisus* embryos.

The removal of the suspensor showed a limited effect on the development of early globular embryos, causing a 10 % reduction of the embryo survival percentage, while no effects were observed in relation to older developmental stages. We hypothesise that the suspensor plays an important function during the first steps of embryo formation, and that its presence becomes less important with the going on of embryo maturation. Liu *et al.* (1993) showed that in *Brassica* the removal of the suspensor had only a limited effect on proembryo survival frequency and it was clearly detectable only in very young embryos. On the contrary, other experiments performed on *Phaseolus coccineus* showed that heart-shaped embryos deprived of the suspensor give rise to a drastic reduction of the embryo development (Cionini *et al.* 1976, Yeung and Sussex 1979). This hypothesis on the involvement of the suspensor in the embryo development may be also supported by the finding of different hormones in suspensors of *Cytisus* and *Phaseolus* (Alpi *et al.* 1979, Ceccarelli *et al.* 1981, Piaggesi *et al.* 1989, Picciarelli *et al.* 1991, 2001).

In conclusion in this work we have established a successful system for *in vitro* culturing early globular embryos of *Cytisus laburnum* until complete maturity. With this technique we obtained a 45 % survival of early globular embryos without adding to the medium any exogenous growth regulators. This system will allow us to perform further investigations in order to better understanding the mechanisms that govern the establishment of embryo morphology.

References

Alpi, A., Lorenzi, R., Cionini, P.G., Bennici, A., D'Amato, F.: Identification of gibberellin A_1 in the embryo suspensor of *Phaseolus coccineus*. - Planta **147**: 225-228, 1979.

Breton, C., Chaboud, A., Matthys-Rochon, E., Bates, E., Cock, M., Fromm, H., Dumas, C.: PCR-regenerated cDNA library of transition stage maize embryos: cloning and expression of calmodulin genes during early embryogenesis. - Plant mol. Biol. **27**: 105-113, 1995.

Ceccarelli, N., Lorenzi, R., Alpi, A.: Gibberellin biosynthesis in *Phaseolus coccineus* suspensor. - Z. Pflanzenphysiol. **102**: 37-44, 1981.

Chu, C.C., Wang, C.C., Sun, C.S., Chen, H., Yin, K.L., Chu, C.Y., Bi, F.Y.: Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. - Sci. sin. **18**: 659-668, 1975.

Cionini, P.G., Bennici, A., Alpi, A., D'Amato, F.: Suspensor, gibberellin and *in vitro* development of *Phaseolus coccineus* embryos. - Planta **131**: 115-117, 1976.

Fischer, C., Neuhaus, G.: *In vitro* development of globular zygotic wheat embryos. - Plant Cell Rep. **15**: 186-191, 1995.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

García, M.D., Molina, C.: Embryo rescue and induction of somatic embryogenesis as a method to overcome seed inviability in *Zea mays* ssp. *mays* × *Zea mays* spp. *parviglumis* crosses. - Biol. Plant. **44**: 497-501, 2001.

Goldberg, R.B., Barker, S.J., Perez-Grau, L.: Regulation of gene expression during plant embryogenesis. - Cell **56**: 149-160, 1989.

Goldberg, R.B., De Paiva, G., Yadegari, R.: Plant embryogenesis: zygote to seed. - Science **266**: 605-614, 1994.

Jurgens, G.: Axis formation in plant embryogenesis: cues and clues. - Cell **81**: 467-470, 1995.

Letham, D.S.: Regulators of cell division in plant tissues. XX. The cytokinins of coconut milk. - Physiol. Plant. **32**: 66-70, 1974.

Liu, C.M., Xu, Z.H., Chua, N.H.: Proembryo culture: *in vitro* development of early globular stage zygotic embryos from *Brassica juncea*. - Plant J. **3**: 291-300, 1993.

Matthys-Rochon, E., Pioloa, F., Le Deunff, E., Mol, R., Dumas,

C.: *In vitro* development of maize immature embryos: a tool for embryogenesis analysis. - J. exp. Bot. **49**: 839-845, 1998.

Mayer, U., Buttner, G., Jurgens, G.: Apical-basal pattern formation in *Arabidopsis*: studies on the role of the genome gene. - Development **117**: 149-162, 1993.

Meinke, D.W.: Perspectives on genetic analysis of plant embryogenesis. - Plant Cell **3**: 857-866, 1991.

Monnier, M.: Culture *in vitro* de l'embryon immature de *Capsella bursa-pastoris* Moench. - Rev. Cytol. Biol. vég. **39**: 1-20, 1976.

Monnier, M.: Survival of young immature *Capsella* embryos cultured *in vitro*. - J. Plant Physiol. **115**: 105-113, 1984.

Monnier, M.: Culture of zygotic embryos. - In: Thorpe, T.A. (ed.): *In Vitro* Embryogenesis in Plants. Pp. 117-153. Kluwer Academic Publishers, Dordrecht 1995.

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

Piaggesi, A., Picciarelli, P., Lorenzi, R., Alpi, A.: Gibberellins in embryo-suspensor of *Phaseolus coccineus* seeds at the heart stage of embryo development. - Plant Physiol. **91**: 362-366, 1989.

Picciarelli, P., Ceccarelli, N., Paolicchi, F., Calistri, G.: Endogenous auxins and embryogenesis in *Phaseolus coccineus* L. - Aust. J. Plant Physiol. **28**: 73-78, 2001.

Picciarelli, P., Piaggesi, A., Alpi, A.: Gibberellins in suspensor, embryo and endosperm of developing seeds of *Cytisus laburnum*. - Phytochemistry **30**: 1789-1792, 1991.

Raghavan, V.: Embryogenesis in Angiosperms. A Developmental and Experimental Study. - Cambridge University Press, Cambridge 1986.

Smith, J.G.: Embryo development in *Phaseolus vulgaris*. Analysis of selected inorganics ions, ammonia, organic acids, amino acids and sugars in the endosperm liquid. - Plant Physiol. **51**: 454-458, 1973.

Van Staden, J., Drewes, S.E.: Identification of zeatin and zeatin riboside in coconut milk. - Physiol. Plant. **34**: 106-109, 1975.

Yeung, E.C., Sussex, I.M.: Embryogeny of *Phaseolus coccineus*: the suspensor and the growth of the embryo-proper *in vitro*. - Z. Pflanzenphysiol. **91**: 423-433, 1979.