BIOLOGIA PLANTARUM 42 (4): 499-504, 1999

Factors influencing the induction and viability of somatic embryos of *Quercus robur* L.

M. ENDEMANN and E. WILHELM

Division of Life Sciences, Austrian Research Centers Seibersdorf, A-2444 Seibersdorf, Austria

Abstract

To induce somatic embryogenesis in *Quercus robur* L. immature zygotic embryos at different developmental stages were collected in weekly intervals from June until September in three consecutive years from four open pollinated trees at two Vienna sites. Acorns were surface sterilised and cultured firstly on P24 medium with 5μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM 6-benzylaminopurine (BAP) or on hormone-free P24 medium and secondly on P24 medium with 0.9 μM BAP. The formation of white-yellow globular structures of somatic embryos started during the fourth week after the induction treatment. High induction frequencies of 30 - 80 % were achieved on 2,4-D/BAP medium, whereas rates on hormone-free medium were below 20 %. The initiation of somatic embryogenesis was favoured in the heartshaped and early cotyledonary stage of the zygotic embryo in all three years and lasted until the acorns reached maximum size in August.

Additional key words: plant growth regulators, moisture content, acorn size, developmental stages.

Introduction

In oak, conventional propagation deals very often with a low rooting ability and seed storage is limited to two years due to high susceptibility to infections related to the high water content of the acorns. Therefore, somatic embryogenesis has become important for *in vitro* propagation. Chalupa (1990) showed the initiation of embryogenic cell lines of immature embryos of *Quercus robur* and *Q. petraea* on woody plant medium (WPM) and Murashige and Skoog (MS) medium. Gingas and Lineberger (1989) reported somatic embryogenesis of immature embryos of *Q. rubra* on hormone-free WPM medium as well as on medium supplemented with auxins and cytokinins. Embryogenic cell lines of immature zygotic embryos of *Q. suber* were

Received 24 September 1998, *accepted* 25 May 1999.

Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid.

Acknowledgements: This work was supported by the Austrian Federal Ministry of Agriculture and Forestry.

Fax: (+43) 2254 780 3653, e-mail: eva.wilhelm@arcs.ac.at

established by Bueno *et al.* (1992) and Manzanera *et al.* (1993). Kim *et al.* (1994) initiated somatic embryogenesis of immature embryos of *Q. acutissima*. Evers *et al.* (1995) was the first that determined a time window of four weeks for the induction of somatic embryogenesis of *Q. robur* that is correlated to a certain developmental stage of the zygotic embryo. Initial problems with low frequencies of somatic embryo conversion into plantlets seemed to be overcome by a combination treatment with increased agar concentration and desiccation (Wilhelm *et al.* 1996).

But there is still little knowledge about the induction process itself. In this work, the effect of different parameters like developmental stage of the zygotic embryo, supplements of plant growth regulators, size and moisture content of the zygotic embryo on induction and viability of somatic embryos were investigated over three consecutive years.

Materials and methods

Plants: Four open pollinated trees of *Quercus robur* L. at two sites in Vienna, Austria, were selected. Both oaks on location 1 (Inzersdorf) were approximately 200 years old, trees on location 2 (Prater) approximately 20 - 40 years old. Acorns at different stages of maturity were collected in weekly intervals over three growing seasons. In 1995 collection started at the end of June and lasted till early September. In 1996 and 1997 collection started in the beginning of July and lasted till the end of August. Ten to twenty acorns were taken per tree at each sampling date.

Induction: Acorns were surface sterilised (2 × 10 min) in an ultrasonic bath containing 2 % NaOCl. Then the acorns were rinsed twice in a sterile solution (Na_2HPO_4 + citric acid + KJ + starch), then transferred to a sterile solution of starch and rinsed twice in sterile distilled water (Rodler 1996). Acorns collected in June were only cut in length, whereas in July, August and September the zygotic embryos were excised and cut in length. The acorns and later the zygotic embryos were put on P24-medium (Patent No. 92902531.0. Robert Teasdale/Forbio) with 0.8 % agar (*Daishin, Brunschwig Chemie*, Amsterdam, The Netherlands), 3 % sucrose, 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5 µM 6-benzylaminopurine (BAP) or without hormones. The pH of all media was adjusted to 5.9 before autoclaving at 121 °C for 20 min. To avoid inhibitory influence of polyphenols, all acorns and later the excised zygotic embryos were transferred to a fresh induction medium within the first week. After 4 weeks they were transferred to a P24 medium with 0.9 µM BAP. The latter medium was used for maintenance of embryogenic cell lines. Subculturing intervals were six weeks. Cultures were kept at temperature of 24 °C ± 2 °C and white light (*TLD 36W/33, Philips*, irradiance 50 µmol m^{-2} s^{-1}, 16-h photoperiod).

Acorn size and moisture content: Length of acorns and zygotic embryos were measured weekly from July until September on location 2. Moisture content of the zygotic embryos was determined gravimetrically.

Statistics: In order to determine the most important factor for induction of somatic embryogenesis a non-parametric test (Kruskal-Wallis) and a Spearman Correlation Analysis was used ($P < 0.05$).

Results

The best results for induction of somatic embryogenesis in *Q. robur* were obtained when the acorns were collected from the end of June until the beginning of August ($P < 0.05$; Fig. 1). Induction rates of 30 - 80 % on 2,4-D/BAP induction medium

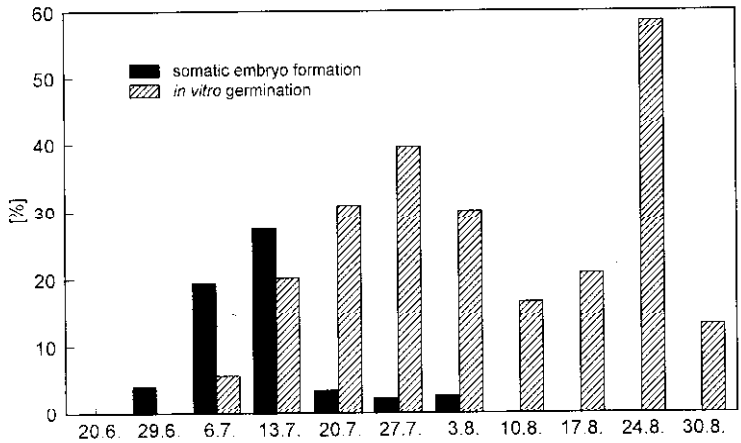

Fig. 1. Percentage of somatic embryo formation and *in vitro* germination of zygotic embryos from acorns collected from the end of June until September in weekly intervals over three consecutive years starting in 1995. The induction was focused on 6 week period starting in the end of June.

could be achieved (Fig. 2*A*). Frequencies on hormone-free medium ranged from 10 to 17 % (Fig. 2*B*). An increasing *in vitro* germination of zygotic embryos ($P < 0.001$) was observed for samples collected from the end of July until August. After middle of August no further induction of somatic embryogenesis was possible (Fig. 1).

Embryo-like structures (white and yellow globular objects) had been formed on the cotyledon surface and on the radicle of the zygotic embryo. No visible callus phase was observed suggesting direct somatic embryogenesis.

During the third and fourth week after the beginning of the induction treatment most of all somatic embryos had been formed on 2,4-D/BAP medium ($P < 0.05$). Later formation occurred in the 8th week after the start of the induction treatment. Different stages of somatic embryos developed side by side on the proliferation medium. Plant growth regulators used in the induction medium had also an influence on viability of the somatic embryos over a 6 month period ($P < 0.01$). Embryogenic potential of 42 % of all induced cell lines has retained up to the present.

Sizes of seeds differed from year to year. In 1995, the acorns were 6 mm long in June and 28 mm in August. In 1996 and 1997 the zygotic embryos were 4 mm long

in July and 30 mm in August. In 1995 growth started early in May and continued slowly over a long period. In 1996 and 1997 the growth started late and continued rapidly (Fig. 3*A*). Acorns reached mature size by mid of August. Shedding took place from September until early October. The developmental stages of the zygotic embryos were the heart-shaped phase in July and the cotyledonary stage at the beginning of August. Moisture content was similar in 1996 and 1997, starting with 97 % in July and decreasing to 38 % in September (Fig. 3*B*).

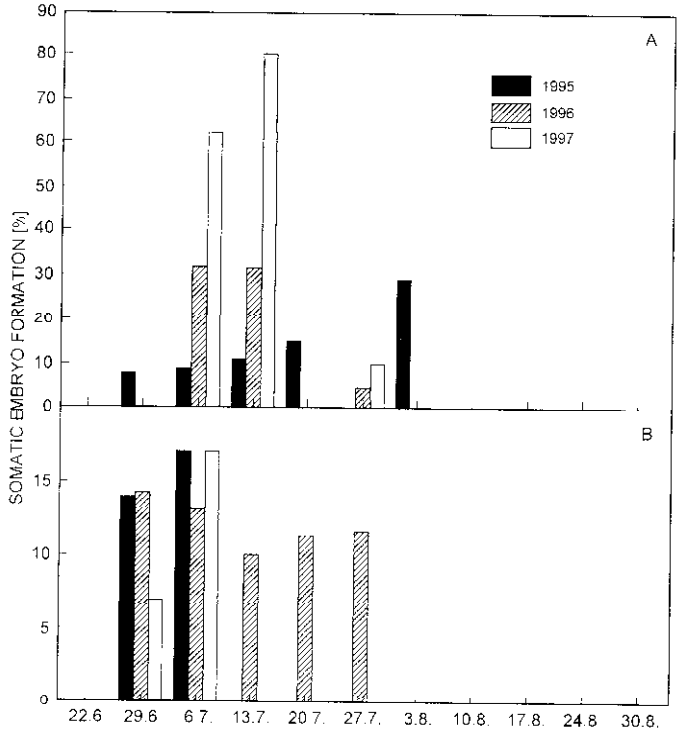

Fig. 2. Percentage of somatic embryo formation on P24 induction medium with 2,4-D and BAP (*A*), and on hormone-free medium (*B*) in three consecutive years.

Discussion

The protocols on induction of somatic embryos differ widely with respect to media types, plant growth regulators and collection times (Gingas and Lineberger 1989, Chalupa 1990, 1995, Manzanera *et al.* 1992). Ostrolucka *et al.* (1995, 1996) suggested, that zygotic embryo stage and media composition have both great influence on the induction process. In our study the usage of plant growth regulators in two step induction process significantly promoted induction in the first 4 weeks after starting the treatment and led to long viability of the cell lines up to the present (42 %).

In 1995 seeds reached 5 mm length about one month earlier than in 1996 and 1997 (Fig. 3). Seeds harvested in 1995 therefore behaved similar to "rapid developers" observed by Finch-Savage *et al.* (1994) but seeds harvested in 1996 and 1997 showed growth pattern like "slow developers". The induction took place only in the growing phase of the zygotic embryo. When the zygotic embryo reached nearly maximum size, it was not possible to induce somatic embryogenesis any more.

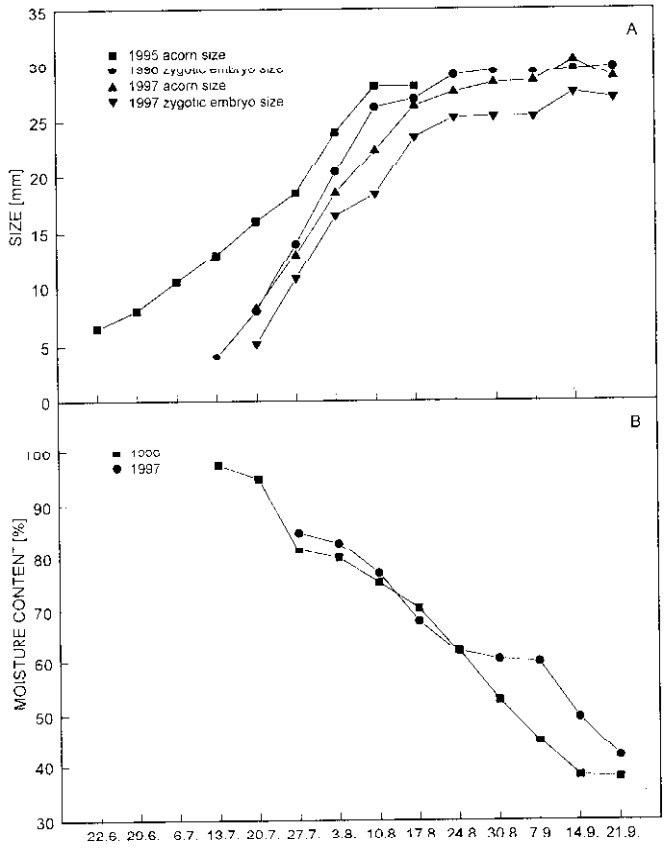

Fig. 3. Size (*A*) and moisture content (*B*) of zygotic embryos during development from June until September in 1995, 1996 and 1997.

The initiation of somatic embryogenesis was significantly related to the heartshaped and early cotyledonary stage of the zygotic embryo in all three years. These results correlate to Evers *et al.* (1995), who observed that initiation of somatic embryogenesis was limited to a four week period in July. Patterns like slow or rapid developers are helpful for prediction of the starting point for acorn collection. If acorns are still below 5 mm length throughout June (slow developers) then a rapid development can be expected for July, thus dramatically shortening the time window for induction. Our results determined significantly that the time of collection (developmental stage), pattern like slow or rapid developers (seasonal influence) and

induction medium supplements (viability) were the main factors that should be considered when attempting to reach mass propagation of *Quercus robur* via somatic embryogenesis.

References

Bueno, M.A., Astorga, R., Manzanera, J.A.: Plant regeneration through somatic embryogenesis in *Quercus suber*. - Physiol. Plant. **85**: 30-34, 1992.

Chalupa, V.: Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). - Plant Cell Rep. **9**: 398-401, 1990.

Chalupa, V.: Somatic embryogenesis in oak (*Quercus* spp.). - In: Jain, S., Gupta, P., Newton, R. (ed.): Somatic Embryogenesis in Woody Plants. Vol. 2. Pp. 67-87. Kluwer Academic Publishers, Dordrecht - Boston - London 1995.

Evers, P., Van Laar, J., Vermeer, E.: The small time window to obtain somatic embryogenesis in *Quercus robur*. - In: Identification and Control of Phase Changes in Rejuvenation. Cost 822 Working Group, Eisenstadt 1995.

Finch-Savage, W.E., Blake, P.S.: Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. - Seed Sci. Res. **4**: 127-133, 1994.

Gingas, V.M., Lineberger, R.D.: Asexual embryogenesis and plant regeneration in *Quercus*. - Plant Cell Tissue Organ Cult. **17**: 191-203, 1989.

Kim, Y.W., Lee, B.C., Lee, S.K., Jang, S.S.: Somatic embryogenesis and plant regeneration in *Quercus acutissima*. - Plant Cell Rep. **13**: 315-318, 1994.

Ostrolucká, M.G.: Asexual embryogenesis and application of this process in the reproduction of oaks. - Forest Sci. **1**: 12-18, 1995.

Ostrolucká, M.G., Krajmerová, D.: Manifestation of embryogenic potential in culture of zygotic embryos of *Quercus robur* L. - Acta Soc. Bot. Pol. **65**: 37-41, 1996.

Rodler, R.: Somatische Embryogenese von *Quercus robur* L. - Diplomarbeit, Universität Wien, Wien 1996.

Teasdale, R.: Formulation of plant culture media and applications therefore. - International Publication No. WO 92/07460. Patent No. Europe: 92902531.0. Forbio PTY Ltd., Queensland 1992.

Wilhelm, E., Burg, A., Berenyi, M., Endemann, M., Rodler, R.: Plantlet regeneration via somatic embryogenesis and investigations on *Agrobacterium tumefaciens* mediated transformation of oak (*Quercus robur*). - In: Ahuja, M., Boerjan, W., Neale, D.B. (ed.): Somatic Cell Genetics and Molecular Genetics of Trees. Pp. 119-125. Kluwer Academic Publishers, Dordrecht - Boston - London 1996.