Using Polysaccharides and Galactomannans as Gelling Agents in Capsule Formation of Artificial Seeds

Seppo Sorvari

Agricultural Research Centre, Institute of Horticulture, Toivonlinnantie 518, FIN-21500 Piikkiö, Finland

Otto Toldi

Agricultural Biotechnology Center, Institute for Plant Sciences, Szent-Györgyi Albert Street 4, P.O. Box 411, H-2101 Gödöllő, Hungary

Katriina Ahanen and Tuula Viinamäki

Agricultural Research Centre, Institute of Horticulture, Toivonlinnantie 518, FIN-21500 Piikkiö, Finland

Tiina Hakonen

Leiras OY, Clinical Research, Pasilanraittio 9, FIN-00240 Helsinki, Finland

Risto Tahvonen

Agricultural Research Centre, Institute of Horticulture, Toivonlinnantie 518, FIN-21500 Piikkiö, Finland

ADDITIONAL INDEX WORDS. carrot, somatic embryogenesis, starch

ABSTRACT. Somatic embryos that are produced in cell and tissue culture conditions do not form endosperm and testa around the embryo. For many envisioned applications, delicate embryos need to be covered with an artificial endosperm-testa matrix that allows for respiration, contains nutrients and protectants, and is hard enough but allows the vague embryo to germinate and grow into a plant. A number of industrially produced and commonly available compounds were tested to determine their efficacy in supporting the germination of encapsulated somatic embryos of carrot (*Daucus carota* L.). Most of the combinations of galactomannans and polysaccharides were at least as effective as calcium alginate alone in germinating the embryos. The disappearance of blue color in Lugol staining around embryos indicates that starch might also provide nourishment to embryos, thus serving as an artificial endosperm and a supportive matrix.

Synthetic seed technology is an advanced method of using totipotency of somatic cells for cloning plants. In this technique, the original genome remains unaffected although the propagation takes place in the same way as with true seeds. Because of the rearrangement of genetic information, true seeds cannot be used to propagate vegetatively propagated plants. Other reasons for using artificial seeds can be, for example, high seed price or poor seed set.

One of the features in somatic embryogenesis is the lack of endosperm and testa developing in vivo around the embryo. The exposed somatic embryo is too small for practical use in mechanized sowing, and its survival possibilities in the soil under normal growing conditions are poor. Therefore, the delicate embryos should be encapsulated with an artificial endosperm matrix to cover and support them in the germination stage. Calcium alginate gels, which have been used to immobilize microbial cells, cell organelles, and enzymes (Kierstan and Bucke, 1977) or produce secondary metabolites from plant cells (Brodelius and Nilsson, 1980), is one of the solutions for hydrated artificial seed technology (Redenbaugh et al., 1986). Other components that have been used for the encapsulation of cells are, for instance, polyethylene oxide (Kitto and Janick, 1985), fluid drilling gel (Cantliffe et al., 1987), chitosan (Nigam et al., 1988), and cellulose (Linko et al., 1980).

Calcium alginate has only supportive functions and a low nutritive value for the embryo in the artificial endosperm matrix. However, starch, which is one of the most common components in the endosperm, especially in monocotyledonous plants, can function as a supportive and nutritive compound (Sorvari, 1986a,

1986b; Sorvari and Schieder, 1987). The value of starch for plant cell tissue has since been shown in tobacco (*Nicotiana tabacum* L.) and wild carrot (*Daucus carota* L.) cell cultures (Henderson and Kinnersley, 1988), potato (*Solanum tuberosum* L.) anther cultures (Calleberg et al., 1989; Calleberg and Johansson, 1993), wheat (*Triticum aestivum*, L.) haploid production (Fadel and Wenzel, 1990; Foroughi-Wehr and Zeller, 1990; Rybczynski et al., 1991), immature embryo cultures (Simonson and Baenziger, 1992), and in apple (*Malus domestica* Borkh.) and red raspberry (*Rubus idaeus* L.) cell cultures (Zimmerman et al., 1995).

Starch has a softer texture than Gelrite, agar, or calcium alginate. To overcome this problem in petri dishes, a polyester net has been used to prevent the sinking of the tissue into the starch medium (Sorvari, 1986a). An alternative solution is to mix starch with some harder gelling agent like agarose (Lashermes, 1992; Tiwari and Rahimbaev, 1992) or Gelrite (Calleberg and Johansson, 1993; Zimmerman et al., 1995). These types of combinations offer a firm support for plant tissue without losing the advantages of starch, as well as lowering costs to only 10% to 15% of that for agar (Zimmerman et al., 1995).

Nevertheless, the disadvantage of Gelrite, agar, and agarose is that they require heating before molding and in beadmaking for sensitive plant tissue this can be quite detrimental. Therefore the purpose of this work was to study the ability of different galactomannans to combine with each other and wheat starch in the production of beads without heating and to record the germination rate of the somatic embryos after entrapment in these matrices.

Materials and Methods

PLANT MATERIAL AND PREPARATION OF SEEDS FOR GERMINATION. The method used was a modification of the methods described by

Received for publication 3 Oct. 1996. Accepted for publication 30 June 1997. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Kamada and Harada (1979), Sung and Okimoto (1981), and Sorvari et al. (1994). Plant samples consisted of hypocotyl sections from seedlings of the commonly cultivated domestic carrot variety Duke. Carrot seeds were prepared for germination by placing them into a tube with a 500- μ m net bottom and washed with distilled water. The seeds were disinfected by immersing the tube briefly into 95% (by volume) ethanol, washing once with distilled water, and incubating for 30 min in 5% (by volume) (active chlorine) sodium hypochlorite with a drop of Triton X-100. Seeds were thereafter washed 3 times with sterile distilled water and germinated on moist sterile cellulose wadding in sterile petri dishes (20 seeds/petri dish) for 4 d at 25 °C in the dark and then 4 d in light (16-h day, 40 to 50 μ mol·s⁻¹·m⁻²).

INITIATION AND MAINTENANCE OF SUSPENSION CULTURES. Suspension cultures were initiated with $\approx$ 1-cm-long hypocotyl sections of seedlings by placing 15 pieces into 100-mL Erlenmeyer flasks containing 20 mL of liquid nutrient medium. The nutrient medium was composed of macro- and microsalts and sucrose described by Murashige and Skoog (1962) supplemented with (in mg·L⁻¹) 1.0 2,4-D, 100.0 meso-inositol, 0.5 thiamine-HCl, 0.5 nicotinic acid, 0.5 pyridoxine-HCl, 2.0 glycine, and 1000.0 casein hydrolysate. The initial cultures were incubated on a gyratory shaker (100 rpm) for 4 weeks under a 16-h day (40 to 50 μ mol·s⁻¹·m⁻²) at 25/23 $\pm$ 1 °C day/night temperature. The first subculture was prepared by sieving the suspension through a stainless steel mesh of 355- μ m pore size. The cells passing through the 355- μ m mesh were collected by centrifugation (100 g_a), and $\approx$ 0.5 to 0.7 mL of the

packed cell volume was resuspended in 20 mL of fresh Murashige and Skoog (MS) medium supplemented with 1.0 mg·L⁻¹ of 2,4-D. For subsequent subcultures, the same procedure was repeated every 2 weeks.

INDUCTION OF EMBRYOGENESIS. The 8-d-old subculture suspension was successively sieved through a series of 355-, 200-, 100-, and 45- μ m mesh, and finally through a 27- μ m mesh. The fraction remaining on the 27- μ m mesh was washed four times with an MS-solution free of plant growth regulators (PGRs) and then suspended in 20 mL of PGR-free MS medium in a 100 mL Erlenmeyer flask to the density of 3 $\times$ 10⁴ cells/mL. The cultures were kept on a gyratory shaker (100 rpm) for 2 weeks at 25 °C in the dark until the embryos had reached polarized stage with clearly visible shoot and root initials, which is called torpedo stage.

GELLING AGENTS. All gelling agents used were commonly available industrial products (Table 1). Sodium alginate, kappa-carrageenan, and wheat starch was purchased from Fluka Chemie AG (Buchs, Switzerland); E410 locust bean gum extract, E415 Xanthan gums, E412 guar gum, Cegalum S 500, and E440 low methoxyl pectin were produced by Sanofi Bio-Industries (Paris, France) and provided by Taru Karikoski, Finnsugar Inc. (Jokioinen, Finland).

LUGOL STAINING. The Lugol staining experiment was carried out in 9-cm petri dishes. The medium was composed of PGR-free one-third-strength MS salts, supplemented with 30 g·L⁻¹ sucrose, 0.1 mg·L⁻¹ thiamine-HCl, 0.5 mg·L⁻¹ nicotinic acid, 0.5 mg·L⁻¹ pyridoxine-HCl, and 2.0 mg·L⁻¹ glycine gelled with 3 g·L⁻¹ Sigma-

Table 1. Compilation of origin, structure, concentrations, and properties of ungelled mixtures and capsules of some commercial available polysaccharides and galactomannans.²

No	SOURCE	GALACTOMANNANS/ POLYSACCHARIDES	ORIGIN	STRUCTURE	CONC. IN %	PROPERTIES OF UNGELLED MIXTURES AND CAPSULES
1	Fluka	-Sodium alginate, (Category Nr.71238)	<i>Laminaria hyperborea</i>	-D-mannuronic acid 25-30%, L-glucuronic acid 65-70%	2.0	Ungelled mixture: low viscosity Capsules: clear, elastic, resistant, round shaped
2	Sanofi Fluka	-E410 Locust bean gum extract (Lygomme® 6) -Sodium alginate	-Endosperm of the seeds of <i>Ceratonia siliqua</i> L. -see above	-1 galactose unit for 4 mannose residues -see above	1.0 1.0	Ungelled mixture: high viscosity Capsules: quite clear, brittle, round shaped
3	Sanofi Fluka	-E415 Xanthan gum (Satiexane® CX 90) -Sodium alginate	-Cultures of <i>Xanthomonas campestris</i> -see above	-Glucose, mannose and glucuronic acid residues -see above	1.0 1.0	Ungelled mixture: high viscosity, grey Capsules: light grey, unelastic, medium resistant, round shaped
4	Sanofi Fluka	-E415 Xanthan gum (Satiexane® CX 91) -Sodium alginate	-Cultures of <i>Xanthomonas campestris</i> -see above	-Glucose, mannose and glucuronic acid residues -see above	1.0 1.0	Ungelled mixture: high viscosity, grey Capsules: light grey, unelastic, medium resistant, round shaped
5	Sanofi Fluka	-E412 Guar gum (Viscogum® HV 3000 A) -Sodium alginate	-Endosperm of the seeds of <i>Cyamopsis tetragonolobus</i> -see above	-1 galactose unit for 4 mannose residues -see above	1.0 1.0	Ungelled mixture: high viscosity Capsules: clear, unelastic, medium resistant, irregular shaped
6	Fluka Fluka	-Kappa-carrageenan, (Category Nr. 22048) -Sodium alginate	- <i>Chondrus crispus</i> -see above	-Sulfated galactose residues and 3,6-anhydrogalactose -see above	0.5 1.0	Ungelled mixture: low viscosity Capsules: clear, elastic, very resistant, irregular shaped
7	Fluka Sanofi	-Wheat starch, Category Nr.85649 -Cegalum S 500 (=Sodium alginate)	-Endosperm of the seeds of <i>Triticum aestivum</i> L. -Fucaceae, Laminariaceae, <i>Macrocystis</i>	-Amylose 20-30%, amylopectin 70-80% -D-mannuronic acid units / L-glucuronic acid units	5.0 2.0	Ungelled mixture: low viscosity Capsules: clear, elastic, very resistant, round shaped, small
8	Fluka Fluka	-Wheat starch -Kappa-carrageenan	-see above -see above	-see above -Sulfated galactose residues and 3,6-anhydrogalactose	5.0 1.0	Ungelled mixture: clear, high viscosity at pH6.8 Capsules: clear, nonelastic, brittle, irregular shaped
9	Fluka Fluka Sanofi	-Wheat starch -Kappa-carrageenan -E440 low methoxyl pectins (Unipectine® 325 NH 95)	-see above -see above - <i>Malus domestica</i> Borkh. pomace or <i>Citrus</i> sp. peels	-see above -see above -Galactopyranosyluronic acid units partially sterified with methanol (degree of esterification < 50%)	5.0 0.5 4.0	Ungelled mixture: Clear, low viscosity at pH4.5 Capsules: cloudy, brittle, irregular shaped
10	Fluka Fluka Fluka	-Wheat starch -Kappa-carrageenan, -Sodium alginate	-see above -see above -see above	-see above -see above -see above	5.0 0.8 0.5	Ungelled mixture: Clear, low viscosity at pH6.9 Capsules: clear, semielastic, brittle, irregular shaped

²Components are mixed together before autoclaving after that capsules were performed by dropping gelling mixture through a Pasteur pipette into sterile 100 mM Ca(NO₃)₂ solution (pH 5.5).

Fig. 1. A carrot somatic embryo at the torpedo stage encapsulated in a calcium alginate bead. The bead was formed by mixing 4 mL of gelling agent with 1 mL of embryo suspension, and dropping the mixture with a Pasteur pipette into 100 mM $\text{Ca}(\text{NO}_3)_2$ solution (pH 5.5).

agar (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) plus 40 g·L⁻¹ wheat-starch. The starch was stained by pipetting 10 mL of 10 mM iodine-potassium iodide (Lugol solution) into the medium and incubated for 30 s. During incubation the petri dishes were shaken several times in order to obtain a more even distribution of the color. The Lugol solution was discarded, and the gel surface washed once with 10 mM potassium phosphate buffer and surface dried in a laminar airflow cabinet. Late torpedo-stage embryos were placed into the medium and incubated for one day at 25 °C in the dark. The control medium was incubated without somatic embryos.

ENCAPSULATION OF SOMATIC EMBRYOS. The gelling agents were first dissolved in distilled water and then autoclaved. The concentration of the gelling agent varied from 0.5% to 5.0% in the stock solution. However, because the final encapsulation solution was made by mixing 4 mL of gelling agent with 1 mL of embryo suspension, the final gelling agent concentration in the beads was 20% lower than that in the stock solution. Embryos were prepared for the entrapment by sieving them first through 500- μm and then 355- μm meshes. The embryos remaining on the 355- μm mesh were collected, washed twice with PGR- and sucrose-free one-third-strength MS medium, and finally mixed with PGR- and sucrose-free one-third-strength MS-medium.

The beads, each containing one torpedo-stage embryo (Fig. 1), were formed by mixing 4 mL of gelling agent with 1 mL of embryo suspension and dropping the mixture with a Pasteur pipette into 100 mM $\text{Ca}(\text{NO}_3)_2$ solution (pH 5.5). The beads were polymerized in the $\text{Ca}(\text{NO}_3)_2$ solution for 25 min and subsequently washed for 60 min in PGR- and sucrose-free one-third-strength MS-medium. The diameter of the beads was 4 to 5 mm, depending on the viscosity of the gelling agent.

The effect of ungelified sodium alginate on the torpedo-stage somatic embryos was tested by incubating them from 0 to 24 h at room temperature in one-third-strength MS-medium mixed with 2% of sodium alginate. The control medium lacked sodium alginate. Embryos (100) were extracted from the incubation and control media at the start and after 2, 4, 8, 16, and 24 h, washed with PGR-free one-third-strength MS-medium, and transferred to a germination medium composed of PGR-free one-third-strength MS, 0.7% Difco Bacto Agar (Difco Laboratories, Detroit), 3% sucrose, and 0.2% activated charcoal. Incubation was performed under a 16-h day (40 to 50 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) at 25/23 $\pm$ 1 °C day/night temperature for 2 weeks, after which the quantity of converting embryos was recorded.

GERMINATION OF ARTIFICIAL SEEDS. The nutrient medium used for germination of embryos encapsulated in different matrices was continuously composed of PGR-free one-third-strength MS medium, 0.7% w/v Difco Bacto Agar, 3% w/v sucrose, and 0.2% w/v activated

Difference significant between:

NAK vs. No 1 ($p=0.0025$)

NAK vs. No 5, 6 and 7 ($p=0.01$)

NAK vs. No 8 ($p=0.01$)

NAK vs. No 9 ($p=0.001$)

Difference not significant between:

NAK vs. No 2, 3, 4 and 10 ($p<0.1$)

1 = sodium alginate (SA)

2 = SA + locust bean gum (LBG)

3 = SA + xanthan gum (XG) CX90

4 = SA + XG CX91

5 = SA + guar gum (GG)

6 = SA + kappa-carrageenan (KC)

7 = Cecalculum S500 (CEC) + wheat starch (WS)

8 = KC + WS

9 = WS + KC + low methoxyl pectins (LMP)

10 = WS + KC + SA

Fig. 2. Germination rate (GR; %) of carrot somatic embryos after inserting in matrices combined of sodium alginate and other polysaccharides or galactomannans; NAK = exposed, noncapsulated embryos.

Fig. 3. Differentiated carrot somatic embryo (torpedo stage) after incubation of several hours on Lugol stained starch-agarose gelled medium. The disappearance of the color is visible as a bright halo around the embryo.

charcoal. The inner diameter of the petri dish was 50 mm and the amount of the medium 11 mL. The total number of beads was 1000, with 100 beads being used per treatment. The density of beads per petri dish was 10. Incubation was performed under a 16-h day (40 to 50 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) at $25/23 \pm 1$ °C day/night temperature for 2 weeks. After the incubation period, the number of converted

embryos was recorded. The experiments used a completely randomized design. The differences between treatments were tested using the log-linear model of the SAS Insight statistical program (SAS Institute, Cary, N.C.).

Results

After 2 weeks of incubation under in vitro conditions, exposed embryos retained a satisfactory germination capacity of 72% (Fig. 2). Sodium alginate alone reduced the survival rate to 51%, significantly lower ($p = 0.0025$) than the germination rate for exposed embryos. If sodium alginate was mixed with locust bean gum, xanthan gum, or wheat starch-kappa-carrageenan, the germination rate rose to 63%, 73%, 74%, and 66%, respectively (Table 1). Such rates were as high as those found for exposed embryos (72%), with no significant difference being detected ($p > 0.1$) between these groups. The mixtures of sodium alginate with guar gum, kappa-carrageenan, and wheat starch reduced the germination rate to 18% (Table 1), a level that was significantly lower ($p = 0.01$) than that for exposed embryos (Fig. 2).

It was also possible to produce beads without sodium alginate if wheat starch was mixed with kappa-carrageenan or kappa-carrageenan plus low methoxyl pectins (Table 1). Low methoxyl pectins, however, had a detrimental effect on the germination rate, with only 12% of the embryos being able to germinate; the difference was significant ($p = 0.001$). In the beads mixed with wheat starch and kappa-carrageenan, the germination rate of embryos was 16% lower than that for the exposed embryos; the difference was significant ($p = 0.02$). When wheat starch and kappa-carrageenan were combined with sodium alginate, the germination rate was 66%, a rate equivalent to that of the exposed embryos, because no significant difference was found ($p = 0.36$). The presumed degradation of the starch during the germination process was studied by using Lugol staining. After transferring embryos onto Lugol-stained starch-medium, within a few hours the blue color started to disappear around the embryo, suggesting possibly degradation of the starch (Fig. 3).

Ungelled sodium alginate proved to be quite detrimental to somatic embryos (Fig. 4). Within 2 h, the germination rate of the embryos had decreased from 87% to 66%. The germination rate after 4 h was 54%, but only 19% after 8 h. An incubation period of ≥ 16 h completely killed all embryos. In the controls without sodium alginate, the germination rate decreased only from 87% to 81% after 24 h of incubation.

Discussion

Hydrocolloids that are used for hydrated systems are derived from higher plants (pectins and galactomannans, guar gum, locust bean gum, cellulose), seaweeds (carrageenans and alginates), animals (gelatin and chitosan), and microorganisms (xanthan gum) (Barbotin et al., 1993). Many of the hydrocolloids are metabolically inert, al-

Fig. 4. Germination rate (GR; %) of carrot somatic embryos after incubation for 0 to 24 h in the MS nutrient medium with or without (control) ungelled sodium alginate.

Fig. 5. A sprouting carrot somatic embryo trapped in a calcium alginate bead. Germination was carried out on plant-growth-regulator-free one-third-strength MS medium, 0.7% w/v agar, 3% w/v sucrose, and 0.2% w/v activated charcoal.

though polyacrylamide, for example, is toxic to plant cells (Brodelius and Nilsson, 1980; Barbotin et al., 1993), and they need high melting temperatures. Good encapsulating material should be nondamaging, protective, durable, and also allow good germination (Fig. 5) (Redenbaugh et al., 1991).

Starch is the most common endosperm nutrient in nature, and it is inexpensive, hydrocolloid-forming, nontoxic, and biodegradable by the plant. In addition to its colloid-forming characteristic, it can function as an energy and carbon source for the developing embryo (Sorvari, 1986a, 1986b; Sorvari, and Schieder, 1987). Starch is composed of amylose and amylopectin, although the ratio of these two components can vary, especially, the amylose content, which can greatly influence gelatinization characteristics. It is for this reason that the waxy type with essentially no linear amylose is a weak gelling agent (National Starch and Chemical, year unknown). This problem can be resolved by mixing starch with other stronger gelling agents to thus form a supportive and nutritive matrix in a petri dish, as has been done, for example, with starch and agarose (Tiwari and Rahimbaev, 1992) and starch and Gelrite (Calleberg and Johansson, 1993; Zimmermann et al., 1995).

Bead formation should be accomplished without resorting to heating because of possible damage to the plant tissue. Therefore, in this experiment one of the goals was to find combinations of starch and other gelling agents with good bead formation but requiring no heating or molding. Sodium alginate is very suitable for this purpose since it can be stabilized in various ways. Besides calcium nitrate, glutaraldehyde alone or combined with gelatin or persimmon tannin (Birnbbaum et al., 1981) can also stabilize sodium alginate. With respect to glutaraldehyde, however, Brodelius and Nilsson (1980) reported that *Cantharantus roseus* L. cells lost their respiration capacity after immobilization in gels treated with glutaraldehyde. Furthermore, some nutrient media may not be suitable for calcium alginate, because the presence of complexing anions such as citrate, EDTA, lactate, and phosphate can cause instability in the gel structure (Birnbbaum et al., 1981). On the other hand, the reversibility of the calcium alginate gel can also be an advantage, because it allows free movement of cells back to the suspension (Brodelius and Nilsson, 1980).

In all combinations studied here, cross-linking was performed at room temperature by pipetting mixtures at the same temperature

into 100 mM calcium nitrate solution. The gelling ability of the calcium alginate was not considerably altered in any of the combinations in which it was used, only in some the size or shape was not as ideal as that produced by calcium alginate beads. It was, however, possible to form beads without alginate. In two cases, when starch was combined with kappa-carrageenan alone or with kappa-carrageenan and low methoxyl pectins, beads formed. The addition of low methoxyl pectins, however, seems to prevent germination. Consequently, only 12% of those beads containing low methoxyl pectins germinated, whereas as much as 56% of similar beads free of low methoxyl pectins germinated.

The question how or even whether starch is degraded still remains unresolved. One sign of the enzymatic degradation of starch is the softening of the gelled starch during cell culture (Sorvari, 1986). In contrast, spontaneous degradation of starch has also been observed in cell culture-free media (Tremblay and Tremblay, 1989). In barley (*Hordeum vulgare* L.) anther cultures, Kuhlman and Foroughi-Wehr (1989) reported a halo in the starch surrounding tissue. Another indication of the use of starch as an energy and carbon source was the successful culture of barley anthers on a medium comprised of barley starch but lacking in any other carbohydrate source (Sorvari and Schieder, 1987). In this study, the putative degradation of starch was visualized by Lugol staining. The disappearance of the stain from around carrot somatic embryos presumably indicates either hydrolysis or some other changes in starch structure, which should be studied more closely. Although starch alone is limited in its suitability for bead making, it seems to be very flexible when used together with other stronger gelling agents.

Literature Cited

- Barbotin, J., J. Nava Saucedo, C. Bazinet, A. Kersulec, B. Thomasset, and D. Thomas. 1993. Immobilization of whole cells and somatic embryos: coating process and cell-matrix interactions, p. 65–103. In: K. Redenbaugh (ed.). *Synseeds: Application of synthetic seeds to crop improvement*. 1st ed. CRC Press, Boca Raton, Fla.
- Birnbbaum, S., R. Pendleton, P.-O. Larsson, and K. Mosbach. 1981. Covalent stabilization of alginate gel for the entrapment of living whole cells. *Biotechnol. Lett.* 3:393–400.
- Brodelius, P. and K. Nilsson. 1980. Entrapment of plant cells in different matrices. *FEBS Lett. Fed. Eur. Biochem. Soc.* 122:312–316.
- Calleberg, E. and L. Johansson. 1993. The effect of starch and incubation temperature in anther culture of potato. *Plant Cell Tissue Organ Cult.* 32:27–34.
- Calleberg, E., I. Kristjandottir, and L. Johansson. 1989. Anther culture of tetraploid *Solanum* genotypes—The influence of gelling agents and correlations between incubation temperature and pollen germination temperature. *Plant Cell Tissue Organ Cult.* 19:189–197.
- Cantliffe, D., J. Liu, and J. Schultheis. 1987. Development of artificial seeds of sweet potato for clonal propagation through somatic embryogenesis, p. 183–195. In: W. Smith and J. Frank (eds.). *Methane from biomass, A system approach*. Elsevier Applied Sciences, New York.
- Fadel, F. and G. Wenzel. 1990. Medium-genotype interaction on androgenetic haploid production in wheat. *Plant Breeding* 105:278–282.
- Foroughi-Wehr, B. and F. Zeller. 1990. *In vitro* microspore reaction of different German wheat cultivars. *Theor. Appl. Genet.* 79:77–80.
- Henderson, W. and A. Kinnersley. 1988. Corn starch as an alternative gelling agent for plant tissue culture. *Plant Cell Tissue Organ Cult.* 15:17–22.
- Kamada, H. and H. Harada. 1979. Studies on the organogenesis in carrot tissue cultures. I. Effects of growth regulators on somatic embryogenesis and root formation. *Z. Pflanzenphysiol.* 91:255–266.
- Kierstan, M. and C. Bucke. 1977. The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gels. *Biotechnol. Bioeng.* 19:387–397.

- Kitto, S. and J. Janick. 1985. Production of synthetic seeds by encapsulating asexual embryos of carrot. *J. Amer. Soc. Hort. Sci.* 110:277-282.
- Kuhlmann, U. and B. Foroughi-Wehr. 1989. Production of doubled haploid lines in frequencies sufficient for barley breeding programs. *Plant Cell Rpt.* 8:78-81.
- Lashermes, P. 1992. Improved anther culture method for obtaining direct regeneration in wheat (*Triticum aestivum* L.). *J. Genet. Breeding* 49:99-102.
- Linko, P., K. Poutanen, L. Weckström, and Y. Linko. 1980. Preparation and kinetic behavior of immobilized whole cell biocatalysts. *Biochimie* 62:387.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- National Starch and Chemical. (Year unknown). Food starch technology. Natl. Starch and Chemical, Manchester, Great Britain.
- Nigam, S., I. Tsao, A. Sakoda, and H. Wang. 1988. Techniques for preparing hydrogen membrane capsules. *Biotechnol. Tech.* 2:271.
- Redenbaugh, K., J. Fujii, D. Slade, P. Viss, and M. Kossler. 1991. Artificial seeds—Encapsulated somatic embryos: Biotechnology in agriculture and forestry, p. 395-416. In: Y.P.S. Bajaj (ed.). *High-tech and micropropagation*. Springer-Verlag, New York.
- Redenbaugh, K., B., Paasch, J. Nichol, M. Kossler, P. Viss, and K. Walker. 1986. Somatic seeds: Encapsulation of asexual plant embryos. *Bio/Technology* 4:797-801.
- Rybczynski, J., R. Simonson, and P. Baenziger. 1991. Evidence for microspore embryogenesis in wheat anther culture. *In Vitro Cell. Dev. Biol.* 27:168-174.
- Simonson, R. and P. Baenziger. 1992. The effect of gelling agents on wheat anther and immature embryo culture. *Plant Breeding* 109:211-217.
- Sorvari, S. 1986a. Comparison of anther cultures of barley cultivars in barley-starch and agar gelatinized media. *Ann. Agr. Fenn.* 25:249-254.
- Sorvari, S. 1986b. The effect of starch gelatinized nutrient media in barley anther cultures. *Ann. Agr. Fenn.* 25:127-133.
- Sorvari, S., K. Ahanen, H. Hiirsalmi, R. Pessala, and S. Tuominen. 1994. Conversion of ABA treated carrot somatic embryos after desiccation, p. 301. *Abstr. XXIVth Intl. Hort. Congr., Intl. Soc. Hort. Sci., Kyoto, Japan.*
- Sorvari, S. and O. Schieder. 1987. Influence of sucrose and melibiose on barley anther cultures in starch media. *Plant Breeding* 99:164-171.
- Sung, Z. and R. Okimoto. 1981. Embryonic proteins in somatic embryos of carrot. *Proc. Natl. Acad. Sci.* 78:3683-3687.
- Tiwari, S. and I. Rahimbaew. 1992. Effect of barley starch in comparison and in combination with agar and agarose on anther culture of *Hordeum vulgare* L. *Curr. Sci.* 62:430-432.
- Tremblay, L. and F. Tremblay. 1991. Effects of gelling agents, ammonium nitrate, and light on the development of *Picea marina* (Mill) B.S.P. (black spruce) and *Picea rubens* Sarg. (red spruce) somatic embryos. *Plant Sci.* 77:233-242.
- Zimmerman, R., S. Bhardwaj, and I. Fordham. 1995. Use of starch-gelled medium for tissue culture of some fruit crops. *Plant Cell Tissue Organ Cult.* 43:207-213.