FAL
Bundesforschungsanstalt
für Landwirtschaft

Aus dem Institut für Pflanzenbau und Grünlandwirtschaft
Und dem
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Manuskript, zu finden in www.fal.de

Published in: Landbauforschung Völkenrode 53(2003)1, pp. 53-59

Braunschweig
Bundesforschungsanstalt für Landwirtschaft (FAL)
2003

Regeneration of plants from cell suspension cultures and encapsulated cell suspension cultures of *Solanum tuberosum* L. cv. Clarissa

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Abstract

Callus cultures of the cultivar Clarissa were initiated from the end pieces of internode explants producing somatic embryos. The chopped somatic embryos and the basis produced soft callus when cultured on solid Murashige and Skoog media containing 1 mg/L 2,4-dichlorophenoxyacetic acid. Cell suspension cultures were established from these calli. They contained small cell clusters and single large vacuolated cells of variable shape. The embryogenic potential was tested on solid media. Four cell lines could be selected which maintained their embryogenic potential over a long period of time. Regenerates transferred to the greenhouse produced plants and tubers not distinguishable from the control plants.

The cell lines did not develop somatic embryos when cultured in hormone-free medium. Somatic embryos formed only in those cultures that were inoculated with a low number of cells. Important were a high concentration of zeatin (2.5 mg/L) and a low level of polyphenols. After transfer on hormone-free medium the somatic embryos regenerated in vitro plants.

The yield of somatic embryos could be raised considerably when cells had been encapsulated in hollow beads. Capsules were cultivated in liquid media that contained first auxins in high concentration and then cytokinines. After 4 weeks capsules were cut in halves and cultivated further. Embryo formation was not only observed on solid medium but also in shaken cultures in which they developed further into plantlets.

The encapsulation system offers simple means to test phytohormone combinations and concentrations. In addition, the medium can be dosed better and inhibiting substances produced by the cells removed more easily when cells are encapsulated. The entrapment of cells in hollow alginate beads allows testing conditions for the mass production of somatic embryos in bioreactors.

Keywords: cell suspension culture; encapsulation; hollow beads; potato; regeneration; Solanum tuberosum L.; somatic embryos

Zusammenfassung

Regeneration von Pflanzen aus Zellsuspensionskulturen und verkapselten Zellsuspensionskulturen von *Solanum tuberosum* L., Sorte Clarissa

Aus den Endstücken von Internodiensegmenten, an denen somatische Embryonen erzeugt worden waren, wurden Calluskulturen angelegt. Dazu wurden die zerschnittenen Embryonen und die abgetrennte Ansatzstelle der Embryonen am Internodienexplantat auf Murashige und Skoog Festmedium aufgelegt, das 1 mg/L (2,4-Dichlorphenoxy)-essigsäure enthielt. Die daraus hergestellten Suspensionskulturen bestanden aus kleinen Zellaggregaten und großen vakuolisierten Einzelzellen. Das embryogene Potential wurde auf Festmedium getestet. Von der Sorte Clarissa konnten 4 stabile Zellinien selektiert werden. Regenerierte Pflanzen, die im Gewächshaus angezogen wurden, unterschieden sich nicht von den Kontrollpflanzen.

Die selektierten Zellinien bildeten keine somatischen Embryonen in hormonfreien Medien aus. Sie entwickelten sich nur dann, wenn die Kulturen mit einer niedrigen Zellzahl überimpft worden waren. Entscheidend waren eine hohe Zeatinkonzentration (2,5 mg/L) und eine niedrige Polyphenolbildung. Die daraus regenerierten In-vitro-Pflanzen hatten ein normales Aussehen.

Eine große Anzahl an somatischen Embryonen wurde erhalten, wenn die Zellen zuvor in Hohlkugeln verkapselt worden waren. Die Kapseln wurden in einem Flüssigmedium mit hoher Auxinkonzentration kultiviert, das dann durch ein Medium mit hoher Cytokininkonzentration ersetzt wurde. Nach insgesamt 4 Wochen wurden die Kapseln halbiert und weiterkultiviert. Somatische Embryonen bildeten sich nicht nur auf Festmedium aus, sondern auch in großer Anzahl in den geschüttelten Kulturen, in denen sie sich auch zu Pflänzchen weiterdifferenzierten.

Die Verkapselung der Zellen ist ein einfaches System, Phytohormonkombinationen und -konzentrationen zu testen. Darüber hinaus können die Kulturmedien besser und dosierter ausgetauscht werden. Auch hemmende Stoffe, die von den Zellen produziert werden, lassen sich leichter entfernen. Das Verkapselungssystem ist daher geeignet, die Kulturbedingungen für eine Massenproduktion von somatischen Embryonen in Bioreaktoren vorher zu untersuchen.

Schlüsselworte: Hohlkugeln; Kartoffel; Regeneration; Solanum tuberosum L.; somatische Embryonen; Verkapselung; Zellsuspensionskultur

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1 Introduction

Potato is a vegetatively propagated crop. Therefore, the development of efficient methods for a large-scale production of plants is the aim of several groups. Somatic embryos could have the potential for industrial application.

There are several reports on the formation of somatic embryos on asexual explant tissues of tetraploid potato cultivated on solid medium: tuber discs (Bragdø-Aas, 1977; Lam, 1977), node explants (García and Martínes, 1995), and leaf tissue (JayaSree et al., 2001). The formation in liquid medium has been described for the first time by Fiegert et al. (2000). High yield of somatic embryos on various potato tissues from a range of genotypes and ploidy levels has been reported by Seabrook and Douglass (2001). The strong link of the somatic embryos with the original explant tissue, however, does not allow a large-scale production in bioreactors. For this process cell suspension cultures producing somatic embryos would be more suitable.

This paper shows that it is possible to obtain somatic embryos from cell suspension cultures of potato and offers means to optimize the medium requirements.

2 Material and Methods

2.1 Culture media and chemicals

Culture media were obtained from Duchefa. The full medium (MS) contained the macro elements, micro elements and vitamins (Murashige and Skoog, 1962) and 30 g/L sucrose. The modified medium (MS3) contained half of the concentration of NH_4NO_3 and KNO_3 of the original MS medium. The propagation medium (VM) contained the salts of the MS medium and 20 g/L sucrose. Bacto^{TM} Agar DIFCO was obtained from Becton Dickinson and was used in the concentration of 7 g/L. The phytohormones 6-benzylaminopurine (BA), gibberellic acid A_3 (GA_3), indole-3-acetic acid (IAA), zeatin and zeatin riboside were obtained from Sigma, 2,4-dichlorophenoxy-acetic acid (2,4-D) from Merck. Media containing IAA, zeatin or zeatin riboside were filter sterilized.

Sodium alginate (Protanal LF 20/40) was obtained from FMC BioPolymer and was used in the concentration of 0.5 % (w/v). Carboxymethylcellulose (Blanose 7MXF) was obtained from Hercules. A 2.5 % (w/v) solution was prepared in 2.5 % (w/v) $CaCl_2$. Both solutions were autoclaved for 15 min allowing fast cool down.

2.2 Plant material and culture conditions

In vitro plants of the cultivar Clarissa were propagated by single-node cuttings. Four explants were cultured in 175 ml sterile polystyrene plant container (Greiner) containing 20 ml solid VM medium. The culture room had a temperature of 20-22 °C and a 14 h (day)/10 h (night) photoperiod. Plants used for embryo production were precultured 3 weeks on solid MS medium. The same medium was used for regenerated plantlets. Before planting out, shoot tips of the regenerates and of in vitro control plants were transferred to solid VM medium and cultured for 2 weeks. Plantlets were directly planted into pots (ø 16 cm) and covered with translucent plastic cups during the first week. The greenhouse had a temperature of 22 °C (day) and 18 °C (night). For documentation photographs were taken during growth and after harvest.

2.3 Initiation and maintenance of cell suspension cultures

Embryo production on internode explants was induced according to Seabrook and Douglass (2001) by placing them in a horizontal position on solid MS medium containing 3.3 mg/L IAA and 30 µg/L BA. After one week they were transferred to solid MS medium containing 2.5 mg/L zeatin, 10 µg/L IAA and 0.2 mg/L GA_3. After culture for three weeks the end pieces of the internode explants producing somatic embryos (Fig. 1) were cut off. The chopped somatic embryos and the basis on which embryos developed were cultured on solid MS medium containing 1 mg/L 2,4-D. After 3-4 weeks heterogeneous calli were transferred to fresh medium. After further culture of 3-4 weeks calli (2-3 g) were suspended in 10 ml of MS3 medium containing 0.5 mg/L 2,4-D in100 ml Erlenmeyer flasks. Plantlets regenerated were removed before starting the cell suspension cultures and also during culture. After one week 5 ml fresh medium was added and after an additional week 10 ml. After a total culture time of three weeks the cultures were transferred to 200 ml Erlenmeyer flasks containing 25 ml of the same medium. One week later the cultures were passed through sieves (12 mesh) to remove large cell aggregates. Aliquots of the filtrate were tested for their ability to produce somatic

Fig. 1:
End piece of internode segment developing somatic embryos

embryos. The filtrate was concentrated down to 5 ml by removing the supernatant after settling of cells and cultured in 100 ml Erlenmeyer flasks after 10 ml fresh medium had been added. Fresh medium was added in weekly intervals. Contents of flasks were split or transferred to 200 ml Erlenmeyer flasks depending on the volume and cell concentration. Those cell lines producing somatic embryos on the test plates were selected and subcultured in two weekly intervals using a quarter of the culture as inoculum. The culture room had a temperature of 23 ± 1 °C and a 16 h (day)/8 h (night) photoperiod. Erlenmeyer flasks were cultured on a gio gyratory shaker (New Brunswick Scientific) at 150 r/min.

2.4 Agar test to detect cell lines with embryogenic potential

2 ml of cell suspension culture are dropped evenly on filter paper (ø 8 cm) that is firmly placed on solid MS3 medium containing 3.3 mg/L IAA and 30 µg/L BA. After culture for 3-4 days the filter paper is placed upside down on solid MS3 medium containing 2.5 mg/L zeatin, 10 µg/L IAA and 0.2 mg/L GA_3. The filter paper is pressed gently and evenly to the surface and then pulled off slowly leaving cells and cell clusters on the surface of the medium. Cell clusters containing cells with embryogenic potential start to develop visible somatic embryos after 3 weeks of culture.

2.5 Encapsulation of cell suspension cultures

The encapsulation technique has been described (Patel et al., 2000). The gelation time was 10 ± 2 min. The hollow beads (capsules) had a diameter between 5.2-5.7 mm and a wall thickness of 0.35 - 0.48 mm.

3 Results and Discussion

Suitable tissues to initiate callus cultures for cell suspension cultures were the end pieces of internode explants producing somatic embryos (Fig. 1). The chopped somatic embryos and the basis produced soft callus when cultured on solid MS media containing 1 mg/L 2,4-D. The calli were heterogeneous and produced plantlets (Fig. 2). They were removed when starting the cell suspension cultures. When placed on hormone-free medium they developed into in vitro plants not distinguishable from the in vitro stock plants. During the first cycles of the cell suspension cultures plant regeneration was not completely suppressed by 0.5 mg/L 2,4-D but decreased with every subculture until it finally ceased. Also those plantlets developed into normal in vitro plants when plated on hormone-free solid medium.

The resulting cell suspension culture was very heterogeneous containing large aggregates comprising parts of the original callus surrounded by a meristematic layer, small cell clusters and single large vacuolated cells of variable shape. Free plasmatic cells supposed to be precursors of embryogenesis could not be detected. However, they were found in the meristematic layer and in small cell clusters. By simple microscopic observation it could not be determined whether they were pro-embryogenic centers or meristematic centers giving rise to callus cells. If the findings of the well-studied carrot system (Jones, 1974) are true also for potato cultures then it cannot be expected that transition from one cell type to another will occur.

After the large parts had been removed by sieving the cultured filtrate contained mainly small cell aggregates and single vacuolated cells. The cultures kept this kind of cell distribution during subcultures thus staying finely dispersed. The growth rate was moderate requiring subculture every other week when a quarter of the culture was used as inoculum.

Since sieving of the culture and random sampling on subculture could result in the loss of those meristematic cells with embryogenic potential aliquots of the cell suspension cultures were routinely tested. Fig. 3 shows a positive result of a selected cell line. Without doubt, it is a disadvantage to have to maintain the cultures for 3 or 4

Fig. 2:
Embryogenic end pieces producing callus

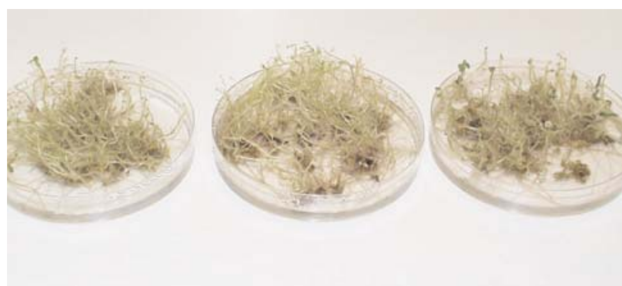
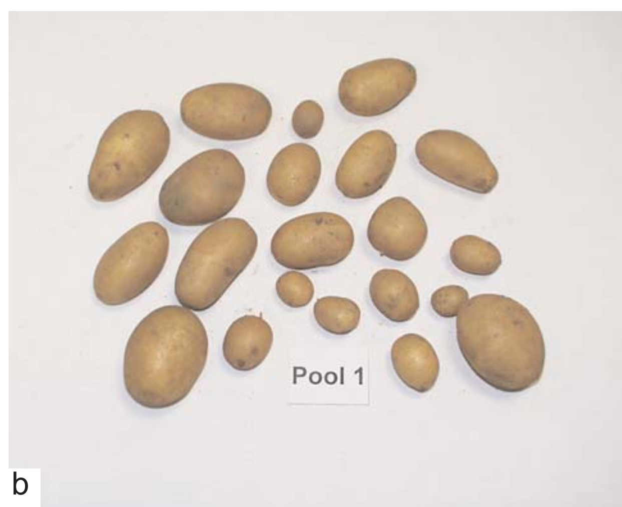
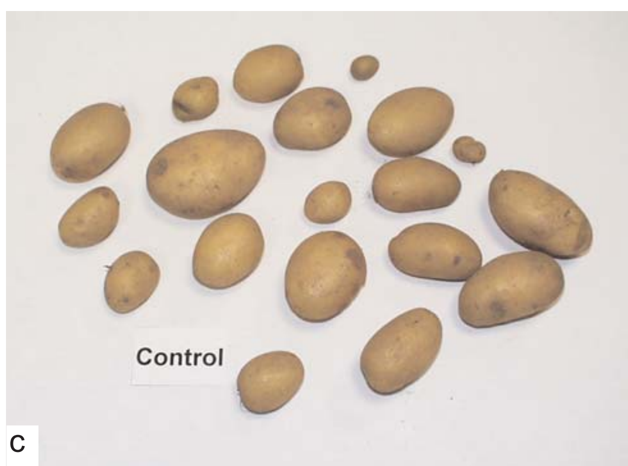


Fig. 3
Positive test result of cell line 2-5





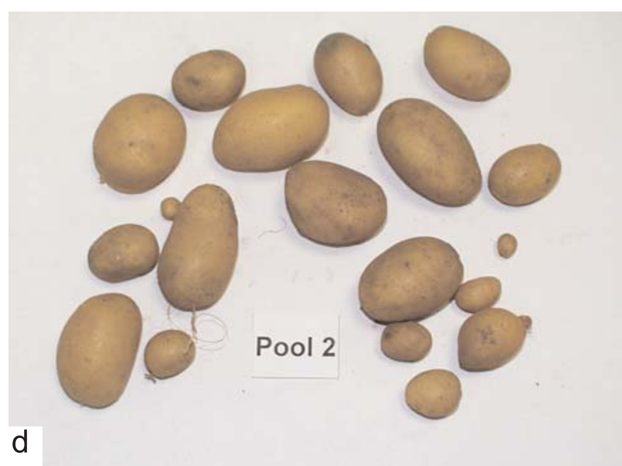


Fig. 4:
Regenerates planted out
a: 4 weeks after planting
b: harvested tubers of "normal" regenerates
c: harvested tubers of control plants
d: harvested tubers of initially tiny regenerates

weeks until the somatic embryos are visible but there is no faster test available at the moment. In this way four cell lines of the cultivar Clarissa could be selected which maintained their embryogenic potential over a long period of time.

To test how the regenerates develop further a randomly selected bunch of developing plantlets shown in Fig. 3 was transferred to hormone-free solid medium. 68 of the 76 plantlets in total grew normally, two regenerates did not grow further and 6 regenerates gave rise to only dwarfish in vitro plants. These six plantlets were planted out in pots (ø 16 cm), likewise 4 in vitro plantlets of the stock and 20 of the 76 normally growing regenerates. Pots were arranged randomly and not rearranged during culture. They were covered with translucent plastic cups during the first days. All plants grew vigorously. Already after 2 weeks no differences could be detected between the originally dwarfish plantlets, the normal plantlets and the control plantlets. Fig. 4a shows a photo taken 4 weeks after planting. Also the tubers of the three groups did not differ from each other (Fig 4b, c, and d). Though only a limited number had been planted out the experiment shows that the danger to obtain abnormal plants from these cell suspension cultures is not very high provided that the regenerates develop normally in vitro.

Other than embryogenic carrot cell suspension cultures the potato cultures did not develop recognizable somatic embryos when cultured in hormone-free medium. Either they contain endogenous auxins inhibiting embryogenesis or they might even require a boost of a high concentration of auxins for induction of embryogenesis. Most likely potato cultures require a different protocol than carrot cultures. For the development of somatic embryos in potato cultures cytokinines in the medium seem to be beneficial. Though BA has been used successfully zeatin or zeatin riboside may be more powerful. Not quite clear is the role

of GA_3. Jarret et al. (1981) found an inhibitory effect on tuber explants when used in the initial phase. However, during maturation of somatic embryos it can be beneficial or at least has no negative effect.

First attempts to produce somatic embryos in cell suspension cultures considered these parameters. Cell suspensions in the late log-phase were sieved through filters (25 mesh) to remove cell debris and the major part of the highly vacuolated single cells. The residues were mixed and distributed evenly on the different trials. Trials were performed both with and without induction. For induction cells were either plated on filter paper on solid MS-medium containing 3.3 mg/L IAA and 30 µg/L BA or suspended directly into the corresponding liquid medium. After 3-4 days of culture the cells were either placed directly in the test medium when the induction had been performed on solid medium or were concentrated first by filtration when the induction took place in liquid medium. The test media contained different concentrations of either zeatin or zeatin riboside either in combination with GA_3 or without GA_3. After two weeks the media were exchanged against media containing the same concentration of zeatin or zeatin riboside and GA_3.

Characteristic for all experiments was the browning of cultures and media probably due to polyphenols. The intensity of browning correlated rather with the cell number than with the treatment. Since high concentrations of polyphenols can inhibit growth and development, it was not surprising that somatic embryos developed only in those experiments that were inoculated with a low number of cells. Fig. 5 shows a small cell cluster undergoing differentiation. Somatic embryos developed irrespective of induction with auxins or presence of GA_3 in the medium. Important were a high concentration of zeatin (2.5 mg/L) and a low level of polyphenols. After transfer on hormone-free medium the somatic embryos developed into in vitro plants not distinguishable from in vitro control plants.

Fig. 5:
Somatic embryos developing in cell suspension cultures

To facilitate medium exchange cells were encapsulated in hollow beads. This was also done to possibly reduce polyphenols under the assumption that they can diffuse through the capsule wall. That calcium alginate hollow beads do not have any negative effect on growth and differentiation of plant cells and explants has been reported by Patel et al. (2000).

The experiment was performed with 3 cell lines doing the cell line 2-5 twice. They were finely dispersed, were light green or light beige-green in color, and were used one week after the last subculture. The same protocol was used for all 4 experiments. For the first week they were cultured in a high concentration of auxins (3.3 mg/L IAA and 30 µg/L BA). In the second week they were treated with a high concentration of zeatin (2.5 mg/L zeatin and 10 µg/L IAA). In the third week the medium contained also GA_3 (2.5 mg/L zeatin, 10 µg/L IAA, 0.2 mg/L GA_3). For the fourth week the same medium was used.

After 4 weeks culture time in total, the capsules were harvested. Capsules of all 4 experiments were densely packed with cells. Cells were brown in color. No kind of differentiation was visible under the stereomicroscope neither on the surface nor in the inside after cutting them in halves.

To see whether the cells were still alive or would even be able to develop somatic embryos 50 capsules of each experiment were cut in halves which were placed on solid MS3 medium containing 2.5 mg/L zeatin, 10 µg/L IAA and 0.2 mg/L GA_3 (A). 50 capsules of each experiment were further cultured in fresh liquid medium containing the same concentration of phytohormones (B) and 50 capsules of each experiment were first cut in halves before culturing in fresh liquid medium (C). The results are summarized in Table 1.

All cell lines had kept the potential to produce somatic embryos. In treatment A first development of somatic embryos could be observed macroscopically after three weeks of culture. Development continued and reached the maximum after six weeks of total culture time. An example can be seen in Fig. 6. Only 7 half-capsules of the cell line 3-3 produced somatic embryos. Also in the first

Table 1:
Embryo and plant formation of encapsulated cell suspension cultures of the cultivar Clarissa

Cell Line	A	B	C
3-3	7 / 100	27 / 60	cont.
2-4	80 / 100	39 / 40	115 plants/20
2-5	22 / 100	19 / 20	598 plants/40
2-5	75 / 100	78 plants/100	>500 plants/100

Numbers represent half-capsules producing somatic embryos per cultured half-capsules or developing plantlets per cultured half-capsules.

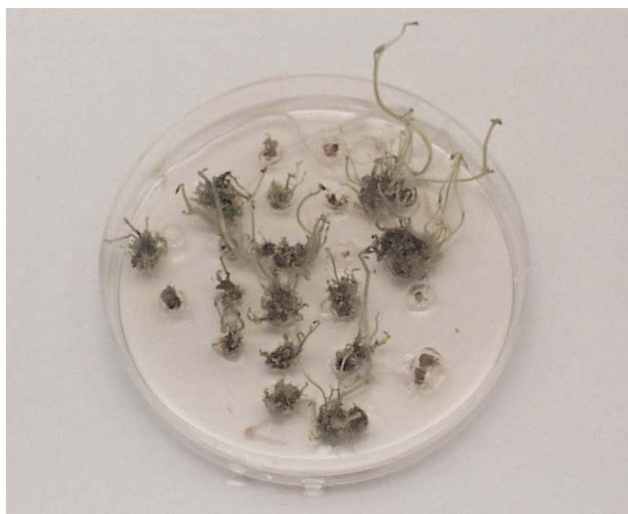


Fig. 6:
Half-capsules developing somatic embryos and plantlets

experiment with cell line 2-5 the production rate was low. The highest number of half-capsules with somatic embryos was found using cell line 2-4 and in the second experiment with cell line 2-5.

Those capsules cultured in liquid medium (treatment B) were harvested after another two weeks of culture since no kind of differentiation could be observed and since due to secondary infections some cultures had already been lost. The capsules of the remaining flasks were cut in halves and those of cell lines 3-3, 2-4 and the first experiment with cell line 2-5 were cultured in the same way as treatment A on solid medium. Only the half-capsules of the second experiment of cell line 2-5 were cultured in liquid medium containing 0.1 mg/L zeatin ribosid and GA_3. After 4 weeks of culture a high number of half-capsules regenerating somatic embryos were found on solid medium for all cell lines. Embryo formation was not only observed on solid medium but also in the shaken culture of the cell line 2-5. On further culture they developed into plantlets (Fig. 7). 78 well-developed in vitro plantlets could be counted.

At the same time plantlet development was also observed in the cultures of treatment C. The cultures of cell line 3-3 had to be discarded due to infection. This was also the case for several cultures of the other two cell lines. Due to limited space on the shaker the half-capsules of cell line 2-4 and the first experiment of cell line 2-5 were cultured stationary in petri dishes containing 2.5 mg/L zeatin, 10 µg/L IAA and 0.2 mg/L GA_3. 115 developing plantlets could be counted in the dish containing 20 half-capsules of cell line 2-5 and 598 in total of cell line 2-5 from which 40 half capsules were cultured.

The half-capsules of the second experiment with cell line 2-5 were cultured in portions of 50 in 1 L Erlenmeyer flasks in MS3 medium containing 0.1 mg/L zeatin riboside and 0.1 mg/L GA_3. Numerous somatic embryos were

Fig. 7:
Plant regeneration in liquid culture

Fig. 8:
Mass production of somatic embryos

Fig. 9:
Developing plantlets from somatic embryos produced in liquid culture

found in both flasks, which developed further into plantlets (Fig. 8). According to a rough estimate there were more than 500 plantlets. 112 selected plantlets were transferred to hormone-free solid medium. 88 of them developed very fast (Fig. 9) whereas 24 showed in the beginning a retarded root growth and accumulation of anthocyanins in the leaflets. When the shoot tips of these plantlets were transferred to fresh medium the newly growing plantlets had a normal appearance not distinguishable from the in vitro control plants.

The experiments with the encapsulated cell suspension culture show that selected cell lines are capable of producing somatic embryos in high amount. Why the yield is much higher than in the same cell lines not encapsulated is not completely understood. One reason might be that an artificial cell tissue is formed by the encapsulation promoting the differentiation of somatic embryos. More likely, however, is the explanation that the medium can be better balanced and inhibiting substances produced by the cells removed more easily when cells are encapsulated.

Since there is still so little knowledge about the medium requirements for the production of somatic embryos in cell suspension culture of potato, the entrapment of cells in hollow calcium-alginate beads offers simple means to test phytohormone combinations and concentrations. In addition, the encapsulation system allows a controlled medium exchange that makes the method useful to test conditions for the mass production of somatic embryos in bioreactors.

Acknowledgements

We thank Bioplant GmbH, Ebstorf, Germany for providing in vitro plants of the cultivar Clarissa. This research project (12209 N/1) was funded by the Federal Ministry of Economics and Labour (BMWA) through the German Federation of Industrial Cooperative Research Associations "Otto von Guericke" e.V. (AiF).

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