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Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* L.

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Summary

A new method is presented for anther culture. Anthers are cultivated on cubes of solid medium, surrounded by liquid medium. This allows changes of media composition at any time. When embryos or callus were produced, the liquid initiation medium was removed and replaced with regeneration medium. The best yield of embryos and the best regeneration frequency were obtained when the initiation medium contained 2,4-dichlorophenoxyacetic acid (2,4-D). Regeneration of shoots from callus was stimulated on double layer medium, with the callus placed on top of a solid medium, partly submerged in the overlaying liquid medium. The best gelling agent for shoot multiplication media was agarose, but gellan gum was a good alternative. Both the production of flower buds and embryogenesis in anther cultures was inhibited by an increased concentration of CO_2 in the air surrounding the donor plants.

Introduction

Low yield of embryos and regenerated shoots has often been a problem in the production of haploids by anther culture of potato. This is probably caused by genetical differences between genotypes but also by non-optimal cultivation methods (Johansson, 1986). It is thus important to consider all events during anther culture and regeneration. In earlier papers, we have demonstrated the importance of an increased concentration of CO_2 in the atmosphere surrounding the cultured anthers (Johansson et al., 1982, Johansson & Eriksson, 1984). Here, results are presented from experiments where the CO_2 concentration had been increased in the greenhouse in which donor plants were cultivated.

It is necessary to avoid physical injuries to anthers or embryos during inoculation or subculturing and we present a method which allows for changes of media at any time during incubation without disturbing anthers or embryos. The effects of the growth substances 2,4-D and zeatin in the anther culture medium, and of different gelling agents in the shoot multiplication, are also demonstrated.

Material and methods

Plant material

a) Flower buds from cvs Elin and Stina were used for anther cultures. The buds were picked when most of the pollen grains were at late tetrad-stage or uninucleate, and

cold treated for 7 days at 7 °C (Johansson, 1986).
b) Callus from anther cultures of several dihaploid clones (produced in anther cultures of cv. Pito) was used in the regeneration experiments.
c) Sterile shoot cultures of cv. Elin and the breeding line P134 were used when the effects on growth of different gelling agents were studied.

Experiments

1. The cube method for anther culture and regeneration. MS-medium (Murashige & Skoog, 1962) with 6 % sucrose was solidified wih 0.4 % gellan gum (Catalogue entry K9A40, Kelco Co Ltd, Rahway, USA) and 5 cubes (10×10×8 mm) of this medium were placed in petri dishes (Ø = 5 cm) to which was also added 5 ml liquid MS-medium with different concentrations of 2,4-D or zeatin. An anther of cv. Elin was placed on top of each cube, and the petri dishes were sealed and incubated in a 12-h light (7 W/m^2) regime at 25 °C.

After 7 days the liquid medium was replenished and when embryos or callus appeared it was replaced with K3-medium (Nagy & Maliga, 1976) with 1 % sucrose, 1 mg/l zeatin, 1 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l indoleacetic acid (IAA).

2. The double layer method for regeneration. Petri dishes (Ø = 5 cm) were provided with 5 ml K3-medium with the same composition as described above but solidified with 0.4 % gellan gum. On top of this solid medium 3 ml liquid K3-medium was poured. Callus, which had failed to regenerate when cultivated only on solid regeneration medium of the same composition, was placed on top of the solid medium, partly submerged in the liquid medium. The cultures were incubated as described under 1. and were subcultured at intervals of 3 weeks.

3. The importance of different gelling agents in shoot multiplication medium. MS-medium, with 1 % sucrose but without growth substances, was solidified with 0.3 % gellan gum, 0.4 % agarose (Sigma) or 0.8 % agar (Difco-Bacto). Unrooted shoots of 30 mm length were cultivated on these media to compare shooting and growth. The cultures were incubated in a 12-h light (7 W/m^2) regime at 25 °C for 4 weeks.

4. Effects of an increased concentration of CO_2 on donor plants and anther cultures. The concentration of CO_2 was increased to 2 % in the glasshouse, where donor plants of cv. Elin and cv. Stina were cultivated. Equal numbers of control plants were simultaneously cultivated in the same conditions, but without increased CO_2-concentration. Anther cultures were started from this material, according to the double layer method for anther cultures described by Johansson et al. (1982). Anthers from surface-sterilized flower buds from these plants were transferred aseptically to petri dishes, where they floated on the liquid medium, 5 anthers/dish; the dishes were sealed with Nescofilm and the cultures incubated for 10 weeks in a 12-h light (7 W/m^2) regime at 25 °C.

Results and discussion

The cube method

The best yield of embryos was obtained when anthers were cultured on hormone-free medium for one week and on medium with 2,4-D during the remaining incubation time. Less good results were obtained if 2,4-D was present during the whole culture period or only during the first week, and also when zeatin was used instead of 2,4-D. When zeatin was present during the entire culture period, embryogenesis was totally inhibited. Anthers, cultivated on hormone free medium during the whole incubation period, produced few embryos (Table 1).

During the following regeneration period, all embryos produced on media with 2,4-D continued to grow and 65 % developed shoots whereas on media without 2,4-D, only 5 % produced shoots. These results can also be compared to earlier experiments with cv. Elin, when more than 400 embryos only produced 1 shoot (Johansson, 1986). I conclude that 2,4-D stimulated both the development of proembryos that were initiated during the first week of incubation, and, later, shoot production.

The cube method offers several advantages compared to conventional anther culture methods: a) there is no need to transfer anthers or embryos and the hormone balance may conveniently be adjusted at any time during the incubation; b) when the liquid media are changed, inhibitors will be removed; c) due to the comparatively slow diffusion in the cube, the living tissues are not exposed to the chemical 'shock' that otherwise may occur when they are transferred to media with different composition; d) physical damage is minimized.

Regeneration on double layer medium. After 8 weeks of incubation and 2 subcultures, more than 80 % of the calluses had regenerated shoots. The success of this method, compared to incubation on solid medium, is probably caused because: a) the living tissues have increased access of nutrients, due to the presence of liquid medium; b) if inhibitors (e.g., phenolics) are produced by the living tissues, they diffuse away more rapidly in the liquid medium, compared to the solid medium.

Table 1. Number of embryos (E) per 100 flower buds produced in anther culture of cv. Elin using the cube method.

Week 1		Remaining period		E
2,4-D	zeatin	2,4-D	zeatin	
-	-	-	-	3.6 a
+	-	-	-	6.9 b
-	-	+	-	27.6 c
+	-	+	-	11.1 d
-	+	-	-	6.5 b
-	-	-	+	11.1 d
-	+	-	+	0.0 e

Hormone concentrations are at 1 mg/l. Letters indicate E values which differ significantly (t-test, $P < 0.05$).

Table 2. Growth of shoot segments of cvs Elin and P 134 on shoot multiplication media solidified with agarose, gellan gum or agar. Growth was measured after 4 weeks.

Clone	Gelling agent	Increase of weight (%)	Number of shoots/culture	Number of roots/culture	Root length/ culture
Elin	Agarose	278	15.5	8.3	51
	Gellan gum	156	15.5	4.8	34
	Agar	105	8.8	2.8	26
P 134	Agarose	159	9.0	7.8	68
	Gellan gum	97	9.3	5.8	37
	Agar	91	7.3	5.0	33

Table 3. Embryogenesis in anther cultures of cvs Elin and Stina, where the donor plants were cultivated in a glasshouse with two CO_2 concentrations.

Cv.	CO_2 conc.	Number of flower buds	Number of embryos	Number of embryos/100 buds
Elin	Normal	246	58	23.6
	2 %	104	12	11.5
Stina	Normal	180	28	15.6
	2 %	88	0	0.0

The importance of different gelling agents. Shoot segments of both clones rooted more rapidly and the growth was promoted on both agarose and gellan gum, compared to agar (Table 2). The development of leaves was retarded on agar and the shoots were thin and elongated. This is not surprising, as agar often contains impurities (Kohlenbach & Wernicke, 1978; Johansson, 1983).

The best total growth was obtained on agarose, but gellan gum is a good alternative, especially as it is much cheaper.

Effects of an increased CO_2-concentration. Both the production of flower buds and, later, embryogenesis in anther cultures of the two cultivars were significantly inhibited (t-test, $p < 0.05$) by the increase of CO_2 (Table 3). I conclude that the concentration of CO_2 surrounding the donor plants affects embryogenesis in subsequent anther cultures, although the optimal concentration of CO_2 has not yet been sought.

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