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Comparison of agar and microcrystal cellulose as gelling agents for *in vitro* culture of *Nicotiana tabacum* stem explants

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Abstract

Investigation was made on a use of microcrystal cellulose as a new and inexpensive gelling agent instead of agar. Microcrystal cellulose in concentration 20 % forms a suitable structure of nutrient medium for *in vitro* cultivation. The higher humidity in the culture container with microcrystal cellulose causes partial vitrification of *Nicotiana tabacum* L. plants, cv. Zlatna arda. It is proved by reduced chlorophyll content, changes in protein synthesis and strongly reduced isoenzyme spectrum of peroxidase.

Introduction

In vitro cultivation requires a nutrient medium with a definite mechanical stability. Agar and gelatine are ones of the most common used components of the coloid system which gave an appropriate structure of the nutrient medium. We propose a substitution of agar for a new gelling agent - microcrystal cellulose, which has got a limited stage of polymerisation. The gelling agent often causes morphological and physiological changes of in vitro plants. This study was undertaken to identify some differences between plants cultivated on agar solidified medium and those ones grown on microcrystal cellulose solidified medium. It was discussed the effect of repeated use of microcrystal cellulose gel.

Material and methods

Gelling agents: Agar (*Laborant*, Austria); microcrystal cellulose (Institute of Chemical Technology, Sofia, Bulgaria).

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Abbreviations used: d.m. - dry mass; EHF - easy-hydrolyzing fraction; HHF - hardly-hydrolyzing fraction; MDP - medium degree of polymerization; MCC - microcrystal cellulose.

The investigations were performed with natural microcrystal cellulose (Table 1) produced from sulphate cellulose by means of hydrolytic destruction with 1 % HCl, hydromodule 1:10 at 130 °C for 60 min (Sokolov *et al.* 1973). The obtained suspension of microcrystal cellulose (Sokolov 1988) was washed out with distilled water to neutral reaction. The natural microcrystal cellulose was characterized with the content of α-cellulose (Obolenska *et al.* 1975), easy hydrolyzing fraction and hardly hydrolyzing fraction (Emelianova 1976), medium degree of polymerisation (Obolenska *et al.* 1975) and humidity.

Table 1. Characteristics of microcrystal cellulose.

Characteristics		Value
Content of α-cellulose [%]		99.95
Content of EHF [%]		-
Content of HHF [%]		99.95
MDP		180
Crystalline degree		0.70
Sulphate ash [%]		0.05
Dimensions of particles:	length [μm]	92.2
	width [μm]	10.1
Form of particles		needle shape
Specific surface [$cm^2 g^{-1}$]		26

Plant material: Stem explants of *in vitro* plants of *Nicotiana tabacum*, cv. Zlatna arda were cultivated *in vitro* in the solid medium of Murashige and Skoog (1962) without growth regulators. The medium was stabilized by 0.7 % Agar, 20 % MCC gel - first use or by 20 % MCC gel - second use. The second MCC gel is prepared by washing MCC gel after first use with distilled water to neutral reaction (pH 7.0).

The pH of all the media was approximately 5.2 when prepared and all were adjusted to 5.8 prior to adding the gelling agents. Cultures were grown for three weeks under 16-h photoperiod and day/night temperature of 24 ± 1 °C.

In vitro plants were characterized by height (cm), colour of leaves (visually), vitrification (based on appearance), rooting (visually), and biochemical analyses.

Protein was determined using the protein dye binding method described by Bradford (1976). Bovine serum albumin (*Serva*) was used as protein standard. Chlorophyll content was determined as previously described (Shlyk 1968) and reading A_{663} and A_{645} in the resulting extracts. PAGE of proteins was carried out using the method of Krochko and Bewley (1988) in a vertical slab apparatus, using a constant current of 4 mA per lane. Peroxidase patterns were stained with 4-chloro-1-naphtol (*BRL*, USA). SDS-PAGE of proteins was performed in 10 % polyacrylamide gel (165 × 120 × 1 mm) according to the method of Laemmli (1970). Separations were carried out for 4 h at the constant current of 4 mA per lane. The gel was stained with Coomassie Brilliant Blue *R 250*. The molecular mass standards (*Serva*, Germany: M_r 29 - 92.5 kDa) were used as markers.

Results and discussion

The nutrient medium with MCC makes the tube walls partially untransparent during the autoclaving. For this reason the explants and *in vitro* plants receive less light than on agar nutrient medium. On the other hand MCC retains more water after autoclaving which results in higher humidity in the tube atmosphere as compared with agar medium. These peculiarities influence on the phenotypical characteristics of *in vitro* plants and cause specific morphological and physiological disorders (Ziv 1991).

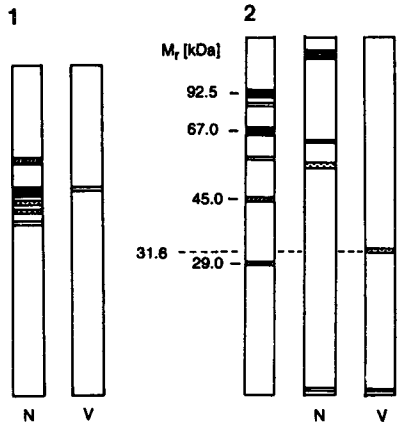

Fig. 1. PAGE of acid peroxidase extracted from leaves of *Nicotiana tabacum*, stained with 4-chloronaphtol. *N* - normal leaves; *V* - vitreous leaves.
Fig. 2. Proteins extracted from normal (*N*) and vitreous (*V*) leaves of *Nicotiana tabacum* separated on SDS - PAGE. M_r markers are indicated.

In vitro plants cultivated on MCC medium are higher than those on agar medium and have got more nodes (Table 2). This is, probably, the result of less light and of higher humidity in the tubes. The growth of *in vitro* plants cultivated on repeatedly used MCC medium is more intensive than on MCC used for the first time. It may be due to the absorption properties of MCC. Some nutrient components saturate the MCC absorption centers which are locked during the second use. It is to be confirmed by additional experiments.

Rooting of *in vitro* plants is better in agar medium than in MCC medium. There is a higher number of rooted plants in medium with MCC - "second use". We suggest this effect is due to decreased MCC absorption of the nutrient components from the medium.

Twenty percent of the plants cultivated on MCC medium are vitreous (Table 2). Vitrification caused by gelling agent was discussed (Pasqualetto 1988). The synthesis of chlorophyll *a* and *b* is reduced and their content in vitreous leaves is about 2.5 times smaller than in normal leaves (Table 3). Another result is a change in electrophoretic spectrum of peroxidase (Fig. 1). Protein level is lower in vitreous

Table 2. Characteristic of *in vitro* plants of *Nicotiana tabacum* L. cv. Zlatna arda, cultivated on MS nutrient medium, solidified by different gelling agents. Means of three replicates of all experiments with minimum twenty plants per each kind of gelling agent. Statistical significance by INSTAT: *** $p<0.001$.

Gelling agent	Average plant height [cm] ***	Plants with *n* number of nodes [%]								Plants with definite leaf colour [%]		Vitreous plants [%]	Well rooted plants [%]	Plants with definite kind of internodes [%]	
		n=1	2	3	4	5	6	7	8						
Agar 0.7 % (m/v)	4.2 ± 0.15	6	12	53	23	6	-	-	-	green pale green yellow	100 - -	-	94	normal short long	29 71 -
MCC 20 % (m/v) first use	5.9 ± 0.19	-	8	23	46	15	8	-	-	green pale green yellow	77 23 -	23	77	normal short long	31 54 15
MCC 20 % (m/v) second use	7.1 ± 0.21	-	-	-	53	35	-	12	-	green pale green yellow	71 29 -	17	82	normal short long	53 24 23

leaves and a 31.6 kDa protein is found to be present in vitreous but not in normal leaves (Fig. 2).

Table 3. Chlorophyll and protein content in leaves of *Nicotiana tabacum* cv. Zlatna arda cultivated *in vitro*. Means of 4 replicates. Statistical significance by INSTAT: * - $p < 0.05$, **** - $p < 0.0001$.

Physiological state of leaves	Chlorophyll *a* [mg g^{-1}(d.m.)]	Chlorophyll *b* [mg g^{-1}(d.m.)]	Protein [mg g^{-1}(d.m.)]
Normal	6.93 ± 0.13	3.29 ± 0.11	17.7 ± 1.46
Vitreous	2.76 ± 0.09 ****	1.33 ± 0.10 ****	13.8 ± 0.70 *

Our conclusion is that 20 % MCC can be used as a gelling agent forming a suitable structure of a nutrient medium. These results present a basis for further investigation to clarify the MCC properties as a new substrate for *in vitro* plant cultivation.

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