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Induction of Microtuberization for One-bud Potato Cuttings *in Vitro*

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ABSTRACT

The patterns and features of *in vitro* microtuberization of three *Solanum tuberosum* L. cultivars, 'Zhukovskij early', 'Nevskij' and 'Nikulinskij' were investigated. The optimum concentration of carbon sources (sucrose, glucose and fructose, singly or in combination) to maximize microtuberization in explants consisting of a single axillary bud and leaf (i.e. one-bud cuttings) were established. 'Zhukovskij early' responded best to sucrose, 'Nikulinskij' to fructose and 'Nevskij' to glucose or any 2-carbon mixture, with the lowest tuberization percentage in any case being 88%. These results were for solid, agarized medium. 'Nikulinskij' was then selected, base don the agarized medium trials, as the model plant and similar experiments were conducted in liquid medium with surprising observations: The tuberization index could be increased by incorporating glucose or fructose in the medium for stolon formation, resulting in more tubers and by incorporating sucrose larger and heavier tubers could form.

Keywords: multiplication factor, plant growth regulator, regeneration, *Solanum tuberosum*, stolon, sucrose, tuberisation
Abbreviations: 6-BAP, 6-benzylaminopurine; **CMP**, clonal micropropagation; **MS**, Murashige and Skoog medium

INTRODUCTION

Tuberization is the predominant type of reproductive development for tuberous plants. Tuberization in potato represents a composite morphogenetic process consisting of successive stages (Ewing and Struik 1992). The initiation and intensity of tuberization *in vitro* are primarily determined by cultivar genotype, photoperiod and medium composition (Melis and van Staden 1984; see review in this Special issue by Dobránszki *et al*.).

The weak link in the clonal propagation of potato plants by microcuttings is the transfer of plantlets from sterile culture to soil cultivation. The root system developed *in vitro* almost inevitably dies in soil where a new root system will develop, and at this stage plants may be most vulnerable, exhibiting poor growth and development. Under standard conditions, the planting of regenerated plants is accomplished manually, since greenhouse work cannot be mechanized. Teixeira da Silva *et al*. (2005) demonstrated, however, in a closely related plant, sweet potato, *Ipomoea batatas*, that by exposing developing plantlets growing *in vitro* to high CO_2 concentrations (3000 ppm) through a gas-permeable container, that the rooting and overall growth and morphology of plantlets could improve, that there were no special acclimatization requirements, and that *in vitro* plantlets could be transplanted to pots in a greenhouse with 100% survival. Another way of overcoming these difficulties is by using microtubers instead of plantlets. A microtuber refers to a potato tuber obtained under sterile conditions *in vitro* and having a size up to 10 mm.

The process of microtuber formation *in vitro* is an interesting physiological model for the analysis of potato growth and development in the case of healthy plantlets obtained through meristem cultivation (Yu *et al*. 2000). Two types of potato tuberization *in vitro* occur: on stolons or on axillary buds. Sometimes both are able to form plants. Axillary tuberization can occur depending on several factors: the level of sucrose in the medium, a sharp fall in temperature up to +2°C, the duration of the dark period or the physiological age or condition of cuttings (Schilde *et al*. 1984). Since the tuberization process on the whole plant or on stem cuttings occurs equally successfully and at an almost identical speed, the latter can be used for simulation and analysis of the tuberization process.

Thirty-five years ago Palmer and Smith (1973) indicated that one-bud stem cuttings are prefered to 2-3-bud cuttings for obtaining tubers since axillary tubers will only develop from one bud, i.e., the developmental process can be more strictly controlled. These authors found, surprisingly, that one-bud cuttings developed more tubers than 6-bud explants, although they had a smaller mass. However, results have been inconsistent in the literature when potato one-bud cuttings were used *in vitro* (Gopal *et al*. 1998; Al-Safadi *et al*. 2000; Singh *et al*. 2001; Gopal *et al*. 2004; Singh *et al*. 2007).

Work by Estrada *et al*. (1986) at the International Potato Center received wide-spread attention. Their technology allowed them to obtain microtubers after the following stages: 1) multiplication of basic material by cuttings; 2) "liquid culture" (containing MS salts + 0.4 mg/l thiamine HCl, 2.0 mg/l Ca-pantothenic acid, 0.4 mg/l gibberellic acid, 0.5 mg/l 6-benzylaminopurine (6-BAP), 0.01 mg/l naphthalene acetic acid, 100 mg/l *myo*-inositol, and 2% sucrose) on a shaking apparatus in the light (16-hour photoperiod); 3) induction of tuberization by full replacement with liquid MS medium supplemented with 0.4 mg/l thiamine-HCl, 100 mg/l *myo*-inositol, 5.0 mg/l 6-BAP, 500 mg/l chlorocholine chloride (CCC) and 8% sucrose, or by inserting small amounts of concentrated solutions of CCC, 6-BAP and sucrose (maximum 8%) in initial medium. Culture vessels were then placed in the dark at 22°C to complete tuberization.

When microtubers are induced, this allows re-infection by all pathogens of multiple microcuttings to be eliminated and to maintain healthy plant material over a long period of time without the need to cultivate it in a greenhouse. Since developing microtubers are deposited in sterile conditions,

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this would eliminate the possibility of contamination. More importantly, when planting microtubers to soil no special covers or agrotechnical tools are required as in the case of regenerated plantlets. Plants from microtubers develop faster and give a greater net reproduction than when regenerated plantlets are planted (Yu *et al*. 2000).

Tuberization *in vitro* is affected by temperature, light, the level of plant growth regulators in media and some other factors (Melis and van Staden 1984; Xu *et al*. 1998; Vecchio *et al*. 2000; Alix *et al*. 2001; Seabrook 2005; reviewed in this Special issue by Dobránszki *et al*.). However, the most important factor in a cultivar's response *in vitro* is the level and source of carbohydrate (Ewing and Struik 1992). However, concrete data about the influence of carbon source on tuberization in different potato cultivar microcuttings is scarce, or missing. The aim of this study was to observe the initial tuberization stages on one-bud potato cuttings *in vitro* and to assess the effect of choice and level of carbohydrate in the tuberization process for three different potato cultivars.

MATERIALS AND METHODS

Reagents and chemicals

All reagents were purchased from Russian suppliers when the level of purity was labeled as 'chemically pure', except for the following: 6-BAP (Sigma, >99%); sucrose (Panreac, farm); D(-)-fructose (Merck, minimum 99%); D(+)Glucose (Sigma, anhydrous; mixed anomers); agar microbiological (Sigma). For the percentage to molar conversion of carbohydrate sources referred to throughout this paper please refer to **Table 1**.

Plant material

Thee cultivars – 'Zhukovskij early', 'Nevskij' and 'Nikulinskij' – of *in vitro*-regenerated potato (*Solanum tuberosum* L.) plants maintained in the All-Russia Research Institute of Potato were used as starting materials. These plants were free of bacteria, fungi and viruses by the culture of apical meristems. Broadly speaking, apical meristems (0.1-0.2 mm) are excised from etiolated sprouts under sterile conditions and placed on Murashige and Skoog (MS; 1962) medium. The growth of isolated meristems is accompanied usually by the formation of callus, from which shoots with leaflets develop spontaneously. The shoots are separated from callus and transplantated onto fresh MS medium. After rooting the plants grow before the appearance of 7-8 leaves. These plants were then used as the starting material for this study.

The three cultivars were clonally propagated by microcuttings placed in 21 mm-wide test tubes on agarized (0.7%) medium (pH 5.8-5.9) with full MS micro- and macronutrients, supplemented with 2% (58 mM) sucrose and 1 mg/l of the vitamins thiamin and pyridoxine. Plants were cultured at 18-20°C, with constant lighting and an illuminating intensity of about 160 μmol photons m^{-2} s^{-1} by cool white fluorescent lamps (TLD 36W/89, Philips, 36 W).

Table 1 Conversion (% (w/v) ↔ mM) table for the three carbohydrate sources used in this study.

% (w/v) / Carbohydrate	2	4	6	8	10
Sucrose	58	117	175	234	292
Glucose	111	222	333	444	556
Fructose	111	222	333	444	556

Microcuttings consisting of a stem segment with one leaf and its associated axillary bud were the starting explants for tuberization experiments. Tuberization *in vitro* was induced in two ways, as described next.

Tuberization method 1: agarized medium

One-bud cuttings were embedded in agarized MS medium supplemented with different single carbohydrate sources (all in equimolar amounts, 234 mM): sucrose (80 g/l), glucose (42.12 g/l), or fructose (42.12 g/l), or their combinations: sucrose + glucose, sucrose + fructose, or fructose + glucose (each at 117 mM). When sucrose was used as the sole carbohydrate source, its concentration was varied from 2 up to 10% (**Tables 1, 2**). In addition, 0.5, 1.0, 1.5 or 2.0 mg/l of 6-BAP was supplied to the sample set of media. The tubes were placed in the dark at 14°C and cultured for 8 weeks after which microtuber yield and the number of microtubers per explant, diameter and weight were determined, while the location where microtubers formed was marked.

Tuberization method 2: liquid medium

Fifteen one-bud cuttings of 'Nikulinskij' (the model cultivar) were placed in a 250-ml vessel with 3 ml of liquid medium containing full-strength MS supplemented with a different carbohydrates and cultured *in vitro* (without shaking) as follows (depending on the developmental stage):

Stage of stolon elongation. At this stage vessels with 15 one-bud cuttings were placed in the dark at 23-24°C. The nutrient MS medium contained 2% sucrose (58 mM) or glucose or fructose (both at 111 mM). After two weeks' culture the number and length of stolons, and the number of internodes on stolons were determined.

Tuberization stage. Medium for stolon growth was removed and replaced by medium for tuberization (full-strength MS supplemented with 1 mg/l of the vitamins thiamin and pyridoxine), 234 mM of a carbohydrate (sucrose, glucose or fructose) as well as 2 mg/l 6-BAP. The carbohydrate concentrations and the addition of 6-BAP differentiate this medium from the medium for stolon growth. Tuberization and subsequent increase in tubers took place

Table 2 Effect of sucrose and 6-BAP concentrations on tuberization process in one-bud potato cuttings assessed at 8 weeks in culture.

Cultivar	Sucrose concentration in medium (%)	% Cuttings with tubers				Average tuber mass (mg)			
		6-BAP concentration (mg/l)				6-BAP concentration (mg/l)			
		0	0.5	1.0	2.0	0	0.5	1.0	2.0
Nikulinskij	2	13.3 ± 1.2 g	26.6 ± 1.7 f	26.0 ± 2.5 f	33.3 ± 2.1 e	55.6 ± 4.5 f	63.3 ± 6.5 e	71.6 ± 4.5 d	84.3 ± 8.8 h
	4	20.0 ± 2.5 f	33.3 ± 2.1 e	33.3 ± 1.4 e	40.0 ± 4.3 d	67.4 ± 2.1 e	74.3 ± 4.2 d	74.3 ± 4.2 g	92.2 ± 7.1 b
	6	33.3 ± 3.2 e	26.6 ± 2.5 f	46.7 ± 4.6 d	60.0 ± 4.5 c	62.7 ± 3.3 e	89.8 ± 9.8 c	89.8 ± 4.9 c	84.3 ± 3.8 c
	8	86.7 ± 5.2 a	87.3 ± 4.5 a	86.7 ± 6.5 a	100.0 ± 3.3 a	63.5 ± 8.2 e	97.7 ± 9.5 b	100.8 ± 6.2 b	112.3 ± 3.8 a
	10	60.0 ± 4.5 c	66.7 ± 3.5 c	60.0 ± 4.5 c	73.3 ± 6.5 b	63.9 ± 4.7 e	98.7 ± 7.3 b	97.4 ± 8.7 b	98.7 ± 4.7 b
Zhukovskij early	2	13.3 ± 1.5 g	26.6 ± 1.2 f	13.3 ± 1.5 g	33.3 ± 2.5 e	36.8 ± 4.5 h	42.8 ± 6.7 g	54.7 ± 8.8 f	51.6 ± 7.2 f
	4	13.3 ± 1.3 g	13.3 ± 2.1 g	26.6 ± 2.1 f	26.6 ± 1.7 f	33.8 ± 6.4 h	58.7 ± 7.3 f	49.7 ± 8.7 g	57.8 ± 9.2 f
	6	26.6 ± 3.5 f	60.0 ± 4.5 c	73.3 ± 3.5 b	73.3 ± 4.5 b	78.3 ± 5.1 d	74.4 ± 6.8 d	77.5 ± 8.4 d	74.6 ± 3.1 d
	8	86.3 ± 5.8 a	80.0 ± 3.5 ab	73.3 ± 5.2 b	86.7 ± 5.1 ab	73.0 ± 3.1 d	84.6 ± 7.4 c	97.8 ± 4.5 b	79.3 ± 6.9 d
	10	60.0 ± 3.1 c	73.3 ± 4.2 b	72.3 ± 5.5 b	73.3 ± 3.9 b	60.0 ± 3.4 e	73.9 ± 6.1 d	72.7 ± 2.8 d	75.4 ± 9.5 d
Nevskij	2	0.0 h	0.0 h	0.0 h	0.0 h	0.0 i	0.0 i	0.0 i	0.0 i
	4	26.6 ± 1.5 f	13.3 ± 1.5 g	26.6 ± 2.0 f	26.6 ± 2.5 f	36.8 ± 2.1 h	65.2 ± 3.3 e	70.3 ± 7.4 d	70.4 ± 5.7 d
	6	26.6 ± 2.0 f	60.0 ± 2.4 c	73.3 ± 4.5 b	80.0 ± 7.2 ab	46.8 ± 5.8 g	80.6 ± 2.4 c	90.8 ± 5.2 b	95.6 ± 8.4 b
	8	80.0 ± 4.5 ab	80.0 ± 5.2 ab	73.3 ± 5.1 b	93.3 ± 6.5 a	81.6 ± 6.7 c	95.8 ± 5.7 b	90.3 ± 3.4 b	100.8 ± 3.5 b
	10	66.7 ± 3.1 c	60.0 ± 4.5 c	80.0 ± 6.5 ab	73.3 ± 4.5 b	82.8 ± 2.8 c	75.3 ± 6.8 d	80.9 ± 6.3 c	78.4 ± 9.6 d

Different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test, $n = 15$.
Separate tests were conducted for both parameters: 1) % Cuttings with tubers; 2) Average tuber mass.

Table 3 Tuberization on one-bud potato cuttings in agarized medium with different single carbohydrates (all at 234 mmol) assessed at 8 weeks.

Cultivar	Carbon source	Cuttings with tubers (%)				Cuttings with tubers* of various diameters (%)			Mass of one tuber (mg)
		Whole plant	On stolon tip	At first internode	Axillary bud	1-3 mm	4-5 mm	6-9 mm	
Nikulinskij	Sucrose	90.1 ± 4.2 a	31.2 ± 3.1 g	10.2 ± 3.1 k	59.6 ± 6.1 de	25.4 ± 3.1 f	60.2 ± 3.7 c	14.04 ± 1.1 g	65.3 ± 8.1 c
	Glucose	80.3 ± 3.2 bc	55.2 ± 2.2 e	10.9 ± 2.3 k	33.9 ± 3.8 g	85.2 ± 4.9 b	14.8 ± 2.4 g	0.0 j	35.9 ± 3.1 d
	Fructose	90.2 ± 4.1 a	50.0 ± 4.1 e	22.2 ± 4.3 h	27.8 ± 2.1 h	83.3 ± 7.1 b	16.7 ± 2.1 g	0.0 j	21.7 ± 4.8 e
Zhukovskij	Sucrose	95.4 ± 3.1 a	5.9 ± 1.1 l	4.1 ± 1.1 m	90.0 ± 9.3 a	25.0 ± 3.2 f	65.0 ± 3.5 c	10.0 ± 1.1 h	74.6 ± 8.3 b
early	Glucose	60.2 ± 3.4 d	33.3 ± 4.3 g	25.5 ± 2.1 h	41.7 ± 5.1 f	91.7 ± 8.9 a	8.3 ± 3.1 i	0.0 j	24.5 ± 2.7 e
	Fructose	75.2 ± 2.7 c	50.0 ± 6.5 e	14.3 ± 3.4 i	35.7 ± 2.7 g	92.9 ± 5.5 a	7.1 ± 1.3 k	0.0 j	25.4 ± 2.2 e
Nevskij	Sucrose	85.1 ± 4.2 b	5.9 ± 1.4 l	5.9 ± 1.2 l	88.2 ± 3.1 ab	0.0 j	100.0 ± 8.1 a	0.0 j	81.6 ± 6.6 a
	Glucose	85.1 ± 3.3 b	52.9 ± 3.1 e	47.1 ± 8.1 f	0.0 n	52.9 ± 4.1 d	47.1 ± 3.3 e	0.0 j	34.3 ± 3.7 d
	Fructose	65.3 ± 2.1 d	60.1 ± 6.8 d	20.1 ± 5.1 h	19.8 ± 3.1 h	90.0 ± 3.9 a	10.0 ± 2.5 h	0.0 j	20.0 ± 2.6 e

Note: different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test, $n = 15$.
Separate tests were conducted for both parameters: 1) % Cuttings with tubers; 2) % Cuttings with tubers of various diameters; 3) Weight of one tuber (mg).
* Tubers were assessed from ALL parts of the plant, independent of tuber location.

over 6 weeks at 14°C in the dark. Thereafter, the yield in microtuber crop was determined by the number of microtubers per explant, their diameter, mass, and the place where they formed was marked.

Acclimatization

The microtubers were placed at 4°C for 4 weeks to enhance the germinability of microtubers and further good development of seedlings *ex vitro*. Then the microtubers were sown in soil in 50 × 30 × 10 cm boxes, which were covered by polyethylene film to increase air humidity at the first stage (critical for germinated microtubers). The percentage germination was estimated 4 weeks after microtubers were sown.

Statistical analyses

All experiments were conducted in triplicate, and each experiment consisted of 15 plants with 3-4 vessels with microcuttings. For all measurements averages and standard errors were calculated by standart mathematical methods using Microsoft Excell 2000, Biostat, Statistica V. 2.6. Significant differences between the means were assessed by Tukey's method at $P = 0.05$.

RESULTS AND DISCUSSION

The process of potato tuberization *in vitro* is complex (see review in this Special issue by Dobránszki *et al*.) but consists primarily of two stages: 1) stolon formation and growth; 2) tuber production and increase in size.

We studied microtuber induction on one-bud potato cuttings *in vitro*, which is a preferred method for the following reasons. First, stem cuttings can be used for simulating the process since analysis of the tuberization process on separate cuttings occurs in much the same way and with identical speed as on the whole plant. Secondly, the use of one-bud stem cuttings is preferential to microtubers because the tubers produced on a whole plant develop only from a single bud, and therefore the tuber formation ratio of whole and on-cutting plants is about 1:7 (Estrada *et al*. 1986).

Tuberization response to single carbohydrate source

Induction of tuberization is affected by photoperiod, temperature, medium composition and other factors. One of the major factors influencing microtuber induction is carbon source or type and its concentration in the medium.

The majority of work on the analysis of the role of carbohydrates in potato tuberization was conducted with sucrose (Guinazu and Tizio 1987; Garner and Blake 1989; Levy *et al.* 1993; Khuri and Moorby 1995). An earlier study showed shown that the use of etiolated potato shoots on solid medium with 8% sucrose induced tuberization (Barker 1953). A linear correlation between tuberization intensity *in vitro* and sucrose content in the medium was confirmed by Levy *et al.* (1993) and Yu *et al.* (2000).

On medium supplemented with 2% sucrose and various concentrations of 6-BAP the percentage tuberization was very low (from 13.3 up to 32.3% in 'Nikulinskij' and from 13.6 up to 33.3% in 'Zhukovskij early') or in general was absent in 'Nevskij' (**Table 2**). An increase in sucrose concentration in the medium up to 4% promoted tuberization in all three cultivars; a further increase in sucrose concentration from 4 up to 8% increased tuberization efficiency from 31 up to 100% in all cultivars. However, an increase in sucrose concentration up to 10% resulted in a decrease of this index (not more than 80% in any cultivar).

Waring (1984) showed that high sucrose contents (> 5%) is necessary for tuberization. Similarly Garner and Blake (1989) cultivated shoots with one axillary bud of 'Pentland javelin' and 'Maris Piper' on MS medium with 4, 8 or 12% sucrose. They showed that cultivation on medium with 8% sucrose enchanced (numbers and size) microtuber formation than 4% sucrose. However, a very high sucrose concentration (12%) did not increase the number of tubers formed, and the tubers were smaller than at 8% sucrose.

The mass of microtubers was also influenced by sucrose concentration. The average maximum tuber mass occurred at 8% sucrose (with or without 6-BAP) (**Table 2**). 6-BAP was only effective, however, when combined with a high (8%) sucrose concentration.

We used different carbohydrates (sucrose, fructose, glucose) as a carbon source in an attempt to increase tuberization. However, since tuberization depends not only on the carbon source, but also on medium osmosis (Yu *et al*. 2000), media with equal osmosis were used; microtuber yield was estimated after 8 weeks (**Table 3**).

The percentage response depended on the cultivar and on the period of cultivation. On medium supplemented with 234 mM sucrose tubers formed in the first week of cultivation for all cultivars with maximum percentage (80%) in 'Nevskij' (**Fig. 1**). By the third week more than 80% of explants of all three cultivars had formed mirotubers and by the fourth week 'Zhukovskij early' showed maximum tuberization percentage.

In the second variant of the experiment one-bud cuttings were cultivated on medium supplemented with 234 mM fructose (**Fig. 2**). In this medium microtubers formed in the first week of cultivation only for two cultivars, 'Nikulinskij' and 'Zhukovskij early'. For 'Nevskij' this process started only in the second week of cultivation. Most intensive tuberization on this medium was in 'Nikulinskij' cuttings (up to 90%). The percentage tuberization was lowest (62%) in 'Nevskij'. Maximum number of tubers formed in the third week of cultivation for all three cultivars (**Fig. 2**). Medium with 234 mM fructose is worst for tuberization in 'Nevskij', but not so for the other two cultivars.

In the third variant we used medium supplemented with 234 mM glucose (**Fig. 3**). On this medium tuberization began in the first week of cultivation for 'Nikulinskij' and 'Zhukovskij early' but the process was delayed by one week in 'Nevskij'. The initial percentage tuberization varied from 12% in 'Nevskij' up to 56% in 'Nikulinskij'. In the third

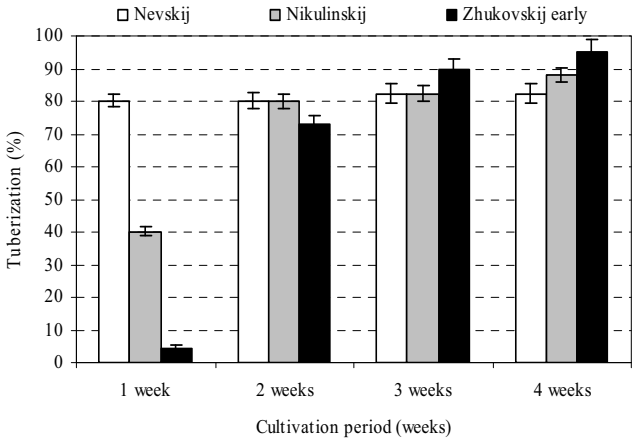

Fig. 1 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with 234 mM sucrose.

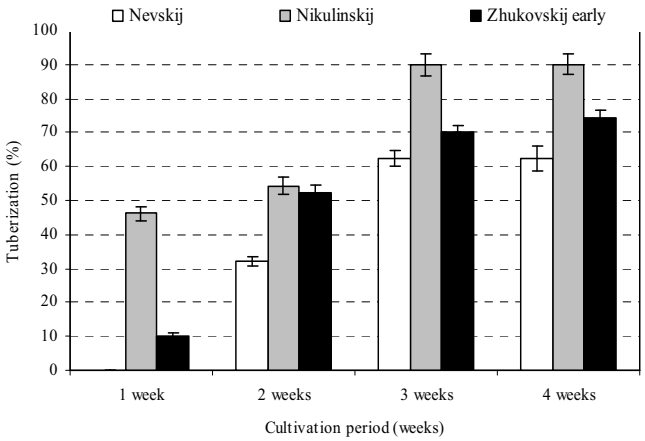

Fig. 2 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with 234 mM fructose.

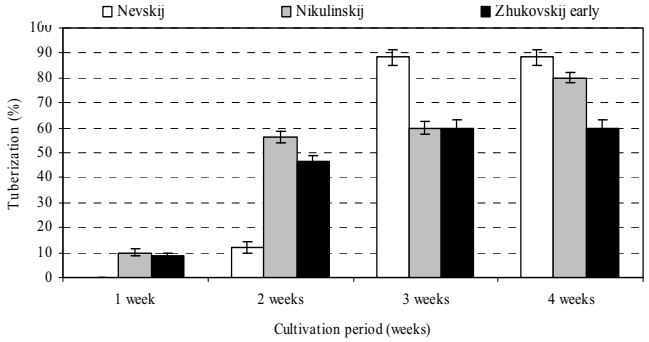

Fig. 3 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with 234 mM glucose.

week of cultivation a sharp (6-fold) increase in the number of formed microtubers was noted for 'Nevskij'. However, subsequent cultivation saw a plateau in tuber formation for this cultivar. Similar, but not as pronounced, trends were observed for the other two cultivars. By the fourth week only 'Nikulinskij' showed a 20% increase while the other two cultivars plateaued. Thus, medium supplemented with 234 mM glucose could support increased tuberization up to the 3rd or 4th week, but this depended on the cultivar.

The choice of single carbohydrate influenced three factors of micro-tuberization: the location at which tubers formed, the diameters of the tubers and the individual tuber mass (**Table 3**). Only two clear (i.e. statistically supported) cultivar-independent trends could be observed with respect to the influence of carbohydrate source on the localization of tuber formation: the first was that fructose supported the formation of more tubers on stolon tips than the other two carbohydrate sources; the second was that sucrose supported the formation of more tubers on axillary buds than either fructose or glucose. All other results, despite having significant differences, were all cultivar-independent.

Sucrose was clearly the carbohydrate source that most positively influenced tuber size (**Table 3**) with 6-100% of tubers falling into the medium-sized (4-5 mm) category. In fact only sucrose supported the formation of large (6-9 mm) tubers, 14% in the case of 'Nikulinskij'. For all three cultivars, fructose impacted tuber diameter most negatively, with most (83-93% for all three cultivars) tubers falling into the 1-3 mm diameter range. Glucose followed a similar trend (i.e. 85-92% of tubers being 1-3 mm in size for 'Nikulinskij' and 'Zhukovskij early') except for 'Nevskij' where there was an almost even balance between small (1-3 mm) and medium-sized (4-5 mm) tubers. As would be expected the trends observed in tuber diameter also were mirrored in tuber mass, with sucrose supporting the heaviest tubers in all three cultivars.

In conclusion, sucrose is the best carbohydrate source for obtaining the largest (widest) and heaviest micro-tubers for any of these three cultivars.

Ovtchinnikova (1992) established that the inhibition of plant growth and stimulation of tuberization with increasing sucrose concentration was cultivar-specific. Sucrose plays a double role in microtuber formation: it is used as a carbon source which is easily metabolized, and also serves as an osmotic agent without inhibiting the increase in tuber size (Khuri and Moorby 1995). Sucrose plays a role, primarily, as a trigger for tuberization; secondly, it is an indispensable source of energy for axillary tuber growth; thirdly, it osmotically prevents axillary bud elongation (Ewing and Struik 1992; Yu *et al*. 2000).

Tuberization response to double carbohydrate source

Unlike the single carbohydrate applications (**Table 3**) where sucrose did not appear to be superior to either glucose or fructose in increasing tuber formation at the whole plant level, except for 'Zhukovskij early', the application of a double carbohydrate source (any) generally increased tuber formation at the whole plant level (lowest percentage = 67% as opposed to 60% in single carbohydrate application). Although there were individual significant differences between the level of tuber formation from different parts of the plant (stolon tip, first internode, axillary bud) when either individual (**Table 3**) or multiple (**Table 4**) carbohydrate sources were used, no specific trend could be observed for any combination of for any of the three cultivars. What was striking, however, was how tuberization was completely inhibited at the first internode when sucrose + fructose were used in 'Nevskij'; similarly, tuberization was completely inhibited in 'Zhukovskij early' axillary buds when sucrose and glucose were used. In general, tuber diameter was decreased by the application of two carbohydrates (**Table 4**) compared to the single application of carbohydrates (**Table 3**), except for the application of sucrose and fructose which saw a massive jump in the number of large (6-9 mm) diameter tubers forming in 'Nevskij'. This of course directly affected the average mass of a single tuber.

In almost all cultivars, when a double carbohydrate source was applied, tuberization began in the second week after the start of treatment. Although the increase in percentage tuberization peaked at the fourth week for the fructose + glucose (these are products of sucrose hydrolysis) combination (**Fig. 4**), the level remained unchanged from weeks 2-4 for the sucrose + fructose and sucrose + glucose (**Figs. 5** and **6**, respectively) mixes, indicating the importance of sucrose for the process. This then confirms the finds for the single carbohydrate applications observed in **Fig. 1** and **Table 2**.

Trophic regulation of stolon and tuber formation in one-bud potato cuttings in liquid medium

Table 4 Tuberization on one-bud potato cuttings in agarized media with a mixture of carbohydrates (all at 117 mmol) assessed after 8 weeks.

Cultivar	Carbon source	Cuttings with tubers (%)				Cuttings with tubers* of various diameters (%)			Mass of one tuber (mg)
		Whole plant	On stolon tip	At first internode	Axillary bud	1-3 mm	4-5 mm	5-9 mm	
Nikulinskij	Sucrose + Glucose	74.3 ± 3.1 c	26.4 ± 3.1 g	18.2 ± 2.1 i	55.4 ± 3.1 e	72.7 ± 3.4 b	27.3 ± 1.1 e	0.0 g	33.9 ± 3.2 d
	Sucrose + Fructose	85.2 ± 4.2 b	46.2 ± 2.1 f	7.7 ± 3.4 j	46.1 ± 5.3 f	76.9 ± 2.2 b	23.1 ± 2.3 e	0.0 g	41.9 ± 3.3 c
	Fructose + Glucose	85.7 ± 5.3 b	53.3 ± 4.5 e	23.1 ± 1.2 h	23.1 ± 1.2 h	100.0 ± 1.2 a	0.0 g	0.0 g	19.5 ± 2.5 f
Zhukovskij early	Sucrose + Glucose	86.3 ± 4.3 b	69.2 ± 3.1 d	30.8 ± 3.3 g	0.0 k	46.2 ± 3.5 d	53.8 ± 4.1 c	0.0 g	64.5 ± 6.1 b
	Sucrose + Fructose	73.3 ± 2.2 c	72.2 ± 3.2 c	18.2 ± 4.2 i	9.1 ± 1.1 j	90.9 ± 4.2 a	9.1 ± 2.5 f	0.0 g	25.6 ± 3.2 de
	Fructose + Glucose	66.7 ± 1.6 d	50.0 ± 4.1 e	20.0 ± 3.1 hi	30.0 ± 2.4 g	100.0 ± 3.1 a	0.0 g	0.0 g	17.7 ± 2.5 f
Nevskij	Sucrose + Glucose	92.3 ± 4.3 a	28.6 ± 2.3 g	7.1 ± 1.1 j	64.3 ± 3.1 d	92.8 ± 4.2 a	7.2 ± 3.1 f	0.0 g	23.6 ± 1.7 e
	Sucrose + Fructose	92.1 ± 5.1 a	50.0 ± 3.1 e	0.0 k	50.0 ± 5.3 e	7.1 ± 3.2 f	42.9 ± 2.5 d	50.0 ± 3.1 c	112.4 ± 8.4 a
	Fructose + Glucose	90.3 ± 2.1 a	21.4 ± 4.2 h	14.3 ± 1.6 i	64.3 ± 2.1 d	71.4 ± 3.1 b	28.6 ± 4.1 e	0.0 g	28.5 ± 4.7 d

Note: different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test, $n = 15$ (Separately: 1 - for Cuttings with tubers (%), 2 - for Cuttings with tubers of various diameters (%), and 3 - for Weight of one tuber (mg)).
* Tubers were assessed from ALL parts of the plant, independent of tuber location.

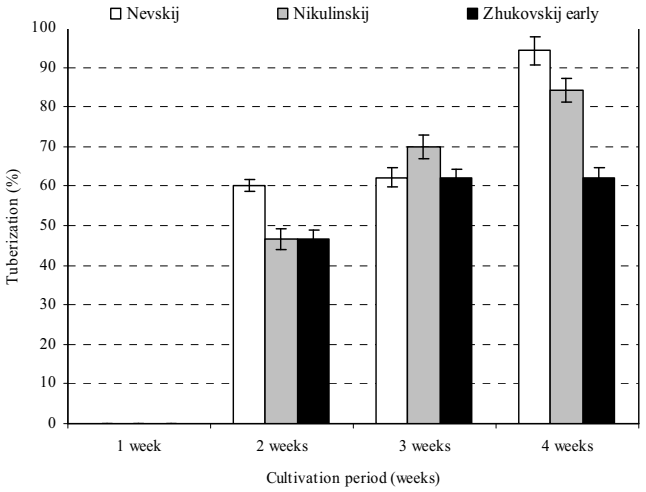

Fig. 4 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with a mixture of fructose and glucose (117 mM each).

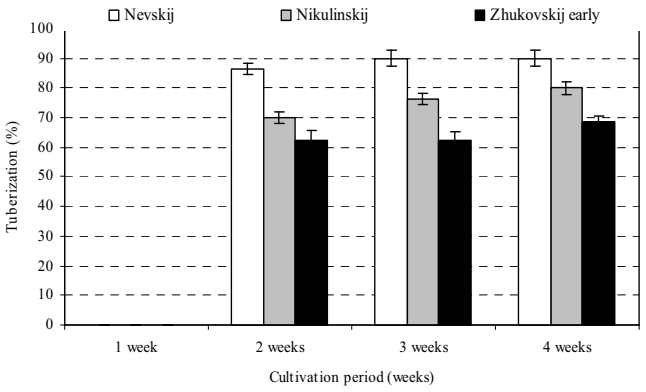

Fig. 5 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with a mixture of sucrose and fructose (117 mM each).

It is possible to cultivate explants both on agarized (**Figs. 1-6; Tables 2-4**) and on liquid (**Figs. 7-9; Table 5**) media. The advantage of liquid medium is the large motility of nutrients that can be provided and changed during cultivation (Xu *et al.* 1998). In a bid to demonstrate the applicability of liquid medium and to show that results could be comparable to those on agarized medium, 'Nikulinskij' was selected as

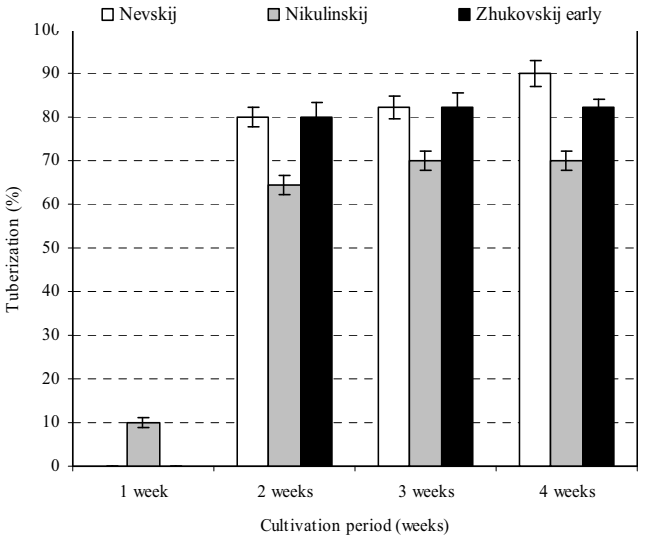

Fig. 6 Induction of tuberization for one-bud potato cuttings on agarized MS medium supplemented with a mixture of sucrose and glucose (117 mM each).

the model cultivar since it performed best on media with separate carbohydrates.

The objective of using explants consisting of cuttings with a leaf and one bud was to eliminate the phototrophic stage with the purpose of increasing the yield of potato microtubers *in vitro* and to reduce the time for their formation. Unlike the studies on agarized medium, these studies on liquid medium tested the effect of different single carbohydrate applications in the medium for stolon formation and for microtuberization. When grown in steady-state conditions of continuous darkness at 24°C (stolon culture), stolons were initiated in PGR-free MS medium supplemented with single carbohydrates, *viz*. 2% glucose, fructose or sucrose, the latter being the control (**Table 5**). Stolon formation was almost similar and equally effectively for all three carbohydrates (**Fig. 7**).

When glucose or fructose were used as the carbohydrate source in the medium for stolon formation, and when this was followed by the use of fructose or glucose in the medium for tuberization, the highest percentage of cuttings with tubers were obtained (**Table 5**). Ironically, sucrose performed most poorly, in contrast to agarized medium. This trend appeared to be true, independent of the location of tuber formation (axillary bud, first internode or stolon tip).

Table 5 Tuberization on one-bud potato cuttings of 'Nikulinskij' in liquid medium with different single carbohydrate applications after 8 weeks culture.

Medium for stolon formation	Medium for tuberization	Cuttings with tubers* (%)	Tuber diameter (mm)	Mass of one tuber (mg)	Tuber location on cuttings (%)		
					Axillary bud	At first internode	On stolon tip
Sucrose	Sucrose	55.2 ± 4.2 c	5.1 ± 0.2 a	112.7 ± 1.1 a	53.2 ± 3.1 c	12.7 ± 1.1 g	34.1 ± 3.1 d
	Fructose	72.2 ± 3.9 b	4.2 ± 0.3 b	103.2 ± 3.7 b	12.7 ± 1.1 g	10.2 ± 2.0 g	76.3 ± 4.1 a
	Glucose	44.3 ± 2.3 d	5.0 ± 0.3 a	106.1 ± 2.2 b	76.2 ± 3.1 a	8.7 ± 1.1 h	25.1 ± 4.7 e
Glucose	Sucrose	85.3 ± 4.1 a	2.1 ± 0.1 d	61.4 ± 2.6 d	53.1 ± 2.5 c	7.6 ± 1.1 h	39.3 ± 4.1 d
	Fructose	74.6 ± 3.8 b	2.2 ± 0.3 d	65.4 ± 4.3 d	20.5 ± 4.1 ef	5.1 ± 0.7 i	74.4 ± 5.8 a
	Glucose	82.2 ± 4.9 a	3.1 ± 0.2 c	72.3 ± 2.8 c	27.8 ± 3.2 e	5.5 ± 1.1 i	66.7 ± 3.2 b
Fructose	Sucrose	82.3 ± 3.2 a	3.2 ± 0.2 c	75.4 ± 2.4 c	55.8 ± 3.7 c	17.0 ± 1.6 f	27.2 ± 1.6 e
	Fructose	83.2 ± 2.1 a	3.1 ± 0.1 c	67.6 ± 2.7 cd	11.3 ± 1.6 g	11.3 ± 4.2 g	70.5 ± 4.3 a
	Glucose	74.5 ± 2.6 b	3.0 ± 0.3 c	68.9 ± 4.2 cd	39.2 ± 4.7 d	9.8 ± 2.1 g	51.0 ± 4.6 c

Note: different letters indicate significant differences at $P \leq 0.05$ according to Tukey's test, $n = 15$ (Separately: 1 - for Cuttings with tubers (%), 2 - for Tuber diameter, 3 - for Tuber weight, and 4 - Tuber location on cuttings).
* Whole plant level.

Fig. 7 Stolon formation in liquid medium with different carbohydrates in potato cuttings of 'Nikulinskij'. 1: 2% sucrose, 2: 2% fructose, 3: 2% glucose.

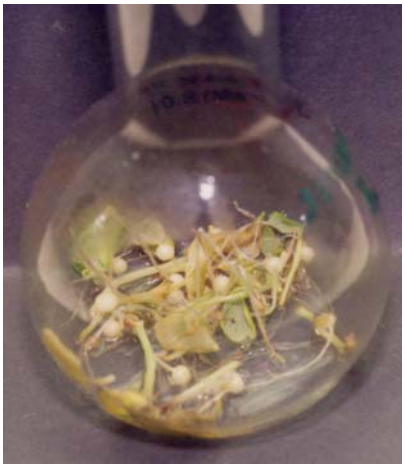

Fig. 8 Microtuber formation by 'Nikulinskij' on liquid medium with 234 mM fructose.

Fig. 9 Microtuberization by 'Nikulinskij' in liquid medium with different carbon sources after replacement of stolon-induction medium for tuberization medium. 1: 234 mM glucose; 2: 234 mM fructose; 3: 234 mM sucrose.

Despite this apparent superiority of glucose and fructose (**Figs. 8, 9**) over sucrose as far as total number of tubers being formed, the average mass of a single tuber and tuber diameter were greatest in all cases when sucrose was used in the medium for stolon formation, mirroring the results for agarized medium.

Three locations of tuberization occurred *in vitro*: on axillary buds, at internodes, or on stolon tips, sometimes all of them occurring simultaneously. Yanchevskaya (1999) showed that axillary tuberization was enhanced by sucrose in the medium.

CONCLUDING REMARKS

1. Microtuberization for all three cultivars started in the first week on agarized media with any carbohydrate source, and the percentage tuberization in any case during this period did not exceed 50%. On media with a double carbohydrates mixture this percentage was only reached in the second week of cultivation.
2. In agarized media with separate carbohydrates the greatest percentage tuberization occurred on medium with 234 mM sucrose for all cultivars. Tubers formed mainly on stolons in media with fructose + glucose while and tubers from axillary buds formed mainly on media with sucrose for all cultivars. On media with separate carbohydrates the axillary bud-derived microtubers had the largest mean weight and diameter than tubers derived from stolons, for all cultivars.
3. 'Nikulinskij' performed best on media with separate carbohydrates, while 'Nevskij' was best on media with double carbohydrate mixes. Thus the formed cultivar was selected as a model plant for the liquid medium studies.
4. The tuberization index can be increased through the use of liquid medium in two ways. By incorporating glucose or fructose in the medium for stolon formation, more tubers can be formed; by incorporating sucrose larger and heavier tubers form.

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