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FORMATION AND MORPHOMETRIC INDICES OF POTATO MICROTUBERS in vitro AT VARIOUS FORMULA OF SUGARS IN MEDIUM

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S u m m a r y

On aseptic culture of potato stolons of the Zhukovskii early and Lugovskoi varieties, isolated in roller in absolute darkness, the authors studied the effect of glucose, sucrose and fructose, supplemented to nutrient medium of Murashige-Skoog (MS-medium), on formation of stolons and tubers. The appearance of only axillary microtubers (40 % — in leaf axil of stem explants and 60 % — in leaf axil of first internode of stolon) the authors observed at the induction of tuber formation by MS-medium with 8 % of sucrose. For the raising of efficiency of tuber formation and the improvement of morphometric indices of microtubers the authors suggest MS-medium with 8 % sucrose, which induces the tuber formation at 7 day of cultivation, replace entirely on analogous in 15-20 days after induction.

Keywords: *Solanum tuberosum* L., microtubers, stolon development, tuberization, sucrose, glucose, fructose.

Conventional modern methods of obtaining potato microtubers (in vitro formed tubers of the diameter less than 10 mm) suggest using test tubes with agar (1) or flasks with liquid (stationary cultivation and/or use of a shaker) medium (2 - 4). Less frequent the procedure is carried out in bioreactors of different types (5-8). All these techniques describe a similar process of growing plants from explants (cuttings with one or more buds) in the light for 4-5 weeks on medium containing 2-3% sucrose with further induction of tuberization (in conditions of darkness or scattered light) by additions of growth regulators and/or raising the sucrose content by 6-9% with further cultivation of microtubers up to their maturation during 5-8 weeks.

Tuberization of potato in vitro is known to be determined by a cultivar's genotype, photoperiod, temperature and composition of nutrient media (9). Formation of tubers is accompanied by anatomical, hormonal and biochemical changes in the subapical zone of stolons. Biochemical changes in cells of developing microtubers are concerned with accumulation of starch and storage proteins. These processes are subjected to key enzymes of carbohydrate metabolism - invertase and saccharosesynthase: the activity of invertase is very high at the start of tuberization and decreases with maturation of microtubers, while the second enzyme shows a controversial trend of activity (10).

The influence of a medium carbohydrate component, its type, form and content inducing tuberization, are being carefully studied for many decades along with duration and rate of microtubers' growth. Since the middle of the XX century, it has been performed a number of researches to optimize carbohydrate composition (sucrose, glucose, fructose, etc.) and content (2-10%) in the medium providing enhanced tuberization in vitro of potato different genotypes (11-14). The greatest number of these studies is focused on sucrose. In contrast to monosaccharides (glucose, fructose), sucrose has a lower osmotic pressure while the equal carbon "load" (7, 15). Autoclave treatment leads to partial hydrolysis of sucrose to glucose and fructose (6, 7, 16), which increases the osmotic potential of a medium (14). To improve the efficiency of tuberization, V.N. Ovchinnikov (17) recommends to optimize the content of carbohydrates by adding monosaccharides to the inducing media with sucrose. Other authors assume that the mix medium containing 4% glucose+4% fructose provides high osmotic pressure leading to the development of smaller microtubers compared to those on the medium keeping only 8% sucrose (7). At present time, it is generally accepted that 6-8% sucrose is an optimal concentration for inducing and growth of potato microtubers in vitro (14, 18), while the higher or lower contents slow down tuberization and result in formation of microtubers smaller in number and/or in size. Under in vitro conditions, sucrose is the trigger of tuberization, the energy source for growth of plant and microtubers, and it is also supposed to be the optimum osmotic component of a culture medium (9).

In vivo tuberization in individual plants (and even stolons) of potato is known to be a hierarchical process affected by both the genotype and environmental factors (19). It has been shown that the number of microtubers induced in vitro is positively correlated with number of stolons, while the share of tuber-forming stolons depends on the genotype (20). Earlier, the authors have found (21) in potato varieties Zhukovskii rannii and Lugovskoi the formation of microtubers in stolon culture during the first 3 weeks after induction. This process was periodic in nature, and the dynamics of microtubers' daily growth in both species corresponded to a damped curve with two distinct peaks close to a sinusoid. Potato tuberization in vitro was found to be a discrete process (21), whose efficiency can be improved by the sufficient supply of tubers with nutrients (primarily sucrose) (7).

The purpose of this study was to reveal the influence of carbohydrate component and the re-replacement of a nutrient medium on the efficiency of tuberization and morphometric characteristics of microtubers grown in vitro.

Technique. The object of study – stem explants with one axillary bud and leaf, cut from the middle part of the 4-week aseptic potato plants (*Solanum tuberosum L.)* the cultivars Zhukovskii rannii (early-ripening), and Lugovskoi (mid-ripening). These improved plants were obtained from the culture of apical meristem in the A.G. Lorch All-Russia Research and Development Institute of Potato Growing (Moscow province).

Initiation and growth of stolons were performed on the modified liquid hormone-free medium of Moorashige-Skoog (MS) (22) with 2% sucrose, glucose or fructose added depending on the variant of experiment. Explants (20 pcs.) were placed in cylindrical vessels of 300 ml containing 6 ml MS-medium. The optimal ratio of a vessel, medium and number of explants was selected previously (23). A prototype of the used culture vessels was a roller bioreactor (5). The vessels were fixed on a roller apparatus (laboratorial experimental device) perpendicular to the disk planted on metal semiaxis, which was inclined at an angle of 3 ^{o} to the horizon

thereby providing a uniform interflow of a liquid medium; speed of disk rotation - 4 rpm. The device was placed in a chamber providing the conditions of a continuous darkness at 20±1 °C during all cycle of tuberization (from the initiation of stolon growth until obtaining microtubers). The optimum time for induction of tuberization - the 7th day - was found earlier (23); by this time, over 80% stolons have two metamers (21). MS-medium was replaced with a close composition containing 8% sucrose, glucose or fructose, and in 10 weeks after that, the efficiency of tuberization was accounted as a percentage: the number of microtubers to the number of explants in culture vessels (24). The other controlled parameters were: diameter of microtubers (mm), their location (in the leaf axils or at the end of stolon) and the weight of microtuber fractions (diameter < and ≥ 5 mm) (mg).

The obtained data were statistically processed in the program "t-test" (MS DOS) based on the Student's t-test (P = 0.05).

Results. To accelerate the procedure of tuberization, its first stage was missed - growing plants from stem explants in the light. Instead of it, the explants were cultured in continuous darkness inducing stolon growth (MS medium with 2% sucrose, glucose or fructose) until the formation of etiolated shoots with two internodes, which fact was detected on the 7th day. These shoots morphologically corresponded to stolons (9), but they manifested diatropic orientation of growth owing to violation of geotropism at periodic rotation of explants on the roller disc. In all variants (using different forms of carbohydrates), there were formed morphologically normal stolons.

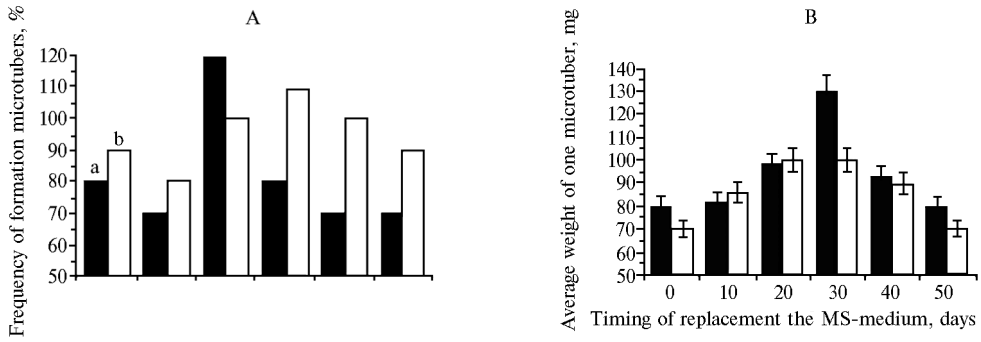
Hierarchy of tuberization in vitro developed by potato cv Lugovskoi in 10 weeks after the induction of tuber formation depending on carbohydrate composition of the MC-medium

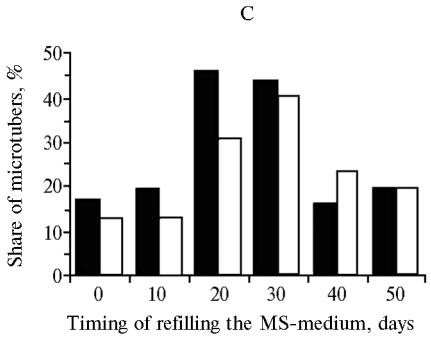
Variant of experiment	Share of formed microtubers, %				
	in leaf axils			on the end of stolon	
	initial stem explant	1st internode of stolon	2nd internode of stolon	1st order	2nd order
Sucrose, 2 % (stolon formation):					
sucrose, 8 % (tuberization)	40	60	0	0	0
glucose, 8 % (tuberization)	0	25	25	15	35
fructose, 8 % (tuberization)	30	60	0	0	10
Fructose, 2 % (stolon formation):					
sucrose, 8 % (tuberization)	55	20	0	0	25
glucose, 8 % (tuberization)	5	40	10	10	35
fructose, 8 % (tuberization)	15	35	10	10	30
Glucose, 2 % (stolon formation):					
sucrose, 8 % (tuberization)	50	20	5	5	20
glucose, 8 % (tuberization)	20	45	10	15	10
fructose, 8 % (tuberization)	15	30	15	10	30

Note. The data are shown for 5 biological replicates and 25 analytical replicates.

During the tuberization, development of stolons wasn't inhibited - they continued to grow and formed new metamers. The hierarchy of formation microtubers on stolons was influenced by the medium carbohydrate component (Table). Regardless of the carbohydrate used during stolon formation, the induction of tuberization with MS-medium keeping 8% sucrose resulted in formation of 40-55% microtubers in leaf axils of source explants. Using sucrose as a carbon source at both stages of formation stolons and tubers contributed to development of axillary microtubers as well (40% in leaf axils of explants and 60% on the 1st internode of stolon). The presence of microtubers only in leaf axils of initial explants and on its 1st internode indicates that the 7-days period of stolon formation was sufficient for subsequent induction of tuberization. In variants with 8% monosaccharides (glucose or fructose), microtubers developed in leaf axils of explants and on apexes of stolons the 1st and 2nd orders regardless of the carbohydrate applied during stolon formation. When the media containing monosaccharides was used at both stages, no hierarchy was detected (Table). In the variant with MS-medium containing 8% sucrose, microtubers were visually larger than those on the media with 8% glucose or 8% fructose.

According to the literature, formation of potato microtubers in vitro occurs during 2 weeks after the induction (6). In previous investigations of the authors, it has been shown that duration of tuberization directly depends on ripening properties of varieties: in early-ripening cultivars (Zhukovskii rannii), the process takes than 2 weeks, in the mid-ripening ones (Lugovskoi) - no more than 3 weeks after the induction with MS medium keeping 8% sucrose; at the same time, saccharides - a major substrate for starch synthesis in tubers - were intensely absorbed from the MS-medium (21).





The efficiency of tuberization (A), **average weight of one microtuber** (B) **and the share of microtubers larger than 5 mm diameter** (C) **during the 10-weeks cultivation of potato stolon culture the varieties Zhukovskii rannii** (a) **and Lugovskoi** (b) **depending on terms of replacement the MS-medium on the similar one.** Cultured on the roller device in continuous darkness at 20±1 °C.

The sufficient carbohydrate nutrition of stolons during tuberization was supplied by complete replacing the MS-medium containing 8% sucrose with an equal volume of the fresh one – on the 10^{th}, 15^{th} 20^{th}, 35^{th} or 45^{th} days after the induction. This procedure significantly increased tuberization parameters in both studied cultivars (Fig). In cv Zhukovskii rannii, tuberization efficiency increased by 50% after refilling the MS-medium on the 15^{th} day (during the active formation of microtubers on cultured stolons) (21). In cv Lugovskoi, the peak of this indicator (by more than 20%) was detected when the culture medium was refilled on the 20^{th} day, which was accompanied by raise (almost 40%) in share of microtubers larger than 5 mm. Refilling the culture medium on the 15^{th}-20^{th} days after the induction contributed to a 60% increase in average weight of microtubers in both cultivars. The developed microtubers were normally shaped, without morphological deviations. In both varieties, refilling the MS-medium in earlier (day 10) or later (day 35 of 45) terms caused almost no effect on morphometric characteristics of microtubers. However, 5-10% microtubers manifested a secondary tuberization, i.e. the process of secondary growth. Thus, to improve the efficiency of tuberization and obtain more mature microtubers, it is necessary to provide unlimited supply of plants with carbohydrate component (sucrose). This can be performed by complete refilling of a culture medium in optimal time (for the early-ripening cv Zhukovskii rannii – the 15^{th} day, for the mid-ripenin cv Lugovskoi –the 20^{th} day after the induction of tuberization).

These findings are consistent with results of W.-C. Yu et al. (7) on mass production of microtubers in the bioreactor. It has been shown the decrease in sucrose content by nearly 0% by the end of the 2^{nd} week after the induction, while glucose and fructose contents in media exceeded 4%. This fact allowed to suggest that sucrose is a soluble disaccharide preferable for tuberization. Apparently, sucrose is not only the best carbon source for potato plants in vitro, but it also provides (the content of 8%) the optimum osmotic potential for development of microtubers (14). At the same time, the activity of acid insoluble invertase located in apoplast is known to be the important factor affecting carbohydrate composition of a liquid medium (in addition to autoclaving leading to a partial hydrolysis of sucrose) during cultivation of vegetable cells, tissues and organs in vitro (7, 25). Stolon culture was constantly in contact with a nutrient medium, and it is possible than the apoplast invertase actively stimulated hydrolysis of sucrose to glucose and fructose, which resulted in reducing the content of this disaccharide in the MS-medium. This circumstance must be considered when developing the automated technology for production of potato microtubers with the use of liquid media.

Thus, the authors have obtained in vitro the improved potato microtubers during the period of 3-4 weeks faster than at the use of conventional technique. These results were achieved by exclusion of the "adult" test-tube plant stage from the scheme combined with induction of tuberization by the use of MS-medium containing 8% sucrose in the 7-day stolon culture grown in continuous darkness on the MS medium keeping 2% sucrose. As the result, more than 60% microtubers were formed on the first two metamers of stolons. The authors suggest a total replacement of culture medium with the similar one (MS medium with 8% sucrose) performed on the 15^{th}-20^{th} day (depending on variety) after the induction of tuberization, which provides the stolon culture with sufficient sucrose supply and improves the efficiency of tuberization.

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