Floriculture and Ornamental Biotechnology ©2007 Global Science Books

Cultivation of *Dioscorea nipponica* Makino *in Vitro* and *ex Vitro*

A. S. Lukatkin[1*] • S. V. Bikunova[1] • Jaime A. Teixeira da Silva[2]

[1] Department of Botany and Plant Physiology, Mordovian State University, Bolshevistskaya Str., 68, Saransk, 430010, Russia
[2] Department of Horticulture, Faculty of Agriculture, Kagawa University, Ikenobe, Miki-Cho, 761-0795, Japan
Corresponding author: * **aslukatkin@yandex.ru**

ABSTRACT

The patterns of introduction of *Dioscorea nipponica* Makino to *in vitro* culture as well as the cultivation of cuttings *in vitro* and multiplication have been investigated. The optimal culture conditions *in vitro* were determined and a 90-98% acclimatization of plants established.

Keywords: seed sterilization, regeneration, multiplication factor, growth regulators
Abbreviations: 2,4-D, 2,4-dichlorphenoxyacetic acid; **6-BAP**, 6-benzylaminopurine; **CMP**, clonal micropropagation; **IAA**, indole-3-acetic acid; **MS**, Murashige and Skoog medium

INTRODUCTION

Dioscorea nipponica Makino is a plant from the *Dioscoreaceae* Family. The genus *Dioscorea* is large and integrates more than 250 species, most of which are widespread in the tropics and subtropics. In Russia two species, *D. caucasica* Lipsky and *D. nipponica* Makino (until recently known as *D. polystachya* Tures) grow naturally.

D. nipponica, a perennial herbaceous plant, is also a dioecious liana (**Fig. 1A**). Its climbing stems can reach 5-7 m and more. The rhizome is horizontal, up to 2.5 m in length and up to 3 cm in diameter, brown, and bears rigid roots along the entire length. Stems are numerous, simple, bare (Tachtadjan 1982). Simple leaves are 6-12 cm of length, wide-ovoid, with a heart-shaped base, on short petioles, alternate. The lower leaf blades are seven-lobed, upper leaves 3-5-lobed. Flowers are small-sized, light-green. Staminal flowers are collected 3-7 in semi-umbraculiferous inflorescences and will result in single, axillary brushes. Pistillate flowers are grouped together in a simple brush (**Fig. 1B**). The fruit is a capsule. Flowering begins in July-August (in the Northern Hemisphere) and the seeds ripen from August to October.

With respect to the chemical composition, pharmacological properties and application, the rhizomes contain steroid glycosides called saponins, whose content is about 8%, the most abundant of which are dioscin, dioscinin and gracillin They are used as a prophylactic and medical remedy in the treatment of atherosclerosis (Blinova and Yakovleva 1990). "Polysponin", a Galenic preparation from *D. nipponica* is also known as "Diosponin", which is in fact made from *D. caucasiana* rhizomes. In oriental medicine *Dioscorea* preparations are used to cure nephritic diseases and impotence.

D. nipponica is widely spread in the Amursky Region, Primorskiy and Khabarovskiy Krai of Russia, as well as in China, Korea, and Japan. *Dioscorea* grows in broad-leaf and mixed forests, on forest glades, edges, in rivers valleys, streams and old river-beds; these plants are often found among wormwood brakes and bushes (Kharkevich and Kachura 1981).

The limiting factors of *D. nipponica* propagation are low seed productivity, low seed germination, slow seedling growth and development, as well as non-limited rhizome harvest (storage) as medicinal raw material (Red Book of RSFSR 1988).

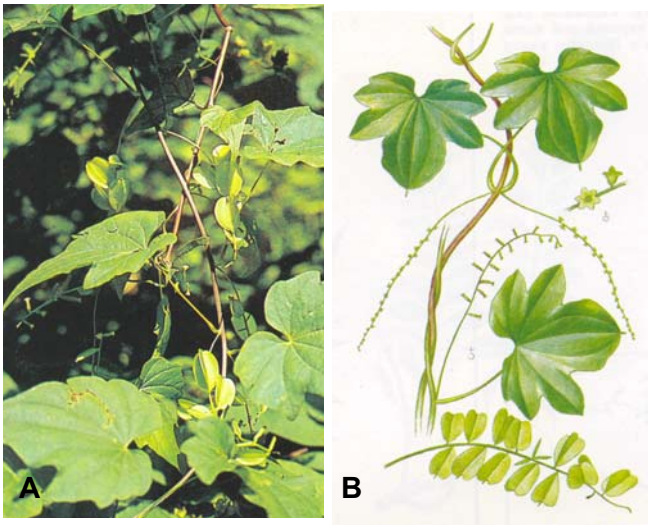

Fig. 1 ***Dioscorea nipponica*** **plant in natural conditions (A), showing stem, leaves, inflorescence, male and female flowers (B).** (**B**) from Red Book of RSFSR (1988) Rosagropromizdat, Moscow.

Other *Dioscorea* species – agricultural or for synthesizing steroid glycosides – have been established in cell culture (Osifo 1988; Kageyama *et al*. 1988; Watanabe 2002; Borthakur and Singh 2002; Mizuki *et al*. 2005). *D. deltoidea* Wall, naturally growing in the mining regions of India, Nepal, Afghanistan and China, contains up to 3.8% diosgenin in the rhizomes and is widely cultured *in vitro* (Karanova 2006; Titova *et al*. 2006).

Some endangered, endemic plants from the genus *Dioscorea* (e.g., *D. caucasia* Lipsky and *D. balcanica* Kosanin) were introduced *in vitro* to reveal differences in morphogenetic paths of regeneration and to preserve these species (Tchulafich *et al*. 1991). The microclonal breeding of these plants by air tubers was also carried out (Grubishich *et al*. 1993). Data about *D. nipponica in vitro* culture, however, is practically absent.

In this article optimal *D. nipponica* cultivation factors *in vitro* were established, as well as the growth of regenerated plants *ex vitro*.

Received: 28 January, 2007. Accepted: 22 March, 2007.

Original Research Paper

MATERIALS AND METHODS

The seeds of *Dioscorea nipponica* Makino, obtained from a botanic Garden of the Mordovian State University, served as the basic material.

Part of the seeds was subjected to scarification by means of abrasive sand (other part was without scarification), following which seeds were superficially sterilized by different concentrations of several agents: ethyl alcohol (70%), Domestos (50%), $KMnO_4$ (0.1%), calcium hypochlorite (7%), H_2O_2 (from 8 to 15%), chloramine B (from 5 to 10%) as well as combinations of some of these. In the main experiment the seeds were sequentially sterilized by a $KMnO_4$ solution (2-3 min), 70% alcohol (1 min), then 6% calcium hypochlorite (15-20 min). After this procedure the seeds were rinsed with sterile distilled water, placed in tubes on hormone-free medium with the mineral (micro- and macronutrients) base of Murashige and Skoog (1962) (MS) and placed to germinate in a thermostat at 25°C in the dark. Infection and germinating of seeds were checked in a few days. Once seeds began to germinate, the tubes were placed in factor-regulated room with light intensity 120-150 micromol·m^{-2}·s^{-1}, temperature from 18 up to 30°C (in main experiments 23-25°C), and grew until the appearance of 1-2 true leaves.

Hormone-free medium with the mineral (micro- and macronutrients) base of Murashige and Skoog (1962) were used as the basic medium. Some variants of the seed germination medium were tested: MS 1, solid agar (0.7%) medium with MS basic salts; MS 2, solid agar (0.7%) medium with MS basic salts + 2.0 g/l activated charcoal; MS 3, solid agar (0.7%) medium with ½MS basic salts (both macro- and micronutrients) + 2.0 g/l activated charcoal; MS 4, liquid medium with MS basic salts; MS 5, liquid MS with ½MS basic salts.

After autoclaving, 15-20 mg/l claforan (cephatoxim), an antibiotic, was added as a powder to the medium.

Obtained sterile plants were clonally micropropagated by cuttings (about 3 mm) included one node with a leaf. Explants were kept for 4-6 h in a solution of antioxidant ascorbic acid (1 mg/l), then placed in tubes (one cutting per tube) on MS medium supplemented with 2% (w/v) sucrose, 0.7% (w/v) agar ("ICN", USA), the following additives all at 1 mg/l – vitamins B_1 (thiamin) and B_6 (pyridoxines), biotin, Ca-pantetonat, riboflavin, myo-inositol – and different concentrations (from 0.1 until to 5.0 mg/l) of growth regulators (2,4-dichlorphenoxyacetic acid – 2,4-D; 6-benzylaminopurine – 6-BAP; 3-indoleacetic acid – IAA).

Some variants of the medium were used to test the rooting of explants and the proliferation of axillary buds: M1, MS supplemented with 0.5 mg/l 2,4-D and 0.1 mg/l 6- BAP; M2, MS supplemented with 1.0 mg/l 2,4-D and 0.7 mg/l 6- BAP; M3, MS supplemented with 5.0 mg/l 2,4-D and 2.0 mg/l 6-BAP; M4, MS supplemented with B_1 and B_6 (up to 2.0 mg/l), 5.0 mg/l niacin, 4.0 mg/l 2,4-D and 2.0 mg/l 6-BAP; M5, MS supplemented with 2.0 mg/l B_1, 1.7 mg/l B_6, 1.8 mg/l IAA and 0.2 mg/l 6-BAP.

The tubes were placed at constant 25°C in the dark for 4 days. Plants were then grown for a 16 hour photoperiod with light intensity 120-150 micromol·m^{-2}·s^{-1} and a 25/18°C day/night temperature. Morphological measurements (proliferating axillary buds, root formation intensity, shoot length) were make every two fortnight for 4 months.

Fully formed *in vitro*-regenerated plants were transplanted to pots with soil (peat-sand-soil, 1:1:1). The pots were covered with polyethylene film, and relative humidity in pots was about 90%. The percentage of surviving plants was measured, and the morphological uniformity of *in vitro*-regenerated plants was compared to that of control, wilt type *D. nipponica* plants.

The experiments *in vitro* and *ex vitro* were repeated three times each, each experiment consisting of 15-20 plants. For all measurements averages and standard errors were calculated by means of standard mathematics on the base of the Microsoft Excel programs.

RESULTS AND DISCUSSION

Seed germination after sterilization

The first stage of *Dioscorea nipponica* introduction to *in vitro* culture involved the sterilization of seeds for obtaining plants in test-tubes. The seeds of *D. nipponica* have a dense seed coat making germination difficult. For improving embryo germination the seeds were to scarified, and then sterilized with the help of a number of sterilizing agents (see Materials and Methods). In this experiment we noted different percent infection, viability of seeds as well as germination percentage (**Table 1**). We found that the best results combining embryo decontamination and viability were by a stepwise treatment of seeds by: 1) a solution of $KMnO_4$, 2) 70% alcohol, 3) 5-6% calcium hypochlorite. This treatment was used in all ensuing experiments.

Table 1 Effects of sterilization agents on infection percent and viability of *Dioscorea nipponica* seeds.

Sterilization agent	Exposition duration (min)	Infection percentage	Seed viability (%)
Ethyl alcohol (70%)	3	52.0	89.0
Domestos (50%)	25	44.3	78.6
$KMnO_4$ (0.1%)	25	86.7	66.2
Calcium hypochlorite (6%)	25	60.4	91.3
H_2O_2 (8%)	25	72.1	92.4
H_2O_2 (12%)	25	59.8	72.1
H_2O_2 (15%)	25	32.4	56.5
Chloramine B (5%)	25	36.3	88.4
Chloramine B (10%)	25	28.1	62.0
Ethyl alcohol (70%) + chloramine B (2%)	3 + 20	20.8	83.4
Ethyl alcohol (70%) + Domestos (50%)	3 + 20	23.3	77.2
$KMnO_4$ (0.1%) + ethyl alcohol (70%) + calcium hypochlorite (6%)	3 + 1 + 20	14.9	90.7

Table 2 Duration of *Dioscorea nipponica* seed germination affected by growth medium composition.*

Growth medium composition	Duration of seed germination (weeks)
MC 1	2
MC 2	5-8
MC 3	2
MC 4	8-12
MC 5	2

*Germination conditions: temperature 25°C; darkness

The speed of seed germination depended on a medium and planting date and varied from 1 to 12 weeks (**Table 2**). The planting date influenced the speed of seed germination, but not to a marked degree. Differences were noticed within 1-2 weeks. Fastest germination was in seeds planted in February and March.

In the case where seeds were not scarified (on all growth media) seed germination was much slower. Scarification is necessary to destroy the testa (= seed coat), impro-

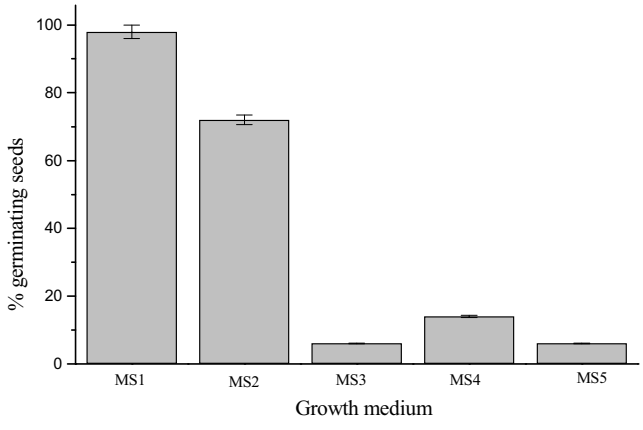

Fig. 2 Effect of growth medium composition on germination percent (± standard deviation) of *Dioscorea nipponica seeds*. Media composition: see Materials and Methods.

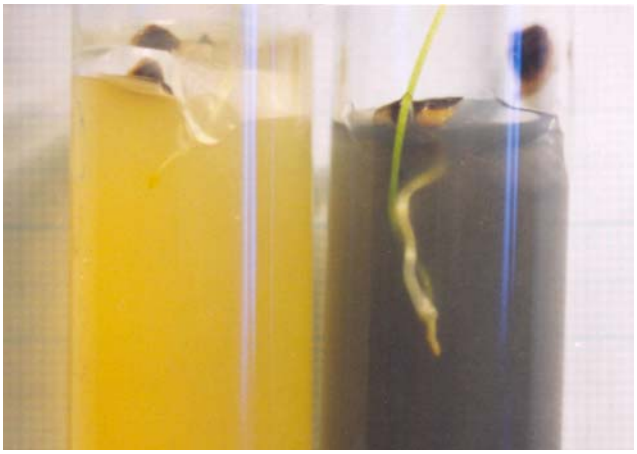

Fig. 3 Germination of *Dioscorea nipponica* scarified seeds on MS medium (left) and MS with activated charcoal (right).

Fig. 4 Germination of *Dioscorea nipponica* seeds on MS medium with activated charcoal (after 5 weeks of cultivation).

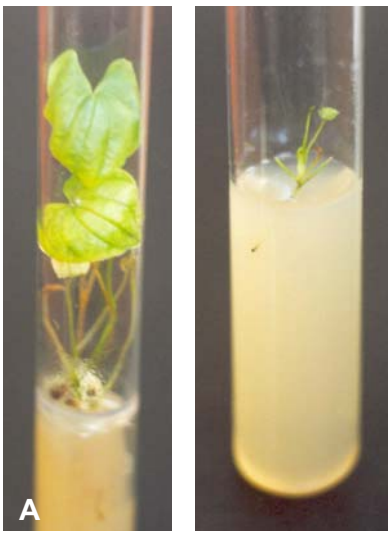

Fig. 5 *Dioscorea nipponica* tuber-plant on MS medium (A). Multiple axial shooting of *Dioscorea nipponica* explant *in vitro* (B).

Table 3 Effect of growth medium composition on axillary buds proliferation in single bud cuttings of *Dioscorea nipponica* after 2 weeks of cultivation.

Medium constituents (mg/l)	Growth medium	% Plants with proliferating axillary buds	Root formation intensity*
2,4-D 0.5 + 6-BAP 0.1	M1	100	++
2,4-D 1.0 + 6-BAP 0.7	M2	90	++
2,4-D 5.0 + 6-BAP 2.0	M3	0	+
Thiamin 2.0 + pyridoxines 2.0 + niacin 5.0 + 2,4-D 4.0 + 6-BAP 2.0	M4	0	+
Thiamin 2.0 + pyridoxines 1.7 + IAA 1.8 + 6-BAP 0.2	M5	73	++

* + rhizogenesis is absent; ++ rhizogenesis is low.
Growth conditions: temperature 25/18°C (day/night); illuminating intensity 120-150 micromol·m^{-2}·s^{-1}; photoperiod 16/8 hour (light/dark).

ving the access of water and nutrients to the embryos. The process of seed germination is fine-adjusted by plant growth regulators. Since nucleases and proteases are at work, there is a disintegration of nucleic acids and proteins, resulting in nucleotides and amino acids, in particular tryptophan, in seeds. Cytokinins derive from nucleotides, auxins from amino acids, and together they influence the start of division and elongation of embryo cells, and as a result, the growing embryo breaks through the testa.

Highest (almost 100%) and shortest (2 weeks) germination were observed on solid agar MS1 medium (**Fig. 2**), particularly from scarified seeds (**Fig. 3**). High seed germination (75%) on MS2 medium supplemented with activated charcoal (2.0 g/l) was also observed (**Fig. 4**). Activated charcoal acts as an antioxidant, adsorbing toxic materials which are secreted by germinating seeds and inhibiting the development of an embryo (Takayama and Misawa 1982). Lowest seed germination was registered in liquid MS4 medium (about 12%).

It is possible to conclude that medium composition plays an essential role in seed germination. Optimum medium for *D. nipponica* seed germination is solid agar (0.7%) medium with MS basic salts.

Growth of *Dioscorea nipponica* plants *in vitro*

Tubes holding seed-germinated plants were placed in factor-controlled rooms (see Materials and Methods for details). The growth of plants was delayed at lower (18-20°C), and especially at higher (27-30°C) temperatures. Plant growth varied with photoperiod (data not shown). Therefore in further experiments *D. nipponica* plants with 1-2 true leaves were placed in suitable growth conditions for maximum and normal growth: light intensity = 120-180 micromol·m^{-2}·s^{-1}, photoperiod 14 h, 23-25°C/16-18°C (day/night). In these conditions plants grew well (**Fig. 5A**).

Clonal micropropagation of *D. nipponica* plants

Clonal micropropagation of *D. nipponica* plants in tubes was achieved by first removing apical dominance (i.e. the apical bud), thus being able to proliferate the emerging axillary shoots.

In particular shoots of normal length as single nodal cuttings were used as secondary explants for recurring propagation. In addition apical dominance could be removed by supplementing the medium with cytokinins (6-BAP, 0.1-2.0 mg/l), resulting in shoot formation with shortened internodes and axillary buds with meristematic meristems, the new shoots.

Clonal micropropagation was achieved on solid agar medium with MS basic salts (full or half), varying a composition and quantity of growth regulators (2,4-D, 6-BAP, IAA) and vitamins. Growth may be inhibited by toxic substances at this stage exuded by explants in the medium, in particular damage induced by cutting activates the enzymes oxidizing plant phenols (Vysozkij 1998). The products of phenol oxidation, in turn, inhibit cell division and elongation (Butenko 1999). Following this logic, and in order to improve the growth of explants, leaves with petioles or shoot cuttings with axillary buds were washed within 4-6 hours in a solution of antioxidant, ascorbic acid (1 mg/l). Ascorbic acid (1 mg/l) was also added to the medium in some experiments. To evaluate the efficiency of development of regenerated plants the beginning of bud proliferation was recorded and shoot length was measured.

In the first week after bud proliferation shoots grew quickly, reaching 0.8-1.0 cm (**Fig. 5B**). The proliferation of axillary buds clearly depends on the medium composition (**Table 3**). Intensive development of axillary buds was observed on media supplemented with growth regulators: M1 containing 0.5 mg/l 2,4-D + 0.1 mg/l 6-BAP (100%); M2 containing 1.0 mg/l 2,4 D + 0.7 mg/l 6-BAP (90%); medium M5 containing 1.8 mg/l IAA + 0.2 mg/l 6-BAP (73%). On M3 and M4 media axillary buds did not develop.

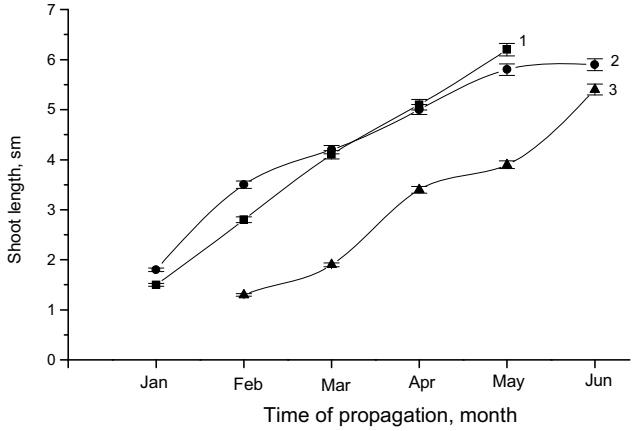

Fig. 6 Dynamics of shoot growth from axillary buds of tubes *Dioscorea nipponica* plants. 1, MS medium supplemented with 2,4-D 0.5 mg/l + 6-BAP 0.5 mg/l; 2, MS medium supplemented with 2,4-D 1.0 mg/l + 6-BAP 0.7 mg/l; 3, MS medium supplemented with IAA 1.8 mg/l + 6-BAP 0.2 mg/l. Growth conditions: temperature 25/18°C (day/night); illumination intensity ~150 micromol·m^{-2}·s^{-1}; photoperiod 16/8 hour (light/dark).

Table 4 Effect of growth medium composition on shoot development and rhizogenesis in single bud cuttings of *Dioscorea nipponica* after 3 weeks of micropropagation.*

Plant growth regulator + vitamin concentrations (mg/l)	Growth medium	Shoot length (mm)	№ shoots /explant	Root formation intensity*
2,4-D 0.5 + 6-BAP 0.1	M1	7.5 ± 0.8	3.0 ± 0.2	–
2,4-D 1.0 + 6-BAP 0.7	M2	5.8 ± 0.6	2.2 ± 0.3	–
Thiamin 2.0 + pyridoxines 1.7 + IAA 1.8 + BAP 2.0	M5	6.3 ± 0.7	3.2 ± 0.4	–

– rhizogenesis is low.
* Growth conditions: temperature 25/18°C (day/night); illumination intensity ~150 micromol·m^{-2}·s^{-1}; photoperiod 16/8 hour (light/dark).

In all growth media root development was low or mediocre (**Table 2**).

Shoots developed most intensively following transplantation onto fresh medium: shoots reached 0.8-1.0 cm in length (**Fig. 6**) and increased 3-4 mm every ten-days. After 5-6 months shoots had reached 5-6 cm. The intensity of shoot growth depended on the medium composition.

Cultivation of *D. nipponica* is ideally done on medium with 2,4-D from 0.5 up to 1.0 mg/l or IAA at 1.8 mg/l, and 6-BAP at 0.1 to 0.7 mg/l. On such media shoots form practically without callus formation thus eliminating the risk of somaclonal variation evolving from callus-derived tissue. Our technique results in the production of uniform material with a high multiplication factor (7-fold in each sub-culture, i.e. 6-8 weeks).

By supplementing the medium with cytokinins apical dominance is removed, resulting in shoots with shortened internodes, and the sprouting of axillary buds into new shoots. On these media the explants form clusters of small shoots, each of which can be clonally multiplied (**Table 4**). In the presence of 2,4-D (0.5-1.0 mg/l), or IAA 1.8 mg/l, and 6-BAP (0.1 mg/l-0.7 mg/l) 95% of axillary buds could develop. The shoots were well turgid, with dark green leaves and reached 2-3 up to 6-8 mm in 3 weeks. An increase of 6-BAP from 0.1 mg/l up to 0.7 mg/l in the medium did not result in increased multiplication, and the growth of formed shoots was not inhibited.

Cultivation of *Dioscorea nipponica* plants *ex vitro*

Regenerated *D. nipponica* plants were micropropagated and grown *in vitro*, and after reaching 5-7 cm in height were directly transplanted into non-sterile conditions. Pots (0.7 L) contained a layer of gravel and soil (peat:sand:soil, 1:1:1). A high percentage of regenerated plants acclimatized (90-98%) with morphologically normal shoots and roots.

The use of healthy regenerated *in vitro* plants as initial transplant material can reduce the propagation period and increase the number of plants.

REFERENCES

* in Russian

Blinova KP, Yakovleva GP (Eds) (1990) *Botanical-Pharmacognostical Dictionary*, Moscow, Wysschaja Shkola, 272 pp*

Borthakur M, Singh RS (2002) Direct plantlet regeneration from male inflorescences of medicinal yam (*Dioscorea floribunda* Mart and Gal.) *In Vitro Cellular and Developmental Biology – Plant* **38**, 183-185

Butenko RG (1999) *Biology of Higher Plant Cells In Vitro and Biotechnologies on its Basis*, FBK-PRESS, Moscow, 160 pp*

Grubishich D, Tchulafich L, Boevich-Zvetich D (1993) Manifestation of air tubers formation sign in relict species *Dioscorea balcanica* Kosanin and *Dioscorea caucasica* Lipsky. *Russian Journal of Plant Physiology* **40**, 283-287

Kageyama K, Yabe K, Iicha T, Washida S (1988) Plant regeneration and acclimatization from meristem of yam (*Dioscorea japonica* Thunb.). *Plant Tissue Culture Letters* **5**, 11-14

Karanova SL (2006) Use of cell selection and induced mutagenesis methods for obtaining *Dioscorea deltoidea* strains with the increased steroid biosynthesis. *Biotechnologia (Moscow)* **2**, 16-19*

Kharkevich SS, Kachura NN (1981) *Rare Species of Plants in Soviet Far East and Their Preservation*, Nauka, Moskow, 231 pp*

Mizuki I, Ishida K, Kikuzawa V (2005) Sexual and vegetative reproduction in the aboveground part of a dioecious clonal plant, *Dioscorea japonica* (*Dioscoreaceae*). *Ecological Research* **20**, 387-393

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Osifo EO (1988) Somatic embryogenesis in *Dioscorea*. *Journal of Plant Physiology* **133**, 378-380

Red Book of RSFSR (1988) Rosagropromizdat, Moscow, 591 pp*

Tachtadjan AL (Ed) (1982) *Life of Plants*. *Flower-Bearing Plants 6*, Prosvetshenie, Moscow, 543 pp*

Takayama S, Misawa M (1982) Differentiation of *Lilium* bulb scales grown *in vitro*. Effect of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. *Physiologia Plantarum* **48**, 121-125

Tchulafich L, Grubishich D, Boevich-Zvetich D (1991) Regeneration of *Dioscorea caucasica* Lipsky and *Dioscorea balcanica* Kosanin in culture *in vitro*. *Russian Journal of Plant Physiology* **38**, 1018-1012

Titova MV, Shumilo NA, Kulichenko IE, Korostelev VV, Oreshnikov AV, Nosov AM (2006) Prolonged cultivation of *Dioscorea deltoidea* Wall cell suspension under semi-continuous conditions in bioreactors. *Biotechnologia (Moscow)* **2**, 28-31*

Vysozkij VA (1998) Biotechnological methods in system of healthy planting material production and of fruit and berry plants selection. PhD Dissertation, Moscow, 44 pp*

Watanabe KN (2002) Challenges in biotechnology for abiotic stress tolerance on root and tubers. *JIRCAS Working Reports* **23**, 75-83