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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Obtaining and Study of Callus and Suspension Plant Cell Cultures of *Tribulus terrestris* L., a Producer of Steroidal Glycosides

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Abstract—Callus and suspension plant cell cultures of *Tribulus terrestris* L., a valuable medicinal plant producing steroidal glycosides, were obtained. The seeds from an American population of *T. terrestris* were used as explants. Regulation of the production and growth of cell cultures, as well as the biosynthetic characteristics of the cell lines, were studied. The combination of phytohormones of 2,4-D (2.0 mg/L) and BAP (1.0 mg/L) was found to be optimal for callus induction and cultivation. Suspension cell culture obtained in liquid medium of the same composition showed such high growth characteristics during prolonged cultivation (more than 2 years) as a maximum accumulation of dry biomass of 13 g/L, specific growth rate at exponential phase of 0.24 day^{-1}, and economical coefficient of 0.39. A semicontinuous mode of cultivation was used to grow the plant cell suspension in a lab-scale bioreactor. Screening of the steroidal glycosides in the obtained cell cultures was carried out. Steroidal glycosides were not found in the callus cultures. However, as was demonstrated by TLC and UPLC ESI MS methods, the suspension culture contained furostanol glycosides, and their amount increased during the cultivation process. These results support the hypothesis of the autoselection of cultivated cells containing compounds promoting their proliferation *in vitro*.

Keywords: callusogenesis, steroidal glycosides, suspension plant cell culture, *Tribulus terrestris* L.

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INTRODUCTION

The cell culture of higher plants is traditionally considered an experimentally created biological system representing a heterogeneous population of dedifferentiated cells [1]. In biotechnology, the use of plant cell cultures *in vitro* is an effective way to produce environmentally friendly renewable plant materials with a high content of targeted biologically active substances, regardless of climatic and weather conditions. In the presence of a highly effective producer strain, this technology ensures the production of original compounds, the manufacturing of which from traditional raw materials is unprofitable or unrealizable. In this aspect, the cell culture of the medicinal plant *Tribulus terrestris* L. may be promising.

It is known that extracts from these plants are safe and effective agents in the treatment of female sexual dysfunction, and they are also used as an aphrodisiac for men [2, 3]. The wound-healing effect of the external application of water extract of *T. terrestris* has been demonstrated [4]. In many studies, biologically active compounds *Tribulus* spp. were shown to have an antitumor effect. In particular, the aqueous extract of *Tribulus* spp. blocked proliferation and induced apoptosis of human liver cancer cells via inhibition of the NF-kB signaling pathway [5–7]. A number of drugs and more than twenty nutraceuticals were produced based on the intact *T. terrestris* plant. In particular, Tribestane and Vitanone drugs are used for the treatment of impotence, and Tribusaponins and Xin-SAK Shutong are used for the treatment of cardiovascular diseases [8, 9]. The production of Tribestane and Tribusaponins has almost stopped these days due to the lack of raw material.

It was established that the biological activity of *Tribulus* spp. extracts in most cases was due to the

Abbreviations: BAP-6-benzylaminopurine; 2,4-D-2,4-dichlorophenoxyacetic acid; NAA—α-naphthylacetic acid; TLC—thin layer chromatography; UPLC ESI MS—ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry.

presence of the wide variety of steroidal glycosides [6, 10, 11] with the following main aglycons: tigogenin, neotigogenin, gitogenin, neogitogenin, gecogenin, neogecogenin, diosgenin, chlorogenin, ruscogenin, and sarsasapogenin [8]. The composition of steroidal glycosides of an intact plant depends on the area of growth [12].

There are reports about production of *Tribulus* spp. callus cultures [13–16]. One of the studies demonstrated the presence of spirostanol glycosides in *Tribulus terrestris* calluses [14]. According to an analysis of available literature, *T. terrestris* suspension culture was not previously obtained. Systematic studies of *Tribulus* spp. cell cultures also have not been performed previously.

Thus, the goal of the present study was the production of callus and suspension plant cell cultures of *Tribulus terrestris* L., elucidation of features of callusogenesis, and study of the growth and biosynthetic characteristics of the obtained cell cultures.

MATERIALS AND METHODS

Seeds of *Tribulus terrestris* L. plant of the American population were used as explants (Forever Seeds Company, the United States). The seeds were washed with Tween-80 detergent (Sigma, Germany) and sterilized with 0.1% solution of mercury bichloride (Sigma) for 5 min. After sterilization, the material was rinsed and then washed three times for 20 min in sterile distilled water. After that, the seeds were placed on agar nutrient medium and used as explants.

For the experiments, MS medium prepared in the standard formulation [17] with the addition of casein hydrolyzate (0.5 g/L) (Merck, Germany), inositol (0.1 g/L) (Merck), 3% sucrose (Merck), and agar (0.5%) (Merck) was used. Nine variants of media differing in the composition of growth regulators were used. The following growth regulators were used in the study: α-naphthylacetic acid (Merck) (0.2–2.0 mg/L), 2,4-dichlorophenoxyacetic acid (2.4-D) (Merck) (1.0–2.0 mg/L), kinetin (Merck) (0.15–1.0 mg/L), and 6-benzylaminopurine (BAP) (Merck) (0.15–2.0 mg/L).

Cultivation was carried out in the dark at 26°C. The subcultivation cycle for callus cell cultures was 4 weeks, and the cycle for suspension cell cultures was 2 weeks. Petri dishes ($d = 60$ mm) were used to cultivate callus cultures, and the callus was divided into 4–6 parts for passages. Suspension cell cultures were grown in flasks with a volume of 250 mL (30–40 mL of suspension in the flask) on a shaker (100 rpm). For inoculation, the ratio of inoculum to fresh medium was 1 : 10.

The growth in a lab-scale bioreactor was performed in a 7 L New Brunswick Scientific laboratory bioreactor (working volume of 5 L), with a mechanical agitator of the "marine-screw" type (stirring speed of 200 rpm). For the bubbling, an original single-point aerating device based on a silicone hose with microholes was used. The aeration rate was 1.0 L/min (0.2 volume of air/volume of medium/min); the cultivation temperature was 26°C.

Such parameters as the content of dry and raw biomass in 1 L of medium, the concentration of cells in the medium, and the viability of the culture were determined to characterize the suspension cultures.

To determine the wet and dry biomass content, a fixed volume of cell suspension (not less than 15 mL in triplicates) was filtered under a vacuum through a paper filter with a Buchner funnel. The biomass was dried to a constant weight in a stream of warm air at 30°C [18].

To determine the cell concentration (X), 0.5 mL of the suspension was incubated with 2.0–2.5 mL of 20% chromic acid solution (Merck) at 60°C for 15–20 min, depending on the age of the suspension [19].

The viability of cell cultures was determined via intravital staining with phenosafranine (Merck) (0.1% solution) by counting live (unstained) and dead (stained) cultured units under a microscope [20].

The parameters of culture growth efficiency were calculated based on the obtained results: the growth index (I), the specific growth rate at exponential phase (μ), the economical coefficient of sucrose (Y), the doubling time (τ), and the dry biomass productivity (P).

The following formulas were used for calculations [21]:

$$I = (X_{max} - X_0)/X_0,$$

where X_{max} and X_0 are the maximum and initial content of dry biomass per liter of nutrient medium.

$$\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1),$$

where X_2 and X_1 represent the content of dry biomass per liter of nutrient medium at the time points equal to t_2 and t_1, respectively (calculated for exponential growth phase).

$$\tau = \ln 2/\mu;$$

$$Y = (X_{max} - X_0)/S,$$

where X_{max} and X_0 are the same as above and S is the initial concentration of the substrate (sucrose) in the medium;

P is the maximal value,

$$P_i = (X_i - X_0)/(t_i - t_0),$$

where X_0 and X_i is the content of dry biomass at the beginning of cultivation and at the time point t_i, respectively.

Analysis of the presence of steroid glycosides in the cell culture was carried out by thin layer chromatography (TLC) in silica gel (see below) and ultra-perfor-

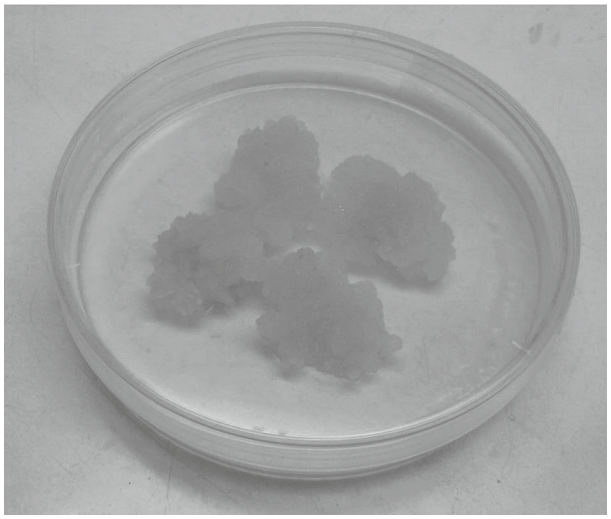

Fig. 1. Callus culture of *T. terrestris* cells.

mance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC ESI MS).

For sample preparation, 100 mg of freeze-dried cell culture biomass were extracted with 70% (by volume) ethanol (ratio biomass-solvent = 1 : 40 (wt/vol) three times for 30 min at room temperature under the action of ultrasound (ultrasound bath Sapfir, Russia). The combined ethanol extracts were evaporated to dryness under vacuum (55°C) and dissolved in water. The resulting solution was applied to a Supelclean ENVI-18 cartridge for solid-phase extraction (Supelco, the United States). The cartridge was successively washed with water and 70% ethyl alcohol. The alcohol fraction was evaporated to dryness and subjected to analysis.

For TLC analysis, the purified glycoside fraction was dissolved in ethanol (70%), and 50 μL of the obtained solution was applied onto a Kieselgel 60 plate (Merck). Furostanol glycosides were eluted by chloroform-methanol-water solvent system (65 : 35 : 10 by volume). Chromatograms were stained with Ehrlich's reagent (1% solution of para-dimethylaminobenzaldehyde (Merck) in a mixture of concentrated hydrochloric acid (Merck)–methanol (Merck) = 34 : 66 vol/vol), followed by plate heating at 100°C for 3–5 min. Furostanol glycosides were detected as red spots.

An ethyl acetate-glacial acetic acid-water system (32 : 9 : 9 by volume) was used to separate the spirostanol glycosides. The chromatograms were developed with a 1% vanillin solution (Merck) in a mixture of ethanol-concentrated sulfuric acid (Merck) (2 : 1 by volume).

For UPLC ESI MS the sample was dissolved in a mixture of acetonitrile (Merck) –water (50 : 50 by volume) and filtered through a nylon filter with 0.2 μm pores (Acrodisc, Germany).

UPLC ESI MS was performed with a Waters Aquity UPLC (Waters, the United States) chromatograph. The sample (0.5 μL) was applied to an ACQUITY UPLC BEH Phenyl column (50 × 2.1 mm, 1.7 μm, Waters, Ireland). The column temperature was 40°C, and the flow rate of the mobile phase was 0.4 mL/min. As the mobile phase, 0.1% (vol/vol) solution of formic acid (Merck) in water and a 0.1% (vol/vol) solution of formic acid in acetonitrile were used. Chromatographic separation was carried out in the gradient elution mode. During the analysis, the composition of the mobile phase was changed as follows (concentration of acetonitrile, vol %): 0–7 min—19%, 7–17 min—19 → 35%, 17–23 min—35 → 45%, 23–27 min—45 → 55%, 27–33 min—55 → 65%, 33–33.5 min—65 → 95%, 33.5–35 min—95%, 35–35.5 min—95 → 19%, 35.5–37—19%.

The analysis was carried out in the positive ion detection mode (range m/z 100–1200) with the following process parameters: ionization source temperature of 120°C, desolvation temperature of 250°C, capillary voltage of 3.0 kV, sample cone voltage of 30 V, and a nitrogen supply rate (desolvation gas) of 600 L/h.

RESULTS AND DISCUSSION

As a result of the experiments on the effect of various growth regulators on callusogenesis, it was established that callus cultures of the studied *Tribulus* spp. of the anchor are formed only on medium containing 2.0 mg/L of 2,4-D and 1.0 mg/L of BAP. The efficiency of synthetic auxin (2,4-D) for induction of callusogenesis of *Tribulus* spp. was described earlier [14–16].

Primary callusogenesis started directly from the seeds on the 10th day of cultivation without the formation of roots and shoots. The intensity of callusogenesis was 20%. The resulting callus cultures were characterized by light yellow color, loose structure, and high degree of hydration (Fig. 1).

On a medium containing NAA and BAP, the formation of somatic embryoids on seeds was observed in the first weeks of cultivation; however, these embryoids died out during the second to third passages. However, when embryogenic cultures were transferred to a medium containing 2,4-D as auxin, they also formed callus with morphological characteristics similar to those of calli obtained initially on the medium with 2,4-D and BAP. Growth processes were not detected on other variants of the used media.

As a result of the study, a callus cell culture of *T. terrestris* that retained a high growth potential for more than 30 cycles of subcultivation (more than two years of cultivation) was obtained.

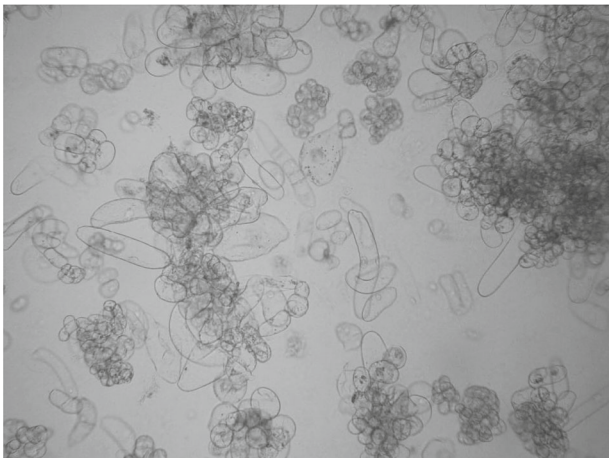

Fig. 2. Suspension culture of *T. terrestris* cells.

A 4-week callus culture after the third growth cycle was used as the explant for the production of the suspension cell cultures. About 30 g of callus cells (raw biomass) were placed on the liquid medium used for growing calli (MS containing 2.0 mg/L of 2,4-D and 1.0 mg/L of BAP), but without agar. After 14 or 21 d of cultivation, the resulting primary suspension cell culture with different densities (dilutions from 1 : 5 to 1 : 10) was transferred on the fresh medium. By the fifth cycle of cultivation, the optimal mode of subculture was determined: the use of a 14-d culture and 10-fold dilution (3 mL of inoculum per 30 mL of fresh medium); the initial density of the culture was about 1.5 g of dry biomass per 1 L of the medium. The resulting suspension cell culture was yellow and contained small aggregates of meristem-like cells, single parenchymal-like and elongated cells, and pear-shaped cells with a large amount of starch grains (Fig. 2).

During the eighth growth cycle, the growth characteristics of the obtained suspension culture of *T. terrestris* cells were analyzed. The dynamics of cell culture growth in normal and semilogarithmic coordinate systems is shown in Fig. 3. Analysis of the presented growth curves demonstrated the absence of lag phase at the initial culture density of 1.5 g/L by dry weight. The derivative growth characteristics are shown in Table 1. These data demonstrated that the culture can be attributed to cultures with strong growth (the maximal accumulation of dry biomass was 13 g/L, the growth index $I = 8.6$, the specific growth rate at the exponential phase for dry biomass was 0.24 day^{-1}, and the economical coefficient ~0.4, dry biomass productivity—1 g/L/day). It should be noted that the culture is characterized by stable growth; it does not decrease in more than two years of cultivation.

The cultivation of a suspension culture of *T. terrestris* cells in a lab-scale bioreactor was performed with a 7-liter New Brunswick Scientific laboratory bioreactor in semicontinuous mode with mechanical stirring. Three cycles of cultivation were performed, and the results are shown in Fig. 4. The data from these experiments showed that cultivation in a lab-scale bioreactor led to an increase in the growth characteristics of the culture. During cultivation of the cell suspension in the flasks, the growth index and the specific growth rate for the dry weight were 8.6 and 0.24 day^{-1}, but these indices increased to 11.3 and 0.38 days^{-1}, respectively, by the third cycle of growth in the bioreactor (Table 2). The high rates of cell culture growth in the bioreactor as compared with those in flasks can be due to the intensity of cell aeration. A rigorous proof of such regularity is complicated; however, it is known that the concentration of dissolved oxygen during cul-

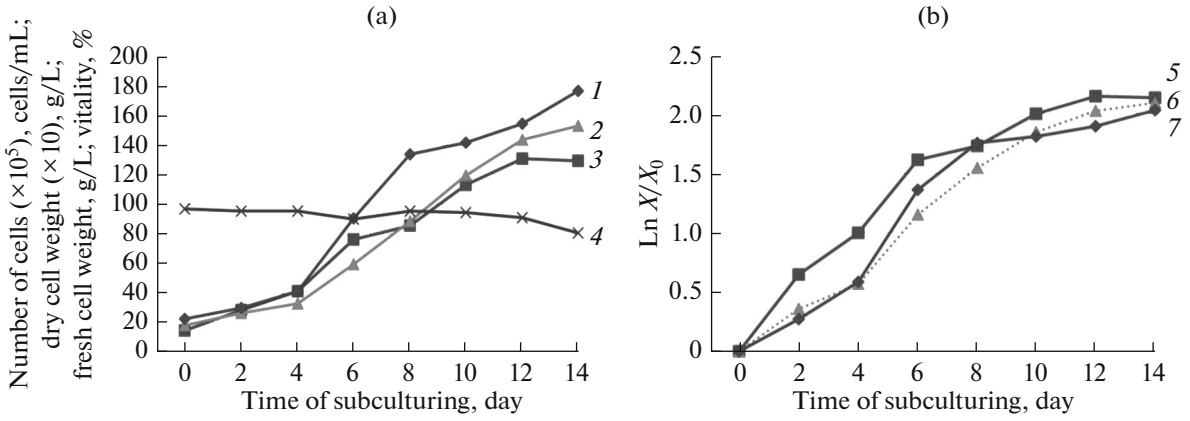

Fig. 3. Dynamics of *T. terrestris* suspension culture growth in flasks (250 mL) in normal (a) and semilogarithmic (b) coordinate systems: (*1*) number of cells x 10^5, cells/mL; (*2*) fresh cell weight, g/L; (*3*) dry cell weight × 10, g/L; (*4*) viability, %; (*5*) dry cell weight, lnX/X_0; (*6*) fresh cell weight, lnX/X_0; (*7*) number of cells, lnX/X_0 (X is current value of the parameter during culturing cycle, and X_0 is initial value of the parameter; initial culture density was 1.5 g/L).

Table 1. Parameters of growth efficiency of *Tribulus terrestris* suspension culture in flasks

Initial parameters of growth efficiency	Derived parameters of growth efficiency					
	I	μ, day^{-1}	τ, day	M_{max}, g/L	*Y*	*P*, g/L/day
Cell concentration in suspension	7.7	0.29	2.39	13.1	0.39	1.0
Fresh cell weight	8.1	0.24	2.89			
Dry cell weight	8.6	0.24	2.89			

I—growth index; μ—specific growth rate in exponential growth phase; τ—doubling time ; M_{max}—maximum accumulation of dry cell weight; *Y*–economical coefficient; *P*—productivity of dry cell weight.

Table 2. Growth characteristics of *Tribulus terrestris* suspension culture growing in a bioreactor

Cycle number	Initial parameters of growth efficiency	Derived parameters of growth efficiency					
		*I**	μ, day^{-1}	τ, day	M_{max}, g/L	*Y*	*P*, g/L/day
1	Fresh cell weight	5.3	0.21	3.30	13.0	0.39	1.3
	Dry cell weight	9.8	0.24	2.89			
2	Fresh cell weight	10.3	0.37	1.87	11.4	0.32	1.4
	Dry cell weight	6.5	0.46	1.51			
3	Fresh cell weight	13.7	0.28	2.48	13.8	0.42	1.2
	Dry cell weight	11.3	0.38	1.82			

* For designations, see note to Table 1.

tivation in flasks is reduced, and it is maintained in the bioreactor at a constant level of 10–15% [22].

The screening of cell extracts of callus cultures by TLC did not reveal steroid glycosides.

Preliminary phytochemical analysis of extracts from the biomass of the primary suspension *T. terrestris* cell culture (third to sixth cultivation cycles), spirostanol or furostanol glycosides were not detected by TLC. However, repeated analysis after 6 months of cultivation (12th–15th cultivation cycles) allowed the detection of furostanol glycosides in *T. terrestris* culture. Four spots of substances positively reacting with Ehrlich's reagent were found on chromatograms (Fig. 5). During the further cultivation of the suspension culture (more than two years), a stable formation of at least two furostanol glycosides remained.

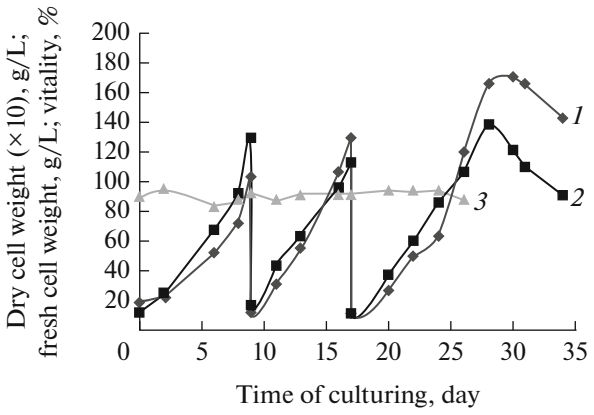

Fig. 4. Growth characteristics of plant cell suspension culture of *T. terrestris* in normal coordinate system during bioreactor cultivation (*1*) fresh biomass, g/L; (*2*) dry biomass × 10, g/L; and (*3*) viability, %.

UPLC ESI MS analysis of the extract from the cell culture biomass was performed for the identification of the detected furostanol glycosides. The presence of peaks of the two main compounds with a retention time of 5.6 and 5.9 min was shown. Analysis of the mass spectra of the positive ions of these compounds (Table 3) allowed us to conclude that they both have tigogenin as aglycone (the presence of a characteristic ion with *m/z* 417) [23]. The presence of an intense ion with *m/z* 1065, which corresponds to the protonated molecule of a glycoside that has lost the water ($[M\text{-}H_2O+H]^+$), indicates that two detected glycosides belong to the furostanol series. A similar phenomenon is very typical for the furostanol forms of steroidal glycosides, in the molecules of which a labile hemiacetal C22-OH group is

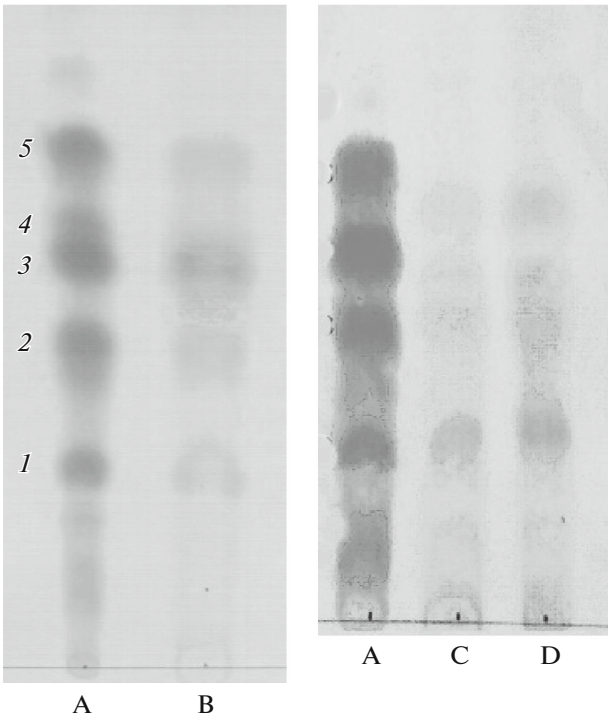

Fig. 5. Thin-layer chromatography of biomass extracts of *T. terrestris* suspension cell culture: A—standard, a sum of steroidal glycosides from *Dioscorea deltoidea* culture biomass; B, C and D—extracts of *T. terrestris* cell culture biomass: B—15th cycle of growth; C—45th cycle; D—47th cycle; *1–5*, designations of furostanol glycosides according to a decrease in their polarity.

present [23]. This assumption was also confirmed by the presence of the $[M+Na]^+$ ion-adduct with *m/z* 1105 in the mass spectra of both glycosides. Analysis of fragment ions (see Table 3) showed that both compounds had four hexose residues (neutral losses of 162 Da). The identity of the mass spectra of two glycosides indicates that they are isomers. Two types of isomerism are known for plant steroidal glycosides [8]: isomerism of the structure of the aglycone and/or the structure of carbohydrate chains. Comparison of the chromatographic behavior of glycosides found in the biomass of *T. terrestris* cell culture with that of the isomeric glycosides of diosgenin [24] suggested the presence of isomeric aglycone structures (probably, 25R/S-isomers, i.e. derivatives of tigogenin and neotigogenin).

Based on a comparison of our results with the literature data [8], it can be concluded that the detected glycosides correspond to 25R/S isomers of terrestrosin H, which was previously isolated from intact *T. terrestris* plants. However, further studies are required to confirm this conclusion.

Analysis of patterns of the formation of steroidal glycosides during long-term cultivation of *T. terrestris* cells *in vitro* allows the assumption that their formation begins as a result of the autoselection of cells containing furostanol glycosides. Such development of the plant cell population *in vitro* can be explained by the antioxidant activity of furostanol glycosides, promoting cell proliferation. A similar phenomenon was previously observed in *Dioscorea deltoidea* cell cultures [24].

Thus, a suspension culture of *T. terrestris* cells was obtained for the first time. The peculiarities of the production of *T. terrestris* cell culture and its growth characteristics were described. The plant cell suspension was grown in a lab-scale bioreactor. A stable synthesis of furostanol glycosides in the cell suspension culture was obtained, and their preliminary structural identification was carried out.

Table 3. Mass-spectrometry of positive ions of the two main compounds detected in the biomass of *T. terrestris* suspension cell cultures by UPLC ESI MS

Retention time, min	MS data		
	$[M-H_2O+H]^+$, *m/z*	$[M+Na]^+$, *m/z*	Other ions, *m/z*
5.6	1065.5695	1105.5447	903.4985 $[M-H_2O-Hex^*+H]^+$
			741.4469 $[M-H_2O-2Hex+H]^+$
			579.3880 $[M-H_2O-3Hex+H]^+$
			417.3363 $[M-H_2O-4Hex+H]^+$
5.9	1065.5592	1105.5492	903.5082 $[M-H_2O-Hex+H]^+$
			741.4478 $[M-H_2O-2Hex+H]^+$
			579.3897 $[M-H_2O-3Hex+H]^+$
			417.3444 $[M-H_2O-4Hex+H]^+$

* Loss of hexose residue ($C_6H_{10}O_5$, 162 Da).

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