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In Vitro Micropropagation of *Drosera rotundifolia*

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ABSTRACT

The aim of the study was to determine an optimal composition of growth media for *Drosera rotundifolia* L. cultured *in vitro*. Adventitious shoots of *Drosera rotundifolia* originating from an established *in vitro* culture were placed on complete Murashige and Skoog (MS) (1962) medium, MS medium with nutrient content reduced to ¾ MS, ½ MS, ¼ MS, $^1/_6$ MS and MS medium with selected macronutrient (KNO_3, KNO_3 + NH_4NO_3, KH_2PO_4 or $CaCl_2$) content reduced by ½. The medium containing ¼ of standard MS nutrients was found the most effective, as plants in this variant had the greatest weight and developed the greatest number of adventitious shoots (37.1). It was also conducive to root growth and development. Flowering was only observed on complete MS, ¾ MS and ½ MS media and MS with reduced content of KNO_3 or KNO_3 + NH_4NO_3. Reducing mineral content to ¼ MS or more resulted in red coloration of glandular tentacles.

Keywords: micropropagation, carnivorous, plants, phytohormones, MS medium

1. INTRODUCTION

Sundew is a widespread genus comprising 194 carnivorous species found nearly everywhere in the world. Its largest populations grow in Oceania, and there are over 40 varieties in southwest Australia alone. It is common in North and South America, and European peat bogs, particularly in taiga and tundra. It also grows in Africa. Sundews are

herbaceous perennial (rarely annual) plants, the life cycle of which may be even 50 years long. Depending on species, the plants form vertical, 1 cm to 1 m high, rosettes. Some species are even creepers with up to 2 m long shoots.

Sundew is so effective in deriving nutrients from its prey that it does not produce nitrate reductase, an enzyme commonly used by other plants to absorb substances from soil (Karlsson and Pate, 1992). As sundew has low soil requirements, it is mainly growing in areas poor in nutrients, such as bogs, swamp forests, wet heaths and shores of dystrophic lakes. However, it has also been confirmed at sites covered with general vegetation (Lloyd, 1942).

Since sundew grows in nitrogen-depleted environment, nitrogen deficiency is made up for with carnivorousness. It attracts its prey with glittering drops of sweet liquid secreted at the end of the tentacles surrounding the leaf blade. Anthocyanins make the sundew leaves red, thus improving their attractiveness to insects. When the prey sits on a leaf, the story of its slow and precise killing begins. First, the liquid covering the leaf surface glues the insect's legs. While the insect moves, vibrations stimulate sensory receptors in the tentacles. The tentacles then start to elongate and bend towards the prey. The section of the leaf on which the insect has been immobilized begins to fold and slowly wraps the prey up. After securing the prey on the leaf surface, formic acid is released from the tentacle tips and it dissolves the insect's body. Protein molecules released from its body again stimulate the tentacle receptors. The appearance of proteins triggers the secretion of proteolytic enzymes that transform the insect into a nutrient rich (predominantly nitrogen compounds) juice. Once the nutrients are absorbed, the tentacles open up and undigested remnants are blown by the wind. Glandular tentacles are extremely sensitive to touch. The more protein the prey contains, the more abrupt the plant reaction is (Stichmann-Marny, 1997).

Special properties of sundew have been for long known in conventional and herbal medicine. Its extract containing glycosides, choline and cyanogenic compounds is an effective painkiller. Sundew leaf tincture used to be a very popular remedy in folk medicine and was used to alleviate the symptoms of women's diseases. Toothache was cured with sundew wash and herbal infusions brought relief in eye and headache and stomach and heart diseases. Sundew flowers used to be dried up and smoked instead of tobacco (Banasiuk et al. 2012). Apart from proteolytic enzymes, sundew herb contains naphthoquinones (plumbagin, ramentaceon, ramenton, hydroplumbagin glucoside and 7-methylhydrojuglone) and flavonoids. Plumbagin shows weak haemostatic and strong bactericidal activity against staphylococci and streptococci, and mycobacteria and diplococci that cause respiratory diseases. Aqueous extracts of sundew have antibacterial, antitussive, antispasmodic (*spasmolyticum*) and sedative properties. Sundew herb is recommended for the treatment of influenza, whooping cough, tonsillitis, tuberculosis, scarlet fever, tonsillitis, laryngitis, bronchitis, sore throat and pneumonia, asthma, and sinusitis. Sundew extracts have also antiatherogenic and antianginal properties. In papers from 19th century, sundew was described as an antiepileptic medicine. Its cytotoxic and anticancer activity has also been confirmed (Kawiak et al. 2012 a, Kawiak et al. 2012 b). There are three sundew species in Poland, round-leaved sundew, great sundew and spatulate-leaved sundew that is a cross of the first two.

Due to medicinal properties of sundew listed above, the attempts at increasing its population with available alternative methods have recently been increased. As *Drosera* populations have significantly dwindled over the last few decades as a result of degradation of its natural habitats, the possibilities of collecting plant extracts are limited (Krenn and

Karting, 2005). Research showed that plants grown in sterile in vitro cultures had six times higher levels of active substances than those grown in the wild (Wawrosch, 2005). However, effective propagation of sundew *in vitro* requires precise determination of culture conditions, and particularly mineral composition of the medium. Therefore, the aim of the study was to figure out optimal composition of the medium for *in vitro* culture of *Drosera rotundifolia* L.

2. MATERIAL AND METHODS

The experiment was conducted in 2014, in the Department of Plant Genetics, Breeding and Biotechnology, West Pomeranian University of Technology in Szczecin. In the first step, plant material was multiplicated to be used in further research. Adventitious shoots of *Drosera rotundifolia* L. were placed on MS medium (Murashige and Skoog, 1962) without any plant growth regulators. When the adventitious shoots reached a diameter of 0.5 to 1 cm, they were placed on MS media with different content of macro- and micronutrients. The control variant was a complete MS medium, and in the experimental variants the content of macro- and micronutrients was reduced to ¾ MS, ½ MS, ¼ MS, $^1/_6$ MS, and 1/8 MS (Table 1). The shoots were also placed on MS media in which the levels of selected macronutrients were reduced by half, i.e. ½ KNO_3, ½ KNO_3 + ½ NH_4NO_3, ½ KH_2PO_4 or ½ $CaCl_2$.

The media contained also 8 g·dm^{-3} agar, 30 g·dm^{-3} sucrose and 100 mg·dm^{-3} inositol. pH was adjusted to 5.7 with 0.1M NaOH and HCl. Then, aliquots of 30 ml were poured into 300 ml jars and autoclaved for 20 min at 121 °C. The plants grew in a phytotron under 16 h photoperiod (16 h light / 8 h darkness) at 24 ± 1 °C. Phytotron shelves were illuminated with 36W cool-light fluorescent lamps generating light with intensity 35 mmol m^{-2}s^{-1}. Each jar contained five plants and each variant was established in 10 repetitions.

After eight weeks the following parameters were assessed: leaf rosette width, root length [cm], number of roots, plant weight [g], number of adventitious plants, percent of flowering plants [%] and plant color. The measurement results were analyzed statistically. The experiment was set in a one-factor completely randomized design. The significance of differences was determined by analysis of variance and the Duncan's Multiple Range Test at P = 0.05.

Table 1. Mineral composition of the media.

Component	Mineral content [mg dm^{-3}]									
	MS	**¾ MS**	**½ MS**	**¼ MS**	**$^1/_6$ MS**	**$^1/_8$ MS**	**MS ½ KNO_3**	**MS ½ KNO_3 ½ NH_4NO_3**	**MS ½ KH_2PO_4**	**MS ½ $CaCl_2$**
KNO_3	1900	1425	950	475	316,7	237,5	950	950	1900	1900
NH_4NO_3	1650	1237,5	825	412,5	275	206,3	1650	825	1650	1650

KH_2PO_4	170	127,5	85	42,5	28,3	21,3	170	170	85	170
$MgSO_4 \cdot 7H_2O$	370	277,5	185	92,5	61,7	46,3	370	370	370	370
$CaCl_2 \cdot 2H_2O$	440	330	220	110	73,3	55	440	440	440	220
$NaH_2PO_4 \cdot H_2O$	170	127,5	85	42,5	28,3	21,3	170	170	170	170
H_3BO_3	6,2	4,7	3,1	1,6	1	0,8	6,2	6,2	6,2	6,2
$ZnSO_4 \cdot 7H_2O$	8,6	6,5	4,3	2,2	1,4	1,1	8,6	8,6	8,6	8,6
KI	0,83	0,6	0,4	0,2	0,1	0,1	0,8	0,8	0,8	0,8
$CuSO_4 \cdot 5H_2O$	0,025	0	0	0	0	0	0	0	0	0
$CoCl_2 \cdot 6H_2O$	0,025	0	0	0	0	0	0	0	0	0
$FeSO_4 \cdot 4H_2O$	27,8	20,9	13,9	7	4,6	3,5	27,8	27,8	27,8	27,8
$MnSO_4 \cdot 4H_2O$	22,3	16,7	11,2	5,6	3,7	2,8	22,3	22,3	22,3	22,3
$Na_2MoO_4 \cdot 2H_2O$	0,25	0,2	0,1	0,1	0	0	0,3	0,3	0,3	0,3
Na_2EDTA	37,3	28	18,7	9,3	6,2	4,7	37,3	37,3	37,3	37,3
Nicotinic acid	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Inositol	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Thiamine	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,4
Pyridoxine	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5	0,5
Glycin	2	2	2	2	2	2	2	2	2	2

3. RESULTS AND DISCUSSION

Sundews, rich in cyanogenic compounds, glycosides and choline, are precious and unique plants. Secondary metabolites derived from them are used in the pharmaceutical, cosmetic and food industry. Research showed that metabolite levels in the plants grown *in vitro* may be even a few times higher than in those growing in natural conditions. Therefore, *in vitro* multiplication of sundew has become very popular recently. In vitro cultures provide secondary metabolites of high quality, do not require pesticides and are independent of atmospheric conditions (Burbidge, 1994).

In vitro management of *Drosera* L. has been investigated in many studies aimed at determining the most favorable conditions for its growth (Kukulczanka and Czastka 1991; Bobák et al. 1995; Kawiak et al., 2003). However, the reported findings are highly contrasting, particularly with regard to the content of mineral components in the growth medium. A study by Kawiak et al. (2003) identified MS medium with mineral content reduced by half as the one most effectively meeting sundew requirements. However, there seem to be no universal medium for all *Droseraceae* species. Królicka et al. (2008) claimed that the best substrate for *Drosera binata* propagation was Vacin and Went medium devoid of plant growth regulators. A study on the effects on various levels of macronutrients in MS medium on the growth of spatulate-leaved sundew (*Drosera intermedia* L.) revealed the most intense plant growth at $^1/_3$ MS medium (Jang, 2003). Grevenstuk et al. (2010), who researched the same species, reported as the most effective the MS medium with mineral content reduced by 75% and not supplemented with any plant growth regulators.

Our results indicated that the medium most favorable for the growth of round-leaved sundew was ¼ MS (Table 2). The plants grown on this medium had the greatest weight (0.725 g) and produced the greatest number of progeny plants (37.1). The number of progeny plants produced by sundews cultured on different media was significantly lower and ranged from 12.9 for 1/8 MS to 20.1 for ½ MS. Significant effects of mineral content reduction on the width of leaf rosette was observed only in 1/6 and 1/8 MS variants, as compared with complete MS medium.

The study showed a clear correlation between reduced content of minerals and increased number of roots. The greatest number of fairly long roots was developed on ¼ MS medium. Numerous roots were also produced by sundew plants growing on the media with the content of nitrogen compounds reduced by half, i.e. ½ KNO_3. According to Rejthar et al. (2014), development of root system during multiplication is highly beneficial from an economic perspective, as it allows for skipping the rooting stage constituting a standard step of micropropagation.

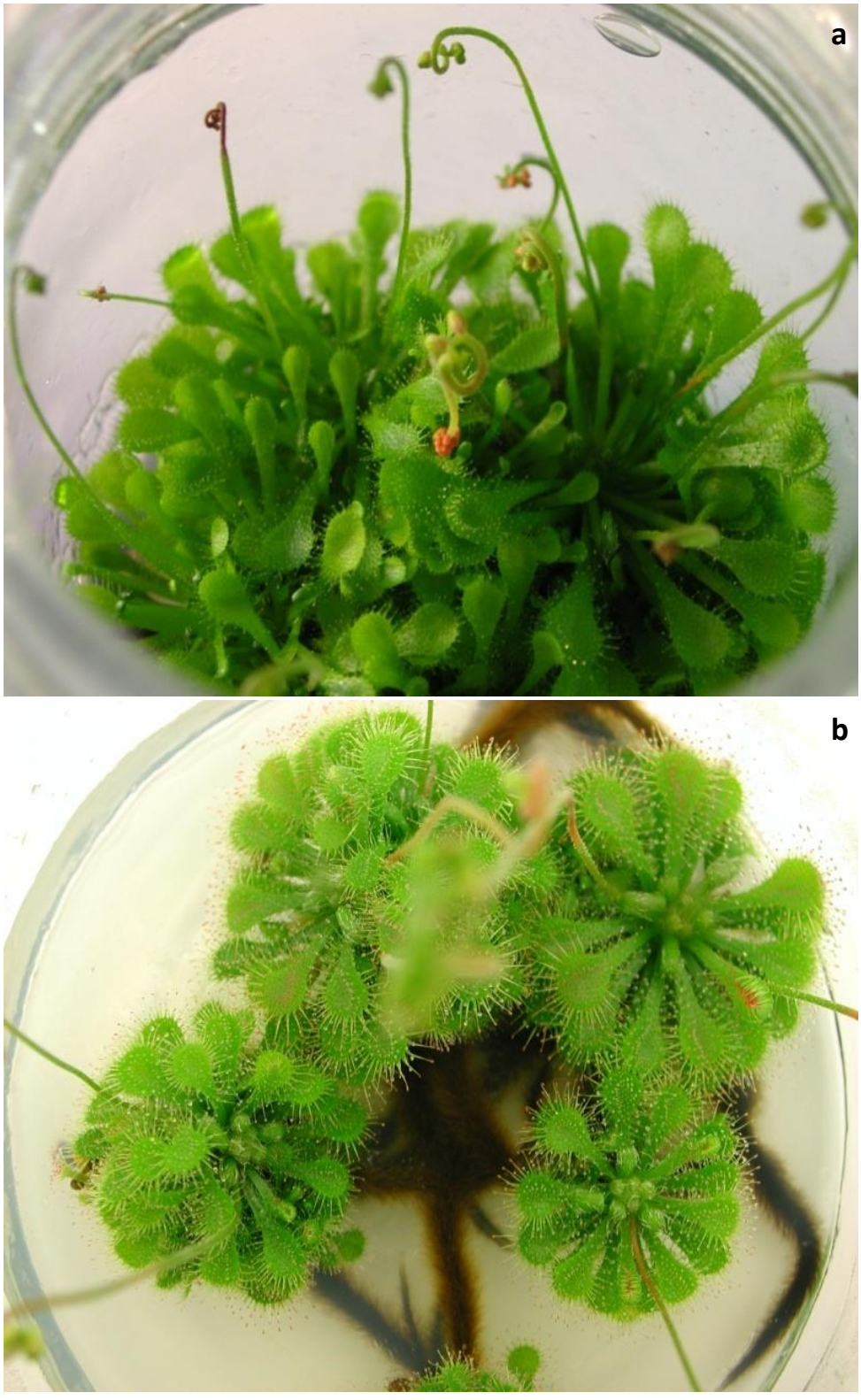
Research reports present flowering as a complicated process controlled by genetic and environmental factors that is rarely initiated *in vitro*. Vásquez-Collantes et al. (2014) investigated sundew flowering *in vitro* and tested standard MS media, MS media with ten and five times lower content of minerals, media enriched with 0.1 mg/l gibberellic acid, 0.1 mg/l IAA and BAP, and different photoperiods. An addition of 0.1 and 1 mg/L of GA_3 induced flowering in 50% of the plants exposed to 20 h photoperiod but the presence of BAP did not initiate flowering at all. In our study, sundews developed flower shoots only when grown on the media with the highest concentrations of macro- and micronutrients (Table 2). Flowering was only observed on complete MS, ¾ MS and ½ MS media and MS with reduced content of KNO_3 or KNO_3 + NH_4NO_3. The percentage of plants producing inflorescence stems decreased along with the reduction of mineral content in the media. They were developed in 96% of plants grown on complete MS medium and 90% and 62% plants grown on ¾ MS and ½ MS, respectively.

An interesting plant response to the reduction of mineral content in the medium was a synthesis of bright red anthocyanins (Foot et al. 2014). This phenomenon was investigated by Ichiishi et al. (1999), who researched the effects of different levels of macro- and micronutrients and sucrose in MS medium. The authors concluded that lowered concentration of NH_4NO_3 caused a change in the color of tentacles but not the leaves. Moreover, removal of NH_4NO_3 and KNO_3 from the medium resulted in other changes in plant coloration. In our

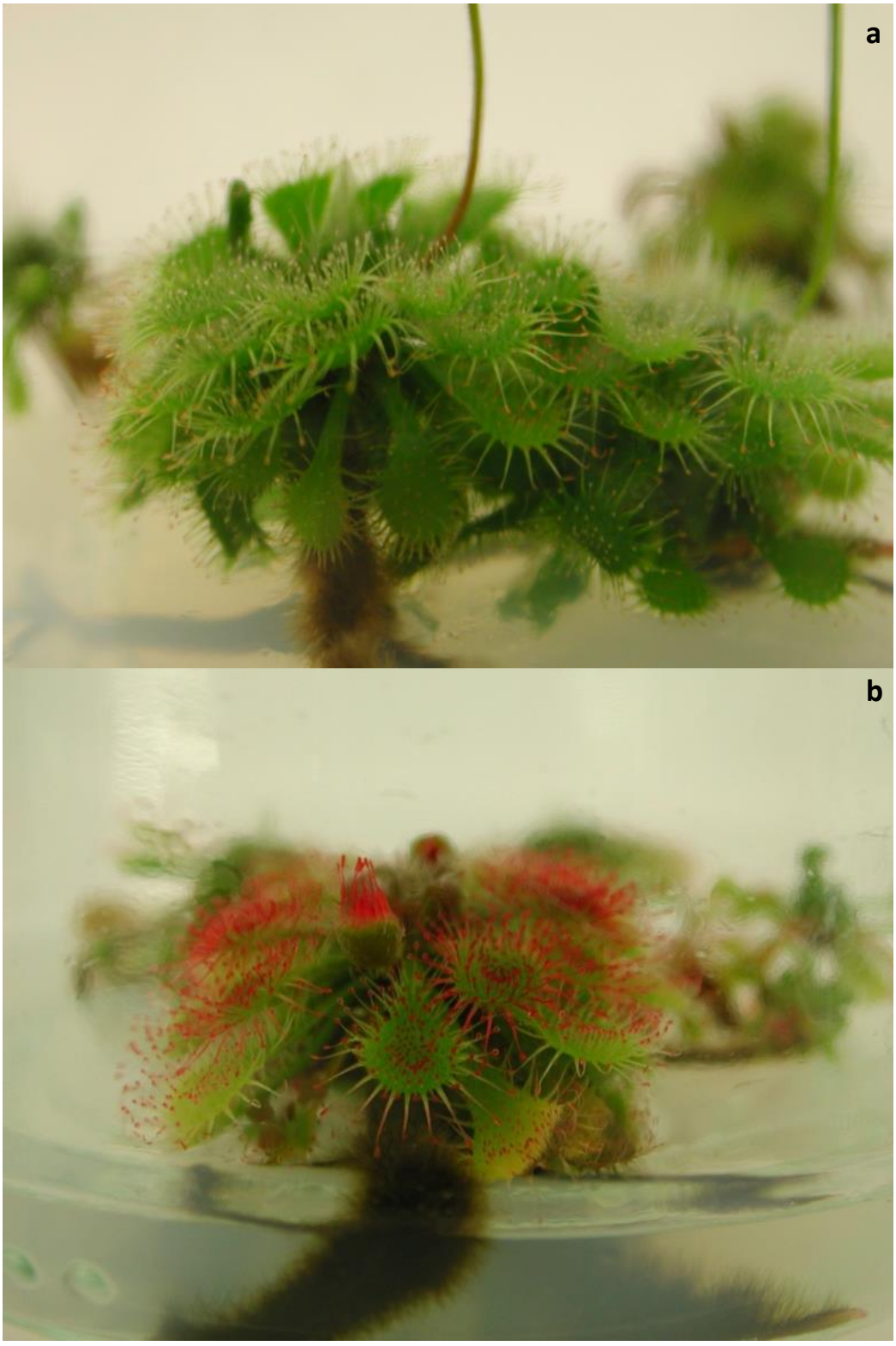
study, the change in color from dark to pale green was observed when medium mineral content was reduced by half (½ MS). When mineral content was further decreased, the glandular tentacles on the leaf blade margins turned dark red. This change in color was also noticed in sundews multiplicated on MS medium with reduced levels of KNO_3 and NH_4NO_3 (MS - ½ KNO_3 – ½ NH_4NO_3). Reducing the content of KNO_3 alone only made the dark green tentacles pale green (Table 2).

Table 2. Morphological characteristics of round-leaved sundew micropropagated on media with different content of minerals

Mineral composition of the media	Leaf rosette width	Root length [cm]	Root number	Plant weight [g]	Number of adventitious plants	Percentage of flowering plants [%]	Plant color
MS	4.21	1.53	1.54	0.312	15.1	98	dark green
¾ MS	3.52	1.65	2.09	0.325	16.4	90	dark green
½ MS	3.68	1.42	2.25	0.398	20.1	62	pale green
¼ MS	2.59	2.13	3.52	0.725	37.1	0	red
1/6 MS	1.58	2.45	3.56	0.326	15.5	0	red
1/8 MS	1.62	2.92	1.68	0.298	12.1	0	red
MS - ½ KNO_3	2.56	2.56	1.79	0.429	16.4	21	pale green
MS - ½ KNO_3 – ½ NH_4NO_3	2.16	2.36	1.89	0.359	15.4	10	pale green
MS - ½ KH_2PO_4	2.56	1.98	3.26	0.365	17.1	0	dark green
MS - ½ $CaCl_2$	2.34	1.47	1.21	0.326	12.9	0	dark green
NIR $_{0.05}$	2.35	0.65	1.36	0.112	6.31	50.6	



Photograph 1. Spatulate-leaved sundew (*Drosera* x *intermedia*) growing on complete MS media (Murashige and Skoog 1962) (**a** and **b**).



Photograph 2. Spatulate-leaved sundew (*Drosera* x *intermedia*) growing on complete MS medium (Murashige and Skoog 1962) with the content of macro- and macronutrients reduced to ¼ and $^{1}/_{6}$ MS (**a** and **b**).

Photograph 3. Sundew flowering on the media with the content of macro- and macronutrients reduced to ½ MS (Murashige and Skoog 1962).

4. CONCLUSIONS

- A recommended medium for micropropagation of *Drosera rotundifolia* L. is MS with the content of minerals reduced by 75% (¼ MS). Sundew plants growing on this media had the greatest weight.
- MS medium containing ¼ of macro- and micronutrients was the most effective for rooting of round-leaved sundew plants. The plants growing on this medium produced the greatest number of fairly long roots.
- Reducing mineral content to ¼ MS or more resulted in red coloration of glandular tentacles on leaves and plants multiplicated on these media did not produce inflorescences.

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