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IN VITRO ALLELOPATHY BETWEEN *DROSERA ROTUNDIFOLIA* L. AND *CYMBIDIUM HYBRIDUM*

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ABSTRACT: The purpose of this study is to investigate allelopathy relationships, for *in vitro* cultures, between *in vitro* seedlings of *Drosera rotundifolia* L. and protocorms of *Cymbidium hybridum* – plant species belonging to phylogenetically distant families – to establish the tolerability of one to the other in order to organize *in vitro* floral arrangements, which are increasingly demanded and appreciated worldwide (**Halevy,** 1999). At the same time, some peculiarities regarding morphological features of *in vitro* co-culture regime have been analysed. As a result mutual synergistic allelopathy effects which allow *in vitro* association when cultivated on original MS62 medium of *Drosera rotundifolia* L. and *Cymbidium hybridum* species, particularly after a 30 days period of conditioning have been identified. These "aesthetic fireworks" maintain viability and robustness for at least 3 months, representing a source of germplasm with high efficiency in relationship to the monocultures for each of the two species used in this study.

Keywords: *Drosera rotundifolia*, *Cymbidium hybridum*, allelopathy, *in vitro*, plant biotechnology.

INTRODUCTION:

The theory of organisms influence by releasing certain chemical substances into the environment , on other organisms in their vicinity, was documented in 1832 by the French botanist Augustin Pyramus De Candolle (Harper, 1977). Later, the German botanist Mollish (1937) defined this phenomenon "allelopathy" as: "the ability of superior or inferior plants to produce substances that released into the environment can favourably or unfavourably influence other plants' development" (Wegmann, 2007). The substances by means of which plants interacts with each other, vary from simple gases to complex aromatic compounds, including some phenolic acids, alkaloids, flovonoide, aliphatic compounds, terpenes, etc. (Harbonah, 1980; Rice, 1987).

Drosera rotundifolia L. – is a herbaceous, perennial, hemicryptophytes, insectivorous and hydrophilic plant, which reaches in the wild heights of about 4-25 cm. It thrives in turbicole, marshy wetlands, where it vegetates in sphagnum mat, which constitutes a moist substrate with acid pH, low in nitrogen salts, phosphorus and sulfur and high altitude over 1000 m in Romania (Pârvu, 2006). The *Drosera rotundifolia L.* plants present features that allow them to adapt to a mixotroph lifestyle, such as various types of bristles. Stanescu (2008) says that there are three types of bristles on the leaves of Drosera, namely: secretory, gland-tentacle, bludgeon the top or sessile bristles. In the natural life environment, these bristles justify their presence, being some "weapons" involved in nutrition; *in vitro* culture condition there was a regression of their presence (Turcuş, 2009).

Given these particularities the speciesbecame a subject of intense study, including *in vitro* cultures (Crouch *et al.*, 1990; Matusiková *et al.*, 2005). One of the *in vitro* cultures pioneers who studied on *D. rotundifolia* L. species was van Waes (1985), who managed the multiplication by using leaf explants. Also, van Waes initiated *in vitro* cultures of *Drosera* from caulinar apex and from axillary buds (van Waes, 1985). In 2001, Yamato and Nakagawa succeeded *in vitro* cultivation of the genus without growth regulators.

Hook (2001) proved the presence of some bioactive compounds, both in the leaves and in the roots of in vitro cultivated *Drosera* plants on culture medium without hormones such as naphthoquinone 7-methyljuglona (0.6%) (Hook, 2011). Marczak *et al.* (2005) also conducted a number of experiments to highlight the presence of some secondary metabolites identified in the body of these plants, resulting that these are good sources of phenolic secondary metabolites as type of flavonoids and naphthoquinone, like ramentaceone or plumbagin - substances that were considered to be responsible for the allelopathy nature of these plants. The elicitor plays an important role in the production of these secondary metabolites, being responsible for initiating the triggering biosynthesis enzyme of their production. These metabolites induce the installation of defense responses in plants, by accumulating and releasing them into the environment (Banasiuk *et al.*, 2012). Plumbagina (2-methyl juglone) is a naphthoquinone, which is, from a structural point of view, similar to juglone (5-hydroxy-1,4-naphthoquinone) (Durand et Zenke, 1971) - an allelopathy toxic compound contained in large amounts in both the leaves and fruitsof the black walnut (*Juglans nigra* L.) confering it inhibiting properties on the neighboring plants (Bode, 1940). Plumbaginawas also isolated from the roots of *Diospyros sylvatica* within several experiments trying to demonstrate the toxic effect on some termite quinones (*Odontotermes obesus*). The study confirmed the termicidal effect of several quinones, including plumbagina, showing a high level of mortality on termite workers (Seru *et al.*, 2004). Also, studies have been made on the detrimental effect of flavonoids on several species of insects, such as *Drosophila melanogaster*, which can be used as bioinsecticide against pests (Mitchell *et al.* 1993).

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Orchids of the *Cymbidium* genus were first described by the Swedish botanist Swartz in 1799 (Navalinskienë *et al.*, 2005). The genus includes perennials, herbaceous, succulent or non-succulent species, forming a family of plants that inhabit forests from great heights in the temperate regions of China and Japan being distributed all the way to south-east Asia and not being excluded from the landscape of Australia or New Zealand (Du Puy et Cribb, 1988).

Starting with the 52 species of the *Cymbidium* genus, horticulturists succeeded in getting countless interspecific hybrids, which today can be performed by using *in vitro* culture or following molecular genetic techniques (Yang *et al.*, 1999; Tsai *et al.*, 2004). Such a hybrid is the *Cymbidium hybridum* , which into *in vitro* cultures appears in a special form of crowded minituberule protocorms, forming clusters, whose sizes vary in shape thus giving them a particular aspect. The first such hybrid was produced in 1889 in England (Turner, 1981).

Morel and Martin (1952) proved that by cultivating orchid meristems on aseptic artificial media, within a year, from each apex can get more than 1 million plants genetically identical. Blidar *et al.*, (2009) demonstrated that the multiplication of *Cymbidium hybridum* protocorms on solid culture medium, overlapping a liquid culture medium, represented by distilled water, led to the best results in terms of number, fresh and dried weight of the protocorms.

The main factor that determines ecological diversification and specialization of orchids is considered to be the existence of associative interrelations with the mycelium of some fungi species in the development cycle of most species of orchids (Cameron *et al.*, 2006; Waterman and Bidartondo, 2008). Weston *et al.* (2005) is the one who describes the recognition "capacity" of fungi by compatible orchid seeds (Weston et al., 2005). This relationship established between orchid seeds and mycorrhiza fungi is considered as a case of allelopathy (Grodzinski, 1991). This "capacity" of the orchid seeds to attract active fungus hyphae and furthermore to maintain a balance between the growth of the orchid protocorms and fungus are mentioned in a small number of works (Gowland *et al.*, 2007). Buyun and Grakhov 2015 published an article referring to the study of the allelopathy relation to 10 different species of orchids, which were grown in greenhouses and mycorrhizal fungus that invades plants, being indispensable to life cycle. The specificity of secondary endometabolites from orchid seeds, and complementarity of orchid and fungus, is the proof of allelopathic fitting of the partners formed in the course of coevolution (Buyun and Grakhov, 2015). In 2008, Uşvat *et al.* observed that by including in the culture medium of the secondary metabolism - caffeine product, in the 0.5 mg / l - 5 mg / l concentration range, had a stimulatory effect on the *Cymbidium hybridum* protocorms.

Other studies on the biochemical composition of the *Cymbidium* orchid genus proved the strong similarity in terms of the molecular structure of lectin isolated from the *Cymbidium* orchid genus and the lectin specific to the *Amaryllidaceae* genus (Van Damme *et al.*, 1991) and therefore contained in leek or onion (Van Damme *et al.*, 1993). Some lectins (glycoprotein) can be very toxic, such as ricin that can be found in castor seeds (*Ricinus communis*) (Butterworth and Lord, 1983) and others were incorporated in genetically modified plants for improving pests resistance (Duca, 2008). These glycoproteins may be responsible for the allelopathy property, which is specific to the *Cymbidium* orchid genus.

Taking into consideration all above discussions related to allelopathy this study aims to highlight the mutual influences, beneficial or harmful, between *C. hybridum* and *D. rotundifolia* in order to perform *in vitro* floral arrangements and for a more intense proliferation of these.

MATERIALS AND METHODS:

The plant material. The inocula that initiated the experiments consisted of *Drosera rotundifolia* L. mini seedlings and solitary protocorms of *Cymbidium hybridum*, originating from *in vitro* germplasm collection of plant biotechnology laboratory of the University of Oradea, functioning for more than 20 years.

Growing conditions and inoculations. The mini seedlings were multiplied based on a solid Murashige-Skoog (1962) (MS62) culture medium supplemented with 6 g / l agar, without, amino acids and hormons and the pH was adjusted to the value 5.7 prior autoclaving. The medium was distributed in heat-resistant vials, with a height of 8 cm and inner diameter of 4.5 cm. Each culture vial contained 9 ml of culture medium, which forms a column of the substrate in the container with a height of 8 mm. The sterilization of the vessels was performed by autoclaving at the temperature of 121°C for 25 minutes.

Each container has a unique rosette inoculated, respectively protocorm în case of V_0D variant – *in vitro* monoculture of *Drosera rotundifolia* L. and V_0C – *in vitro* monoculture of *Cymbidium hybridum*, and in case of the V_1 variant the vitro cultures containing both species in the same culture containers - one single inoculum of each species. For each experimental variant, were used by 100 jars.

Plant incubation and growing was done in the board of vegetation, exposing them to cold fluorescent lighting tubes emitting white light of 6500 K (Kelvin) placed at 33 cm from the surface of the culture shelves, with a measured brightness of 4.22 Klux, using a Delta OHM HD 8366 luxmeter model, equipped with a Delta OHM sensor, LP 8366 PHOT model and in photoperiodic regime, which corresponded to 16 hours of light / 24-hour, ambient temperature oscillating between 24 °C and 26 °C.

Morphometry Every 30 days, respectivelly at 30, 60 and 90 days after inoculation, measurements were carried out, observing the evolution of several parameters, as follows: on Drosera *rotundifolia* L.: the number of roots, the maximum length of the roots, the number of newly formed rosettes, the number of leaflets, the length of the limb, the weight of the fresh biomass and of the dry biomass; on *Cymbidium*

hybridum: the overall size of the glomerule, the number of protocorms, the weight of the fresh biomass and of the dry biomass.

In this experiment it was used two experimental control variants, one for each plant species: V_0D - for the vitro plants of *Drosera rotundifolia* L. from the trial version (V_1) and the V_0C version - for *in vitro* plantlets of *Cymbidium hybridum*, from the same experimental variant.

Statistical analysis. For each of the above mentioned biometric parameters and at every experimental date, the values recorded on monoculture were considered as reference (100%) for the corresponding parameters belonging to the same species of co-culture vitroplantlets. All statistical analyses were made using Microsoft Excel. Each experiment was repeated three times.

RESULTS AND DISCUSSIONS:

Except for the first 30 days of the *in vitro* culture, we found an allelopathic stimulating influence on both plant species, respectively *Drosera rotundifolia* L. and *Cymbidium hybridum.* The proof is given by the difference in average values, which are higher than those of the control. The results are discussed below.

Biometric measurements and morphologic aspects at 30 days.

At the end of the first 30 days of culture, we noted the negative allelopathic effect of the *Cymbidium hybridum* on *Drosera rotundifolia* L.seedlings, regarding the rooting , and their growth in length, but also a stimulatory effect on the growth and development of the stem organs and accumulation of fresh and dry biomass at vitroplantlets of *Drosera*.

On the other hand, we registered a negative influence exerted on the *Cymbidium* protocorms by *Drosera* plants, the proliferation of the protocorms being reduced by 3.2% compared to the control (*C. hy*. monoculture), but the difference was not supported statistically (Table 2). Regarding the two gravimetric parameters that we analysed - fresh and dry weight - there was an inhibitory influence exerted by *Drosera* plantlets over those of *Cymbidium*, but only on the accumulation of water in the protocorms, not on their development degree or mineral accumulation (Table 2). However, considering the fact that the fresh vegetable biomass suffered only 0.7%, compared to the control group, all the other aspects (morphological, biometric and gravimetric aspects of dry biomass) are superior to the control, we can state that *Drosera* plantlets exert on the whole a positive allelopathic effect on *in vitro* cultures of *Cymbidium*.

Biometric measurements and morphologic aspects at 60 days.

At the second time of the experimental observations, it was noted an increase of the stimulative allelophatic influence exerted by the *Cymbidium* protocorms on the *Drosera seedlings*, both in terms of morphogenesis and plant biomass accumulation. The only parameter where the data marked in the *Drosera* monoculture (**V_0D**) was superior to those from biculture (**V_1**), was represented by the average length of the roots, case in which the difference was not statistically significant and of only 7.7% (0.2 mm / root in absolute terms) (Table 1). However, taking into consideration that at 30 days the difference was of 75%, it can be concluded that the allelopathic effect induced by the presence of orchid protocorms is an important one and also in the case of the *Drosera root growth.*

Also, there was an increase in the number of protocorms in case of in vitro culture in a allelopathic relationship (var. **V_1**) in relation to the number marked on the control variant (**V_0C**). The registered differences were of 14.1% in favour of co-culture *Cymbidium* protocorms (1.5 protocorms / glomere more in group **V_1**). Looking at the dynamics of this parameter, it is noted that in the bicultural variant (var. **V_1**) its value almost doubled, increasing from 6.2 at 30 days, to 12.1 protocoms / glomerule at 60 days, while in monoculture (**V_0C**), the growth rate was lower (from 6.4 at 30 days, to 10.6 at 60 days). The stimulative allelopathic effect exerted by *D. rotundifolia* seedlings on the *C. hy*. protocorms, was also noted on the accumulation of fresh and dry biomass, both being above the control group (Table 2).

Following the above, it can be stated that at 60 days of biculture, the association of the two species led to better results, the value of the analyzed parameters increasing more intensely within 30-60 days compared to those belonging to the corresponding monocultures.

Biometric measurements and morphologic aspects at 90 days.

Throughout the experimental period (90 days), there was an incentive caulogenesis on vitroplantlets of *Drosera* by protocorms of *Cymbidium*, a phenomenon that has increased with the age of in vitro culture, leading, for example, to increases of even 120% in case of the parameter number of neoformed rosettes at *Drosera* neoformed in biculture (**V_1**), compared to the corresponding control (**V_0D**) (Table 2). Thus, if 30 days after inoculation of orchid protocorms inhibited rootedness, at this experimental date was obtained elevated values on all biometric parameters belonging to the species *Drosera rotundifolia* L., cultivated in the same culture vessel with orchids protocorms.

At 90 days after installing the experiments, the stimulating influence of *D. rotundifolia* vitroplants on the proliferation of *Cymbidium* protocoms, was deeper comparing to the results at 60 days *in vitro* co-culture, leading to the registration of a higher number of orchid protocorms, compared to control (**V_0C**), to 110.3% (with 12.7 protocorms/glomerules more) (Table 1). In terms of weight parameter values, they were superior compared to control (**V_0D** and **V_0C**), values being a proof of positive mutual allelopathy influences, induced by the two plant species. As a result of those findings, we conclude that the inhibitory allelopathy effect induced by vitroplants of *D. rotundifolia* on *Cym. hy.*, highlighted in the first 30 days, was reversed within 30-90 days, moving in a stimulating mutual relationship.

The presence of the *Cymbidium hybridum* orchid in co-culture with *Drosera* plants (**V_1**) clearly exerted a

stimulatory effect on their growth and development, the effect becoming more prominent with the aging of the *in vitro* cultures. The allelopathic positive effect exerted by orchid protocorms stood out on the other plant species, such as *Sequoia sempervirens* L. In this case, the *Cymbidium hybridum* protocorms boosted growth and the branching of the stems, and also increased the number of leaflets in relation to the *S. sempervirens* seedlings under monoculture, a phenomenon increasingly stronger with the aging of the vitroculture (Manci, 2009). On the other hand, as noted, there has been a positive allelopathic influence exerted by the *D. rotundifolia* vitroplantlets on those of *Cymbidium hybridum.* In similar studies, beneficial growth and development aspects of the *Sequoia* seedlings, which were placed in the same container with *Dosera* plants (Rogojan, 2010), were reported. The results of the experiments confirmed our allelopathic properties of the two plant species, respectively the *Drosera rotundifolia* L. and the *Cymbidium hibridum*. Another aspect was observed at the *Drosera rotundifolia* L. vitroplantlets was the neogenesis of the mini rosettes, and later on, plants at the leaf lamina level, giving rise to colonies of rosettes. The phenomenon was more intense in the case of the *Drosera* specimens in the presence of orchid protocorms (at 90 days of culture being recorded 4.56 / culture container on $\mathbf{V_1}$, respectively 1.08 to *Drosera* plants in monocultures).

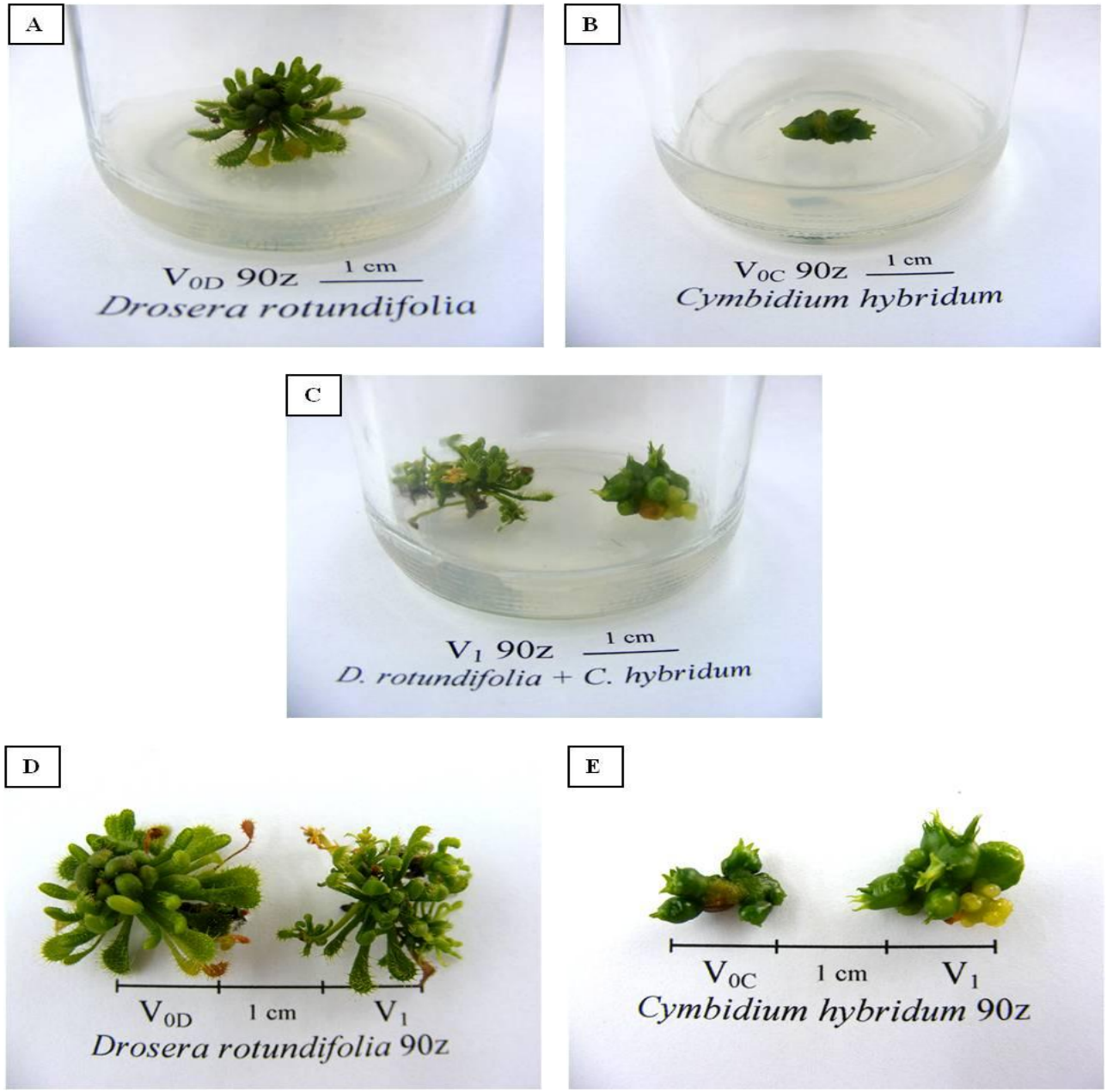

Fig. 1. Comparison of *in vitro* macroscopic aspects at 90 days of *Drosera rotundifolia* L. and *Cymbidium hymbridum*, where: **A** – monoculture of *D. rotundifolia* (V_0D); **B** – monoculture of *C. hy.* (V_0C); **C** – *D. rotundifolia* and *C. hy.* In biculture (V_1); **D** – comparison between *D. rotundifolia* seedlings from monoculture (left – V_0D) and biculture (right – V_1); **E** – comparison between *C. hy.* protocorms from monoculture (left – V_0C) and biculture (right – V_1);

Table 1.
Statistical processing of the data measured in the *in vitro* seedlings of *D. rotundifolia* L. cultivated in monoculture (V_0D) and in biculture with *C. hybridum* protocorms (V_1)

		V_0D (control) (monoculture)		**V_1** (biculture of *D. rotundifolia* with *C. hy.*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signifi-cance**
30	Roots no.	0.9 ± 1.48	2.19	0.4 ± 0.76	0.57	-0.5	-55.6	ns
	Root length (mm)	1.2 ± 1.98	3.92	0.3 ± 0.62	0.38	-0.9	-75	**
	Rosettes no.	1.9 ± 0.49	0.24	2.8 ± 0.98	0.96	0.9	47,3	***
	Rosettes diameter (mm)	16.5 ± 0.43	0.18	19.3 ± 0.54	0.29	2.8	16.9	**
	Leaf no.	21.6 ± 0.93	0.86	28.2 ± 0.85	0.72	15.6	23.8	**
	Fresh weight (mg)	634.1 ± n/a	n/a	988.3 ± n/a	n/a	354.2	55.8	n/a
	Dry weight (mg)	166.9 ± n/a	n/a	231.6 ± n/a	n/a	64.7	38.7	n/a
60	Roots no.	1 ± 1.09	1.18	1.3 ± 0.83	0.68	0.3	30	ns
	Root length (mm)	2.6 ± 2.72	7.39	2.4 ± 0.72	0.51	-0.2	-7.7	ns
	Rosettes no.	3.2 ± 1.85	3.42	4.6 ± 1.97	3.88	1.4	64.2	**
	Rosettes diameter (mm)	14.2 ± 0.54	0.29	17.8 ± 0.59	0.34	3.6	25.3	**
	Leaf no.	37.6 ± 1.61	2.59	48.6 ± 0.89	0.79	11	29.2	***
	Fresh weight (mg)	1187.7 ± n/a	n/a	1598.2 ± n/a	n/a	410.5	34.5	n/a
	Dry weight (mg)	281.9 ± n/a	n/a	369.9 ± n/a	n/a	88	31.2	n/a
90	Roots no.	1.6 ± 1.81	3,27	1.9 ± 1.16	1.34	0.3	18.7	ns
	Root length (mm)	2.6 ± 2.91	8.46	4.6 ± 0.97	0.94	2	76.9	*
	Rosettes no.	3.5 ± 2.04	4.16	7.7 ± 1.5	2.25	4.2	120	***
	Rosettes diameter (mm)	17.1 ± 0.65	0.42	23.6 ± 0.7	0.49	6.5	38	***
	Leaf no.	45 ± 2.76	7.61	70.2 ± 2.79	7.78	25.2	56	***
	Fresh weight (mg)	1873 ± n/a	n/a	2441 ± n/a	n/a	568	30.3	n/a
	Dry weight (mg)	447.5 ± n/a	n/a	601.3 ± n/a	n/a	153.8	34.3	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

Table 2.
Statistical processing of the data measured in the *in vitro* protocorms of *C. hybridum* cultivated in monoculture (V_0C) and in biculture with *D. rotundifolia* L. seedlings (V_1)

		V_0C (control) (monoculture)		**V_1** (biculture of *C. hy.* with *D. rotundifolia*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signifi-cance**
30	Protocorms no.	6.4 ± 1,36	1.84	6.2 ± 1.24	1.53	-0.2	-3.2	ns
	Glomerule diam. (mm)	5.6 ± 1.43	2.04	5.4 ± 1.26	1.58	-0.2	-3.6	ns
	Fresh weight (mg)	882.3 ± n/a	n/a	876.9 ± n/a	n/a	-5.4	-0.7	n/a
	Dry weight (mg)	107.9 ± n/a	n/a	130.8 ± n/a	n/a	22.3	21.2	n/a
60	Protocorms no.	10.6 ± 1.45	2.1	12.1 ± 2.35	5.52	1.5	14.1	ns
	Glomerule diam. (mm)	7.7 ± 1.76	3.09	7.9 ± 1.9	3.61	0.2	7.9	ns
	Fresh weight (mg)	1714.5 ± n/a	n/a	2156.2 ± n/a	n/a	441.7	25.7	n/a
	Dry weight (mg)	193.3 ± n/a	n/a	265.3 ± n/a	n/a	72	37.2	n/a
90	Protocorms no.	10.6 ± 1.96	3.84	22.3 ± 2.59	6.07	11.7	110.3	***
	Glomerule diam. (mm)	8.6 ± 1.9	3.61	10.5 ± 3.3	10.89	1.9	10.3	**
	Fresh weight (mg)	1989.9 ± n/a	n/a	3310.6 ± n/a	n/a	1320.7	66.3	n/a
	Dry weight (mg)	262.7 ± n/a	n/a	412.4 ± n/a	n/a	149.7	56.9	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

CONCLUSIONS:

After the adjustment period (the first 30 days), the allelopathic influences, of mutually synergistic type, which allow the *in vitro* association of the *Drosera rotundifolia* L. and *Cymbidium hybridum* species, culturing them in the same culture vessel being successful. Therefore, we recommend the *in vitro* biculture of the two plant species, on MS62 culture

medium without growth regulators, if you aim for a stronger proliferation and, consequently, a faster multiplication of them, compared to the vitroplantlets of the appropriate monocultures, and also if you intend to meet some economic aspects regarding the marketing of *in vitro* floral arrangements.

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