ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 154-167
doi: 10.25083/rbl/24.1/154.167

Received for publication: April 24, 2017
Accepted: July 4, 2017

Original paper

In vitro Allelopathy at Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni

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Abstract

So far, the allelopathy is relatively little studied for in vitro cultures. The present study aims to highlight the allelopathic interactions of in vitro cultures between two plant species cultivated on one hand for their economic importance, and on the other for their beauty, respectively Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni; the research purpose was to determine the tolerability of one towards the other, in order to make in vitro floral arrangements, which are more and more popular and appreciated worldwide. Also, we plan to contribute in highlighting possible morphological and anatomical particularities of plants, which are in vitro co-culture, depending on the presence of growth regulators in the growing substrate. The analysis of this research revealed the existence of mutual allelopathy influence, synergistic from sequoia towards stevia, and antagonistic from stevia towards sequoia, the in vitro association being possible only until the age of 20 days of the in vitro cultures, up to which the allelopathy is mutually stimulating in the presence of 1 mg/l IBA. Keeping in vitro of both species in the same container of culture, over the age of 20 days, proved to be beneficial only for stevia plants, independently of the presence of growth stimulants.

Keywords : Sequoia sempervirens, Stevia rebaudiana, allelopathy, in vitro, plant biotechnology

To cite this article: BLIDAR CF, POP IF, FENESI B. In vitro Allelopathy at Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni. *Rom Biotechnol Lett*. 2019; 24(1): 154-167. DOI: 10.25083/rbl/24.1/154.167

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Introduction

The theory of influence exerted by some chemical compounds released into the environment by certain species on other species found in their vicinity was grounded since 1832 by the French botanist Augustin Pyramus De Condolle (J.L. HARPER [1]). After almost 100 years, the term "allelopathy" was launched in 1937 by the German botanist Hans Molisch (H. MOLISH [2]), who defines this process as including "any biochemical interaction, whether positive or negative, among plant of all levels of complexity" (Molish 1937 cited by J.V. LOVETT & A.H.C. HOULT [3]). This type of "socialization of superior plants" (R. KNAPP [4]) is achieved via biochemical compunds, such as phenolic acids, acetic acid, butyric acid, alkaloids, flovonoids, aliphatic compounds, hydroqiniones, terpenes, etc. (J.B. HARBONE, 1980 [5]; E. RICE [6]).

If millions of years ago, Sequoia trees genre were widespread, especially on southern continents, currently two species survived: Sequoia sempervirens (D. Don) Endl. and Sequoia giganteum (giant sequoia) (J.A. WOLFE [7]). Sequoia sempervirens (D. Don) Endl. (redwood) is a species belonging to gymnosperms from Pinophyceae class (Coniferophytes), order Pinales (Coniferales) widespread on the northern coast of California and Oregon coast (USA). It is the only hexaploid conifer (2n = 6x = 66) living today (W.L. LIBBY & al. [8]; M.R. AHUJA & D.B. NEALE [9]). Redwood is considered the highest tree in the world, reaching an average height of 60 meters and a trunk circumference of 3.6 - 4.8 meters. They are longevous trees, the oldest was reported as having 2,200 years; they reach maturity at 400-500 years old (H.A. FOWELLS [10]). Sequoia wood is used in timber industry, being mainly oriented to furnishings manufacture, construction elements (i.e. doors, windows, etc.), railway sleepers, structures of wooden houses (L. POP [11]).

This evergreen species, member of Taxadoiaceae family, unlike the others, has the ability to generate sprouts at the base of the stem that after cutting the trunk each sprout forms its own roots (L. POP [11]).

In vitro cultures from Sequoia sp. have been initiated since 1950, when explants taken from cambial tissue explants were in vitro cultivated, subsequently explants taken from various organs of plants were used (Y.P.S. BAJAJ [12]).

Boulay (M. BOULAY [13]) found that following repeated subcultivation, S. sempervirens inocula become more reactive, which is beneficial for micropropagation, by shortening the regeneration time of the plantlets. In order to obtain sprouts, positively reacting for in vitro cultures, it was recommended to handling parent trees that have been grown in vivo, by pruning, grafting, chemical treatment, etc. (A. FRANCLET [14]; A. FRANCLET [15]).

In vitro cultures initiated from stem fragments were widely studied by Boulay (M. BOULAY [13]) and Ball (E.A. BALL & al. [16]). By using in vitro multiplication and rooting, it was found that plants can be easier obtained starting from meristematic tissues than clones similar to those resulting from stem fragments. Small amounts of callus were also obtained at basal level of inocula, and the roots developed at the base of stem (M. BOULAY [13]).

Walker (N. WALKER & al. [17]) studied the initiation of in vitro culture originating from stem fragments, and they discovered that meristematic dome (showing attached the first two leaf primordia) develops well on the culture medium without growth regulators and supplemented with activated charcoal.

The animal-derived activated carbon added into the culture media in a concentration of 2% boosts the production of both hairy roots, multiple, and the generation of basal shoots at the level of in vitro plants of Sequoia sempervirens (D. Don) Endl. (L. POP [11]).

In their experiments on S. sempervirens, Pop and Cachiță (L. POP & C.D. CACHIȚĂ [18]) using culture media prepared with deuterium-depleted water (87.5 ppm. deuterium), proved to be more effective for increasing the stem of in vitro plantlets, reported to the usual double distilled water (150 ppm deuterium). On contrary, the complete replacement of the double distilled water in culture medium with deuterium depleted water (25 ppm deuterium), exerted an inhibitory effect on stem growth.

Among first studies on in vitro allelopathy tested S. sempervirens mini seedlings in relations with

protocorms of Cymbidium hybridum to determine the tolerability of a species towards the other was that published by Manci (A.M. MANCI [19]). The author found that the presence of cymbidium protocorms in the same jar stimulated the branching and stem growth in sequoia. Moreover they observed an increase in the number of leaflets compared to monoculture, a process that is more and more strong with age of in vitro culture. This partnership proved to be beneficial only up to 60 days age of the in vitro cultures, after which a process of slowing down of general development was observed.

Rogojan (P.A. ROGOJAN [20]) analyzed the reaction of mini cuttings of S. sempervirens in allelopathic conditions with another species: Drosera rotundifolia L. The authors reported that the in vitro association of these two plant species proved to be successful for long term cultivation.

Ward et al. (B.B.WARD & al. [21]) studied the effect of some monoterpenes produced by S. sempervirens on the in vitro development of Nitrosomonas europaea and identified adverse effects induced by four of them (i.e. limonene, sabinene, myrcene, and γ-terpinene compounds). Just one monoterpene (i.e. β-pinene) had a stimulatory effect on the in vitro development of N. europaea.

Stevia rebaudiana Bertoni is a herbaceous perennial plant and member of the Asteraceae family, Eupatorieae class, being known as Eupatorium rebaudianum. It can reach one meter height having many leaves of approx. 2-3 cm length. It is a short-day species, with an essential requirement of light for flowering, of about 13 hours. The aboriginal people in Brazil and Paraguay used stevia leaves for hundreds of years as a sweetener, but also as a remedy for various diseases (J. POL & al. [22]). Now, it is widely cultivated because of its sweet taste conferred by stevioside - a glycoside of the diterpene derivative steviol, especially contained in the leaves (A. DOSSIER [23]), a substance that is 300 times sweeter than sugar, which can successfully replace it (J.O. ATTEH & al. [24]). The plant is a good source of carbohydrates (61.93%), proteins (11.41% D.W.), crude fiber (15.52%) and minerals (i.e. K, Ca, Na, Mg, Cu, Mn, Fe, Zn); also essential amino acids were found in large quantities (A.E. ABOU-ARAB & al. [25]). This valuable natural sweetener is used worldwide as a substituting sugar, whose excessive consumption leads to multiple medical problems, such as obesity (J. POL & al. [22]; S. STRAUSS [26]).

Stevia seeds have a very low germination rate, and in vitro tissue culture is the fastest way for mass propagation of plants (M.B. AHMED & al. [27]).

Subsequently, Tawara (A.S. TAWARE & al. [28]) concluded that the MS62 culture medium containing 0.3 mg/l kinetin is most effective for in vitro cultivation of the stevia seedlings. The root induction was optimized on a MS62 culture medium supplemented with 0.2 mg/l IBA. The plantlets and the regenerated callus cultivated on a modified MS62 culture medium supplemented with 2,4-D and IBA proved to increase the production of stevioside. For these studies the authors used nodal explants that produced multiple shoots when cultivated on a modified MS62 culture medium supplemented with different combinations and concentrations of growth regulators, the supplemented variant with 3 mg / l BAP being the optimal (K.M. PANDA & al. [29]).

Badawi and collaborators (A.M. BADAWI & al. [30]) conducted a study that had as purpose the use of steviosides, the natural sweetener extracted from S. rebaudiana plants, as a substitute for sugar in dairy product for people suffering from diabetes.

Taware (A.S. TAWARE & al. [31]) studied the influence of various extracts of callus and seedlings of S. rebaudiana on seed germination of some agricultural crops, such as Triticum aestivum, Sorghum vulgare, Arachis hypogea, Glycine max, Cajanus cajan and Cicer arietinum. The experiments were carried out in Petri dishes and sterilized for five days at an average temperature of 28 °C. Callus proved to contain some metabolites showing some biological activities such as a significant inhibition of seed germination of C. cajan and C. arietinum. These inhibitory effects were proved by the browning of the roots' peak and a certain inhibition of their growth.

Tadhani (M.B. TADHANI & R. SUBHASH [32]) proved the antimicrobial effect of the in vitro stevia

leaves cultivation on two microbes: B. subtilis, S. aureus. Already in vivo, stevia proved to have an inhibitory effect on the development of some bacteria and other microorganisms, thereby successfully using the disinfection of wounds and gum disease (A.S. TAWARE & al. [27]).

Materials and Methods

Plant material. The used inocula consisted in mini seedlings of Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni, with a size of approx. 1 cm length, taken from vitroplants, having attached small leaves at the level of the nodes, shortened by half, from the Plant Biotechnology Laboratory of the University of Oradea.

Growing and inoculation conditions. The mini seedlings were multiplied on a Murashige-Skoog (1962) (MS62) (T. MURASHIGE & F. SKOOG [33]) modified medium, supplemented with 7 g/l agar, 3-indoleacetic acid (IAA) and kinetin (K) and, the pH was adjusted to 5.7 before autoclaving. The medium was distributed in heat-resistant vials, with a height of 7 cm and 2.5 cm inner diameter. The medium sterilization was performed by autoclaving at 121 °C for 20 minutes.

Each vial was inoculated with one single redwood mini seedling, in case of the **VxSs**, or stevia to **VxSr** variants, and in the case of the **VxSsS**r variants the in vitro cultures contained both species in the same containers, one single inoculum of each plant.

To find the influence of the growth regulators on the associative vitro cultures, 3 variants of culture medium supplemented with growth regulators and one control, without regulators were organized as follows:

- **V0X** - basal medium (MB) - MS62 without growth regulators (control);
- **V1X** - MB-MS62 + 1 mg / l indole-3-butyric acid (IBA) (4.92 μM);
- **V2X** - MB-MS62+ 1 mg / l Kinetin (K) (4.64 μM);
- **V3X** - MB-MS62+ 1 mg / l IBA + 1 mg / l K;

where "**X**" represents the type of crop (monoculture sequoia - **Ss**, or stevia - Sr, or bi culture with both species - **SsSr**) (Table 1).

Table 1. Experimental variants used *in vitro* experiments of allelopathy between *S. sempervirens* and *S. rebaudiana*

The content of media in growth regulators	**Type *in vitro* culture**		
	V_x**Ss** (monoculture of *S. sempervirens*)	V_x**SsSr** (bi culture of *S. sempervirens* with *S. rebaudiana*)	V_x**Sr** (monoculture of *S. rebaudiana*)
V_0X (MB-MS without growth regulators)	**V_0Ss**	**V_0SsSr**	**V_0Sr**
V_1X (MB-MS + 1 mg/l IBA)	**V_1Ss**	**V_1SsSr**	**V_1Sr**
V_2X (MB-MS + 1 mg/l K)	**V_2Ss**	**V_2SsSr**	**V_2Sr**
V_3X (MB-MS + 1 mg/l IBA + 1 mg/l K)	**V_3Ss**	**V_3SsSr**	**V_3Sr**

The in vitro cultivation of inocula was conducted in growing room, under a lighting with fluorescent white day-light (6500K), with light intensity of 20 μM m-2s-1 PAR, under photoperiodical which corresponded to 16 h day light/ 24 hours, a temperature ranging from 22 °C to 24 °C.

Measurement of growth. After 20, 40 and 60 days of inoculation, observations following the monitoring of morphological parameters were: number of roots, root length, number of stems, length of stems and number of leaflets. Two gravimetric parameters were compared: fresh weight and dry weight (g).

We used two experimental variants as control, one for each plant species: VxSs – for the vitroplants of Sequoia sempervirens (D. Don) Endl., respectively VxSr variant - for the vitroplantlets of Stevia rebaudiana Bertoni from the sample variant (VxSsSr).

In total 12 experimental variants were developed and each was used in 60 vials.

Statistical analyses. For each of biometric parameter, at every experimental date, the values recorded in case of monocultures were considered as reference (100%) for the corresponding parameters in vitro plants belonging to the same species of co-culture. All statistical analyses were made using Microsoft Excel; the values are significantly different at $p<0.05$ according to t test. The experiment was repeated three times under the same conditions.

Results and Discussions

Biometric measurements and morphologic aspects at 20 days.

After the biometrizations performed at 20 days of in vitro cultures, we noticed that the seedlings of S. sempervirens exerted a stimulating allelopathic effect on S. rebaudiana. However, it was noticed an inhibitory activity exerted by the stevia on the sequoia seedlings development.

In case of culture medium without growth regulators, 93% of the S. sempervirens plantlets under co-culture had a weaker growth, showing obvious signs of senescence, consisting in a general mustard colour, with stem buds underrepresented or even absent, compared with V0Ss variant (Table 2, Figure 1).

Table 2. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **20 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in co-culture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.2 ± 0.71	0.50	1.0 ± 0.56	0.31	-0.2	-16.6	ns
	Strains length (mm)	8.3 ± 3.19	10.23	5.0 ± 2.13	4.55	-3.3	-39.7	***
	Leaf no.	6.6 ± 2.43	5.93	2.0 ± 0.99	0.99	-4.6	-69.6	***
	Fresh weight (mg)	23.5 ± n/a	n/a	10.6 ± n/a	n/a	-12,5	-54.8	n/a
	Dry weight (mg)	2.4 ± n/a	n/a	1.4 ± n/a	n/a	-1	-41.6	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.7 ± 0.91	0.84	2 ± 0.88	0.79	0.3	15.0	ns
	Strains length (mm)	6 ± 1.52	2.31	5.5 ± 1.73	3.00	-0.5	-8.3	ns
	Leaf no.	2.5 ± 1.02	1.05	2.5 ± 1.08	1.17	0.0	0.0	ns
	Fresh weight (mg)	13.6 ± n/a	n/a	30.4 ± n/a	n/a	16.8	123.5	n/a
	Dry weight (mg)	3.8 ± n/a	n/a	4.6 ± n/a	n/a	0.8	21.0	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.8 ± 0.86	0.74	1.6 ± 0.89	0.80	-0.2	-11.1	***
	Strains length (mm)	12.6 ± 2.63	6.96	7.6 ± 2.22	4.94	-5.0	-39.6	***
	Leaf no.	10.0 ± 2.24	5.03	5.2 ± 1.63	2.66	-4.8	-48.0	***
	Fresh weight (mg)	66.5 ± n/a	n/a	63.6 ± n/a	n/a	-2.9	-4.51	n/a
	Dry weight (mg)	16.5 ± n/a	n/a	13.5 ± n/a	n/a	-3.0	-18.1	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.0 ± 1.14	1.30	1.5 ± 0.98	0.96	-0.5	25.0	*
	Strains length (mm)	9.7 ± 2.29	5.25	7.7 ± 2.38	5.70	-2.0	-20.6	***
	Leaf no.	8.0 ± 1.72	2.96	5.2 ± 1.71	2.95	-2.8	-35.0	***
	Fresh weight (mg)	84.4 ± n/a	n/a	55.4 ± n/a	n/a	-29	-34.3	n/a
	Dry weight (mg)	7.2 ± n/a	n/a	5.2 ± n/a	n/a	-2	-27.7	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.

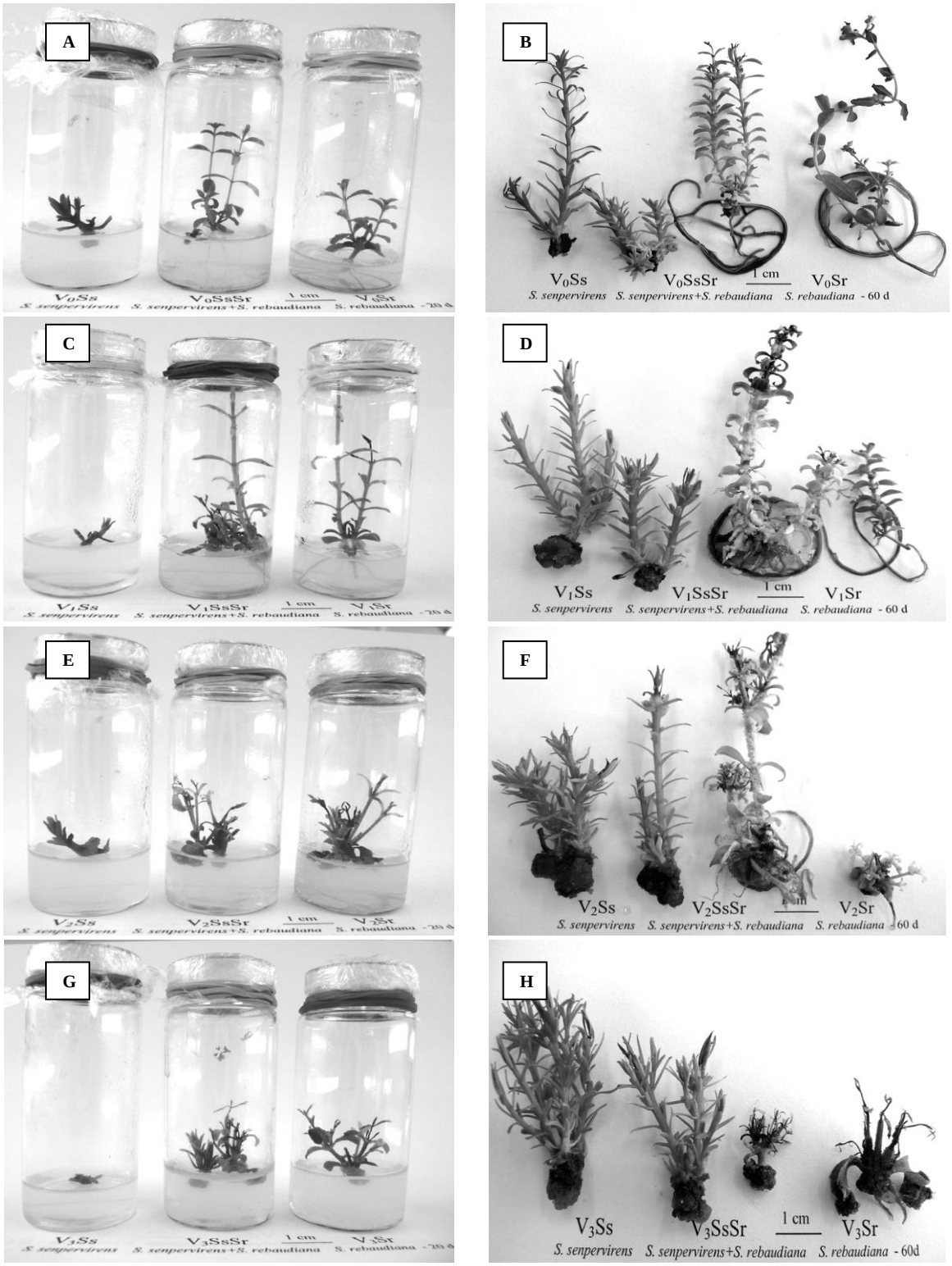

Fig. 1. Comparative morphological aspects of *in vitro* plantlets of *S. sempervirens* and *S. rebaudiana* under monoculture (VxSs respectively VxSr) and those of co-culture (VxSsSr) at 20 days - left column (**A, C, E, G**) and 60 days - right column (**B, D, F, H**) from the mounting experiments on culture media MS62 modified without added growth regulators (variants V_0X) with the addition of 1 mg / l IBA (var. V_1X) with the addition of 1 mg / l K (var. V_2X) or with the addition of 1 mg / l IBA and 1mg / l K (var. V_3X).

In case of co-cultivating plantlets of S. rebaudiana with those of S. sempervirens, a high positive reactivity was noticed compared to that recorded for the same plants separately as monoculture of stevia (V0Sr) (Table 3, Figure 1); the main morphological differences were a greater number of stems (8%) and an increased length (52,9%) and their degree of branching for the V0SsSr variant, the addition to control (V0Sr) (Table 3, Fig. 1A).

Table 3. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (V_xSr) and in co-culture with *S. sempervirens* (V_xSsSr) at **20 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	1.6 ± 0.84	0.72	2.5 ± 1.29	1.68	0.9	56.2	***
	Roots length (mm)	24.6 ± 2.86	8.23	23.0 ± 3.23	10.49	-1.6	-6.5	*
	No. of strains	2.5 ± 0.87	0.75	2.7 ± 1.13	1.27	0.2	8	ns
	Strains length (mm)	15.5 ± 3.35	11.25	23.7 ± 2.74	7.54	8.2	52.9	***
	Leaf no.	10.2 ± 2.14	4.59	15.0 ± 2.53	6.42	4.8	47.0	***
	Fresh weight (mg)	59.1 ± n/a	n/a	47.9 ± n/a	n/a	-11.2	-18.9	n/a
	Dry weight (mg)	8.1 ± n/a	n/a	6.3 ± n/a	n/a	-1.8	-22.2	n/a
V_1	Roots no.	2.4 ± 0.87	0.77	3.0 ± 1.35	1.84	0.6	25.0	**
	Roots length (mm)	9.8 ± 2.01	4.05	8.2 ± 2.17	4.73	-1.6	-16.3	***
	No. of strains	2.6 ± 0.87	0.76	2.0 ± 1.01	1.03	-0.6	-23.0	**
	Strains length (mm)	24.4 ± 2.02	4.10	31.2 ± 3.37	11.35	6.8	27.8	***
	Leaf no.	19.4 ± 2.56	6.55	20.2 ± 2.48	6.17	0.8	4.1	ns
	Fresh weight (mg)	88.4 ± n/a	n/a	151.2 ± n/a	n/a	62.8	71.0	n/a
	Dry weight (mg)	9.8 ± n/a	n/a	20.8 ± n/a	n/a	11.0	112.2	n/a
V_2	Roots no.	1.0 ± 0.47	0.22	1.2 ± 0.46	0.21	0.2	20	**
	Roots length (mm)	6.6 ± 2.48	6.17	4.2 ± 1.08	1.17	-2.4	-36.3	***
	No. of strains	2.2 ± 0.76	0.58	1.8 ± 0.87	0.76	-0.4	-18.1	*
	Strains length (mm)	24.3 ± 1.84	3.41	20.8 ± 1.90	3.63	-3.5	-14.4	***
	Leaf no.	10.3 ± 2.39	5.72	9.2 ± 2.08	4.35	-1.1	-10.6	*
	Fresh weight (mg)	149.3 ± n/a	n/a	128.3 ± n/a	n/a	-21.0	-14.0	n/a
	Dry weight (mg)	16.9 ± n/a	n/a	29.3 ± n/a	n/a	12.4	73.3	n/a
V_3	Roots no.	0.0 ± n/a	n/a	0.0 ± n/a	n/a	0.0	0.0	n/a
	Roots length (mm)	0.0 ± n/a	n/a	0.0 ± n/a	n/a	0.0	0.0	n/a
	No. of strains	3.0 ± 0.91	0.84	2.5 ± 1.18	1.41	-0.5	-16.6	ns
	Strains length (mm)	16.0 ± 2.03	4.14	17.5 ± 2.20	4.87	1.5	9.3	**
	Leaf no.	10.8 ± 1.94	3.78	6.0 ± 1.67	2.81	-4.8	-44.4	***
	Fresh weight (mg)	189.0 ± n/a	n/a	200.6 ± n/a	n/a	11.6	6.13	n/a
	Dry weight (mg)	22.6 ± n/a	n/a	25.9 ± n/a	n/a	3	14.6	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.

The number of leaflets of the plantlets reached the highest values of the V0SsSr variant compared to control (V0Sr), the percentage differences were of 47% and 4.8 leaves/vitroplant (Table 3, Fig. 1A). Also, the number of roots registered values of 56.2% higher for stevia cultivated on culture medium without growth regulators (V0SsSr) compared to V0Sr (control).

In case of V2SsSr variant 55% of the stevia plantlets presented signs of senescence, at the level of the leaves in a proportion of 20-30% (Table 2, Figure 1). In these cultures, the plantlets of S. rebaudiana had a higher rate of growth compared to those which were viable, without senescence (Table 3, Fig. 1).

Regarding the viability of in vitro plantlets of sequoia, positive results were obtained on the culture medium MB-MS supplemented with 1 mg/l IBA + 1 mg/l K (V3SsSr). The plantlets had an overall color of deep green (Fig. 1G). The largest accumulation of fresh weight and dry matter for the plants of sequoia were recorded in case of the V1SsSr experimental variant, the percentage differences compared to control being of 123.5% for fresh weight and 21% for dry weight (Table 2). For in vitro plantlets of stevia on these two parameters, higher values than the control variants, were registered in the experimental V1SsSr variant, the differences being of 71% and 112.2% (Table 3, Fig. 1C).

Biometric measurements and morphologic aspects at 40 days. In the second stage of experimental observations, an increased inhibitory allelopathic influence exerted by the stevia plantlets on sequoia plantlets was observed, both in terms of morphogenesis of plants and biomass accumulation. We could not find any parameter where the values recorded at the sequoia co-cultivated with stevia to be higer compared to those of monoculture as a control, whatever the composition of the used culture medium (Table 4).

Table 4. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **40 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (**V_2**), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in biculture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.5 ± 0.75	0.75	1.1 ± 0.56	0.32	-0.4	-26.6	*
	Strains length (mm)	17.2 ± 2.65	7.02	5.3 ± 1.89	3.58	-11.9	-69.1	***
	Leaf no.	23.5 ± 3.17	10.10	2.0 ± 0.99	0.99	-21.5	-91.4	***
	Fresh weight (mg)	123.4 ± n/a	n/a	18.8 ± n/a	n/a	-104.6	-84.7	n/a
	Dry weight (mg)	15.9 ± n/a	n/a	2.3 ± n/a	n/a	-13.6	-85.5	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.3 ± 1.19	1.42	2.1 ± 0.80	0.64	-0.2	-8.6	ns
	Strains length (mm)	17.6 ± 3.81	14.5	14.4 ± 3.25	10.5	-3.2	-18.8	***
	Leaf no.	29.5 ± 4.0	16.18	7.0 ± 1.84	3.41	-22.5	-76.27	***
	Fresh weight (mg)	98 ± n/a	n/a	78.4 ± n/a	n/a	-19.6	-20.0	n/a
	Dry weight (mg)	12.1 ± n/a	n/a	9.6 ± n/a	n/a	-2.5	-20.6	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.9 ± 1.15	1.32	1.7 ± 0.77	0.59	-1.2	-41.3	***
	Strains length (mm)	26.6 ± 3.14	9.87	18.4 ± 3.75	14.11	-8.2	-30.8	***
	Leaf no.	20.9 ± 1.94	3.77	9.7 ± 2.21	4.90	-11.2	-53.5	***
	Fresh weight (mg)	240.7 ± n/a	n/a	57.4 ± n/a	n/a	-183.3	-76.1	n/a
	Dry weight (mg)	23.2 ± n/a	n/a	17.1 ± n/a	n/a	-6,1	-26.2	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.3 ± 1.17	1.38	1.8 ± 0.85	0.73	-0.5	-21.7	ns
	Strains length (mm)	33.5 ± 2.19	4.80	12.8 ± 2.53	6.40	-20.7	-61.7	***
	Leaf no.	25.9 ± 2.58	6.68	15.5 ± 2.21	4.89	-10.7	-40.1	***
	Fresh weight (mg)	217.5 ± n/a	n/a	161.5 ± n/a	n/a	-56.0	-25.7	n/a
	Dry weight (mg)	21.3 ± n/a	n/a	13.1 ± n/a	n/a	-8.2	-38,4	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 5. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (**V_xSr**) and in co-culture with *S. sempervirens* (V_xSsSr) at **40 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	2.6 ± 0.89	0.80	5.2 ± 1.36	1.84	2.6	100	***
	Roots length (mm)	65.3 ± 2.47	6.10	56.8 ± 2.76	7.64	-8.5	-13.0	***
	No. of strains	3.5 ± 1.02	1.04	3.2 ± 1.16	1.34	-0.3	-8.5	ns
	Strains length (mm)	25.3 ± 1.53	2.36	34.3 ± 2.34	5.51	9.0	35.5	***
	Leaf no.	15.6 ± 2.49	6.24	24.4 ± 2.63	6.96	8.8	56,.4	***
	Fresh weight (mg)	82 ± n/a	n/a	198.9 ± n/a	n/a	116.9	142.5	n/a
	Dry weight (mg)	8.6 ± n/a	n/a	19.6 ± n/a	n/a	11.0	127.9	n/a
V_1	Roots no.	4.1 ± 1.62	2.64	3.2 ± 1.16	1.35	-0.9	-21.9	**
	Roots length (mm)	18.3 ± 2.4	5.7	15.5 ± 2.63	6.92	-2.8	-15.3	***
	No. of strains	2.7 ± 0.88	0.78	2.4 ± 1.03	1.06	-0.3	-11.1	ns
	Strains length (mm)	72.4 ± 2.75	7.58	52.2 ± 2.38	5.67	-20.2	-27.9	***
	Leaf no.	22.7 ± 1.95	3.80	21.1 ± 1.95	3.80	-1.6	-7.04	***
	Fresh weight (mg)	298.9 ± n/a	n/a	542.0 ± n/a	n/a	243.1	86.9	n/a
	Dry weight (mg)	35.7 ± n/a	n/a	47.6 ± n/a	n/a	11.9	33.3	n/a
V_2	Roots no.	4.4 ± 1.45	2.10	4.5 ± 1.50	2.25	0.1	2.2	ns
	Roots length (mm)	25.3 ± 2.36	5.57	20.9 ± 1.99	3.96	-4.4	-17.3	***
	No. of strains	2.3 ± 0.81	0.67	2.8 ± 0.98	0.96	0.5	21.7	**
	Strains length (mm)	34.2 ± 2.06	4.24	35.5 ± 2.72	7.44	1.3	3.8	*
	Leaf no.	11.1 ± 2.33	5.46	13.7 ± 1.77	3.15	2.6	23.4	***
	Fresh weight (mg)	532.5 ± n/a	n/a	594.6 ± n/a	n/a	62.1	11.6	n/a
	Dry weight (mg)	52.6 ± n/a	n/a	62.3 ± n/a	n/a	9.7	18.4	n/a
V_3	Roots no.	0.1 ± 0.44	0.20	0.9 ± 1.82	3.32	0.8	800	**
	Roots length (mm)	0.3 ± 1.19	1.43	0.2 ± 0.46	0.21	-0.1	-33.3	ns
	No. of strains	3.2 ± 1.28	1.66	3.4 ± 1.34	1.79	0.2	6.25	ns
	Strains length (mm)	26.6 ± 2.32	5.39	26.2 ± 2.1	4.41	-0.4	-1.5	ns
	Leaf no.	8.5 ± 1.5	2.25	6.4 ± 1.47	2.17	-2.1	-24.7	***
	Fresh weight (mg)	377.1 ± n/a	n/a	267.4 ± n/a	n/a	-109.7	-29.0	n/a
	Dry weight (mg)	46.7 ± n/a	n/a	34.9 ± n/a	n/a	-11.8	-25.2	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.

In the case of the co-culture of in vitro plants of stevia, the most increased rootedness was recorded when cultivated on V3SsSr culture medium that was with 800% higher compared to control (V3Sr). The values of number and length of stems, was higher observed in stevia seedlings obtained in the case of V2SsSr, compared to control (Table 5). Regarding the number of leaflets, the highest value was found at the experimental V0SsSr variant, due to the stimulating effect exerted by the sequoia plants (Table 5).

Regarding gravimetric parameters, for sequoia cultivated with stevia, the recorded values were lower than those registered for control as monocultures, which might be due to the allelopathic inhibitors influence exerted by the biochemical compounds released by stevia plants on that of sequoia (Table 4).

In the case of the S. rebaudiana plants, the fresh and dry weights reached the highest differences values was recorded on the culture medium without growth regulators (V0SsSr): being of 142.5% of the fresh weight and 127.9% for dry weight (Table 5).

As a result, we can conclude that at 40 days of co-cultivating stevia and sequoia, the most effective variant was found to be the control without growth regulators, but only for S. rebaudiana.

In the case of the Sequoia plants, the allelopathic inhibitor influences induced by stevia led to recording some negative effect of the development compared to control, no matter the used experimental variant.

Biometric measurements and morphologic aspects at 60 days.

Throughout the experimental period (60 days), the presence of the S. rebaudiana in co-culture with S. sempervirens inhibited their growth and development, a process increasingly stronger with the aging of the in vitro cultures, being able to record during the measurement of the parameter value leaflets number value -63.8% on variant V0SsSr, and -59% for V3SsSr variant, in plants co-culture compared to controls (Table 6, Fig. 1B).

Table 6. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **60 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in biculture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.0 ± 0.78	0.61	2.0 ± 0.59	0.35	0.0	0.0	ns
	Strains length (mm)	30.3 ± 2.32	5.38	25.1 ± 1.60	2.58	-5.2	-17.1	***
	Leaf no.	38.7 ± 2.34	5.50	14.0 ± 1.85	3.43	-24.7	-63.8	***
	Fresh weight (mg)	187.5 ± n/a	n/a	116.0 ± n/a	n/a	-71.5	-38.1	n/a
	Dry weight (mg)	21.8 ± n/a	n/a	11.5 ± n/a	n/a	-10.3	-42.2	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0 ± n/a	0.00	0	0	ns
	No. of strains	3.0 ± 1.41	2	2.8 ± 0.81	0.66	-0.2	-6.66	ns
	Strains length (mm)	35.5 ± 2.13	4.57	18.2 ± 2.61	6.83	-17.3	-48.7	***
	Leaf no.	38.1 ± 2.04	4.18	17.8 ± 2.05	4.22	-20.3	-53.2	***
	Fresh weight (mg)	293.7 ± n/a	n/a	167.4 ± n/a	n/a	-126.3	-43.0	n/a
	Dry weight (mg)	16.5 ± n/a	n/a	21.9 ± n/a	n/a	5.4	32.7	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	3.5 ± 1.26	1.59	2.2 ± 0.97	0.95	-1.3	-37.1	***
	Strains length (mm)	32.6 ± 2.00	4.02	21.9 ± 1.66	2.78	-10.5	-32.8	***
	Leaf no.	47.8 ± 1.76	3.11	23.2 ± 2.28	5.20	-24.6	-51.4	***
	Fresh weight (mg)	448.2 ± n/a	n/a	193.8 ± n/a	n/a	-254.4	-56.7	n/a
	Dry weight (mg)	48.7 ± n/a	n/a	20.7 ± n/a	n/a	-28	-57.4	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	4.3 ± 1.70	2.9	2.6 ± 1.00	1.01	-1.7	-39.5	***
	Strains length (mm)	35.8 ± 2.28	5.20	23.2 ± 2.67	7.16	-12.6	-35.1	***
	Leaf no.	68.3 ± 2.83	8.01	28.0 ± 2.67	7.14	-40.3	-59.0	***
	Fresh weight (mg)	440.9 ± n/a	n/a	339.7 ± n/a	n/a	-101.2	-22.9	n/a
	Dry weight (mg)	44.8 ± n/a	n/a	44.3 ± n/a	n/a	-0.5	-1.1	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.

At 60 days of starting the experiments, the plantlets of stevia cultivated on V2SsSr variant were characterized by strong vitality marked by a broad caulogenesis, showing a large number of stems and high lengths.

The most intense caulogenesis in case of the stevia plant in coculture was recorded in the variant V2SsSr (medium MB-MS supplemented with 1 mg/l K). Regarding the number of roots, the highest value was found in the experimental variant V0SsSr variant (Table 7, Fig. 1F).

Regarding the parameters of fresh and dry weight, in case of the stevia plantlets, reaching the highest values at the in vitro plants cultivated on the culture

medium supplemented with 1 mg/l K (V2SsSr), increases recorded compared to group control being seven times higher for fresh biomass and three times higher in case of dry biomass (Table7, Fig. 1F).

Table 7. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (V_xSr) and in co-culture with *S. sempervirens* (V_xSsSr) at **60 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	3.1 ± 1.15	1.33	6.8 ± 1.10	1.21	3.7	119.3	***
	Roots length (mm)	71.7 ± 2.38	5.68	66.6 ± 2.30	5.31	-5.1	-7.11	***
	No. of strains	3.9 ± 0.99	0.99	3.5 ± 1.02	1.04	-0.4	-10.2	ns
	Strains length (mm)	34.4 ± 2.86	8.18	42.2 ± 3.06	9.42	7.8	22.6	***
	Leaf no.	21.4 ± 2.16	4.68	35.2 ± 2.03	4.13	13.8	64.4	***
	Fresh weight (mg)	158.2 ± n/a	n/a	311.0 ± n/a	n/a	152.8	96.5	n/a
	Dry weight (mg)	17.1 ± n/a	n/a	24.0 ± n/a	n/a	6.9	40.3	n/a
V_1	Roots no.	22.4 ± 2.21	4.92	5.2 ± 1.44	2.10	-17.2	-76.7	***
	Roots length (mm)	54.5 ± 2.30	5.29	47.8 ± 2.41	5.85	-6.7	-12.2	***
	No. of strains	3.6 ± 1.06	1.13	3.1 ± 0.98	0.96	-0.5	-13.8	ns
	Strains length (mm)	73.3 ± 3.20	10.30	88.6 ± 2.94	8.67	15.3	20.8	***
	Leaf no.	29.3 ± 2.12	4.55	32.4 ± 2.45	6.03	3.1	10.5	***
	Fresh weight (mg)	314.1 ± n/a	n/a	692.4 ± n/a	n/a	378.3	120.4	n/a
	Dry weight (mg)	39.5 ± n/a	n/a	55.3 ± n/a	n/a	15.8	40.0	n/a
V_2	Roots no.	5.5 ± 1.39	1.95	5.8 ± 1.18	1.41	0.3	5.45	ns
	Roots length (mm)	27.6 ± 2.31	5.35	30.3 ± 2.49	6.23	2.7	9.78	***
	No. of strains	2.5 ± 0.88	0.77	3.8 ± 0.81	0.67	1.3	52.0	***
	Strains length (mm)	37.2 ± 2.17	4.71	46.8 ± 2.01	4.05	9.6	25.8	***
	Leaf no.	11.5 ± 2.42	5.88	14.6 ± 1.54	2.39	3.1	26.9	***
	Fresh weight (mg)	43.4 ± n/a	n/a	357.2 ± n/a	n/a	313.8	723.0	n/a
	Dry weight (mg)	9.6 ± n/a	n/a	39.7 ± n/a	n/a	30.1	313.5	n/a
V_3	Roots no.	-	-	-	-	-	-	-
	Roots length (mm)	-	-	-	-	-	-	-
	No. of strains	-	-	-	-	-	-	-
	Strains length (mm)	-	-	-	-	-	-	-
	Leaf no.	-	-	-	-	-	-	-
	Fresh weight (mg)	-	-	-	-	-	-	-
	Dry weight (mg)	-	-	-	-	-	-	-

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.

In conclusion, at 60 days from the experiments, in the case of S. sempervirens and S. rebaudiana, was recorded a mutual allelopathy influence, synergistic in part sequoia on stevia, and antagonistic in part stevia on sequoia.

Keeping into in vitro culture under allelopathy conditions both plant species, over 20 days, it is effective only in the case of stevia, which is positively influenced by redwoods plantlets, while stevia plants have had a negative allelopathic effect on the redwoods, wherever the used culture medium.

In similar studies, the allelopathic effects exerted by sequoia plants on those of cultivated Drosera rotundifolia L. were reported, which were grown in the same container on the MS62 medium without growth regulators. The negative effects of Sequoia plants exerted in the first 60 days of the study generated the inhibition, but not by stopping the increase of the number of roots, leaflets and rosettes belonging to Drosera rotundifolia L. species.

Subsequently, between 60 to 90 days to have a reversal effects exerted by the Sequoia seedlings,

respectively to evolve from antagonistic to synergistic (P.A. ROGOJAN [20]). A reversible allelopathic effect was recorded by Blidar (C.F. BLIDAR & al. [34]) at in vitro cocultures of Cymbidium hybridum protocorm like bodies (PLB) and Drosera rotundifolia L., where up to 30 days the orchid exerted a negative effect on the drosera plantlets, and then an synergistic one.

It was also reported negative in vitro allelopathic effects of stevia plantlets on the seed germination of C. cajan and C. arietinum (A.S. TAWARE & al. [31]), as well as on some bacteria development (A.S. TAWARE & al. [28]).

Manci (A.M. MANCI [19]) analyzed the reactivity of the S. sempervirens mini seedlings under allelopathic conditions with protocorms of C. hybridum. In this case, the redwood plants stimulated the growth and development of protocorms, the combination of both species, being beneficial up to the age of 60 days of in vitro cultures for orchids.

The results obtained in our experiments confirmed the in vitro allelopathic properties of the two species, S. sempervirens respectively S. rebaudiana.

After 60 days, choosing a modified MS62 culture medium supplemented with kinetin (K) at a concentration of 1 mg/l (V2SsSr) was the first choice, but only for the plantlets of S. rebaudiana. Taware (A.S. TAWARE & al. [28]) also showed the effectiveness of the use of 0.3 mg/l kinetin in the culture medium in which the explants of stevia are transferred.

Conclusions

The present study showed that the identified allelopathic synergistic influences, allow the in vitro association of S. sempervirens with S. rebaudiana, but only until the age of 20 days of in vitro culture, the positive growth and the development being recorded, but it was relatively insufficient for possible methods of sub-cultivation or efficient acclimation.

However, the use of a modified MS62 culture medium supplemented with 1 mg/l IBA, was found to be most favourable for the proliferation and growth of the plantlets for both species.

Economically speaking, namely marketing the in vitro floral arrangements containing both Sequoia sempervirens and Stevia rebaudiana species is not recommend due to the inhibitory allelopathic influences exerted by the stevia plantlets over the sequoia. However, the phenomenon of inhibition induced by the stevia plantlets, may represent a possibility of in vitro conservation of this, thus adding the conservation variants in the literature (A. PĂUNESCU, [35]). This requires studies to revive the culture maintained for a long term under inhibition (A. MANOLE & C. BANCIU [36]) and genetic, too (A. KARP & al. [37]).

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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