Scientific Papers. Series B, Horticulture. Vol. LXVII, No. 1, 2023
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

EVALUATION OF BIOMASS PRODUCTION OF *STEVIA REBAUDIANA* BERTONI USING CLASSICAL *IN VITRO* CULTURE AND TEMPORARY IMMERSION BIOREACTOR SYSTEM

Doina CLAPA[1], Ana Maria RADOMIR[2], Adrian George PETICILĂ[3], Monica HÂRȚA[1]

[1]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
Faculty of Horticulture and Business in Rural Development, BIOCERA Research Centre,
3-5 Mănăștur Street, 400372, Cluj-Napoca, Romania
[2]National Research and Development Institute for Biotechnology in Horticulture Ștefănești-Argeș,
37 Bucharest-Pitești Road, 117715 Ștefănești, Argeș, Romania
[3]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Horticulture,
59 Mărăști Blvd, District 1, 011464 Bucharest, Romania

Corresponding author email: monica.harta@usamvcluj.ro

Abstract

In vitro cultures can provide a sufficient quantity of high-quality uniform biomass under controlled conditions. The aim of this study was to develop an efficient micropropagation system for biomass production from S. rebaudiana. In vitro shoot proliferation of S. rebaudiana was compared in classical agar-gelled solid culture medium (SM) and the Plantform temporary ımmersion bioreactor system (TIS). Murashige and Skoog 1962 (MS) medium was used in both culture systems and was supplemented with 0, 0.1, 0.2, and 0.3 mg/L 6-Benzyladenine (BA). The maximum biomass production (1396.72 ± 54.03 mg) was recorded using the TIS in the variant with a concentration of 0.2 mg/L BA. The lowest biomass production (229.96 ± 29.33 mg) was obtained on MS solid culture media without BA. It is noteworthy that the water content tended to decrease in TIS compared to SM. In vitro-grown plantlets were screened for possible genetic differences using Start Codon Targeted (SCoT). The PCR amplification products were monomorphic in micropropagated plants and their mother plant, thus proving the genetic fidelity and uniformity of the plants grown in vitro for biomass production.

Key words*: fresh weight, genetic fidelity, solid media, Plantform, Start Codon Targeted Polymorphism*

INTRODUCTION

The development of plant tissue cultures for the production of secondary metabolites has been underway for more than three decades (Yue et al., 2016) because it offers a number of advantages over conventional plant cultivation methods: independence from geographical and seasonal variations, tissue cultured plants are generally free from fungal and bacterial diseases, relatively short growth cycles, mass propagation of a uniform plant material and lack of use of pesticides and herbicides (Debnath et al., 2006).

Therefore, different groups of metabolites have been examined in the biomass of shoots grown in vitro in several plant species such as: biomass of in vitro shoots of *Eryngium species*, shoot cultures of *Ruta graveolens*, tissue cultures of six genotypes of *Deschampsia antarctica*, biomass from in vitro shoots cultures of *Aronia melanocarpa* (Clapa et al., 2022). Likewise, plant tissue culture techniques are increasingly used for the mass propagation of *Stevia rebaudiana* Bertoni and establishing optimal conditions for in vitro cultivation is essential for obtaining high biomass and secondary metabolites production (Miladinova-Georgieva et al., 2023).

S. rebaudiana is a perennial herbaceous plant (family Asteraceae) known as sweet leaf, sweet herbs and honey leaf due to the presence of more than 30 different steviol glycosides found mainly in the leaves (Miladinova-Georgieva et al., 2023). Steviol glycosides are 250-300 times sweeter than sucrose (Peteliuk et al., 2021). Stevia is a valuable source of vitamins, minerals (K, Ca, Na, Mg, Cu, Mn, Fe, Zn), proteins, carbohydrates, dietary fibers, essential amino acids, fatty acids as well as other bioactive compounds such as flavonoids, phenolic compounds, phytosterols, chlorogenic acids

and triterpenes (Wölwer-Rieck, 2012; Peteliuk et al., 2021). The benefits of *S. rebaudiana* are numerous, it is used to treat diseases such as diabetes, obesity, tooth decay, hypoglycemia and hypertension (Faramayuda et al., 2022).

In vitro propagation of *S. rebaudiana* has been studied by many researchers to develop different micropropagation protocols in semisolid media gellified with agar (Sivaram & Mukundan, 2003; Tadhani et al., 2006; Kalpana et al., 2009; Sairkar et al., 2009; Pratibha et al., 2010; Ali et al., 2010; Singh & Dwivedi, 2014; Razak et al., 2014; Soliman et al., 2014; Kaplan et al., 2019; Yesmin, 2019) or in temporary immersion systems (TIS) (Norazlina et al., 2012; Sacco et al., 2013; Ramírez-Mosqueda et al., 2016; Vives et al., 2017; Rosales et al., 2018; Bayraktar, 2019; Melviana et al., 2019; Saptari et al., 2022). Furthermore, several plant tissue culture techniques have been developed for mass propagation, biomass and secondary metabolite production of *S. rebaudiana,* such as: callus culture, suspension cultures, adventitious root culture (Kazmi et al., 2019).

Hence, plant tissue culture techniques can provide the production of plantlets which are genetically uniform and have homogeneous secondary metabolite content within a short time under controlled physical conditions (Bayraktar et al., 2016). Currently, molecular markers are valuable biological tools for analysing the genetic fidelity of micropropagated plants (Pandey et al., 2021). The aim of this study was to develop an efficient micropropagation system for biomass production from *S. rebaudiana* and to evaluate the clonal fidelity of in vitro-grown shoots through DNA-based molecular markers. In vitro biomass production of Stevia was compared in classical agar-gelled solid culture medium (SM) and the Plantform temporary immersion bioreactor system (TIS). The genetic stability of in vitro-grown plantlets was assessed by Start Codon Targeted (SCoT) molecular markers.

MATERIALS AND METHODS

Biomass production in solid and TIS culture systems

The explants used for this experiment were excised from in vitro cultures of *S. rebaudiana* (4 weeks old) which had been cultured on Murashige and Skoog medium (MS) Murashige & Skoog, 1962) with 3% (w/v) sugar, 5 g/L Plant agar, pH 5.8, without growth regulators.

For the production of biomass, two *in vitro* culture systems were used: solid (SM) and liquid (TIS). For solid media, 370 ml jars were used as culture vessels, which were 7 cm in diameter and 12.7 cm high, with screw caps, containing 50 ml media/jar. TIS culture systems used Plantform bioreactors (Welander et al., 2014), with a volume of 400 ml media/ bioreactor. The immersion time was one minute at four-hour intervals and the aeration time was four minutes at one-hour intervals. The basal culture medium used for both types of culture systems was MS with four concentrations of BA, 0, 0.1, 0.2, and 0.3 mg/L, respectively. The SM was solidified with 5 g/L Plant Agar. The liquid culture media and those jellified with agar were sterilized for 20 minutes in the autoclave at 121°C and 0.11 MPa. All the components of the culture media were purchased from Duchefa (Biochemie B.V, Netherlands), and they were added prior to sterilization, as well, the pH was adjusted to 5.8.

The culture vessels with solid media were inoculated with 5 shoot fragments, whereas the bioreactors were inoculated with 20 shoot fragments, each of them 1.5-2 cm in length.

The in vitro cultures were incubated in the growth room at 16-hour photoperiod with 32.4 µmol $m^{-2}s^{-1}$ light intensity (Philips Core Pro LED tube 1200 mm 16 W 865 CG, 1600 lm Cool Daylight) at 23 ± 3°C and 50 ± 2% humidity.

Genetic fidelity analysis using SCoT markers

The genetic fidelity between the in vitro obtained stevia shoots and their mother plant was assessed using SCoT markers. The biological material was represented by leaves of the in vitro proliferated shoots from the two different culture systems and from their mother plant as control. The harvested leaves were dried, ground into a fine powder (Tissue Lyser II, Qiagen, Germany) and kept at 4°C until the genetic analyses were carried out.

Total genomic DNA isolation. DNA was isolated from four randomly selected shoots for

each culture media variant and each in vitro culture system. The dry material represented by 0.2 g of leaf powder was processed using a protocol based on the CTAB (cetyltrimethylammonium bromide) method as published by (Lodhi et al., 1994) and improved by (Pop et al., 2003) and (Bodea et al., 2016). The DNA concentration and purity were determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Prior to performing the PCR (polymerase chain reaction) amplifications, all the DNA samples were diluted to 50 ng/ µL using nuclease-free water.

SCoT analysis. For the SCoT analysis, the PCR amplifications were carried out using the protocol described by (Collard & Mackill, 2009). The reaction mixture (a total volume of 15 µL) consisted of 50 ng/ µL of gDNA, distilled H_2O for the PCR reactions, 5X GoTaq Flexi Green buffer (Promega, Madison, WA, USA), 1.5 mM $MgCl_2$ (Promega, Madison, WA, USA), 0.2 mM of dNTP mix (Promega, Madison, WA, USA), 1 µM SCoT primer (GeneriBiotech, Hradec Králové, Czechia), and 1U of GoTaq polymerase (Promega, Madison, WA, USA). The PCR temperature cycling conditions were: (a) 1 cycle of 5 min at 94°C for initial denaturation, (b) 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, and (c) the final elongation step of 7 min at 72°C. The SCoT primers used in this study are shown in Table 1.

Table 1. The list of SCoT primers used

Primer name	The 3'-5'nucleotide sequence of the primer
SCoT 9	CAACAATGGCTACCAGCA
SCoT 10	CAACAATGGCTACCAGCC
SCoT 12	ACGACATGGCGACCAACG
SCoT 15	ACGACATGGCGACCGCGA
SCoT 16	ACCATGGCTACCACCGAC
SCoT 18	ACCATGGCTACCACCGCC
SCoT 19	ACCATGGCTACCACCGGC
SCoT 21	ACGACATGGCGACCCACA
SCoT 22	AACCATGGCTACCACCAC
SCoT 23	CACCATGGCTACCACCAG

The PCR amplifications were repeated twice for each SCoT primer to ensure the reproducibility of the results. Separation of the PCR amplified products was performed by electrophoresis on 1.4% agarose gels (Promega, Madison, WA, USA) stained with RedSafeTM Nucleic Acid staining solution (iNtRON Biotech, Seoul, South Korea) in 1X TBE (Tris Borate-EDTA buffer), at 110 V and 136 mA for 2.5-3 h. The electrophoretic profiles were visualized under UV (ultraviolet in UVP Biospectrum AC Imaging System (UVP BioImaging Systems, Upland, CA, USA).

Data Collection and Statistical Analysis

Each *in vitro* experiment was repeated three times, more exactly three jars per repetition and three bioreactors per repetition. Stevia shoots grown *in vitro* on solid and liquid media were collected for analysis after 4 weeks of culture. Data analysed refer to the average length of shoots (SL), the fresh weight (FW), dry weight (DW) and water content (WC). FW was obtained by weighing the shoots immediately after the material was removed from the in vitro culture medium. To obtain DW the material was dried for three days at 45°C and weighed again. WC percentage was calculated using the formula (Mazurek et al., 2021):

WC (%) = [(Fresh Weight-Dry Weight)/Fresh Weight] *100

ANOVA was performed followed by Tukey's HSD test ($P \leq 0.05$) to determine the statistically significant differences between the means. Values shown (in text and figures) are means ± SE (standard error).

RESULTS AND DISCUSSION

For biomass production, two different culture systems (solid-gellified with agar and TIS) were compared in terms of growth parameters in *S. rebaudiana*.

Regarding shoot lengths (Figures 1 and 2), they recorded maximum values in TIS, on MS medium supplemented with 0.2 mg/L BA (11.91 ±0.54 cm), while the shortest shoots were obtained in MS solid media supplemented with 0.3 mg/L BA (4.40 ± 0.19 cm).

Previous studies used BA (BAP) in various concentrations for shoot induction in the MS solid media or TIS (Kalpana et al., 2009; Ali et al., 2010; Norazlina et al., 2012; Hassanen & Khalil, 2013).

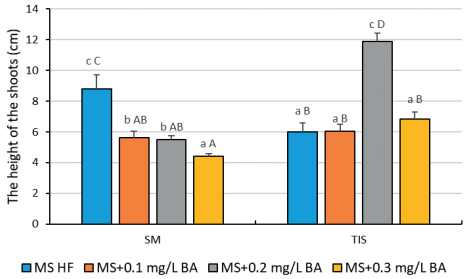


Figure 1. Shoots length (cm) of *S. rebaudiana* cultivated on MS medium supplemented with 0, 0.1, 0.2 and 0.3 mg/L BA, in two different culture systems: solid culture medium (SM) gelled with 5g/L agar, and liquid culture medium through temporary immersion (TIS) system in Plantform bioreactor. Lowercase letters above the bars indicate significant differences between the means of the shoots length obtained on the same culture system; capital letters above the bars represent significant differences between the means of the shoots length obtained on the both culture systems according to Tukey's HSD test (P≤0.05)

On a culture medium similar to ours but solidified with 0.8% (w/v) agar and supplemented with higher concentrations of BAP (0.5, 1.0, 1.5 and 2 mg/L) Hassanen & Khalil (2013) reported that shoot length of stevia decreased from 3.84 cm to 0.74 cm with increasing BA concentration.

In general, these studies show that BA, in low concentrations, is an adequate cytokinin for *in vitro* multiplication in *S. rebaudiana*, a fact supported by our results.

Fresh weight is a very important parameter in biomass production for obtaining bioactive compounds. Growth measurements in terms of fresh weight were taken at the end of the four-week culture cycle. After weighing the freshly removed inocula from the cultured media it was noticed that all the concentrations of BA increased fresh weight in both culture systems, but FW increase of the cultures differed between bioreactor and agar medium. The fresh weight proved to be statistically significantly higher on 0.2 mg/L BA in TIS, 1396.72±54.03 mg/inoculum, respectively (Figure 3a).

However, in TIS, higher concentration of BA (0.3 m/L) resulted in a decrease in fresh weight (491.36 ± 52.05 mg/inoculum).

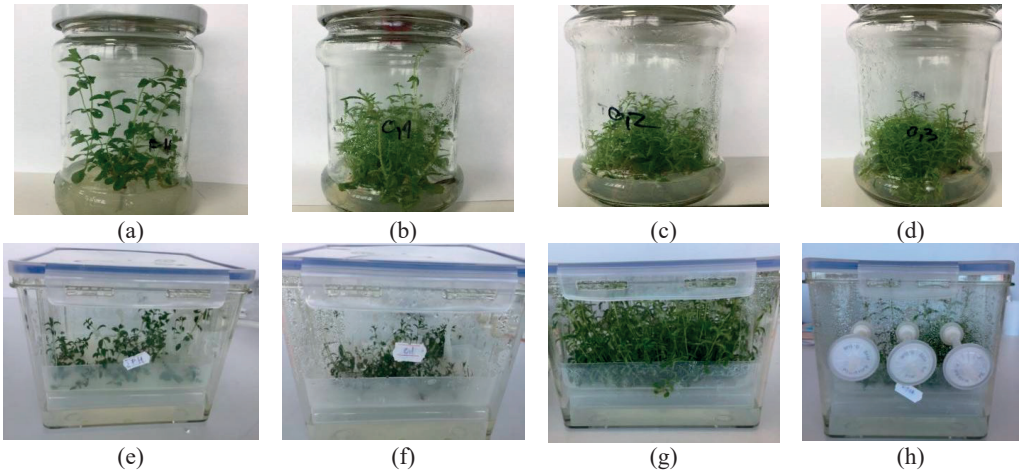
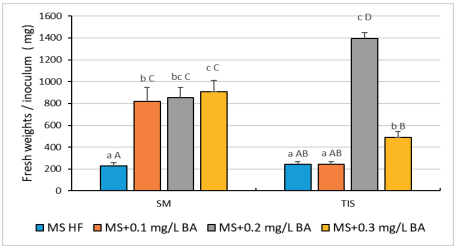
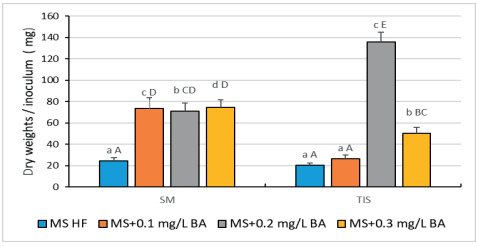


Figure 2. *In vitro* cultures of *S. rebaudiana*, cultured on MS medium supplemented with different concentrations of BA, in two culture systems: solid culture medium gelled with 5 g/L agar (SM) without BA (a), SM supplemented with 0.1 mg/L BA (b), SM supplemented with 0.2 mg/L BA (c), SM supplemented with 0.3 mg/L BA (d); liquid culture medium through TIS in Plantform bioreactor without BA (e), TIS supplemented with 0.1 mg/L BA (f), TIS supplemented with 0.2 mg/L BA (g), TIS supplemented with 0.3 mg/L BA (h)



(a)



(b)

Figure 3. Fresh weights /inoculum (mg) (a) and dry weights /inoculum (mg) (b) of *S. rebaudiana* cultured on MS medium supplemented with 0, 0.1, 0.2 and 0.3 mg/L BA, in two different culture systems: solid culture medium (SM) gelled with 5g/L agar, and liquid culture medium through TIS system. Lowercase letters above the bars indicate significant differences between the means of the fresh weights obtained on the same culture system; capital letters above the bars represent significant differences between the means of the fresh weights obtained on the both culture systems according to Tukey's HSD test (P≤0.05)

The maximum FW production on the solid medium was achieved at a concentration of 0.3 mg/L BA, 909.31±101.37 mg/inoculum, respectively. In both culture systems the lowest FW was obtained on culture medium without BA, respectively 229.96 ± 29.34 mg/inoculum on SM and 244.32 ± 22.71 mg/inoculum on TIS (the difference is not statistically significant). The dry weight ranged from 20.46 ± 2.06 to 135.69 ± 9.39 mg/inoculum for TIS and 24.41 ± 2.88 to 74.42 ± 7.45 mg/inoculum for agar medium (Figure 3b). After drying the leaves the total water content was determined. The determinations revealed an increasing tendency in water content with the increasing of BA concentrations for both culture systems (Figure 4).

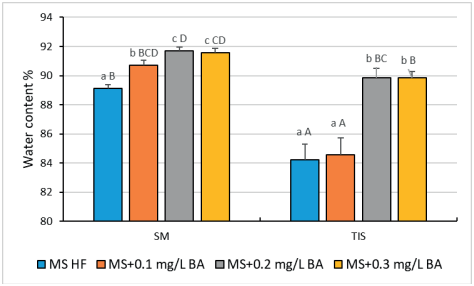


Figure 4. Water content (%) of *S. rebaudiana* cultivated on MS medium supplemented with 0, 0.1, 0.2 and 0.3 mg/L BA, in two different culture systems: solid culture medium (SM) gelled with 5g/L agar, and liquid culture medium through temporary immersion (TIS) system in Plantform bioreactor. Lowercase letters above the bars indicate significant differences between the means of the shoot length obtained on the same culture system; capital letters above the bars represent significant differences between the means of the shoot length obtained on the both culture systems according to Tukey's HSD test (P≤0.05)

In this case the WC of the proliferated shoots in the in SM showed an increase from 89.10 ± 0.28% for MS without BA to 91.69 ± 0.24 % on media supplemented with 0.2 mg/L BA. The proliferated shoots in TIS culture system showed an increase of WC from 84.21 ± 1.07% (MS without BA) to 89.86 ± 0.61 % on media supplemented with 0.2 mg/L BA. Our results show that the shoots obtained in TIS had a higher WC compared to those obtained in SM. It should be noted that WC is higher in TIS compared to SM at all concentrations of BA. Previous studies have also shown significant differences in fresh and dry weight or water content in *S. rebaudiana* or other species grown in TIS and agar medium. Thus, Vives et al (2017) reported a 6.6 times higher FW production in temporary immersion bioreactor (BIT®) compared to solidified medium with agar after 21 days of culture of *S. rebaudiana* on MS medium with 0.25 mg/L BA. Welander et al. (2014) show that Digitalis and Echinacea gained significantly more weight during cultivation in TIS compared to agar medium, while Rubus showed the opposite result. These results might be due to the fact that TIS provides efficient ventilation of the plant tissues due to the intermittent contact with the medium, because most of the time the plant material is not submerged but surrounded with plenty of sterile air. When submerged, there is direct contact between the liquid medium and the leaves; this makes possible nutrient uptake by the leaves, unlike in semisolid media (Jesionek et al., 2017; Vives et al., 2017; Monja-Mio et al, 2021).

SCoT genetic analysis

In total, 15 SCoT primers were used in this study for the initial screening between the field-grown mother plant and in vitro-derived Stevia shoots cultured on MS medium supplemented with different concentrations of BA, in two culture systems, but only ten SCoT primers had amplified clearly and reproducible bands. The number of scorable bands for each SCoT primer varied from 4 (SCoT 9) to 7 (SCoT 18) (Table 2).

Table 2. The level of polymorphism detected with SCoT primers in *Stevia rebaudiana* shoots grown *in vitro* in two culture systems on MS medium supplemented with different concentrations of BA

Primer name	Size of bands (bp)	Number of monomorphyc bands
SCoT 9	300-800	4
SCoT 10	400-1000	6
SCoT 12	400-1800	6
SCoT 15	450-1300	5
SCoT 16	400-1700	6
SCoT 18	400-2000	7
SCoT 19	400-1500	6
SCoT 21	400-800	5
SCoT 22	400-1200	6
SCoT 23	400-3200	5

Each SCoT primer generated amplification products ranging in size from 300 bp (SCoT 9) to 3200 bp (SCoT 23). The results of this study showed that the primers produced amplification products which were monomorphic across all the analysed samples and no polymorphism was detected in in vitro shoots obtained in two culture systems on MS medium supplemented with different concentrations of BA (Figure 5).

Maintaining the clonal fidelity of in vitro-cultured plants is an important aspect of commercial micropropagation (Rai, 2023).

Evaluation of genetic fidelity of in vitro-grown plants is essential before their commercialization, especially in superior genotypes in which the regenerated plants are expected to be true-to-type with their mother plants (Bairu et al. 2011; Krishna et al. 2016; Arora et al. 2022). In the last decade, SCoT markers either alone or in combination with other traditional molecular markers such as RAPD and ISSR have been used to detect somaclonal variation or to assess genetic homogeneity in tissue culture-raised plants of many plant species (Rai, 2023).

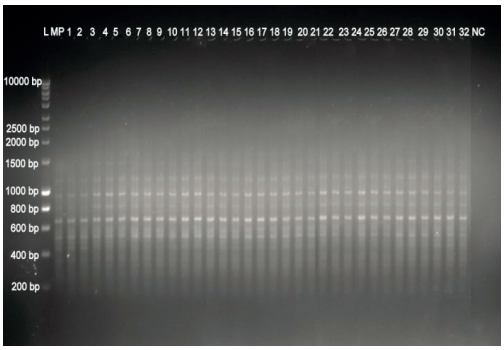


Figure 5. The SCoT profiles of the mother plant and *in vitro* shoots obtained in two culture systems on MS medium supplemented with different concentrations of BA generated by primer SCoT 18. Lanes: MP – SCoT bands from the field-grown mother plant; 1-16 - SCoT bands from shoots grown on solid agar-gellified MS media supplemented with different concentrations of BA (4 shoots x 4 culture media variants); 17-32- SCoT bands from shoots grown on temporary immersion system in liquid MS media supplemented with different concentrations of BA (4 shoots x 4 culture media variants); L - molecular marker (1Kb, Invitrogen, USA); NC - sample controls without DNA

In addition, several DNA-based molecular markers such as RAPD and ISSR markers have been used to assess the genetic fidelity of *S. rebaudiana* of tissue culture-raised plants (Modi et al., 2012; Lata et al., 2013). To our knowledge, the present study is the first report confirming the genetic stability of micropropagated Stevia plants and their mother plant using SCoT markers.

CONCLUSIONS

The experiments presented in this study demonstrate that TIS can be used to increase in vitro biomass production of *S. rebaudiana*. Maximum biomass production (1396.72 ± 54.03 mg/inoculum) was recorded using medium MS with a concentration of 0.2 mg/L BA in TIS. Also, the water content of Stevia biomass tended to decrease in temporary immersion system (TIS) compared to solid agar-gelled MS media (SM).

The genetic uniformity of the *in vitro*-grown shoots with their mother plant was confirmed in both culture systems using SCoT molecular

markers, thus revealing the sustainability of the culture systems for *in vitro* biomass production of *S. rebaudiana*.

ACKNOWLEDGEMENTS

This research was funded by Romanian Ministry of Agriculture, contract ADER 5.2.3/2023 and National Research Development Projects to finance excellence (PFE)-14/2022-2024 granted by the Romanian Ministry of Research and Innovation.

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