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INFLUENCE OF MEDIUM TYPE AND CULTURE METHOD ON THE MULTIPLICATION EFFICIENCY OF *Chlorophytum comosum* (Thunb.) JACQUES IN *in vitro* CONDITIONS

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ABSTRACT

In response to the challenges posed by modern plant micropropagation techniques, a promising technology for growing shoots temporarily immersed in nutrient solution (temporary immersion system, TIS) using SETIS™ bioreactors has been developed. In this experiment, the suitability of this technology for the propagation of *Chlorophytum comosum* (Thunb.) Jacques was assessed. *In vitro* culture was carried out using a conventional technique on solid and liquid media using the SETIS™ bioreactor. The study has demonstrated that the efficiency of liquid culture using the SETIS™ bioreactor is higher than the conventional culture. The highest multiplication coefficient, fresh weight of regenerants, and RGR index value in bioreactor cultures were recorded on Rugini OM medium. No statistically significant differences between MS medium and Rugini OM medium regarding shoot length and vigour with this culture method were found. When using the conventional way, better results can be achieved with MS medium.

Key words: *in vitro* propagation, MS medium, Rugini OM medium, SETIS™ bioreactor, temporary immersion system (TIS)

INTRODUCTION

Chlorophytum comosum (Thunb.) Jacques is a species native to South and Central Africa, whose cultivation has spread worldwide [Bjorå et al. 2008]. It found application in traditional Chinese medicine, where the tuberous roots extracted from it were used to treat burns, bone fractures, or bronchitis [Alisha et al. 2014]. It is widely cultivated in tropical regions of the world as a garden plant with ornamental functions. *C. comosum* (Thunb.) Jacques is also popular for its properties that remove toxic pollutants in the air [Hubai et al. 2023]. In addition, there is increasing research conducted on extracts obtained from its tubers, which show potential anti-proliferative activity against human cell lines: HeLa, HL-60, U937, CCRF-HSB-2 [Alisha et al. 2014]. The anti-cancer activity of tuber methanol extract was also confirmed by the SRB test in the HL60 leukaemia cell line [Deore et al. 2015]. The same authors, based on the conducted research, showed the presence of saponins as well as carbohydrates, flavonoids, steroids, triterpenoids, and saponin glycosides in the tubers of this plant. An ethanolic extract from the leaves of *C. comosum* (Thunb.) was also demonstrated to exhibit antibacterial activity against *Staphylococcus epidermidis* [Sabrina 2022]. Proceedings of modern biotechnology have introduced significant changes in *in vitro* plant tissue

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cultures. Conventional methods of plant tissue cultures are an excellent tool to support plant propagation, but they are time-consuming and not sufficiently effective for many species. Typically, closed containers with a solid medium are used to carry out this process. On the other hand, micropropagation conducted in bioreactors uses liquid culture media and can be implemented in various systems [Mamun et al. 2015]. One such system is the temporary immersion system (TIS) using the SETISTM bioreactor, in which a brief contact between the plant material and liquid medium occurs several times a day. TIS brings many benefits, including fast growth of cultures, better transfer of nutrients and gases, stable micro-environmental conditions, and also allows for automation of the cultivation process, thereby reducing labour costs and, consequently, the price of propagated plants [Eibl and Eibl 2008, Mirzabe et al. 2022]. Optimisation of medium composition, as well as control and monitoring of parameters such as immersion time, temperature, aeration, and the quality of the incoming light, can contribute to the increase in culture efficiency, which may affect the result of micropropagation or increased production of specific secondary metabolites [Yancheva et al. 2019]. Some disadvantages, such as plant malformations or somaclonal variation, could be overcome by selecting the most optimal bioreactor design for each propagated genotype [Steingroewer et al. 2013]. Continuous improvement of the TIS technique offers the possibility of rapid micropropagation of many plant species, including *C. comosum*. The main objective of this study was to present two methods of propagation of *C. comosum* (Thunb.) Jacques in *in vitro* cultures, as well as to test the effect of culture media on the efficiency of the micropropagation process. Given the increased interest in the therapeutic potential of the species under study, this work describes the use of a temporary immersion system using the SETIS™ bioreactor in the micropropagation of *C. comosum* for the first time.

MATERIAL AND METHOD

Plant material. The experimental material for establishing the *in vitro* culture consisted of plants of the species *C. comosum* (Thunb.) Jacques. The research was carried out in the in vitro culture laboratory of JMP Flowers Producers Group sp. z o.o. in Stężyca. The starting materials for establishing the culture were cuttings obtained from the flower stalk of *C. comosum*. Mother plants (Fig. 1A) from which cuttings were collected were placed for two weeks in an isolated room, during which they underwent three chemical treatments with a fungicidal solution (0.1% Previcur Bayer). The cuttings were cut from the mother plant, washed under running tap water, and then decontaminated. Decontamination stages are shown in Table 1.

Table 1. Explant decontamination procedure

Stage	Time	Decontamination solution
1.	20 minutes	sterile H_2O + drop of detergent
2.	1 minute	ethyl alcohol 70%
3.	30 minutes	H_2O_2 10% + drop of detergent
4.	2 × 10 minutes	sterile H_2O

Preparation of culture media. The experiment used media developed by Murashige and Skoog [1962] (MS Medium) and Rugini [1984] (Rugini Olive Medium, OM) supplemented with sucrose (30.0 g·dm^{-3}) and the following plant growth regulators (PGR's) – BA (6-benzylaminopurine – 1 mg·dm^{-3}), IBA (indole-3-butyric acid – 0.1 mg·dm^{-3}), GA3 (gibberellic acid – 0.1 mg·dm^{-3}). For the experiment, the pH of the medium was adjusted to 5.8 with 1 M NaOH and 1 M HCl before autoclaving at 121°C for 20 min. Two different culture systems were tested, one using solid media (conventional) solidified with agar (agar-agar Sigma-Aldrich – 6.0 g·dm^{-3}) in a plastic vessel and the other using liquid medium in the SETIS™ bioreactor (Vervit, Belgium). In the solid culture, 100 ml of medium was dispensed into each vessel, while in the SETIS™ bioreactor, 2000 ml of liquid medium was used. The media contained the same set of plant hormones but differed in the micro- and macronutrient contents.

Initiation and stabilisation of *in vitro* culture. Sterile explants (Fig. 1B) were placed on solid MS and Rugini OM media and transferred to the phytotron to

Fig. 1. Preparation of plant material for initiation of the culture: (A) – mother plant; (B) – primary explants; (C) – cultures on solid media; (D) – cultures in the SETISTM bioreactor

detect possible contamination. The culture stabilised within two weeks of placing the plants on the media, resulting in explants capable of propagation.

Plant propagation. Two weeks after initiating the culture, the explants were removed from the vessel, fresh weight (FW initial; g) and length (cm) were determined, and subsequently placed on fresh solid media and in a bioreactor for propagation (Fig. 1C, D). The explants were cultured in the phytotron under 23–25 µmol m^{-2} s^{-1} light provided by LED at a photoperiod of 16 h light/8 h dark and temperature of 21°C ±2°C. The immersion and aeration periods of the SETIS™ culture were controlled using air pumps and a timer placed in the growth chamber; the frequency of immersion was 5 min every 6 h (5 min/wet; 6 h/dry), with 15 min aeration every 4 h. A total of 50 explants were used for each of the four treatments – conventional system with solid MS or Rugini OM medium and – SETIS™ bioreactor system using liquid MS or Rugini OM medium. In the conventional system, five shots per plastic vessel were used, while 50 shots per SETIS™ bioreactor. The experiment was repeated three timesassessing a total of 600 explants.

Data collection and statistical analysis. To evaluate shoot multiplication, the following data were collected after 28 days (first subculture) and 56 days (second subculture) in both the conventional (Fig. 2A) and SETIS™ (Fig. 2B) systems: number of new shoots (pcs), shoot length (cm) and fresh weight (FW final; g). Shoot vigour was assessed on a scale of 1–8, according to Benelli and De Carlo [2018]. ANOVA was performed, and differences between the means were tested using Tukey's test, $P \leq 0.05$ (TIBCO Statistica 13.3 software package TIBCO Software Inc., 2020). The RGR index of shoot cultures was recorded after 28 and 56 days of culture and calculated as follows [Gatti et al. 2017]:

$$[\ln \text{FW final} - \ln \text{FW initial}] \times 100/\text{days of culture}$$

where: ln = natural logarithm.

RESULTS AND DISCUSSION

Various methods are used to achieve efficient plant regeneration under in vitro conditions during tissue culture. Many authors working on various plants, including Rosales et al. [2018] (*Stevia rebaudiana*),

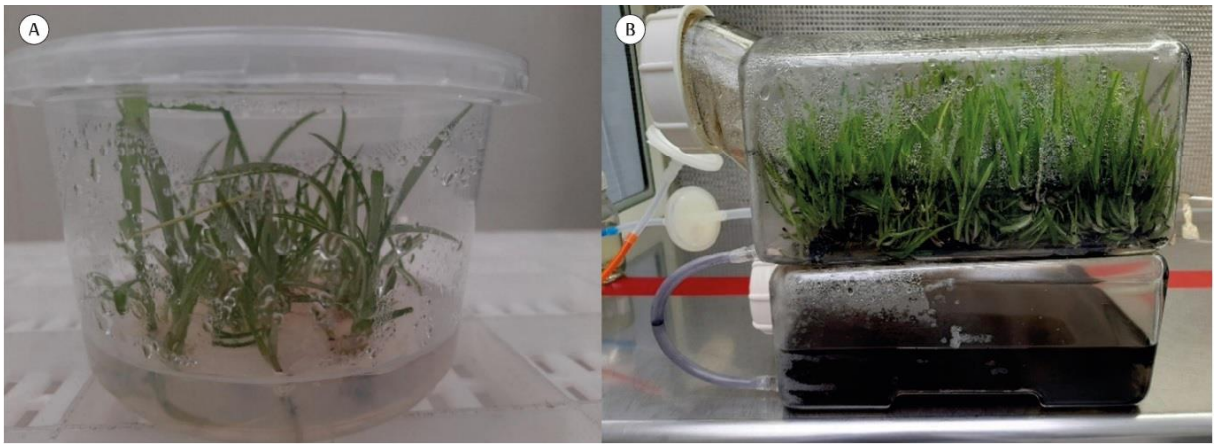

Fig. 2. *C. comosum* in *in vitro* conditions after the second subculture: (A) culture on solid medium, (B) culture in a bioreactor

Martínez-Estrada et al. [2019] (*Anthurium andreanum*), Marco et al. [2021] (*Vanilla planifolia*), and Vendrame et al. [2023] (*Brassavola nodosa*) have argued that the SETIS™ bioreactor is more efficient compared to conventional methods utilising solid or semi-solid media. It has been fully confirmed by the results of the present work on *C. comosum* (Figs. 3, 4, 5, 6). However, due to the lack of literature on in vitro culture using a bioreactor for the studied species, the findings of this study were compared with the results obtained in other species. In the current study, the number of propagated shoots in both the first and second subcultures (Tab. 2) was significantly higher in the bioreactor system. Furthermore, for this culture method, the Rugini OM medium proved to be more effective compared to the MS medium, with a greater impact observed in the first passage than in the second one. The analyses also revealed a different reaction of the explants to the applied medium during the culture, regardless of the culture method. On MS medium, the number of multiplied shoots increased markedly with the duration of the culture, amounting to 2.0 and 3.3 shoots per explant for the conventional method and 2.3 and 3.5 for the SETIS™ method in the first and second subcultures, respectively. However, on Rugini OM medium, the multiplication rate achieved after four weeks of culture remained practically unchanged during the next four weeks and was 1.4 and 1.6 shoots per explant for the conventional and 3.5 and 3.6 for the SETIS™ method, respectively. Other authors also indicated the possibility of obtaining a higher multiplication rate using the bioreactor. The use of PlantForm TIS bioreactor culture of myrtle (*Myrtus communis* L.) by Aka et al. [2020] allowed for approximately double the multiplication rate compared to a solid medium. Marco et al. [2021], using the SETIS™ temporary immersion system, achieved nearly a 3-fold higher multiplication rate of *Vanilla planifolia* compared to the culture conducted on a semi-solid medium. However, in the case of the *Spathiphyllum* 'Chico' genotype, Aka Kaçar et al. [2020] obtained a multiplication rate of 8.08 shoots per explant for the solid medium, and 5.05 shoots per explant for the bioreactor system, and considered both methods equally effective in the multiplication of this species. The number of shoots per explant obtained by Kaushal et al. [2021] during the optimisation of *C. borivilianum* micropropagation on a solid medium with the use of flower stalk as an explant was 15.27 ±1.14. Samantaray et al. [2009], in a micropropagation study involving *Chlorophytum* spp., used immature inflorescence as explants on an MS semi-solid medium and obtained a wide range of variation in the number of multiplied shoots. It ranged from 0.75 to 14.55 for *C. arundinaceum* and from 1.05 to 16.7 for *C. borivilianum*, depending on the type of explant and the concentration of growth regulators. Maghiar [2005], studying *C. comosum* using a mineral base medium Murashige-Skoog and apical explants drawn from stolons, achieved only a 20% increase in the multiplication rate after 30 days of culture and only on one of the 12 tested media. He notes, however, that as the culture continues, this value may increase to 80% after 90 days. The present data indicate that different species of *Chlorophytum*, such as *C. borivilianum*, and

Fig. 3. Shoots regenerated on the Rugini OM medium in the conventional (A) and SETISTM (B) systems after the first subculture

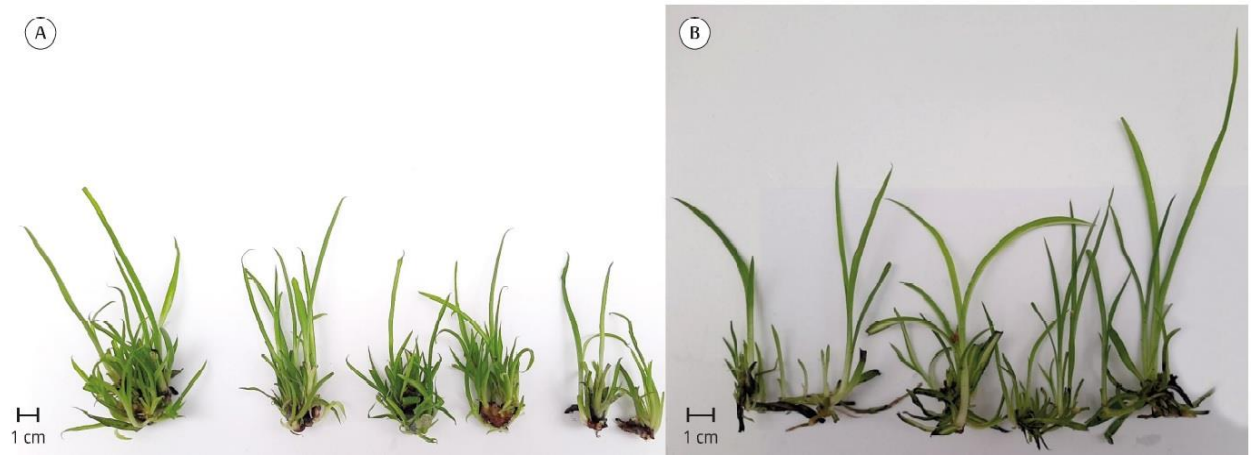

Fig. 4. Shoots regenerated on MS medium in the conventional (A) and SETISTM (B) systems after the first subculture

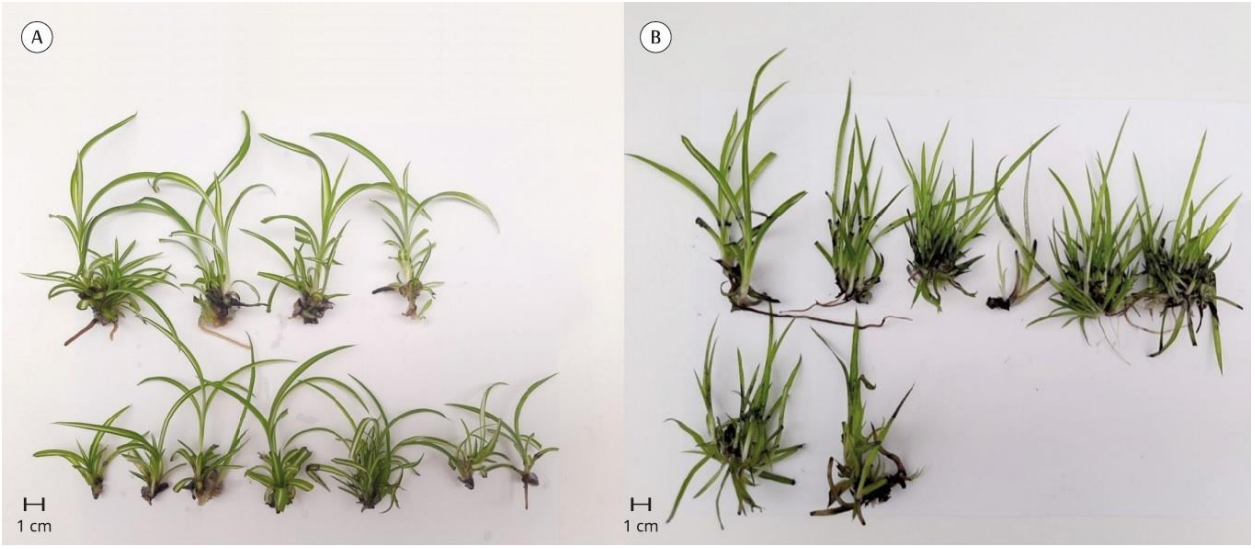

Fig. 5. Shoots regenerated on Rugini OM medium in the conventional (A) and SETISTM (B) systems after the second subculture

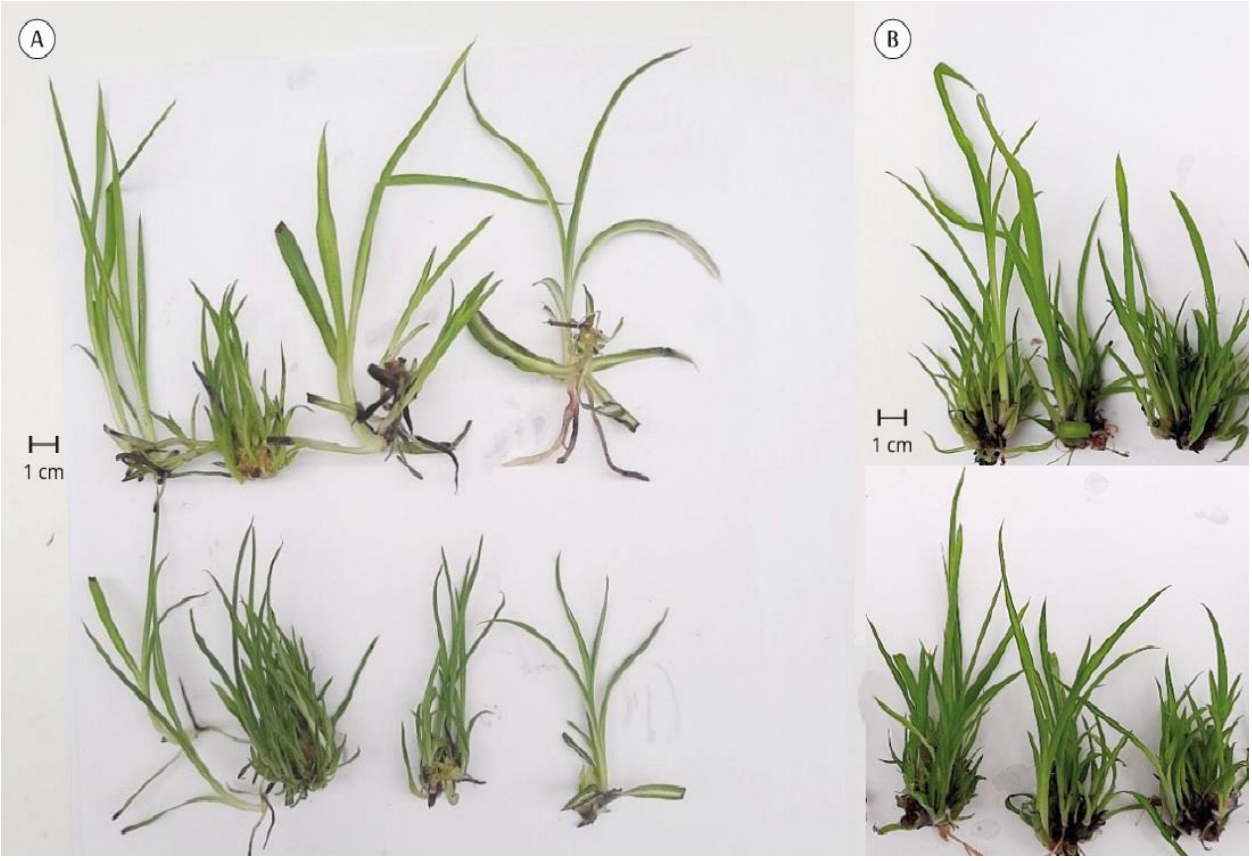

Fig. 6. Shoots regenerated on MS medium in the conventional (A) and SETISTM (B) systems after the second subculture

Table 2. Effects of multiplication using the conventional and SETIS™ systems after the first and second subcultures

Culture system	Medium	Number of shoots per explant (pcs.)		Shoot vigour (scale 1–8)	
		first subculture	second subculture	first subculture	second subculture
Conventional	MS Medium	2.0 b*	3.3 a	7.2a	6.4b
	Rugini OM	1.4 b	1.6 b	5.0b	6.0b
	Mean	1.7 B	2.4 B	6.1B	6.2B
SETIS™	MS Medium	2.3 ab	3.5 a	7.0a	7.5a
	Rugini OM	3.5 a	3.6 a	6.8a	7.3a
	Mean	2.9 A	3.5 A	6.9A	7.4A

*Different letters (a–b) in the columns indicate a significant difference between the experimental factors: conventional system × medium and the SETIS™ × medium. Different letters (A, B) in the columns indicate a significant difference between the conventional and SETIS™ systems

Table 3. Evaluation of shoot length in cultures in the conventional and SETIS™ systems after 4 and 8 weeks

Culture system	Medium	Shoot length (cm)	
		first subculture	second subculture
Conventional	MS medium	7.5 ab*	11.1 a
	Rugini OM	6.7 b	7.9 b
	Mean	7.1 B	9.1 B
SETIS™	MS medium	10.0 a	11.0 a
	Rugini OM	7.9 ab	11.6 a
	Mean	8.9 A	11.3 A

*Different letters (a–b) in the columns indicate a significant difference between the experimental factors: conventional system × medium and the SETIS™ × medium. Different letters (A, B) in the columns indicate a significant difference between the conventional and SETIS™ systems

C. arundianceum, using a similar type of explant, allow to obtain similar multiplication rates, much higher than those obtained in the present study on both solid media and in a bioreactor. In a recent study, Nath et al. [2023] report the possibility of obtaining much higher values of the multiplication rate, up to 8.33 shoots per explant, after testing BAP at a concentration of 2.0 mg in *C. comosum* var. *comosum using* MS solid medium. The observed variation in results may be due to the different types of culture media, different types of explant used in this study, and cultures with different phytohormone levels. It is consistent with the report of Bouzroud et al. [2022], who suggested that each species may require different concentrations of phytohormones to achieve the best multiplication results. The analysis of the vigour of regenerants primarily revealed a positive impact of bioreactor culture compared to culture on solid medium. However, plants regenerated on solid MS medium also exhibited high vigour. According to Gianguzzi et al. [2019], the TIS system provides better contact between the explant and the culture medium. It allows for more effective absorption of nutrients, which in turn leads to greater vigour [Benelli and De Carlo 2018]. Furthermore, according to Georgiev et al. [2014], the disadvantages of cultures conducted on solid media include high humidity, poor gas exchange, and accumulation of gases detrimental to growth.

Similarly to the number of multiplied shoots, their length was also significantly greater using the SETIS™ system compared to the conventional method at both culture analysis time points (Tab. 3). After completion of the first subculture, longer shoots were obtained on MS medium than Rugini OM in both the conventional and SETIS™ systems. However, at the end of the second subculture, the MS medium remained more effective in the conventional system, while better results were achieved on the Rugini OM medium in the SETIS™ system, with no statistically significant difference between these effects. Alister et al. [2005] indicated that the use of the temporary immersion bioreactor system RITA® resulted in enhanced shoot elongation in *Eucalyptus* clones compared to semi-solid media. On the other hand, Vendrame et al. [2023] research in *Brassavola nodosa* (L.) indicated that there were no significant differences in shoot length and leaf number between culture systems, including SETIS™ temporary immersion bioreactors and conventional semi-solid methods, which was consistent with the findings of Kaçar et al. [2020].

The observed positive effect of the SETIS™ method on the shoot multiplication rate and their length also resulted in higher weight of the obtained regenerants. The data clearly showed a significantly higher weight of regenerants obtained with the SETIS™ system compared to the conventional method in both subcultures (Fig. 7). Higher shoot weight was observed on Rugini OM medium in both the first and second passages compared to MS medium, but the differences were not statistically significant (Fig. 8). However, it should be noted that when using solidified media, higher shoot weight was observed in regenerants from MS medium compared to Rugini OM medium. On the other hand, the use of liquid media resulted in higher efficiency of OM medium, which was particularly evident in the se-

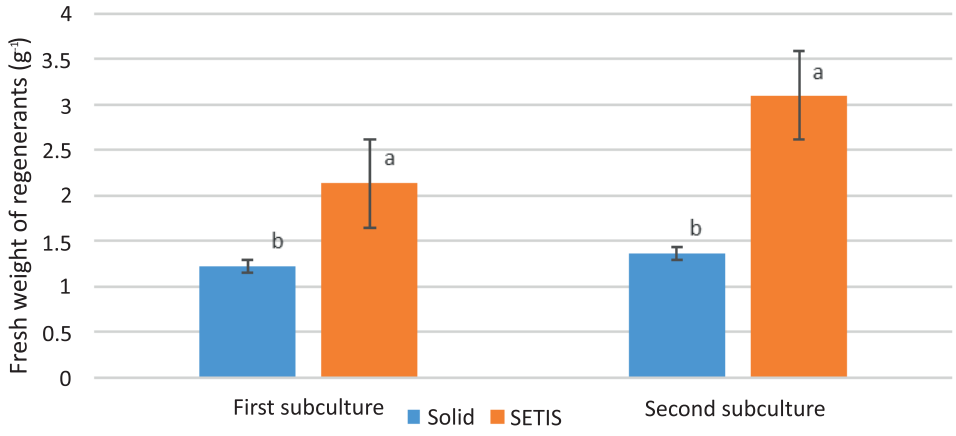

Fig. 7. Effect of culture method on fresh weight of regenerants at the two-time points analysed. Bars represent the standard error. Different letters above the bars indicate statistically significant differences (Tukey's test, $p \leq 0.05$)

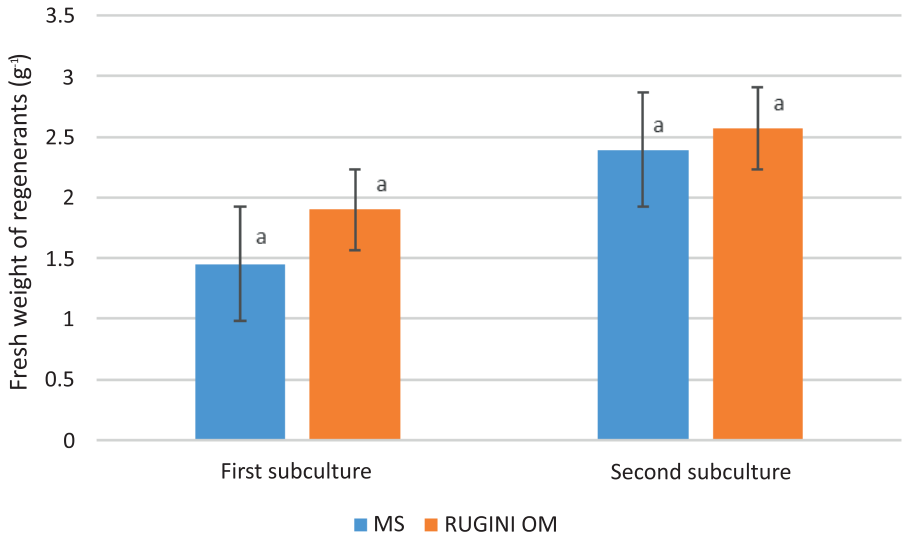

Fig. 8. Effect of culture method on fresh weight of regenerants at the two-time points analysed. Bars represent the standard error. Different letters above the bars indicate statistically significant differences (Tukey's test, $p \leq 0.05$)

cond subculture, where the fresh mass of regenerants reached an average of 4.29 g (Fig. 9). As reported by Jo et al. [2008], the most remarkable plantlet growth, expressed as higher fresh mass, was achieved using an immersion system with a net (raft method), which provided a constant supply of nutrients and aeration to the explants in comparison to the Ebb and flood system, which was not suitable for the propagation of *Alocasia amazonica*. Alister et al. [2005] have observed that the temporary immersion system provides a highly aerobic environment for plant growth due to forced ventilation through the vessel lid. However, they also pointed out that its efficiency was dependent on the immersion time, i.e. duration, frequency, or both. In this study, a 5-minute immersion time every 6 hours was used. According to Zhang et al. [2018], the optimal immersion system for multiplying *Bletilla striata* was a 3-minute immersion every 6 hours, while Regueira

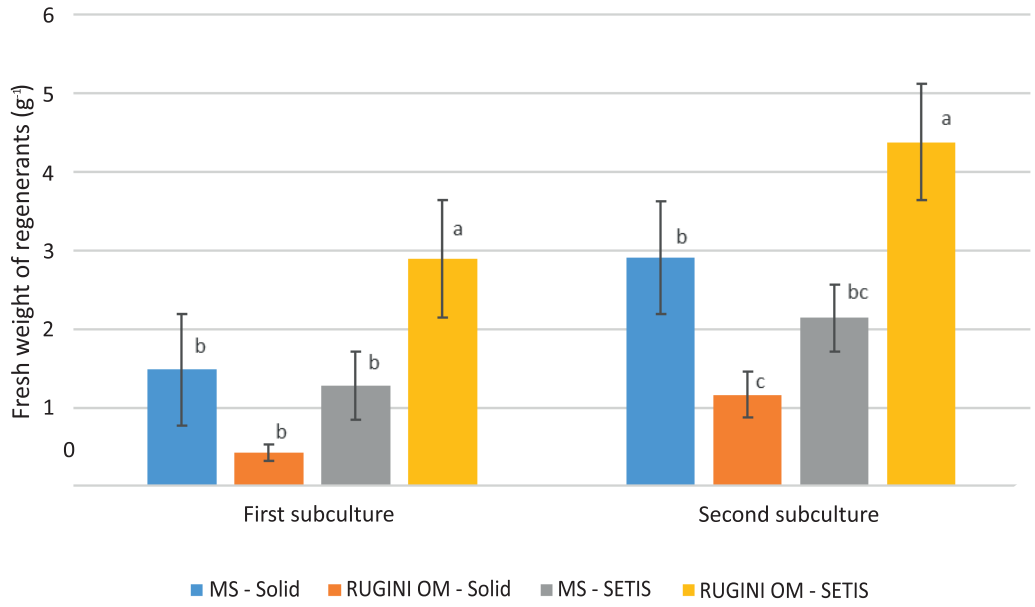

Fig. 9. Effect of medium and culture method on the weight of regenerants at two-time points analysed. Bars represent standard error. Different letters given above the bars indicate statistically significant differences (Tukey's test, $p \leq 0.05$)

Table 4. Relative growth rate (RGR) index of *C. comosum* shoots after the first and second subcultures in different culture systems

Culture system	Medium	RGR	
		first subculture	second subculture
Conventional	MS medium	4.31b*	5.19a
	Rugini OM	1.44c	3.39b
	Mean	2.80B	4.29A
SETIS™	MS medium	3.57b	3.50b
	Rugini OM	7.22a	5.87a
	Mean	5.40A	4.67A

*Different letters (a–c) in the columns indicate a significant difference between the experimental factors: conventional system × medium and the SETIS™ × medium. Different letters (A, B) in the columns indicate a significant difference between the conventional and SETIS™ system

et al. [2018] considered a 1-minute immersion every 6 hours to be sufficient for *Salix viminalis*. Similarly to the present study, Simsek et al. [2017] investigated the effects of different media on micropropagation of *Myrtus communis*. They obtained a higher mean shoot weight on MS solid medium compared to Rugini OM solid medium. In contrast, the results for *Pfaffia glomerata* were the opposite, with higher weights obtained on Rugini OM solid medium [Silva et al. 2019]. Thus, it should be recognised, as argued by Gianguzzi et al. [2019], that it is impossible to develop a universal nutrient solution composition for all species. Therefore, it is necessary to optimise a micropropagation protocol specific to the studied genotype.

The highest RGR index was observed in the bioreactor culture on Rugini OM medium in both the first and second passage (Tab. 4). The lowest values of this parameter were observed for the same medium variant in solid form at both culture evaluation time points. It clearly indicated the high efficiency of this medium in

the bioreactor technique. Similar results were obtained by Gianguzzi et al. [2019], who conducted a culture of *Capparis spinosa* using a TIS system while pointing out the need to optimise parameters that have a decisive impact on culture efficiency. The results obtained by Benelli and De Carlo [2018] for *Olea europea* also confirmed the increase of the RGR index when applying a liquid culture in a PlantForm bioreactor. This type of bioreactor also promoted biomass production more efficiently than solid media in the case of *Quercus robur* [Gatti et al. 2016]. The above reports suggest, and the results obtained in this study confirm, that the temporary immersion system, in which explants are periodically immersed in a liquid medium, significantly improves the efficiency of in vitro culture of *C. comosum.*

CONCLUSION

The present study applied the SETIS™ bioreactor system, and this is the first report on its potential use for the micropropagation of *C. comosum* (Thunb.) Jacques. Based on the results, it can be concluded that the SETIS™ bioreactor system demonstrates high suitability for the micropropagation of the investigated species, significantly surpassing the conventional system in terms of efficiency. It is reflected in the higher number of shoots per explant, their length, and the RGR index. In this system, a slightly higher number of new shoots was obtained on Rugini OM medium; however, the differences compared to the MS medium were statistically insignificant. Nevertheless, due to the higher fresh weight and vigour of shoots obtained on this medium, the acclimatisation of the obtained plants to *ex vitro* conditions will be more effective. Therefore, it can be concluded that the use of the SETIS™ bioreactor system in the micropropagation process will improve *in vitro* plant quality. The MS medium will be more suitable for the conventional system of propagating *C. comosum* (Thunb.) Jacques under *in vitro* conditions. Taking into account the obtained results, in order to increase the multiplication effect in SETIS™ bioreactor, further research should focus on optimising the composition of the culture medium, in particular with regard to the type and concentration of PGRs. We also suggest considering having more than two subcultures.

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