

Effect of a double phase culture system and activated charcoal on *in vitro* propagation of *Malus sylvestris* (L.) Mill.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: The effectiveness of a double phase (solid/liquid) culture system (DPS) in comparison to a conventional (solid) system (CS) as well as, the role of various concentrations of activated charcoal in both systems on the enhancement of micropropagation of *Malus sylvestris* (L.) Mill. were investigated. In this study, lateral shoots were used as primary explants and a comparison for shoots regeneration and rooting abilities was assessed between DPS and CS micropropagation systems. Also, the effect of activated charcoal concentration (0, 250, 500, 1000 mg l⁻¹) during rooting stage was evaluated for both micropropagation systems. All assessed biometric parameters were higher in the DPS propagation system. The addition of activated charcoal induced effectively rhizogenesis in both systems, whereas the highest value of roots length (13.16 cm) was in the DPS system supplemented with activated charcoal at 500 mg l⁻¹. The DPS culture system represents a promising low-cost and time-saving technique which may improve micropropagation efficiency in producing a large quantity of homogenous wild apple plants.

1. Introduction

Malus sylvestris (L.) Mill. represents an autochthonous species of Albania. This distinct species is of great importance not only as a primary wild relative and potential gene donor for the domesticated apple (Stephan *et al.*, 2003), but also for its medicinal values (Stojiljković *et al.*, 2016). For years in Albania, no accurate inventory has been conducted for the vulnerable populations of this species which is classified in the category of threatened species, and maybe soon, it will be at possible risk of extinction (FAO, 2015; Gixhari and Ramadani, 2016). In Europe also, there is a lack of information regarding its geographical distribution and for this reason, in The IUCN Red List of Threatened Species (IUCN, 2019), it is

included in the category of 'data deficient' species. Beside this, it is also reported as endangered in some European countries (Larsen *et al.*, 2006; Wagner *et al.*, 2014). The specific need for agricultural yield is one of the key reasons for the development of tissue culture technology worldwide. Indeed, plant micropropagation is an efficient method of propagating disease-free, genetically uniform and massive amounts of plants under *in vitro* conditions (Gupta *et al.*, 2020). Over the years, the number of plant species which have been clonally propagated through tissue culture was increased and the most commercially important species have been studied. *In vitro* propagation techniques have found wide use because of their effectiveness in terms of the high-quality product obtained and of reduced cost (Jain and Ishii, 2003; Debnath *et al.*, 2006; Damiano *et al.*, 2008; Lambardi *et al.*, 2013). Clonal propagation creates the possibility of obtaining a large quantity of homogeneous plant material, which can be conserved for short/mid-term periods through minimal growth methods, or long-term period, through cryopreservation (Kameswara, 2004; Day and Stacey, 2007; Benelli *et al.*, 2012). For all these advantages, and also because of the current situation on the geographical distribution and importance of *M. sylvestris*, it is of strategic importance to develop an effective micropropagation protocol, to obtain significant numbers of clonal plantlets which can be used for *ex situ* conservation strategies or other purposes.

In all micropropagation methods, the main goal is to optimize a successful protocol that ensures a rapid clonal propagation and results also as a time-saving technique (Lambardi *et al.*, 2013). In most reports, the protocols implemented for *Malus* sp. micropropagation are based on conventional micropropagation systems in semisolid culture media which typically include explants inoculation/proliferation, subculture, and rooting steps. Several authors mentioned the effective stabilization of wild apple micropropagation using conventional micropropagation system consisting in a monophasic/agarized medium (Modgil *et al.*, 1999; Boudabous *et al.*, 2010; Dobránszki *et al.*, 2011; Kereša *et al.*, 2012; Zhang *et al.*, 2020). But Teixeira da Silva *et al.* (2019), in a review regarding tissue culture of *Malus* sp., mentioned that most reports aimed to find alternative gelling agents other than agar, in order to reduce costs.

Although agarized media are successfully used for plant micropropagation, nowadays it has become absolutely important to improve the productivity and

uniformity of valuable vegetal materials with economic values by reducing the cost of production, space, time or, optimizing other issues related to micropropagation coefficient, rooting index, etc. In addition to solid media, several techniques have been successfully practiced for the micropropagation of economically important plants such as 1) the use of liquid cultures for the micropropagation of two apple rootstocks (Mehta *et al.*, 2014), pineapple (Dal Vesco *et al.*, 2001) and *Dioscorea* sp. (Jova *et al.*, 2011); 2) the use of continuous immersion bioreactors for apple rootstock (Chakrabarty *et al.*, 2003), eucalyptus (Mendonça *et al.*, 2016), chestnut (Vidal *et al.*, 2017), hybrid chestnut (Cuenca *et al.*, 2017); 3) the use of temporary immersion bioreactors for wild apple (Sota *et al.*, 2021), apple rootstocks (Chakrabarty *et al.*, 2003; Zhu *et al.*, 2005), and oak (Gatti *et al.*, 2017).

An alternative for improving *in vitro* micropropagation protocols is the use of double-phase nutrient media (DPS). In this method, the explants are fixed in a solid medium, while the liquid medium is periodically added during the culture, therefore eliminating the need for subcultures. In this way, the propagation costs and the chances of contamination are reduced (Senapati, 2015). There are very few reports on the use of double-phase media for *in vitro* propagation of plants (Scherwinski-Pereira *et al.*, 2012; Lopez and Suarez, 2018). In most reports, the same culture medium was used in solid and liquid phases (except agar presence) to increase the plantlets' mass production during the cultures. But it would also be useful and interesting to test various media for propagation and rooting in the same culture container. In this case, the solid phase would have a hormonal content effective for rooting induction, while the liquid one would have a hormonal content to induce lateral shoots development. Furthermore, the addition of activated charcoal (AC) in the culture media enhances *in vitro* rooting induction/development in some fruit-trees species (Wang and Huang, 1976; Thomas, 2008), and also acts as an adsorbent of phenolic compounds to avoid oxidation phenomena (Boudabous *et al.*, 2010; Shinde *et al.*, 2010).

This study aimed to evaluate the efficiency of DPS (solid/liquid culture system) in comparison with the conventional CS (solid) propagation system for improving *in vitro* shoots regeneration of *Malus sylvestris* (L) Mill. In addition, the effect of various concentrations of AC in both systems on rooting abilities was evaluated.

2. Materials and Methods

Plant material and micropropagation systems

Axillary buds of wild apple [*M. sylvestris* (L.) Mill.] were excised from scions of the population of Maminas at Durrës County in western Albania and were used as initial explants. The explants were disinfected with 70% ethanol for 3 min, followed by the treatments with 0.2% of 50% carbendazim (Bavistine) for 7 min and 0.01% HgCl₂ for 10 min., and multiple rinses with sterile distilled water were performed.

In this research, two micropropagation systems for *in vitro* regeneration of *M. sylvestris* plantlets were compared:

- *Conventional micropropagation system* (CS), consisting in the explants culture on solid medium (monophase system);
- *Double phase system* (DPS), consisting in the explants culture on solid medium plus a liquid phase at the same time.

For DPS micropropagation systems, the liquid medium was added every week in the culture vessels, specifically 1 ml in the test tubes (proliferation stage) and 3 ml in Erlenmeyer flasks (rooting stage). All procedures were performed under aseptic conditions.

Media composition and culture conditions

In vitro shoots proliferation. MS medium (Murashige and Skoog, 1962) was used, supplemented with 1 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.1 mg l⁻¹ α-naphthalenacetic acid (NAA) for both liquid and solid phase.

Rhizogenesis induction. The liquid phase was the same as in the proliferation stage, while for both systems under study the solid medium was supplemented with 1 mg l⁻¹ NAA and different concentrations (0, 250, 500, 1000 mg l⁻¹) of activated charcoal (AC).

In all cases, in the medium was added sucrose at 3%, while, for solid medium preparation, 7 g l⁻¹ of agar (Sigma-Aldrich) was also supplemented. The pH was adjusted to 5.7 before medium autoclaving at 120°C for 20 min.

The cultures were maintained in the growth chamber at 25±2°C in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m⁻² s⁻¹.

Data elaboration

Leaves number, shoots number, shoots length, roots number and roots length, were evaluated after 42 days of culture for each micropropagation stage.

Experimental data were elaborated by Tukey-Kramer test, and the analysis of variance (ANOVA) with JMP 7.0 statistical software.

3. Results and Discussion

In vitro regeneration wild apple shoots in the DPS and CS systems

After 42 days of *in vitro* culture, the growth dynamic of wild apple explants in the CS and DPS micropropagation systems was evaluated. During proliferation, for both systems, the PGRs ratio was such that induced lateral shoots regeneration. The comparative growth dynamic between the two propagation systems for leaves number, shoots number and shoots length, is presented in the variability charts (Fig. 1), and it clearly shows that the micropropagation system highly affected growth parameters (Fig. 2 a-e). The contact of the explants with the liquid medium (Fig. 2 c), facilitated and increased the amount of nutrients absorbed by the explants while in the solid medium (Fig. 2 a; b), the solid consistency itself slowed down the rate of absorption.

In our findings, the number of leaves for shoot in both systems, was high, specifically 18.75 during the culture in the CS system, and 19.25 in the DPS system (Fig. 1 a).

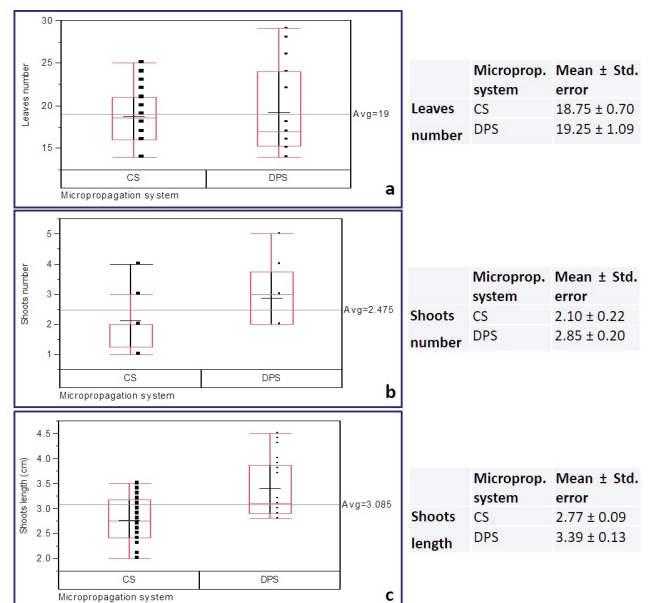


Fig. 1 - Variability chart: leaves number (a); shoots number (b); shoots length (cm) (c), in the DPS and CS micropropagation systems.

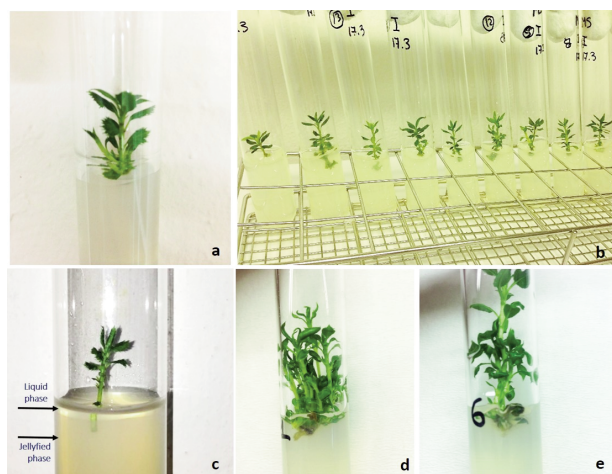


Fig. 2 - *In vitro* regeneration of wild apple shoots in CS micropropagation system (a, b) and in DPS micropropagation system (c, d, e).

DPS showed a higher efficiency for shoots number and shoot length, 2.85 and 3.39 cm, respectively, compared to CS, with 2.10 shoots number and 2.77 cm shoots length (Fig. 1 b; c). Overall, the explants grown in the CS propagation system, showed a lower regeneration potential for the monitored biometric parameters, which can be related to the absorption of substances from the nutrient medium. The greater thickness of the shoots cultivated in the DPS system was evident during the proliferation phase (Fig. 2 d; e).

The efficiency of the DPS system due to the presence of the liquid phase that enhanced the contact area of the explant with the nutrient medium, leading to an increase in the rate of diffusion, absorption and, continuous replacement of nutrients consumed over the days of culture is reported by various authors stressing the efficiency of the double phase micropropagation system (Moraes *et al.*, 2004; Pullman and Skryabina, 2007; Scherwinski-Pereira *et al.*, 2012; Dorić *et al.*, 2014; Senapati, 2015). In this regard, Rodriguez *et al.* (1991) found that the micropropagation rate of *Pyrus communis* L., especially in terms of axillary shoots formation, was higher when a liquid medium was added onto the jellified one. The superiority of DPS compared with CS system is also reported by Oliveira *et al.* (2013) on *in vitro* propagation of vanilla. In this species, axillary shoot multiplication was greatest in DPS, with an increase over 2.5-fold in comparison to the solid medium system, after 90 days of cultivation. Similarly, Couselo *et*

al. (2006) noted that the micropropagation rates of Albariño plants were significantly higher when culturing in a DPS system with the same concentration of BA (8.88 μ M) in both phases, in comparison to a monophasic one. The same trend on the efficiency of DPS cultivation system was reported for the micropropagation of arrow cane (Lopez and Suarez, 2018) ananas (Scherwinski-Pereira *et al.*, 2012), *Pyrus* sp. (Moraes *et al.*, 2004), and *Rauwolfia serpentine* (Senapati, 2015). On the contrary, Barceló-Muñoz *et al.* (1999) during micropropagation of avocado, reported that continuous culturing under DPS conditions induced succulence in shoot bases and hyperhydric of the cultures.

Together with the effectiveness for the growth dynamic, DPS has another advantage due to the periodic addition of the liquid medium. In this condition, the period from one subculture to the other can be longer than in a monophasic liquid or solid medium, where this period is up to 3-4 weeks, as reported also by Mahmud *et al.* (2014).

The CS system needs to change the medium in culture vessels after some time to avoid nutrient deficiencies. All this requires a series of laborious operations in terms of costs, both of hand labor and chemicals. Moreover, there is more working time in the cabinet laminar flow which creates possibilities for increasing the percentage of culture contamination. So, it can be said that in DPS propagation system, the cost of plant production is reduced. This finding is also accurately reported by Lopez and Suarez (2018) who calculated the costs of production per plant and found that, for arrow cane, the cultivation in the DPS system reduced the micropropagation costs by 20%. A similar estimation in terms of production cost was also realized by Senapati (2015) who found that DPS system was much more effective due to the costs reduction specifically with 33.36% on the nutrients used, 39.28% on the energy used and 33.33% on the labor costs. Optimization of such technique that, in addition to being low cost, also provides rapid *in vitro* plantlets regeneration, is of great interest for commercial use.

Biomass production during the rooting stage

After the proliferation stage, the shoots were transferred in DPS system and CS system for rooting. The first system had in the liquid phase the same type and concentration of PGRs of the proliferation stage, to promote the shoots development and at the same time, the solid phase was prepared to give

a rhizogenic induction. The medium of CS system was supplemented with rooting induction hormone (IAA). The biometric parameters were greatly affected, during this stage, not only by the propagation system but also by the concentration of activated charcoal in the media (Table 1).

not show significant differences between DPS and CS. This indicated that AC, with concentrations > than 250 mg l⁻¹, was the determining factor to improve rooting response. In this propagation stage, also DPS system resulted most advantaged in comparison to CS one.

Table 1 - The effect of the micropropagation system and activated charcoal concentration on roots and shoots development during the rooting phase of *M. sylvestris*

Propagation system	Activated charcoal (mg l ⁻¹)	Roots		Shoots	
		length (cm)	number	length (cm)	number
DPS	0	2.46 ± 0.16 e	2.66 ± 0.21 e	3.40 ± 0.15 c	3.53 ± 0.38 e
	250	5.23 ± 0.27 d	4.86 ± 0.31 d	4.61 ± 0.62 b	5.61 ± 0.36 b
	500	13.16 ± 0.67 a	8.73 ± 0.51 b	5.58 ± 0.17 a	7.46 ± 0.48 a
	1000	11.03 ± 0.30 b	9.80 ± 0.29 a	4.76 ± 0.17 b	5.40 ± 0.32 bc
CS	0	2.22 ± 0.11 e	2.46 ± 0.24 e	2.74 ± 0.11 d	2.40 ± 0.19 f
	250	4.81 ± 0.22 d	4.33 ± 0.28 d	3.18 ± 0.14 c	3.93 ± 0.31 de
	500	9.93 ± 0.33 c	7.33 ± 0.39 c	3.37 ± 0.12 c	4.60 ± 0.25 cd
	1000	10.40 ± 0.39 bc	7.53 ± 0.29 c	3.02 ± 0.12 cd	4.26 ± 0.30 de

Data represents mean ± standard error. Within each column, data followed by different letters are significantly different at P≤0.05 by Tukey-Kramer test.

Rhizogenesis induction

From a comparison between the two propagation systems for the same concentration of AC, in general, the best result was achieved in the DPS propagation system. Overall, rooting induction was highly affected by the concentration of AC in the culture media, and it was observed a positive correlation between the concentration of AC in the media and the rhizogenesis rate (Table 1; Fig. 3 a, b).

For roots length parameter, during the DPS culture, the best result (13.16 cm) was obtained at 500 mg l⁻¹ of AC concentration; while the higher roots number (9.80) was showed at 1000 mg l⁻¹ of AC concentration. In the CS, for both parameters, the best results were achieved at 1000 mg l⁻¹ of AC, respectively with 10.40 cm for roots length and 7.53 for roots number. There were no significant differences for the roots number parameter in CS propagation system between culture media supplemented with 1000 or 500 mg l⁻¹ of AC.

From an overall evaluation and comparison for the rooting response depending on both propagation system and AC concentration, the optimal condition for *in vitro* rooting of wild apple shoots was on DPS propagation system supplemented with 1000 mg l⁻¹ or 500 mg l⁻¹ of AC.

On culture medium supplemented with 250 mg l⁻¹ of AC or AC-free medium, the rooting response did

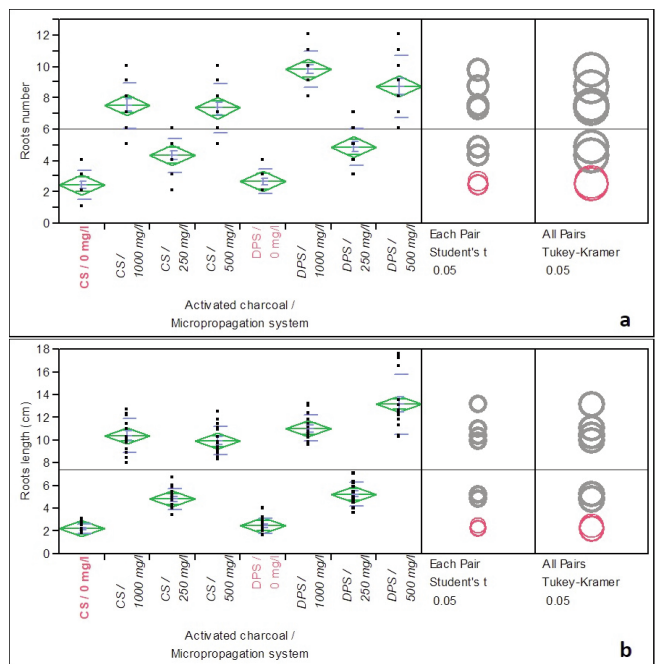


Fig. 3 - One-Way ANOVA analysis of roots number (a) and roots length (cm) (b) depending on the activated charcoal (AC) concentration and micropropagation system.

New shoots regeneration during the rooting stage

Since the liquid phase in the DPS had a hormonal ratio improving the development of lateral buds, at this stage also the response of plants in terms of mass formation of new shoots was evaluated. The

solid phase in both CS and DPS was optimized only for the induction of rhizogenesis.

As it can be seen from data presented (Table 1; Fig. 4 a, b and Fig. 5) the differences regarding shoots length and number between DPS and CS cultivation systems are highly significant.

The comparison between the DPS and CS propagation systems showed the considerable DPS efficiency for shoots length and shoots number. In particular, in AC-free media, the differences were highly significant, 3.40 cm (shoots length) and 3.53 (shoots number) for DPS in comparison with 2.74 cm and 2.40 shoots, respectively, in the CS. Obviously, DPS was more effective due to the supplementation of liquid phase with hormones responsible for shoots proliferation. Also, this proved further that the double phase system was effective for the growth of plantlets and at the same time for their rooting. During the rooting stage, the DPS allowed the simultaneous formation of roots and new shoots at a higher rate, thus leading to the possibility to reduce the micropropagation cost by combining in a single one the last stage of subculture and rooting.

The presence of AC supported also the development of additional shoots, in both propagation systems, but in DPS this effect was more pronounced and significant in comparison to CS. Moreover, the AC influence increased until 500 mg l⁻¹ concentration, and, indeed at this AC concentration, the DPS

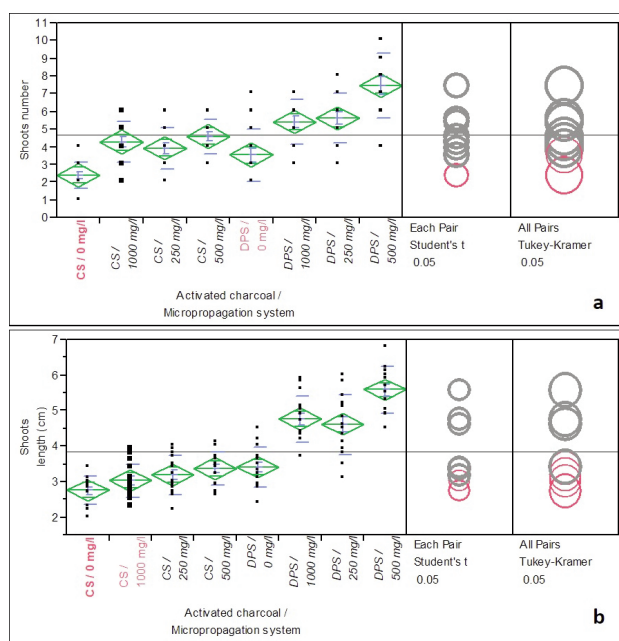


Fig. 4 - One-Way ANOVA analysis of shoots number (a) shoots length (cm) (b) depending on the activated charcoal (AC) concentration and micropropagation system.



Fig. 5 - Rhizogenesis induction and mass plantlets production during the rooting stage: culture in AC-free media (a); Wild apple cultures grown in various concentrations of AC (b); roots measurements (c); mass production of rooted plantlets (d).

showed the higher rate of shoots number (7.46) and shoots length (5.58 cm). The same tendency was observed even in CS where the concentration of AC at 500 mg l⁻¹ gave higher efficiency, with 3.37 cm of the shoots length and 4.60 for the shoots number.

The results indicated that AC concentration in the media was an important factor that highly affected micropropagation rate. Roots and new shoots formation responses were positively correlated and were dependent on the AC concentration in the nutrient media. In AC-free media, the roots formation was not at a high rate in comparison to the other AC concentrations.

Addition of activated charcoal in culture medium may affect *in vitro* plants growth, in terms of multiplication ratio, shoot elongation, rooting and embryogenesis (Pan and Van Staden, 2001; Thomas, 2008; Abdulwahed, 2013). Most publications have focused on the effects of activated charcoal on tissue response during *in vitro* propagation, and it was shown that its use may either promote or inhibit growth under *in vitro* conditions, depending on different factors. Boudabous *et al.* (2010) reported that the use of MS-half medium supplemented with 200 mg l⁻¹ of AC and 3.0 mg l⁻¹ of IBA, was highly effective on *in vitro* rhizogenesis of apple. On the other hand, Magyar-Tábori *et al.* (2002), in their study didn't find any favourable effect of activated charcoal on rooting characteristics of apple, but the plants originated from media that contained activated charcoal grew more vigorously during rooting and acclimatization.

In cotton (*Gossypium hirsutum*), the addition of AC in the medium enhanced shoots and roots induc-

tion as well as shoots length from split embryo axes as compared to MS basal medium (Hazra *et al.*, 2002). Also, Dev *et al.* (2015) mentioned that the use of 200 mg l⁻¹ of AC, significantly improved *in vitro* multiplication of some grape genotypes, meanwhile Hassan *et al.* (2011) found that the presence of AC in the medium enhanced microtuberization and *in vitro* regeneration of potato plantlets. Moreover, it is widely accepted that some of the beneficial effects of activated charcoal can be attributed to the removal of inhibitory substances from the nutrient medium. This phenomenon is mostly considerable for *M. sylvestris* *in vitro* propagation, because shoot explants of this plant species even after establishment and several subcultures shows browning at the shoot base (Sota *et al.*, 2021).

Roots grow according to negative phototropism and, in many cases, light is considered as a stress factor for roots induction (Silva-Navas *et al.*, 2015) and it is reported that the combination of darkness and exogenous auxins enhance rooting response (Monteuuis and Bon, 2000; López-Pérez and Martínez, 2015). In our research, the presence of AC in the nutrient medium created a state of darkness, thus enabling the formation of a well-developed roots system. This ensured a higher absorption of the nutrients, which can lead also to new shoots formation and development. Furthermore, the presence of a higher concentration of cytokinins vs. auxins in the liquid phase also was responsible for lateral shoots development. In this context, in our study, both the propagation system types and the presence of AC in the media, highly affected the micropropagation rate and the quality of regenerated plantlets.

Moreover, another advantage of DPS in comparison to CS system that can explain enhanced growth is the weekly addition of the liquid media into the vessel during the culture period, since the plantlets did not suffer mineral deficiencies.

4. Conclusions

Malus sylvestris, wild apple, is a very important plant species and is properly considered a threatened one; in such situation, the constant optimization of the micropropagation protocols is a necessity. The specific type of propagation system plays a key role in enhancing growth parameters. In the present study, our findings sufficiently indicated that the use of the DPS propagation system in comparison to CS

one was highly effective during *in vitro* shoots' regeneration and rooting stage. Moreover, the addition of activated charcoal for rooting induction obviously improved not only roots formation but also the growth of wild apple shoots. Among various concentrations of AC examined, the addition of 500 mg l⁻¹ AC in culture medium was found to be the best concentration for this process. The obtained results demonstrated a direct correlation between the propagation system used and the concentration of AC in the culture media. The application of DPS propagation system for *in vitro* propagation of wild apple, therefore represents more time-saving and a low-cost technique in comparison to the CS. These findings could provide a platform for progressively improving the clonal propagation of wild apple plantlets grown under *in vitro* conditions, leading so to the possibility for the effective use of these plantlets for its *ex situ* conservation or other scientific or practical purposes.

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