https://doi.org/10.17221/56/2023-JFS

Reducing costs, improving profits: A low-cost culture media for woody plants micropropagation

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Citation: Castro-Camba R., Vielba J.M., Covelo P., Vidal N., Sánchez C. (2023): Reducing costs, improving profits: A low-cost culture media for woody plants micropropagation. J. For. Sci., 69: 377–388.

Abstract: Micropropagation has enormous potential in the forestry industry for the mass production of elite genotypes. A limiting factor that hinders a wider adoption of this technique is its high associated cost, dissuading small producers and laboratories from its use. Thus, it is important to develop low-cost protocols to increase the competitiveness of micropropagation. The goal of the present study was to elaborate a low-cost culture medium that would allow plant growth without diminishing plant quality and production. The replacement of laboratory sucrose with commercial sugar as a carbon source, and laboratory agar with locally produced agar as a gelling agent was tested. To validate the effects of the medium for woody species micropropagation, two relevant forest species, chestnut and birch, were used and several phenotypic characteristics were recorded. Our data indicated that the substitutions made in the media composition did not alter growth or rooting parameters. Substitution of laboratory sucrose and Bacto agar by table sugar and local commercial agar provides a valuable alternative for forest species micropropagation and for increasing their potential profitability.

Keywords: birch; carbon source; chestnut; gelling agent; microshoots; woody species

The European sweet chestnut (*Castanea sativa* Mill.) is a woody plant species widely distributed across the Mediterranean basin since the Last Glacial Maximum, during which the three large Mediterranean peninsulas (Balkan, Italian, and Iberian), the Atlantic coast of France and Spain, and the southern coast of the Black Sea acted as refugia for this species (Roces-Díaz et al. 2018). Traditional management of chestnuts has been related to a higher plant species richness (Guitián et al. 2012) and to an increase in the number of bird species (Morelli et al. 2019), among other ecological parameters. In addition to contributing to mitigating climate change through carbon sequestration (Castaño-Santamaría et al. 2013), chestnut is also a highly profitable species (Martín et al. 2012). In fact, chestnut production has an increasing economic importance (Braga et al. 2015), with the main purpose of chestnut plantations being timber production (Carbone et al. 2020). However, fruit production is also a major traditional objective of chestnut cultivation, and new

Supported by Xunta de Galicia (Spain) through the projects IN607A and 'Contrato Programa' 2021 (AGI/CSIC I + D + I 2021, Ref-ACAM 20210200033).

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https://doi.org/10.17221/56/2023-JFS

advances are allowing to obtain bioactive compounds from chestnut shells, burs and leaves (Vella et al. 2018). Therefore, sustained and low-cost mass propagation of elite or plus genotypes could provide significant advantages for the chestnut-related industry.

The European white birch (*Betula pubescens*) is a short-lived tree species broadly distributed in northern and eastern Europe (Beck et al. 2016). The wood of *B. pubescens* is a very cost-effective source of pulpwood and fuel wood (Hynynen et al. 2010), although birch plantations have also been used for high-quality timber production (Cameron 1996). White birch has a high migration potential facilitating its adaptation ability to climate change conditions (Truong et al. 2007). Due to its resiliency, high productivity, wood properties and relations with the environment, *B. pubescens* is an interesting species for the forest-based industry in the context of climate change (Dubois et al. 2020). Moreover, birch also provides ecological benefits. The introduction of birches into spruce monocultures has a positive effect on avian biodiversity (Felton et al. 2011). On the other hand, birch mycorrhization is a promising technique for soil phytoremediation due to its capacity to sequestrate Mn and Zn from contaminated soils (Fernández-Fuego et al. 2017).

The potential economic market of both species, besides their biological and ecological values, demands the adoption of cost-effective methods for the mass propagation of disease-resistance and highly productive trees. *In vitro* culture techniques provide alternative tools to conventional methods of vegetative propagation for large-scale production of plants as well as for *ex vitro* conservation and long-term preservation of valuable and endangered germplasm (Singh et al. 2014; Corredoira et al. 2017). Furthermore, *de novo* regeneration protocols can be developed *in vitro* for plant improvement by genetic transformation or genome editing (Maher et al. 2020; Che et al. 2022). Micropropagation allows the rapid production of a large number of genetically uniform plants from a chosen genotype throughout the year, in a short period of time and in a reduced space. This approach has been used for the large-scale production of forest trees (Giri et al. 2004). It has a clear potential in the management and quality of forestry plantations by the propagation of trees with desired traits, the so-called *plus* genotypes (Sedjo 2001).

One of the major limiting factors for the micropropagation of woody species is their recalcitrance to *in vitro* culture, particularly at the adult stage, which hampers the mass production of proven selected genotypes. In line with this, the age-related decline in adventitious rooting capacity is a major obstacle in the vegetative propagation of adult trees, preventing the regeneration of complete plants in rooting-recalcitrant species (Legué et al. 2014; Vielba et al. 2020). Therefore, the development of optimized protocols for multiplication and adventitious rooting is mandatory.

A detailed protocol for micropropagation of basal shoots from mature trees of European chestnut through proliferation of axillary buds in semisolid medium has been previously reported (Vieitez et al. 2007). However, morphogenetic responses are highly dependent on the chestnut species and genotypes (Vielba et al. 2020). For instance, in a recent report of the micropropagation of *C. sativa* Italian cultivars, the highest rooting rate was 35% (Pavese et al. 2022). Protocols for chestnut micropropagation in liquid medium by using temporary immersion systems (TIS) have been reported in chestnut species (Vidal et al. 2015; Cuenca et al. 2017; Gago et al. 2022). Systems of this sort reduce production costs by using agar-free and low-sucrose containing media, and have proven efficient for chestnut proliferation and rooting (Gago et al. 2022). However, the best rooting rates were achieved with the highest concentration of sucrose, regardless of the genotype. Furthermore, the implementation of TIS sometimes derives in a higher rate of explant hyperhydricity, besides the requirements for a more specific equipment needed for CO_2 enriched air supply. Micropropagation of birch species from juvenile and mature trees has been previously reported (Chalupa 1981; Welander 1993; Covelo et al. 2019). Micropropagated birch plantlets from mature trees were used in commercial production and tested in the field (Meier-Dinkel 1992; Jones et al. 1996; Viherä-Aarnio, Velling 2001), but for different reasons their commercial use ended unprofitable (Häggman et al. 2007).

Besides biological constraints, other economic reasons prevent the application of micropropagation techniques to a higher number of species. The high costs associated with *in vitro* culture techniques (Kodym, Zapata-Arias 2001), which include equipment, infrastructure, loss of plants during acclimatization to *ex vitro* conditions, etc.,

https://doi.org/10.17221/56/2023-JFS

discourage many companies and laboratories from using these methods to produce plants, particularly in developing countries. The main goal of commercial laboratories is to maximize benefits with minimal investment (at minimum cost). Therefore, a challenge for making profitable *in vitro* production of plants is to decrease their final cost, which will also improve the resilience of laboratories during economic recessions, when cuts in budgets are the rule in most countries (Pellens et al. 2018; Ahmad et al. 2022).

Although there are fixed expenses in micropropagation which are difficult to reduce, looking for more affordable consumable products could be an easy and fast strategy to improve the competitiveness of *in vitro* culture. Local available commercial agar (LC agar) and table/market sugar (commercial white sugar) can be used instead of Difco agar and laboratory sucrose (lab-sucrose), respectively, in order to validate their suitability as reagents in the semisolid media for proliferation and rooting of profitable woody species. Theoretically, commercial white sugar could be used as a carbon source without any negative effect on micropropagation since it has a content in pure sucrose equal to or higher than 99.5%, as defined by several European laws (Directive 2001/111/EC of the Council of the European Union of 20 December 2001; Regulation (EU) No. 1308/2013 of the European Parliament and of the Council of 17 December 2013). This replacement could reduce production costs, increasing the profitability of large-scale micropropagation and facilitating the use of *in vitro* culture techniques in companies and developing countries. Locally produced agar obtained from the waste of food industries, like those producing algae for human consumption, can lead to the harnessing of the complete product, reducing the generation of by-products, algae overexploitation and representing another source of profit for this industry. Moreover, the use of locally produced agar strengthens supply chains and allows more advantageous contracts with suppliers due to the lack of competitors.

In the present work, we have developed a low-cost culture medium which allows for chestnut and birch micropropagation and rooting without any loss in plant quality, making this technique more profitable for small to medium companies and developing countries, therefore improving economic, social and ecological benefits.

MATERIAL AND METHODS

Plant material and root induction. Stock shoot cultures of chestnut and birch were used in this study. Chestnut shoots were established *in vitro* from basal shoots of an 80-year-old field grown tree of chestnut *(Castanea sativa)* and cultured *in vitro* for more than 30 years. Chestnut shoots were routinely grown on GD medium (Gresshoff, Doy 1972) containing 0.075 mg·L^{-1} benzyladenine (BA), 7 g·L^{-1} Bacto agar and 30 g·L^{-1} purified sucrose (lab-sucrose). Healthy and well-formed shoots developed after a 4-week subculture cycle were either used for multiplication through the proliferation of axillary buds on fresh medium or for rooting experiments. For rooting experiments, the callus and basal leaves of shoots were removed and then shoots were placed for 5 days in root induction medium consisting of one-third strength GD macronutrients (1/3 GD), 30 g·L^{-1} sucrose, 7 g·L^{-1} Bacto agar, and 5 mg·L^{-1} indole-3-butyric acid (IBA). During this period, shoots were incubated in darkness at 24 °C, and at the end of the auxin induction period they were transferred to auxin-free medium (1/3 GD) under standard conditions for 25 days. In the case of birch (*Betula pubescens*), material derived from a monumental tree ('Piñoi') was used. Shoots were routinely cultured in MS (Murashige, Skoog 1962) medium with modified vitamins, supplemented with 10 mg·L^{-1} Fe-EDTA, 10 mg·L^{-1} ascorbic acid, 10 mg·L^{-1} citric acid, 1 mg·L^{-1} folic acid, 0.1 mg·L^{-1} naphthaleneacetic acid (NAA) and 0.4 mg·L^{-1} meta-topolin. At the end of the proliferation cycle, roots developed at the base of shoots, thus further auxin treatments for root induction were not performed in birch. In order to test the effects of the carbon source (sucrose or table sugar) and the gelling agent [Bacto agar or locally produced commercial (LC) agar], proliferation of shoots was performed in the culture medium containing (*i*) purified laboratory sucrose (lab-sucrose) purchased from Duchefa, and Bacto agar (CM medium); (*ii*) table/household sucrose and Bacto agar (M1 medium), (*iii*) lab-sucrose and LC agar (M2 medium), and (*iv*) table sucrose and LC agar (M3 medium). The composition of these media is shown in Table 1. The effects of the carbon source and gelling agent were also evaluated in the rooting media (RM) of chestnut. The agar and sucrose of the rooting media designated RM, R1, R2, and R3 correspond to those used in the MC, M1, M2, and M3 media, re-

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Table 1. Effect of the carbon source and gelling agent on the final cost of 1 L of media

Medium	Carbon source	Gelling agent	Cost (EUR)	Difference (%)
CM–RM	lab sucrose	Bacto agar	5.22	–
M1–R1	table sugar	Bacto agar	4.67	–10.54
M2–R2	lab sucrose	LC agar	1.50	–71.26
M3–R3	table sugar	LC agar	0.95	–81.80

LC – local commercial agar

spectively. The pH of the different media was adjusted to 5.6–5.7 and then autoclaved at 121 °C for 20 min.

Chestnut shoots of the clone P2 were cultured in test tubes (1 shoot per tube) with a subculture cycle of four weeks. For each treatment, there were 24 replicate tubes, and each experiment was repeated 6 times (144 shoots). Birch shoots were cultured in glass jars, seven explants per jar and with three replicates per treatment, and the experiment was repeated 3 times (63 shoots). They were subcultured every two months into fresh medium, leading to an 8-week proliferation cycle. In chestnut, rooting experiments were carried out in three glass jars with 6 explants per jar and treatment, and the experiment was repeated three times (6 explants × 3 jars × 3 repeats = 54 explants per treatment). The plants were kept in growth chambers at 25 °C and 80–90% relative humidity, under a 16-h photoperiod and with a light intensity of 90–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps.

At the end of the 4 or 8 week proliferation cycle, the number of new shoots, the length of the longest shoot and the number of new leaves were recorded. In the rooting experiments, the following parameters were evaluated at the end of the rooting period: the percentage of rooted shoots, the average root number per rooted explant and the length of the longest root. Normality of the data was tested by Shapiro-Wilk test of normality. Homogeneity of variance was analysed by Bartlett's test, when normality of the data was proved, and Levene's test, when normality was not detected. Then, the data were analysed by ANOVA or Kruskal-Wallis test, depending on the normality. All statistical analysis were performed in R software (4.2.2, 2022).

RESULTS

Culture media cost and potential savings. Total price per litre of each medium was calculated using the average price of different trading houses (Table 1). In the case of Bacto agar, the average cost was EUR 266.92 for a 454 g container according to the prices of Midland scientific, Bacto, Fischer Scientific and VWR. Locally available commercial agar cost was EUR 11.40 for a 200 g container. On the other hand, the average cost of sucrose was EUR 19.16 per 1 kg according to the prices of Duchefa, Bacto and Fischer Scientific. Locally available table sugar cost EUR 0.67 per kg. Both elements were the most expensive compounds of the culture medium. Furthermore, potential savings with the substitution of CM with any other of the three tested media were estimated. The final cost of media prepared with any of the alternative compounds tested in the present study was markedly reduced comparing with the standard media, with the greatest savings occurring when Bacto agar was replaced by LC agar. In M3, the medium in which Bacto agar as well as lab-sucrose were replaced by the locally available substitutes, the potential savings of total cost of the medium were almost 82% (Table 1).

Calculations were performed only considering carbon source and gelling agent for two reasons: other compounds are not replaceable in culture media composition and their cost can be considered as negligible (less than EUR 0.05 per L).

Effect of the carbon source and gelling agent on the proliferation of shoots. The suitability of LC agar as a gelling agent and table sugar as a carbon source for chestnut micropropagation was tested in this investigation. To assess the shoot performance at the end of the proliferation cycle, several parameters were evaluated, including the number of shoots, the length of the longest shoot and the number of new developed leaves. No significant differences ($P \leq 0.001$) were detected for any of the evaluated parameters between the different treatments. Average shoot number ranged from 1.89 ± 0.06 (CM medium) to 1.95 ± 0.36 (M2 medium; Figure 1A), whereas average shoot length ranged from 3.07 ± 0.49 cm (CM medium) to 3.11 ± 0.22 cm (M3; Figure 1B). Regarding the

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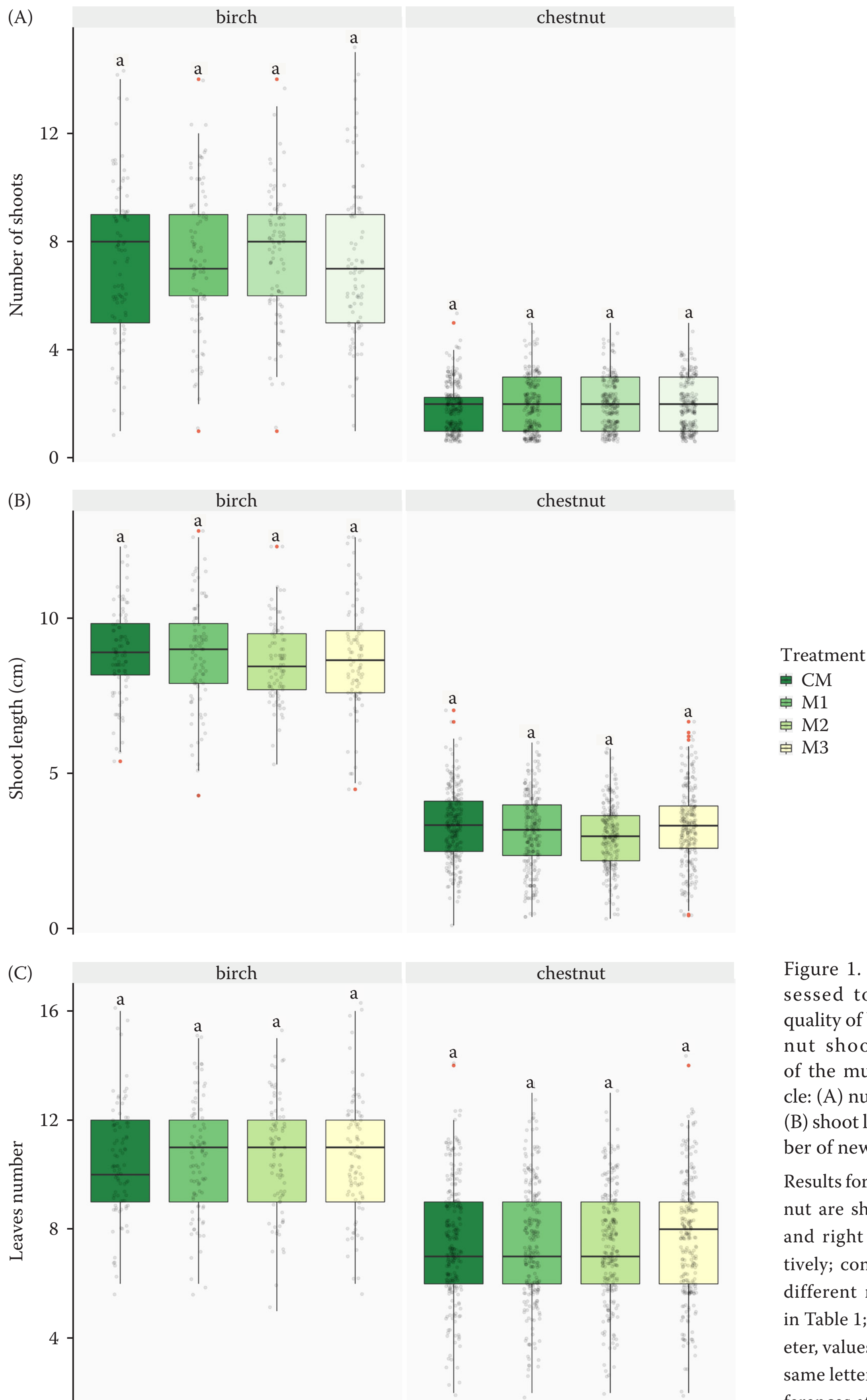

Figure 1. Parameters assessed to evaluate the quality of birch and chestnut shoots at the end of the multiplication cycle: (A) number of shoots; (B) shoot length; (C) number of new shoots

Results for birch and chestnut are shown on the left and right panels, respectively; composition of the different media is shown in Table 1; for each parameter, values followed by the same letter indicate no differences at $P \leq 0.001$

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average number of leaves, the data were also consistent through all the treatments, with the lowest number of leaves recorded in M2 (7.46 ± 0.31) and the highest number observed in M3 (7.65 ± 0.22; Figure 1C). In summary, CM provided the lowest number of shoots as well as the lowest size of tallest shoot, and M3 the highest number of leaves, although not statistical differences between the treatments were found.

Birch cultures were not affected by the carbon source and/or gelling agent used in culture media (Figure 1) since not statistically significant differences ($P \leq 0.001$) were detected for any of the evaluated parameters among treatments. Although the lowest number of shoots (7.19 ± 0.31) was achieved in the M3 medium, and the longest shoot (8.89 ± 0.36) was produced in the regular medium supplemented with lab agar and lab sucrose (CM), the greatest number of shoots (7.47 ± 0.25) and number of leaves (10.71 ± 0.33), were obtained in M2 and M3 medium, respectively (Figure 1A–C).

These results reinforce the hypothesis that the culture medium composition could be modified to reduce costs without any negative impact on the new shoots, maintaining their growth ability. Moreover, the overall phenotype of both chestnut and birch microshoots was not affected by the treatments, as they showed equal characteristics even when grown in different media (Figure 2).

Effect of the carbon source and gelling agent on adventitious rooting. The use of table sugar and LC agar instead of laboratory sucrose and agar in the chestnut rooting medium was investigated. Rooting percentage, the number of roots and the length of the longest root were used as parameters to determine the effect of the treatments on rooting rates (Figure 3).

Chestnut rooting rates were kept stable for all the tested treatments without significant differences ($P \leq 0.001$) for any of the evaluated parameters (Figure 3). The rooting percentage of chestnut varied between 95.83 ± 0.61 (RM and R2 medium) and 97.91 ± 0.53 (R1 and R3 medium; Figure 3A), whereas the average root number ranged from 4.14 ± 0.26 (R2 medium) to 4.33 ± 0.2 (R3 medium; Figure 3B). The longest root length varied from 4.23 ± 0.34 cm (R2 medium) to 4.55 ± 0.57 cm (R1 medium; Figure 3C). Based on the rooting

Figure 2. Shoots of chestnut (top) and birch (bottom) grown on a proliferation medium supplemented with: (A, E) laboratory sucrose and Bacto agar (CM); (B, F) table sugar and Bacto agar (M1); (C, G) laboratory sucrose and locally produced commercial agar (M2); (D, H) table sugar and locally produced commercial agar (M3)

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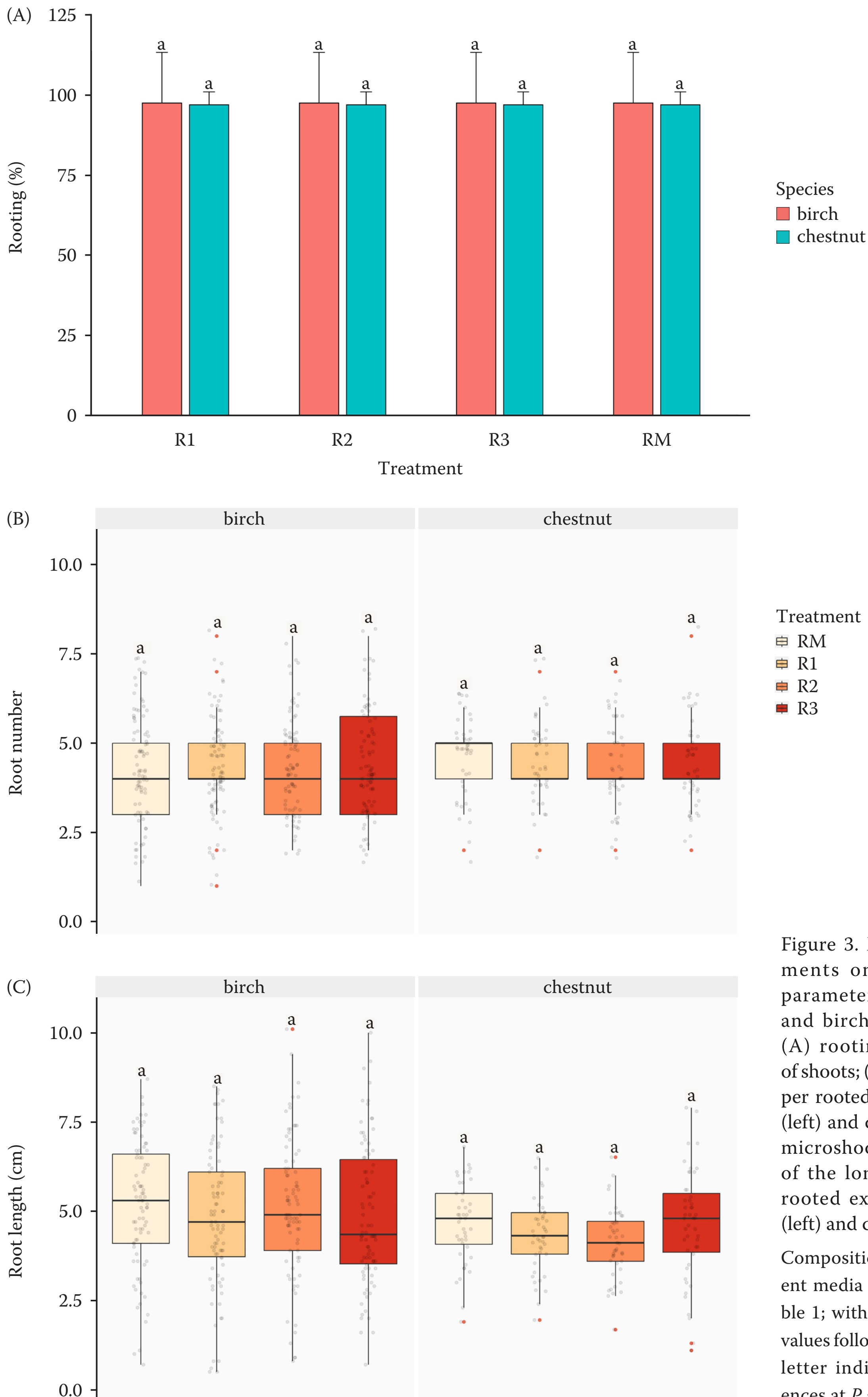

Figure 3. Effect of treatments on the rooting parameters of chestnut and birch microshoots: (A) rooting percentage of shoots; (B) root number per rooted shoot in birch (left) and chestnut (right) microshoots; (C) length of the longest root per rooted explant in birch (left) and chestnut (right)

Composition of the different media is shown in Table 1; within each species, values followed by the same letter indicate no differences at $P \leq 0.001$

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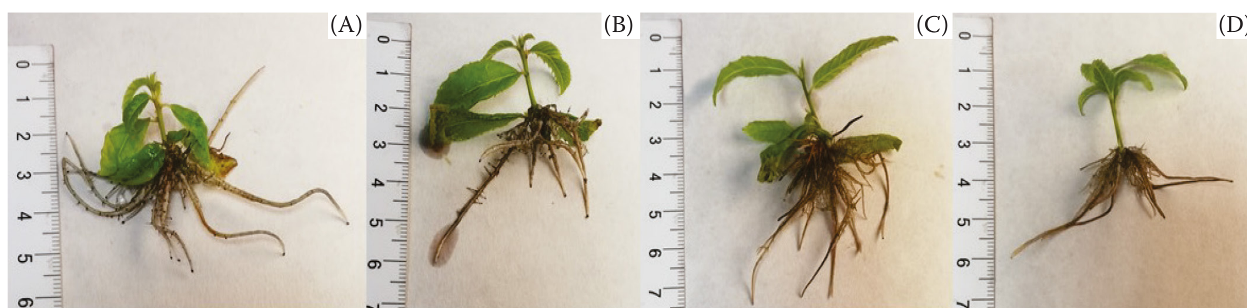

Figure 4. Rooted chestnut shoots on a medium supplemented with: (A) laboratory sucrose and Bacto agar (RM); (B) table sugar and Bacto agar (R1); (C) laboratory sucrose and locally produced commercial agar (R2); (D) table sugar and locally produced commercial agar (R3)

percentage and the root number, the medium supplemented with table sugar and LC agar provided the best results, whereas the longest root length was found in the medium containing table sugar and Bacto agar.

Birch rooting performance was evaluated at the end of the multiplication cycle, as these shoots develop adventitious roots spontaneously, probably due to the presence of NAA in the proliferation medium. Rooting percentages ranged from 96.59 ± 1.6 (CM and M2 media) to 97.91 ± 0.53 (M1 and M3 media; Figure 3A). The average root number varied between 4.19 ± 0.16 (M2 medium) and 4.33 ± 0.17 (M3 medium; Figure 3B). The values of the longest root length fluctuated between 4.69 ± 0.22 cm in M1 medium and 5.01 ± 0.31 cm in CM medium (Figure 3C). As shown in Figure 3, there are no statistically significant differences for any of the variables analysed ($P < 0.001$) in the tested rooting media.

Therefore, these results indicate that both laboratory-grade sucrose and agar could be substituted with table sugar and LC agar without any decrease in rooting percentage. As shown for chestnut (Figure 4) and birch (Figure 2E–H), the root system architecture was not affected by the different treatments.

DISCUSSION

Improving protocols for multiplication and rooting of *in vitro* cultured plants is a key step in research and micropropagation to produce low-cost plants on a large scale without compromising proliferation rates and plant quality, therefore increasing the profitability of mass production. To achieve this objective, we focused on the most expensive compounds of culture medium for which lower-cost substitutes are readily available in local markets: carbon source and gelling agent. Previous studies have shown a cost reduction that varied between 58.57%, when the laboratory agar was substituted by Isubgol and sucrose was substituted by market sugar (Agrawal et al. 2010), and 86.40% when Sago was used as gelling agent (Saraswathi et al. 2016). In our study, replacing LC sucrose with table sugar and using LC agar as gelling agent lead to significant cost savings (82%), thus achieving values close to the best alternatives previously published.

The substitution of laboratory sucrose as carbon source by commercial sugar had been previously reported in liquid medium in pineapple (Dutta et al. 2013), as well as in semi-solid media in crops like *Pogostemon cablin* (Swamy et al. 2010) and *Solanum tuberosum* (Kuria et al. 2008). However, our study is the first report on micropropagation of chestnut and birch that evaluates the effect of this switch on shoot proliferation and rooting rates. We found similar results to those obtained in previous studies in non-woody species (Demo et al. 2008; Swamy et al. 2010), with no substantial changes neither in micropropagation nor in rooting rates, maintaining explants quality and root architecture. Moreover, results found for rooting parameters in the woody species *Balanites aegyptica* and *Phyllantus emblica* (Gour, Kant 2011) also support our findings, showing that no major phenotypical effect is found when using commercial sugar.

In contrast to the easily replaceable carbon source, finding a gelling agent substitute is a more difficult task. However, there are multiple substances, particularly starch, which can be suitable substitutes for laboratory agar (Gordo et al. 2012). In our study, LC agar was used, instead of other more common substitutes such as guar gum or Isubgol, due to its low cost and availability. Moreover, in comparison

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to other potential substitutes, LC agar has a similar composition to laboratory agar, as it is also derived from the same class of algae (Rhodophyta). Studies performed in species like *Solanum tuberosum* (Kuria et al. 2008) or *Vanilla planifolia* (Mengesha et al. 2012), in which cassava and enset starch were used as gelling agents, or in *Dendrobium chrysotoxum* (Jain, Babbar 2005), *Isatis tinctorial* (Saglam, Cifici 2010) and *Nicotiana tabacum* (Ozel et al. 2008), where laboratory agar was substituted by Isubgol, showed that the presence of these compounds in the culture medium increased the number of shoots compared to the use of laboratory agar. Similar results were achieved in some woody plant species such as *Vaccinium corymbosum* (Clapa et al. 2008) or *Crataeva nurvala* (Jain, Babbar 2006), growing in culture media containing as gelling agents Isubgol and guar gum, respectively. While agar is mainly composed of a mixture of agarose and agaropectin, Isubgol shows the presence of other different polysaccharides, including D-xylose, aldobiuronic acid and L-arabinose (Babbar, Jain 1998). Whether this is the reason for the differences found when using Isubgol requires further research. However, other parameters, including the number of opened leaves, were analysed regarding to the gelling agent, without remarkable changes in response to the culture media (Agrawal et al. 2010; Ullah et al. 2015). On the other hand, other studies performed in *Faidherbia albida* and *Uapaca kirkiana* (Maliro, Lameck 2004) or in banana (Buah 2014; Saraswathi et al. 2016) have noted the positive effect of alternative gelling agents, such as cassava flour and sago, when they were combined with laboratory agar. In contrast, our results did not show any significant differences in any of the analysed parameters during the proliferation phase, thus confirming previous results in woody and non-woody species (Babbar, Jain 1998; Jain, Babbar 2006; Clapa et al. 2010; Raina, Babbar 2011).

In the present work, rooting ability and rooting rates of chestnut and birch were not affected by the presence of table sugar and/or LC agar in the culture medium. Similar findings were reported in non-woody species such as *Nicotiana tabacum* (Ozel et al. 2008), or woody species like *Syzygium cuminii* (Babbar, Jain 1998) or banana (Saraswathi et al. 2016). On the other hand, a positive effect of enset agar on the rooting percentage was observed in *Vainilla planifolia* (Mengesha et al. 2012). A beneficial effect of using low-cost compounds on the root length has been reported in *Curcuma longa* (Tyagi et al. 2007) as well as in woody plant species (Babbar et al. 2005), whereas the negative effect of using Isubgol on root length has also been reported (Ullah et al. 2015). In contrast, our results are consistent with earlier studies showing that root length can be maintained in a lower-cost culture medium (Ozel et al. 2008; Raina, Babbar 2011). Regarding the root number, a detrimental effect of table sugar on the micropropagation of *Solanum tuberosum* has been reported by Demo (Demo et al. 2008). On the other hand, an increase of the number of roots was observed in *Curcuma longa* when agar was complemented with cassava starch (Buah 2014) or completely substituted by Isubgol (Agrawal et al. 2008). In the chestnut and birch experiments, the root system was not affected by the presence of LC agar or table sugar in the culture media, in agreement with the results previously obtained in other species like *Nicotiana tabacum* (Ozel et al. 2008), *Curcuma longa* (Jain, Babbar 2006) or banana (Agrawal et al. 2010), therefore suggesting that the low-cost substitutes provide a valuable resource for the development of micropropagation protocols.

In conclusion, the substitution of sucrose and laboratory agar with white sugar and locally produced commercial agar allows for a 82% reduction in culture medium cost without any damages neither to the quality of the shoots nor to the root system development of chestnut and birch. The results obtained imply significant savings, which can lead to the improvement of the *in vitro* culture of chestnut and birch competitiveness and making the micropropagation of chestnut genotypes *plus* an actual profitable business. Thus, the expansion of micropropagation techniques in chestnut and birch can be used to support the present and future demand of these woody plant species with improved selected clones.

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Received: May 15, 2023
Accepted: July 7, 2023
Published online: August 7, 2023