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Assessment of mint (*Mentha* spp.) species for large-scale production of plantlets by micropropagation

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ABSTRACT. Species of the genus *Mentha* produce essential oils which are widely used in pharmaceutical and cosmetic industries. Current study evaluates the potential for *in vitro* propagation and estimates mass production of plantlets of *Mentha* species. Nine species (*M. piperita, M. suaveolens, M. canadensis, M. longiflora, M. aquatica, M. arvensis, Mentha x gracilis, M. gracilis* and *M. spicata*) were propagated with five successive 30-day subcultures in MS medium supplemented with NAA (0.05 μM) and BAP (4.4 μM). Shoots were rooted in MS with IBA, IAA or NAA (0.0; 0.25; 0.5; 2.5 or 5.0 μM). The rooted plantlets were finally acclimatized in a greenhouse. Studied species increased in multiplication rates between 4.2 and 9.0-fold per month. *M. piperita*, *M. longiflora*, *M. arvensis*, *M. x gracilis* and *M. gracilis* showed the greatest potential for plantlet production since the estimated production varied between 6,000 and 27,000 plantlets after five 30-days subcultures. The addition of auxin to the medium did not influence root induction. However, IAA at a concentration of 5 μM provided the best results for root length and fresh weight, with averages 11.1 cm and 0.16 g, respectively. Survival of plantlets reached 100% during the greenhouse acclimatization process.

Keywords: peppermint, medicinal plants, *in vitro* propagation.

Avaliação de espécies de menta (*Mentha* spp.) para a produção de mudas em larga escala por micropropagação

RESUMO. Espécies do gênero *Mentha* produzem óleos essenciais largamente usados na indústria farmacêutica e de cosméticos. O estudo avaliou o potencial de propagação *in vitro* e estimou a produção de mudas de espécies de menta. Nove espécies (*M. piperita, M. suaveolens, M. canadensis, M. longiflora, M. aquatica, M. arvensis, Mentha x gracilis, M. gracilis* e *M. spicata*) foram propagadas por até cinco sucessivos subcultivos de 30 dias em meio de MS adicionado de ANA (0,05 μM) e BAP (4,4 μM). Os brotos foram enraizados em meio de MS com AIB, AIA ou ANA (0,0; 0,25; 0,5; 2,5 ou 5,0 μM). Finalmente, as mudas foram aclimatizadas em casa de vegetação. As espécies estudadas apresentaram aumentos nas taxas de multiplicação, variando entre 4,2 e 9,0 vezes por mês. *M. piperita*, *M. longiflora*, *M. arvensis*, *M. x gracilis* e *M. gracilis* mostraram os melhores potenciais para propagação, uma vez que a produção variou entre 6.000 e 27.000 mudas após cinco subcultivos de 30 dias. A adição de auxina no meio não influenciou a indução de raízes. Entretanto, o AIA na concentração de 5 μM promoveu os melhores resultados quanto ao comprimento e massa fresca das raízes, com médias de 11,1 cm e 0,16 g, respectivamente. No processo de aclimatização houve 100% de sobrevivência das mudas.

Palavras-chave: hortelã, plantas medicinais, propagação *in vitro*.

Introduction

Mentha plants produce a large amount of essential oils of high commercial value, such as carvone, dihydrocarvone, menthone, menthol, pulegone, linalool and linalyl acetate. Biochemical and pharmacological studies have attributed antioxidant, anti-allergic, antiviral, antibacterial, antimicrobial and cancer-cell inhibiting effects to these compounds (McKAY; BLUMBERG, 2006).

Among the essential oils, menthol, produced in specialized glands in the leaves, stems and flowers of plants of the genus, is economically the most important of them all. The compound is renowned for its anesthetic and anti-inflammatory properties, widely used in the production of drugs used in cases of sore throats. Menthol is also used in the production of cosmetics, food and sanitary products (CHAKRABORTY; CHATTOPADHYAY, 2008).

Due to the increasing economic importance of essential oils derived from the species *Mentha*, associated with their great scientific interest (McKAY; BLUMBERG, 2006), the demand for raw material for producing these compounds is on the

increase. It is thus highly relevant that efficient methodologies be developed for large-scale production of *Mentha* spp. plantlets (MAITY et al., 2011).

However, production of *Mentha* species plantlets for the establishment of commercial plantations that use traditional farming methods has been hampered by the species high nutrient and water requirements, which are associated with several diseases faced by these cultures, such as infections caused by the pathogen *Verticillium dahliae* (POOVAIAH et al., 2006; WANG et al., 2009).

Micropropagation has emerged as an excellent alternative for the mass production of *Mentha* plantlets. This method can maximize the proliferation of shoots and buds by means of growing successive subcultures to obtain the largest number of high health and genetic quality of plantlets in the shortest time possible (BISWAS et al., 2014). When compared to traditional propagation methods, other advantages attributed to the micropropagation of plants are that it can be done in small physical spaces, at any time of the year (RAJA; AROCKIASAMY, 2008; WANG et al., 2009; MEHTA et al., 2012; SANTORO et al., 2013).

In the case of *M. viridis*, the micropropagation of nodal segments and shoot tips in an MS nutrient medium (MURASHIGE; SKOOG, 1962), supplemented with 6-benzylaminopurine (BAP), with a multiplication rate of about 40 buds per initial explant after 45 days of cultivation, demonstrated the high efficiency of the method in species of the *Mentha* genus (RAJA; AROCKIASAMY, 2008).

On the other hand, the micropropagation of nodal segments and shoot tips of *M. spicata*, also in an MS nutrient medium supplemented with BAP, yielded an average of only 1.2 shoots per explant after 30 days of culture (BISWAS et al., 2014). According to Silva et al. (2006) the effectiveness of *in vitro* cultivation of the *Mentha* genus may vary significantly among different species.

However, although there are many studies in the literature regarding the micropropagation of *Mentha* species (RAJA; AROCKIASAMY, 2008; VASILE et al., 2011; MAITY et al., 2011; MANIK et al., 2012; BISWAS et al., 2014), there have not yet been reports of studies comparing the propagation rates of these species or quantifying the potential for producing plantlets for each one of them.

Current study evaluates the *in vitro* behavior of nine mint species for large-scale production of plantlets by micropropagation.

Material and methods

Nodal segments approximately 1.0 cm long, containing two axillary buds from the species *Mentha piperita*, *M. suaveolens*, *M. canadensis*, *M. longiflora*, *M. aquatica*, *M. arvensis*, *Mentha* x *gracilis*, *M. gracilis* and *M. spicata* were used as starter explants. The source of the explants was obtained from the *in vitro* Germplasm Collection of Embrapa Recursos Genéticos e Biotecnologia (Embrapa Genetic Resources and Biotechnology), Brasilia, DF.

The *in vitro* growth of the *Mentha* species comprised inoculation of nodal segments in test tubes (25 x 150 mm), each containing 10 mL of culture medium, closed with plastic stoppers and sealed with transparent plastic film. The culture medium was composed of MS salts and vitamins (MURASHIGE; SKOOG, 1962), supplemented with 0.05 μM of NAA (naphthaleneacetic acid), 4.4 μM of BAP and 20 g L^{-1} sucrose, according to protocol by Biswas et al. (2014), modified. Prior to autoclaving at 12°C and at a pressure of 1.3 atm for 20 minutes, the culture medium was solidified using 2.5 g L^{-1} Phytagel, with pH adjusted to 5.7 ± 0.1.

The explants were cultivated using five successive 30-day subcultures. After each subculture, the average height (cm) and number of new shoots were evaluated, in addition to the multiplication rate of the explants. Multiplication rate was calculated as the average number of buds produced per bud initially inoculated, along each subculture. After the 5 subcultures, and based on the multiplication rates obtained for each one, the *in vitro* production of plantlets of the *Mentha* species was also estimated. The experimental design was completely randomized, composed of 20 repetitions per treatment, with each parcel consisting of an explant.

After five subcultures, shoots of the *M.* x *gracilis* and *M. arvensis*, selected at random, were used for the rooting experiments. Nodal segments, approximately 1.0 cm long and with two axillary buds, were vertically inoculated in 250-mL glass flasks containing 40 mL of MS medium, closed with plastic stoppers and sealed with transparent film. Concentrations of 0.0; 0.25; 0.5; 2.5 and 5.0 μM of IBA (indole butyric acid), IAA (indole acetic acid) or NAA were evaluated in this basic culture medium. The latter was solidified with 2.5 g L^{-1} Phytagel and pH was adjusted to 5.7 ± 0.1 before autoclaving.

After a period of 30 days, the variables number of roots, fresh weight (g) and length of the longest root (cm) were evaluated. The experimental design was completely random, consisting of four repetitions per treatment, each parcel being composed of 6 explants.

The maintenance of *in vitro* cultivated plant material was conducted in a growth chamber at a temperature of 25 ± 2°C, a photoperiod of 16 hours, and a light radiation of 40 μmol m^{-2} s^{-1} provided by daylight-type fluorescent lamps.

Acclimatization was accomplished by removing the plantlets from their respective cultivation flasks (treatment) and planting them in 200 mL plastic cups containing Bioplant® commercial substrate, after washing the roots with running water to remove residues of the culture medium. The plants were then kept in a greenhouse, where they remained for up to 30 days, when the plants´ survival rate was determined.

The greenhouse was covered by transparent polyethylene film (150 microns), with a relative humidity of 75 ± 5%, a temperature of 27± 4°C, a luminosity of 450-500 μmol m^{-2} s^{-1} and a photoperiod of 12 hours. The plants were watered every 6 hours for 15 minutes at the rate of 6 L h^{-1} m^{-2}, with the nozzles at a distance of 1.5 m.

All experimental data underwent analysis of variance and averages were compared with Scott-Knott test at 5% probability. Data obtained by count (number of shoots and number of internodes + tip) were transformed by $(x + 1)^{0.5}$.

Results and discussion

Mentha species displayed different responses to the *in vitro* propagation process, and showed statistical differences in height, number of shoots and multiplication rate of the cultures (Table 1) (Figure 2-A). Biswas et al. (2014) also observed that, in the micropropagation of *Mentha* spp., the *in vitro* morphogenesis of the cultures varied significantly among the different species analyzed. According to Gamborg et al. (1976) and Gaspar et al. (1996), different plant species have different potentials for absorbing and metabolizing the endogenous hormones and compounds of the nutrient medium, thus presenting different responses to *in vitro* cultivation.

In the case of height of shoot, *M. x gracilis* and *M. gracilis* showed better results, with an average of 5.8 and 5.3 cm, respectively, differing statistically from the other species with averages between 2.3 and 3.8 cm (Table 1).

Similar results were obtained for *M. viridis* by Raja and Arockiasamy (2008) and for *M. arvensis* by Maity et al. (2011), who took into account *in vitro* propagation of nodal segments in an MS medium supplemented with 4.4 μM BAP and verified the formation of shoots at an average height of 3.2 cm and 4.4 cm, respectively. Mehta et al. (2012) also reported the formation of shoots of about 3.7 cm in height in the micropropagation of *M. piperita* nodal segments in an MS medium supplemented with 2.2 μM BAP and 2.3 μM Kinetin. According to Poovaiah et al. (2006), the formation of shoots with a height greater than 2 cm in *in vitro* cultivation of *Mentha* is satisfactory, since they are suitable for the subsequent *in vitro* rooting step.

Regarding the number of shoots, all species showed an average of more than 1.5 shoots per explant, except *M. canadensis* and *M. spicata*, which showed significantly lower averages, i.e. 1.2 and 1.1 shoots per explant, respectively (Table 1). Biswas et al. (2014) also obtained *in vitro* multiplication of *M. spicata* nodal segments in an MS medium supplemented with 4.4 μM BAP, a low rate of shoot

Table 1. Height, number of shoots, and multiplication rate per subculture during *in vitro* culture of *Mentha* species.

Shoot height (cm)										
Nº Subculture	*M. piperita*	*M. suaveolens*	*M. canadensis*	*M. longiflora*	*M. aquatica*	*M. arvensis*	*M. x gracilis*	*M. gracilis*	*M. spicata*	Average
1	1.2 bB	0.4 cB	1.6 bB	0.4 cB	0.9 dB	1.6 bB	1.2 cB	3.7 bA	1.0 cB	1.3 b
2	2.1 bC	1.7 bB	1.6 bB	0.8 cC	2.2 cC	2.1 bC	6.5 bA	4.0 bB	1.2 cC	2.5 b
3	2.2 bC	3.2 aC	2.0 bC	2.6 bC	5.0 bB	3.0 aC	6.2 bA	6.8 aA	2.0 bC	3.7 a
4	2.3 bC	3.7 aB	4.3 aB	4.5 aB	3.9 bB	2.9 aC	6.8 bA	4.7 bB	2.8 bC	4.0 a
5	3.6 aC	3.9 aC	4.0 aC	4.1 aC	6.7 aB	3.7 aC	8.5 aA	7.3 aB	5.0 aC	5.2 a
Average	2.3 C	2.7 C	2.5 C	2.8 C	3.8 B	2.7 C	5.8 A	5.3 A	2.5 C	
Number of shoots per explant										
1	1.1 cA	1.0 dA	1.2 aA	1.0 cA	1.1 cA	1.0 bA	1.1 cA	1.4 cA	1.0 bA	1.1 b
2	1.4 cB	1.7 cA	1.2 aB	1.2 cB	1.1 cB	1.2 bB	1.5 bA	1.8 bA	1.1 bB	1.4 b
3	2.3 aA	2.6 aA	1.2 aC	2.7 aA	1.2 cC	1.6 aB	1.6 aB	1.9 bB	1.6 aB	1.9 a
4	2.0 bB	1.9 bB	1.3 aC	2.0 bB	1.6 bB	1.8 aB	2.0 aB	2.6 aA	1.0 bC	1.8 a
5	1.8 bB	1.4 cC	1.0 aD	1.3 cC	2.5 aA	2.0 aB	1.5 bC	2.7 aA	1.0 bD	1.7 a
Average	1.7 A	1.7 A	1.2 B	1.7 A	1.5 A	1.6 A	1.5 A	2.1 A	1.1 B	
Multiplication rate per explant										
1	1.4 bB	1.0 cB	1.6 bB	1.0 eB	1.8 dA	2.0 dA	1.4 dB	2.6 dA	1.1 cB	1.5 c
2	8.6 aA	6.1 bB	4.4 aC	4.1 dC	2.9 dC	6.0 cB	5.8 cB	6.3 cB	2.9 bC	5.2 b
3	8.8 aC	10.8 aB	4.4 aD	14.6 aA	8.3 bC	9.3 bC	10.2 bB	10.8 bB	6.1 aD	9.2 a
4	8.2 aB	5.0 bC	5.9 aC	12.2 bA	6.6 cC	11.6 aA	8.7 bB	10.8 bA	5.5 aC	8.2 a
5	10.3 aB	6.6 bC	4.5 aC	8.4 cB	11.4 aB	9.3 bB	12.1 aB	14.5 aA	4.8 aC	9.1 a
Average	7.5 A	5.9 B	4.2 B	8.1 A	6.2 B	7.6 A	7.7 A	9.0 A	4.2 B	

Averages followed by the same letter (uppercase for horizontal comparison and lowercase for vertical), are not statistically different from each other using the Scott-Knott test at 5% probability.

formation that reached about 1.2 shoots per explant. According to Chaturvedi et al. (2007), it is normal for some species to respond only slightly to *in vitro* induction of multi-shoots, thus creating the need for developing *in vitro* propagation protocols specific for them.

For multiplication rate, the *Mentha* species showed indexes ranging between 4.2 and 9 shoots per explant (Table 1). Similar results were obtained by Santoro et al. (2013) who verified a multiplication rate of about 13 buds per explant in *in vitro* growth of *M. piperita* nodal segments in a nutrient medium supplemented with 2.6 μM BAP. Current study showed the greatest potential for plantlets production in species *M. piperita, M. longiflora, M. arvensis, M. x gracilis* and *M. gracilis,* with no statistical differences among them. Estimated *in vitro* plantlets production after 5 successive 30-day subcultures ranged between 6,000 and 27,000 (Figure 1) in these species. In the micropropagation of the nodal segments of *M. arvensis* in an MS medium supplemented with 4.4 μM BAP, Biswas et al. (2014) obtained an estimated production of mint species plantlets close to 4,000 after 3 successive subcultures.

According to Vujovic et al. (2012), the effect of subculture in *in vitro* propagation varies, depending on the species. For these authors, during *in vitro* multiplication, a decrease in potential shoot production can be seen in the last subcultures due to successive cuttings and the cumulative effect of the cytokinins, which, over time, can cause damage to the tissues in cultivation. However, in the present study, it was observed that shoot height and formation, besides the multiplication rate, presented averages that were significantly higher in the last three subcultures, thus demonstrating that achieving 5 subcultures is possible without affecting the growth rates.

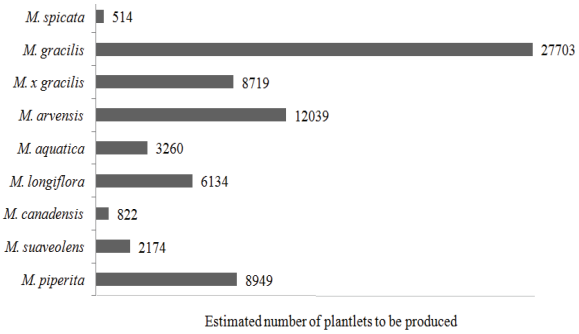

Figure 1. Estimated *in vitro* production of plantlets of different *Mentha* species after five 30-days subcultures.

For *in vitro* rooting of shoots obtained during the multiplication phase, it was found that adding auxin to the culture medium did not influence the induction of root formation, since there were no statistical differences between the various treatments. The mean number of roots that formed per explant was 6.3 for all treatments and both species (Table 2). According to De Klerk et al. (1999), in some species the formation of adventitious roots is spontaneous, and may be caused, among other factors, by root primordia and/or endogenous auxins present in the tissues of the explants in cultivation.

Regarding length of the longest root, best results were obtained in the treatment with the highest IAA concentration, with an average of 11.1 cm. On the other hand, shorter roots were obtained in treatments with the highest concentrations of IBA and NAA, with averages 5.4 and 4.9 cm, respectively. Senthil and Kamaraj (2012) also found that culture medium supplemented with the highest auxin concentrations decreased root length in the *in vitro* root formation of *M. viridis*.

According to De Klerk (2002), the addition of auxins to the process of *in vitro* root formation may

Table 2. Number of roots, length (cm) and fresh root weight (g) of *M. x gracilis* and *M. arvensis* during *in vitro* rooting.

Auxin	Concentration (μM)	Number of roots			Length of main root (cm)			Fresh root weight (g)		
		M. x gracilis	*M. arvensis*	Average	*M. x gracilis*	*M. arvensis*	Average	*M. x gracilis*	*M. arvensis*	Average
Control	0	7.0	4.5	5.8 a	8.2	6.7	7.4 b	0.36	0.36	0.21 a
IBA	0.25	13.0	6.3	9.0 a	7.2	5.4	6.1 c	0.15	0.07	0.095 b
	0.5	5.3	4.8	4.9 a	4.8	5.4	5.1 d	0.12	0.06	0.056 b
	2.5	5.9	4.6	5.1 a	7.5	4.8	5.8 d	0.13	0.02	0.1 b
	5.0	5.9	5.8	5.9 a	6.0	5.0	5.4 d	0.26	0.08	0.14 b
IAA	0.25	6.5	5.8	6.0 a	7.0	12.5	10.3 a	0.14	0.06	0.09 b
	0.5	11.0	7.1	8.3 a	6.5	7.3	7.0 b	0.51	0.13	0.25 a
	2.5	7.0	---⋆	7.0 a	5.9	---⋆	5.9 c	0.12	---⋆	0.12 b
	5.0	8.8	3.6	5.7 a	6.1	14.5	11.1 a	0.17	0.15	0.16 a
NAA	0.25	5.5	5.3	5.4 a	4.0	7.8	5.9 c	0.08	0.03	0.05 b
	0.5	6.3	6.6	6.5 a	4.4	5.1	4.8 d	0.10	0.02	0.06 b
	2.5	7.6	5.7	6.6 a	3.6	6.3	4.9 d	0.11	0.04	0.08 b
	5.0	5.9	5.8	5.9 a	4.1	5.3	4.9 d	0.04	0.05	0.045 b
Average		7.1 A	5.4 B		5.6 B	7.1 A		0.16 A	0.06 B	

Averages followed by the same letter (uppercase for horizontal comparison and lowercase for vertical), are not statistically different from each other, according to the Scott-Knott test at 5% probability.⋆Treatment lost due to contamination.

often inhibit the growth of the cultures since, as a rule, auxins stimulate ethylene synthesis, which in turn regulates plant growth.

For fresh root weight, the best results were provided by the control (0.21 g root biomass) and by IAA auxin concentrations of 0.5 and 5.0 μM (average of 0.20 g fresh biomass). However, it was found that the treatments containing the IBA and NAA auxin presented, in addition to variable root length, results significantly lower than the control results (average of 0.08 g fresh weight). These results demonstrate the adverse effect of these growth phytoregulators on the *in vitro* rooting of these *Mentha* species. In agreement with Pacurar et al. (2014), the type of auxin used during the *in vitro* rooting stage is one of the factors that most influence the outcome of the process, and can even produce harmful effects when used in high concentrations, thus compromising the root formation.

Among the species, *M. x gracilis,* with an average formation of 7.1 roots per explant and 0.16 g fresh weight, was significantly superior to *M. arvensis,* with an average of 5.4 roots per shoot and 0.06 g fresh biomass (Figure 2-B; C). Raja and Arockiasamy (2008) obtained a root formation in *M. viridis* which was similar to that of *M. arvensis,* averaging 5.7 roots per shoot. In the case of *M. piperita*, Manik et al. (2012) had lower results, or rather, about 3.2 roots per explant. On the other hand, *M. arvensis* showed significantly better results in root length than *M. x gracilis,* with an average of 7.1 cm. In the *in vitro* rooting phase of *M. piperita*, Mehta et al. (2012) obtained significantly shorter root length of about 1.7 cm. According to Souza and Pereira (2007), the formation of *in vitro* roots does not occur homogeneously in the different plant species. According to these authors, genetic factors are strongly linked to the ability of species to form adventitious roots, a fact that could explain the differences observed in current study.

Regarding the acclimatization process, it was found in the present study that, regardless of the treatment used in the rooting process, the survival rates of both the plantlets species studied was 100% (Figure 2-D). Similar results were obtained by Raja and Arockiasamy (2008), i.e., the acclimatization of micropropagated *M. viridis* plants had survival rates between 90 and 95%. In the acclimatization of the *M. spicata* plantlets produced *in vitro* Poovaiah et al. (2006) also had survival rates of approximately 100%. During the acclimatization of *M. piperita* shoots formed *in vitro*, the survival rate was about 90% (Manik et al., 2012). Lastly, regarding *M. arvensis*, Maity et al. (2011) also obtained survival rates of about 90% in the acclimatization of shoots produced via *in vitro* propagation.

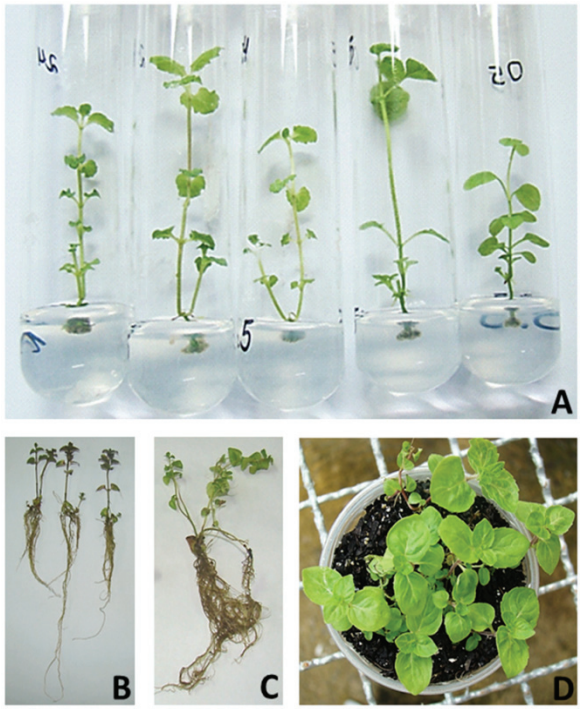

Figure 2. A) Fifth subculture of the *in vitro* multiplication of *Mentha arvensis* (first three test tubes) and *M. canadensis* (last two test tubes). B) *In vitro* rooting of *M. arvensis* in the MS + 5.0 μM of IAA. C) *In vitro* rooting of *M. x gracilis* in the MS + 5.0 μM of IAA. D) Acclimatization of *M. arvensis*.

Conclusion

Species of the genus *Mentha* present different potentials for *in vitro* propagation, with *M. piperita, M. longiflora, M. arvensis, M. x gracilis* and *M. gracilis* featuring the greatest propagation potential.

In general, the greatest multiplication rate for *Mentha* species is obtained starting from the 3rd subculture. It is estimated that, for these species, there is an *in vitro* mass production ranging between 500 and 27,000 plantlets, depending on the species.

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