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Gelation of Culture Medium with K-Carrageenan Improves and Reduces the Cost of *in vitro* Propagation of *Comanthera mucugensis* (Giul.) L. R. Parra & Giul

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HIGHLIGHTS

- Agar is the most used gelling agent for plant culture medium, but it is expensive.
- Gelling of culture medium with κ-carrageenan improves *in vitro* growth and direct and indirect organogenesis of *Comanthera mucugensis*.
- The use of κ-carrageenan represents a 23.2% reduction in the cost of producing the culture medium compared with agar.
- New studies are needed to improve the *in vitro* rooting of *C. mucugensis*.

Abstract: *In vitro* multiplication is the main method for seedling production of *Comanthera mucugensis*, an endangered ornamental plant. The technique consists of cultivating plant tissues under aseptic conditions, controlled environment, and using appropriate culture medium. The physical characteristics of the medium are mainly determined by the presence of a gelling agent. Agar is the most used substance on the *in vitro* cultivation of *C. mucugensis*, however, it is one of the most costly components to manufacture the medium. The objective of the present study was to evaluate the effect of κ-carrageenan as an alternative gelling agent in the propagation of *C. mucugensis*. The seeds and stem explants were cultured on medium gelled with agar or κ-carrageenan, both at 7 g L^{-1} concentration. The results indicated that the plants established in medium with κ-carrageenan presented an increase in length and induced formation of shoots. Direct organogenesis was also improved with the use of this gelling agent. In comparison, agar culture presented

the lowest rate of direct regeneration and the lowest number of shoots. In addition, gelation with κ-carrageenan was efficient in increasing the frequency of callogenesis, as well as, the highest callus regeneration and number of shoots per callus. Rooting was not affected by the type of gelling agent. The substitution of agar for κ-carrageenan can represent a reduction of 23.2% in the cost of manufacturing the culture medium for *in vitro* propagation of *C. mucugensis*.

Keywords: germination; organogenesis; callogenesis; agar.

INTRODUCTION

Comanthera mucugensis (Giul.) L. R. Parra & Giul. (Eriocaulaceae) is a species of "everlasting flowers" endemic to the Chapada Diamantina - Bahia, with high commercial value for ornamental use. This plant has been overexploited since the 1970s, causing the reduction of natural populations and consequent decrease in production for national and international trade [1]. Currently, the species is considered to be at high risk of extinction in the wild [2]. The growth of *C. mucugensis* occurs under restricted environmental conditions, which makes it difficult to establish *ex situ* cultures and makes plant tissue culture the main technique for cultivation and propagation of the species [3]. However, this is a very expensive method of *ex situ* conservation and many germplasm collections can have low genetic variability [4].

The *in vitro* propagation of plants is a complex process composed of different steps, such as: initial establishment of the culture, regeneration of plant tissue via organogenesis or somatic embryogenesis (both can occur directly from the explant or indirectly via callus formation), elongation and rooting of the obtained shoots [5]. In addition, it involves several variables, such as the control of environmental conditions (asepsis, luminosity, photoperiod and temperature) and the composition of the culture medium (nutrients, carbon source, vitamins, regulators and gelling agent) [5-7].

The control of the chemical and physical characteristics of the culture medium is determinant for the success of *in vitro* cultivation and is influenced by the type of gelling agent [8-9]. The gelation confers consistency and support to the plants, regulates the absorption of water and other substances, and can influence morphogenesis at different stages of *in vitro* propagation [9]. Traditionally, agar is the most popular gelling agent used in media preparation [5], being employed in the *in vitro* propagation of *C. mucugensis* [10]. However, it is a high-cost substance and can represent up to 90% of the cost for manufacturing the culture medium [11].

Over the years, several types of gelling agents have been developed and incorporated into *in vitro* culture protocols, including with superior results compared to agar [12-14]. For example, gelation with κ-carrageenan, a polysaccharide also obtained from red macroalgae composed of galactose and ester sulfate subunits, has been reported in studies involving direct and indirect organogenesis, and somatic embryogenesis in several plant species [9,15,16]. It has, however, not yet been tested in the propagation of *C. mucugensis*.

The gelling of the culture medium for *C. mucugensis* cultivation is restricted to the use of agar, and there are no data in the literature about the effects of other reagents. Therefore, this study investigated the viability of using κ-carrageenan as an alternative gelling agent in the germination, organogenesis and *in vitro* rooting of *C. mucugensis*.

MATERIAL AND METHODS

Germination and initial growth

The *C. mucugensis* seeds were collected from inflorescences of mature plants from Mucugê Municipal Park in Chapada Diamantina, Bahia State, Brazil (12º99’21’’S and 41º34’19’’W) in October 2021. In laminar flow cabinet, the seeds were disinfected in 70% ethyl alcohol for 30 s, followed by immersion for 10 min it a solution of sodium hypochlorite – NaOCl (2.5% active chloride) with an added drop of neutral detergent (Ypê®), with subsequent rinsing three times in sterile distilled water. These were sown on standard medium for the species [10]: half-strength MS (Murashige & Skoog 1962) (MS½) and 15 g L^{-1} sucrose. The media were gelled with 7 g L^{-1} of agar (Himedia®, type I) or κ-carrageenan (PhytoTechnology Laboratories®).

After 30 days, the germination rate was evaluated. In addition, the full lengths, number of responsive plants for shoots and number of shoots per plant were recorded after 90 days of cultivation.

Multiplication via direct organogenesis

The *C. mucugensis* stem with approx. 0.5 cm length, previously obtained from microplants cultivated on agar-gelified and plant regulator-free medium, were used as explants for induction of new shoots. The explants were inoculated on regulator-free MS½ gelled with 7 g L^{-1} of agar (Himedia®, type I) or κ-carrageenan (PhytoTechnology Laboratories®).

After 30 days, the responsive explant rate and the number of shoots per explant were registered.

Multiplication via indirect organogenesis

The *C. mucugensis* stem with approx. 0.5 cm length, previously obtained from microplants cultivated on agar-gelified and plant regulator-free medium, were used as explants for induction of callus. The explants were inoculated on MS½ supplemented with 4.44 μM of 6-benzylaminopurine (BAP) [10] and gelled with 7 g/L of agar (Himedia®, type I) or κ-carrageenan (PhytoTechnology Laboratories®).

After 30 days, the responsive explant rate was evaluated. Then, these were transferred to regulatory-free medium, and the regeneration rate and the number of shoots per callus were estimated after 30 days.

Rooting of shoots

The last stage of *in vitro* propagation consisted of rooting. The explants used were shoots obtained in the process of direct organogenesis after 120 days of cultivation. The selected shoots were standardized, presenting a height between 1.5 and 2 cm, and had their roots removed. Afterwards, they were inoculated with in MS½ gelled with 7 g L^{-1} of agar (Himedia®, type I) or κ-carrageenan (PhytoTechnology Laboratories®).

After 120 days, the rooting rate, the length of largest root and number of roots were evaluated.

General cultivation conditions

All media were supplemented with 15 g L^{-1} sucrose [10]. *In vitro* cultures were performed in test tubes (25 x 150 mm), closed with polyvinyl chloride (PVC) film. The pH was adjusted between 5.6-5.8 and autoclaved for 15 minutes at 121 °C and 1.0 atm of pressure. The experiments were then maintained in growth rooms under a 16 hour photoperiod, temperature of 25 ± 3 ºC, and fluorescent white light at 60 μmol $m^{-2}s^{-1}$. All *in vitro* experiments were performed in the Plant Tissue Culture Laboratory of the Universidade Estadual de Feira de Santana (UEFS).

Experimental design and statistical analyses

The experimental design was randomized and each experiment consisted of ten repetitions with five samples per treatment (gelling agent type). The results were examined for normality using the Shapiro-Wilk test followed by analysis of variance (ANOVA) with Tukey test or Mann–Whitney *U* test. All of the analyses were performed considering a 5% level of significance, using PAST software [17].

Cost Analysis

To analyze the cost of gelling agents in the production of the medium, agar and κ-carrageenan were compared using products with the same amount of powder. In this case, the gelling agents were quoted in the 5Kg version. The product values were consulted in the official online stores of each manufacturer on the same day to avoid price variations. Both products were quoted in US dollars. To obtain the real cost value, all decimal places of the numbers were considered in the calculations.

RESULTS

The *in vitro* cultivation of *C. mucugensis* can be performed in gelled medium with agar or κ-carrageenan at a concentration of 7 g L^{-1}. The gel structure formed by κ-carrageenan is firm and clarified, allowing better observation of plant root development compared to agar that has an opaque appearance (Figure 1A). No hyperhydricity of the plants occurred on the media solidified with these gelling agents. Furthermore, the results obtained showed that the germination rate of the medium gelled with agar and κ-carrageenan did not differ from each other, indicating that the type of gelling agent does not alter the germination potential of *C. mucugensis* seeds (Table 1). However, gelation with κ-carrageenan stimulated greater growth (3.5 ± 0.9) (Figure 1B) and induced shoots in 90-day-old microplants, contributing to the increased success of *in vitro* propagation.

Table 1. Germination rate after 30 days and growth and multiplication parameters of *Comanthera mucugensis* after 90 days on gelled media with agar or κ-carrageenan.

Gelling agent	Germination rate (%)	Full length (cm)	Shoot responsive plant rate (%)	Number of shoots per plant
Agar	100 ± 0 [A]	1.7 ± 0.7 [B]	0 ± 0 [B]	0 ± 0 [B]
κ-Carrageenan	95 ± 0 [A]	3.5 ± 0.9 [A]	33.5 ± 9.5 [A]	1.5 ± 0.5 [A]

Data correspond to means ± SD.
Equal letters in the same column do not differ by Tukey's test ($p < 0.05$).

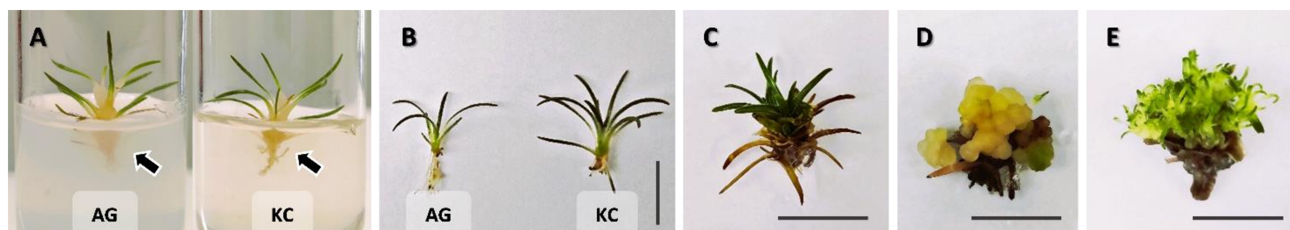

Figure 1. *In vitro* propagation of *Comanthera mucugensis* on gelled medium with agar or κ-carrageenan. (a) General aspect of the culture medium. (b) Initial growth; (c) Direct organogenesis from a stem explants; (d) Callogenesis from stem explants in the presence of BAP; (e) Regeneration of shoots via indirect organogenesis. AG = agar; KC = κ-carrageenan. Bar= 1 cm.

In addition, it was observed that multiplication via direct organogenesis of *C. mucugensis* was influenced by the type of gelling agent (Table 2). The explants grown on medium with κ-carrageenan showed the highest rate of responsive explants, being 30% greater compared to agar (Figure 1C). The use of traditional gelation promoted the mean of 2.3 ± 1.2 shoots per explant, while, the alternative gelling agent doubled the production (4.5 ± 1.8).

Table 2. Evaluation of multiplication via direct organogenesis of *Comanthera mucugensis* on gelled medium with agar or κ-carrageenan.

Gelling agent	Responsive explant rate (%)	Number of shoots per explant
Agar	50 ± 12.45 [B]	2.3 ± 1.2 [b]
κ-Carrageenan	80 ± 16.32 [A]	4.5 ± 1.8 [a]

Data correspond to means ± SD.
Equal uppercase letters in the same column do not differ by Tukey's test ($p < 0.05$).
Equal lowcase letters in the same column do not differ by Mann–Whitney U test ($p < 0.05$).

Cultivation of stem explants in medium supplemented with 4.44 µM BAP induced callogenesis, independently of the type of gelling agent. However, indirect multiplication of *C. mucugensis* was improved by gelling the medium with κ-carrageenan (Table 3). It promoted the highest callus proliferation rate (51.6 ± 11), about 20% more than agar. The callus presented an organogenic aspect, friable texture and with a coloration that varied between green and brown, with few necroses (Figure 1D). The regeneration of shoots occurred via indirect organogenesis on medium without growth regulator (Figure 1E). Gelling the medium with κ-carrageenan increased the rate of regeneration and shoot numbers per callus by 21.5% and 7.3%, respectively, compared to the results obtained using agar (Table 3).

Table 3. Evaluation of multiplication via callogenesis and indirect organogenesis of *Comanthera mucugensis* on gelled medium with agar or κ-Carrageenan.

Gelling agent	Callus responsive explant rate (%)	Regeneration rate (%)	Number of shoots per callus
Agar	30.7 ± 7.3 [B]	32 ± 8.8 [B]	14 ± 7 [b]
κ-Carrageenan	51.6 ± 11 [A]	53.5 ± 8.5 [A]	21.3 ± 9.1 [a]

Data correspond to means ± SD.
Equal uppercase letters in the same column do not differ by Tukey's test ($p < 0.05$).
Equal lowcase letters in the same column do not differ by Mann–Whitney U test ($p < 0.05$).

After 120 days of culture of the microplants, it was observed that the type of medium gelling did not promote significant difference in the induction and growth of roots of *C. mucugensis* shoots (Table 4).

Table 4. Evaluation of the rooting of *Comanthera mucugensis* on gelled medium with agar or κ-Carrageenan.

Gelling agent	Rooting rate (%)	Length of largest root (mm)	Number of roots
Agar	29.1 ± 6 [A]	5.5 ± 0 [A]	3 ± 1 [A]
κ-Carrageenan	31.5 ± 4 [A]	5 ± 0 [A]	4 ± 1.5 [A]

Data correspond to means ± SD.
Equal letters in the same column do not differ by Tukey's test ($p < 0.05$).

The analysis of the prices of these gelling agents showed that agar has the highest acquisition value and, therefore, the most expensive for the gelation of the medium (Table 5). Its use represents a value of approximately $1.39 for manufacturing one liter of culture medium. The substitution of agar for κ-carrageenan means an approximate reduction of 23.2% in the cost of gelling the culture medium for *C. mucugensis*.

Table 5. κ-Carrageenan and agar values and cost of gelling the culture medium for *Comanthera mucugensis*.

Gelling agent	Price per 5 Kg	Value per g	Value per 7g
κ-Carrageenan	$ 765.29 [1]	0.153058	1.071406
Agar type I	$ 996.50 [2]	0.1993	1.3951

[1] Price consulted in January 30, 2023. Product ID: C257-5KG (https://phytotechlab.com/carrageenan.html)
[2] Price consulted in January 30, 2023. Product ID GRM666-5KG2 (https://www.himediastore.com/agar-agar-type-i-10191)

DISCUSSION

The agar is the most frequently used gelling agent for plant tissue culture media. The substance is a sulfated hydrocolloid extracted from red seaweed composed of agarose and agaropectin, which possesses desirable characteristics of high gel clarity, stability and resistance to metabolism during *in vitro* cultivation [18]. However, besides being a costly reagent, the high demand for micropropagation has resulted in the overexploitation of certain species of agarophyte seaweeds (such as the genera *Gelidium* and *Pterocladiae*) and in a threat to the conservation of the natural resource [19]. Other substances have been proposed as alternatives to agar, such as gelan gum, commercialized under the names Gelrite and Phytagel [13], and κ-carrageenan [9].

The κ-carrageenan, besides being a gelling agent, is considered as a plant biostimulant and has been associated with growth improvement, propagation and stress mitigation in several plants [20,21]. Previous studies have shown that polymers of this polysaccharide were able to enhance key physiological and/or biochemical processes in plants, including photosynthesis, cell division, nitrogen, carbon and sulfur assimilation, and NADPH synthesis [22–24]. In addition, it has the ability to attenuate heat stress in seeds of *C. mucugensis* [25]. In the present study, its application as a substrate in the *in vitro* cultivation of this species was responsible for promoting the greatest length and formation of shoots in microplants.

Another study conducted on tobacco (*Nicotiana tabacum*) demonstrated that gelling with κ-carrageenan was efficient in enhancing callus growth and regeneration compared to other gelling agents [9]. The use of this sulfated polysaccharide has also been reported in the solidification of the media that make up the callus induction protocols in agave [26] and *Dendrocalamus hamiltonii* [27], in apple embryogenesis [15] and in potato, gladiolus, and tulip propagation [16, 28, 29].

The gelling with κ-carrageenan is also advantageous for culture media that need to be supplemented with antibiotics. It was reported that high concentrations of the antibiotic kanamycin inhibited the *in vitro* regeneration of tobacco and gladiolus, however, the medium gelled with κ-carrageenan attenuated the effects of the substance, allowing an increase in the regeneration rate of the explants [16]. Regarding the physicochemical properties, its use does not alter the pH of the medium after adjustment and autoclaving [30], although the gel strength is low compared to agar [9]. The gelation with κ-carrageenan, at a concentration of 7 g L^{-1}, permitted the formation of a firm gel structure capable of supporting the microplants and explants of *C. mucugensis*.

The *in vitro* organogenesis of *C. mucugensis* was studied by Lima-Brito [10] which demonstrated regeneration of stem explants directly in medium without growth regulator and indirectly in medium supplemented with BAP. The experiments were performed on agar-gelled media, in which a direct regeneration rate of 58.75% was observed after 60 days of culture. In the present study, 80% of explants

grown on κ-carrageenan medium regenerated in only 30 days (Figure 1B). The gelling agent may have altered the gel matric potential of medium and, possibly, interfered with *in vitro* morphogenesis [31].

The previous studies demonstrated that callus induction in *C. mucugensis* can occur from whole plants and nodal segments in media supplemented with 1.78 and 3.55 µM of BAP [32] and from leaf explants cultured with 4.44 µM BAP [10]. These experiments used agar as the gelling agent. The present results suggest that optimization of the indirect organogenesis protocol can be obtained by cultivating the stem explants in medium gelled with κ-carrageenan and supplemented with 4.44 µM BAP. Despite the great potential for callogenesis of the species, callus regeneration is little discussed in the literature.

Rooting is one of the limiting steps for the propagation of "evergreens" due to the low response of plants to the formation of roots *in vitro*. Previous investigations showed that the use of auxin is not efficient for inducing root growth of *C. mucugensis* [3], which contributes to the low success of *ex vitro* cultivation. In the present study, the type of medium gelling did not promote a significant difference in the induction and root characteristics of the shoots. Therefore, the importance of new methods to improve *C. mucugensis* rooting is highlighted.

The reduction in the cost of micropropagation of plants through the partial and total substitution of agar has been reported in several studies and has generated promising results [14,33–35]. The main low-cost alternatives are: corn starch, sago, cassava starch and isabgol. The present results showed that the use of κ-carrageenan as a gelling agent is favorable and represents a 23.2% reduction in the production cost of the culture medium for *C. mucugensis*. It is a very exploited substance in the manufacture of food and cosmetics and is widely available in the market in several brands, however, quality and purity must be considered.

CONCLUSION

The present study demonstrated that gelling the culture medium with 7 g L^{-1} of κ-carrageenan is a viable alternative for germination, organogenesis and callogenesis of *C. mucugensis*. In addition, its use as a substitute for agar reduces the cost of manufacturing the medium. *In vitro* rooting was not affected by the type of gelling agent, indicating the need for new protocols to improve rhizogenesis in *C. mucugensis*.

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Conflicts of Interest: The authors declare no conflict of interest.

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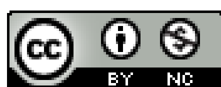

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