



# Micropropagation of *Campomanesia xanthocarpa* O. Berg (Myrtaceae), a medicinal tree from the Brazilian Atlantic Forest

Jean Santos Machado<sup>1</sup> · Juliana Degenhardt<sup>2</sup> · Fabiano Rodrigo Maia<sup>1</sup> · Marguerite Quoirin<sup>1</sup>

Received: 26 April 2019 / Accepted: 22 January 2020 / Published online: 5 February 2020  
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## Abstract

**Key message** Our study shows for the first time a complete guabiroba micropropagation system with acclimatized plants. BA was the best plant growth regulator for multiplication and IBA for rooting.

**Abstract** *Campomanesia xanthocarpa* O. Berg (Myrtaceae), popularly known as guabiroba, is a woody species native to Brazil, important for its potential as a medicinal plant and fruit tree. For the first time, this study shows a complete system of micropropagation for this species. For this, nodal segments with two axillary buds each were used as starting material. Plant Preservative Mixture™ (0.1%) added to culture media was efficient in controlling contamination throughout the culture process. Two formulations of culture medium were compared for in vitro establishment. 2-Isopentenyladenine, 6-benzyladenine, kinetin and zeatin were tested during the multiplication step. Woody plant medium (WPM) was appropriate for all culture steps and, during the multiplication process, 2.2 μM BA induced the best numbers of new shoots per explant during three subcultures (3.0–3.5 per month). For rooting of microcuttings, a rate of 53% was reached in the WPM supplemented with 4.9 μM indol-3-butyric acid. A plastic box containing a mixture of commercial substrate and vermiculite (1:1 v/v) was used for plantlet acclimatization, allowing 52% of survival after two months. In conclusion, a complete micropropagation protocol was developed providing healthy plants. Further studies are needed to improve multiplication and survival rates.

**Keywords** Cytokinin · Guabiroba · Plant preservative mixture · Woody plant medium

## Introduction

*Campomanesia xanthocarpa* O. Berg (Myrtaceae) is a woody species native to the Savanna and Atlantic Forest of Brazil, popularly known as guabiroba or gabirola (Sobral et al. 2016). This species has medicinal potential and is used in folk medicine for the treatment of gastric ulcers (Markman et al. 2004) and to control blood glycemia (Biavatti et al. 2004; Vinagre et al. 2010; Vicili et al. 2014) and cholesterol levels (Klafke et al. 2010). Its medicinal properties are due to the phenolic compounds, such as flavones and chalcones, present in the leaves, fruits, and bark (Schmeda-Hirschmann

1995). Other important compounds are essential oils, found in the leaves, flowers and fruits, and pigments in the seeds, which are exclusive to *Campomanesia* genus (Cardoso et al. 2010). This species is not currently cultivated and the fruits and wood are harvested by extracting the trees growing in natural conditions (Carnevali et al. 2015). This activity along with the lack of domestication can reduce the native populations of *C. xanthocarpa*.

The seeds of *C. xanthocarpa* are recalcitrant and cannot be stored, so propagation by seed is difficult (Melchior et al. 2006). Propagation by cutting or grafting techniques is unsuccessful, with high death rates and low rooting percentages (Brunner Scutti 2000; Teleginski et al. 2018). Hence micropropagation may be a way to propagate this species on a large scale. To ensure the survival of plant tissues in these conditions, asepsis is essential and antibiotics or biocides are sometimes added to the culture media. Plant Preservative Mixture (PPM™) is a mixture of two biocides (isothiazolone type), used to avoid contamination of in vitro cultures by microorganisms. According to the manufacturers, PPM™ (Plant Cell Technology, Washington, D.C.) can kill bacteria

Communicated by Merkle.

✉ Jean Santos Machado  
jean\_jsm@hotmail.com

<sup>1</sup> Programa de Pós-Graduação em Botânica, Universidade Federal do Paraná, Av. Cel. Francisco H. dos Santos, Curitiba, PR 81530-000, Brazil

<sup>2</sup> Embrapa Florestas, Colombo, PR 83411-000, Brazil

and fungi cells, prevent fungal spore germination and eliminate endogenous contamination by inhibiting some important enzymes of the citric acid cycle and electron transport chain of these microorganisms. Other advantages of this biocide are that it is liquid and resistant to high temperatures, which allows its autoclaving, is not toxic and does not damage in vitro cultured explants (George and Tripepi 2001).

The crucial factors for promoting organogenesis during the micropropagation process are the type and concentration of the plant growth regulators (PGR) added to the culture medium (Machakova et al. 2008). Auxins and cytokinins are the most important PGR for initiating shoot or root organogenesis and are also the most critical factors for determining the morphogenic potential of the plant tissue (Kumar et al. 2016). Among the effects of PGR, the auxins promote apical dominance, cell growth and rhizogenesis while the cytokinins promote cell division and induce bud proliferation (Machakova et al. 2008; George et al. 2008). During micropropagation the auxins most commonly used are 2,4-dichlorophenoxyacetic acid (2,4-D), indolacetic acid (IAA), indol-3-butyric acid (IBA) and  $\alpha$ -naftaleneacetic acid (NAA). The cytokinins are 2-isopentenyl adenine (2-iP), 6-benzyladenine (BA), kinetin (Kin) and zeatin (Zea) (Kumar et al. 2016).

Although micropropagation studies using wild Myrtaceae species are still scarce, they show that the use of PGR varies according to the species. In most protocols BA is added to the medium to promote shoot proliferation while IBA is used for rooting. The few studies conducted to date indicate satisfactory results for *Eugenia pyriformis* (Nascimento et al. 2008), *Acca sellowiana* (Ross and Grasso 2010) and *Eugenia uniflora* (Silva et al. 2014). However, efficient protocols of vegetative propagation of *C. xanthocarpa* have not yet been developed. Therefore, due to the importance of this plant and the difficulties of its propagation, the aim of this study was to establish a complete protocol for its micropropagation by multiplication of axillary buds.

## Material and methods

### Plant material and culture conditions

Mature fruits of *C. xanthocarpa* were harvested from a single source tree in the National Forest (FLONA) of Irati, Parana, Brazil. A sample of this source tree was also harvested and taxonomically identified. The seeds were removed from fruits and disinfested with ethanol 70% for 1 min and sodium hypochlorite 5% with three drops per 100 mL of Tween 20 for 10 min and then rinsed three times in distilled sterile water. The seeds were germinated in test tubes containing 12 mL of Woody Plant Medium-WPM (Lloyd and McCown 1980) gelled with 0.6% agar (SIGMA, USA), with PPM<sup>TM</sup>

(0.1%) without sucrose and with a pH adjusted to 5.8. The tubes were closed with plastic caps and autoclaved at 120 °C for 20 min. After 3 months of culture (Fig. 1A), 1 cm stem segments with one nodal segment were excised from the plantlets and used as explants (Fig. 1B) for the establishment of aseptic cultures and shoot multiplication.

For the establishment of aseptic cultures, shoot multiplication and rooting experiments, the culture media were poured into glass flasks 8.5 cm in height and 5.5 cm in diameter, each containing 40 mL of medium. All media were supplemented with sucrose (3%) and 0.6% agar (SIGMA, USA). The pH was adjusted to 5.8 prior to autoclaving at 120 °C for 20 min. The cultures were maintained at  $26 \pm 2$  °C, under cool white fluorescent bulbs, with an irradiance of 50  $\mu\text{mol}/\text{m}^2/\text{s}$  and a 16 h photoperiod.

### Establishment of aseptic cultures

The basal medium was WPM (salts and vitamins) supplemented with 2.2 or 4.4  $\mu\text{M}$  benzyladenine (BA) and PPM<sup>TM</sup> (0.1%) which were compared to control media containing the same BA concentrations without PPM<sup>TM</sup>. Thirty days after establishment of nodal segments in vitro, the new shoots were excised from the initial explant and subdivided into explants with two axillary buds and transferred into fresh media for one subculture (sc).

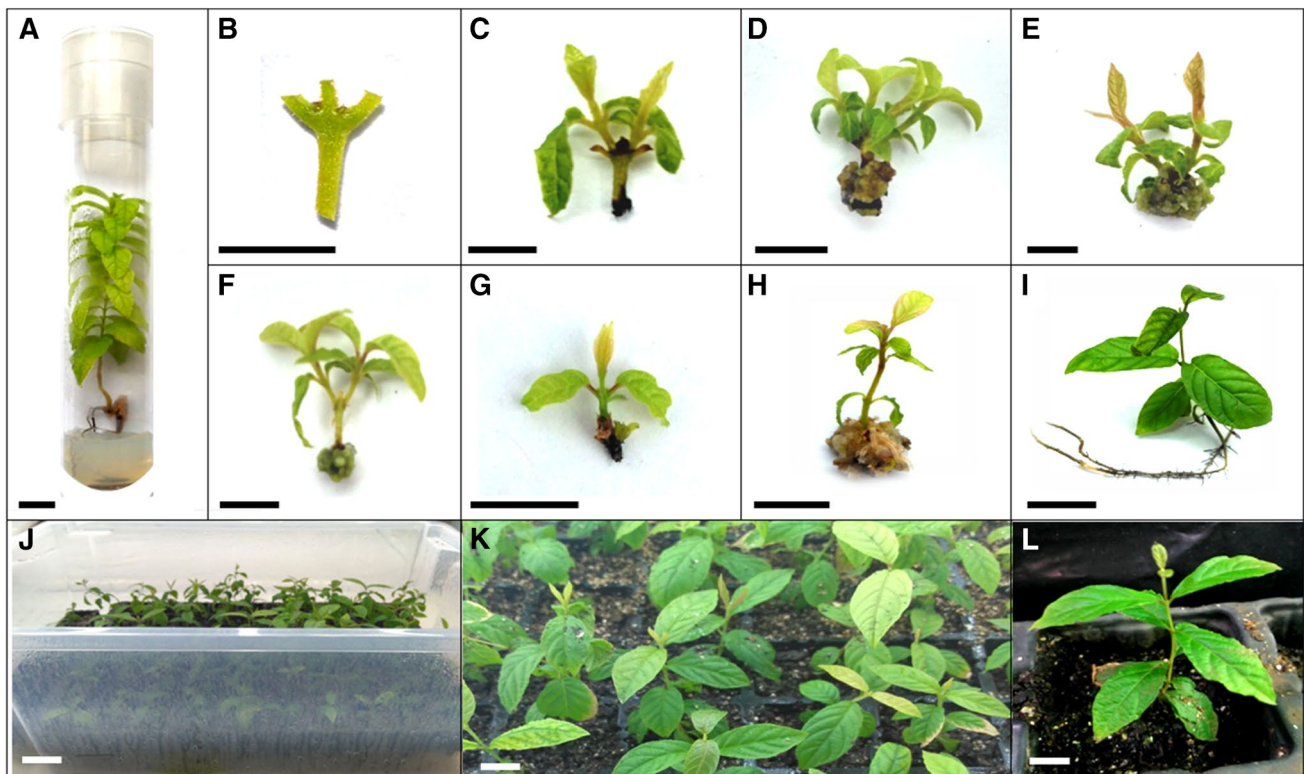
### Shoot multiplication

In the first experiment, the MS medium (Murashige and Skoog 1962) and WPM were supplemented with 2.2 and 4.4  $\mu\text{M}$  BA and PPM<sup>TM</sup> (0.1%) and a control without BA was added. Nodal segments (shoots) were cultured and, after 30 days, two scs of 30 days each were carried out.

In the second experiment, the effects of additional cytokinins were investigated. For this, BA, zeatin (Zea), 2-isopentenyladenine (2-iP) and kinetin (Kin) were added (2.2 or 4.4  $\mu\text{M}$  of each) into the WPM supplemented with PPM<sup>TM</sup> (0.1%) before autoclaving and a cytokinin-free medium was also used. Three scs on the same media were performed every 30 days. After 30 d on the establishment medium and at the end of every sc, the following parameters were evaluated: percentage of explants forming new shoots and numbers of new shoots per explant and, when needed, the necrosis percentage.

### Rooting

Two experiments were conducted with 1 cm long microcuttings with intact apices, obtained from all treatments in the multiplication stage, randomly transferred to the different media. In the first experiment, MS, WPM and QL (Quoirin and Lepoivre 1977) media supplemented with PPM<sup>TM</sup>



**Fig. 1** Micropropagation of *Campomanesia xanthocarpa*. **A** Ninety-day-old seedling grown in vitro; **B** nodal segment with axillary buds; **C** new shoots in WPM without plant growth regulator. Shoot proliferation in WPM supplemented with 2.2  $\mu\text{M}$  BA (**D**), zea (**E**), kin (**F**)

and 2-iP (**G**); rooting in WPM supplemented with 37.6  $\mu\text{M}$  NAA (**H**) and 34.3  $\mu\text{M}$  IBA (**I**, **J**); acclimatization in a plastic box; acclimatized plants in a greenhouse after 30 (**K**) and 120 d (**L**). Bars: 1 cm

(0.1%) without PGR were used to determine the best culture media for rooting. The cultures were maintained in the conditions described above apart from keeping them in the dark for the first week. After 30 d, the rooting percentage and mean number of roots per rooted explant were evaluated. In a second experiment, IBA (4.9, 9.8, 14.7, 24.5 and 34.3  $\mu\text{M}$ ) or NAA (5.37, 10.7, 16.1, 26.8 and 37.6  $\mu\text{M}$ ) were added to WPM supplemented with PPM<sup>TM</sup> (0.1%).

### Acclimatization

Vigorous rooted microcuttings without calli (Fig. 1I) obtained on media supplemented with IBA and on plant growth regulator-free media were removed from the culture medium, washed in running water and randomly transplanted into plastic trays divided into 3.5  $\times$  3.5 cm<sup>2</sup> cells containing a commercial Florestal Plantmax<sup>TM</sup> substrate and a mixture of this substrate with vermiculite in a proportion of 1:1 (v/v) (Fig. 1J).

Forty microcuttings were transplanted in each substrate (Fig. 1K). The trays were placed inside a transparent polypropylene plastic box (46  $\times$  30  $\times$  16 cm) covered with its own lid (same material) and maintained in the growth room

during 7 d. After this period they were transferred into a greenhouse with a maximum temperature of 20  $\pm$  2  $^{\circ}\text{C}$  (day) and minimum of 18  $^{\circ}\text{C}$  (night), under mercury vapor lamps giving an irradiance of 20  $\mu\text{mol}/\text{m}^2/\text{s}$  and a 16 h photoperiod. After 15 d, the box was gradually opened with total removal after 30 d. The survival rate was calculated after 60 d.

### Experimental design and statistical analysis

In all in vitro experiments there were five explants per flask and 12 flasks per treatment. The experimental design was a randomized block with treatments in a factorial scheme (5  $\times$  12), resulting from the combination of culture media and BA concentrations (in the establishment step); cytokinin type and its concentrations (multiplication step) and culture media. To explore the results of the response variables (shoot formation percentage and mean number of new shoots) and predictor variables (PGR and concentrations and culture media) the data were adjusted to the Generalized Linear Model (GLM) according to the distribution (binomial or linear) of the response variables. Variable differences were analyzed using Poisson distribution and the logit link function, which associates the expected response

values with the linear predictors in the model. Categorical variables (presence/absence) such as in the rooting and acclimatization, were analyzed with binomial distribution (Oksanen et al. 2016). The results of auxin type and concentrations (rooting step) as well as the survival rates during acclimatization were independently analysed by non parametric Kruskal–Wallis test. All the statistical analyses were made in R platform, version 3.2.5 with the *vegan* (Oksanen et al. 2016), *car* (Fox and Weisberg 2011), *lme4* (Bates et al. 2015) and *nlme* (Pinheiro et al. 2016) packs. The post-hoc tests (Tukey's test with  $p < 0.001$ ) were made with the *glht* pack (Hothorn et al. 2017).

## Results and discussion

### Establishment of the aseptic cultures

No contamination was observed in the media containing PPM™ throughout the period analyzed. However, in PPM™-free media, 33% of the explants were contaminated after 30 d and 42% after the sc (data not shown). Previous studies reported problems in *C. xanthocarpa* micropropagation due to the endophytic bacterial contamination that was resistant to surface disinfection (Brunner Scutti 2000). In our experiments this contamination did not occur at any stage of the culture which indicates that PPM™ was effective. Endophytic bacterial contamination is considered as one of the main problems of in vitro culture and was also observed in another Myrtaceae species, *Psidium cattleianum* (Freire et al. 2018). In that case endophytic bacterial contamination developed and it was necessary to supplement the culture media with ampicillin. In the present study we did not need to use another powerful and environmentally toxic disinfection agent such as an antibiotic or heavy metal. This fact makes the protocol safer in relation to biosafety standards.

The percentage of shoot formation reached more than 90% in the media containing PPM™ and BA, at the end of the initial culture and of the subcultures. During the initial culture, the highest number of new shoots per explant was 2.0 in the media supplemented with PPM with or without BA. After 30 d of sc the highest values were 2.5 and 2.7 in media with PPM and 2.2  $\mu\text{M}$  BA and in the PPM-free medium with 4.4  $\mu\text{M}$  BA, respectively (data not shown). These results suggest that the presence of 0.1% PPM™ in the media did not negatively affect *C. xanthocarpa* establishment. However, in other species, a reduction and even inhibition of new shoot production or elongation were observed when PPM was present in the culture medium (George and Tripepi 2001; Miyazaki et al. 2010; Huh et al. 2015). PPM™ can be effective in avoiding contamination but new

experiments need to be carried out to verify whether it can affect multiplication and rooting.

### Effects of basal culture media and BA on axillary shoot production

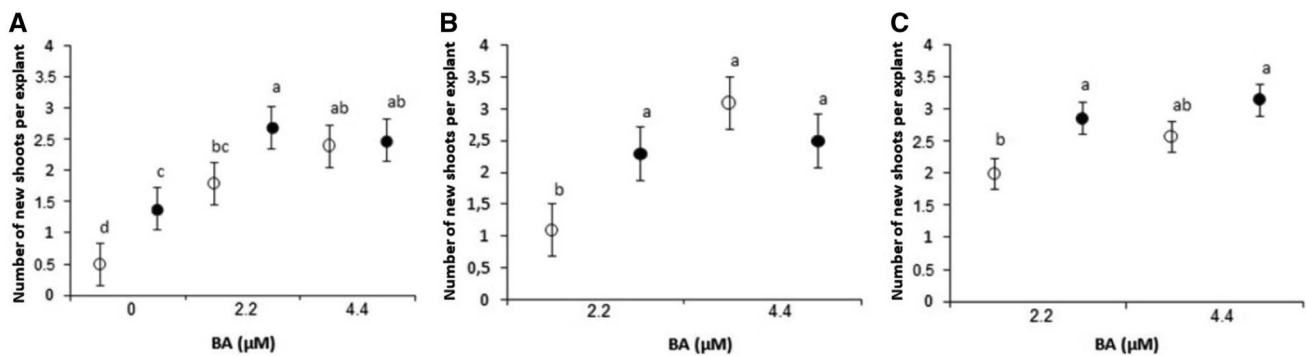
There was interaction between BA concentration and culture medium formulation for shoot formation and number of new shoots per explant during both periods of culture ( $p < 0.001$ ). After 30 d of culture, the explants formed new shoots in both media supplemented with BA, ranging from 60 to 100% ( $\chi^2 = 11.38$ ;  $df = 5$ ;  $p < 0.001$ ) and, at the end of the first sc, 87 and 80%, ( $\chi^2 = 4.462$ ;  $df = 3$ ;  $p < 0.001$ ) in the WPM supplemented with both BA concentrations (data not shown). At the end of the 2nd sc, WPM and MS medium with 2.2  $\mu\text{M}$  BA had the highest percentage of segments forming new shoots ( $\chi^2 = 3.692$ ;  $df = 3$ ;  $p < 0.001$ ), ranging between 85 and 93% (data not shown). In the initial culture, both BA concentrations were better in combination with WPM, while only the highest BA concentration (4.4  $\mu\text{M}$ ) was better in combination with MS medium ( $\chi^2 = 24.620$ ;  $df = 3$ ;  $p < 0.001$ ) (Fig. 2A). After the 1st sc, the rate was slightly higher (3.1) in MS medium supplemented with 4.4  $\mu\text{M}$  BA than in the other treatments (Fig. 2B). In the second sc also, higher numbers of new shoots per explant were reached in WPM (3.3 and 3.0) as well as in MS medium with 4.4  $\mu\text{M}$  BA (2.7) ( $\chi^2 = 24.620$ ;  $df = 3$ ;  $p < 0.001$ ) (Fig. 2C). These results show that the addition of BA to MS medium and WPM improved the formation of new shoots and the number of new shoots per explants.

Considering the results obtained in the two culture media formulations, we suggest that *C. xanthocarpa* explants do not need a high nitrogen concentration in the culture media such as that of MS medium. WPM was specially developed for woody plant tissue culture and has lower nitrogen content, a higher concentration of calcium and thiamine than MS medium and does not contain iodine or cobalt (Lloyd and McCown 1980). It is therefore difficult to determine which of these elements or vitamin promotes better in vitro growth of *C. xanthocarpa*.

In contrast many authors successfully used the MS medium in Myrtaceae micropropagation. This was the case for *Eugenia myrtifolia* (Blando et al. 2013), *P. cattleianum* (Freire et al. 2018), *Psidium guajava* (Mishra et al. 2007) and *Syzygium cordatum* (Dewir et al. 2011). However, culture media with diluted mineral salt concentrations were more suitable for *E. pyriformis* (Nascimento et al. 2008), *E. uniflora* (Silva et al. 2014), *Eugenia involucra* (Golle et al. 2012) and *A. sellowiana* (Ross and Grasso 2010).

The explant response to culture medium varies greatly in these Myrtaceae species even among species belonging to the same genus, as occurs with *Eugenia*, suggesting that





**Fig. 2** Effect of basal media and BA concentration ( $\mu\text{M}$ ) on the number of new shoots per explant of nodal segments of *Campomanesia xanthocarpa* at the end of the second subculture of 30 d. **A** initial culture; **B** first subculture; **C** Second subculture. White circles:

MS medium; dark circles: WPM. All culture media contained 0.1% PPM™. Data with the same letter did not differ by Tukey's test with  $p < 0.001$ . Bars represent mean  $\pm$  standard error

inherent characteristics can influence the response of these species to in vitro culture media.

### Effect of the cytokinins

All the cytokinins and its concentrations induced the formation of new shoots after 30 d without difference between the treatments ( $\chi^2 = 4.605$ ;  $df = 7$ ;  $p > 0.001$ ). Only two axillary buds developed when the nodal segments were cultured on the control medium (Fig. 1C). The highest numbers of new shoots per explant (3.6 and 3.7) were obtained in the media containing 2.2 and 4.4  $\mu\text{M}$  BA at the end of the 1st sc (Fig. 3) and these values were higher than for the other treatments ( $\chi^2 = 2.417$ ;  $df = 7$ ;  $p < 0.001$ ). In the media supplemented with Zea the number of new shoots per explant did not exceed 3.0 (Fig. 3) but was higher than for 2-iP and Kin treatments ( $\chi^2 = 2.674$ ;  $df = 7$ ;  $p < 0.001$ ). The lowest values of number of new shoots per explants were observed in the media containing 2-iP and Kin (Fig. 3). Although the leaf size and photosynthetic pigments were not evaluated, visual observation indicated that the explants cultivated in media with 2-iP and Kin were chlorotic and leaves were reduced (Fig. 1F, G).

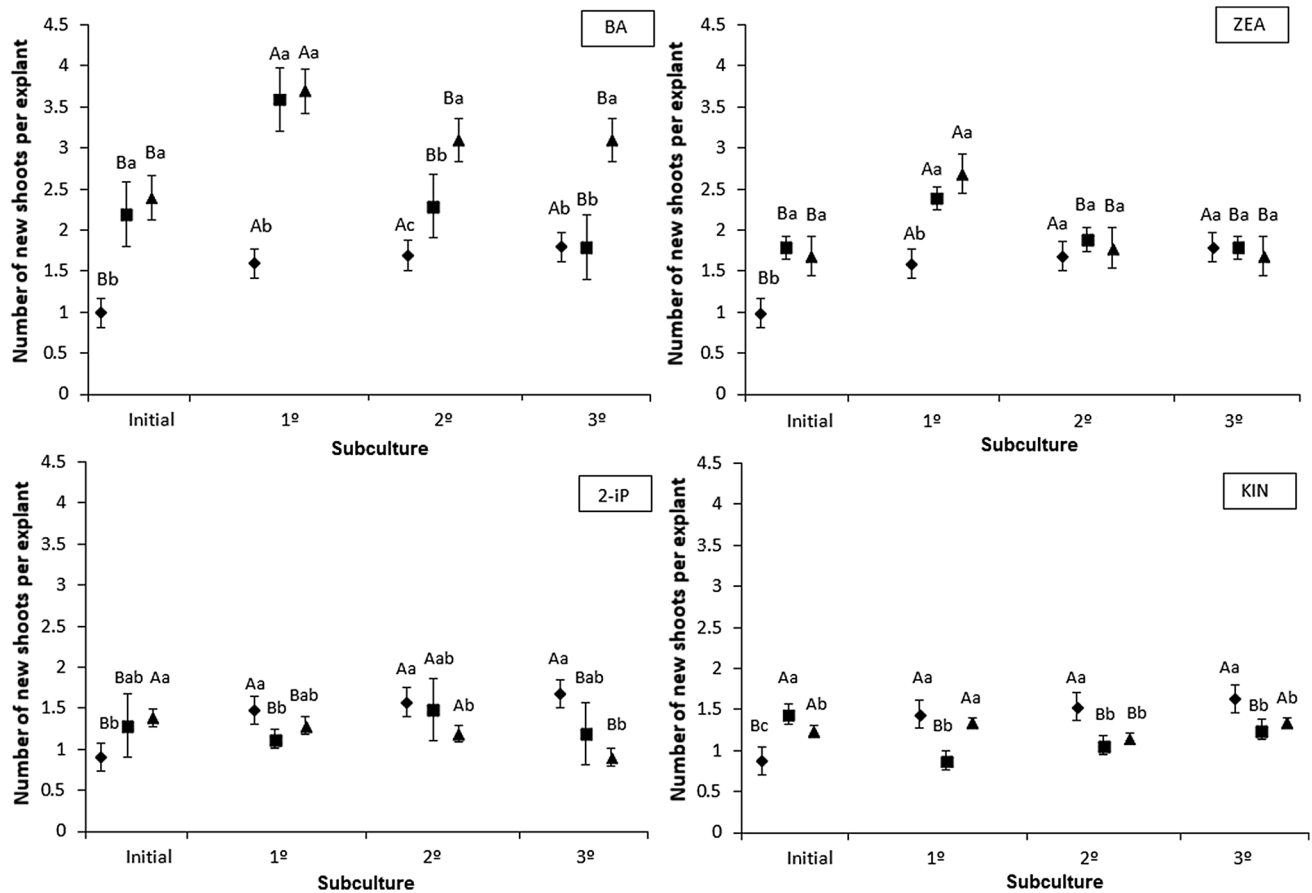
The formation of new shoots varied as a result of the type and concentrations of cytokinins supplemented to the culture medium. BA promoted slightly higher number of new shoots per explant than the other cytokinins tested ( $p < 0.001$ ). This cytokinin has often been used for multiplication of axillary buds in woody plants (Oliveira et al. 2013) and was the most suitable for other Myrtaceae species: *Campomanesia adamantium* (Rossato et al. 2015), *E. pyriformis* (Nascimento et al. 2008), *E. uniflora* (Silva et al. 2014), *E. myrtyfolia* (Blando et al. 2013), *P. cattleianum* (Freire et al. 2018), *P. guajava* (Mishra et al. 2007; Liu and Yang 2011), *Myrtus communis* (Parra and Amo-Marco 1996), *Myrcianthes pungens* (Souza et al. 2011) and *Syzygium alternifolium* (Khan

et al. 1999). In contrast, no exogenous cytokinin (BA, 2-iP and Kin) was effective for *A. sellowiana* multiplication (Oltamari et al. 2000; Ross and Grasso 2010).

Concerning BA concentrations, similar results were obtained in the presence of 4.4  $\mu\text{M}$  BA with a number of new shoots per explant of 4.0 for *E. pyriformis* (Nascimento et al. 2008) and 2.0 for *C. adamantium* (Rossato et al. 2015). However, high BA concentrations were more efficient for *S. alternifolium* with a rate of 3.8 for 17.4  $\mu\text{M}$  BA (Khan et al. 1999) and for *P. guajava*, with 2.7 for 12  $\mu\text{M}$  (Mishra et al. 2007). The latter number of new shoots per explant were similar to our results obtained with lower concentrations of BA but when the explants were cultivated in the presence of 6.6  $\mu\text{M}$  BA the rate decreased and was equal to the control. The same result was observed for *E. involucra* (Golle et al. 2012).

Studies on the effect of Zea on multiplication of wild Myrtaceae species are scarce. In the present study, the explants cultivated in the presence of Zea had normal morphology with green leaves (Fig. 1E) and the second highest number of new shoots per explant. For *E. uniflora* the number of new shoots per explants was 1.8 and did not differ from the numbers obtained with BA and 2-IP (Souza et al. 2008). Kin and 2-iP were not suitable for *C. xanthocarpa* as they induced a low number of new shoots per explant, a reduced size and a frequent oxidation of the explants.

The number of new shoots per explant obtained for explants cultured on BA media varied little through the scs and the values were almost constant except in the 1st sc when they were higher than in other culture periods (Fig. 1D). In *S. alternifolium* the number of new shoots per explant increased from 4.6 to 8.0 until the 3d sc and were constant after that period (Khan et al. 1999). Other studies on Myrtaceae micropropagation did not report scs. Therefore, for the first time, this study shows the results of several scs for a wild species of Myrtaceae. The low number of new



**Fig. 3** Effect of four cytokinins on the number of new shoots per explant of *Campomanesia xanthocarpa* nodal segments during three subcultures in WPM. Diamonds: control without cytokinins; squares: 2.2  $\mu\text{M}$  and triangles: 4.4  $\mu\text{M}$ . Data with the same letter did not dif-

fer by Tukey's test with  $p < 0.001$ ; capital letters: between subcultures and lower-case letters: between concentrations in each culture. Bars represent mean  $\pm$  standard error

shoots per explant obtained may be due to PPM<sup>TM</sup> addition to the culture medium and need to be investigated. These results can help to understand the micropropagation of species of this family and allow new insights into this process.

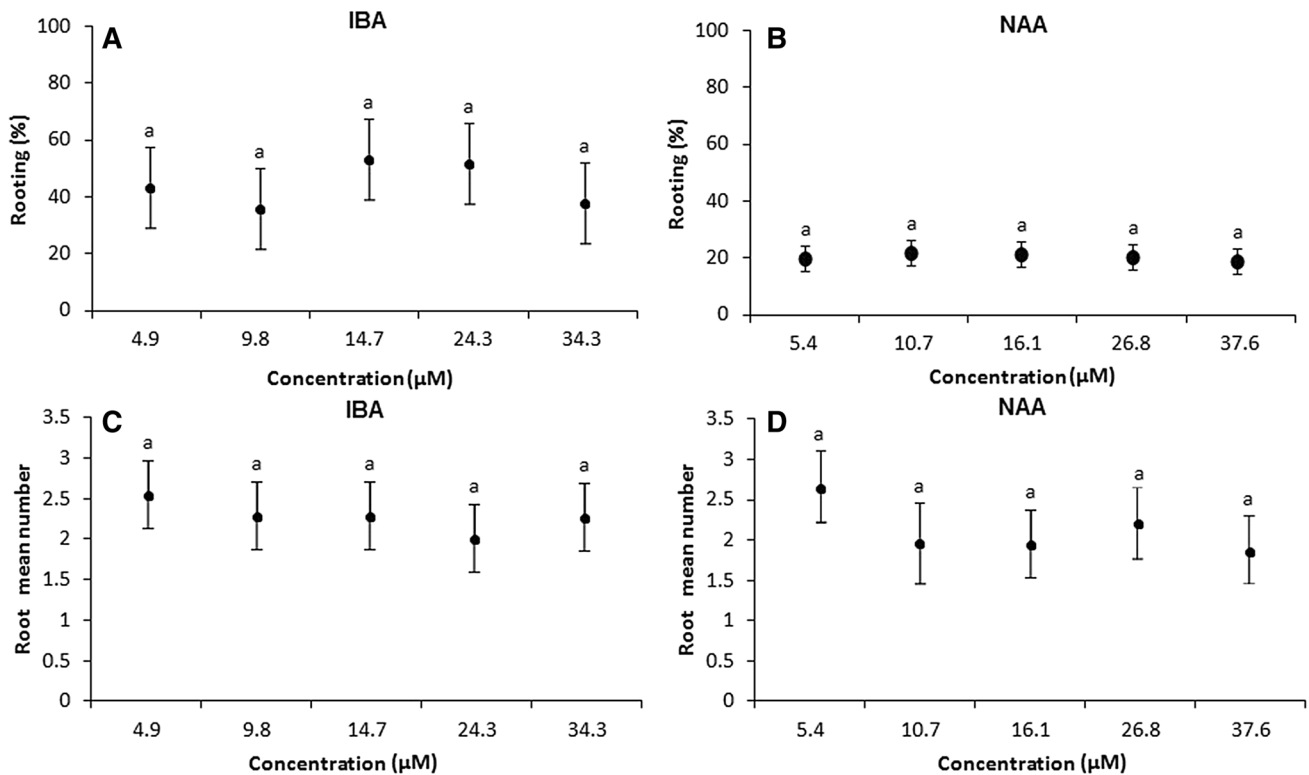
## Rooting

After 60 d of culture in auxin-free WPM, the rooting rate of microcuttings was 49% and differed from other treatments ( $\chi^2 = 351.56$ ;  $df = 2$ ;  $p < 0.001$ ). The rooting rate in the QL and MS media was very low or nil, with a high necrosis percentage (data not shown). These results are in accordance with many studies which report that a diluted culture medium is more efficient for rooting and this kind of medium is generally used for rooting of Myrtaceae species (Oltramari et al. 2000; Mishra et al. 2007; Nascimento et al. 2008; Oliveira et al. 2013). Rooting percentage did not vary between half-strength MS, WPM, and half-strength WPM for *E. involucra* (Golle et al. 2012), *P. guajava* (Mishra

et al. 2007; Liu and Yang 2011) and *M. communis* (Parra and Amo-Marco 1996).

Root formation in an auxin-free medium was also observed in previous studies on *C. xanthocarpa* carried out by Brunner Scutti (2000) and in other Myrtaceae species such as *A. sellowiana* (Oltramari et al. 2000), *E. myrtifolia* (Blando et al. 2013) and *E. pyriformis* (Nascimento et al. 2008). This phenomenon may be due to the endogenous auxins present in the microcuttings.

In the experiment testing IBA for 60 d, the highest rooting percentages were 52 and 53% in WPM supplemented with 14.7 and 24.5  $\mu\text{M}$  IBA respectively (Fig. 4A). However, these values did not differ from those obtained with the other concentrations ( $H = 3.451$ ;  $df = 4$ ;  $p > 0.001$ ). The highest mean number of roots per rooted microcutting was 2.6 in the lowest IBA concentration (Fig. 4C) although there was no statistical difference between the results ( $H = 4.534$ ;  $df = 4$ ;  $p > 0.001$ ). In the medium supplemented with NAA, the rooting percentage was 20% (Fig. 4B) and did not differ from the percentages obtained with the other concentrations



**Fig. 4** Rooting of microcuttings of *Campomanesia xanthocarpa* in function of type and concentration of auxin added to WPM after 60 d of culture. All media contained 0.1% PPM™. **A** Rooting percentage in indol-butyric acid (IBA) treatments; **B** rooting percentage in naphthalene acetic acid (NAA) treatments **C** root mean number per

rooted microcutting in IBA treatments; **D** root mean number per rooted microcutting in NAA treatments. Data with the same letter did not differ by Tukey's test with  $p < 0.001$ . Bars represent mean  $\pm$  standard error

( $H = 0.006$ ;  $df = 4$ ;  $p > 0.001$ ). The mean number of roots per rooted microcutting was 2.7 in the lowest NAA concentration (Fig. 4D) again without statistical difference between the results ( $H = 1.534$ ;  $df = 4$ ;  $p > 0.001$ ). The microcuttings cultivated in media supplemented with IBA were normal and more vigorous (Fig. 1I) than microcuttings cultivated in the presence of NAA. In this last case, a callus was formed at the stem base, the plant size was reduced and the leaves were chlorotic (Fig. 1H).

In Myrtaceae fruit trees, IBA is generally used to promote rooting without callogenesis. For *C. xanthocarpa*, different concentrations of this auxin were efficient and induced a high rooting percentage and root mean number without callus formation during all cultures. Similar results were found for *E. involucra* where 70% of rooting was reached after 60 d in a medium containing 4.9 μM IBA (Golle et al. 2012), for *E. pyriformis* with 60% after 40 d (Nascimento et al. 2008) and for *S. cordatum* with 70% after 30 d (Dewir et al. 2011). In contrast, in *P. guajava*, root formation was observed in a medium containing 49 μM IBA after 17 d (Mishra et al. 2007). This PGR is metabolized quickly in the plant tissues so this phase needs low concentrations of auxins to induce rhizogenesis (Kumar et al. 2016). NAA can

also promote rhizogenesis; however, this PGR is also commonly used to induce rapid cell proliferation (Machakova et al. 2008; Kumar et al. 2016) and this fact can explain the callus formation at the base of all microcuttings that were cultivated in the presence of NAA even at the lowest concentration. Therefore, IBA is more appropriate for *C. xanthocarpa* rooting.

### Acclimatization

All plants cultured for 15 d in both substrates survived in the fully covered plastic box. The micropropagated plants' survival did not differ between the two substrates after 30 days ( $H = 1.113$ ;  $df = 1$ ;  $p > 0.001$ ). The same occurred for *P. cattleianum* (Freire et al. 2018) with 80% of survival irrespective of the substrate used. However, in our study, after 45 d, 92% of the plants cultivated in the mixture were alive while only 44% survived in the commercial substrate ( $H = 16.36$ ;  $df = 1$ ;  $p < 0.001$ ). The same difference occurred after 60 d with a plant survival rate of 52% in the mixture (Fig. 1K, L) and 19% in the commercial substrate ( $H = 7.918$ ;  $df = 1$ ;  $p < 0.001$ ). The substrate composition and characteristics are greatly variable and a mixture of

two or more substrates may be suitable for plant development due to the combination of the various components, which can complement each other (Oliveira et al. 2013). Vermiculite has high porosity which allows easier water flow; however, it does not contain mineral nutrients. The commercial forest substrate (Plantmax®) has a low porosity, retains water and provides some nutrients. Our results showed that a combination of both was advantageous for *C. xanthocarpa* acclimatization. Mixtures were also more suitable for *S. cordatum* (Khan et al. 1999) and *P. guajava* (Mishra et al. 2007) acclimatization, with 70 and 80% of survival in treatments with soil, sand and coconut fiber.

When the cover was fully removed, some plants died. The main factor that hampered plant survival may be the stress caused by the difference between the relative air humidity of in vitro culture and a greenhouse environment (Pospisilová et al. 1999). The leaves of some plants wilted as a response to this stress and new leaves with adapted anatomy which could help to control water loss did not develop during 60 days.

Certainly, the new leaf formation was affected by the stress of acclimatization but this slow development was similar to the initial development of non-micropropagated guabiroba plants cultivated in a greenhouse and in the field (Carnevali et al. 2015). According to those authors, the most efficient factors to evaluate the guabirobeira initial development are the dry mass, seedling height and stem diameter. We therefore suggest that the formation of new leaves may be an important factor for improving plant survival during acclimatization.

Longer periods in the fully covered plastic box, reducing air humidity in the box by modifications to the lid, changing the greenhouse conditions by fogging and using a water loss control agent such as abscisic acid (Pospisilová et al. 1999; 2007) may be more efficient for ensuring a high survival rate for acclimatized plants.

## Conclusions

For the first time, a complete micropropagation protocol by multiplication of axillary buds was developed for *C. xanthocarpa*. Moreover, this study provides information about the in vitro development of a wild Myrtaceae species. Future studies aiming at multiplication and acclimatization improvement are widely encouraged to achieve a larger number of micropropagated plantlets and observe the effect of PPM on multiplication and rooting. The differences of results between *C. xanthocarpa* and other Myrtaceae species vary during all steps of micropropagation and need to be further studied.

**Author contribution statement** JM, MQ, JD: concept and design of the experiments; MQ, FM: supervision, interpretation of the data and revision of the manuscript; JM: performed all the experiments, collected, analyzed, interpreted the data; JM and MQ: wrote the manuscript.

**Acknowledgements** The authors thank the Coordenação de Aperfeiçoamento de Pessoa de Nível Superior (CAPES, Brazil) for a grant to the first author, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for research funding, Duane F. Lima for taxonomy identification and Eileen Bagyary for editing the manuscript.

**Funding** This study was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Brazil.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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