Ciência e Agrotecnologia, 42(4):372-380, Jul/Aug. 2018
http://dx.doi.org/10.1590/1413-70542018424011018

In vitro propagation of *Campomanesia rufa*: An endangered fruit species

Propagação *in vitro* de *Campomanesia rufa*: Uma espécie frutifera em extinção

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Received in May 5, 2018 and approved in July 25, 2018

ABSTRACT

The *Campomanesia rufa* is a fruitful species native from Brazil considered as endangered by the IUC (International Union for Conservation of Nature) and low information regarding its propagation is available. In this context, the aim of the present study was to develop *in vitro* germination, micropropagation and callogenesis protocols for the species. For *in vitro* germination, seeds were inoculated in MS medium supplemented with GA_3 (gibberellic acid) and for shoot induction, the medium was supplemented with three different cytokinins BA (benzyladenine), BAP (6-Benzylaminopurine) or TDZ (Thidiazuron). For shoot growth, culture medium containing BA, BAP, TDZ were maintained under white fluorescent lamps and blue:red light-emitting diodes (LED). GA_3 was also tested on *in vitro* shoot elongation. For oxidation control, the medium was supplemented with PVP (polyvinylpyrrolidone) and for callus induction, 2.4-D (dichlorophenoxyacetic acid). The results showed 68% germination regardless of GA_3 concentration. In the propagation stage, BAP at 4.5 µM induced a higher number of shoots (4.53) and LED lamps as the light source combined with the culture medium with 1 µM BAP induced a higher number of shoots (4.08). The highest average of *C. rufa* length (31.9 mm) was obtained using 8.8 µM GA_3. The use of PVP (584.3 uM) controls up to 27.3% oxidation in young leaf explants. The use of 10 µM 2.4-D leads to a higher callus formation (58.7 %). Therefore, it can be concluded that the use of BAP is efficient in the induction of shoots, PVP controls oxidation leaf segments, and 2.4-D induces callus in *C. rufa.*

Index terms: Tissue culture; micropropagation; Myrtaceae; casaqueira.

RESUMO

A *Campomanesia rufa* é uma espécie frutífera nativa do Brasil considerada como vunerável pela IUCN (International Union for Conservation of Nature) e poucas informações sobre a propagação são encontradas. Neste contexto, o objetivo do presente estudo foi desenvolver protocolos de germinação *in vitro*, micropropagação e calogênese para a espécie. Para germinação *in vitro*, as sementes foram inoculadas em meio MS suplementado com GA_3 (Ácido Giberelico), para a indução da parte aérea, o meio de cultura foi suplementado com BA (benzilamina), BAP (6-Benzilamina Purina) ou TDZ (Thidiazuron). Para o crescimento da parte aérea, o meio de cultura contendo BA, BAP e TDZ foi mantido sob lâmpadas fluorescentes brancas e sob diodos emissores de luz (LED). O GA_3 também foi testado para elongação de brotações *in vitro*. Para o controle da oxidação, o meio foi suplementado com PVP (polivinilpirrolidona) e para a indução de calos, 2,4-D (Ácido 2,4-diclorofenoxiacético) . Os resultados mostraram 68% de germinação, independentemente da concentração de GA_3. No estágio de propagação, BAP a 4,5 µM induziu maior número de brotações (4,53) e lâmpadas de LED como fonte de luz combinada com o meio de cultura com 1 µM de BAP induziu maior número de brotações (4,08). A maior média de comprimento de *C. rufa* (31,9 mm) foi obtida usando 8,8 µM de GA_3. O uso de PVP (584,3 µM) controla até 27,3% de oxidação em explantes de folhas jovens. O uso de 10 µM de 2,4-D leva a uma maior formação de calos (58,7%). Portanto, pode-se concluir que o uso de BAP é eficiente na indução de brotações, o PVP controla a oxidação de segmentos foliares e o 2,4-D induz calos em *C. rufa*.

Termos para indexação: Cultura de tecidos; micropropagação; Myrtaceae; casaqueira.

INTRODUCTION

Brazil is considered as the country with the greatest diversity in the world, since it shelters a vast genetic heritage and among its biomes, the savanna occupies 22% of the territory. Its plant physiognomy houses several species with medicinal properties and of economic interest (Bailão et al., 2015; Ministério do Meio Ambiente, 2017).

However, a significant portion of these savanna plants are threatened with extinction or need further studies due to high endemism and predatory extractivism (Ferreira et al., 2017). Among the species, it can be mentioned *Campomanesia rufa*, belonging to the Myrtaceae Family, locally known as *Casaqueira*, *Gabiroba*, *Guabiroba*, *Guaviroba* or *Guavira* (Campos et al., 2017). It is a fruit tree of restricted occurrence to the savanna, very little known in the scientific context and, due to this lack of

information; it is considered as a vulnerable species by IUCN (IUCN 2017). The *C. rufa* shows great potential for commercial exploitation, whether for *in natura* consumption of fruits, production of sweets and jellies, besides its medicinal properties.

Despite the afore mentioned relevance, the studies reporting the *in vitro* or *ex vitro* propagation of this species efficiently are scarce in the literature, since it shows difficulties of propagation in its natural habitat (Zhang et al., 2017). This characteristic is reported for some species from the genus *Campomanesia*, showing seeds with low longevity due to the high water content when the fruit is detached from the mother plant, (Vallilo et al., 2006) contributing to the increase of respiration and hence to the proliferation of microorganisms, leading to the deterioration of plant material (Pinhal et al., 2011).

In this context, *in vitro* culture appears as a viable and efficient alternative to propagate threatened or vulnerable plants, such as *C. rufa.* Based on the above, the objectives of this study were to develop protocols for germination, micropropagation and callogenesis in *C. rufa* aiming its *in vitro* propagation.

MATERIAL AND METHODS

Plant material

Ripe fruits were used as seed source. After collection, the fruits were immersed in a solution of distilled water and 50% (v/v) sodium hypochlorite with 2% active chlorine for 20 min, passing through three washes with distilled water. Subsequently, the fruits were placed to dry in a laminar flow cabinet for 30 min. The mucilage adhered to the seeds was extracted and the seeds were washed in running water.

Germination

The seeds were submerged in 70% alcohol for 30 s and immersed in sodium hypochlorite (2% active chlorine) for 20 min under constant stirring and then washed three times in sterile distilled water. Seeds were then inoculated into test tubes with 15 mL MS medium (Murashige; Skoog, 1962) supplemented with 30 g L^{-1} sucrose, different concentrations (0, 2, 8, 32, and 128 µM) of GA_3 and solidified with 7 g L^{-1} agar. The pH was adjusted to 5.8 before autoclaving for 20 min at 121 °C. After inoculation, the explants were maintained in the growth room at 25 ± 2 °C. The germination (%) was evaluated 90 days after inoculation.

Micropropagation

Different sources and concentrations of cytokinins in the induction of shoots

Stem segments (~10 mm) of plants established *in vitro*, leafless and containing two lateral buds were inoculated in MS medium supplemented with 30 g L^{-1} sucrose, different cytokinins: 6-benzylaminopurine (BAP), 6-benzyladenine (BA) or thiadizuron (TDZ - 1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea) at concentrations of 0, 2.25, 4.5, and 9.0 µM and solidified with 7 g L^{-1} agar. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 20 min. After the inoculation, the explants were maintained in the grow room at 25 ºC, 16 h photoperiod and 36 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The number of shoots and buds, and the average length of shoots (mm) were evaluated at 30, 60 and 90 days after inoculation.

Light quality in the development of shoots

Shoots (~30 mm) containing the apical bud were inoculated in MS medium supplemented with 30 g L^{-1} sucrose, 1 µM BAP, BA or TDZ and solidified with 7 g L^{-1} agar. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 20 min. After inoculation of shoots, the explants were maintained in a grow room at 25 ± 2 ºC, having as light sources the conventional white fluorescent light (20 W) with a value of 44 µmol m^{-2} s^{-1} PPFD and red-blue (70:30) light-emitting diodes (LED) with a value of 98 µmol m^{-2} s^{-1} PPFD. After 90 days of inoculation, the number of shoots, shoot length (mm) and number of leaves and buds were evaluated.

In vitro elongation of shoots using GA_3

In order to elongate shoots lower than 15 mm length, these explant were inoculated in MS medium supplemented with 30 g L^{-1} sucrose, different concentrations (0, 0.5, 1.0, 2.0, 4.0, and 8.0 µM) of GA_3 and solidified with 7 g L^{-1} agar. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 20 min. After inoculation, explants were maintained for 30 days in a grow room at 25 ± 2 °C under a 16 h photoperiod with irradiance of 36 µmol m^{-2} s^{-1} provided by white light. After 30 days, the shoot length (mm) and the number of leaves were evaluated.

Callogenesis

Oxidation control in leaves

Young leaf explants (~50 mm^2) had the lateral and apex of the leaf blade excised. Three cross-sections on

the midrib were performed to increase the contact surface with the culture medium. The explants were inoculated in MS medium supplemented with 30 g L^{-1} sucrose, different concentrations (0, 100, 200, 400 and 800 µM) of polyvinylpyrrolidone (PVP) and solidified with 7 g L^{-1} agar. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 20 min.

The explants were maintained for 30 days in a grow room at temperature of 25 ± 2 °C under 16 h photoperiod with irradiance of 67 µm m^{-2} s^{-1}. The oxidation rate (%) under the leaf area was evaluated based on a scale for different levels of oxidation (0, 25, 50 and 100%), being standardized the following scale for evaluation: 0% - absence of oxidation in the leaf blade, 25% - laterals of the blade oxidized, 50% - laterals and half of the blade oxidized, and 100% - blade fully oxidized.

Callus induction

Leaf explants (50 mm^2) were excised on the laterals and at the apex of the leaf blade, with three cross sections on the midrib to increase the contact with the culture medium. The explants were inoculated with the abaxial portion in contact with the culture medium. The used culture medium was MS supplemented with 30 g L^{-1} sucrose, 584.3 uM PVP, different concentrations (0, 0.1, 1.0, 5, 0 or 10 µM) of 2,4-dichloro-phenoxyacetic acid (2,4-D) and solidified with 7 g L^{-1} agar. The medium was adjusted to pH 5.8 and then autoclaved at 121 °C for 20 min. The explants were maintained in grow room for 30 days in the absence of light and temperature of 25 °C. Subsequently, the rate of callus formation in the explants (0, 25, 50, 75 and 100%) was evaluated, being considered: 0% - absence of callus, 25% - onset of callus formation, ¼ leaf with callus formation, 50% - half of leaf with callus formation, 75% - almost total leaf showing callogenesis, ¾ leaf with calluses, and 100% - leaf with total callus formation.

Statistical analyses

The experiments consisted of four replicates, being each one with five test tubes containing one explant per tube, followed by a completely randomized design. For statistical analysis, the ANOVA was applied, being that the data were evaluated by the Scott-Knott test ($p<0.05$) for the germination, different sources and concentrations of cytokinins in the induction of shoots, and light quality in the shoot development experiments. *In vitro* elongation of shoots using GA_3 and callogenesis was analyzed by the regression ($p<0.05$), bouth using the SISVAR® software (Ferreira, 2014).

RESULTS AND DISCUSSION

Germination

C. rufa seeds exposed to GA_3 at different concentrations (0, 2, 8, 32 and 128 µM) in the culture medium showed no significant difference. Similar behavior was shown in *Genipa americana* L., a Brazilian fruit tree species in which no response to the application of exogenous GA_3 during its *in vitro* germination process was observed (Souza et al., 2016).

Exogenous application of gibberellin is used commonly in order to overcome physiological dormancy. The presence of ideal concentrations of this plant hormone in the seed induces the production of hydrolytic reserve enzymes (Wang et al., 2016). However, when the endogenous levels of gibberellins are already close to the optimum, the exogenous use of this plant hormone will not contribute to increase the germination. In some cases, the use of doses above the optimum level for the species may even cause phytotoxicity, leading to a reduced germination, as reported for seeds of *Pilosereus aurisetus*, *Lippia graveolens* and *Lippia javanica* treated with GA_3 (Mattana et al., 2017; Reis et al., 2012).

C. rufa seeds showed a *in vitro* germination rate of 68%, which may be considered as low when compared to other species from the genus *Campomanesia*, as *C. xanthocarpa*, *C. adamantium* and *C. pubescences,* which have been reported to show germination above 90% (Dresch et al., 2015; Dousseau et al., 2011; Gogosz et al., 2010; Melchior et al., 2006; Scalon et al., 2009). The low *in vitro* germination rates observed for *C. rufa* may be due to seed quality since the seeds were collected in areas of natural occurrence, where they are exposed to variations of environment and adverse conditions. Such environmental conditions may influence the quality of seeds, generating great variation in the seed size and/or the presence of empty seeds, without the formation of the embryo (Bruno et al., 2014).

Different sources and concentrations of cytokinins in the induction of shoots

A significant difference in the number of shoots, both for the interaction of concentrations and types of cytokinin, and for inoculation time and type of cytokinin was observed. In the interaction between concentrations and type of cytokinins, the use of the BAP provided an increase in the final number of shoots, showing higher formation at the concentration of 4.5 µM with an average of 4.53 shoots, not differing statistically from the concentration of 9.0 µM. For BA and TDZ, no significant difference was observed among the used concentrations (Figure 1A).

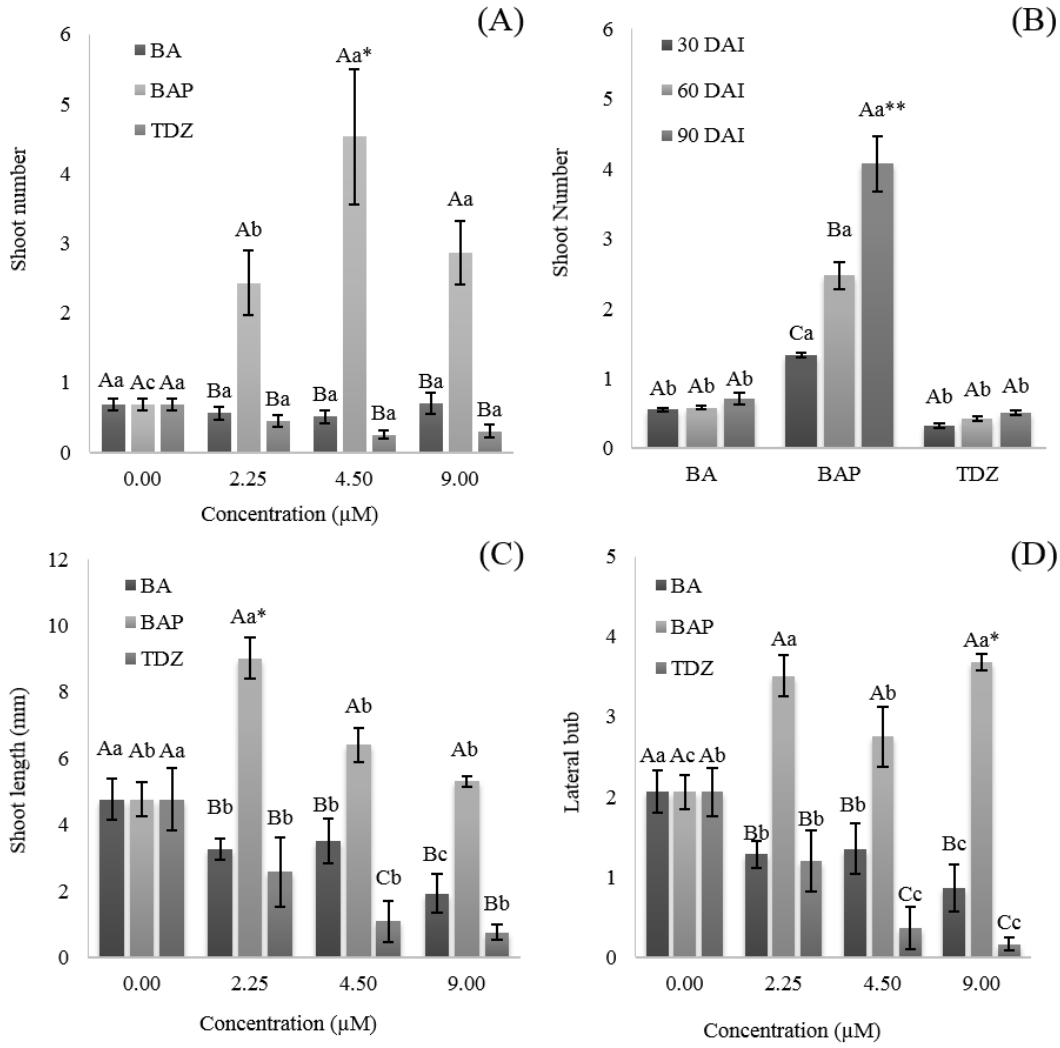

Figure 1: Number of shoots formed in MS medium at different concentrations (µM) and sources of cytokinins (BA, BAP and TDZ) (A) and type of cytokinin (BA, BAP and TDZ) at different inoculation times (30, 60 and 90 days) (B); shoot length (C) and formation of lateral shoots (D) formed in MS medium at different concentrations and type of cytokinin. *Capital letters compare at each cytokinin concentration and lowercase letters compare at each type of cytokinin. **Capital letters compare at each type of cytokinin and lowercase letters compare at each time after inoculation.

In the interaction between inoculation time and cytokinin, the BAP growth regulator was the only one that showed a difference of inoculation over time, showing an increase in the number of shoots reaching the maximum at 90 days, averaging 4.08 shoots (Figure 1B).

For the analyzed variables, length of shoots and number of buds, only the interaction between the factors concentrations and type of cytokinin was observed. Larger shoot length averages were obtained when 2.25 µM BAP was added to the culture medium, averaging 9.01 mm (Figure 1C). For the number of buds, the highest averages (3.5 axillary buds) were observed when BAP was used at the concentrations of 2.25 and 9.0 µM, not differing from each other (Figure 1D). For the other types of cytokinins, a decrease in shoot length and number of buds was observed, demonstrating a possible phytotoxicity of BA and TDZ sources.

Cytokinins are widely used for plant propagation by participating in different plant growth and development processes. However, the addition of different cytokinins to the culture medium may induce different responses in each species. In general, the cytokinin BAP, compared to

TDZ and BA, induced buds with better characteristics in all analyzed parameters (Figure 2A) being recommended for propagation of *C. rufa*.

The use of BAP in the propagation of plants from the family Myrtaceae has been reported for several species, such as *Eugenia pyriformis* Cambess, *Myrcianthes pungens* O. Berg and *Eucalyptus grandis* W. Hill promoting an expressive induction of shoots favoring the propagation of these species (Andrade; Almeida; Goncalves, 2006; Nascimento et al., 2008; Souza et al., 2011).

The use of TDZ formed microshoots whose length was less than 3 mm (Figure 2 B) which may have influenced the low number of shoots with smaller size. The TDZ prevents cytokinin degradation by inhibiting an important cytokinin degradation enzyme (cytokinin oxidase/dehydrogenase, CKX), thus prolonging its effects. In the literature, the descriptions for TDZ applications in the propagation of woody plants are considered as vestigial, i.e., less than 1 µM, since it can inhibit the elongation of shoots at higher concentrations (Huetteman; Preece, 1993). In a study performed with *Withania somnifera* L., different concentrations of TDZ were applied for the *in vitro* propagation of this species and the highest averages for the number of shoots were maintained at concentrations below 1 µM (Fátima; Anis, 2011).

Although BAP and BA are considered as similar growth regulator, some studies have demonstrated differences in their responses, e.g., the use of BAP has been shown as more effective in relation to the BA in the micropropagation of hybrid of almond and peach (Arab et al., 2014).

Ligth quality during shoot growth

Higher formation of new shoots was observed when subjected to fluorescent light (white) in the presense of BAP and TDZ. Under LED (blue:red) light source an increase on shoot number was observed in the presence of both BAP and TDZ, with the use of BAP promoting higher formation (~4.9 shoots) (Figure 3A).

For the number of buds, a significant difference was observed only when the conventional white light was used. This difference was observed when of 1 µM BAP was used, resulting in larger number of axillary buds, with an average of 7.4 buds/explant (Figure 3B). Similar results were observed for number of leaves in which the referred treatment provided the formation of 9.5 leaves/explant when such explants were cultured in medium supplemented with 1 µM BAP under conventional white light (Figure 3C). As also shown in the present study, BAP has been widely used in *in vitro* culture of woody species due to its efficiency in the induction of adventitious buds and leaves (Prudente et al., 2016; Sa et al., 2016).

The use of colored LED lamps, despite having shown a response to the number of shoots, did not demonstrate effective formation of new buds and leaves in the interaction with different cytokinins. For the variable shoot length, no significant difference was observed among treatments showing and average of 22.13 mm length. The use of LEDs and white (fluorescent) light were not effective in increasing the length of blackberry (*Rubus sp*), a woody fruit tree (Rocha et al., 2013), presenting results similar to the observed for *C. rufa*.

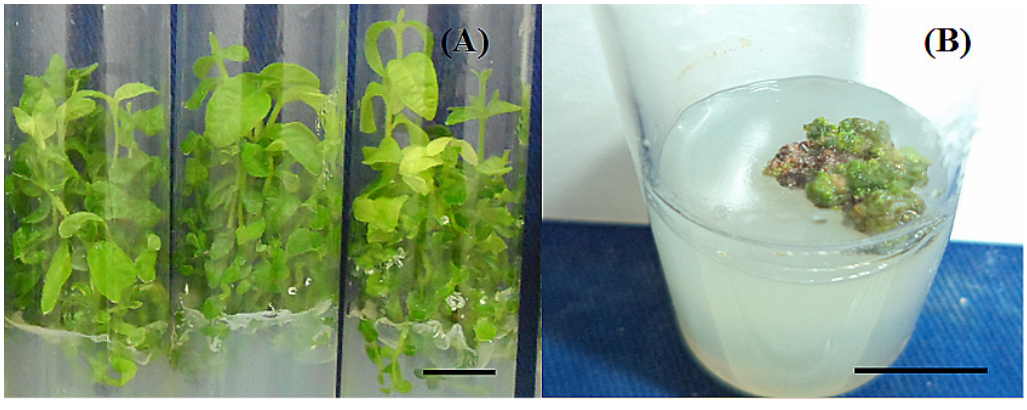

Figure 2: Shoots induced from nodal segment of *C. rufa* cultured in MS medium with addition of 4.5 µM BAP (A) and microshoots (smaller than 3.0 mm) formed from nodal segment of *C. rufa* cultured in MS medium with 2.25 µM TDZ (B). Bar = 1 cm.

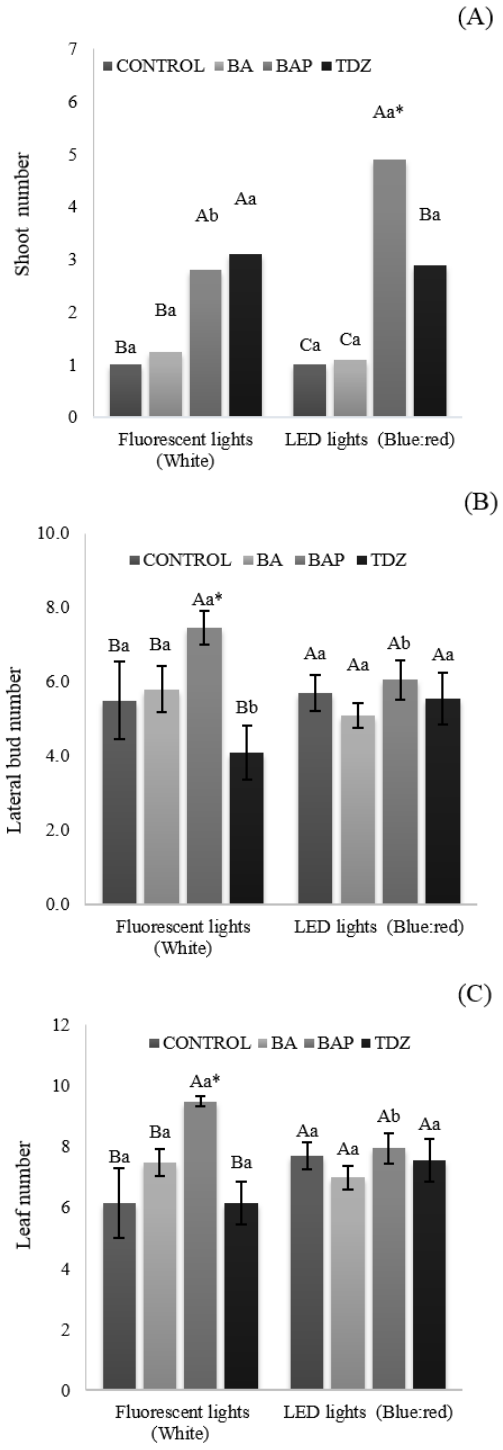

Figure 3: Number of shoots (A), lateral buds (B) and leaves (C) of *C. rufa* formed in MS medium with different sources of cytokinins (BA, BAP and TDZ) fluorescent and LED lights. *Capital letters compare within different sources of light and lowercase letters compare at each type of cytokinin.

In vitro shoot elongation using GA_3

The *C. rufa* shoots inoculated in MS medium supplemented with different concentrations GA_3 showed significant elongation ($p<0.05$) after 30 days of culture. The elongation of *C. rufa* behaved linearly in as much as the concentration of GA_3 was increased, having the maximum point reached at the highest concentration of GA_3 (8 µM). The found value was 31.92 mm, being 33.5% higher than the average found in plants without the addition of GA_3 (Figure 4).

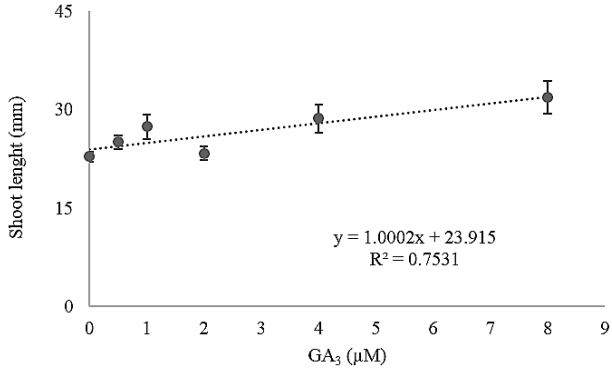

Figure 4: Regression analysis of C. rufa shoots length cultured in vitro in MS medium supplemented with different concentrations of GA_3.

GA_3 stimulates the internode elongation, so that it is widely used in *in vitro* culture that show growth in rosette or formation of small shoots, mainly due to its action in cell division and/or expansion (Hedden; Sponsel, 2015; Gupta; Chakrabarty, 2013). However, the use of GA_3 concentrations above the optimum can cause the emergence of malformed plants, as observed in *Annona emarginata*, a native fruit tree from the Brazilian savanna (Freitas et al., 2016).

For the number of leaves, there was no significant difference among treatments with the different concentrations of GA_3 used, obtaining an overall average of 6.2 leaves per explant.

Callogenesis

For the callus formation, a trend curve was observed where the increase of PVP concentration showed a decrease in the oxidation rate up to the minimum point (584.3 µM), reaching the lowest oxidation rate (27.3%). Which demonstrates reduction of approximately 54% oxidation in *C. rufa* leaf explants cultured *in vitro* (Figure 5A).

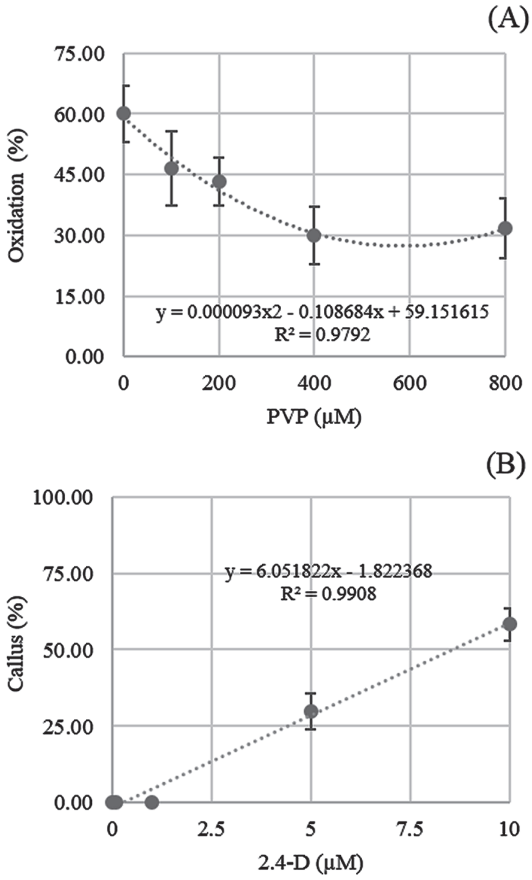

Figure 5: Regression analysis of oxidation rate in *C. rufa* leaf explant cultured *in vitro* in MS medium supplemented with PVP (A) and at callogenesis rate formed in leaf explants in MS medium supplemented with 2,4-D (B).

In the callogenesis, the results showed a linear behavior, with an increasing growth until reaching the maximum point at the highest concentration of 2,4-D tested, i.e., the highest callus formation occurred at the concentration of 10µM 2,4-D with an average rate of 58.7% calluses (Figure 5B). The application of PVP in culture medium as antioxidant in the *in vitro* propagation of woody species has been previously reported in *Boswellia serrata* (Nikam et al., 2013) and *Jatropha curcas* L (Pequeño-Granado et al., 2015).

The PVP is a water-soluble polymer formed by chains of multiple N-Vinylpyrrolidones, which absorb the phenols by hydrogen bonding, thus limiting the oxidation/darkening of the explant (Figueireido; Albarello; Viana, 2001; Sathyanarayana; Varghese, 2007). 2,4-D may induce both organogenic and embryogenic calluses and the explant response will depend not only on its genetic potential for regeneration, but also on the concentration of the regulator, culture conditions and explant type (Al-Khayri; Naik, 2017).

The *C. rufa* calluses showed friable appearance (Figure 6 A-C) and coloration in the yellowish-white band (Figure 6 A) and scanning electron microscopy images demonstrated that induced calluses showed granular aspects (Figure 6 A, B).

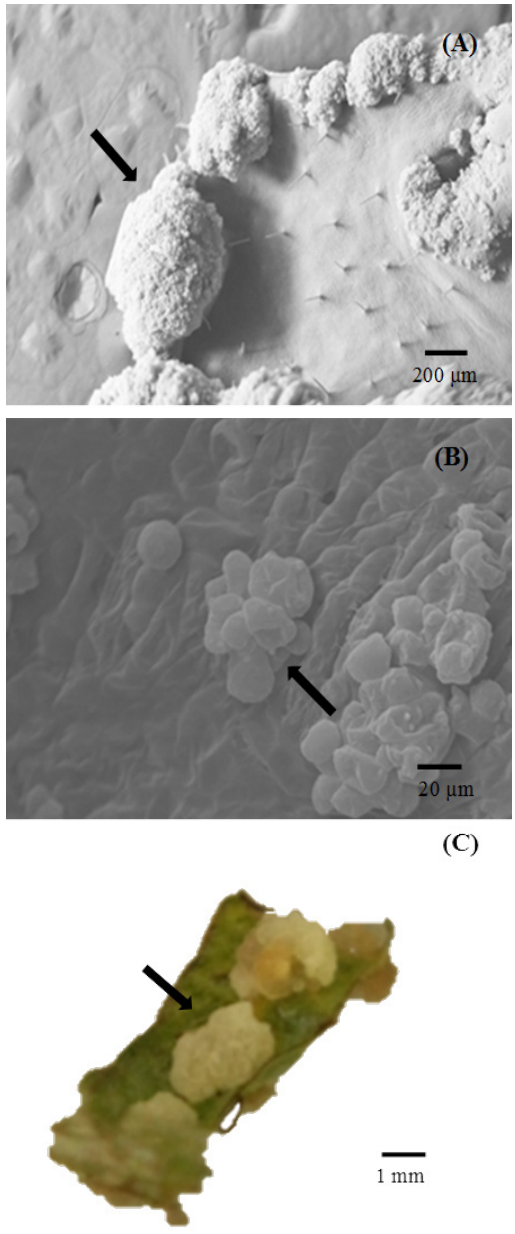

Figure 6: Callus aspects analyzed by scanning electron microscopy (A, B) images and callus aspects developing in the adaxial part of *C. rufa* leaf explants cultured *in vitro* (C).

CONCLUSIONS

For the *in vitro* establishment of *C. rufa*, the use of gibberellin does not affect the germination rates. The cytokinin BAP (4.5 μM) showed high effectiveness on shoot induction. For the development of shoots and to increase the number of leaves and buds, it is suggested to maintain BAP treated explants under fluorescent lamp (white). For shoot elongation, the use of 8 μM GA_3 is recommended. The use of PVP reduces the oxidation of leaf explants while 10 μM 2,4D provide higher callus formation.

ACKNOWLEDGMENTS

We would like to thank the National Council for Scientific and Technological Development (CNPq), Grant No. 442678/2014-2, for the financial support and the Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarship.

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