Abstract

In vitro culture techniques are recognized as efficient strategies for large-scale plant production, as well as providing alternatives for plant conservation. In this study the micropropagation of *Tarenaya rosea* was established using petiole and foliar blade segments cultivated on MS medium with 6-benzyladenine (BA) and/or 6-furfurylaminopurine (KIN). The regeneration rate from explants was evaluated after 30-days in culture, as well as the proliferation rate from explant-derived shoots, reached after four subcultures performed at 30-days in culture. In vitro propagation occurred by both direct (DO) and indirect (IO) organogenesis. The highest regeneration rates by DO (50% to 100%) were reached on media containing only BA, while morphogenic calluses (IO) were mainly formed with BA+KIN. Explants on media with BA showed the presence of small black nodules on their surface, and histological analysis revealed the presence of trichomes with anthocyanin content. Elongation and rooting were reached on growth regulator-free MS. Acclimatization rates around 80% were achieved and the in vitro-regenerated plants were successfully maintained under field conditions. Results show significant morphogenetic potential of *T. rosea* from leaf explants, mainly when cultivated in the presence of 4.4 µM BA, providing a new alternative source of plant material for biotechnological and in vitro conservation studies.

Key words: anthocyanin, *Cleome rosea*, cytokinins, organogenesis.

Original Paper

Micropropagation of *Tarenaya rosea* (Cleomaceae) from leaf explants

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Introduction

Biotechnological tools have been increasingly used to explore the medicinal potential of natural products. The exploitation of populations of medicinal plants grown in the wild has increased, demonstrating the need to apply in vitro methods to increase the supply of germplasm of interest. Among these methods, the most successful is micropropagation, which involves the mass multiplication of plants in a relatively short time, under axenic conditions, through tissue samples (Kumar & Reedy 2011). In addition, plant cell culture strategies allow the production of active compounds for the herbal and pharmaceutical industries independent of seasonal factors and under controlled and constant in vitro conditions (Gandhi et al. 2015).

In particular, species from the Cleomaceae family have been evaluated for the presence of bioactive compounds owing to their use in traditional medicine (Sivanesan & Begum 2007; Abdullah et al. 2016; Alamilla-Fonseca et al. 2018). The Cleomaceae family comprises 18 genera and about 350 species, mostly annual, but sometimes perennial herbaceous plants and shrubs widely distributed in tropical and subtropical regions (Patchell et al. 2014).

_Tarenaya rosea_ (Vahl ex DC.) Soares Neto & Roalson, formerly named _Cleome rosea_ (Soares Neto et al. 2018), is a native Brazilian herbaceous species occurring in coastal sandy plains (“restingas”). These ecosystems are now undergoing intense anthropogenic impact (Rocha et al. 2017). The species has been evaluated for its medicinal potential (Simões et al. 2006, 2010a; Simões-Gurgel et al. 2012a), and protocols aiming at its in vitro production and conservation have already been established, including in vitro germination (Castro et al. 2014), calllogenesis (Simões et al. 2009a), cell suspension culture (Simões-Gurgel et al. 2011) and cryopreservation (Cordeiro et al. 2015a,b, 2016, 2017, 2020). With respect to in vitro propagation studies (Simões et al. 2004, 2009b, 2010b), the morphogenetic potential of leaves, an important source of explants has not been explored so far. Many studies reported the use of leaves as a source of explants owing to the possibility of obtaining a high number of explants per donor plant (Sreedhar et al. 2008; Naing et al. 2016). Leaves of _T. rosea_ have 3 to 5 leaflets and long petioles, which provide large and suitable amounts of plant material for in vitro cultures. Therefore, the present work aimed to evaluate the in vitro morphogenetic capacity from petiole and foliar blade explants, as well as from explant-derived shoots over time in culture, in order to establish new and efficient protocol for large-scale plant production.

Material and Methods

Plant material

The fruits of _Tarenaya rosea_ were collected between February and March 2006 from populations located at Maricá, Rio de Janeiro, Brazil (22°58'01"S, 42°58'36"W). The authenticity of the species was previously confirmed, and a voucher was deposited in the Herbarium of the Rio the Janeiro State University, Rio de Janeiro, Brazil (HRJ7185). Seeds were inoculated in a mixture of soil and sand (1:1) and maintained at 28 ± 2°C under a 12-h photoperiod. Two-month-old seedlings were used as the source of explants. They have leaves formed by 3 to 5 leaflets and long petioles (5–7 cm). Plants were washed under running tap water, roots were removed, and the remaining aerial parts were immersed in 0.5% sodium hypochlorite and 0.05% Tween 80 (v/v) for 10 min, with agitation, and rinsed three times (5 min each) in distilled water.

Bud induction and shoot proliferation

Petiole (1 cm length) and foliar blade (1 cm²) segments were inoculated on Murashige & Skoog (1962) medium (MS) containing 30 g.L⁻¹ sucrose, solidified with 8 g.L⁻¹ agar (Merck) and supplemented with different concentrations of 6-benzyladenine (BA) (1.1; 2.2; 4.4 µM) or 6-furfurylaminopurine (KIN) (1.2; 2.3; 4.6 µM) used alone or in combination. The pH of all media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Petiole explants were inoculated vertically, and foliar blades were placed with the abaxial surface in contact with the culture media. Four explants were inoculated per glass flask (60 × 80 mm) containing 30 mL of culture medium, and the flasks were closed with polypropylene caps. The flasks were maintained in a growth chamber at 26 ± 2°C under a 16 h photoperiod provided by cool white fluorescent tubes (45 µmol.m⁻².s⁻¹). Five flasks were used per treatment, and each experiment was repeated twice. The percentage of explants that induced shoots (regeneration rate) and the mean number of shoots per explant obtained by direct (DO) or indirect (IO) organogenesis were evaluated after 30 days of culture.
Primary explant-derived shoot cultures

Shoots developed in petiole and laminar foliar explants after 30-day in culture were isolated and subcultured onto fresh medium of the same composition used to the primary explants cultures. The shoot cultures were performed at 30-day intervals during four subcultures. The percentage of explant derived-shoots with multiplication capacity (proliferation rate) and the mean number of shoots produced by DO or IO were evaluated after each subculture and represented in the results by the average (mean ± standard deviation) of the four subcultures.

Rooting and acclimatization

In vitro propagated shoots (> 0.5 cm) were transferred to flasks containing 30 mL of solid MS medium devoid of growth regulators (MS0) to induce rooting. The flasks were maintained for 30 days under the same physical conditions as those described above. After this period the percentage of rooting was evaluated. Fifteen flasks with two shoots each were used and the experiment was repeated twice.

Whole plants were transferred to plastic pots (7.5 × 7 cm) containing a mixture of garden soil and sand (2:1). The pots were placed into glass chambers (80 cm × 40 cm × 40 cm) at 28 ± 2º C under a 12-h photoperiod for 30 days. In order to reduce relative humidity inside the chambers, covers were gradually opened after the second week and completely removed 30 days after transplanting. Ten pots with two plants each were used, and the experiment was repeated twice. Ex vitro establishment was assessed through the percentage of acclimatized plants surviving three months after planting.

Histological analysis

Petiole and foliar blade explants were fixed in CRAF III (Sass 1958) and dehydrated through a graduated series of ethyl alcohol (ethanol) and distilled water. After that, the ethanol was replaced by xylol and the material was gradually infiltrated by solutions of xylol and paraffin wax 56 ºC. Finally, the material was embedded in paraffin and allowed to solidify in block form at room temperature (Johansen 1940). Serial sections (8–10 μm) were obtained with Leica rotary microtome (Model RM2025), deparaffinized in xylol and double-stained in astra blue and basic fuchsin (Roeser 1972). Slides were mounted with synthetic resin and then analyzed under an Olympus BX41-BF-I-20 microscope. Images of histological sections were obtained with a Q Color R3 video camera and Image-Pro Express 6.0 software.

Pigment identification

To determine the chemical nature of pigment produced in the explants surface, the material was extract using an acidified methanol solution (0.1% hydrochloric acid). After that, a few drops of the alkaline solution sodium hydroxide (0.1%) were dripped into the extraction solution and then a few drops of the acidic solution hydrochloric acid (0.1%) were used. The change in color as a function of pH was followed to determine the nature of the pigment produced (Vankar & Bajpai 2015).

Statistical analysis

The experiments were organized in a completely randomized design and were repeated twice. BA and KIN treatments (isolated or in combination) were evaluated at the same time (10 treatments × 5 flasks × 2 replications) and the percentage values were subjected to arcsine transformation prior to analysis. The data were submitted to analysis of variance (ANOVA), and the means were compared by Tukey test. The statistical analysis was performed at the 5% level of significance using the GraphPad Prism 5 statistical software package.

Results

Petiole and foliar blade cultures

The micropropagation of T. rosea was efficiently induced when leaf explants were cultivated on MS medium supplemented with cytokinins (Fig. 1). Explants from petioles and foliar blades showed the development of adventitious buds after 5 to 9 days in culture. At the end of the first week, petiole explants showed swelling in the region directly in contact with the culture medium, followed by shoot formation by indirect organogenesis (IO). In addition, direct organogenesis (DO) was observed in the apical region of the explants (Fig. 1a).

The morphogenic response of foliar blades was first observed by DO at the cut ends of the explants (Fig. 1b) with no differences in terms of regeneration capacity between apical and basal explant regions. During the second week in culture, calluses were produced near the cut ends, followed by the development of shoots through IO (Fig. 1b). Shoots were induced by DO and IO along the period of culture from both the abaxial and adaxial surfaces.
Figure 1 – a-f. Micropropagation of *Tarenaya rosea* from leaf explants cultivated on MS medium supplemented with 4.4 µM BA – a. shoot regeneration on petiole explants during the second week in culture by direct (black arrow) and indirect (red arrow) organogenesis; b. shoot regeneration on foliar blade explant during the second week in culture by direct (black arrow) and indirect (red arrow) organogenesis; c. direct (black arrow) and indirect (red arrow) shoot multiplication from petiole-derived shoot during the third subculture; d. fasciated shoot developed from foliar blade explant-derived shoot during the fourth subculture; e. *in vitro* root system developed after 30 days in culture on MS medium devoid of growth regulators; f. three month-old acclimatized plants. Bars: a = 0.58 cm; b = 0.20 cm; c = 0.28 cm; d = 0.44 cm; e = 0.87 cm; f = 4.90 cm.
Supplementation with phytoregulators was essential to induce morphogenic response since explants cultivated on medium devoid of growth regulators were nonresponsive. Media supplemented with BA were the most efficient to induce organogenesis from both types of explants, reaching regeneration rates up to 100% (Tab. 1).

Petiole explants cultivated on media supplemented with only BA reached the highest regeneration rates by DO, while the combination of BA + KIN was more suitable to the induction of regeneration by IO (Tab. 1). In cultures of foliar blade, the combination of BA + KIN induced the highest regeneration rates by both DO and IO.

Cultures established on medium supplemented with 4.4 µM BA displayed the highest number of shoots per explant, both from petiole (4.40 ± 0.52) and foliar blade (8.50 ± 1.65) explants. On the other hand, morphogenic calluses were mainly produced on media supplemented with the combination of 2.2 µM BA + 2.3 µM KIN (5.40 ± 0.75/petiole and 4.27 ± 1.32/foliar blade) (Tab. 1).

The supplementation of culture medium with only KIN could not efficiently induce morphogenesis. The lowest concentration tested (1.2 µM) was nonresponsive for both types of explants, while the highest concentration (4.6 µM) resulted in regeneration rates up to 30% (Tab. 1). Furthermore, the main response of foliar blade explants maintained on media supplemented with KIN was an enlargement in size.

Primary explant-derived shoot cultures

Newly developed shoots obtained from petiole and foliar blade explants showed propagation capacity by both DO and IO (Fig. 1c) during four subcultures. Shoots derived from petiole explants reached higher proliferation rates when compared to the regeneration rates obtained directly from the explants, especially considering propagation by DO. The maximum number of shoots per explant was achieved through DO on medium supplemented with 4.4 µM BA (5.03 ± 0.65) (Tabs. 1; 2).

Shoots derived from foliar blade explants reached higher proliferation rates when compared to the regeneration rates by IO alone. However, the maximum number of shoots after four subcultures was achieved through DO on medium supplemented with 4.4 µM BA + 4.6 µM KIN (5.40 ± 1.74) (Tab. 2).

As observed in cultures initiated with primary explants, explant-derived shoots showed low proliferation capacity on media supplemented with only KIN. Cultures maintained in these conditions achieved proliferation rates up to 30% and low production of shoots per explant (Tab. 2).

The development of shoots with abnormal morphology was observed along the subcultures in the presence of BA alone or in combination with KIN. These shoots were fasciated with a wide stem, and they exhibited undulations resembling the fusion of stem axis of several shoots, albeit slightly compressed to a flattened state (Fig. 1d). The percentage of fasciated shoots was low and they were not considered a factor in determining the mean number of shoots per treatment.

Pigment identification

During the third week of culture on media supplemented with BA alone or in combination with KIN, small black nodules were formed on the surface of explants. These nodules were observed mainly at the top of the ribs on the adaxial surface of foliar blades (Fig. 2a), but they also occurred in the petiole explants (Fig. 2b). Initially, the nodules resembled buds, once buds are also known to present a dark color at the beginning of their development. However, unlike buds, these nodules were not attached to the explants and could be easily removed without visual tissue damage. The chemical composition of these nodules was identified as anthocyanin after the extraction of pigments in the presence of acidified methanol and a typical color change induced by pH variation (Delpech 2000).

The histological analysis of petiole and foliar blade explants revealed the absence of any internal structures that could be associated with pigment production or storage. Leaf surface analysis showed the presence of emergences and trichomes (Fig. 2c,e). The emergences were non-glandular and constituted by epidermal and subepidermal tissues (chlorenchyma cells) (Fig. 2d), while the trichomes were glandular, formed just by the epidermal layer and occurring only on the adaxial surface at the top of the ribs (Fig. 2f).

Rooting and acclimatization

The shoots transferred to MS medium devoid of growth regulators (MS0) reached an average length of about 2 cm and presented rooting rates higher than 90%. The root development started after 3 to 5 days in culture and after 30 days, the plants showed long root systems (Fig. 1e). The plants transferred to ex vitro conditions developed
Table 1 – Effect of BA and KIN on the regeneration rate and mean number of shoots produced from petiole and foliar blade explants of *Tarenaya rosea* after 30 days in culture.

| BA/KIN (µM) | Petiole | Foliar blade |
|-------------|---------|--------------|
|              | Direct Organogenesis | Indirect Organogenesis | Direct Organogenesis | Indirect Organogenesis |
|              | Regeneration rate (%) | Mean number of shoots/explant | Regeneration rate (%) | Mean number of shoots/explant | Regeneration rate (%) | Mean number of shoots/explant | Regeneration rate (%) | Mean number of shoots/explant |
| 0.0/0.0     | 0       | 0            | 0       | 0            | 0       | 0            | 0       | 0            |
| 1.1/0.0     | 60 ± 20a | 0.80 ± 0.16bc | 30 ± 5bc | 0.30 ± 0.26d | 50 ± 5b | 1.20 ± 0.16c | 25 ± 5c | 0.50 ± 0.24f |
| 2.2/0.0     | 60 ± 25a | 1.60 ± 0.43b | 30 ± 10ce | 0.85 ± 0.19c | 65 ± 10b | 1.80 ± 1.77c | 40 ± 10ab | 2.48 ± 0.65b |
| 4.4/0.0     | 75 ± 5a  | 4.40 ± 0.52a | 65 ± 15ab | 2.65 ± 0.25b | 100 ± 0a | 8.50 ± 1.65a | 40 ± 5b | 3.10 ± 0.75a |
| 0.0/1.2     | 0       | 0            | 0       | 0            | 0       | 0            | 0       | 0            |
| 0.0/2.3     | 0       | 0            | 15 ± 5f | 0.35 ± 0.34f | 0       | 0            | 0       | 0            |
| 0.0/4.6     | 25 ± 10b | 0.65 ± 0.81bc | 25 ± 5c | 2.00 ± 0.59b | 30 ± 5b | 0.45 ± 0.28f | 0       | 0            |
| 1.1/1.2     | 25 ± 10b | 0.40 ± 0.28bc | 55 ± 10p | 1.10 ± 0.53bc | 80 ± 5p | 3.25 ± 0.53p | 50 ± 15a | 1.60 ± 0.25p |
| 2.2/2.3     | 20 ± 5b  | 0.20 ± 0.16b | 80 ± 20a | 5.40 ± 0.75a | 100 ± 0a | 7.10 ± 1.37a | 55 ± 15a | 4.27 ± 1.32a |
| 4.4/4.6     | 45 ± 20b | 1.10 ± 1.00b | 50 ± 5p | 4.65 ± 1.84b | 90 ± 5p | 3.30 ± 0.68b | 60 ± 20a | 2.74 ± 1.05b |

Data represent mean ± standard deviation.
Same letters in each column are not significantly different by Tukey test at 5%.
Table 2 – Effect of BA and KIN on the proliferation rate and mean number of shoots produced from primary explant-derived shoots of *Tarenaya rosea* after four subcultures.

| BA/KIN (µM) | Petiole-derived shoots | Foliar blade-derived shoots |
|-------------|-------------------------|-----------------------------|
|             | Direct Organogenesis     | Indirect Organogenesis       |
|             | Proliferation rate (%)   | Mean number of shoots/explant | Proliferation rate (%) | Mean number of shoots/explant | Proliferation rate (%) | Mean number of shoots/explant |
| 0.0/0.0     | 0                       | 0                           | 0                       | 0                           | 0                       | 0                           |
| 1.1/0.0     | 70 ± 10<sup>a</sup>     | 1.06 ± 0.11<sup>c</sup>    | 60 ± 10<sup>b</sup>     | 1.06 ± 0.31<sup>c</sup>    | 65 ± 15<sup>b</sup>     | 0.55 ± 0.42<sup>c</sup>    | 35 ± 5<sup>c</sup>     | 1.55 ± 0.39<sup>е</sup> |
| 2.2/0.0     | 90 ± 5<sup>a</sup>      | 3.59 ± 0.53<sup>б</sup>    | 90 ± 5<sup>a</sup>      | 3.44 ± 0.68<sup>б</sup>    | 80 ± 10<sup>б</sup>     | 1.00 ± 0.70<sup>б</sup>    | 35 ± 15<sup>е</sup>    | 3.01 ± 0.68<sup>е</sup> |
| 4.4/0.0     | 85 ± 20<sup>а</sup>     | 5.03 ± 0.65<sup>в</sup>    | 75 ± 15<sup>б</sup>     | 3.84 ± 0.70<sup>в</sup>    | 85 ± 10<sup>а</sup>     | 4.36 ± 1.70<sup>а</sup>    | 80 ± 5<sup>а</sup>     | 4.21 ± 0.76<sup>е</sup> |
| 0.0/1.2     | 0                       | 0                           | 0                       | 0                           | 0                       | 0                           | 0                       | 0                           |
| 0.0/2.3     | 0                       | 0                           | 5 ± 5<sup>е</sup>       | 0.04 ± 0.08<sup>е</sup>    | 0                       | 0                           | 0                       | 0                           |
| 0.0/4.6     | 30 ± 5<sup>е</sup>      | 1.09 ± 0.38<sup>д</sup>    | 15 ± 5<sup>е</sup>      | 0.26 ± 0.19<sup>д</sup>    | 20 ± 5<sup>е</sup>      | 0.55 ± 0.39<sup>е</sup>    | 0                       | 0                           |
| 1.1/1.2     | 65 ± 5<sup>б</sup>      | 1.28 ± 0.38<sup>д</sup>    | 50 ± 15<sup>б</sup>     | 0.90 ± 0.23<sup>д</sup>    | 60 ± 5<sup>б</sup>      | 1.03 ± 0.22<sup>д</sup>    | 70 ± 5<sup>б</sup>     | 1.33 ± 0.31<sup>д</sup> |
| 2.2/2.3     | 85 ± 5<sup>a</sup>      | 3.73 ± 1.60<sup>в</sup>    | 60 ± 20<sup>в</sup>     | 1.60 ± 0.80<sup>в</sup>    | 75 ± 10<sup>а</sup>     | 1.61 ± 0.66<sup>в</sup>    | 65 ± 10<sup>в</sup>     | 2.84 ± 0.58<sup>в</sup> |
| 4.4/4.6     | 70 ± 25<sup>а</sup>     | 2.63 ± 0.78<sup>е</sup>    | 85 ± 5<sup>а</sup>      | 5.73 ± 0.94<sup>а</sup>    | 60 ± 10<sup>а</sup>     | 5.40 ± 1.74<sup>а</sup>    | 90 ± 5<sup>а</sup>     | 2.34 ± 0.68<sup>а</sup> |

Data represent mean ± standard deviation.

Same letters in each column are not significantly different by Tukey test at 5%.
Figure 2 – a-f. Leaf surface of *Tarenaya rosea* – a. detail of anthocyanin secretion (arrow) on the adaxial surface of foliar blade explant; b. detail of anthocyanin secretion (arrow) in petiole explant; c. emergences on the adaxial leaf surface; d. histological section of an emergence showing epidermal (arrow) and subepidermal (asterisk) tissues; e. glandular trichomes at the top of the ribs on the adaxial leaf surface; f. microscopic view of a glandular trichome (arrow). Bars: a = 0.12 cm; b = 0.10 cm; c = 0.05 cm; d = 30 µm; e = 0.05 cm; f = 30 µm.
new leaves and three months after the beginning of the process, they reached acclimatization rates around 80%. The in vitro-regenerated plants were phenotypically normal and were successfully maintained under ex vitro conditions (Fig. 1f).

Discussion

Supplementation with BA alone, or in combination with KIN, resulted in shoot formation from petiole and foliar blade explants. Cultures established on medium supplemented with 4.4 µM BA achieved the highest number of shoots per explant by DO, while morphogenic calluses were mainly produced on media supplemented with the combination of 2.2 µM BA + 2.3 µM KIN. Previous work of in vitro propagation of T. rosea using stem explants also reached the largest number of shoots regenerated by DO on medium containing 4.4 µM BA, although the combination of 4.4 µM BA + 4.6 µM KIN resulted in the highest production of shoots by IO (Simões et al. 2004). Organogenesis from leaf explants has also been evaluated in other Cleomaceae species. Indirect shoots were obtained from Cleome viscosa L. explants cultivated on MS medium supplemented with a combination of BA and 1-Naphthaleneacetic acid (NAA) (Naseem & Jha 1994). On the other hand, leaf explants of Cleome spinosa Jacq. failed to present any morphogenic response when cultivated on MS medium supplemented with different concentrations of BA and KIN alone or in combination (Albarello et al. 2006).

Shoots were induced from both the abaxial and adaxial surfaces of foliar blades devoid of petioles. Similar results were achieved in another species (Vij & Pathak 1990; Kaur & Vij 2000), although some studies showed the production of shoots on only one of the surfaces (Nayak et al. 1997; Kaur & Bhutani 2009). Shoot induction from foliar blades of T. rosea was more intense near the petiole region, suggesting the occurrence of a physiological gradient of phytohormones and other metabolites leading to differences in shoot regeneration (Karam & Al-Majathoup 2000; Sreedhar et al. 2008). The presence of the petiole was also essential to induce regeneration in other species (Corredoira et al. 2008; Jain et al. 2011).

Supplementation with KIN alone did not sufficiently induce shoot propagation. Similar results were also reached in cultures of stem explant of T. rosea cultivated on solid MS medium (Simões et al. 2004). However, when stem explants were cultivated in liquid MS medium supplemented with KIN alone, high regeneration rates were achieved, both by DO and IO (Simões et al. 2009b). These responses reflect a significant difference between culture conditions on solid and in liquid media. The higher propagation capacity verified in liquid media could be explained by the better contact of the tissues with nutrients and growth regulators (Suthar et al. 2011).

Although the supplementation with KIN alone was not efficient to shoot propagation in T. rosea, the foliar blade explants cultivated in the presence of this phytoregulator showed a significant increase in size. The ability of cytokinins to promote cell enlargement is well known, especially in leaf disks (van Staden & Zazimalova 2008). Studies report the enlargement of foliar blade on media supplemented with cytokinins, as observed for C. spinosa on media with KIN (Albarello et al. 2006) and Stevia rebaudiana (Bertoni) Bertoni (Sreedhar et al. 2008) on media containing BA and KIN in various combinations.

The distinct proliferation capacity observed between petiole and foliar blade explants, as well as between explants and primary explant-derived shoots, reflects not only anatomical and physiological features, but also the differential endogenous hormone balance in these materials. Moreover, this response may be related to distinct distribution of protein receptors for the phytoregulators used exogenously and the differential acquisition of morphogenetic competence (Almeida et al. 2015). Other studies also reported differential morphogenetic responses from leaf explants, as in Kalanchoe blossfeldiana, Poelln. the foliar blade explants of which were responsive to in vitro propagation, but not the petiole (Thomé et al. 2004).

Explants cultivated on media supplemented with BA alone or in combination with KIN developed small black nodules containing anthocyanin pigments. Secretion of anthocyanins was also observed in the leaflets of in vitro shoots of Hypericum perforatum L., and histological analysis showed that the pigment was stored in glands localized in the mesophyll and formed by a small cluster of cells (Mulinacci et al. 2008). In T. rosea, the presence of specialized internal structures to store the pigment was not observed. However, some glandular trichomes that accumulated anthocyanin pigments were observed mainly at the top of the ribs. The presence of glandular trichomes has been frequently reported in the literature, and the importance of these structures has been
highlighted in taxonomic, pharmacognostic and/or phytochemical studies (Metcalf & Chalk 1957; Williams et al. 2003; Edeoga et al. 2009; Gupta & Rao 2012; Khuntia et al. 2013).

Because of the commercial importance of anthocyanins, enhanced production of these pigments from plant cell and tissue culture strategies has been extensively explored (Simões et al. 2012b). The induction of anthocyanins was previously reported in callus (Simões et al. 2009a) and cell suspension cultures (Simões-Gurgel et al. 2011) of T. rosea. In addition, some shoots propagated from stem explants took on a transitory violaceus coloration after the subcultures (Simões et al. 2004). However, the presence of anthocyanins in trichomes is reported here for the first time for the species. These glandular trichomes could be the origin of the small black nodules observed in the leaf surface. The mechanical and hydric stresses suffered by plant material during in vitro manipulation could have contributed to the induction of these pigments.

The development of fasciated shoots was also observed along the subcultures of explant-derived shoots. A similar response was verified from observation of in vitro shoots originated from stem explants of T. rosea (Simões et al. 2004). Numerous studies have reported on the appearance of fasciated plants in natural environmental conditions (Stange et al. 1996; Reboredo & Silvares 2007). However, little experimental evidence can confirm the effect of a particular environmental factor or treatment that causes fasciation (Iliev & Kitin 2011). Several studies also reported the development of in vitro fasciated shoots induced by exogenously applied cytokinins (Iliev & Kitin 2011; Kitin et al. 2005; Mitras et al. 2009). In the present work, the number of fasciated shoots was directly related to the increase in phytoregulator concentrations. Similar to T. rosea, media supplementation with BA induced fasciation in cultures of Prunus avium (L.) L. (Kitin et al. 2005) and Fraxinus excelsior L. (Mitras et al. 2009). The presence of the cytokinin-like compound thidiazuron also was reported to cause fasciation in some woody species (Bosela & Michler 2008; Durkovic 2008). Under in vitro conditions, Iliev & Kitin (2011) reported that shoot fasciation is a direct result of abnormally enlarged shoot apical meristems and changes in the developmental control of meristematic cells.

Shoots of T. rosea achieved high rooting rates on culture medium devoid of growth regulators. The efficiency of in vitro rooting and elongation on MS0 was also observed in micropropagation protocols previously established from stem and root explants of the same species (Simões et al. 2004, 2009b). Similar results were achieved with C. spinosa (Albarello et al. 2006). On the other hand, supplementation with auxins was necessary for root regeneration in C. viscosa (Naseem & Jha 1994) and C. gynandra L. (Naseem & Jha 1997).

Rooted plants reached acclimatization rates around 80%. Similar rates were obtained for plants propagated from stem explants (Simões et al. 2004), results which demonstrate the effectiveness of the micropropagation process using petiole and foliar blade explants.

This work presented a new protocol for assessing the efficacy of micropropagation of T. rosea using leaf explants. The propagation capacity was achieved from both petiole and foliar blade, contributing to large-scale in vitro production of the species and providing an alternative source of plant material for biotechnological studies. The occurrence of propagation by direct organogenesis, the preferential pathway for micropropagation, was more expressive in the presence of 4.4 µm BA for both explants. Moreover, the maintenance of propagation capacity of primary explant-derived shoots over time in culture demonstrates the feasibility of long-term in vitro proliferation of T. rosea.

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