Original Paper

An efficient method for total RNA extraction from leaves of arboreal species from the Brazilian Cerrado

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Abstract

Considering the lack of information on RNA extraction from arboreal species, especially from the Brazilian Cerrado, the aim of this study was to test RNA extraction methods for a wide variety of native plant species from this biome. The methods tested consisted of: (i) TRizol® reagent, (ii) TRizol® reagent with modifications, (iii) CTAB buffer, and (iv) Modified CTAB buffer, initially for leaf samples of Xylopia aromatica and Piper arboreum. Later the procedure with the best results was used to obtain purified RNA from 17 other native species. Based on A260/A280 absorbance ratio the Modified CTAB method was the best for total RNA extraction for those woody species. Ten out of eleven species tested through RT-PCR generated fragments of the expected size from the total RNA extracted by the selected method, confirming it as the best option to obtain high-quality RNA for molecular analyses and for use in the detection of viruses infecting these tree species.

Key words: Cerrado Biome, cDNA, RT-PCR, woody species.

Introduction

Isolation of good quality RNA or DNA is the first step in studies related to plant molecular biology. The methods used in this process were established using the herbaceous Arabidopsis species as model plant, showing chemical and biological properties far different from Neotropical woody plants (Sánchez et al. 2016). Transcriptome studies of tree species help understand numerous plant processes under different conditions, such as gene expression associated with desirable agronomic characteristics (Deng et al. 2016; Ouyang et al. 2016; Shiraishi et al. 2016) related or not to physiological processes such as flowering (Liu et al. 2016) and wood generation (Wang et al. 2016). These studies are dependent on obtaining relatively large amount of high-quality RNA. Thus, efficient protocols for total RNA extraction...
from trees are important for genomic analysis of these species, and also for the purpose of detecting infection by plant viruses.

Just a few total RNA extraction methods have proven to be efficient in tree species, such as Quercus robur L. (Kiefer et al. 2000), Hevea brasiliensis (Willd. ex A.Juss.) Müll.Arg. (Deng et al. 2012) and Neolamarckia cadamba (Roxb.) Bosser (Ouyang et al. 2014). In many cases, the efficiency of these methods was not confirmed for trees from the Neotropical savanna (the Cerrado). Most species in this biome are rich in phenolic compounds and polysaccharides that negatively interfere with the quantity and quality of extracted RNA (Cordeiro et al. 2010). As a result, there is some difficulty in studying these plants at the molecular level, despite the fact that some species show desirable characteristics in different areas, including medical interest, production of antimicrobial compounds (Calixto-Júnior et al. 2016; Correia et al. 2016), and allelopathic potential in weed control (Candido et al. 2016).

Given the importance of obtaining substantial amounts of high quality RNA from our native Cerrado trees, especially the non-cultivated species, the aim of this study was to establish the most efficient method for such extractions, since the difficulty in extracting quality RNA from these plants needs a solution to advance the knowledge of the cellular mechanisms present in this unexplored flora.

Material and Methods

To verify which method would be applied for total RNA extraction from leaf samples of Cerrado arboreal plants, four methods were initially tested for two different species. Of these four methods, the one that showed the best results was chosen for testing in another 17 species of plants native to the Cerrado.

Plant material

Mature leaves recently collected from three different protocols of each species were used. Four different protocols for total RNA extraction were first tested in Xylopia aromatica (Lam.) Mart. and Piper arboreum Aubl. leaves, with three repetitions for each method. For all methods, approximately 100 mg of leaf tissue was ground in 1.5 mL or 2.0 mL microtubes using a pestle and liquid nitrogen. Specific amounts of extraction buffer, depending on the method, were added before the samples were thawed to avoid RNA degradation.

The solutions used in all methods were prepared in RNase-free water:

Method 1 - TRIzol®: 1 mL of TRIzol reagent (Invitrogen®) was added to each ground sample. The tube was vortexed for one minute, and then incubated at room temperature for 5 minutes. 200 µL of chloroform were added to each sample and vortexed for 2 minutes, followed by centrifugation at 15,294 rcf for 15 minutes at 4 ºC. 600 µL of the supernatant were transferred to new tubes and 420 µL of isopropanol was added. The sample was incubated on ice for 5 minutes, followed by centrifugation at 15,294 rcf for 10 minutes. The supernatant was discarded, followed by washing with 600 µL of 70% ethanol (cold) and centrifugation at 15,294 rcf for 5 minutes at 4 ºC. The ethanol was discarded and the tube was kept at room temperature for 2 hours or until the pellets were completely dry. Each sample was resuspended in 30 µL of autoclaved Milli-Q water and stored at -80 ºC.

Method 2 - modified TRIzol® (Xiao et al. 2015): 1 mL of TRIzol® reagent (Invitrogen) was added to ground samples, vortexed vigorously for approximately 30 seconds and incubated at room temperature for 5 minutes. 200 µL of chloroform were added to each sample and vortexed once again. The sample was incubated at room temperature for 3 minutes, followed by centrifugation at 17,949 rcf for 3 minutes. Approximately 500 µL of the supernatant was transferred to new tubes and 500 µL of isopropanol was added. The sample was maintained at room temperature for 10 minutes and centrifuged at 17,949 rcf for 10 minutes at 4 ºC for pellet formation, followed by disposal of the supernatant. The pellet was washed with 1 mL of 70% ethanol (cold). The tube was briefly vortexed, followed by centrifugation at 10,621 rcf for 5 minutes at 4 ºC. The supernatant was discarded and the tube was kept at room temperature until the pellets were completely dry. Finally, the pellets were resuspended with 30 µL of autoclaved Milli-Q water, incubated at 55 ºC for 10 minutes and stored at -80 ºC.

Method 3 - CTAB (Cordeiro et al. 2010): The extraction buffer (2% CTAB; 2% PVP; 100 mM Tris/HCl, pH 8.0; 25 mM EDTA; 2M NaCl) was pre-heated at 60 ºC and 2% Beta-mercaptoethanol was added immediately before use. 600 µL of this buffer were added to each ground sample. The tube was briefly vortexed and incubated for 15 minutes at 60 ºC. 600 µL of chloroform:isoamyl alcohol (24:1) were added. The tube was vortexed
for 5 minutes followed by centrifugation at 7,700 rcf for 10 minutes. Approximately 450 µL of the supernatant was transferred to a new tube and 600 µL of chloroform:isoamyl alcohol (24:1) was added. The tube was centrifuged at 7,700 rcf for 10 minutes and 350 µL of the supernatant was transferred to a new tube and added with a 7.5 M lithium chloride solution to the equivalent of 1/3 of the volume of the tube. The sample was maintained for overnight precipitation at 4 °C. After precipitation, the tube was centrifuged at 7,700 rcf for 30 minutes. The supernatant was discarded and 900 µL of Milli-Q water, 0.2 volumes of 2 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol were added. The sample was kept at -20 °C for 2 hours for additional precipitation, followed by centrifugation at 7,700 rcf for 30 minutes. The supernatant was discarded and the pellet was washed with 300 µL of 70% ethanol (cold) and centrifuged at 7,700 rcf for 10 minutes. The pellet was dried at room temperature and resuspended with 30 µL of autoclaved Milli-Q water.

Method 4 - CTAB (Chang et al. 1993): Three solutions are needed for this method: CTAB extraction buffer (2% CTAB; 2% PVP 30000; 100 mM Tris/HCl; 25 mM EDTA; 2 M NaCl; spermidine 108 µL for 200 mL of buffer; 2% Beta-mercaptoethanol) autoclaved without Beta-mercaptoethanol; SSE (1.0 mM NaCl; 0.5% SDS; 100 mM Tris-HCl pH 8.0; 1.0 mM EDTA pH 8.0); Lithium chloride: EDTA (7.5 M LiCl; 50 mM EDTA). Beta-mercaptoethanol was added to the CTAB buffer and heated at 65 °C immediately before use. 1 mL of CTAB buffer was added to each ground sample and vortexed vigorously. The tube with the buffer and sample was heated at 65 °C for 45 minutes and resuspended through agitation every 10 minutes during heating. Then, 700 µL of chloroform was added and the tube was agitated vigorously in a vortex for 5 minutes, followed by centrifugation at 10,621 rcf for 10 minutes. Approximately 600 µL of the supernatant was transferred to new tubes (1.5 mL) and 700 µL of chloroform was added, agitated vigorously and centrifuged for 10 minutes at 10,621 rcf. 400 µL of supernatant were transferred to new tubes and immediately placed on ice. The same volume of supernatant was added with LiCl2·EDTA solution and mixed, and the sample was maintained overnight at 4 °C. After precipitation, the sample was centrifuged at 10,621 rcf for 20 minutes. The supernatant was discarded and the pellet resuspended with 100 µL of SSE and 50 µL of chloroform. The tube was vortexed vigorously for 6 minutes, followed by centrifugation at 15,294 rcf for 10 minutes. Approximately 120 µL of supernatant was transferred to new tubes and 2x the volume of 95% ethanol was added. The sample was kept at -80 °C for 20 minutes for the purpose of precipitation. The supernatant was discarded and the pellet washed with 200 µL of 70% ethanol (cold) and centrifuged at 10,621 rcf for 5 minutes. The pellet was dried at room temperature and resuspended with 30 µL of autoclaved Milli-Q water.

After comparing the efficiency of the methods tested for the two species mentioned (X. aromatica and P. arboreum), Method 4 was selected and used to obtain purified RNA from 17 other native woody species (Tab. 1), covering fifteen botanical families.

Assessment of quality and quantity of total RNA extracted and RT-PCR: the purity of extracted RNA was analyzed in a NanoDrop Lite spectrophotometer (Thermo Scientific) adjusting yield values in µg.mL^-1 and the A260/A280 absorbance ratio. Integrity was determined based on visualization through 1.2% agarose gel electrophoresis in TBE buffer, in which were applied 5 µL of RNA (above 10 ng.mL^-1) in Milli-Q water.

The total RNA extracted with the protocol that showed the best results in NanoDrop and electrophoresis was used in RT-PCR assays. For this step, 11 species were selected, based on different values of RNA yield to verify the viability of these samples in RT-PCR analysis. One sample of each species (Aspidosperma discolor A.DC; Cedrela fissilis Vell.; Emmotum nitens (Benth.) Miers; IXora sp.; Mabea fistulifera Mart.; Matayba elaeagnoides Radlk.; Nectandra lanceolata Nees; Piper arboreum; Styrax camorum Pohl; Xylpopia aromatica; Zanthoxylum rhoifolium Lam.) was used. A forward (5'-AAACGGCTACCAATCCCAAG-3') and reverse (5'-CCCTTCAATGGATC-CATCGTTA-3') Gm18SrRNA primer pair, which aligns with the 18S gene region (Stolf-Moreira et al. 2010), present in most eukaryotic cells, was used in the reactions. For positive control in RT-PCR, total RNA extracted from soybean by method 1, which is effective for this type of plant, and RNA extracted by method 4 were used, since the primers were designed based on the genome of this cultivated plant species.

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Prior to cDNA (complementary DNA) synthesis, the obtained RNA was DNAse treated, according to the manufacturer specifications (Promega®). The method (cDNA synthesis) was performed using M-MLV reverse transcriptase (Invitrogen®). The reaction (for a final volume of 19.5 µL) was prepared with 6.5 µL of Milli-Q water, 1 µL of dNTPs (10 mM), 1 µL of reverse primer, 100-200 ng of RNA, 4 µL of 5X RT buffer, 2 µL of DTT and 1 µL of M-MLV reverse transcriptase. The reaction was kept at 37 ºC for 60 minutes and inactivated through heating at 70 ºC for 15 minutes. After cDNA synthesis, PCR was performed with 6.5 µL of Milli-Q water, 1 µL of 5X Buffer, 0.3 µL of MgCl2, 0.7 µL of dNTPs (10 mM), 0.2 µL of each primer (forward and reverse) and 1µL of Taq DNA polymerase (Invitrogen®). The PCR was carried under the following conditions: initial denaturation at 94º for one minute, followed by 35 cycles at 95 ºC for two minutes, 62 ºC for 30 seconds, 72 ºC for 30 seconds and, after 35 cycles, a final extension step at 72 ºC for 6 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel in TBE 1X buffer.

Table 1 – Species used for total RNA extraction using Method 4.

| Family        | Species                                      | No. of samples |
|---------------|----------------------------------------------|----------------|
| Annonaceae    | Cardiopetalum calophyllum Schltdl.           | 3              |
| Apocynaceae   | Aspidosperma discolor A.DC.                  | 3              |
| Araliaceae    | Schefflera morototoni (Aubl.) Maguire et al. | 3              |
| Burseraceae   | Protium heptaphyllum (Aubl.) Marchand        | 1              |
| Chrysobalanaceae | Hirtella glandulosa Spreng.                  | 3              |
| Chrysobalanaceae | Hirtella gracilipes (Hook.f.) Prance        | 2              |
| Euphorbiaceae | Mabea fistulifera Mart.                      | 3              |
| Lauraceae     | Nectandra lanceolata Nees                    | 3              |
| Melastomataceae | Miconia chamissonis Naudin                  | 1              |
| Meliaceae     | Cedrela fissilis Vell.                       | 3              |
| Meliaceae     | Trichilia pallida Sw.                        | 3              |
| Metteniusaceae | Emmotum nitens (Benth.) Miers                | 1              |
| Myristicaceae | Virola sebifera Aubl.                       | 3              |
| Rubiaceae     | Ixora sp.                                    | 3              |
| Rutaceae      | Zanthoxylum rhoifolium Lam.                  | 3              |
| Sapindaceae   | Matayba elaeagnoides Radlk.                  | 3              |
| Styracaceae   | Styrax camporum Pohl                         | 3              |

Results

The TRIzol® reagent was ineffective to extract RNA from *P. arboreum* and *X. aromatica* plants, through agarose gel electrophoresis analysis, showing no bands linked to 18S and 28S rRNAs. Spectrophotometrical analysis showed below optimal A260/A280 ratios for these samples, ranging out of the ideal rates between 1.8 and 2.0 (Fig. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.12743429.v1>; Tab. 2) (Glasel 1995).

The concentration of RNA extracted with all protocols showed values that varied among the samples. Considering the A260/A280 absorbance values for *P. arboreum*, the values of Methods 1 and 2 were often below 1.5, which is below recommended for RNA quality (Glasel 1995). While for *X. aromatica*, the values showed higher fluctuations, below 1.5 or above 2.5 (Tab. 2). These results were confirmed by agarose gel analysis, in which bands were not visualized (Fig. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.12743429.v1> ; Tab. 2).

For Methods 3 and 4, the A260/A280 ratios for both species resulted in better values (Tab. 2;
Table 2 – Productivity values and absorbance ratio of total RNA extraction from leaf samples of *Piper arboreum* and *Xylopia aromatica* using four different methods.

| Extraction | Sample* | Method 1 | Method 2 | Method 3 | Method 4 |
|------------|---------|----------|----------|----------|----------|
|            | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 |
| P1a        | 24.1    | 1.40     | 98.5     | 1.30     | 43.8     | 1.91     | 37.1     | 2.05     |
| P1b        | 39.8    | 1.43     | 24.0     | 1.44     | 43.8     | 2.04     | 29.9     | 2.09     |
| P1c        | 27.5    | 1.13     | 53.4     | 1.45     | 53.1     | 1.70     | 53.2     | 2.02     |
| P2a        | 3.6     | 1.10     | 29.4     | 1.68     | 77.8     | 2.18     | 96.2     | 2.09     |
| P2b        | 25.2    | 1.46     | 8.8      | 1.65     | 43.3     | 1.80     | 118.5    | 2.03     |
| P2c        | 16.4    | 1.17     | 10.3     | 1.09     | 41.7     | 2.01     | 53.2     | 2.02     |
| P3a        | 6.7     | 1.01     | 28.6     | 1.41     | 45.8     | 1.79     | 43.0     | 1.90     |
| P3b        | 48.4    | 1.47     | 17.2     | 3.54     | 42.0     | 2.08     | 42.6     | 2.01     |
| P3c        | 40.4    | 1.29     | 5.9      | 1.22     | 59.0     | 2.23     | 62.1     | 2.02     |

**Piper arboreum**

| Extraction | Sample* | Method 1 | Method 2 | Method 3 | Method 4 |
|------------|---------|----------|----------|----------|----------|
|            | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 |
| P1a        | 17.0    | 1.08     | 43.2     | 0.92     | 43.8     | 2.18     | 32.4     | 2.02     |
| P1b        | 6.4     | 1.15     | 7.4      | 1.20     | 38.5     | 2.18     | 32.8     | 1.99     |
| P1c        | 16.6    | 0.81     | 8.2      | 1.03     | 38.9     | 2.22     | 33.4     | 2.00     |
| P2a        | 6.0     | 1.12     | 4.4      | 1.09     | 55.3     | 1.97     | 45.1     | 1.94     |
| P2b        | 4.9     | 1.22     | 62.2     | 1.11     | 39.0     | 2.04     | 38.1     | 1.88     |
| P2c        | 7.6     | 0.86     | 4.2      | 1.14     | 45.6     | 1.83     | 11.6     | 2.01     |
| P3a        | 4.1     | 0.99     | 12.2     | 1.09     | 44.5     | 2.09     | 88.9     | 2.01     |
| P3b        | 8.1     | 0.93     | 8.0      | 1.22     | 44.6     | 1.92     | 75.4     | 2.01     |
| P3c        | 1.0     | 1.13     | 96.8     | 1.44     | 27.0     | 2.08     | 97.3     | 2.08     |

**Piper arboreum**

| Extraction | Sample* | Method 1 | Method 2 | Method 3 | Method 4 |
|------------|---------|----------|----------|----------|----------|
|            | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 | Yield   | Absorbance ratio A260/A280 |
| P1a        | 19.6    | 1.09     | 79.6     | 1.24     | 17.1     | 1.34     | 62.1     | 2.02     |
| P1b        | 19.0    | 1.13     | 44.2     | 1.45     | 2.6      | 1.87     | 72.1     | 2.01     |
| P1c        | 31.5    | 1.15     | 37.8     | 1.48     | 2.7      | 1.56     | 73.0     | 2.09     |
| P2a        | 22.4    | 1.19     | 37.1     | 1.51     | 3.4      | 1.65     | 80.2     | 2.00     |
| P2b        | 29.5    | 1.23     | 54.1     | 1.30     | 5.6      | 1.67     | 70.0     | 2.02     |
| P2c        | 6.8     | 1.18     | 49.0     | 0.94     | 6.2      | 1.63     | 79.8     | 2.01     |
| P3a        | 44.5    | 1.23     | 83.2     | 2.24     | 4.2      | 1.60     | 78.8     | 2.08     |
| P3b        | 24.5    | 0.95     | 52.7     | 2.30     | 7.0      | 1.54     | 80.7     | 2.15     |
| P3c        | 37.5    | 1.25     | 54.6     | 2.77     | 3.3      | 1.78     | 111.5    | 2.04     |
### Xylopia aromatica

| Extraction | Sample | Method 1 | Method 2 | Method 3 | Method 4 |
|------------|--------|----------|----------|----------|----------|
|            |        | Yield    | Absorbance ratio A260/A280 | Yield    | Absorbance ratio A260/A280 | Yield    | Absorbance ratio A260/A280 | Yield    | Absorbance ratio A260/A280 |
| 1          | X1a    | 102.5    | 1.59     | 86.3     | 1.29     | 2.5      | 1.94     | 44.7     | 2.02     |
|            | X1b    | 141.5    | 1.30     | 36.6     | 1.30     | 3.2      | 1.90     | 42.4     | 2.09     |
|            | X1c    | 82.5     | 3.73     | 19.5     | 0.73     | 14.2     | 2.01     | 46.5     | 2.05     |
|            | X2a    | 86.4     | 0.94     | 56.3     | 0.99     | 3.1      | 2.03     | 38.5     | 2.05     |
|            | X2b    | 24.0     | 1.82     | 74.6     | 1.02     | 3.1      | 1.90     | 43.3     | 2.12     |
|            | X2c    | 18.6     | 1.36     | 91.2     | 1.26     | 3.1      | 1.70     | 39.4     | 2.09     |
|            | X3a    | 52.1     | 1.26     | 18.1     | 1.26     | 3.5      | 2.01     | 42.1     | 1.90     |
|            | X3b    | 29.4     | 1.15     | 15.6     | 1.10     | 2.4      | 2.28     | 42.6     | 2.17     |
|            | X3c    | 38.5     | 1.53     | 44.9     | 1.56     | 3.4      | 1.79     | 43.2     | 2.09     |
| 2          | X2b    | 24.0     | 0.98     | 33.6     | 1.34     | 3.3      | 2.11     | 34.5     | 2.05     |
|            | X2c    | 50.2     | 0.77     | 14.1     | 1.05     | 8.4      | 2.27     | 26.3     | 1.97     |
|            | X3a    | 32.1     | 1.22     | 98.8     | 0.89     | 3.2      | 1.72     | 28.3     | 2.08     |
|            | X3b    | 52.1     | 1.26     | 18.1     | 1.26     | 3.5      | 2.01     | 42.1     | 1.90     |
|            | X3c    | 29.4     | 1.15     | 15.6     | 1.10     | 2.4      | 2.28     | 42.6     | 2.17     |
|            | X3c    | 38.5     | 1.53     | 44.9     | 1.56     | 3.4      | 1.79     | 43.2     | 2.09     |

*P1 to P3 and X1 to X3 refer to samples from three different plants with three replicates each (‘a’ to ‘c’). The extraction using each method was performed three times (Extraction 1 to 3).
For *P. arboreum* samples, Method 4 proved to be the most effective since it was possible to visualize bands related to rRNAs (Fig. 1) on three extractions, with A260/A280 values within optimal ranges (Tab. 2). For *X. aromatica*, similar results were obtained in two out of three extractions (Tab. 2), different from Method 3 where the results were obtained only for *P. arboreum* (Tab. 2; Fig. S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.12743429.v1>). Method 3 failed to generate visible bands in most samples and was considered inefficient. Thus, Method 4 showed to be more efficient for total RNA extraction for this type of plant and was selected for the tests involving 17 other native species from the Brazilian Cerrado (Tab. 1). When using Method 4, the pellet presented white coloration, slightly transparent, easily resuspended. Differently from Methods 1 and 2 in which the pellet was viscous and yellowish in color, and from Method 3 in which in most samples was not visible.

Spectrophotometer and agarose gel analysis varied among the tested species (Fig. 2; Fig. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.12743429.v1>), which was expected because they belong to different botanical families. After the RNA extraction by method 4, RT-PCR of 11 out of 17 species was performed. The fragments of 151 bp were amplified for 10 species, with soybean samples used as positive controls (Fig. 3).

**Discussion**

The TRIzol®-based methods 1 and 2 were not able to eliminate all contaminants that may interfere in the reactions leading to the extraction of high quality RNA. Such contaminants, probably phenolic compounds and polysaccharides, are present in high concentration in most woody species from the Brazilian Cerrado, such as *X. aromatica* (Stashenko *et al.* 2004). These contaminants can be held responsible for the viscous and yellowish appearance of the obtained pellet, which made the resuspension procedure difficult.

Phenolic compounds can irreversibly bind to nucleic acids, co-precipitating with RNA, thereby reducing the quality of the final product (Chang *et al.* 1993; Peng *et al.* 2014). In Methods 3 and 4, PVP and Beta-mercaptoethanol reagents are used as reducing agents to avoid oxidation of phenolic...
compounds. Furthermore, in Method 4 the high concentrations of NaCl on CTAB buffer and SSTE might have helped in polysaccharide removal (Chang et al. 1993).

Nevertheless, the main difference in efficiency of Method 4 over Method 3 is in the use of spermidine, a polyamine that is involved in cellular metabolism in different tissues. In plants, spermidine, and other polyamines are correlated with the response to abiotic stress and antioxidant activity (Diao et al. 2017; Liu et al. 2017; Zhang et al. 2017). Thus, spermidine may be directly related to maintenance of RNA quality throughout the extraction process by Method 4.

The quality of the extracted RNA is often revealed by the visualization of clear bands in agarose gel when it comes to the 18S and 28S regions of ribosomal RNA. The purity of the bands can be measured by the absorbance ratio A260/A280. This ratio indicates the level of protein contamination, based on the principle that nucleic acids and proteins exhibit optimal absorbance values of 260 nm and 280 nm respectively (Glasel 1995; Mathieson & Thomas 2013). Thus, the best values are between 1.5 and 2.0 in 260 nm, which represents how much nucleic acid is present in the solution in relation to the amount of proteins. The maximum of 2.0 is considered as above that value, the amount of RNA may be so high that it will negatively interfere with RT-PCR (Tattersall & Ergul 2005).

The TRIzol® reagent was feasible for herbaceous plants, such as the RNA extracted from soybean samples used in RT-PCR (Fig. 3). When this reagent was used for RNA extraction of woody species, the result was unsatisfactory. This may be explained by the chemical composition of these tree species, with high concentration of phenolic compounds and polysaccharides, interfering negatively in the reactions (Moreira et al. 2003; Calixto-Júnior et al. 2016).

The lack of phenol on the methods that presented the best results contributed to the production of appropriate samples for cDNA synthesis and subsequent quality of the RT-PCR products (Chang et al. 1993). This was shown when RT-PCR amplifications were satisfactory in ten out of 11 native tree species samples used (Fig. 3). The Zanthoxylum rhoifolium sample probably did not amplify through RT-PCR because of its low RNA concentration and high DNA that was eliminated in the DNAse treatment (Fig. S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.12743429.v1>).

Taking into account the differences in the composition of the Brazilian Cerrado tree species used in this work, it can be concluded that Method 4 showed efficiency for extraction of high-quality RNA from most of the species in this study. Thus, it is possible to recommend Method 4 for wide use.

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Figure 2 – Electrophoresis in 1.2% agarose gel of total RNA extraction by method 4-CTAB for five arboreal species from the Brazilian Cerrado. M = Marker 1 Kb Plus Ladder (Invitrogen®); C.c = Cardiopetalum calophyllum; N.l = Nectandra lanceolata; S.c = Styrax camporum; P.a = Piper arboreum; M.e = Matayba elaeagnoides. 1, 2 and 3 are different samples of each species.

Figure 3 – RT-PCR of total RNA extracted by method 4 from five woody species from the Brazilian Cerrado using the Gm18SrRNA primer pair. M = Marker 1 Kb Plus Ladder (Invitrogen®); P- = negative of PCR reaction; C- = negative control of cDNA synthesis; Gm1 = Glycine max (extraction by method 1); Gm2 = Glycine max (extraction by method 4) Xa = Xylopia aromatica; Pa = Piper arboreum; En = Emmotum nitens; Cf = Cedrela fissilis; Mf = Mabea fistulifera; Ad = Aspidosperma discolor; Sc = Styrax camporum; Isp. = Ixora sp.; Nl = Nectandra lanceolata; Me = Matayba elaeagnoides; Zr = Zanthoxylum rhoifolium.
use in molecular studies involving our native tree species, including the diagnosis of viral diseases via RT-PCR.

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