Efficient Method for Isolation of High-Quality RNA from *Psidium Guajava* L. Tissues

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Methodology

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Title: Efficient method for isolation of high-quality RNA from *Psidium guajava* L. tissues

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Abstract

Background: *Acquiring high-quality RNA in sufficient amounts is necessary in plant molecular biology and genetic studies. Several methods of RNA extraction from plants are available in the literature, mainly due to the great biochemical diversity present in each species and tissue, which can complicate or prevent the extraction. Psidium guajava (Myrtaceae family) is a perennial fruit tree of medicinal and economic value; nevertheless, only few molecular and omics studies are available for the species. One reason for this fact is the difficulty in obtaining the RNA due to the content of the samples, which are rich in polyphenols, polysaccharides and secondary metabolites. Furthermore,*

there is still no tested or standardized method available for the isolation of RNA from guava or *Psidium* samples, which hampers advances in the genus.

Results: Here we compare the quality and yields of RNA isolated using six extraction protocols: two based on the application of cetyltrimethylammonium bromide (CTAB) lysis buffer, two commercial kits (PureLink™ RNA Mini Kit and RNeasy® Plant Mini Kit), one using the TRIzol® reagent, and one applying guanidine thiocyanate lysis buffer followed by organic phase extraction. RNA integrity, quality and yields were assessed by agarose gel electrophoresis and spectrophotometry. The CTAB-based method provided the highest RNA yields and quality for five different tissues (flower bud, immature leaf, young leaf, mature leaf and root), genotypes and stress conditions.

For the most efficient protocol, the average yield of RNA from guava leaf was 210.4 µg/g of tissue, and the A_{260}/A_{280} and A_{260}/A_{230} ratios were 2.1 and 2.2, respectively. RT-PCR analysis demonstrated that the purity of the samples was sufficient for molecular biology experiments.

Conclusion: CTAB-based methods for RNA isolation were found to be the most efficient, providing the highest RNA yields and quality for tissues from *P. guajava*. Additionally, they demonstrated to be compatible for downstream RNA-based applications, besides showing the advantages of lower cost and time investments.

Keywords: RNA, guava, *Psidium guajava*, Myrtaceae, isolation method, CTAB.

Background

Advances in the knowledge of cell and molecular biology – such as the discovery of genes, underlying mechanisms of gene regulation, signal transduction and the factors involved in phenotypic characteristics – require a whole range of techniques, such as

northern hybridization, reverse transcription, polymerase chain reaction (PCR), construction of cDNA libraries, and sequencing. Obtaining high RNA quality and yields is fundamental for the execution and success of these approaches. The isolation of intact RNA is difficult due to the chemical nature of the RNA molecule, which is more susceptible to hydrolysis, and its sensitivity to enzymatic degradation by ribonucleases (RNases), which are very active, widespread, stable, and require no-cofactors [1, 2]. Furthermore, the isolation and purification of high-quality RNA from plant tissues in sufficient amounts has been reported to be difficult in samples rich in polyphenols, polysaccharides and other secondary metabolites that interfere with the quantification and subsequent applications [3–7].

Guava (*Psidium guajava* L., family Myrtaceae) is an important commercial fruit crop cultivated in tropical and subtropical regions of the world. The largest producer countries are India, China, Thailand, Pakistan, Mexico, Indonesia and Brazil, respectively [8]. Different parts of the plant are rich in nutrients and functional elements, such as antioxidants, vitamin C, potassium and fiber [9, 10]. It is also widely used for medicinal purposes owing to its healing, anti-allergic, anti-diabetic, anti-diarrheal, anti-neoplastic, anti-inflammatory and anti-microbial properties [11–17]. Although relevant knowledge is available about the species' biochemical composition, benefits and applications, when compared to other crops, genetic and genomic studies in guava are incipient. Developments in the genetic analysis and functional genomics of guava could complement the conventional breeding process, leading to improvement of crop productivity and addressing the challenges of enhancing fruit quality and tolerance to abiotic and biotic stresses [18–20]. However, the success of many downstream RNA-based applications relies on obtaining high-quality RNA.

The use of cetyltrimethylammonium bromide (CTAB) to isolate nucleic acids was originally described in 1953 for bacterial samples [21]. Since then, this protocol has been extensively used and modified for the extraction of DNA and RNA from several species [22–26]. In recent years, CTAB-based methods have been used for nucleic acid isolation from many plant species, especially those containing a high level of phenolic compounds and polysaccharides [3, 27–29]. Most of the popular RNA isolation protocols are based on guanidine salts, such as the reagent TRIzol® (Invitrogen, USA) and commercial kits like RNeasy® (QIAGEN, Germany) and PureLink™ RNA (Invitrogen, USA). Although this method has been used successfully for the isolation of RNA from tissues of a variety of plants [30–34], for other species, protocols based on the guanidine method have proven to be unable to isolate quality RNA with satisfactory yields; besides, they increase the chances of co-purifying contaminants, which interferes with downstream applications [7, 35, 36].

The present study was motivated by the lack of knowledge about which methods are efficient for the extraction of sufficient quantities of high-quality RNA from *P. guajava*, mainly for use in studies of gene expression and omics analyses. Here, we examined the use of two extraction buffers based on a cationic surfactant, CTAB, and four methods using chaotropic lysis buffers containing guanidine thiocyanate. In the guanidine group, two commercial kits that use silica membrane to isolate RNA were evaluated, the PureLink™ RNA Mini Kit and the RNeasy® Plant Mini Kit. The third method is based on the association of lysis buffer with phenol-chloroform, the TRIzol® reagent. The last protocol in this group is the guanidine thiocyanate method, the only already described for the isolation of *P. guajava* RNA [37]. Our goal was to identify an efficient RNA isolation method for various *P. guajava* tissues that can be used in plant molecular biology studies.

98 **Results and Discussion**

99 RNA isolation plays an important role in various experiments in plant molecular
100 biology, such as gene expression and transcriptomics. However, obtaining RNA with
101 enough quality and quantity may be a great challenge, mainly for plant species, since their
102 biochemical composition can hinder and even prevent the extraction. Besides, due to the
103 rich variety of cellular chemical contents of plants, there are no standard methods for the
104 isolation of RNA applicable to all plant species. Members of the Myrtaceae family are
105 characterized by abundance of essential oils rich in terpenes, tannins, phenolic
106 compounds, polysaccharides and other secondary metabolites that hinder the extraction
107 of nucleic acids from these species [10, 38, 39]. This highlights the need to seek an
108 efficient, uncomplicated and cost-effective method and thus contribute to the advances in
109 molecular studies for the species of this family.

110 In this study, six RNA isolation methods were compared, and the success of each
111 protocol was judged by the quantity, purity and integrity of the recovered RNA. Figure 1
112 shows the outcomes for RNA integrity, assessed by agarose gel electrophoresis. For
113 CTAB₁ and 2 as well as the guanidine thiocyanate protocols, distinct 28S and 18S rRNA
114 bands were visible, with high brightness and no obvious smearing due to degradation,
115 suggesting that these methods ensured RNA integrity. Differently, for the samples
116 prepared with TRIzol[®], the RNeasy[®] Plant Mini Kit and the PureLink[™] RNA Mini Kit
117 protocols, no band or degradation smear could be observed. The A_{260}/A_{280} and A_{260}/A_{230}
118 ratios are often used to indicate RNA sample purity, with ratio values of ~2.0 generally
119 indicating high RNA purity. The values for A_{260}/A_{280} ratio (Table 1) for CTAB₁ ($2.10 \pm$
120 0.03), CTAB₂ (2.13 ± 0.04) and the guanidine thiocyanate protocols (1.95 ± 0.14)
121 indicated that these methods were efficient in preventing mainly protein contamination.

On the other hand, for TRIzol®, the RNeasy® Plant Mini Kit and the PureLink™ RNA Mini Kit protocols, the low values indicate that there were problems in the extraction and a substantial amount of proteins precipitated with the nucleic acid. Importantly, with regard to the ratio A_{260}/A_{230} , only CTAB-based protocols showed values that correspond to high purity (2.24 ± 0.33 and 2.24 ± 0.07). The other samples showed high absorbance at 230 nm, a wavelength at which carbohydrates, phenols and aromatic compounds generally absorb.

Figure 1 – Qualitative analysis of RNA by electrophoresis. Denaturing agarose gel (1%) electrophoresis of total extracted RNA (2 μ L) stained with GelRed® (Biotium, USA). For the positive control (C+), total RNA was extracted from soybean (*Glycine max*) leaves, and extractions using *P. guajava* leaves were performed in duplicate (R1 and R2).

Table 1 – Analysis of RNA yield and purity for different methods

Method	n	Yield (ng/ μ L)	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
CTAB ₁	6	384.15 $\pm$ 69.35	2.10 $\pm$ 0.03	2.24 $\pm$ 0.33
CTAB ₂	6	701.27 $\pm$ 191.96	2.13 $\pm$ 0.04	2.24 $\pm$ 0.07
PureLink™ RNA Kit	5	140.45 $\pm$ 32.13	1.00 $\pm$ 0.20	0.25 $\pm$ 0.06
RNeasy® Plant Kit	6	33.20 $\pm$ 6.92	1.18 $\pm$ 0.14	0.17 $\pm$ 0.05
TRIzol® reagent	6	447.95 $\pm$ 134.98	1.09 $\pm$ 0.13	0.41 $\pm$ 0.10
Guanidine thiocyanate	6	445.80 $\pm$ 257.13	1.95 $\pm$ 0.14	1.63 $\pm$ 0.26

The values correspond to the median of the values and MAD (median absolute deviation) obtained between the replicates (n) of each method in two independent experiments.

The results of the qualitative analyses (Figure 1) are consistent with the quantifications obtained by spectrophotometry shown in Table 1. The RNA concentrations of the samples were obtained by evaluating the absorbance at 260 nm (A_{260}). In this analysis, the RNA samples from CTAB₂ had the highest yields, 210.4 ± 57.6 μ g of RNA for each gram of leaf tissue. CTAB₁ and the guanidine thiocyanate methods also provided good yields (115.25 ± 20.81 and 133.74 ± 77.14 , respectively). However, the yield of the guanidine-based method is possibly not accurate, since the intensity of the rRNA bands is weaker than in the CTAB₁ method. This may be a reflection of contaminants present in the guanidine thiocyanate samples, evidenced by the low 260/230 ratio (1.63 ± 0.26), or genomic DNA contamination. The contaminants also absorb in the 260 nm range, thus distorting the quantification. The same occurred in samples extracted with TRIzol[®], which showed great absorption at A_{260} , but presented very low $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios (1.09 ± 0.13 and 0.41 ± 0.10) and no visible bands in the agarose gel. Additionally, both evaluated commercial kits failed to isolate RNA from *P. guajava* leaves, as demonstrated by the low obtained yields and the analysis of $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios indicating great contamination of the samples.

The comparative evaluation of these results allowed to determine that CTAB-based methods were more efficient for isolating RNA from *P. guajava* leaves, whereby the CTAB₂ protocol was the most efficient, isolating RNA with high purity, integrity and yields. These findings are compatible with other studies which demonstrated that methods based on guanidine salts, TRIzol[®] and commercial kits were not effective for extracting RNA from species rich in secondary metabolites [4, 7, 35]. The CTAB-based methodology has also been used successfully by Jaakola *et al.* (2001) for the extraction of high-quality RNA from *Vaccinium myrtillus* [40], and by Zeng & Yang (2002) in *Cinnamomum tenuipilum* [29], both perennial tree species. More recently, researchers of

species from the Myrtaceae family have isolated RNA by the CTAB method and performed analyses of transcriptome and gene expression, such as Guzman *et al.* (2014) in *Eugenia uniflora* [41], and Vining *et al.* (2015) and Favreau *et al.* (2019) in *Eucalyptus grandis* [42, 43]. In the study involving *P. guajava*, Furlan *et al.* (2012) isolated RNA using the guanidine thiocyanate buffer evaluated in this study [37]. However, in the comparative analysis, this method did not show the best results.

Guava samples have a high content of polyphenols, polysaccharides, tannins, among other metabolites that may interfere with the RNA isolation [44, 45]. In solution, phenolic compounds are easily oxidized to form quinones, which can bind to the RNA and render it insoluble, hindering the RNA isolation and/or downstream applications [45]. During extraction, polysaccharides produce saccharide fragments that may be co-purified with the RNA, owing to their chemical characteristics being very similar to nucleic acids [44, 46]. The greater efficiency of the extraction by the CTAB method may be related to the composition of the respective lysis buffer. CTAB is a cationic surfactant, acting as a strong detergent to help break membranes and separate the nucleic acids from polysaccharides and cellular debris [47]. The oxidation of polyphenols is prevented by the use of soluble polyvinylpyrrolidone polymers (PVP) that immediately bind to those compounds, and this preclude the formation of quinones and their binding to RNA [40]. Also heating of the extraction buffer with a relatively high concentration of β -mercaptoethanol promotes more efficient tissue disruption, removal of tannins and polysaccharides, and prevention of RNase activity [48, 49]. Carbohydrate contamination is reduced by the use of LiCl in the precipitation step, since the saccharides remain in solution while the RNA precipitation occurs [48].

It is known that the composition varies greatly between tissues, not less under stress conditions, owing to the physiological metabolism and remodeling of pathways for

the stress response. Moreover, isolating RNA of adequate quality from tissues exposed to stressful conditions such as salinity and heavy metal toxicity is difficult due to increased accumulation of reactive oxygen species (ROS), secondary metabolites and other compounds that easily degrade nucleic acids or promote chemical changes in nucleic acids that hinder isolation [50, 51]. With the aim of expanding the analysis and verifying the usefulness of the proposed protocol, we also evaluated the ability of the CTAB₂ protocol to gain RNA from different *P. guajava* tissues (Figure 2 and Table 2), from tissues under salt and cadmium stress conditions (Table 2), and from another species of the same genus, *Psidium guineense* (Supplementary information – S1).

In summary, the proposed protocol provided high RNA yields, quality and purity in the assessed tissues and conditions. Even in developing tissues, such as flower buds, which usually accumulate high amounts of secondary metabolites and have been reported with low quality [52], in this study, good yields and quality were observed. The best results were obtained for the immature leaf and root tissues, evidenced mainly by the analysis using TapeStation[®] capillary electrophoresis (Figure 2.B), in which these samples presented RIN (RNA Integrity Number) [53] of 7.2 and 8.8, respectively. For leaf samples from plants under salt and cadmium stress (Table 2), reduction in yield was observed; however, this result was already expected, since these treatments can induce the degradation of nucleic acids as a result of the cell death trigger as well as shift in the chemical content [54, 55].

The presence of contaminants in samples not only hampers the isolation and quantification of the RNA, but may also interfere with the activity of some enzymes, such as reverse transcriptase in the synthesis of cDNA and DNA polymerase in PCR. Substances used in extraction techniques, such as ethanol, phenol, chloroform and salts, are described to inhibit these enzymes and consequently impair these techniques.

213 **Figure 2 – Qualitative analysis of RNA from different tissues.** (A) Denaturing agarose
214 gel (1%) electrophoresis of total extracted RNA (2 μ L) stained with GelRed[®] (Biotium,
215 USA). Extractions using the CTAB₂ protocol were done in duplicate (R1 and R2) for
216 different tissues from two distinct genotypes of *P. guajava*, Cortibel RM and Paluma. (B)

Quality analysis of RNA samples by TapeStation® (Agilent Technologies, USA) using RNA ScreenTape® assay, after DNase I treatment, and RIN (RNA Integrity Number) were calculated. (C) Quality check of cDNA from CTAB₂ samples (Cortibel RM tissues) by amplification of histone H2A gene measured using SYBR® Green qRT-PCR, and melting curve showing specific amplification products.

Table 2 – Analysis of RNA yield and purity using CTAB₂ method

RNA yield and purity for different tissues					
	Tissue	n	Yield (ng/μL) *	A ₂₆₀ /A ₂₈₀ ratio *	A ₂₆₀ /A ₂₃₀ ratio *
Cortibel RM	Flower bud	4	2325.10 ± 520.53	2.08 ± 0.13	2.28 ± 0.07
	Immature leaf	12	1004.48 ± 184.14	2.15 ± 0.04	2.21 ± 0.08
	Young leaf	12	759.94 ± 64.63	2.14 ± 0.01	2.33 ± 0.05
	Mature leaf	12	539.02 ± 88.87	2.11 ± 0.04	2.40 ± 0.07
	Root	12	618.77 ± 118.68	2.19 ± 0.03	2.55 ± 0.35
Paluma	Immature leaf	12	842.80 ± 248.90	2.14 ± 0.03	2.24 ± 0.10
	Young leaf	12	775.80 ± 110.69	2.17 ± 0.04	2.31 ± 0.06
	Mature leaf	12	358.74 ± 67.82	2.22 ± 0.03	2.32 ± 0.07
	Root	12	274.49 ± 31.53	2.21 ± 0.02	2.53 ± 0.10
RNA yield and purity for stressed leaves from different <i>P. guajava</i> genotypes					
	Genotype	n	Yield (ng/μL) #	A ₂₆₀ /A ₂₈₀ ratio #	A ₂₆₀ /A ₂₃₀ ratio #
Control	Paluma	6	629.95 ± 102.41	2.25 ± 0.07	2.40 ± 0.21
	Cortibel LG	6	749.24 ± 298.81	2.22 ± 0.02	2.37 ± 0.04
	Cortibel RG	6	622.35 ± 43.88	2.20 ± 0.02	2.36 ± 0.01
	Cortibel RM	4	681.24 ± 87.74	2.21 ± 0.02	2.36 ± 0.07
	Cortibel XIII	4	805.02 ± 450.30	2.17 ± 0.03	2.24 ± 0.08
Salt Stress	Paluma	6	615.00 ± 52.30	2.15 ± 0.01	2.33 ± 0.05
	Cortibel LG	6	633.40 ± 102.98	2.18 ± 0.03	2.38 ± 0.06
	Cortibel RG	6	601.74 ± 152.71	2.15 ± 0.01	2.10 ± 0.02
Cd	Cortibel RM	3	204.59 ± 146.71	2.09 ± 0.08	1.88 ± 0.38
	Cortibel XIII	4	219.99 ± 87.43	2.08 ± 0.09	2.06 ± 0.23

* The values correspond to the median of the values and MAD (median absolute deviation) obtained between the replicates (n) of each condition in two independent experiments.

The values correspond to the mean of the values and SD (standard deviation) obtained between the replicates (n) of each condition. Cd = Cadmium stress.

However, inherent substances in the sample, such as polyphenols and polysaccharides can also co-precipitate with the RNA and affect downstream applications [56–61]. In that regard, to ascertain the quality of the samples for applications in molecular biology, samples of four different tissues (immature leaf, young leaf, mature leaf and root) were also evaluated via qRT-PCR. All assessed samples showed a characteristic amplification curve and unique peaks in the melting analysis, demonstrating their adequate quality for molecular biology studies (Figure 2C).

In addition, to further investigate the differences between the CTAB protocols, the presence of inhibitors in the samples was inferred by quantitative analysis via qRT-PCR. Linear regression curves based on two-fold serial dilutions containing prepared cDNA synthesized from the same volume of RNA (5 μ L) were used for all samples. This care was taken to enable comparing the interference of the contaminants in the samples, in which the prevalent is the sample volume that directly impacts the inhibitor concentration. On average, 1 μ L of cDNA contained 100 ng ($\pm$ 25 ng). From the cycle threshold (Ct) values obtained by amplification of the Histone H2A gene, a linear equation was generated for each sample. Under ideal conditions, each amplification cycle duplicates the cDNA content, and the efficiency of the reaction is said to be one hundred percent. However, especially for samples obtained from plants, without an additional purification step the presence of contaminants is very frequent [62].

Figure 3 shows the graphs for all samples. The effect of PCR inhibition is reflected on the distance between the dots and lines (linear equation) and the obtained values (black) as well as the ideal values (gray). From these results, it can be inferred that all samples present some level of DNA polymerase inhibition, and the interference is slightly more accentuated in CTAB₂ than in CTAB₁ samples.

Figure 3 – Quantitative analysis by qRT-PCR to compare CTAB methods. Linear regression curves of the real-time reverse transcription polymerase chain reaction assay based on serial dilutions of cDNA (1.0, 0.5, 0.25 and 0.125 μL) synthesized from the same volume of RNA (5 μL) were used for all samples. The log of cDNA volume (μL) is indicated on the x-axis, whereas the corresponding Ct values are shown on the y-axis. Each dot represents the mean result of three replicates. The correlation coefficient (R²) and linear equation of the regression analysis are shown. Values obtained in this assay for each sample are represented in black and ideal values in gray, estimated based on the Ct

values of the highest dilution, considering an efficiency of one hundred percent in each cycle.

These effects probably reflect competitive inhibition, since dilution of the samples was followed by reduction of interference. It is also worth mentioning that, although the CTAB₂ protocol apparently shows a greater amount or greater inhibitory effect, it will most likely not hamper the qRT-PCR analysis, which can be explained by a few aspects. First, as indicated by analysis of the square of the correlation coefficient (R^2), the contamination follows a linear regression. Second, the amount usually applied in the PCR reaction is between 30-10 ng per reaction and, for this range, the values obtained are very close to the ideal. Lastly, although the samples for this test were selected within the same concentration range (Supplementary information S2), those obtained using CTAB₂ are consistently more concentrated, as already discussed and presented in Table 2. Thus, in each PCR reaction, in order to apply the same amount of template, the sample must be further diluted, which will additionally minimize this effect of contaminants.

Conclusion

In the present study, we compared six RNA isolation methods, and CTAB-based protocols were found to be the most efficient, providing the highest RNA yields and quality for different tissues, genotypes, and under stress conditions. Additionally, we demonstrated that samples are compatible for downstream RNA-based applications, besides showing the advantages of lower cost and time investments. From that knowledge, we hope to contribute to the increase and quality of molecular genetic studies for *Psidium guajava* the related species.

Methods

Plant Material

Clonal seedlings of *Psidium guajava* (Paluma and Cortibel RM genotypes) were obtained from the Frucafé Nursery, accredited by the Brazilian Ministry of Agriculture. The specimens used in this study were obtained and maintained in healthy conditions in a greenhouse.

For the comparison of protocols, six leaf discs with diameter of 0.8 cm (70-100 mg) were collected from young leaves of the Paluma genotype when the plants were approximately 100 days old. Each sample represented a pool of tissues obtained from three different plants. All collected biological material was immediately frozen in liquid nitrogen and subsequently stored in an ultra low temperature (ULT) freezer at -80°C.

To evaluate the efficiency of RNA isolation in different tissues and genotypes, flower bud, immature leaf, young leaf, mature leaf and root from the Cortibel RM genotype and immature leaf, young leaf, mature leaf and root from the Paluma genotype were used (Supplementary information – S3). Approximately 100 mg (80-110 mg) of tissue were used for each sample, except the root, for which 200 mg of tissue were used.

RNA Isolation

All reagents were prepared in RNase-free ultrapure water, and glasses, mortars and pestles were treated with 1 M NaOH, autoclaved, and then baked overnight at 60°C. At least five extractions were performed for each protocol, as well as two extractions with soybean (*Glycine max*) as positive control. All samples were extracted starting from the same material and in the same quantity. The tissue samples were ground in liquid nitrogen using a porcelain mortar (60 mL) until becoming a fine powder. Care was taken to ensure

that the material did not thaw in this process. The extraction buffer corresponding to each method was added to samples of the obtained tissue powder.

Cetyltrimethylammonium Bromide (CTAB)-based Methods

Extraction buffer: 2% CTAB, 2% PVP (mol wt 30,000), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.05% spermidine trihydrochloride, 2% β -mercaptoethanol (added just before use).

CTAB Protocol 1 (CTAB₁):

This CTAB protocol was initially adapted by Zeng and Yang (2002) and minor modifications were made here, such as reducing the amount of starting material. Briefly, 900 μ L of extraction buffer were heated at 65°C and added to a mortar containing the sample (~100 mg, powdered in liquid nitrogen as described above), then transferred to a 2.0 mL microtube, and vigorously shaken several times. The mixture was incubated at 65°C for 10 min under shaking. Next, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and the microtube was vigorously mixed. The tube was centrifuged at 10,000 $\times g$ for 10 min at 4°C. The supernatant was recovered and transferred to a new 1.5 mL microtube and re-extracted with an equal volume of chloroform/isoamyl alcohol (~650 μ L). The supernatant was slowly and carefully collected to avoid contamination, and a new centrifugation was performed at 25,000 $\times g$ for 30 min at 4°C to sediment and discard the insoluble material. To precipitate the RNA, 0.5 volume of 5 M LiCl was added to the supernatant, mixed well, and the tube was subsequently incubated at 4°C overnight. The RNA pellet was obtained by centrifugation at 25,000 $\times g$ for 30 min at 4°C. The supernatant was discarded, and the pellet was washed three times with 75% (v/v) ethanol

and centrifuged at 10,000 ×g for 20 min at 4°C. The pellet was dried at room temperature and solubilized in 30 µL of RNase-free ultrapure water. The extracted RNA was stored in an ULT freezer at -80°C for subsequent analyses.

CTAB Protocol 2 (CTAB₂):

This CTAB protocol was adapted by Guzman *et al.* (2014), and here the initial sample amount was reduced from 150 mg to 100 mg of tissue, besides minor changes to some procedures. Initially, 900 µL of pre-warmed extraction buffer (65°C) was added to mortar containing tissue powder, and the mixture was stirred until becoming homogeneous. The mixture was then transferred to a 2 mL microtube and incubated at 65°C for 10 min. Next, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and the tube was vigorously mixed. The microtube was centrifuged at 7,000 ×g for 20 min at 4°C. The supernatant was recovered and transferred to a new 1.5 mL microtube, then re-extracted with an equal volume of chloroform/isoamyl alcohol (~650 µL). Next, 0.5 volume of 5 M LiCl was added to the supernatant, with subsequent incubation at 20°C for 4 hours. The RNA was selectively pelleted by centrifugation at 16,000 ×g for 30 min at 4°C. The pellet was washed with 75% (v/v) ethanol and dried at room temperature. The RNA was solubilized in 30 µL of RNase-free ultrapure water and stored in an ULT freezer at -80°C for subsequent analyses.

PureLink™ RNA Mini Kit

Approximately 100 mg of tissue powdered in liquid nitrogen were used to obtain total RNA. After tissue disruption, 600 µL of the Lysis Buffer (already containing 10 µL of 2-mercaptoethanol for each 1 mL of buffer) was added to promote chemical lysis of the cells. Into the tissue homogenate, 0.5 volume of absolute ethanol was added and the

mixture was vortexed. Next, 700 μ L of the lysate was transferred to silica spin column for the binding of RNA. The column was centrifuged at 12,000 $\times g$ for 15 seconds. This procedure was carried out until all the lysate had passed through the column and the flow-through was discarded. Subsequently, the column was washed with 700 μ L of Wash Buffer I and centrifuged at 12,000 $\times g$ for 15 seconds to remove the residual buffer. A second wash was performed using 500 μ L of Wash Buffer II, and a new centrifugation under the same conditions was performed. That last step was repeated. The column was subjected to additional centrifugation to remove wash buffer residues. The silica spin column was transferred to a new 1.5 mL microtube for performance of RNA elution, in which 30 μ L of RNase-free ultrapure water was used. The obtained RNA was stored in an ULT freezer at -80°C.

RNeasy® Plant Mini Kit

After tissue disruption with liquid nitrogen in a mortar, 450 μ L of Buffer RLC (already containing 10 μ L of 2-mercaptoethanol for each 1 mL of buffer) was added to the powdered sample (~100 mg), and the mixture was homogenized vigorously. To facilitate tissue disruption, the mixture was incubated at 56°C for 2 min. The lysate was transferred to a QIAshredder spin column (lilac) and centrifuged at 14,000 $\times g$ for 2 min. The supernatant of the flow-through was transferred to a new 2 mL microtube without disturbing the pellet. Half a volume (~250 μ L) of absolute ethanol was added to each sample and the mixture was homogenized by pipetting. Next, ~650 μ L of cleared lysate were transferred to the RNeasy® Mini spin column (pink) and immediately centrifuged at 8,000 $\times g$ for 15 seconds. The flow-through was discarded, and the column was washed with 700 μ L of Buffer RW1 and centrifuged again at 8,000 $\times g$ for 15 seconds. The flow-through was discarded and 500 μ L of Buffer RPE was added to the column, followed by

another centrifugation at 8,000 $\times g$ for 15 seconds. To remove any residue from the wash buffer, an additional centrifugation at 10,000 $\times g$ for 1 min was performed. The column was then transferred to a new 1.5 mL microtube, and the RNA was eluted from the column by addition of 30 μ L of RNase-free ultrapure water and centrifugation at 8,000 $\times g$ for 1 min. To improve performance, the flow-through was reapplied to the column and again centrifuged. The RNA was stored in an ULT freezer at -80°C.

TRIzol[®] Method

For the extraction of total RNA, the leaf tissue (~100 mg) was powdered in a mortar using liquid nitrogen and 1 mL of the TRIzol[®] reagent (Invitrogen) was added to the sample, which was homogenized using a pestle. The entire mortar content was transferred to a 1.5 mL microtube and incubated for 5 min at room temperature. A centrifugation step at 12,000 $\times g$ for 10 min at room temperature was performed according to the manufacturer's specifications. The whole supernatant was transferred to a new 1.5 mL microtube. Subsequently, 200 μ L of chloroform was added to the sample, which was shaken for 30 seconds and incubated for 5 min at room temperature under constant slow shaking. The sample was then centrifuged at 12,000 $\times g$ for 15 min at 4°C. The aqueous phase was transferred to a new 1.5 mL microtube. For RNA precipitation, an equal volume of isopropanol (~500 μ L) was added to the sample, which was incubated at room temperature for 15 min. After this step, the sample was centrifuged at 12,000 $\times g$ for 10 min at 4°C and the supernatant was discarded. The pellet was washed using 1 mL of 75% (v/v) ethanol. The microtube was centrifuged at 10,000 $\times g$ for 5 min at 4°C, and the ethanol was completely removed. The pellet was dried at room temperature and the RNA was solubilized in 30 μ L of RNase-free ultrapure water. The RNA was stored in an ULT freezer at -80°C.

Guanidine Thiocyanate Method

Extraction buffer: 4 M guanidine thiocyanate, 100 mM Tris-HCl (pH 8.0), 25 mM sodium citrate pH 8.0, 0.5% N-laurylsarcosine, 1% PVP (mol wt 30,000), 2% β -mercaptoethanol (added just before use).

This method was used by Furlan *et al.* (2012) for RNA extraction from *P. guajava* tissue, but was adapted by Salzman *et al.* (1999). Here, we proposed a small adjustment to the initial buffer volume and amount of tissue. Initially, 900 μ L of extraction buffer was added to 100 mg of powdered tissue, disrupted using liquid nitrogen in a mortar. After vigorous homogenization, the mixture was transferred to a 2 mL microtube and incubated for 1 min at room temperature. Subsequently, 750 μ L of chloroform/isoamyl alcohol (24:1, v/v) was added and the sample was mixed slowly by inversion for 10 min. Next, the sample was centrifuged at 16,000 $\times g$ for 10 min at 4°C. The aqueous phase (~600 μ L) was transferred to a new 1.5 mL microtube and re-extracted with an equal volume of chloroform/isoamyl alcohol. After centrifugation under the same conditions as described above, the aqueous phase was recovered and transferred to a new 1.5 mL microtube. The RNA was then precipitated by the addition of 2 volumes of cold absolute ethanol and 0.1 volume of 5 M NaCl. The sample was centrifuged at 16,000 $\times g$ for 10 min at 4°C and the formed pellet was washed with 75% ethanol. The RNA was solubilized in 800 μ L of RNase-free ultrapure water and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) was added. The mixture was homogenized for 5 min at room temperature, then centrifuged at 12,000 $\times g$ for 10 min at room temperature. The aqueous phase was recovered and transferred to a new 1.5 mL microtube. The RNA was precipitated by addition of 2 volumes of ice-cold absolute ethanol and 0.1 volume of 5 M NaCl, with overnight incubation at -20°C. The samples

were centrifuged at 16,000 ×g for 10 min at 4°C, and the formed pellet was washed with 75% (v/v) ethanol. The pellet was dried at room temperature, and the RNA was solubilized in 30 µL of RNase-free ultrapure water and stored in an ULT freezer at -80°C for subsequent analyses.

RNA Analysis (Yield, Purity and Integrity)

RNA purity and concentration were assessed by determining the absorbance of RNA in RNase-free water at 230, 260 and 280 nm, using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The RNA yield was calculated based on absorbance at 260 nm (A_{260}). The ratios A_{260}/A_{280} and A_{260}/A_{230} were assessed to evaluate RNA purity. RNA integrity was evaluated from the 28S and 18S rRNA bands in 1.0% (w/v) formaldehyde–agarose gel after electrophoresis, staining with 1:20,000 GelRed® (Biotium) and visualization with Gel Doc XR+ System (Bio-Rad, USA). Quality analysis of eighth RNA samples were assessed by TapeStation® (Agilent Technologies, USA) using RNA ScreenTape® assay, after DNase I treatment and RIN (RNA Integrity Number) were calculated.

cDNA Synthesis and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Initially, the RNA was treated with RQ1 RNase-Free DNase I (Promega, USA) following the manufacturer's instructions. The first-strand cDNA was synthesized from 3.0 µg of the total RNA by reverse transcriptase with random primer (50 ng/µL), according to instructions of the SuperScript™ IV First-Strand Synthesis System (Invitrogen, USA). The PCR reaction mix (20 µL) was prepared following instructions of the PowerUp SYBR® Green Master Mix (Life Technologies, USA) in a LightCycler® 96

System (Roche Life Science, Germany) thermal cycler. The cycling conditions were as follows: initial denaturation at 95°C for 2 min; 40 cycles at 95°C for 15 seconds and 72°C for 60 s; and standard dissociation step from 50 to 95°C with increments of 0.5°C for 10 seconds. Real-time PCR analyses were performed to evaluate the quality of the RNA samples from *P. guajava* tissues obtained using the CTAB₂ protocol. Amplification of the samples was performed using a common endogenous gene, Histone H2A, forward primer 5'-AAGCCGGTCTCTCGGTCTGT-3', reverse primer 5'-GCATTACCAGCCAACTCCAG-3' (Supplementary information S4).

Inhibition test

Quantitative analysis was performed to evaluate the presence of inhibitors in samples from *P. guajava* leaves obtained using the tested CTAB protocols. For this test, RNA was treated with RQ1 RNase-Free DNase I (Promega, USA) following the manufacturer's instructions. The first cDNA strand was synthesized from 5.0 µL of the total RNA by reverse transcriptase with random primer (50 ng/µL), according to the instructions of the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). qRT-PCR amplification was performed using a common endogenous gene, Histone H2A. Standard curves were constructed for each sample, in which the largest volume of cDNA was 1 µL (~100 ng per reaction) and the lowest was 0.125 µL (~12.5 ng per reaction), following a dilution factor of 1:2. From the Ct values obtained for each dilution, a graph was plotted relating Ct vs. log (cDNA volume), and the linear equation was obtained for each sample. Using the Ct value of the lowest dilution, a curve was estimated in which the efficiency would be optimal, increasing one Ct value per cycle.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Carpinetti PA, Ferreira MFS and Ferreira A designed the study. Carpinetti PA, Fioresi VS, Cruz TI, Almeida F and Canal D performed the protocol evaluations. Carpinetti PA, Fioresi VS and Almeida F carried out the extraction of different tissues, genotypes and

samples under stress. Fioresi VS and Carpinetti PA are responsible for the qRT-PCR analysis. Carpinetti PA, Ferreira MFS, Fioresi VS, Almeida F and Ferreira A wrote the manuscript. All authors have read and approved the final manuscript.

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Not applicable

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Supplementary information

Analysis of RNA yield and purity of in vitro plant samples from *Psidium guineense*

Samples	Yield (ng/ μ L)	A ₂₆₀ /A ₂₈₀ ratio	A ₂₆₀ /A ₂₃₀ ratio
1	141.43	2.12	2.15
2	185.18	2.08	2.15
3	336.43	2.15	2.15
4	235.04	2.06	2.11
5	330.05	2.19	2.28
Mean	245.63	2.12	2.17
SD	77.45	0.05	0.06

Supplementary information S1 – Analysis of RNA samples from *P. guineense*. Table summarizing the quantification of the RNA samples in a NanoDrop spectrophotometer (Thermo Fischer, USA), as well as the ratios A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀. Denaturing agarose gel (1%) electrophoresis of total extracted RNA (2 μ L) stained with GelRed (Biotium, USA).

RNA yield and purity of samples used in qRT-PCR inhibition test

	Samples	Yield (ng/ μ L)	A ₂₆₀ /A ₂₈₀ ratio	A ₂₆₀ /A ₂₃₀ ratio
CTAB₁	A	294.2	2.07	2.53
	B	260.5	2.16	2.40
	C	339.6	2.13	2.49
	D	460.8	2.13	2.51
	E	339.6	2.13	2.49
	F	301.7	2.17	2.51
CTAB₂	A	420.12	2.23	2.42
	B	525.69	2.04	2.45
	C	378.84	2.22	2.17
	D	339.81	2.23	2.27
	E	435.11	2.12	2.47
	F	622.22	2.19	2.37
	Mean	396.9	2.16	2.42
	SD	99.45	0.06	0.10

Supplementary information S2 – RNA yield and purity of samples used in qRT-PCR inhibition test. Table summarizing the quantification of the RNA samples in a NanoDrop spectrophotometer (Thermo Fischer, USA), as well as the ratios A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀.

Supplementary information S3 - Five different tissues from *P. guajava* used to obtain the total RNA. Flower bud (BF); Immature leaf (IL); Young leaf (YL); Mature leaf (ML); and Root (R).

Supplementary information S4 – Primer efficiency. For the primer efficiency analysis, standard curves were constructed with cDNA quantities of 50 ng (highest), 25 ng, 2.5 ng, 0.25 ng and 0.025 ng (lowest). The efficiency was 98%.

Figures

Figure 1

Qualitative analysis of RNA by electrophoresis.

Figure 2

Qualitative analysis of RNA from different tissues.

Figure 3

Quantitative analysis by qRT-PCR to compare CTAB methods.