http://dx.doi.org/10.1590/0100-2945-278/15

COMUNICAÇÃO CIENTÍFICA

COMPARISON OF RNA EXTRACTION METHODS FOR *Passiflora edulis* SIMS LEAVES[1]

ANNY CAROLYNE DA LUZ[2], IRANY RODRIGUES PRETTI[3], MARIA DO CARMO PIMENTEL BATITUCCI[4]

ABSTRACT- Functional genomic analyses require intact RNA; however, *Passiflora edulis* leaves are rich in secondary metabolites that interfere with RNA extraction primarily by promoting oxidative processes and by precipitating with nucleic acids. This study aimed to analyse three RNA extraction methods, Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA), TRIzol® Reagent (Invitrogen) and TRIzol® Reagent (Invitrogen)/ice -commercial products specifically designed to extract RNA, and to determine which method is the most effective for extracting RNA from the leaves of passion fruit plants. In contrast to the RNA extracted using the other 2 methods, the RNA extracted using TRIzol® Reagent (Invitrogen) did not have acceptable A260/A280 and A260/A230 ratios and did not have ideal concentrations. Agarose gel electrophoresis showed a strong DNA band for all of the Concert™ method extractions but not for the TRIzol® and TRIzol®/ice methods. The TRIzol® method resulted in smears during electrophoresis. Due to its low levels of DNA contamination, ideal A260/A280 and A260/A230 ratios and superior sample integrity, RNA from the TRIzol®/ice method was used for reverse transcription-polymerase chain reaction (RT-PCR), and the resulting amplicons were highly similar. We conclude that TRIzol®/ice is the preferred method for RNA extraction for *P. edulis* leaves.
Index terms: *Passiflora edulis*, RNA extraction, Concert™, TRIzol®, low temperature.

COMPARAÇÃO DE MÉTODOS PARA EXTRAÇÃO DE RNA DE FOLHAS DE *Passiflora edulis* SIMS

RESUMO- Para análises de genômica funcional é indispensável que o RNA esteja íntegro. Folhas de *Passiflora edulis* são ricas em metabólitos secundários que dificultam o processo de extração de RNA, principalmente por desencadearem processos oxidativos e precipitarem juntamente aos ácidos nucleicos. Este estudo objetivou analisar três métodos de extração de RNA: Concert™ Plant RNA Reagent (Invitrogen), TRIzol® Reagent (Invitrogen) e TRIzol® Reagent (Invitrogen)/gelo- produtos comerciais específicos para extração de RNA, e indicar qual é o mais eficaz para uso em folhas de maracujazeiro. O RNA extraído com o método TRIzol® Reagent (Invitrogen) não apresentou razões A260/A280 e A260/A230 satisfatórias, tampouco concentrações ideais, ao contrário dos outros dois métodos. A eletroforese em gel de agarose mostrou, pelo método Concert, banda nítida de DNA em todos os extratos obtidos, o que não ocorreu com os métodos TRIzol e TRIzol/gelo. Com o método TRIzol as amostras apresentaram arraste durante a eletroforese. Devido a menor contaminação com DNA, às razões A260/A280 e A260/A230 ideais e maior integridade das amostras, o RNA proveniente do método TRIzol/gelo foi utilizado para a RT-PCR. Os *amplicons* obtidos apresentaram alta similaridade. Considerou-se o TRIzol/gelo como método preferencial para extração de RNA em folhas de *P. edulis*.
Termos para indexação: *Passiflora edulis*, extração de RNA, Concert™, TRIzol®, baixa temperatura.

[1](Trabalho 278-14). Recebido em: 28-10-2014. Aceito para publicação em: 07-07-2015.
[2]Bióloga, M.Sc., Bolsista FACITEC, Universidade Federal do Espírito Santo, Vitória-ES. E-mail: annydaluz@yahoo.com.br
[3]Bióloga, M.Sc., Bolsista CAPES, Universidade Federal do Espírito Santo, Vitória-ES. E-mail: nyevau@yahoo.com.br
[4]Bióloga, Dr, Universidade Federal do Espírito Santo, Vitória-ES. E-mail: docarmo_batitucci@yahoo.com.br

The Passifloraceae species occur mainly in tropical regions of the Americas (ZERAIK and YARIWAKE, 2010). The culture of *Passiflora edulis* has great economic importance in Brazil as well as in other countries (POMMER and BARBOSA, 2009), consisting significant source of income for producers and promising market for the juice industry (Paiva et al., 2014). The importance of passion fruit crop in Brazil can be measured in the production of fruit, which reached 776.097 tons in 2012 (ANUÁRIO BRASILEIRO DE FRUTICULTURA, 2013). Furthermore, *Passiflora edulis* is a species widely used as a herbal medicine and has anxiolytic action, sedative, anti-inflammatory, diuretic, anti-helmintic, in the treatment of hypertension and the symptoms of menopause (BENINCÁ et al., 2007; DENG et al., 2010; LI et al., 2011).

P. edulis leaves are rich in phenolic compounds, alkaloids, cyanogenic glycosides, monoterpenes and saponins (LORENZI and MATOS, 2002), which inhibit RNA extraction (BITENCOURT et al., 2011). RNA extraction from specific tissues is the first step in gene expression studies and characterization of transcripts (BITENCOURT et al, 2011). Intact RNA is essential for analysing differential expression because RT-PCR is the most sensitive method for detecting mRNA. Problems with sensitivity, reproducibility and specificity can result from a limited or low quality RNA sample (MEISEL et al., 2005). Many studies have reported difficulties isolating high quality RNA at satisfactory quantities (WANG et al., 2011). In plants, these analyses can be technically difficult due to many compounds, which could compromise the extraction and purification of RNA molecules present in these organisms (BITENCOURT et al., 2011) that compounds precipitate with the RNA because of their similar chemical properties, affecting sample quality and yield (ASIF et al., 2000).

Nucleic acids resist degradation to varying degrees (MATHAY et al., 2012). Compared with DNA, RNA is more easily cleaved in alkaline media (DALLAS et al., 2004) and at high temperatures (PASLOSKE, 2001).

The advances in passion fruit culture were obtained by genetic enhancement (MELETTI, 2011). Then, the best method for RNA extraction in *P. edulis* must be determined so that analyses such as complementary DNA (cDNA) library construction, suppressive subtractive hybridisation (SSH), RT-PCR, cDNA-amplified fragment length polymorphism (cDNA-AFLP) and northern blot are not limited by low-quality samples (AINSWORTH, 1994).

Three RNA extraction methods, Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA), TRIzol® Reagent (Invitrogen) and TRIzol® Reagent (Invitrogen)/ice (all are commercial products specifically for RNA extraction), were tested in to determine the best method for passion fruit plant leave RNA extractions. RNA extractions using these methods were analysed using a spectrophotometer and run on an agarose gel, followed by RT-PCR and sequencing.

P. edulis SIMS leaves were obtained from 20 plants per month in 3 different months a commercial nursery in the city of Sooretama (19° 11′ 52″ S, 40° 5′ 29″ W), Espírito Santo, Brazil. After collection, samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

TRIzol® Reagent Protocol

One millilitre of TRIzol® Reagent (Invitrogen) was added to 100 mg of *P. edulis* SIMS leaf tissue in a microcentrifuge tube, ground using liquid nitrogen and homogenised using a vortex. After 5 min incubation at room temperature, 0.2 mL of chloroform was added, and the mixture was agitated for 15 s. After 3 min, the sample was centrifuged at 12000 g for 15 min at 4°C. The aqueous phase was transferred to a new microcentrifuge tube, and 0.5 mL of isopropanol was added. Then, the sample was incubated at room temperature for 10 min and subsequently centrifuged at 12000 g for 10 min at 4°C. The supernatant was removed from the tube, and the RNA pellet was washed with 1 mL of 75% ethanol, vortexed and centrifuged at 7500 g for 5 min at 4°C. The wash was discarded. After the microcentrifuge tube was dried, the RNA pellet was resuspended in 20 µL of ultrapure RNase-free water.

TRIzol® Reagent/ice Protocol

One millilitre of TRIzol® Reagent (Invitrogen) was added to 100 mg of *P. edulis* SIMS leaf tissue, ground using liquid nitrogen and homogenised using a vortex. Then, the mixture was incubated for 5 min on ice, followed by centrifugation at 12000 g for 15 min at 4°C. The supernatant was transferred to a new microcentrifuge tube, and 0.2 mL of cold chloroform (4°C) was added and mixed by inverting the tube for 15 s. After incubation on ice for 3 min, the sample was centrifuged at 12000 g for 15 min at 4°C. The aqueous phase was transferred to a new microcentrifuge tube, and 0.5 mL of cold isopropanol (4°C) was added. The sample was incubated at -20°C for 20 min, with additional 10 min incubation on ice. After 30 min of incubation, the sample was centrifuged at 12000 g for 10 min at 4°C. The

supernatant was discarded, and 1 mL of cold 75% ethanol (4°C) was added to the RNA pellet, vortexed and centrifuged at 7500 g for 5 min at 4°C. The wash was discarded. After the microcentrifuge tube was dried, the RNA pellet was resuspended in 20 µL of ultrapure RNase-free water.

Concert™ Plant RNA Reagent (Invitrogen) Protocol

Five hundred microlitres of cold (4°C) Concert™ Plant RNA Reagent (Invitrogen) was added to 100 mg of leaf tissue, ground using liquid nitrogen in a microcentrifuge tube on ice and vortexed. The mixture was incubated at room temperature for 5 min and then centrifuged at 12000 g for 2 min. The supernatant was transferred to a new microcentrifuge tube, and 0.1 mL of 5 M NaCl was added and mixed. Next, 0.3 mL of chloroform was added, and the mixture was agitated by gently inverting the tube. Then, the sample was centrifuged at 12000 g for 10 min at 4°C. Four hundred microlitres of the aqueous phase was transferred to a new microcentrifuge tube, and 400 µL of cold isopropanol was added, mixed by inverting the tube and incubated at room temperature for 10 min. Subsequently, the sample was centrifuged at 12000 g for 10 min at 4°C. The supernatant was discarded, and 1 mL of 75% ethanol was added to the pellet and centrifuged at 12000 g for 1 min at room temperature. The wash was discarded. After the residual liquid was removed, the pellet was resuspended in 30 µL of ultrapure RNAse-free water.

Quantification and RNA quality estimation

RNA extracts were analysed in a Thermo Scientific NanoDrop 3300 spectrophotometer (Thermo Scientific NanoDrop, NanoDrop Technologies, Wilmington, DE, USA) with absorbances at 230 nm, 260 nm and 280 nm. RNA integrity was analysed by running the samples on a 1% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA) and visualised using an ultraviolet (UV) transilluminator.

RT-PCR

Due to its no DNA contamination, acceptable A260/A280 ratio and higher sample integrity, the RNA sample from the TRIzol® Reagent/ice method was used for RT-PCR to test amplification. cDNA amplification was performed using the following primers, which were specific for the *18S* (18S ribosomal subunit) gene: forward primer (5′-TGACGGAAGAATTAGGGTTCG-3′) and reverse primer (5′-ACTTGCCCTCCAATGGATC-3′). The negative control was performed without the RNA samples, water was used instead. RNA samples were treated with DNAse I (Invitrogen) following the manufacturer's protocol. cDNA was synthesised using oligo(dT), reverse transcriptase and deoxyribonucleotides from a Superscript® III First Strand Synthesis System kit (Invitrogen) and then treated with RNase to purify the final product from the reaction. Transcription reactions were performed following the manufacturer's protocol. The RT-PCR product, which was stained with GelRed™ (Biotium), was visualised in a 1% agarose gel using a UV transilluminator.

SEQUENCING

The RT-PCR products were sequenced to confirm their identity by alignment. The samples were sequenced in the ACTGene laboratory (Centre for Biotechnology, UFRGS, Porto Alegre, RS, Brazil) using an ABI PRISM 3100 Genetic Analyzer automatic sequencer with 50 cm capillaries and POP6 polymerase (Applied Biosystems, Foster City, CA, USA). Template DNAs (30 to 45 ng) were labelled using 3.2 pmol of the 5′-3′ primers (described above) and were used one at a time with 2 µL of the reagent BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems) in a total reaction volume of 10 µL. Labelling reactions were performed using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with an initial denaturation step of 96°C for 3 min, followed by 25 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 min. After labelling, the samples were purified by isopropanol precipitation and washed with 70% ethanol. The precipitated products were diluted in 10 µL of formamide, denatured at 95°C for 5 min, chilled on ice for 5 min and electro-injected into the automatic sequencer. Sequencing data were collected using the Data Collection v1.0.1 software (Applied Biosystems) programmed with the following parameters: Dye Set "Z"; Mobility File "DT3100POP6{BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50 cm_cfv_100"; and Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

The resulting sequences were aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) program (National Center for Technology Information (NCBI), Bethesda, MD, USA).

RNA has a UV absorption spectrum maximum at 260 nm, whereas proteins absorb at 280 nm. Other contaminants, such as phenols and aromatic compounds, absorb UV light at 230 nm. Thus, the A260/A280 and A260/A230 ratios are frequently used as indicators of RNA sample purity (WANG et al., 2011). An A260/A280 ratio between 1.8 and 2.2

indicates a highly pure RNA sample. Ideal values for the A260/A230 ratio range between approximately 2.0 and 2.2 (ALEMZADEH et al., 2005). Table 1 presents the total RNA quality, as determined by the following criteria: the A260/A280 ratio, the A260/A230 ratio and the RNA concentration. The RNA sample from the TRIzol® Reagent/ice extraction had the highest purity and the highest RNA concentration.

RNA sample purity was also analysed by electrophoresis on a 1% agarose gel. The sample obtained from the Concert™ Plant RNA Reagent method appeared to be contaminated with genomic DNA in the electrophoresis; however, the samples obtained using TRIzol® Reagent were not contaminated. Notably, the samples obtained using the TRIzol® Reagent method were smeared on the gel, indicating degradation. In contrast, the RNA samples extracted using the TRIzol® Reagent/ice protocol were not contaminated with genomic DNA and had good integrity in the agarose gel electrophoresis experiment (Figure 1).

According to the manufacturer, TRIzol® Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization, these can explain the better integrity of samples from TRIzol® Reagent.

Nucleic acids are highly sensitive to degradation at high temperatures (PASLOSKE, 2001), and RNA extraction is highly affected by contamination from high concentrations of phenolic compounds (ZHU et al., 2012). Similar to the passion fruit plant, *Zostera marina* L. (eelgrass) is a plant with high levels of phenolic compounds, and high quality RNA was obtained only by maintaining low temperatures during extraction (ZHU et al., 2012). Therefore, low temperatures prevent the inhibition of high-quality RNA extraction in plants containing high phenolic contents.

Because of the acceptable A260/A280 and A260/A230 ratios and low DNA contamination, RNA from the TRIzol®/ice treatment was used for RT-PCR. The *18S* primer set generated a 200 bp amplicon in all of the samples (Figure 2).

The identity of the RT-PCR product was confirmed by alignment with sequences deposited into the Gen Bank database (NCBI) with high levels of homology using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cDNA sequence obtained from the *18S* primer set was 99% similar to the *18*s ribosomal RNA from 50 plant species, including *Ixiolirion tataricum*, *Gladiolus illyricus*, *Hesperocallis undulata*, *Triplostegia glandulifera* and *Polyosma sp.*

Therefore, of the methods tested, we concluded that the TRIzol® Reagent/ice protocol is preferred for extracting RNA from *P. edulis* leaves. The results demonstrate that the TRIzol® Reagent/ice protocol effectively eliminates most of the interfering molecules, including polyphenols, and leads to higher yields for intact RNA and low amounts of DNA contamination, indicating that this method is the best of the methods studied. Due to the high sensitivity of reverse transcription, highly pure RNA must be extracted for constructing cDNA libraries and for measuring gene expression.

TABLE 1-Purity and yield analysis of total RNA from *P. edulis* extracted by different methods.

Method	A260/ A280	A260/ A230	Concentration (ng/µL)
Concert™ Plant RNA Reagent	1.90±0.06	0.92±0.06	1421.22± 192.19
TRIzol® Reagent	1.75±0.08	0.75±0.11	789.50± 86.89
TRIzol® Reagent/ice	1.95±0.04	2.07±0.11	2307.70± 363.70

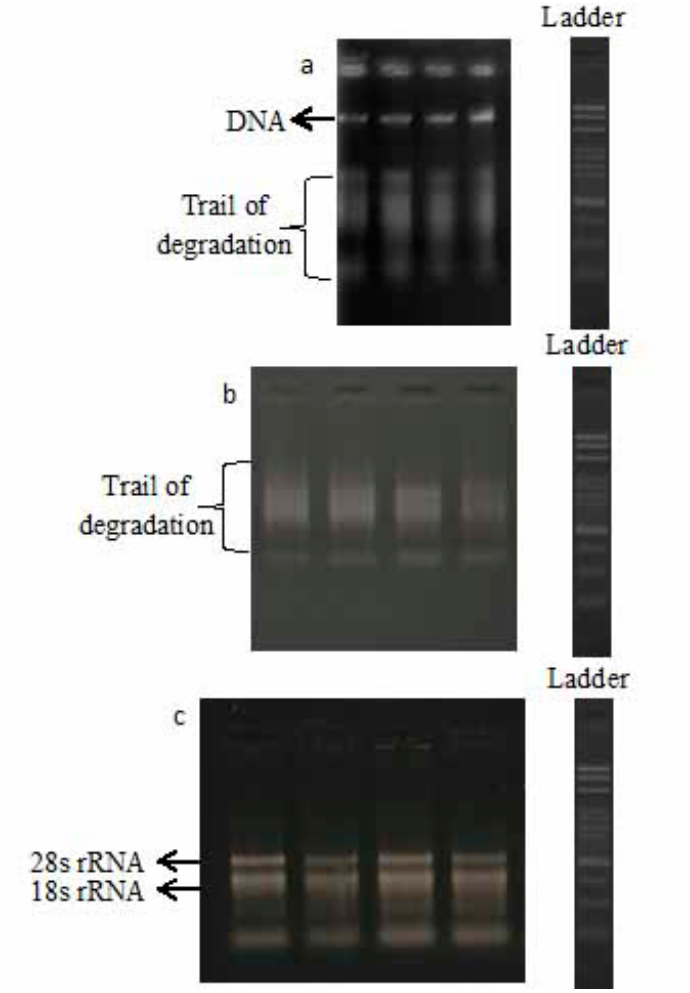

FIGURE 1- Electrophoretic analysis of *Passiflora edulis* RNA isolated using various extraction methods. Each lane indicated different sample. (a) Total RNA from *P. edulis* leaves isolated using *Concert™ Plant RNA Reagent Invitrogen®*. (b) Total RNA isolated from *P. edulis* leaves using TRIzol *Reagent Invitrogen®*. (c) Total RNA isolated from *P. edulis* leaves using TRIzol *Reagent* (*Invitrogen®*)/ice. Total RNA of each sample was loaded into the different well, as well as the 1Kb ladder.Then was run on a 1% agarose gel stained with GelRed™ (BIOTIUM™) in 1X TBE buffer.

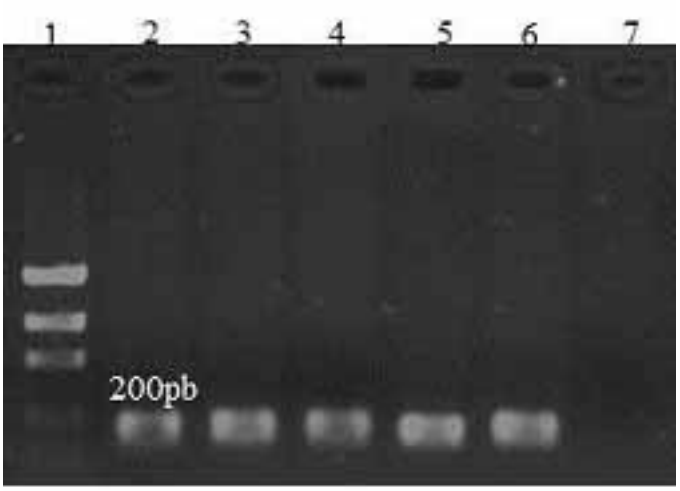

FIGURE 2- Agarose gel showing the result of RT- PCR from samples of *P. edulis* RNA obtained with TRIzol *Reagent* (*Invitrogen*®)/ice. Lane 1 show a 100pb ladder. The lanes 2 to 5 show the fragments of 200 pb resulting from the partial amplification of gene *18s*. Lane 6 shows the negative control.

ACKNOWLEDGMENTS

This research was supported by the National Council for Scientific and Technological Development (Conselho Nacional de desenvolvimento Científico e Tecnológico-CNPQ) through the Integrated Project – CNPq MCT 16/2008 Integração UFES-UFV for consolidation of the Plant Biology Graduate Program and creation of the doctorate program at the Federal University of Espírito Santo. We also thank the Plant Biology Graduate Program at the Federal University of Espírito Santo (PPGBV-UFES).

REFERENCES

AINSWORTH, C. Isolation of RNA from floral tissue of *Rumex acetosa* (Sorrel). **Plant Molecular Biology Reporter,** New York, v.12, n.3, p.198–203, 1994.

ALEMZADEH, A.; FUJIE, M.; USAMI, S.; YAMADA, T. Isolation of high-quality RNA from high-phenolic tissues of eelgrass *(Zostera marina* L.) by keeping temperature low. **Plant Molecular Biology Reporter,** New York,, v.23, n.4, p.421–421, 2005.

ANUÁRIO BRASILEIRO DE FRUTICULTURA 2014. Santa Cruz do Sul: Editora Gazeta, 2013. 140p.

ASIF, M.H.; DHAWAN, P.; NATH, P.A simple procedure for the isolation of high quality RNA from ripening banana fruit. **Plant Molecular Biology Reporter**, New York, v.18, n.2, p.109-115, 2000.

BENINCÁ, J.P.; MONTANHER, A.B.; ZUCOLOTTO, S.M.; SCHENKEL, E.P.; FRÖDE, T.S. Evaluation of the anti-inflammatory efficacy of *Passiflora edulis*. **Food Chemistry**, Berlin, v.104, p.1097–1105, 2007.

BITENCOURT, G.A; CHIARI L; VALLE, C.B; LAURA, V.A; MORO, J.R. **Avaliação de diferentes métodos para extração de RNA total de folhas e raízes de braquiária**. Campo Grande: Embrapa Gado de Corte, 2011. **(**Boletim de Pesquisa e Desenvolvimento, 29)

DALLAS, A.; VLASSOV, A.V.; KAZAKOV, S.A. Transesterification and hydrolytic cleavage of nucleic acids catalyzed by metal ions. In: ZENKOVA, M.A. **Artificial nucleases**. Berlin: Springer-Verlag, 2004. p.61-89.

DENG, J.; ZHOU, Y.; BAI, M.; LI, H.; LI, L. Anxiolytic and sedative activities of *Passiflora edulis* f. *flavicarpa*. **Journal of Ethnopharmacology**, New York, v.128, p.148–53, 2010.

LI, H.;ZHOU, P.; YANGA, Q.; SHENA, Y.; DENG, J.; LI, L., ZHAO, D. Comparative studies on anxiolytic activities and flavonoid compositions of *Passiflora edulis* 'edulis' and *Passiflora edulis 'flavicarpa'*. **Journal of Ethnopharmacology**, New York, v.133, p.1085–90, 2011.

LORENZI, H.; MATOS, F.J.A. **Plantas medicinais no Brasil**: nativas e exóticas cultivadas. Nova Odessa: Instituto Plantarum, 2002. 512p.

MATHAY, C; YAN, W; CHUAQUI, R; SKUBITZ, A.P.N., JEON, J.; FALL, N; BETSOU, F.; BARNES, M. ISBER biospecimen science working group. Short-term stability study of rna at room temperature. **Biopreservation and Biobanking**, Vancuover, v.10, n.6, p.532-542, 2012.

MEISEL, L.; FONSECA, B.; GONZÁLEZ, S.; BAEZA-YATES, R.; CAMBIAZO, V.; CAMPOS, R.; GONZÁLEZ, M.; ORELLANA, A.; RETAMALES, J.; SILVA, H. A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. **Biological Research**, Santiago, v.38, n.1, p.83–88, 2005.

MELETTI, L.M.M. Avanços na cultura do maracujá no Brasil. **Revista Brasileira de Fruticultura**, Jaboticabal, v.33, p.083-091, 2011. Número especial.

PAIVA, C.L.; VIANA, A.P.; SANTOS, E.A.; SILVA, R.N.O; OLIVEIRA, E.J. Diversidade genética de espécies do gênero *Passiflora* com o uso da estratégia WARD-MLM. **Revista Brasileira de Fruticultura,** Jaboticabal, v.36, n.2, p.381 - 390, 2014.

PASLOSKE, B.L. Ribonuclease inhibitors. In: SCHEIN, C.H. **Nuclease methods and protocols**. New Jersey: Humana Press, 2001. p.105-112.

POMMER, C.V.; BARBOSA, W. The impact of breeding on fruit production in warm climates of Brazil. **Revista Brasileira de Fruticultura**, Jaboticabal, v.31, n.2, p.612-634, 2009.

WANG, X; XIAO, H; CHEN, G; ZHAO, X; HUANG, C; CHEN, C; WANG, F. Isolation of high-quality RNA from *Reaumuria soongorica*, a desert plant rich in secondary metabolites. **Molecular Biotechnology,** Oxford, v.48, n.2, p.165-172, 2011.

ZERAIK, M.L.; YARIWAKE, J.H. Quantification of isoorientin and total flavonoids in *Passiflora edulis* fruit pulp by HPLC-UV/DAD. **Microchemical Journal**, New York, v.96, p.86-91, 2010.

ZHU, H; JIANG, K; ZHANG, F; ZHANG, D; PI, Y; MA, L. Improved isolation of good-quality total RNA from the optic stalk of Mud crab, *Scylla paramamosain*. **Electronic Journal of Biotechnology**,Valparaiso,v.15, n.2, p.1–8, 2012.