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# Protocol adjustment improves the extraction of high-quality total RNA from common bean stems infected by *Sclerotinia sclerotiorum*

## Ajuste de protocolos melhora a extração de RNA total de alta qualidade de hastes de feijão infectadas por *Sclerotinia sclerotiorum*

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**ABSTRACT**

The Straw Test is an assay developed to evaluate the resistance of common bean to white mold, in which the plant stems are inoculated and the symptoms of the disease are monitored. It is plausible to admit that investigating gene expression in pathogen-infected tissues may be strategically interesting. However, obtaining a quality RNA is a basic requirement for this purpose. Therefore, the objective of this study was to evaluate adjustments in protocols of commercial kits in the expectation of improving the quality of RNA obtained from bean stems. For this, plants of two lines were inoculated and the stems pathogen-infected were collected 72 hours after. For RNA extraction, two commercial reagents were used following the manufacturer's recommendations and then following adaptations in these protocols. In particular, the proposed modifications relate to volumes of supernatant recovered in purification steps, additional step of chloroform purification and extended time for nucleic acids precipitation. The obtained RNA was analyzed by spectrophotometer, electrophoresis and bioanalyzer, then converted into cDNA and subsequently submitted to PCR. From the obtained data, it was observed that the adaptations made in the protocols contributed to better results and that, when the indicative values of RNA quality are guaranteed, the subsequent reactions are more pure, precise and representative.

**Index terms:** *Phaseolus vulgaris* L.; RNA isolation; white mold; plant-pathogen interaction.

**RESUMO**

O Straw Test é um ensaio desenvolvido para avaliar a resistência do feijoeiro ao mofo branco, no qual as hastes da planta são inoculados e os sintomas da doença são monitorados. É plausível admitir que a investigação da expressão gênica em tecidos infectados por patógenos possa ser estrategicamente interessante. No entanto, obter um RNA de qualidade é um requisito básico para essa finalidade. Portanto, o objetivo deste estudo foi avaliar ajustes em protocolos de kits comerciais na expectativa de melhorar a qualidade do RNA obtido a partir de hastes de feijão. Para tanto, plantas de duas linhagenslinees foram inoculadas e as hastes infectadas pelo patógeno foram coletadas após 72 horas. Para extração de RNA, dois reagentes comerciais foram utilizados inicialmente seguindo as recomendações do fabricante e seguindo as adaptações desses protocolos. Em especial, as modificações propostas referem-se aos volumes de sobrenadante recuperados nos passos de purificação, etapa adicional de purificação por clorofórmio e maior tempo para precipitação dos ácidos nucleicos. O RNA obtido foi analisado por espectrofotômetro, eletroforese e bioanalyzer, posteriormente convertido em cDNA e, por fim, submetido à PCR. A partir dos dados obtidos, observou-se que as adaptações feitas nos protocolos contribuíram para resultados melhores e que quando os valores indicativos de qualidade do RNA são garantidos, as reações subsequentes são mais puras, precisas e representativas.

**Termos para indexação:** *Phaseolus vulgaris* L.; isolamento de RNA; mofo branco; interação planta-patógeno.

## INTRODUCTION

Although bean production is prominent in Brazil, with an estimated production of 3.184 million tons (Conab, 2018), its productivity is affected by diseases (Oliveira et al., 2015). Among them is the white mold caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, which can lead to 100% loss of crop (Vasconcellos et al., 2017). Contrasting with this scenario, obtaining genotypes with high levels of resistance to the pathogen, combined with other traits desirable for the crop, has demanded great effort from the bean breeders (Schwartz; Singh, 2013).

It is well known that knowledge about the molecular basis of plant-pathogen interaction is essential for the elaboration of alternative strategies for genetic breeding (Vleeshouwers; Oliver, 2014), however, for diseases such as white mold, literature is limited (Oliveira et al., 2015). Progress in this aspect has been sought through marker-assisted selection to obtain pathogen-resistant genotypes (Schwartz; Singh, 2013). The challenge is that, in some cases, the results are equivalent to phenotypic selection (Torga et al., 2010).

To improve this scenario, it has become relevant to analyze the transcriptional profiles of genotypes submitted to specific experimental conditions. There are several possibilities, such as the study of individual genes by RT-PCR and qPCR (Ma et al., 2015; Vasconcellos et al., 2017), isolation, cloning and expression of specific genes in model organisms, followed for functional studies (Wang et al., 2013), transcriptomes of a given genotype (Jain et al., 2016), or analyzes of the transcriptional profiles of host and pathogen, simultaneously (Westermann; Gorski; Vogel, 2012). Regardless of the chosen strategy, isolation of high-quality total RNA consists of a critical stage of the research (Ahmad et al., 2017).

RNA is a delicate molecule to be studied. The presence of a 2'OH in ribose enables RNA as nucleophile, making it vulnerable to enzymatic degradation (Lavoie; Elela, 2008). RNA also has a high degree of susceptibility to secondary metabolites (Jordon-Thaden et al., 2015) and, sometimes, co-precipitated inhibitory molecules during the RNA extraction process may interfere with subsequent enzymatic reactions (Kolosova et al., 2004; Ma et al., 2015).

Given the importance of RNA quality for research on gene expression, several studies are conducted to adjust RNA extraction methods (Ma et al., 2015; Pereira et al., 2017). In the case of beans, there are investigated protocols for the extraction of RNA from leaves, roots and seeds (Borges et al., 2012; Pereira et al., 2017), but few reports have been found for the stem, organ in which the symptoms of the white mold are commonly evaluated. It is believed that plant genes induced by *S. sclerotiorum* can be found in the transcriptome of these tissues (Oliveira et al., 2015).

It is important to highlight that the chemical composition of the tissue can affect the quality of the extracted RNA (Gehrig et al., 2000). In the case of the bean plant challenged by white mold, there is lignification and accumulation of phenolic compounds in the plant tissues (Oliveira et al., 2015). So, RNA extraction methods need to be investigated and adapted if necessary (Ahmad et al., 2017), since low quality RNA samples necessarily compromise all subsequent analyzes, representing loss of time and research resources.

In this study, simple adaptations performed according to previous experiments resulted in high-quality RNA, contaminants free and suitable for subsequent enzymatic reactions.

## MATERIAL AND METHODS

### Genotypes

Two lines were selected for this study, since they have been used in parallel in differential gene expression assays. One susceptible and other partially resistant to white mold, Beryl and Cornell 605, respectively (Griffiths, 2009; Lehner et al., 2015). Their seeds were sown in pots with 2 kg of soil fertilized with NPK (04-14-08, 1g / kg soil), and kept in greenhouse in the period from November to December 2017 under controlled temperature and humidity, at 24 ± 2º C and 70 ± 5%, respectively. Each experimental plot consisted of 3 plants per pot. At 35 days after emergence, the plants were inoculated, and 72 hours after inoculation (HAI), the stems were collected and frozen immediately in liquid nitrogen. Padder et al. (2016) selected 0, 24, 72 and 96 hours after inoculation to study the transcriptome of the bean inoculated by *Colletotrichum lindemuthianum*, becoming a parallel reference, given the lack of information about beans inoculated by *Sclerotinia sclerotiorum*.

In parallel, a similar assay was conducted, except for the collection of the stems for RNA extraction, in order to investigate the incidence of differential symptoms in the two lines, validating the inoculation process. The procedure used to evaluate the reaction of the genotypes to *S. sclerotiorum* followed the Straw Test method, described by Petzoldt and Dickson (1996) and modified by Terán et al. (2006).

### *Sclerotinia sclerotiorum* isolate and inoculation

The pathogen used to inoculate the lines Beryl and Cornell 605 was an isolate of *S. sclerotiorum* UFLA 27, originary from Lambari, Minas Gerais State (Silva et al., 2014), kept in mycological collection of the Plant Resistance Laboratory of the Department of Biology, Federal University of Lavras, MG. Initially, the sclerotia were subjected to asepsis and cultured on Petri dishes containing PDA (Potato-Dextrose-Agar) medium for 5 days at 20 °C under 12h photoperiod. So, the mycelium was obtained and a 0.5 to 0.7 mm diameter punch device was used to chop it up. The new Petri dishes with PDA were used for multiplying the mycelium disks in an incubation chamber for 72 h.

Inoculation procedure Straw Test, conducted at 35 days after germination (35 DAG), consists of cutting the stem apex at about 2.5 cm from the node, where a P200 micropipette tip was inserted, with a PDA disk containing agar in the control group, or agar + mycelium of *S. sclerotiorum* in the treatment with inoculums (Silva et al., 2014).

### Total RNA extraction from stems

Initially, fragments of 5 cm from the stem were collected 72 hours after inoculation (HAI) and immediately frozen in liquid nitrogen. Is important highlight that before extraction of the total RNA from bean stems, we followed the symptoms of the white mold, as a necessary condition for both the validation of the inoculation procedure and confirmation of susceptibility and resistance to *S. sclerotiorum* of the lines Beryl and Cornell 605, respectively. The symptoms were evaluated twice (7 and 14 DAI) by the method proposed by Singh, Schwartz and Steadman (2014), using grades from 1 to 9 to quantify the severity of the symptoms in the inoculated stem. Additionally, the advancement of symptoms in affected tissues was measured twice (7 and 14 DAI) by millimeter ruler, and these data were presented. For statistical analysis, the measurements of 3 replicates per treatment were taken and these data were submitted to variance analysis and Tukey test at 5% of probability.

Two commercial reagents were used for total RNA extraction, the PureLink® RNA Kit and RNATRIzol® Isolation Reagent (Invitrogen™). The manufacturer's recommendations were used, however, some modifications have been necessary to improve the RNA quality. As Gasic, Hernandez and Korban (2004) related difficulties to isolate total RNA from apple tissues with enough quality and quantity for cDNA library construction using these reagents, we also decided to evaluate the modifications in the protocols based on methods previously used by our laboratory in occasions of difficult extraction. In order to preserve the identity of the kits, since the objective of this work is not to compare them, they were randomly coded in A and B. Their adapted versions were coded A* and B*, respectively.

The grinding consists of a critical stage of the whole process of RNA extraction, both with respect to the thickness of the macerate and the maintenance of the low temperature of the sample to avoid degradation (Ma et al., 2015). Thus, the plant stems were carefully macerated in liquid nitrogen to the powder consistency and portion equivalent to 100 mg of tissue was transferred to 1.5 ml microtube. From this point on, procedures were applied according to the manufacturer's recommendations for each kit and the adaptations performed are described between parentheses.

### Kit A and (Kit A Adaptation)

1. The ground stems were transferred to 1.5 mL tubes, then 500 µL of kit A reagent (600 µL) were added and the samples were homogeneized by vortexing (Time set at 1').
2. Microtube was kept horizontal at room temperature for 5' (10'), in order to maximize contact between sample and reagent.
3. The samples were centrifuged at 12.000 x g for 2' at room temperature and the supernatant (450 µl of supernatant collected) was transferred to new tubes containing 100 µL 5M NaCl.
4. It was added 300 µL of chloroform and the homogenization was made by inversions (vortex 1').
5. The samples were centrifuged at 12.000 x g for 10' at 4 °C.
6. (Now, 430 µL supernatant was collected and transferred to a tube containing 100 µL of 5M NaCl, to which were added 300 µL of chloroform. The mixture was vortexed at 1' and then centrifuged at 12.000 x g for 10' at 4 °C).
7. The sample was centrifuged at 12.000 x g for 10' at 4 °C to separate the phases e the upper, aqueous phase was transferred to a clean RNase-free tube (400 µL) with equal volume of isopropanol (400 µL), and homogenized gently by slow inversions of the tube (20 inversions).
8. The tubes were kept horizontally for 10' at room temperature (maintenance of tubes vertically at -20 °C for 16h).
9. After this precipitation period of the nucleic acids, the samples were centrifuged at 12.000 x g for 10' at 4 °C (Centrifugation at 12.000 x g for 25' at 4 °C).
10. Thereafter, the supernatant was discarded, 1 mL of 75% RNase-free ethanol was added to wash the pellet obtained (700 µL) and the microtubes were centrifuged at 12.000 x g for 1' at room temperature (centrifugation at 12.000 x g for 10' at 4 °C).
11. After discarding the alcohol, a brief centrifugation 12.000 x g at 4 °C was performed, followed by the removal of the residual alcohol with the aid of a micropipette. Finally, upon drying of the pellet in the hood, the RNA was resuspended in 20 µL of autoclaved Milli-Q-DEPC water and stored in a freezer -80 °C.

### Kit B and (Kit B Adaptation)

1. The ground stems were transferred to 1.5 mL tubes, where 1 mL of kit B reagent was added, followed by incubation at room temperature for dissolution of the cell complex.
2. Were added 200 µl of chloroform and the samples were incubated for an additional 3'.
3. The sample was centrifuged at 12.000 x g for 15' at 4 °C.
4. Then, supernatant was transferred to a new microtube, to which 500 µl of isopropanol was added. 5. The samples

were left on ice for 10' and thereafter, were centrifuged at 12.000 x g for 10' at 4 °C.
6. The supernatant was discarded, the pellet was resuspended in 1 mL of 75% ethanol RNase free and homogenized by vortexing.
7. In sequence, the tubes were centrifuged at 12.000 x g for 5' at 4 °C, and this time, the supernatant was discarded and the pellet set to dry for 10' under room temperature.
8. Finally, the pellet was resuspended with 35 µl of autoclaved Milli-Q-DEPC water, and the microtube was subjected to 55 °C for 10 minutes in a water bath (As adaptation, the same procedure reported above, except for the final incubation step at 55 °C for 10 minutes).

### RNA Quality and cDNA libraries

To verify the quality and quantity of the RNA samples obtained, they were submitted at NanoVue ™ Plus Spectrophotometer (General Electric Company, GE), taking into account the absorbances ratios 260/280 and 260/230 nm, and the RNA integrity number (RIN) was verified with Agilent 2100 Bioanalyzer (General Electric Company, GE). For the DNAse treatment, the Turbo DNA Free Kit was used, following the manufacturer's recommendations. Again, the samples were subjected to the NanoVue™ Plus Spectrophotometer, providing both the total RNA concentration and the absorbances 230, 260 and 280 nm. Finally, for visual quality certification of the RNA, the samples were submitted to 1% agarose gel electrophoresis, taking into account the integrity of the bands corresponding to rRNAs 28 and 18S.

The synthesis of the cDNA libraries was performed from 500 ng mRNA using the SuperScript® IV Reverse Transcriptase Kit, following the manufacturer's recommendations and using oligo$D(T)_{20}$ as primer.

According to Pereira et al. (2017), it was used descriptive statistics to analyze these data, with emphasis on mean and standard deviation.

### PCR

The quality certification of a cDNA library can be observed from the results of subsequent enzymatic reactions (Gehrig et al., 2000; Ma et al., 2015). Among the various possibilities, PCR is one of the most practical. Thus, PCRs were performed using two primer pairs, being the first designed for Actin gene (F-CCTTCACCACCTCAGCAGAG and R-GAACTGGTCCTGGCTGTCTC), and the second for PvFBOX gene (F-ACGACGGAGAATGGCTACAC and R-GCCAGAAGTTCAAGGTCCTG). The reactions were made by using 5 ng of DNA, 5X Green GoTaq® Reaction Buffer (1.5 mM MgCl2), 0.4 µM of each forward primer and reverse, 0.8 mM dNTP mix and 1.25 units of GoTaq® DNA polymerase (5u/µL), completing with $H_2O$ nuclease free the reaction to 25 µL. The PCR cycles were programmed as follow: an initial cycle of 2' at 94 °C for denaturation, followed by 35 cycles of 94 °C for 30", 60 °C for 30", and 72 °C for 30", ending with one step of additional extension at 72 °C for 7' and subsequent incubation at 4 °C until the moment of application of the samples to 1% agarose gel electrophoresis to qualitative evaluation.

## RESULTS AND DISCUSSION

### Validation of the inoculation procedure

In order to certify the bean inoculation procedure by *S. sclerotiorum*, the progress of white mold symptoms in plants was monitored. The data of the control treatment were not presented, since they did not present any symptoms (Figure 1). The Beryl line showed a more pronounced susceptibility to the pathogen than the Cornell 605, both at 7 and 14 DAI, thus corroborating the previous knowledge of (partial) resistance and susceptibility of the lines Cornell 605 and Beryl to *S. sclerotiorum*, respectively (Griffiths, 2009; Lehner et al., 2015). The scale of notes corroborated with the information obtained through the millimeter ruler (data not shown).

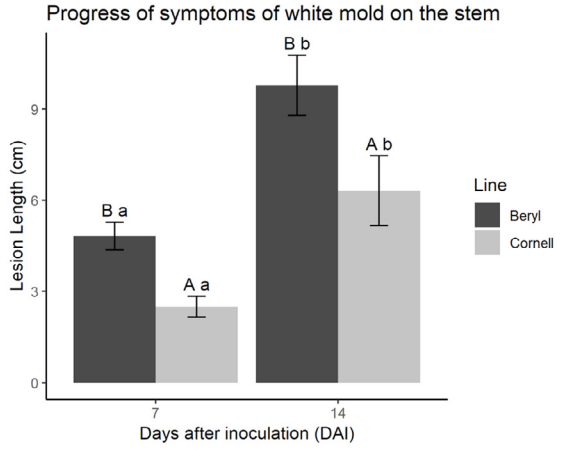


**Figure 1:** Responses of Beryl and Cornell 605 lines to inoculation by Sclerotinia sclerotiorum at 7 and 14 days after inoculation (DAI), measured by symptom progression in centimeters on inoculated stems. The bar represents the standard error of the mean of the replicates of each treatment. Uppercase letters compare DAIs within lines and lowercase letters compare lines within DAIs, at the 5% probability level by the Tukey test.

### Quantification and quality of extracted RNA

Regarding the RNA extraction from the stems, it is important to highlight some difficulties inherent to the morphological characteristics of the tissue under study. The stems are composed of plant support tissues, incorporated by secondary metabolites that provide tissue stiffness and, consequently, resistance to the maceration procedure. In addition, the literature reports the influence of these metabolites on the quality of extracted RNA (Jordon-Thaden et al., 2015; Ma et al., 2015). After RNA extraction from the samples, the total RNA quantification consists of the first diagnosis about the quality of the procedure. Spectrophotometer quantification is an important indicator of RNA quality (Desjardins; Conklin, 2011), considering that they inform both the RNA concentration obtained and the presence of contaminating substances in the sample.

Based on the absorbance readings, the four protocols investigated provided satisfactory amounts of nucleic acids. The mean yield of total RNA of the A protocol ranged from 882.67 to 2743.67 ng/µL, while the kit A* showed more regular results, ranging from 1129.30 to 1580.75 ng/µL. For kit B protocol the amplitudes of variation were higher 614.6 at 1459.65, and 1191.75 and 3019.5 for the kit B* (Table 1). Pereira el al. (2017) also observed high amplitude of variation in the quantifications of the bean seed RNA.

In absolute values, the kit B* produced more RNA, but, admitting the standard deviation as criteria, the kit A* provided more stable results (Figure 2). It is possible to observe that before treatment with DNase, all the kits presented differences of yield among their treatments, except for kit A*. Indeed, regularity can be considered more interesting than quantity due the inconsistencies of some samples.

Regarding absorbance ratios, superiority was observed for the kit A*, considering that these samples showed 260/280 ratio between 2.1 and 2.13, which is in fact desirable for RNA samples (Jazi; Rajaei; Seyedi, 2015; Ma et al., 2015). For the other protocols, A, B and B*, the ratios obtained were generally below 2.08 but above 1.77, which does not indicate lack of quality, but possible presence of more DNA in the sample or protein (Djami-Tchatchou; Straker, 2012). It is important to note that RNA samples with high quality have values close to 2.0, since the uracil absorbance is greater than thymine. In turn, DNA tends to have a ratio of 260/280 nm around 1.8.

The most prominent differences between the protocols were observed for the 260/230 ratio. In general, the 260/230 values for pure nucleic acid are higher than 260/280 values. Samples obtained with the protocol of the kit A presented mean values between 1.81 and 2.45. Dash (2013) associated 260/280 absorbance between 1.9 and 2.7 with RNA of high purity. With the kit B protocols, the relationships obtained in the samples oscillated more drastically, varying from 1.02 to 1.91. From these data, it is plausible to assume that the procedures of the kit A were cleaner and of better quality, since some samples of the kit B - based protocols presented low values for the 230/260 ratio (1.02, 1.08 and 1.14), indicating the presence of contaminants that absorb at 230 nm, such as carbohydrates and phenol (Djami-Tchatchou; Straker, 2012). Among the adaptations performed, the reduction of the volume of supernatant collected seems to have been responsible for the best result of the protocol of the kit A. The recovery of less supernatant from the sample was equivalent to reducing the risk of catching contaminants.

After the DNase treatment, the samples were again subjected to the spectrophotometer. This time, all protocols showed satisfactory values, considering that the concentrations fluctuated within expected range (Table 1). However, it was notable that samples from the kit B produced less RNA than the others.

Regarding the absorbances ratio 260/280 nm, the protocols gave mean values within the range of 1.8 to 2.0, except for some treatments. For the 260/230 ratio, two treatment of the kit A, one of the kit A* method, three of the kit B and three of the kit B* showed values below 1.8, showing that the kit A* was more stable. Additionally, the RIN values ranged from 6.7 to 9.3, indicating RNA high quality (Figure 3), as also observed for Pereira et al. (2017).

The success in the RNA extraction process should be measured as a function of the quantity, quality and integrity of the RNA extracted (Ma et al., 2015). It is acceptable that the visualization of aliquots in agarose gels submitted to electrophoresis is fundamental for certification of the sample integrity (Ahmad et al., 2017; Djami-Tchatchou; Straker, 2012). Thus, all samples of RNA treated with DNase were submitted to 1% agarose gel electrophoresis. By this procedure, it was observed that the protocols presented similar results, according to the patterns of bands visualized in the agarose gel (Figures 4A, B, C, D).

**Table 1:** Quantification (ng / µL) and absorbance ratios (260/280 and 260/230 nm) of RNA samples before and after treatment with DNase measured by spectrophotometer, followed by the corresponding standard deviation.

| | | | Total RNA before DNase | | | Total RNA after DNase | | |
|---|---|---|---|---|---|---|---|---|
| Extraction method | Line | Treatment | [ ] ng/µL | 260/280 | 260/230 | [ ] ng/µL | 260/280 | 260/230 |
| A | Beryl | Mock | 896.67 ± 121.42 | 2.04 ± 0.022 | 2.45 ± 0.07 | 164.67 ± 12.33 | 1.81 ± 0.02 | 1.71 ± 0.06 |
| | | Inoculated | 2313 ± 412.57 | 2.08 ± 0.003 | 2.00 ± 0.10 | 137.47 ± 2.72 | 1.78 ± 0.02 | 1.90 ± 0.36 |
| | Cornell 605 | Mock | 882.67 ± 69.33 | 2.03 ± 0.010 | 2.26 ± 0.28 | 140.80 ± 7.38 | 1.83 ± 0.01 | 1.90 ± 0.15 |
| | | Inoculated | 2743.67 ± 115.17 | 2.06 ± 0.011 | 1.81 ± 0.04 | 134.40 ± 16.55 | 1.89 ± 0.16 | 1.61 ± 0.09 |
| A* | Beryl | Mock | 1404.50 ± 167.00 | 2.10 ± 0.01 | 2.30 ± 0.12 | 152.80 ± 49.15 | 1.79 ± 0.15 | 1.54 ± 0.65 |
| | | Inoculated | 1576.25 ± 427.55 | 2.13 ± 0.005 | 2.37 ± 0.09 | 167.50 ± 8.21 | 1.94 ± 0.02 | 1.99 ± 0.57 |
| | Cornell 605 | Mock | 1129.30 ± 206.52 | 2.12 ± 0.01 | 2.15 ± 0.03 | 145.90 ± 21.56 | 1.94 ± 0.03 | 1.97 ± 0.38 |
| | | Inoculated | 1580.75 ± 476.41 | 2.12 ± 0.002 | 2.41 ± 0.12 | 144.60 ± 17.10 | 1.93 ± 0.05 | 2.06 ± 0.55 |
| B | Beryl | Mock | 614.60 ± 96.95 | 1.98 ± 0.01 | 1.14 ± 0.24 | 139.30 ± 27.58 | 1.81 ± 0.08 | 1.00 ± 0.30 |
| | | Inoculated | 1459.65 ± 624.99 | 2.01 ± 0.02 | 1.91 ± 0.08 | 143.70 ± 10.93 | 1.86 ± 0.07 | 1.90 ± 0.15 |
| | Cornell 605 | Mock | 629.50 ± 302.73 | 1.89 ± 0.09 | 1.14 ± 0.14 | 128.40 ± 13.56 | 1.76 ± 0.10 | 1.08 ± 0.13 |
| | | Inoculated | 1336.75 ± 298.22 | 1.98 ± 0.02 | 1.89 ± 0.18 | 135.30 ± 14.68 | 1.82 ± 0.03 | 1.66 ± 0.17 |
| B* | Beryl | Mock | 1677.50 ± 508.93 | 1.81 ± 0.05 | 1.08 ± 0.25 | 145.60 ± 39.61 | 1.90 ± 0.06 | 1.21 ± 0.41 |
| | | Inoculated | 3019.50 ± 957.19 | 1.93 ± 0.02 | 1.75 ± 0.12 | 146.55 ± 24.38 | 1.91 ± 0.07 | 1.99 ± 0.16 |
| | Cornell 605 | Mock | 1191.75 ± 597.13 | 1.77 ± 0.09 | 1.02 ± 0.11 | 145.00 ± 72.94 | 1.78 ± 0.11 | 1.29 ± 0.30 |
| | | Inoculated | 2320.75 ± 454.78 | 1.90 ± 0.07 | 1.72 ± 0.16 | 151.60 ± 37.29 | 1.92 ± 0.04 | 1.66 ± 0.23 |

*Modified.

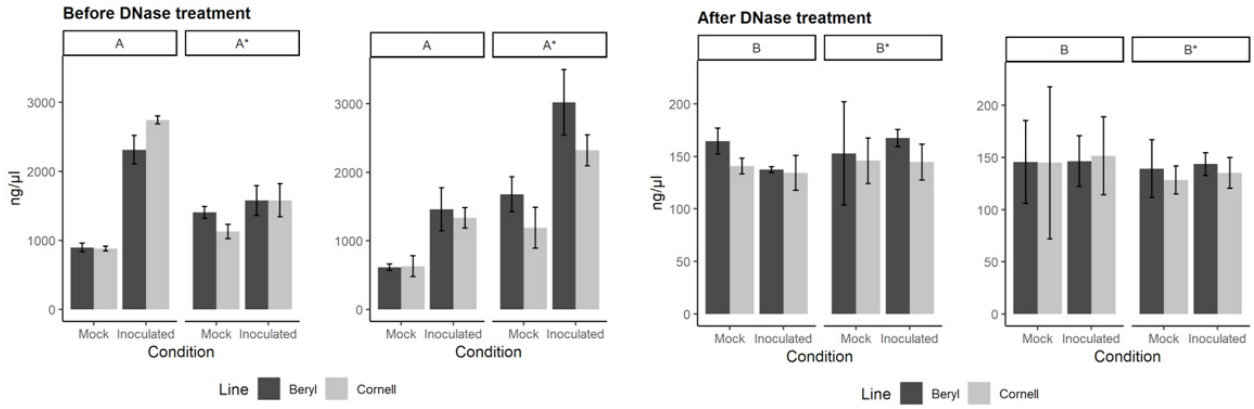


**Figure 2:** Quantification of the RNA (ng/ µL) obtained by protocols A, A* (modified), B and B* (modified), before and after the DNase treatment, and its respective bar of standard error of the mean. In the y-axis is the quantification of RNA in ng / µL. In the x-axis are presented the combinations between the following factors: Condition (Mock or inoculated) and Line (Beryl or Cornell).

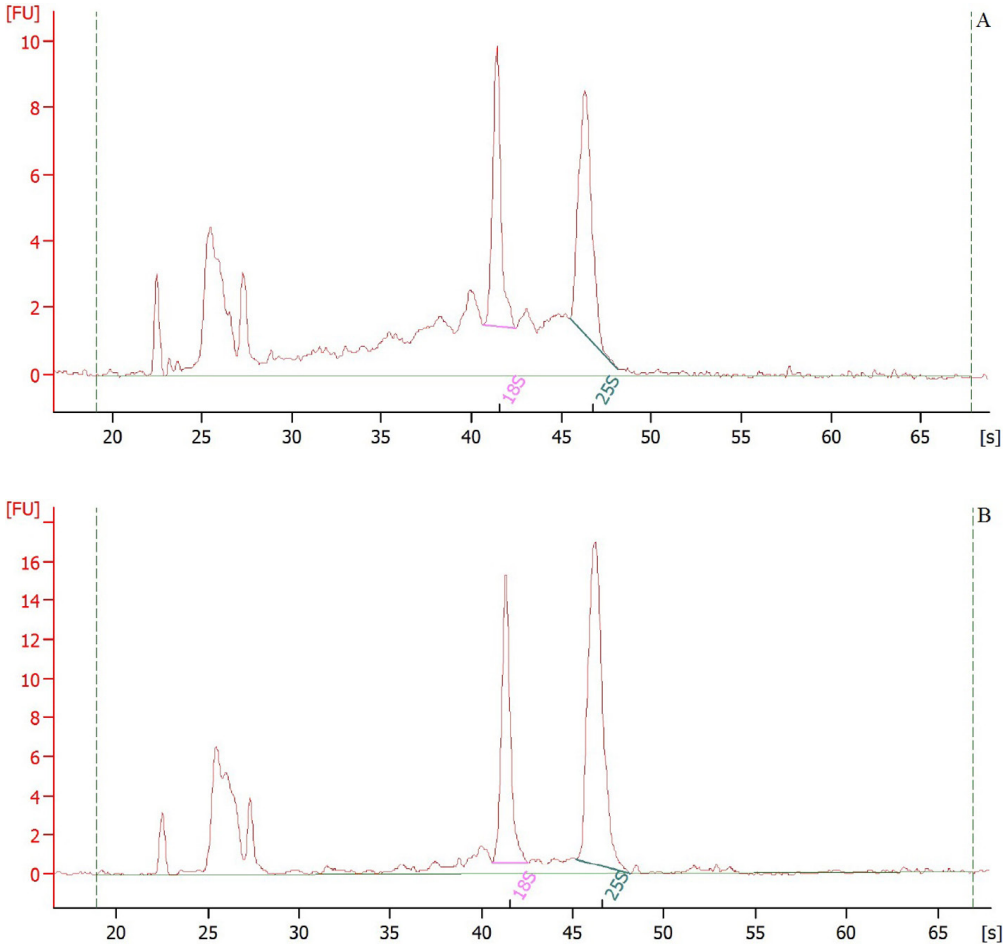


**Figure 3:** Electropherogram and electrophoresis of representative samples of RNA (6.7 and 9.3 RIN) analysed with the Agilent 2100 bioanalyser system with the RNA 6000 Nano™ kits.

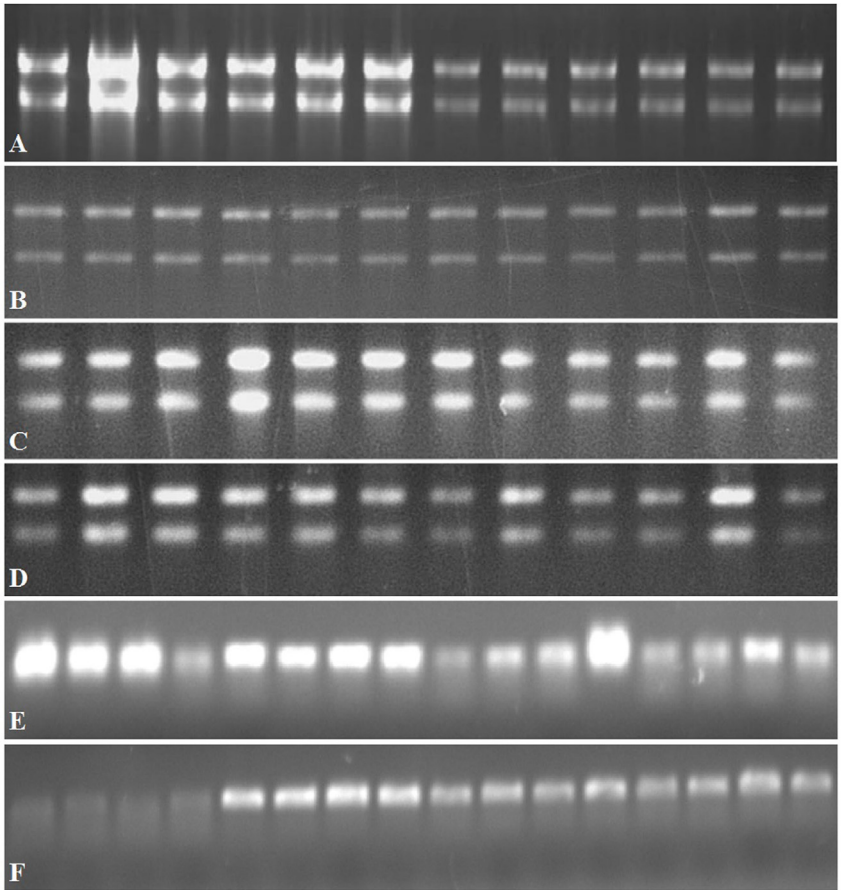


**Figure 4:** Total RNA extracted by methods of RNA isolation, kit A (A), kit A* (B), kit B (C) and kit B* (D). Aliquots (2 µL) of RNA samples were loaded on 1% agarose gel stained with ethidium bromide (0.5 µg mL -1). The order of application of the samples on the gel was as follows: 1, 2, 3 (mock Beryl), 4, 5, 6 (mock Cornell 605), 7, 8, 9 (inoculated Beryl), 10, 11 and 12 inoculated Cornell 605). PCR amplification products targeted by the primers for Actin (E) and PvFBox (F). The samples were distributed on the agarose gel following both the order of the protocols and libraries, respectively: kit A (1 to 4), kit A* (5 to 8), kit B (9 to 12), kit B* (13 to 16); Inoculated Beryl (1, 5, 9 and 13), inoculated Cornell 605 (2, 6, 10 and 14), mock Beryl (3, 7, 11 and 15) and mock Cornell 605 (4, 8, 12 e 16).

However, the similarity found by electrophoresis does not corroborate the differences observed by the spectrophotometer. Although absorbance rates are important to indicate the quality of a nucleic acid sample, its downstream application is best for this function (Desjardins; Conklin, 2011). Thus, to verify the application of RNA extracted by different methods in a downstream enzymatic reaction, the samples were converted into cDNA libraries (Djami-Tchatchou; Straker, 2012) and submitted to PCR using primers for two genes: the first of endogenous expression (actin) and the second of inducible expression (PvFBOX). For the Actin gene, the PCR product profile was strong and regular for 3 and 4 samples obtained with kit A and kit A*, respectively. For samples extracted with both kit B and kit B*, although amplification occurred, it was weak and uneven when compared to those obtained with kit A protocols (Figure 4E).

In the case of the inducible gene (*Pv*FBOX), again the samples obtained with kit A* showed a stronger amplification profile relatively the samples extracted with kit B (Figure 4F). Suzuki, Makino and Mae (2001) questioned whether the difference in expression between samples could be attributed to quality of extracted RNA. In fact, our research leads to this perception. The very weak amplification of this gene in the samples from the kit A protocol is intriguing. While corroborating the potential for modifications made in the protocol, this observation either indicates near absence of transcripts of this gene in

the cDNA library or the inhibition of the PCRs for genes with lower expression levels. Anyway, RNAs with better quality are more adequate for gene expression studies (Ma et al., 2015; Pereira et al., 2017).

To explain this result, it should be noted that the most important modifications in the kit A* method were the reduction of the volume of rescued supernatant at some stages, as well as the repetition of the extract purification procedure, which may have contributed to avoid inhibitory contaminants in the samples. As known, secondary metabolites affect quality, yield and downstream reactions (Ahmad et al., 2017).

These data can be interpreted as a function of the intensity and clarity of fragments amplified in the agarose gel. Based on this principle, the PCR products from kit A* samples were more pronounced. Both the amplification intensity and the absence of nonspecific amplifications or gel drag were indicative of assertiveness. These results are consistent with the 260/280 and 260/230 ratios, where the samples from the kit A* showed the values ~2.0, generally accepted as "pure" for RNA (Desjardins; Conklin, 2011).

In view of the above, it is reasonable to assume that the indicative values of RNA quality should not be neglected and must be strictly observed in order to guarantee the success of subsequent reactions. It is also plausible to assume that the absorbance ratios of some samples are outside the indicative quality range (1.8 to 2.2) is circumstantial. Therefore, since the protocols can be adapted according to species (Gehrig et al., 2000), tissue (Gasic; Hernandez; Korban, 2004) or stage of life (Pereira et al., 2017), it is important to apply the methods according to the manufacturer's recommendations, but checking the need for fine adjustments in the procedure. Gasic, Hernandez and Korban (2004) related that often modifications of the methods are required. This work helps to conclude that, once the indicative quality values of extracted RNA are observed, the subsequent results are more precise and representative.

## CONCLUSIONS

This work allowed to verify that the adaptations carried out in the purification and precipitation steps of the nucleic acids improved the RNA quality indicators. In addition, since indicative values of RNA quality are observed (integrity of 28S and 18S rRNAs in electrophoresis, RNA integrity number - RIN, and absorbance ratios 260/280 and 260/230), the results of subsequent enzymatic reactions, such as PCR, are more accurate and representative.

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