Comparison of DNA extraction methods for detection of citrus huanglongbing in Colombia

Comparisión de métodos de extracción ADN para detección de huanglongbing en Colombia

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ABSTRACT

Four DNA citrus plant tissue extraction protocols and three methods of DNA extraction from vector psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) were compared as part of the validation process and standardization for detection of huanglongbing (HLB). The comparison was done using several criterias such as integrity, purity and concentration. The best quality parameters presented in terms of extraction of DNA from plant midribs tissue of citrus, were cited by Murray and Thompson (1980) and Rodríguez *et al.* (2010), while for the DNA extraction from psyllid vectors of HLB, the best extraction method was suggested by Manjunath *et al.* (2008).

Key words: *Candidatus* Liberibacter, *Diaphorina citri*, Psyllidae, vectors.

RESUMEN

En el proceso de validación y estandarización para la detección de huanglongbing (HLB) se compararon protocolos de extracción de ADN, cuatro a partir de tejido vegetal de cítricos y tres a partir del psílido vector *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). Para la comparación de los protocolos se utilizaron los criterios de integridad, pureza y concentración. Los protocolos citados por Murray and Thompson (1980) y Rodríguez *et al.* (2010) fueron los que presentaron los mejores parámetros de calidad en términos de extracción de ADN a partir de la nervadura central del tejido vegetal de cítricos. En la extracción de ADN a partir del psílido vector de HLB, el método que mejores resultados arrojó fue el reportado por Manjunath *et al.* (2008).

Palabras clave: *Candidatus* Liberibacter, *Diaphorina citri*, Psyllidae, vectores.

Introduction

The disease known as citrus huanglongbing (HLB) was first detected in Asian countries and Africa and more recently in American countries such as Argentina (SENASA, 2013), Costa Rica (SFE, 2011), Belize (Manjunath *et al*., 2010), Cuba (Martínez *et al*., 2009), Mexico (NAPPO, 2009), Dominican Republic (Matos *et al*., 2009), USA (Halberth, 2005; Manjunath *et al*., 2008) and Brazil (Teixeira *et al*., 2005), which implied important losses to the citrus industry (Bové, 2006). HLB is caused by alpha-proteobacteria non-cultivated genus *Candidatus* Liberibacter inhabiting the phloem of citrus plants (Da Graca, 1991; Tsai and Liu, 2000; Tsai *et al*., 2002) and is spread by vegetative propagation and insect vectors, reason why an effective control is difficult (Hung *et al*., 2004; Manjunath *et al*., 2008).

According to the etiological agent, its genome and the influence of temperature on the expression of symptoms in the host plant, HLB has been divided into Asian, African and American variants (Halbert and Manjunath, 2004). The Asian and American variants are transmitted by the psyllid vector *Diaphorina citri* and are worldwide known as *Candidatus* Liberibacter asiaticus (Garnier *et al*., 2000) and *Candidatus* Liberibacter americanus (Teixeira *et al*., 2005), while the African variant *Candidatus* Liberibacter africanus is transmitted by the psyllid *Trioza erytreae* and *Diaphorina citri* (Bové, 2006; Lin *et al*., 2010).

In 2007, the psyllid vector *Diaphorina citri* was first reported in Colombia by the Colombian Agricultural Institute (ICA) in citrus crops and seedlings in the departments of Valle del Cauca and Tolima; later the insect was found in the departments of Risaralda, Caldas, Quindio, Antioquia, Cordoba, Cesar, Bolivar, Atlantico, Norte de Santander, Santander, Casanare, Meta, Huila, Cauca, Nariño and Cundinamarca, infesting 95% of the citrus area in the Central Pacific, Orinoco and Atlantic regions of Colombia (Ebratt *et al*., 2011), due to Colombian´s proximity to countries where the presence of the insect vector and disease has been detected, puts the country at high risk for this serious disease of citrus.

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Conventionally, visual symptoms indicate the presence of HLB (Roistacher, 1991), but over time more reliable detection systems have been developed based on electronic microscopy, specific fluorescent marker substances to HLB (Schwarz, 1968) and enzyme linked immunosorbent assays with monoclonal antibodies (ELISA) (Gao *et al*., 1993). Developed detection methods using PCR and real time PCR, were based on the 16S ribosomal DNA region analysis and several other regions of the bacterium's genome (Hocquellet *et al*., 1999; Lin *et al*., 2010; Morgan *et al*., 2012).

In order to standardize and apply molecular methods for detection of HLB in citrus regions around the country and to establish a rapid and reliable method for diagnosis, four DNA extraction protocols were compared from citrus leaf tissue, and three DNA extraction methods from *Diaphorina citri* psyllids. The basic parameters evaluated in each method were: concentration, purity, efficiency and integrity.

Methods and materials

Plant material

Ten samples of lemon mandarin (*Citrus aurantifolia*) leaf tissue from trees between 10 and 15 years old were collected in the area of the Instituto Colombiano Agropecuario of Mosquera (ICA-Tibaitata), and were then transported in styrofoam coolers with the appropriate cooling conditions until their final storage location. The next day, the leaves were washed with sterilized water and dried with paper towels, to begin the DNA extraction process.

Capture of *Diaphorina citri* psyllids

Adult individuals were collected using entomological nets, while the nymphs of *D. citri* were directly taken from symptomatic trees in different phenological stages (vegetative, flowering or harvest) such as lemons (*Citrus limon*), oranges (*Citrus sinensis*) and mandarins (*Citrus reticulata*) between 4 and 20 years old, located in the municipalities of Sasaima and La Mesa, in the department of Cundinamarca. Afterwards, they were placed in 1.5 mL Eppendorf tubes with alcohol (96% concentration) and taken to the National Laboratory of Phytosanitary Diagnosis LNDF-ICA-Tibaitata, where they were stored at room temperature until the DNA extraction was performed.

DNA extraction from plant tissue of citrus

Four DNA extraction methods previously reported were compared. Three of them were based on CTAB and 2-mercaptoethanol (Thompson *et al*., 1983; Murray and Thompson, 1980; Rodríguez *et al*., 2010) and one reported by Qiagen (a commercial kit). Ten samples were tested by each method. The whole DNA was extracted from the midrib of each leave of the collected samples, which were finely cut and pulverized using liquid nitrogen according to the four protocols. All were compared through four quality parameters: concentration, purity, efficiency and integrity, which depend on detection of the disease during the analytical procedures based on PCR (García-Cañas *et al*., 2004), and the pollutants coming from the extraction process that can inhibit these reactions (Hughes and Moody, 2007).

Extraction of DNA from *D. citri* psyllids

To perform the extraction of DNA from psyllids three methods were compared: Manjunath *et al.* (2008), Aljanabi *et al*. (1998) and Teixeira *et al*. (2005). In all the cases, the sample was made up by six nymphs or six adults. The concentration, purity and integrity of the three quality indicators were evaluated.

Determination of the integrity, concentration, and purity of DNA extracts

Extracted DNA was run on an agarose gel 1.2% to determine its integrity (undegraded DNA, and without DNA presence scanning) according to a scale from one to five (one meaning completely degraded DNA and five non-degraded DNA). The concentration of all samples was determined by a spectrophotometric analysis using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE), according to the manufacturer's instructions and procedure. Its purity was assessed by a 260/280 nm ratio, with and approximate value of 1.8. This value indicates the DNA is free of contaminants. The effectiveness of the DNA extraction process was calculated by the following equation:

$$A = \frac{(B)\,(C)}{D} \qquad (1)$$

where, A is the DNA performance (ng of ADN/mg of plant tissue); B, is the volumen of the DNA extract (µL of H_20 or buffer in which the extracted DNA pellet was resuspended), in accordance to the used extraction method); C, is the DNA concentration in the volume of extract (ng μL^{-1}); D, is the plant tissue weight used in each DNA extraction method (mg).

Statistical analysis

The statistical analysis was performed using the "t-Student" test, specifically the couple comparison products of SAS (Statistical Analysis System) software version 8.0 (SAS Institute, 2002), which integrated both plant and insect

samples, previous verification of the assumptions of normality and homoscedasticity.

Discussion and results

Extraction effectiveness and integrity of the DNA absorbance range 260/280 nm, from leaf tissue

In each of the methods for extracting DNA from leaf tissue different factors were evaluated: concentration (ng μL^{-1}), performance (ng mg^{-1} DNA from plant tissues), the purity by the absorbance ratio 260/280 nm, indicating the presence or absence of protein contaminants, and the integrity of the relation to the non-degradation of the total DNA. Once the parameters were evaluated, it was observed that the DNA obtained from the Murray and Thompson (1980) protocol, showed the required parameters, and its concentration and purity determined via equipment Nanodrop 1000 (Figs. 1 and 2) was adequate. Thompson's protocol (1983), was a good indicator which enabled establishing that although its concentration was optimized for the needs of amplification (396.8 ng DNA/mg plant tissue, on average), its purity (1.2) indicated the presence of polyphenols due probably to the absence of PVP for DNA extraction, and its integrity (2.4) was also deficient, as degradation was evidenced, which can decrease the efficiency of the PCR (Holden *et al*., 2003).

The protocol reported by Rodríguez *et al.* (2010), starting from 500 mg of plant tissue, had optimal quality parameters with a high purity of the DNA (1.77 to 2.06), and an appropriate integrity (4.5) defined by non degraded DNA, the DNA concentrations extracted were relatively high compared with the other extraction methods evaluated (Fig. 2). Figure 3 shows the integrity of DNA extractions obtained by this protocol, which was the best of the four methods analyzed. Finally, it was demonstrated that when assessing the commercial Qiagen method, yield was inferior to the other procedures tested in this study. Additionally, the absorbance rate indicated the presence of contaminants such as not hydrolyzed proteins and polyphenols, which could not be completely removed during the process. However, the integrity of the method had an acceptable value compared to the other protocols (4.1).

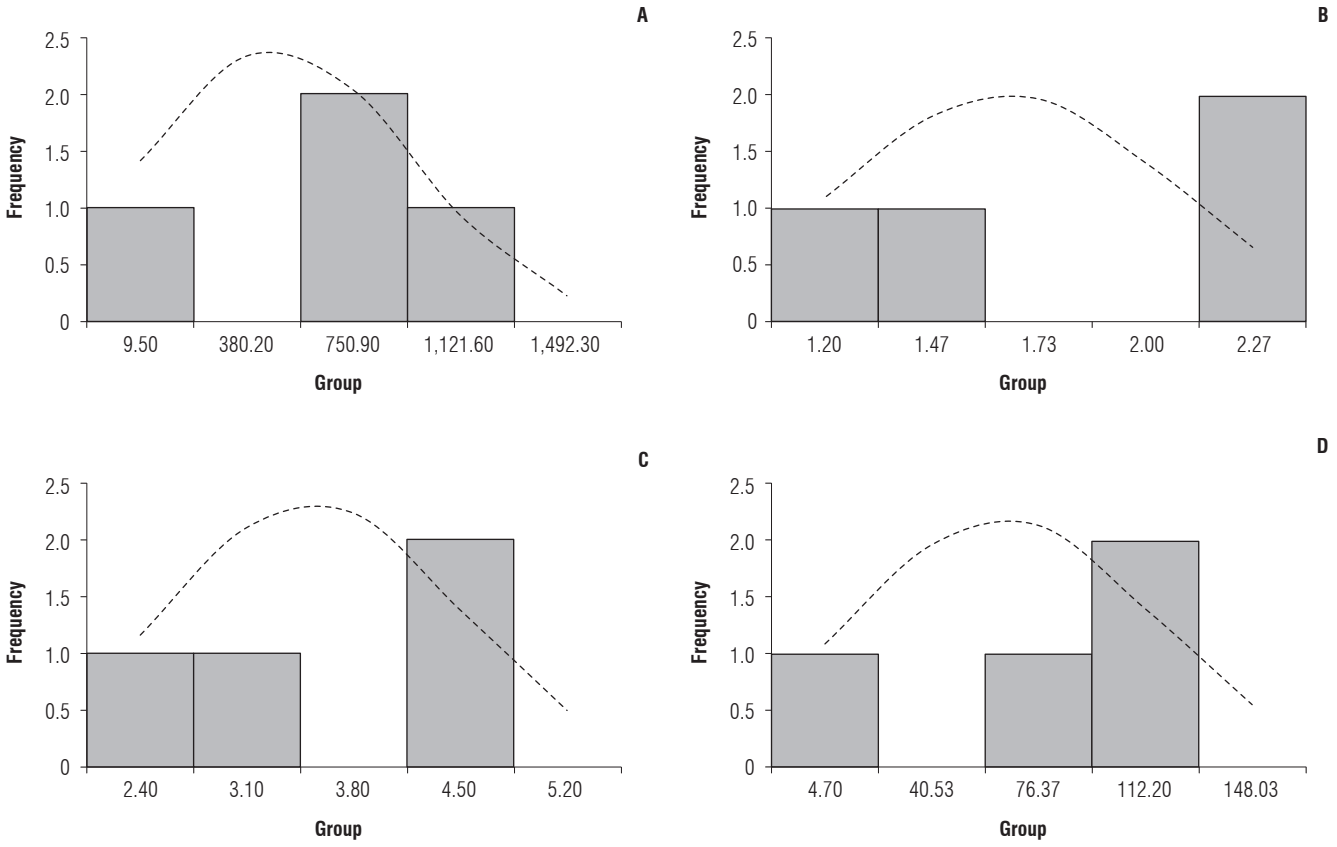

FIGURE 1. Distribution data with respect to the average A, concentration; B, absorbance; C, integrity; D, yield of four DNA extraction protocols from citrus leaf tissue. Positive asymmetric in concentration parameters, integrity and yield was observed, while for the absorbance distribution was symmetrical.

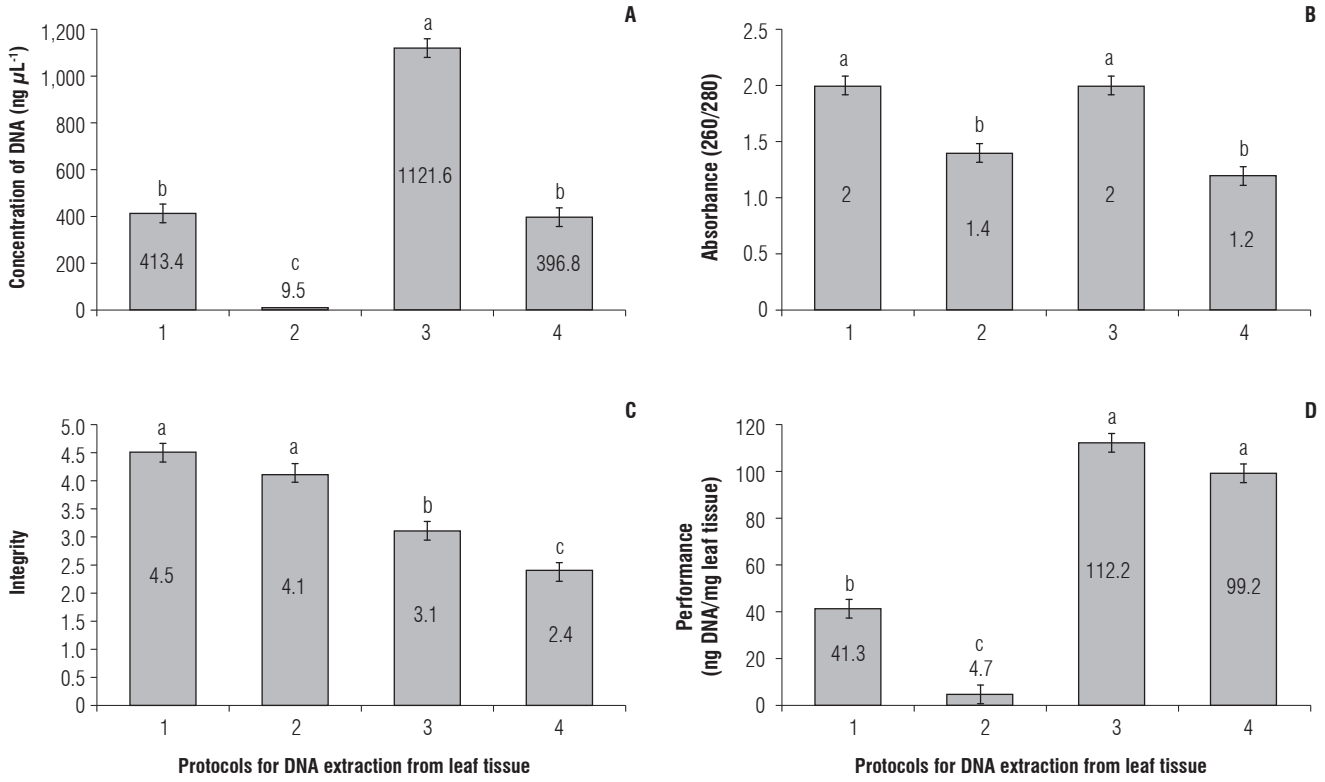

FIGURE 2. Comparison of the concentration parameters A, concentration; B, purity; C, integrity; D, performance of the four DNA leaf tissue extraction protocols cited by Rodríguez *et al.* (2010) (1), Qiagen (2), Murray and Thompson (1980) (3) and Thompson (1983) (4). Means with different letters indicate significant differences according to t-Student test ($P \leq 0.05$) (n = 10). Error bars indicate standart deviation.

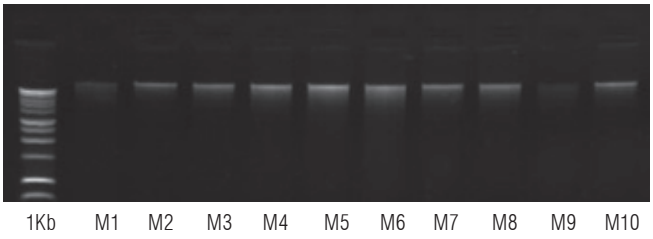

FIGURE 3. Visualization of total DNA from ten samples of citrus plants in agarose gel 1.2%, extracted by the method of Rodríguez *et al*. (2010). Line 1 shows a molecular weight marker (1 kb). Lines M1 to M10, show analyzed samples of citrus plant tissues.

Concentration and integrity of the DNA extractions, and the absorbance range 260/280 nm, from psyllids

The visualization of the results for each of the evaluated parameters (Figs. 4 and 5) to compare DNA extraction methods from psyllid *D. citri,* reported by Manjunath *et al.* (2008), Aljanabi *et al.* (1998) and suggested by Teixeira *et al.* (2005), shows that the latter did not provide good quality parameters, such as the low concentration and poor integrity of the DNA. This is similar to observations reported by Aljanabi *et al.* (1998), where although the concentration of nucleic acids was high (230.6 ng μL^{-1}), the quality of DNA was degraded (1.1) and its absorbance (0.9) indicated the presence of contaminants in the samples.

Finally, the protocol reported by Manjunath *et al.* (2008) allowed establishing the samples proven with this method had the best integrity among all the other tested protocols (Fig. 6), and outstanding concentration of nucleic acids and a better absorbance ratio that indicated that the samples were free of contamination.

Because the concentration of the bacteria's variants that causes HLB can be very low in the insect vector and especially in citrus leaf tissue, causing that the symptoms of this disease are not always easily observed, consequently it is very important to have a method that give the best indicators of purity, quantity and quality, so although certain protocols in this study showed good results in one or more of the parameters evaluated, should not be used as a starting point for the diagnosis of HLB, because contaminants such as polyphenols and no hydrolyzed proteins could lead to inhibition of the PCR reactions and lead to false negatives.

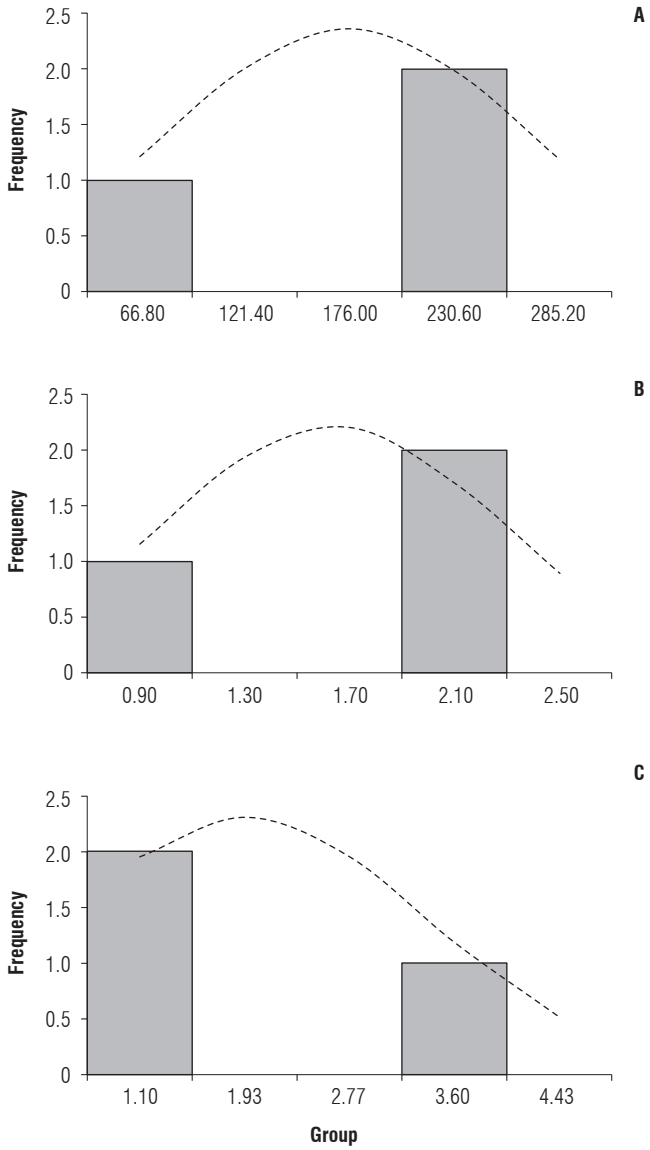

FIGURE 4. Distribution data with respect to the average A, concentration; B, absorbance; C, integrity of three DNA extraction protocols from D. *citri* psyllids. Negative asymmetric in concentration and absorbance parameters was observed, while for the integrity its distribution was positive asymmetrical.

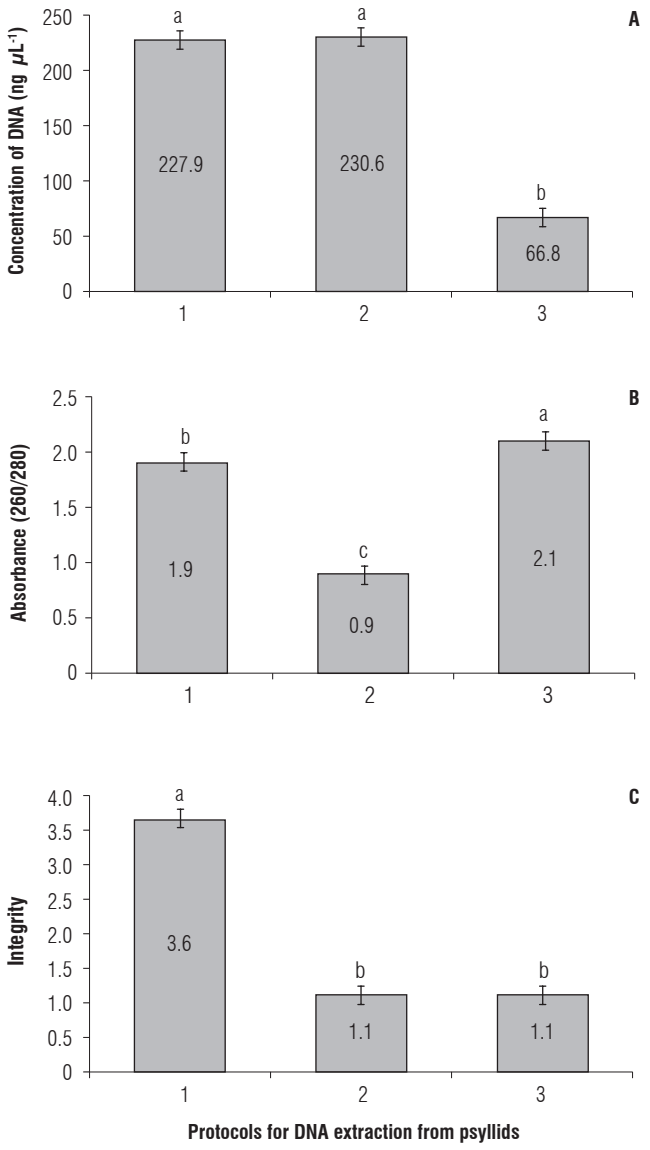

FIGURE 5. Comparison of the concentration parameters A, concentration; B, purity; C, integrity; of DNA extraction protocols psyllids from *D. citri* cited by Manjunath *et al*. (2008) (1), Aljanabi *et al*. (1998) (2) and Teixeira *et al*. (2005) (3). Means with different letters indicate significant differences according to t-Student test ($P \leq 0.05$) (n = 10). Error bars indicate standart deviation.

According this, the statistical analysis conducted by the testing method "t-Student ", significant differences were found between the methodologies evaluated for both DNA extractions made from leaf tissue and those made from *D. citri*. The DNA extraction method for psyllids reported by Manjunath *et al*. (2008), was the best quality indicators obtained and presented significant difference ($P \leq 0.0001$) compared to the methods reported by Aljanabi *et al*. (1998) and Teixeira *et al*. (2005). Regarding the protocols used from plant tissue, the difference between the methods which presented the best quality parameters (Murray and Thompson, 1980; Rodríguez *et al*., 2010), with the protocols

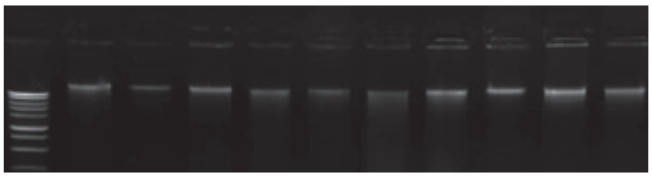

FIGURE 6. Total DNA from *D. citri* psyllids in agarose gel 1.2%, extracted by the method of Manjunath *et al*. (2008). Line 1, shows a molecular weight marker 1 kb. Samples M1-M5, show DNA obtained from adult insect. M6-M10 samples, show DNA obtained from insect nymph.

of Qiagen and the reported by Thompson (1983), was highly significant ($P \leq 0.0001$).

Conclusions

The protocols with best results as quality indicators to perform DNA extractions from leaf tissue of citrus plants were cited by Murray and Thompson (1980) and Rodríguez *et al.* (2010). Also, the method by which the best results were obtained for this procedure from psyllids, was the proposed by Manjunath *et al.* (2008).

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