Extraction and amplification of DNA from Brazilian Red propolis

S. A. Jain[1]; G. M. Marchioro[1]; A. C. Costa[1]; E. D. Araujo[1,2]

[1]*Department of Biology/ Laboratory of Genetics and Conservation of Natural Resources – GECON, Federal University of Sergipe, 49100-000, Aracaju-SERGIPE, Brazil*

[2] *Program of Ecology and Conservation, Federal University of Sergipe, 49100-000, Aracaju-SERGIPE, Brazil*

Sonajain24@yahoo.com

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Propolis is a resinous mixture of plant origin collected by honey bees. The chemical composition and biological activity of propolis depends on its botanical origin thus making it important to connect a particular propolis to its plant source. This manuscript describes DNA extraction from Brazillian red propolis and also shows how the extracted DNA can be utilized as a new alternative to determine the botanical origin of propolis. DNA from propolis was extracted using a CTAB-based method and the extracted DNA was proved to be of good quality for PCR amplification. DNA extraction was carried out from various samples collected independently with similar results proving the successfulness of the described protocol. The extracted DNA was amplified by PCR, using plant specific primers. Sequencing of the PCR amplified bands can be utilized for the identification of its botanical origin.

Key-words: *Apis mellifera*, Botanical origin, molecular approach

Extração e amplificação de DNA de própolis vermelha do Brasil

Própolis é uma mistura resinosa de origem vegetal coletada por abelhas. A sua composição química e atividade biológica dependem das resinas vegetais coletadas pelas abelhas, dessa forma, é importante associar cada tipo de própolis à sua origem botânica. Este trabalho descreve a extração de DNA a partir de própolis vermelha brasileira e também mostra como o DNA extraído de amostras de própolis pode ser utilizado como uma nova abordagem metodológica para determinar a origem botânica de variedades de própolis. O DNA de própolis foi extraído utilizando um método baseado em CTAB e o DNA extraído provou ser de boa qualidade para a amplificação por PCR. A extração do DNA foi realizada a partir de várias amostras recolhidas de forma independente, com resultados consistentes para comprovar o sucesso do protocolo de extração descrito. O DNA extraído foi amplificado por PCR utilizando iniciadores específicos para espécies vegetais. O sequenciamento das bandas amplificadas por PCR pode ser utilizado para a identificação da origem botânica de própolis.

Palavras- chaves: Apis mellifera, origem botânica, abordagem molecular.

1. INTRODUCTION

Propolis is a non-toxic resinous mixture collected by *Apis mellifera* from buds and exudates of plants surrounding the apiary. Bees use propolis for various purposes among them the most important being the use of propolis in the beehive as a protective barrier against both the microorganisms and the intruders [1-3]. Propolis has been used in folk medicines for centuries and its use in food and beverages to improve health and prevent diseases has increased dramatically in recent years [4] because of its proven antimicrobial, anti-inflammatory, anticancer and antioxidant properties [5, 6]. The biological activity of propolis is linked to its chemical composition which in turn is dependent on the plant that is the source of resin. Studies show that propolis produced in different seasons of the year have different chemical compositions due to change in the vegetation surrounding the apiary [7, 8]. Thus it is of great importance to connect a particular propolis to its botanical origin which defines its chemical composition and biological activity. A number of studies have been carried out to identify the botanical origin of propolis. These studies include comparative analysis of the chemical constituents of the plant exudates and propolis using chromatographic methods, palinological and microbotanical methods and direct observation of the bee behavior [9-11].

Chromatographic methods have been used as standard techniques for the identification of botanical origin of propolis. However the presence of contaminants and formation of resin complexes of diverse botanical origins significantly hamper the sensitivity of the process. Buds and plant fragments which are the source of resin collected by the bees can be used as markers for the identification of botanical species as was the case of the botanical origin of Brazilian green propolis [10]. However the morphological analysis of these fragments is very difficult, requiring a profound knowledge of the micromorphology of the plant species, which is an area with great shortage of skilled professionals. Also, this analysis relies a lot on the state of conservation of the morphological structures of the plant material which is generally poor. On the other hand, presence of DNA in these plant fragments can be utilized as a safe and reliable identification method, less dependent on the state of conservation of the morphological structure of the plant material. DNA based method represents a new methodological approach which can also serve as a counter proof to the chromatographic methods utilized currently for the analysis of propolis.

This manuscript describes extraction and amplification of DNA from Brazilian red propolis for the first time. It is based on the fact that propolis apart from containing plant resins also contains pollen and other plant fragments which can be the source of DNA and provide information on its origin. A major obstacle in the extraction of DNA from propolis is the presence of as much as 30% beeswax in propolis [12, 13] which hinders the process of DNA extraction. The presence of phenolic compounds also makes the extraction process difficult. A number of protocols were tested for the extraction of DNA from Brazilian red propolis, including commercially available Macherey- Nagel NucleoSpin® Food, but unsatisfactory results were obtained from all the procedures. None of the protocols tested were able to extract DNA suitable for PCR from propolis. Finally, a CTAB- based protocol [14] for DNA extraction from honey was successfully utilized for the extraction of DNA from this matrix.

1. MATERIALS AND METHODS

Sample preparation

Propolis is hard and brittle at room temperature. It was broken down into small pieces and approximately 5g of propolis was washed with 100-200ml of hexane 2-3 times using a funnel and filter paper. The propolis was then air dried to remove the excess of hexane and stored at -20^{o}C till further use.

DNA extraction

200mg of propolis pre-washed with hexane was homogenized by grinding in a mortar with

liquid nitrogen. Extraction of DNA was carried out as described by Jain et al [14]. Grounded and pre-washed propolis was incubated at 37ºC overnight in lysis buffer containing CTAB, TrisHCl, EDTA, sarcosyl, sorbitol, NaCl, sodiumdisulphite and PVP-40 as recommended by the authors. Next day the mixture was mixed with the same volume of chloroform and isoamyl alcohol and centrifuged. The aqueous phase containing the DNA was then collected in a fresh tube and precipitated with ice cold propanol. Precipitated DNA was washed with 70% alcohol, air dried, dissolved in 25µl TE and stored at-20ºC. The integrity of the extracted DNA was analysed by running approximately 5µl of the extracted DNA on 0.8% agarose gel. The gel was visualized after staining it with SYBR green (Sybr Green I, LCG Biotecnologia, Brazil).

PCR

The DNA extracted from 200mg of propolis was diluted 10^{-1} times and 1µl of the diluted sample was used for PCR amplification in 20µl final volume using 10µl Taq DNA Pol 2X Master Mix Red (Amplicon, Denmark) and 0.5µM ITS2 plant DNA barcoding primers [15]. PCR was performed in a thermocycler using the following conditions: 94ºC/5 minutes, followed by 40 cycles of 94ºC/30 seconds, 65ºC/30 seconds, 72ºC/45 seconds, and a final extention at 72ºC for 10 minutes. 5µl of the PCR product was then run on 2% agarose and visualized after staining with SYBR green under UV. Universal primers for the amplification of beta tubulin gene [16] were also utilized to analyze the quality of the extracted DNA.

2. RESULTS AND DISCUSSION

Propolis is produced by action of various enzymes and substances present in the bee saliva on the plant resin and bee wax mixture [1, 3]. DNA extraction from propolis has never been reported. In this research, the red propolis produced in the coastal regions of the north-east Brazil was treated with hexane to remove the excess wax and then used for the extraction of DNA. The cell lysis buffer apart from CTAB also contained PVP and N-lauroyl sarcosine to remove polyphenols during cell lysis [17]. DNA extraction was carried out many times with samples collected independently at different times of the year, and similar results were obtained all the time. Figure 1 show the total DNA extracted from four different propolis samples. In the figure, lane 6 contains 60ng of DNA from plant as a positive control.

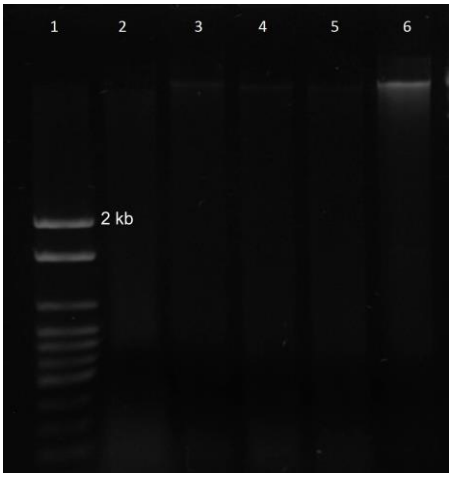

Figure 1: DNA extraction from propolis Lane 1: DNA ladder; lane 2-5: total DNA from propolis Lane 6: total DNA from plant.

DNA extracted from all the four samples could be easily amplified by PCR (Figure 2) using

plant specific primers (15) proving that the DNA extracted using the described protocol is good enough for its amplification by PCR. Four positive controls containing total DNA from plant, fungi, bacteria and honey bee, and one negative control (deionized water) were included during PCR analysis. The amplified DNA fragment from propolis of approximately 450bp (Figure 2, lanes 2-5) is similar in size with PCR fragment produced with plant DNA (lane 6), suggesting that the plant content of the propolis is the source of this PCR fragment. Thus, sequencing of the amplified PCR product from propolis can throw a light of on its plant source. Amplification of propolis DNA with primers based of beta tubulin gene [16] which amplify PCR product in the range of (900bp-2000bp) was unsuccessful (results not shown) suggesting that the amplification of propolis DNA has a size based restriction probably due to fragmentation of its DNA, as is also observed in the case of DNA extracted from processed food. Thus primers which amplify short fragments work much better than primers which amplify large fragments.

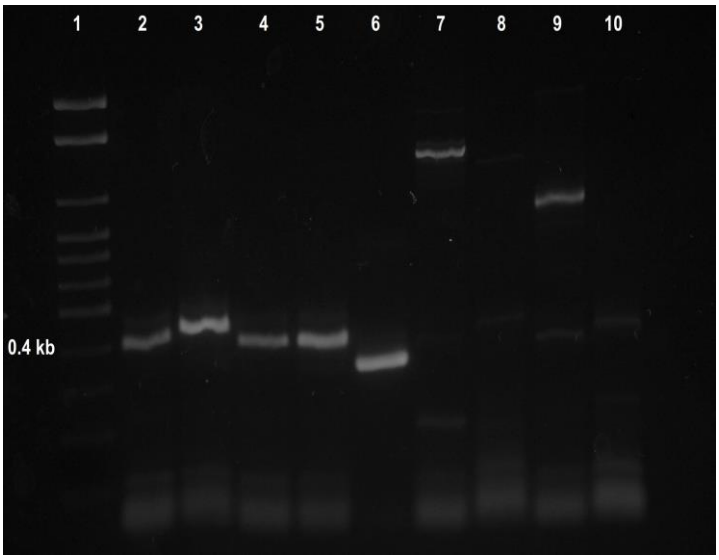

Figure 2: PCR amplificaton using plant barcoding primers. Lane 1: DNA ladder; Lane 2-5 propolis DNA; lane 6: plant DNA; lane 7: fungal DNA; lane 8: bacterial DNA; lane 9: DNA from honey bee; lane 10: deionised water.

3. CONCLUSION

DNA from propolis was successfully extracted. Successful extraction and amplification of propolis DNA is an important achievement which can be used for a variety of analysis on a molecular level including identification of its botanical origin and presence or absence of desired plant species.

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