Optical study of laser biospeckle activity in leaves of *Jatropha curcas* L.: a non-invasive and indirect assessment of foliar endophyte colonization

Maria Fernanda D’Jonsiles, Gustavo Ernesto Galizzi, Andres Ezequiel Dolinko, Maria Victoria Novas, Esteban Ceriani Nakamurakare, Cecilia Cristina Carmarán

Abstract

Currently, the detection of endophytic fungi is mostly determined by invasive methods, including direct isolation of fungal organisms from plant tissue in growth media, molecular detection of endophytic fungi DNA from plant material by PCR, or evaluation using microscopy techniques. In this work, we explore the potential of laser biospeckle activity (LBSA) for the detection of endophytic colonization of leaves of a promising energy crop, *Jatropha curcas* L. We compared the laser biospeckle activity of endophyte infected and uninfected *J. curcas* leaves. The differences between blade and veins (including midrib) of the studied leaves were validated and growth parameters of the studied plants were also analyzed using the normalized weighted generalized differences coefficient (nWGD), which characterizes a particular laser bioactivity behavior. The obtained results showed a relationship between the endophytic burden of leaves and the LBSA, suggesting that LBSA is a useful tool to indirectly detect endophytic colonization in situ. Also, the increased water movements inside leaves promoted by endophytic colonization could be explained by the obtained data.

Keywords Foliar endophyte colonization · Laser biospeckle activity · Normalized weighted generalized differences · Water movement · *Jatropha curcas*

Introduction

The study of biological interactions and their impact, at different biological and ecosystemic levels, has expanded our interpretation and understanding of organisms and their functioning. In plants, biological interactions occurring between functionally distinct symbionts that provide different benefits often result in the promotion of key processes benefiting plant growth and health (Stanton 2003). Examples of this would be providing biotic and abiotic stress resistance and tolerance, enhancing nutrient availability, and degrading toxic substances (Li and Zhang 2015). Fungal endophytes are defined as fungi that live asymptomatically within healthy plant tissues for at least part of their life cycle. This group of fungi has been found in all plant species studied to date (Carroll 1988; Wilson 1995; Jumpponen and Trappe 1998; Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011; Kato et al. 2017), and they are ubiquitous in plants in natural ecosystems. The roles of the endophytes in ecosystems and the nature of the interactions between woody plants and their foliar endophytes are at
an early stage of elucidation. However, some studies have shown that this group of fungi may modulate plant development as well as ecology of plant communities (Herre et al. 2007; Mejia et al. 2008; Yuan et al. 2011). Research interest is particularly focused on the endophytes of leaf and root tissues due to the multiple processes in which they are involved and the interactions of the leaf or root mycobiome with their hosts (Lindow and Brandl 2003; Rodriguez et al. 2009; Persoh 2015; Pandey et al. 2016; Vurukonda et al. 2018; Zhou et al. 2018; Li et al. 2019).

Our ability to visualize complex interactions in the microbiome helps us to understand the different roles that endophytes can play. Researching about the potential benefits of endophytic fungi, such as protection against herbivory or resistance to stress, involves studying endophyte-colonized plants (E+), which need to be monitored and be distinguishable from non-colonized plants (E−). Detection of endophytes is typically achieved through several techniques, including direct isolation of fungi from plant tissue using growth media (culture dependent), molecular detection of endophytic fungi DNA from plant material by PCR (culture independent), or histological techniques for microscopy (Hyde and Soytong 2008; McKinnon et al. 2017). All these methods involve a destructive analysis of the sample and the assumption that the results reflect the existing endophytic communities in all the tissue, both in the part analyzed and in the remaining ones, which for different reasons, cannot be analyzed. In this context, the application of non-invasive techniques represents a great advantage and increases our ability to detect this type of organisms in situ.

The speckle effect occurs when coherent light, such as the one coming from a laser source, is scattered over a rough surface or transmitted through a medium with random variations in its refractive index. The final optical field is the result of the interference of the light scattered by the optical inhomogeneities randomly distributed in the medium. Consequently, the resulting light intensity distribution presents a granular structure and is known as speckle pattern. If the scattering medium shows some type of activity that changes the optical path of the interfering light, e.g., micrometric movements or small variations of refractive index, the resulting speckle pattern changes in time, and the visual appearance is similar to that of boiling liquid. This effect is called dynamic speckle. The study of the temporal evolution of the dynamic speckle is a widely known non-contact and non-destructive technique, used to characterize the parameters involved in the processes that generate the activity of the scattering media (Rabal and Braga Jr 2008). If the scattering media is a biological sample, the effect is known as biospeckle. In this case, the activity observed is the result of movement inside the living tissue and can be attributed to many processes, such as growth and cell division, cytoplasmic movement, biomechanical reactions, or water-related activities (Zdunek et al. 2014; Ansari and Nirala 2015). The study of the biospeckle activity, also known as biospeckle laser or LBSA (laser biospeckle activity), has been widely applied for analyzing different processes, such as plant seed activity, blood flow detection, visualization of tissue perfusion, burn scar perfusion, fruit maturation, and assessment of leaf vein system (Zhong et al. 2014; Zdunek et al. 2014; Ansari and Nirala 2015). Regarding fungi, it was applied for the detection of pathogenic fungi in seeds (Mancini et al. 2016) or to test the efficiency of fungicides (Silva et al. 2018), evaluating their biological activity with promising results.

Jatropha curcas L. (Family: Euphorbiaceae) is a promising energy crop that is being extensively studied to develop biofuel technology from its seeds, as well as for other beneficial uses. Studies in progress on this species suggest that the artificial inoculation of endophytes can improve certain interesting agronomic features of plants (D’Jonsiles et al. 2019). However, the techniques used today do not provide non-invasive tools that demonstrate if the inoculation was successful or not.

The aim of this study was to test the potential of laser biospeckle activity (LBSA) for the indirect detection of foliar endophytes colonization. This approach is based on comparing the biospeckle activity of endophyte infected and uninfected J. curcas leaves. The differences between blade and veins of the studied leaves were validated and growth parameters of the studied plants and their correlation with LBSA parameters were also analyzed.

Materials and methods

Plant material

Seeds of J. curcas L. were provided by Ing. Diego Wassner (Cátedra de Cultivos Industriales, Facultad de Agronomía de la Universidad de Buenos Aires, Argentina). They were collected from experimental plots of J. curcas located in Siete Palmas, Formosa Province, Argentina (25° 13′ 21.04″ S, 58° 17′ 59.67″ W).

Endophyte infection and colonization analyses

In order to reach the proposed objective, different procedures were performed, which allowed obtaining leaves of J. curcas with different status of endophytic colonization (high or low), and thus analyze foliar tissue to determine the percentage of colonization through culture studies, evaluate biomass indicators, and study the LBSA on leaves of J. curcas.

For these procedures, 120 seeds were pre-germinated in plastic trays, and transferred to plastic pots, with commercial soil (Terra fertile S.A.) to obtain young plants. From those, 24 plants whose leaves showed no sign or symptom of any
disease were selected: 10 plants were selected to determine the specific age of the leaves that contained the greatest endophytic burden, and 14 plants were selected for LBSA analysis.

Foliar endophytic colonization is a natural process. Thus, it was necessary to perform the following experimental procedure to obtain plants with low or no colonization: E− plants (endophyte colonization < 25%) were covered with transparent acetate in order to avoid the infection and E+ plants (endophyte colonization > 25%) remained uncovered (Arnold and Herre 2003, D’Jonsiles et al. 2019).

For the different procedures carried out thereafter, leaves (or cotyledons, in the corresponding case) were analyzed. Each leaf was cut into segments of about 2 × 2 cm. In the case of leaves analyzed by LBSA, endophyte isolation was performed on segments corresponding exactly to the evaluated areas (Fig. 1). All segments (including the areas of leaves previously analyzed by LBSA) were sterilized using the following immersion sequence: 1 min in 70% ethanol, 2 min in a 4% bleach solution, and 30 s in 50% ethanol. Each segment was transferred to 90-mm Petri dishes containing MEA: malt extract (OXOID) 2%, agar (Difco) 2% supplemented with chloramphenicol (Sigma) 1%. Plates were incubated at 24 °C for six days and examined periodically. Outgrowing mycelia were isolated, purified, transferred onto slants containing MEA, and stored at 4 °C. Once the isolate fungi were obtained in pure culture, the percentage of endophyte isolated was registered as follows:

\[
\%EI = \frac{\text{Total of endophytes isolated}}{\text{total of segments analyzed per leaf}} \times 100.
\]

**Endophyte leaf colonization**

In order to determine the adequate leaf age for the analysis of endophytic colonization using LBSA, 10 plants were grown for four months to keep them exposed for enough time to the endophyte inoculum present in the environment. Endophyte isolations were carried out from cotyledons (which can persist in the plant for as long as a month) and from 2-, 4-, 6-, 8-, or 10-day-old leaves. The findings (see “Results” for details) indicated that 10-day-old leaves represented the adequate age to perform the following tests. Taking into account these preliminary results, 14 plants (7E+ and 7 E−) were grown for 4 months (from January to April 2017), and they were subsequently used to evaluate plant biomass and growth parameters. Additionally, fourteen 10-day-old leaves (7 E+ and 7 E−) were used for the analyses carried out with LBSA and endophyte isolation.

**Plant biomass and growth parameters**

After four months, all plants were harvested and fresh weights (FW) were registered. The dry weights (DW) of shoots and roots were recorded after oven-drying them at 60 °C to constant weight. Root (RL), stem (SL), and total length (TL) were also measured.

**Biospeckle data analysis**

Among the 14 plants assigned to categories E+ or E− (7 E+ and 7 E−), 7 plants per category were chosen (those that did not show symptoms or signs of any disease), and one leaf per plant was used for LBSA analyses. The determination of biospeckle activity is based on the acquisition of a temporal sequence of images \(\{I_1, I_2, \ldots, I_N\}\) of the illuminated sample, where \(N\) is the total number of images recorded. Then, the detected light intensity for every pixel of the images acquired during the monitoring time is processed by a computational method. Different methods have been developed to characterize biospeckle activity, and the choice of a specific approach depends on the nature of the phenomenon under study. In situations where the biospeckle activity is not uniform over the sample, image-processing-based techniques such as the Fujii method, weighted parameterized Fujii method, generalized differences (GD), or weighted generalized differences (WGD) methods are efficient to characterize the detected activity (Rabal and Braga Jr 2008; Ansari and Nirala 2015; Arizaga et al. 2002).

In order to analyze biospeckle activity in *J. curcas* leaves, we used the WGD coefficient defined at pixel coordinates \((x,y)\) as
WGD(x,y) = \sum_{k=1}^{N-\Delta} \sum_{\delta=1}^{\Delta} |I_k(x,y) - I_{k+\delta}(x,y)| p_\delta

where \( I_k \) is the intensity of the \( k \)-th image, \(|\cdot|\) stands for the absolute value operator, \( p_\delta \) are user-defined weights, \( N \) is the total number of images acquired, and \( \Delta \) is the number of consecutive images to be considered in the computation of the differences. In summary, all the possible differences between the \( \Delta \) consecutive images are added in absolute value for each pixel, and this procedure is repeated for the first \( N - \Delta \) images of the recorded sequence. Finally, the accumulation of the evaluated differences results in the WGD coefficient at pixel coordinates \((x,y)\). In this study, we set \( N = 2000 \), \( \Delta = 10 \), and \( p_\delta = 1 \) for every \( \delta = 1, \ldots, \Delta \).

As every \( I_k(x,y) \) value is subtracted in absolute value from all other values at the same location, the result does not depend on the order of appearance of the intensity values. It results that the WGD is minimum when all \( I_k(x,y) \) are approximately equal, i.e., the activity is low or non-existent, or high when all \( I_k(x,y) \) are very different. Conversely, it can be seen that the WGD value is maximum when the \( I_k(x,y) \) values in the time histogram are evenly distributed near the bounds of the gray-level interval. This is so when the lower value occurs for half the time, and the other half, the higher value appears. In our case, the gray-level interval is between 0 and 255. Thus, if the histogram of the intensity values of a pixel as a function of time shows two or more modes (i.e., maxima), the value of the WGD is greater when the modes are more separated.

If the \( p_\delta \) weights are set to zero for all but the smaller \( \delta \) values, relatively fast variations are enhanced with respect to slower ones. On the other hand, when processes are very slow with respect to the acquisition time and their variations are hidden by noise, it is more convenient to compare only frames that bear a significant time separation. That is to say, the \( p_\delta \) weights are set to zero for small \( \delta \) values and to one for some time interval where the process is expected to show significant variations (Rabal and Braga Jr 2008; Arizaga et al. 2002).

Table 1

| Leaf age | %EI |
|----------|-----|
| Cot      | 0.000 |
| 2        | 0.000 |
| 4        | 6.667 |
| 6        | 9.266 |
| 8        | 26.427 |
| 10       | 60.632 |
**Optical setup**

A diagram of the optical setup used in the experiments is depicted in Fig. 2. In this study, we used a green light laser in order to minimize the light absorbed by the leaves and to be able to get the information from the leaf surface. In this manner, we aimed to minimize the interference with the leaf photosystems. The green light from a Nd:YAG laser (Coherent model Compass 315 M, 100 mW, lambda 532 nm) is expanded by means of the expansor lens (L) and directed to the specimen (S) by means of an illumination mirror (M1). The light scattered by the specimen is directed to the high-speed video camera (Dalsa model CA-D6), externally driven by the frame grabber (Coreco Imaging PC-DIG) by means of an observation mirror (M2). The resolution of the acquired images was 256 × 256 pixels and 256 Gy levels. The camera frame rate was set to 25 images per second and each dataset contained 2000 images. The specimen S (i.e., the plant leaf) lays horizontally on a supporting plate clamped to the optical bench. Additionally, the leaf was covered by a transparent acrylic plate to prevent spurious movements of the leaf. The assembly of the plants in the optical bench is shown in Fig. 3a and Fig. 3b depicts the optical setup during LBSA measurements.

**Image processing**

The sequences of biospeckle images were processed by evaluating the WGD coefficient. As explained in the “Biospeckle data analysis” section, this coefficient was independently evaluated for each pixel of the image sequence. Finally, a matrix containing the WGD coefficient value for each pixel was obtained.

In order to minimize the influence of changes in color and reflectivity of each analyzed leaf, or other unknown surface variables of different leaves, the WGD matrix of each leaf was divided by its maximum value in the same matrix, resulting in a normalized WGD (nWGD) matrix. In this way, we obtained a matrix ranging from 0 to 1, where unity corresponds to the maximum value of nWGD obtained for each image sequence. Afterwards, we computed the histogram corresponding to every nWGD matrix. To construct the histogram, the range of nWGD values was divided into 256 consecutive non-overlapping equal-sized intervals or bins. Then, we counted how many times the nWGD values fell into each bin, and this count value is named frequency. Consequently, the histogram is an estimate of the probability distribution of nWGD because the higher the count (or frequency) is, the greater the probability of occurrence of a nWGD value. Finally, we compared the maximum frequency (MxF) and the corresponding nWGD values for all samples.

**Statistical analysis**

In order to compare differences between E+ and E− plants among all variables, a non-parametric Kruskal-Wallis test was performed: nWGD (normalized weighted generalized differences); MxF (maximum frequency); FW (fresh weight); DW (dry weight); RL (root length); SL (stem length); TL (total length).

A multivariate analysis was applied to LBSA and growing parameters in 10 plants (5 E+/5 E−). Principal component analysis (PCA) was used to identify groups of plants showing similar patterns with respect to those variables. Therefore, this analysis intended to extract the main trends, revealing the structure of the data. As the variables MxF and TL are directly obtained from nWGD and the sum of RL and SL, respectively, they were excluded from the analysis to avoid redundancy. When analyzing the original data set of quantitative variables, no potential outliers were found and all assumptions were met. Since the variables are expressed in different measurement scales, we computed a PCA on the correlation matrix to standardize the variables.

All analyses were performed with R version 3.4.3 2 (R Development Core Team, 2017).
Results obtained from the study of endophytic colonization in function of leaf age (Table 1) showed that cotyledons and 2-day-old leaves did not present endophytic colonization, and 10-day-old leaves carried a larger burden of endophytes than 4-, 6-, or 8-day-old leaves. Therefore, subsequent analyses were performed on 10-day-old leaves of plants with and without exposure to natural infection (E+ and E− plants respectively). Leaves of E− plants registered values of percentage of endophyte isolated (%EI) between 0 and 25%, while E+ plants showed values between 25 and 100% EI (Table 2). The genera of endophytes obtained from E+ and E− leaves of Jatropha curcas were Alternaria, Aspergillus, Colletotrichum, and Nigrospora. The percentage frequencies of the fungal genera obtained are shown in Table 3. Results obtained from LBSA showed that E− leaves registered the higher values of MxF (maximum frequency) but the lower values of nWGD (normalized weighted generalized differences). E− plants showed higher values of FW (fresh weight), DW (dry weight), RL (root length), SL (stem length), and TL (total length) compared with E+ plants (Table 4).

Differences between variables, analyzed through a Kruskal-Wallis test, are shown in Fig. 4. The results for biomass analysis showed that there are significant differences for DW (H = 7.62, p = 0.0057). For FW, there were no significant differences (H = 1.65; p = 0.19). The Kruskal-Wallis test for growing parameters revealed significant differences for RL (H = 9.19, p = 0.0024), but not for SL (H = 0.05174, p = 0.82) and TL (H = 1.97, p = 0.1598) (Fig. 4a).

As a result of the PCA based on growth variables, including nWGD, the proportion of variance representing the first two axes was 85% (axe I = 60%; axe II = 25%). Axis I was positively associated with nWGD and negatively associated with dry weight (DW), foliar weight (FW), length root (RL), and stem root (SL). Axis II was positively associated with nWGD, SL, and WW and this axis was negatively associated with RL. The plot showed that the plants cluster in two groups. The group located on the left was formed by plants 1–5, all E−, which displayed the highest values of DW, FW, RL, and SL and the lowest values of nWGD. The group on the right included plants 6–10, all E+, which displayed the highest values of nWGD and the lowest values of the other variables (Fig. 7). Plant 6 (E+) was located at the positive end of axe I, a short distance apart from the rest of the E+ plants.

The LBSA was carried out on 10-day-old leaves from E− an E+ plants. The results for nWGD and MxF showed significant differences in both cases between E+ and E− leaves (H = 6.86, p = 0.0089; H = 6.81, p = 0.009 respectively) (Fig. 4b and c respectively).

The nWGD images of the speckle image sequences of the evaluated leaves for E+ and E− showed noticeable differences. The nWGD for the E− cases studied showed lower values in the leaf blade than in the veins (Fig. 5a, b). On the contrary, the nWGD for the E+ cases showed values in the same order of magnitude for the veins, and the region of the blade, as shown in Fig. 5c and d. The frequency of value of nWGD is represented by the histograms (Fig. 6), as a function of the nWGD range. The obtained data showed two different peaks, corresponding to E+ vs E− leaves.

Table 4 Values obtained from E− (< 25% of isolation) or E+ (> 25% of isolation) Jatropha curcas leaves for all the variables studied

| Leaves | (•) nWGD | (•) MxF | (••) FW | (••) DW | (•••) RL | (•••) SL | (•••) TL |
|--------|----------|--------|--------|--------|--------|--------|--------|
| E− (<25%) | 0.203 | 547 | 12.38 | 15.41 | 14 | 22 | 36 |
| 0.221 | 634 | 8.59 | 12.31 | 15 | 17 | 32 |
| 0.217 | 609 | 10.53 | 11.51 | 16 | 20 | 36 |
| 0.242 | 498 | 15.47 | 12.38 | 14 | 26 | 40 |
| 0.285 | 439 | 11.84 | 12.52 | 13 | 21 | 34 |
| 0.221 | 557 | 5.9 | 1.09 | 13 | 24 | 37 |
| 0.224 | 501 | 8.59 | 1.82 | 17 | 23 | 40 |
| E+ (>25%) | 0.281 | 399 | 3.04 | 0.65 | 8 | 15 | 23 |
| 0.296 | 498 | 7.21 | 1.31 | 9 | 17 | 26 |
| 0.314 | 403 | 12.81 | 2.76 | 8 | 24 | 32 |
| 0.392 | 368 | 11.92 | 2.32 | 10 | 27 | 37 |
| 0.371 | 373 | 5 | 1.17 | 11 | 19 | 30 |
| 0.285 | 432 | 3.1 | 0.55 | 10 | 14 | 24 |
| 0.342 | 353 | 6.33 | 1.35 | 7 | 21 | 28 |

nWGD, normalized weighted generalized differences; MxF, maximum frequency; FW, fresh weight; DW, dry weight; RL, root length; SL, stem length; TL, total length, (•) adimensional values, (••) values in g, (•••) values in cm.

The LBSA was carried out on 10-day-old leaves from E− an E+ plants. The results for nWGD and MxF showed significant differences in both cases between E+ and E− leaves (H = 6.86, p = 0.0089; H = 6.81, p = 0.009 respectively) (Fig. 4b and c respectively).
This plant showed the highest value of nWGD and higher values of FW, DW, and SL, compared with the rest of the E+ plants.

The Pearson coefficient ($r = 0.91; P = 0.001$) between segment the scores of axe I and endophyte percentage indicates a positive and significant association between these vectors.

**Discussion**

Considering that endophytes play important roles in plant fitness, and traditional techniques to study them are most often destructive, here, we apply a non-invasive technique to explore the LBSA in endophyte-colonized and non-colonized leaves of *J. curcas*. The results suggest that the biospeckle activity could be used as a good indirect indicator of endophytic colonization in leaves.

Our results show that the foliar endophytic community isolated from *J. curcas* is mainly represented by the Ascomycota phylum, composed of a limited number of taxa, such as *Alternaria*, *Aspergillus*, *Colletotrichum*, and *Nigrospora*, all of which were previously reported as endophytes of this host (Jaiswal and Pandey 2013, Kumar and Kaushik 2013). An interesting result is that, in the present study, strains of *Alternaria* were those most frequently isolated in agreement with previous work (D’Jonsiles et al. 2019).

LBSA is a technique that is currently used in different fields of science. Braga Jr et al. (2007) presented results obtained from the application of biospeckle phenomena to detect fungi in beans (*Phaseolus vulgaris* L.). On the other hand,
White et al. (2011) used this methodology for computing functional vascular density within a rodent dorsal window chamber model. In turn, Zhong et al. (2014) used this analysis for surface activity detection of attached leaves to monitor variation of leaf water status. Also Zdunek et al. (2014) reviewed the current stage of biospeckle technique and its applications, particularly for plant tissue evaluation. They concluded that the biospeckle method shows a number of interesting non-destructive applications in agricultural crops. At present, in the area of quality evaluation, this method is still under development and has a chance to be commercially used as it is already applied in medicine (Shvartsman and Fine 2003; Zdunek et al. 2014; Farraro et al. 2016; between others). Our study was based on the analyses of fungal

![Fig. 5](image)

Representative images of biospeckle activity obtained from plants analyzed. a and b represent E− leaves (free of endophyte). c and d represent E+ leaves (infected with endophytes). Note that the highest values are represented in red.

![Fig. 6](image)

Fig. 6 Values of nWGD obtained after biospeckle data analysis. The histogram shows that lower values of nWGD (normalized weighted generalized differences) with high values of MxF (maximum frequency) correspond to E− leaves, while highest values of nWGD with low values of MxF correspond to E+ plants. Y axis represents the frequency, and X axis represents the value of nWGD. Each color represents an analyzed leaf. Note that in the histogram, not all the analyzed leaves are shown. This is because the lines overlap, which would make it difficult to read the graph.
endophytes from 10-day-old leaves of *J. curcas*. We observed that there is a clear relationship between the endophytic burden of leaves and the biospeckle activity. Our data show that leaves with low endophyte colonization (E−) have lower nWGD values, but higher values of MxF, while leaves with high endophyte colonization (E+) have higher values of nWGD but lower values of MxF.

Interestingly, multivariate analyses showed that E+ and E− plants formed two distinctive groups. In particular, this could be related to biomass and growth parameters that differed significantly between those groups and that are correlated with endophyte colonization. E+ and E− plants presented similar fresh weight, but E+ dry weight was significantly lower than in E− plants. These differences may be attributed to higher content of water in E+ plants. Furthermore, roots were significantly longer in E− plants than in E+. Both results suggest that E+ plants would have a more efficient translocation of water than E− plants.

Plant–fungus associations, which are ubiquitous in plant communities, play important and varied roles in different plant physiological aspects (Hyde and Soytong 2008). Fungal pathogens associated with roots, vascular tissue, and foliage may interfere with water uptake and transport, increase rates of foliar transpiration, and induce xylem embolism and tissue death (Agrios 1997). In contrast, rhizosphere mutualists such as ecto- and arbuscular mycorrhizal fungi may benefit hosts by increasing surface area for water uptake, enhancing stomatal regulation of water loss, and increasing root hydraulic conductivity (Augé 2001; Lösch and Gansert 2002). Arnold and Engelbrecht (2007) took an experimental approach to show that endophyte colonization increases minimum leaf conductance, a measure of leaf water loss after maximal stomatal closure under drought stress, in the tropical tree *Theobroma cacao*. They showed that during maximum stomatal closure, leaves infected with a natural density and diversity of endophytes exhibit almost two-fold rates of water loss in relation with uninfected leaves. In contrast, from the studies reviewed by Dastogeer and Wylie (2017), it is evident that endophyte colonization can significantly improve plant drought stress tolerance. These authors suggest that non-mycorrhizal fungi (for example foliar endophytes) may mediate the effects of water stress by adjusting, regulating, or modifying plant physiological, biochemical, and metabolic activities. In accordance with this, our data for DW and RL suggest that E+ plants would have a more efficient translocation of water than E− plants, as previously mentioned. This is also consistent with the results obtained for LBSA analysis, where we observed a greater activity in E+ leaves, and this response would be explained by water movements inside leaves.

An emergent property of the endophytic fungi as a result of the multiple interactions between plants and microorganisms is their great phenotypic plasticity, which means their ability to respond to various environmental signals. Our ability to visualize complex interactions in the microbiome helps us to
understand the different roles that endophytes can play (Porras-Alfaró and Bayman 2011). Employing fast and non-invasive methods of detection of these organisms, such as the one we applied in this work, and encouraging research in this regard can be very useful in the area of agriculture.

Conclusions

LBSA is a promising non-invasive technique that is currently used for medical purposes, and it is considered to have great potential in agronomic areas. The present work represents a first approach for the development of a new tool to detect not only the status of endophytic colonization (both fungal and bacterial) in a crop of interest, mediated by an indirect, non-destructive, and faster technique, but also to detect those that cannot be isolated. The fungal genera identified in this work have already been reported as endophytes of *Jatropha curcas* (Jaiswal and Pandey 2013, Kumar and Kaushik 2013; D’Jonsiles et al. 2019), but the assays performed here did not include the evaluation of endophytic bacteria or epiphytic organisms that could be contributing to the response obtained. Despite this, the evidence accumulated so far (Augé 2001; Lösch and Gansert 2002; Arnold and Engelbrecht 2007; Dastogeer and Wylie 2017) links the movements of water and/or nutrients within the plant with the presence of fungal endophytes, supporting the idea that LBSA is mainly related to plant tissues infected with fungal endophytes. The results obtained in this work constitute a solid starting point to continue exploring the virtues of this promising method. However, more studies are necessary to determine its accuracy.

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