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ORIGINAL PAPER

# Live biospeckle laser imaging of root tissues

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**Abstract** Live imaging is now a central component for the study of plant developmental processes. Currently, most techniques are extremely constraining: they rely on the marking of specific cellular structures which generally apply to model species because they require genetic transformations. The biospeckle laser (BSL) system was evaluated as an instrument to measure biological activity in plant tissues. The system allows collecting biospeckle patterns from roots which are grown in gels. Laser illumination has been optimized to obtain the images without undesirable specular reflections from the glass tube. Data on two different plant species were obtained and the ability of three different methods to analyze the biospeckle patterns are presented. The results showed that the biospeckle could provide quantitative indicators of the molecular activity from roots which are grown in gel substrate in tissue culture. We also presented a particular experimental configuration and the optimal approach to analyze the images. This may serve as a basis to further works on live BSL in order to study root development.

**Keywords** Tissue culture · Biospeckle · Roots · Image analysis · Live imaging

**Abbreviations**

| | |
|---|---|
| BSL | Biospeckle laser system |
| GD | Generalized differences |
| LASCA | Laser speckle contrast analysis |
| LSV | Laser speckle velocimetry |
| PIV | Particle image velocimetry |

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## Introduction

Dynamic laser speckle, also known as biospeckle, are interference patterns formed when a biological specimen is irradiated by a coherent light, such as a laser source.

The biological activity, addressed in the dynamic laser speckle applications, does not refer to a precise definition but relates broadly to a range of processes such as cell growth and division or cellular processes, represented by organelle movement, cytoplasmic streaming, or biochemical reactions. This activity result in the movement of molecules and microscopic structures (scatterers) that creates interference patterns, named dynamic speckle, and this physical phenomenon can be exploited to measure sensitive changes in the biological functioning of cell and tissues. The approach was first introduced by medical scientists to study the perfusion of blood flow in various tissues (Briers 1975; Fujii and Asakura 1985; Rajan et al. 2006; Serov and Lasser 2005; Wardell et al. 1993).

In many cases, however, biospeckle patterns are not the result of well-defined macroscopic fluxes of matter, such as those observed during blood flow (Zhao et al. 1997). This was supported by numerous studies showing correlation between biospeckle patterns and well-identified biological processes: in agriculture, the use of dynamic speckle has been reported in areas related to animal reproduction, measuring the whirl (Carvalho et al. 2009), to fruits analysis of maturation and bruising (Xu et al. 1995; Pajuelo et al. 2003; Rabelo et al. 2005), to seeds evaluation of

moisture and separating endorsperm from embryo (Braga et al. 2003; Sendra et al. 2005), and to fungi or to parasites identifying their presence (Pomarico et al. 2004; Braga et al. 2005).

The analysis of dynamic laser speckle patterns involve the processing of the stochastic patterns of pixel intensity of digital images collected from illuminated samples, which provides numerical descriptions of the biological activity across the tissues. The choice of a specific approach to analyze biospeckle data is determined by the nature of the collected signals. In the case of homogeneous matter, the use of statistics of first or second order is the best approach, e.g., using autocorrelation functions (Xu et al. 1995), Inertia moment methodology (Arizaga et al. 1999), or even Briers contrast (Briers 1975). In most of the other cases where samples are non-homogeneous biological tissues, image analysis techniques (using either back or forward scattering configuration) have proved more efficient to process and interpret the nature of the signals emitted from samples, i.e., Fujii's method (Fujii et al. 1987), generalized difference method (Arizaga et al. 2002), or LASCA (Briers and Webster 1996), or even adopting laser speckle velocimetry (LSV) technique based on particle image velocimetry (PIV) technique (Bazylev et al. 2003; Formin 1998; Pickering and Halliwell 1984) which is particularly related to flow measurements.

The development of quantitative imaging techniques has now become central to understand biological systems (Haseloff 2003). Molecular techniques allow the visualization and the identification of cellular processes (Beemster and Baskin 1998; Kurup et al. 2005; Heisler et al. 2005). Image analysis, in turn, is used to track and quantify them during live experiments (Bengough et al. 2006; Moreno et al. 2006; Dumais and Kwiatkowska 2001). The data is integrated into models to describe how biological systems work of the biological systems as a whole (Dupuy et al. 2008). Unfortunately, live imaging approaches are generally time-consuming, fastidious and highly specific. The simplicity and flexibility of the biospeckle laser (BSL) imaging systems to image plant tissues make them an attractive new technique for modern quantitative biology, in particular to sensitive phenomena present in tissues.

In this paper, we have evaluated the biospeckle technique and its ability to detect changes of the biological activity across plant tissues. Root tissues were seen as an ideal subject: unlike many organs of the shoot, root development is very regular and results in simple and transparent tissues that can be imaged in depth. Three different techniques of analysis have been tested and they were applied to two different plant species. Roots were grown in gel, and the acquisition system was tested with different experimental configurations to optimize the acquisition of images during laser illumination.

## Theory

Biospekle laser systems generate time sequences of light intensities across the illuminated samples. The temporal and spatial stochasticity of pixel intensities from the images collected is related to the motion and flux of matter, and this may provide useful information on the metabolic activity within plant cells and tissues.

The image analysis of biospeckle consists in evaluating, spatially or temporally, the magnitude of pixels variation. It can be subdivided into three main approaches: Fujii, GD or LASCA. Fujii and GD methods on one hand are based on temporal analysis from a collection of frames. The LASCA technique on the other hand uses the spatial variations of a single image (frame) to analyze the nature of the biospekle patterns.

In all cases, the result obtained is an image in which gray level represents the biospeckle activity across the sample. The high gray levels in GD and Fujii's methods indicate high biological activity while in the LASCA technique, they are correlated with low levels of activity.

### Fujii's method

Fujii et al. (1987) presented a method for processing the biospeckle signals from time sequences data. The analysis is based on the summation of the weighted differences between pixels of two consecutives images, $I_k$ and $I_{k+1}$:

$$Fj(i,j) = \sum_{k=1}^{N} \frac{(I_k(i,j) - I_{k+1}(i,j))}{I_k(i,j) + I_{k+1}(i,j)} \quad (1)$$

where $k$ is the image sequence, $i$ and $j$ represent the coordinates of the intensity matrix $I_k$. $F_j$ is the resulting Fujii's image.

In this process, the weighting terms, represented by the summation of the two subsequent images, emphasize small changes, which results in a clearer image if compared with GD method.

### GD method

The generalized difference method was presented by Arizaga et al. (2002) as an alternative to Fujii's approach. The weighting process was eliminated and the difference between pixel intensity was generalized to the full length of the time sequences:

$$GD(i,j) = \sum_{k} \sum_{l} (I_k(i,j) - I_{k+l}(i,j)) \quad (2)$$

$i$, $j$, $k$ and $I$ are as defined in the previous section and GD is the resulting generalized difference image. The double summation means that any pair of images ($I_k$, $I_l$) from a

stack of size $N$ will be compared directly. Although the GD and Fujii's methods result in similar processed images, the GD method is more computationally intensive.

### LASCA

Laser contrast speckle analysis (LASCA) was presented by Briers and Webster (1996). It is based on the concept of contrast proposed by Briers (1975) to evaluate the blood flow from biospeckle. Equation 3 presents the formula for computing LASCA images. It uses the spatial mean of the intensity of the images, $\langle I \rangle$, and the spatial standard deviation, $\sigma_{x,y}$, caused by the intensity variations in the image:

$$C = \frac{\sigma_{x,y}}{\langle I \rangle} \tag{3}$$

One whole matrix, representing just one speckle image I($x$,$y$) with sizes $x$ and $y$, is adopted to measure the contrast resulting in a new matrix, $I_c(i,j)$ of $i$ and $j$ pixels. The size of the new matrix is smaller than the original by a factor related to the width of the window used to compute the contrast using the Eq. 3. The window walks over the whole original image, and the creation of the new matrix can be observed in Eq. 4.

$$I_c(i,j) = \frac{\sigma(x,y)_{7\times7}}{\langle I(x,y)_{7\times7} \rangle} \tag{4}$$

where the figure 7 represents a window with the size of 7 pixels, first suggested by Briers (1975).

The processing time of the method is fast, which makes LASCA the most computationally efficient method. It allows real time observations, although it reduces the image quality. It is generally adjusted to measure velocities observed in typical blood flow systems.

### Image enhancement

Some alternatives exist for pos-processing, one of them related to the frequency approach. The use of wavelets transform was one way to deal with the dynamic speckle data being considered as a time history (Passoni et al. 2005). Braga et al. (2007) showed the availability to create a map of frequencies using the time history of the speckle patterns adopting the wavelets transform, unfolding the possibility to use it as a tool to image enhancement.

## Materials and methods

Roots of *Coffea arabica* and *Eucalyptus grandis* plants were grown in gel tubes (Fig. 1). The *Coffea arabica* roots have one single large tap root whilst the *Eucalyptus grandis* root presents few secondary lateral roots.


**Fig. 1** **a** Roots of *Coffea arabica* growing in gel tubes, **b** Roots of *Eucalyptus grandis* growing in gel tubes

Nodal segments from 8-year-old *Eucalyptus* trees, measuring 5–8 mm in length were cut and rinsed with a Teepol solution (5–10 drops in 100 mL water). Their surfaces were sterilized in 2% solution of sodium hypochlorite for 30 min and rinsed again with distilled water before inoculation. MS basal medium (Murashige and Skoog 1962), which contained 3.0% sucrose, 0.6% agar (Difco-Bacto), was supplemented with BAP, NAA and/or $GA_3$ at different concentrations to be used for multiple shoot induction. Solid MS medium was supplemented with BAP at 1.0 mg $L^{-1}$ for shoot multiplication and for rooting, and then solid one-half MS media was supplemented with BAP 1.0 mg $L^{-1}$. The pH was adjusted to 5.8. The cultures were incubated under 26 ± 2°C with 16 h in the light (PPFD) = 36 μmol $m^2$ s and 8 h in the dark. Imaging was carried out 60 days after inoculation.

Seeds from 10-year-old *C. arabica* trees were surface sterilized and planted on MS medium, agar (Difco-Bacto) 8.0 g $L^{-1}$. Gibberellic acid (GA) 2 mg $L^{-1}$ supplement was used for in vitro germination. Microshoots (10 mm) were subcultured in Erlenmeyer flasks (250 mL) containing 150 mL of solid MS media supplemented with BAP 1.0 mg $L^{-1}$ and IAA 0.5 mg $L^{-1}$. The cultured microshoots were incubated under 26 ± 2°C with 16 h in the light (PPFD) = 36 μmol $m^2$ s and 8 h in the dark.

The microshoots (10 mm) were transferred to test tubes (25 × 150 mm) containing 25 mL of solid half-strength with MS media and sucrose (15 g $L^{-1}$). It was used with

1.0 mg $L^{-1}$ IBA for the root growth stage. The cultured microshoots were maintained under 26 ± 2°C with 16 h in the light (PPFD) = 36 μmol $m^2$ s and 8 h in the dark. Imaging was carried out 60 days after inoculation.

Two samples of each species were illuminated using a forward and backward-scattering configuration as presented in Fig. 2. The illumination is generated by a 10 mW HeNe laser, 632 nm, expanded by a lens large enough to cover the sample. The images were collected in CCD cameras with 640 × 480 pixels sensors, with the shutter set to 1/60 s. The angle of the laser beam with the CCD camera was adjusted in order to obtain high-contrast images with minimal specular reflection of light. A collection of 128 images in gray level (8 bits) were analyzed by using generalized difference, Fujii and LASCA routines (Marcon and Braga 2008).

One set of images was pos-processed using the wavelets transform to evaluate the ability to enhance the image produced by the routine methods. The procedure adopted to the whole image sequence was the same showed in Braga et al. (2007) to only one pixel. The wavelets transform was used to split the frequency composition of each pixel of the images considering its time history, and therefore, to reconstruct the image assembling the frequency components, but without one range of frequency. The filtering produced in this step is used to reduce the influence of some phenomena in the final image such as the case of the gel in this work.

Considering the acquisition rate of 0.08 s, the highest frequency was 6.25 Hz. The filtering process was tested over 10 ranges with the step of 0.625 Hz.

## Results

The forward scattering configuration with an angle of 45° (Cf. Fig. 2) produced the best images. It allowed capturing the biospeckle signals without the interference of the reflected light in the tube. The other configurations induced different degrees of saturation in the images, mostly related to the specular reflection of light at the surface of the tube. This could be filtered through the use of polarizers, but it increased unnecessarily the number of devices and adjustments in the configuration.

The different methods were assessed visually by their ability to discriminate different regions of the roots with different biological activity which can be addressed to a range of sources such as cytoplasmic streaming and macroscopic fluxes. Any relation with metabolic activity such as that observed in seeds and fruits is not proved yet with these results. Figures 3 and 4 show the areas of activity in pseudo-color images that result from the generalized difference method, Fujii's method and LASCA in coffee and eucalyptus trees, with a colormap from blue (low activity) to red (high activity) using DG and Fujii methods, and red (low activity) to blue (high activity) using LASCA method. The roots of the coffee trees (Fig. 3) had large diameters and could easily be detected by any of the methods. However, *Eucalyptus grandis* roots (Fig. 4) had small secondary lateral roots positioned on the main primary root, and these small root structures were hardly detected by any of the methods tested, i.e., GD, Fujii's and LASCA. These limitations are inherent to the current experimental system which has low magnification and uses a standard MS medium.

The analysis of the biospeckle patterns using the GD method provided clear distinct signals for roots and for the gel medium and activity maps of complete root architectures. The analysis could also detect sub-organ variations in the intensity of the activity. In particular, vascular tissues and root meristems that had not reached the tube walls at the time of the experiment showed higher levels of activity. This is not an unexpected result in the root tip, since it is caused by long distance transport in vasculature and meristematic activity. Despite its broad range of application, the Fujii's method was not capable of distinguishing the

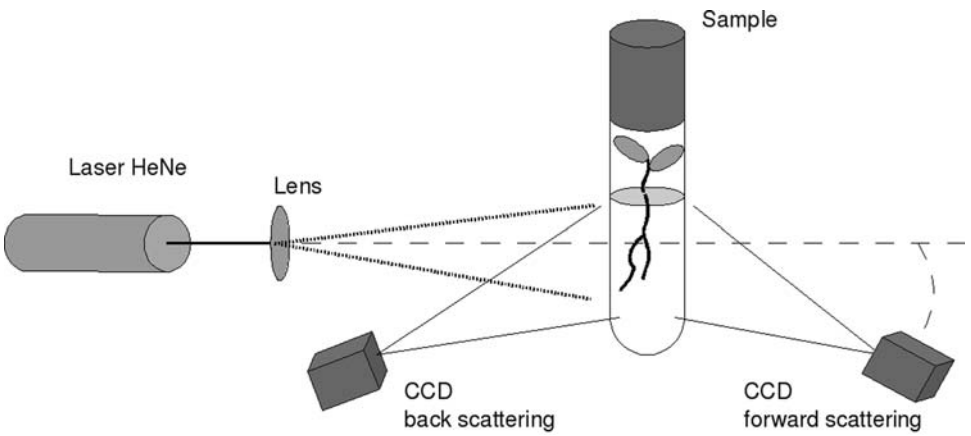


**Fig. 2** Experimental configuration of a forward and a back-scattering approach

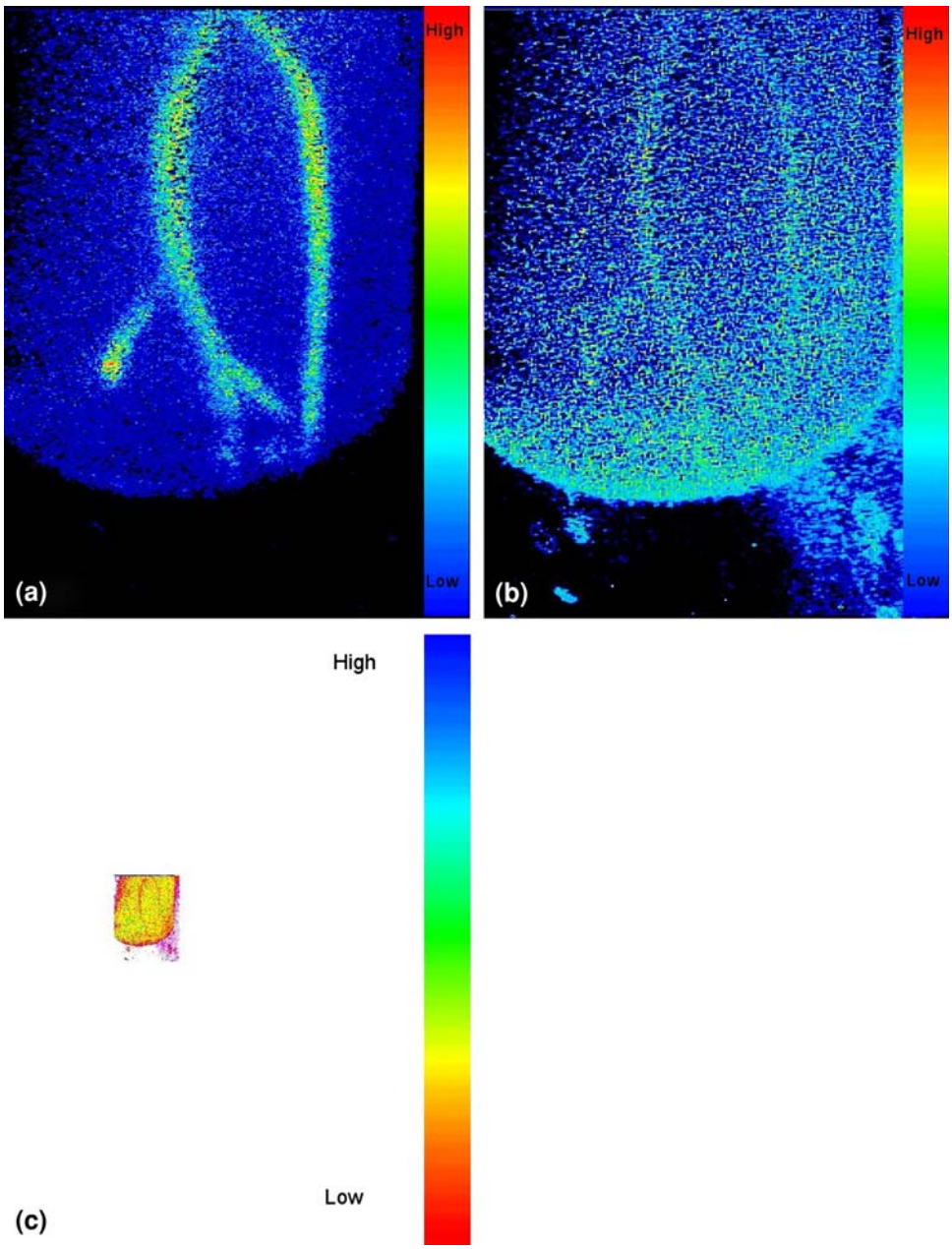


**Fig. 3** Processed images from *Coffea arabica* tree roots using **a** GD method, **b** Fujii's method, **c** LASCA where high and low activity in the color scale mean 255 and 1 in *gray* level

roots from the gel. This is due to the action of the gel, which acts as a diffuser: low levels of activity with the diffused scattered light in the gel were amplified in the Fujii's method and reduced the contrast between the roots and the gel. The LASCA method (7 × 7 pixels window) resulted in images of lower resolution, but was capable of detecting differences in the activity of the main roots. Windows of smaller size, such as 3 × 3 pixels, did not improve the quality of the analysis.

The wavelets transform applied to the sequence of biospeckle images of coffee trees allowed the enhancement of the root profiles, filtering the gel influence. The best range of frequencies that produced the highest contrast and the segmentation of the roots from the gel was from 0.625 to 1.25 Hz which was related to low frequencies. In this case, the filtering occured over the other ranges. In Fig. 5, it is possible to see the enhancement of the root profile analyzed by the Fujii's method after the filtering process when compared with the Fig. 3b.

## Discussion

Currently, most approaches related to imaging from plant biological activity involves the tagging of specific tissues or cellular/molecular structures. For example, combinations of

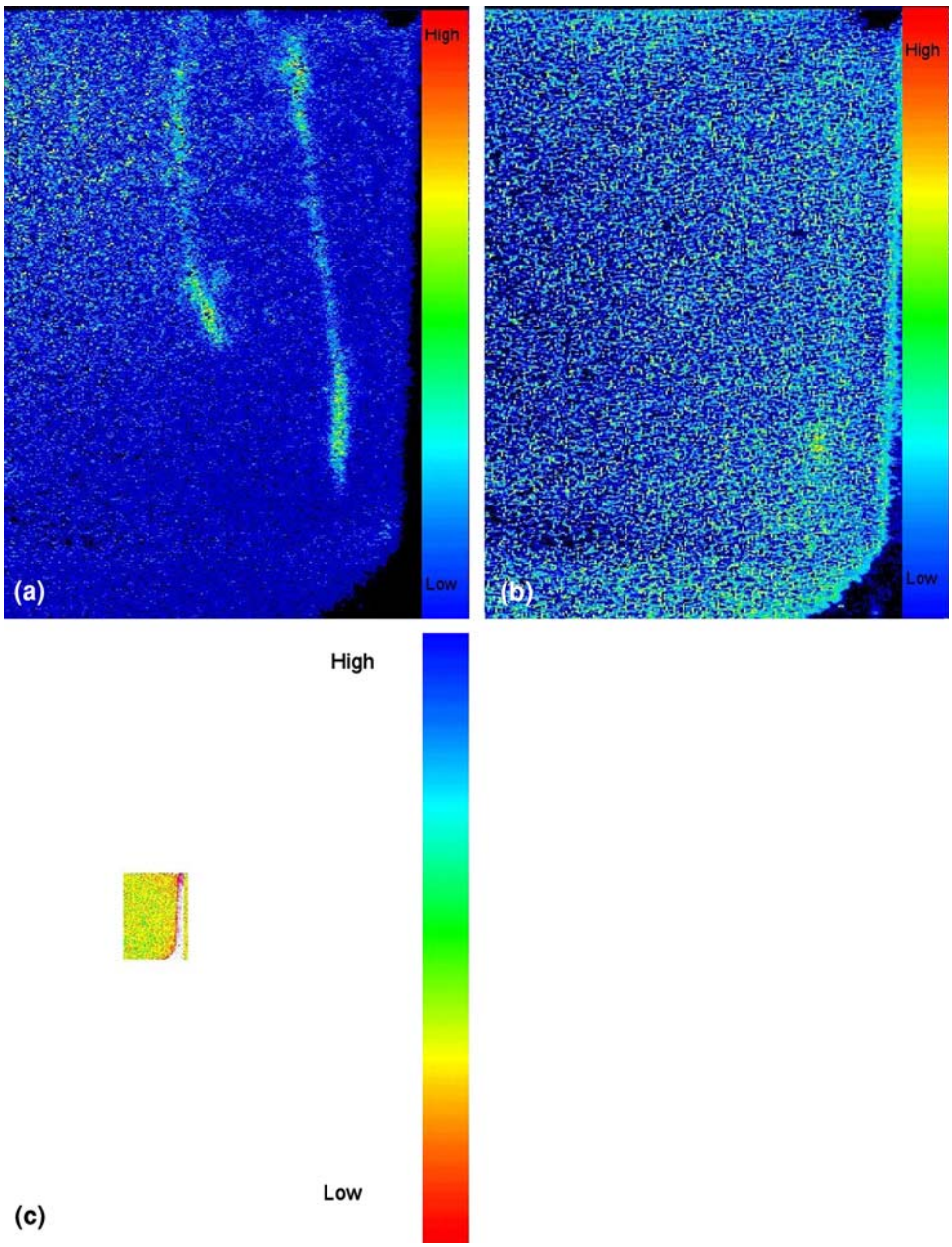


**Fig. 4** Processed images from *Eucalyptus grandis* tree roots using **a** GD method, **b** Fujii's method, **c** LASCA

dyes and clearing agents are used to obtain anatomical descriptions of the plant with high resolution (Truernit et al. 2008). Unfortunately, staining protocols that are suitable for live experiments are limited. Other molecular approaches such as immunolabeling (Reinhardt et al. 2004) are rarely used during live experiments due to their decreasing strength with time and poor in depth penetration of tissues. For these reasons, fluorescence microscopy has become the most common approach to obtain temporal and spatial information on gene activity during development (Heisler et al. 2005). When combined with confocal laser scanning microscopy (Moreno et al. 2006) or optical projection tomography microscopy (Sharpe et al. 2002), the fluorescence microscopy allows accurate reconstructions of the 3D patterns of gene expression across tissues.

In this study, we found that the biospeckle phenomenon can be used to present the outlines of root tissues, and as well to present areas with different activity that can unfold further investigations to correlate with particular phenomena in the root tissues. By using simple tissue culture techniques and classical analysis of biospeckle patterns, it was possible to discriminate different levels of activity in root tissues, and outlines without the need for staining tissues. In the future, this would allow high throughput inexpensive techniques to be developed for genetic screens and characterization of root growth. There are also

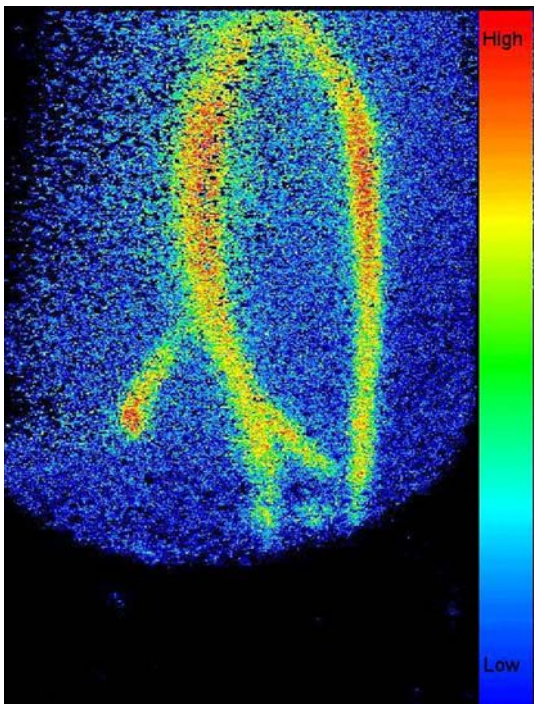


**Fig. 5** Image enhancement of *Coffea arabica* tree roots considering only the 0.625–1.25 Hz range of frequencies using Fujii's method

different ways the current system could be adapted in order to obtain more accurate information on specific processes in roots when necessary. Increasing the magnification would notably provide higher biospeckle signal resolution and could help identify fine tissue-based changes in biological activity. The use of a different gel medium, e.g., Phytagel, Sigma-Aldrich) would provide suitable illumination and would prevent saturation of the camera. Finally, more specific processing algorithm could be designed to emphasize and segment patterns of activities that are related to specific biological processes (Rabal and Braga 2008).

In addition, most modern cell imaging techniques use a type of cellular marker and require genetic transformations which are available only for model species, e.g., arabidopsis. They also use sophisticated and expensive microscopes to allow few fluorescent signals to be monitored during a single experiment. BSL imaging could potentially overcome some of these shortcomings by providing affordable, high-throughput imaging solutions to quantify changes in tissue biological activity. It has a large working distance, can depict larger specimen through transparent medium and allow plants to grow vertically. This would make biospeckle imaging an attractive technique for plant biological studies.

The enhancement of the biospeckle images, using wavelets transform or other frequency tools, is a novel area that was unfolded by a recent research with Butterworth filters (Sendra et al. 2005), and in particular the technique adopted in this work using wavelets transform. The filtering technique can enhance the root area as well as the gel area depending on the chosen frequency. This presents a powerful tool in tissue analysis with a complex displacement of layers with distinct activities under the biospeckle view.

## Conclusions

The BSL phenomenon was used to build a map of biological activity in root tissues. A simple experimental system was developed to allow the acquisition of signals from roots grown in gels. Classical image analysis techniques were used to process biospeckle patterns and the GD method demonstrated to be the best approach to accentuate the biological activity across tissues. Biospeckle laser imaging could be a compelling new system for the imaging of plant tissues.

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