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Evaluation of Maize Seeds Treated with Trichodermil® through Biospeckle

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Abstract: The present work aimed to study the treatment with the bioprotector *Trichoderma harzianum* on maize seeds immediately after treatment and during storage. Maize seeds were analyzed through germination test, vigor tests (cold test, accelerated aging and aerial length), and the viability of the treated seeds was verified through the biospeckle. Seed treatment is recommended because approximately 90% of crops intended for human and animal feeding are seed-propagated. A pathogen present in the seed results in increased diseases in the adult plant, in addition to introducing pathogens in other areas. So the seed treatment can provide better results than chemicals disease control on plants. Bioprotectors have antagonistic fungi that produce metabolites and enzymes that will avoid the proliferation of phytopathogenic fungi. Among the bioprotectors, *Trichoderma* spp., used for phytosanitary control of seeds, stands out. The tests were carried out with treatment in order to evaluate the physiological potential caused by the treatment with the bioprotector. The biospeckle experimental runs were performed in parallel with the sanitation test. The germination percentages of 93.0%, 87.5%, and 87.5%, respectively, showed that they were all above the minimum requirement for commercialization, which is 85%. Analyzing the vigor and sanitation tests, it was verified that the best treatment was on day zero. Among the seeds analyzed through biospeckle, using the laser speckle contrast analysis (LASCA) technique, it was possible to identify fungi activity.

Key words: *Zea mays* L., bioprotector treatment, *Trichoderma harzianum*, germination, sanitation, seed storage, biospeckle.

1. Introduction

Seeds as a culture's main input must be well selected by the producer so that their enterprise succeeds. The propagation of the corn crop occurs through its seed, and this can be attacked by pests and pathogens. Among the pathogens that cause diseases to the plants and are transmitted through the seeds, such as fungi, which besides diseases, reduce the vigor and germination potential of the seeds [1]. So, the phytosanitary control of the seeds is essential for a productive and sustainable agriculture.

Several plant diseases in Brazil have their causal agents introduced through seeds, which may have pathogenic microorganisms, internally or externally. The presence of fungi in the seeds may reduce their quality due to deterioration, which may cause the seed to completely lose its viability [2].

Plague and disease control in agriculture is done basically through the application of synthetic products (agrochemicals), which may cause serious environmental and toxicological problems, exposing the environment and humans to many risks, being sometimes serious pollutants for many agroecosystems [3]. Aiming to reduce the use of synthetic pesticides and the risks to operators, it is possible to replace the use of chemicals by employing treatments with bioprotectors.

Bioprotectors have antagonistic fungi which produce and release substances (metabolites and enzymes) which will degrade the hyphae of the phytopathogenic fungi, avoiding their proliferation. Among the bioprotectors, *Trichoderma* spp. is highlighted, which presents positive results on the

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control of fungi and bacteria. Besides those advantages, they can stimulate the germination, emergence and the aerial growth [4]. Several studies have already demonstrated the efficiency of *Trichoderma*. According to Luz [5], the microbiolization with *T. harzianum* strain T-22 provided a significant increase in the emergence of plants and yield of corn grains. Filho et al. [6] concluded that *Trichoderma* spp. isolate CEN 262 provided higher development index of aerial parts of eucalyptus seedlings. Harman et al. [7] reported that 10-day corn seedlings showed higher biomass production. A pathogenic fungus is recognized by *Trichoderma* spp. through pathogens released molecules. These molecules may attach to G protein-coupled receptors (Gpr1) on the surface of the *Trichoderm*a spp. hyphae, thus inducing a signaling cascade comprising G proteins and mitogen-activated protein kinases (MAPKs), which may modulate the activities of transcription factors (TFs). These elements enhance the expressed genes that encode enzymes for cell wall lysis. Simultaneously, the pathogenic fungus responds by generating secondary metabolites and reactive oxygen species (ROS) that cause a detoxification in *Trichoderma* spp. [8].

These characteristics of *Trichoderma* spp. may be beneficial for agricultural producers who require seeds with a higher sanitary quality avoiding future productivity problems. The use of seeds that have been treated with biocontrol agents is recommended to maintain diseases control and transmission, since some species of *Trichoderma* spp. are potential antagonists of several phytopathogenic fungi that attack the seeds. There are several mechanisms of action that these fungi use, among which, the most important one is the production of metabolites and enzymes that have antifungal properties [7]. In addition, *Trichoderma* spp. contributes to a higher density of plants in the crop [9].

One way to assess seed quality is through biospeckle, which is obtained from an optical phenomenon that occurs when a material (biological or not) is illuminated with a laser. When applied to a biological tissue, there is a speckle pattern which resembles boiling liquid on the illuminated face [10]. The biospeckle is characterized by being an optical phenomenon of interference that carries information of the material illuminated by a coherent light source like the laser. The images were processed through the laser speckle contrast analysis (LASCA) method [11], based on the loss of contrast that occurs when you take a picture of a dynamic scene. Thus, the motion occurs in a time interval shorter than the integration time of the camera, which is finite. Thus, a tissue with high biological activity will have an image formed with less contrast than an image of a static tissue, which comes from a static scene [12].

Many studies applying biospeckle to evaluate the biological activity of microorganisms have been carried out. Grassi et al. [13] used the biospeckle to evaluate the protozoan *Trypanosoma cruzi*. Sendra et al. [14] used biospeckle to detect bacterial activity. In Ref. [15] the authors used biospeckle analysis to detect the infection in apples by fungal cultures. They have compared with reference methods, such as hyperspectral imaging and chlorophyll fluorescence. Some research works involve fungal activities in seeds. Rabelo et al. [10] evaluated bean seeds contaminated by fungi through the biospeckle. Vivas et al. [16] evaluated coffee seed activity through biospeckle.

Usually the evaluation of the physiological quality of the seeds is done by germination and vigor tests. The health quality analyses indicate the need or not of the seed treatment or the efficiency of the treatment [17]. Seed treatment with *Trichoderma* spp. promotes the growth of more vigorous seedlings, besides controlling the presence of pathogenic fungi. Besides being a non-toxic biological control to man and animals, they come from an unlimited natural resource resulting from the multiplication of microorganisms [4].

This paper aimed to assess the efficiency of the treatment with microbiological fungicide, *T. harzianum*, by analyzing the germination, the vigor of the seeds and the sanitation test correlated with biospeckle.

2. Materials and Methods

2.1 Maize Seeds

The work was performed at the Post-Harvest and Optical Laboratories in the School of Agricultural Engineering (FEAGRI) at University of Campinas (UNICAMP). The seeds were acquired from the Agronomical Institute of Campinas from the IAC AIRAN strain of the IA 83/2016 lot.

2.2 Bioprotector Treatment

Initially, a stock solution was elaborated by adding 2 mL of Trichodermil® (Koppert Biological Systems, Brazil) with 3 mL of distilled water, for every 1 kg of seeds. From stock solution, 5 mL was pipetted and mixed until it was uniformly adhered to every seed.

In this work, the initial germination, moisture content and sanitation tests were performed and in parallel tests with no treatment (NT) and with the Trichodermil® treatment (Treatments T-a, T-b and T-c correspond, respectively, to days zero—immediately after the treatment, 15 d and 35 d of storage). The authors aimed to assess the physiological potential caused by the treatment with the bioprotector, through the assessment of the germination index, accelerated aging, cold test and measurement of the length of the aerial part of the seedling. After the initial tests for the control sample, the seeds were packaged in multi-layered paper bags in a controlled chamber at the temperature of 20 °C and relative humidity of 44.0%.

2.3 Initial Assessment

The maize seeds quality was defined from determination of moisture content, germination and sanitation [17, 18].

The initial seed moisture content was determined through the oven method at 105 ± 3 °C during 24 h [18], in which three repetitions of approximately 2 g of seeds each were used. According to Ref. [18], this method aims to obtain the degree of moisture of a seed. It is based on the principle of removing the water contained in the seeds in the form of steam through a source of heat. The seeds were weighed before and after leaving the oven, and the water mass was given by the difference between the initial and final weighings. The conduction of this method, as well as equipment used is described in Ref. [18].

The germination test was performed according to Ref. [18], using four repetitions of 50 seeds. The seeds were placed in plastic boxes (30.0 × 30.0 × 5.0 cm) on dampened sand substrate with 60% of that sand water retention capacity and kept at 30 °C in the germinator. The results were expressed in percentages of germination and height of the aerial part, as a single counting.

The assessment of the seeds regarding sanitation was performed through a method which consisted of the distribution of 8 repetitions of 25 seeds on filter paper dampened with distilled water in plastic boxes [19].

The seeds were dampened in filter paper until the water was completely absorbed, then they were taken to a freezer, so that their germination process was interrupted for 12 h. After this, the seeds were placed in a biochemical oxygen demand (BOD) chamber at 20 ± 2 °C, under an intermittent regimen of 12 h of light/12 h of darkness for 7 d for the incubation. All seeds were assessed with the help of a stereoscopic microscope aiming to identify the developing fungi. The results were expressed in percentages of occurrence of fungi associated to the seeds.

2.4 Assessment Tests

The modified cold test was performed according to the recommendations of Krzyzanowski et al. [20]. After the germination test according to the seed analysis rules (RAS), the paper rolls were kept at 10 °C for 7 d in BOD chamber. After 7 d, they were placed in a germinator adjusted at 30 °C, where they remained for 5 d. The results were expressed in germination percentages for each treatment.

The accelerated aging test was performed according to the methodology of Krzyzanowski et al. [20]. After 24 h/42 °C the seeds were submitted to the germination test in paper roll, where the normal seedlings were counted according to Ref. [18]. The results were expressed in germination percentages for each treatment.

For the biospeckle, the optical trial, a diode laser was used with a 632 nm wavelength (red) and 10 mW of power, a Canon Rebel T6 camera EOS 1300D with a 18-55 mm lens, a PC computer with a core i5 processor with 4 GB of RAM memory with software for image processing, such as ImageJ version 1.50i [21], MATLAB® version 7.12.0.635 (R2011a, The Mathworks, Inc., Natick, MA).

After collecting the images with the camera, using ImageJ, an area of the image was selected for the processing which converted the colors to the eight bit scale (256 levels of gray).

In this work, it was used an application in MATLAB®, LASCA, which was used in order to acquire the activity map of the seeds through the LASCA algorithm, which is the LASCA adapted from Ref. [22].

After processing with the MATLAB® application with the LASCA algorithm, the image presented in Fig. 1 was obtained.

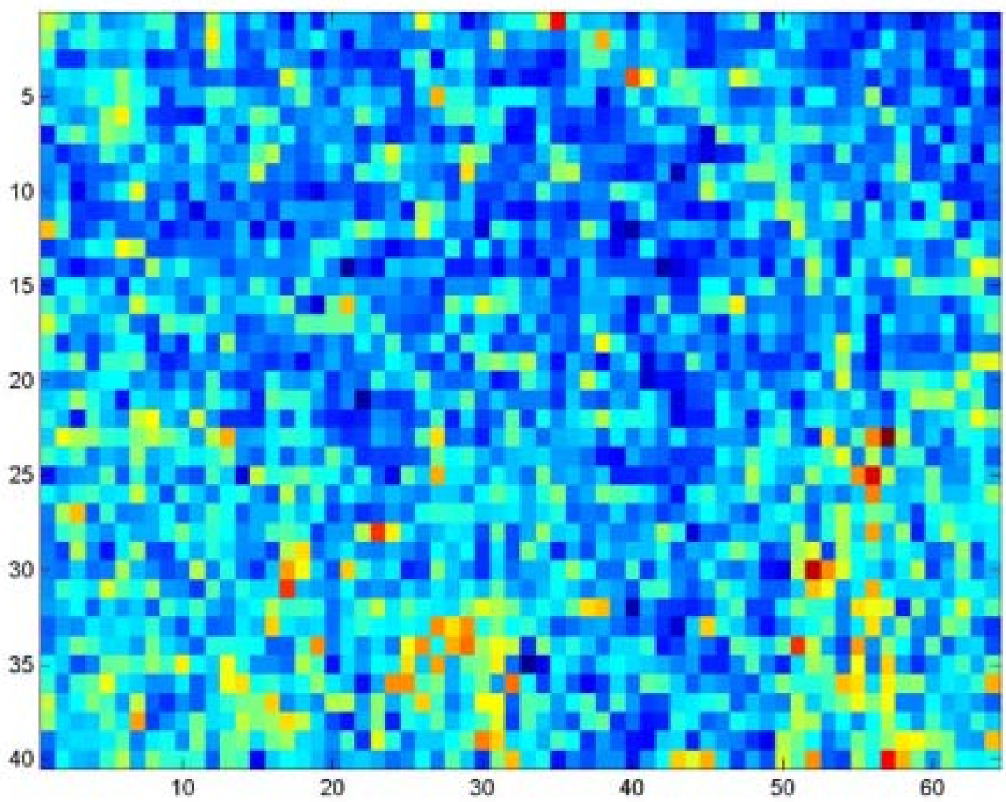

Fig. 1 Activity map obtained through LASCA of the treated seeds with 10 d.
Picture size: 5,184 × 3,456; horizontal resolution: 72 dpi; vertical resolution: 72 dpi.
A region of interest in the images was selected by selecting only the seeds for processing. The MATLAB routine was created based on the algorithm described by Silva [22]. The regions with dark blue tones have high biological activity and the lighter ones, low biological activity.

2.5 Statistical Analysis

For all tests, the analysis of variance was performed and the means compared separately through Tukey test (p < 0.05), for each treatment. For that, the SISVAR software was used [23].

3. Results and Discussion

3.1 Initial Assessment

Initially, it was obtained 93.5% of seed germination, 10.5% of moisture content and 97.5% of *Fusarium* ssp. occurrence.

3.2 Germination Test

The germination test results are presented in Fig. 2, where treatments T-a, T-b and T-c presented 93%, 87.5% and 87.5% of germination, respectively, showing that the treatments did not affect the seed viability, when compared to the test with NT, which obtained a value of 93.5%.

The seedling emergence was high in both genotypes in all treatments, obtaining much higher values than the minimum required for the commercialization of maize seeds in Brazil (85% germination). According to the Tukey test, there was no significant difference (at 5%) among the assessed treatments (NT, T-a, T-b and T-c).

In the research work of Brand et al. [17], applying combined treatments of fungicide and *T. harzianum*, the germination results were higher in relation to the samples without treatment.

3.3 Cold Test

From the cold test, associated to the seed vigor, it was possible to assess the seed vigor treated with Trichodermil®, being possible to observe the significant difference of the T-a (87.0%) and T-b (83.5%) treatment compared to the 66.0% from the samples with NT, except for the T-c seeds, 69.0%, which was close, making it apparent that T-a was the treatment which was closer to the initial germination test (93.5%) (Fig. 3).

Analyzing this test, treatments T-a, T-b and T-c were classified in a scale of highest vigor. Comparing to the seeds with NT, there was an increase on the vigor after the treatment. Thus, as well as Brand et al. [17], a positive effect was observed on the physiological potential of the seeds treated with the bioprotector.

Faria et al. [4] obtained higher results than the seeds not treated with *T. harzianum* in the germination test at low temperature.

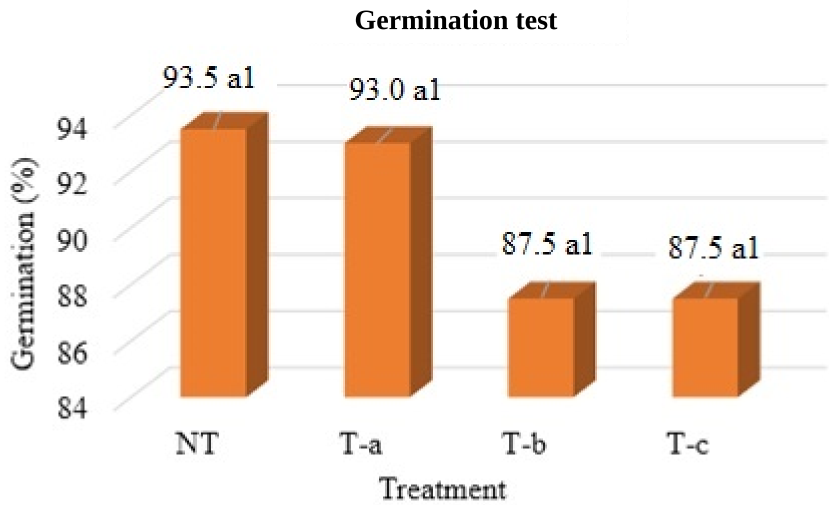

Fig. 2 Average germination result for the four assessed treatments, which were statistically analyzed through Tukey test at 5% probability.

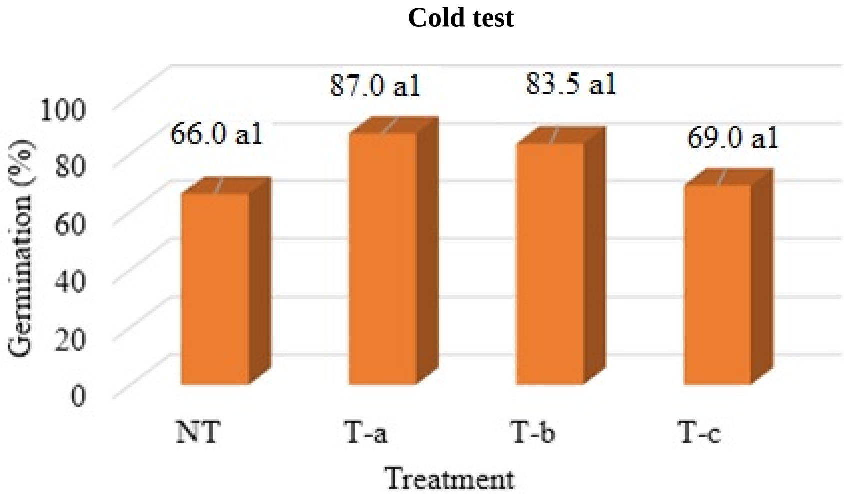

Fig. 3 Average germination results for the four assessed treatments, submitted to cold test, statistically analyzed through Tukey test at 5% probability.

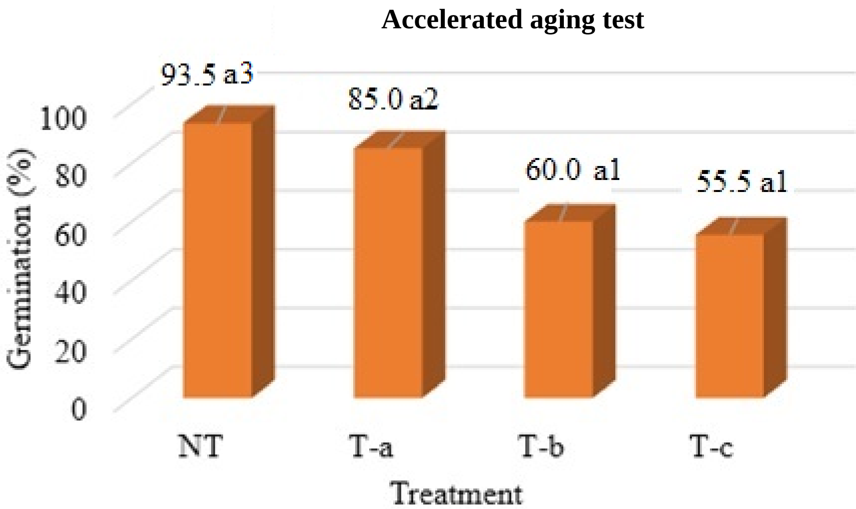

Fig. 4 Average germination results for the four assessed treatments, submitted to the accelerated aging test, statistically analyzed through Tukey test at 5% probability.

3.4 Accelerated Aging Test

Regarding the accelerated aging test study (Fig. 4), treatment T-a presented germination of 85.0%, highlighting the increase on the physiological potential of the seeds relative to the samples with NT. However, for treatments T-b and T-c (60.0% and 55.5%, respectively), the low physiological potential was noticed. Comparing treatment T-a to the initial germination test (93.5%), the treatment was the one which approached the most, thus preserving the initial quality.

The strain imposed from the accelerated aging test was higher than from the cold test, resulting in a drop on germination. Both tests presented statistical variability.

3.5 Length of the Aerial Part

In the germination test counting, the normal emerged seedling parts (hypocotyl, aerial part) were measured using a ruler. The average results per seedling were expressed in centimeters. The data were presented in Fig. 5. There was an increase of the aerial part with the treatment, 18.9 cm for T-a, 21.4 cm for T-b, 19.4 cm for T-c and the test with NT (witness) presented a growth of the aerial part of 18.0 cm. Faria et al. [4] also obtained the highest lengths of seedlings treated with *T. harzianum* when compared to seeds without treatment and seeds treated with fungicides.

3.6 Sanitation Test

Regarding the sanitation test, only the incidence data associated to *Fusarium* spp. were presented, due to the inexpressiveness of the other microorganisms.

The treatment with bioprotector considerably reduced the incidence of *Fusarium* spp. found in the seeds when compared to the results presented by the control, indicating a significant difference for treatments T-a and T-b, 31.5% and 43.5%, respectively. However, for T-c, the results approached the control (97.5%) (Fig. 6).

Treatment T-a presented lower occurrence of pathogens in relation to T-b and T-c. It is observed that the pathogen occurrence increased through time. It is noticed that the treatment is more effective in the beginning of the storage, but it has an effectiveness drop over time. Through the statistical test, up to the 15th day, pathogen occurrences were significantly lower compared to the samples with NT and after 35 d.

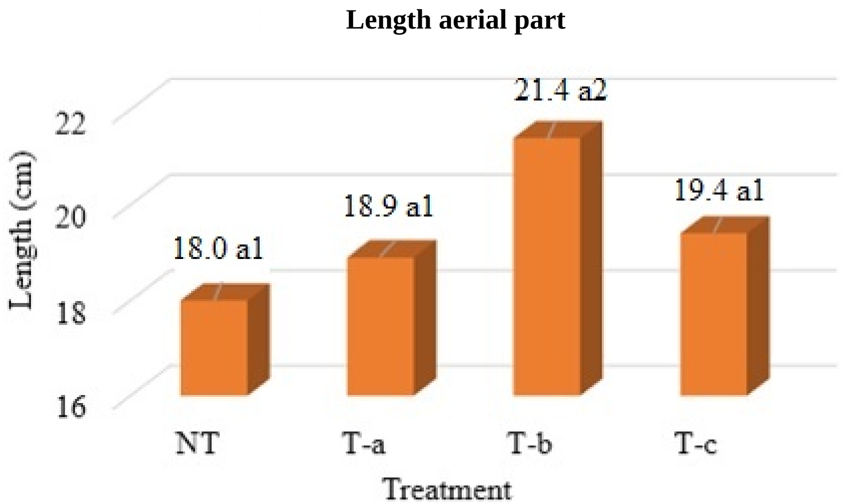

Fig. 5 Average length results of the aerial part for the four assessed treatments, submitted to the germination test, statistically assessed through Tukey test at 5% probability.

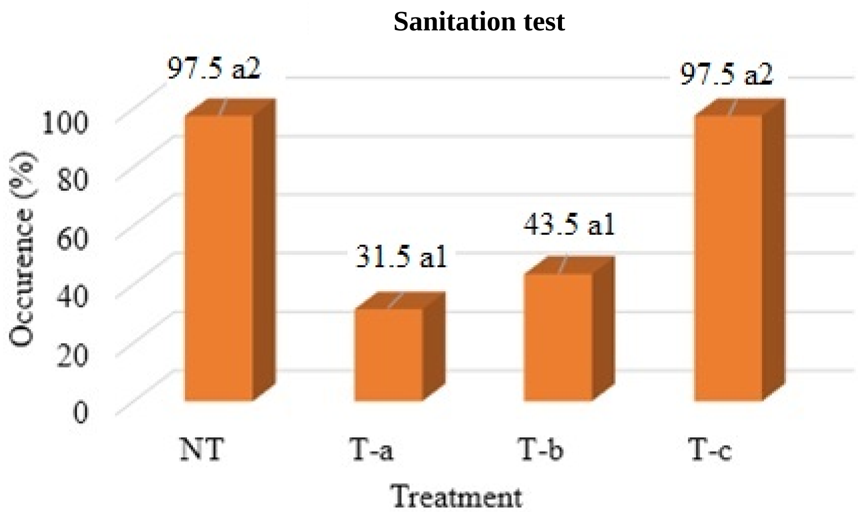

Fig. 6 Occurrence of *Fusarium* spp. for the four assessed treatments, statistically analyzed, through Tukey test at 5% probability.

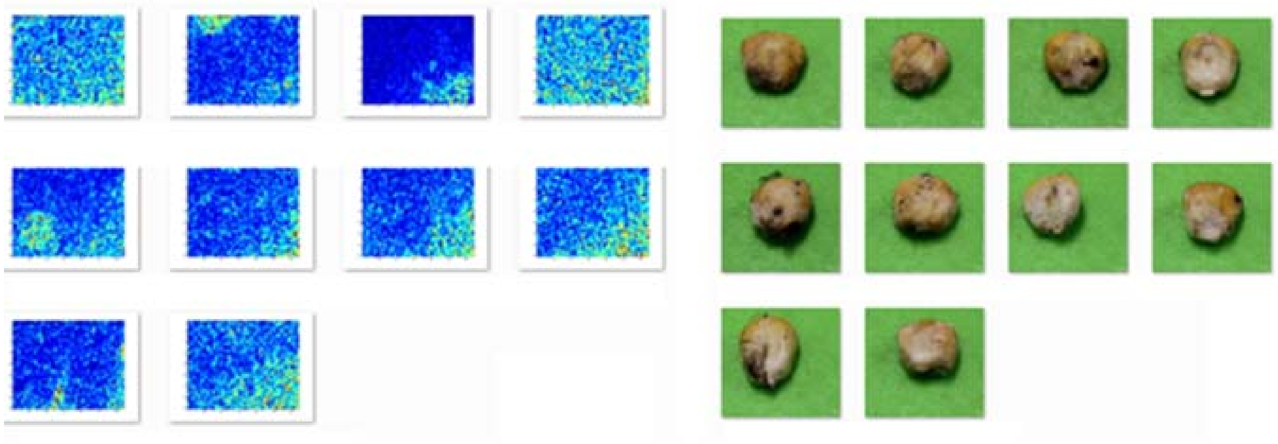

Fig. 7 NT samples.
Left image: after processing LASCA; right: RGB images.

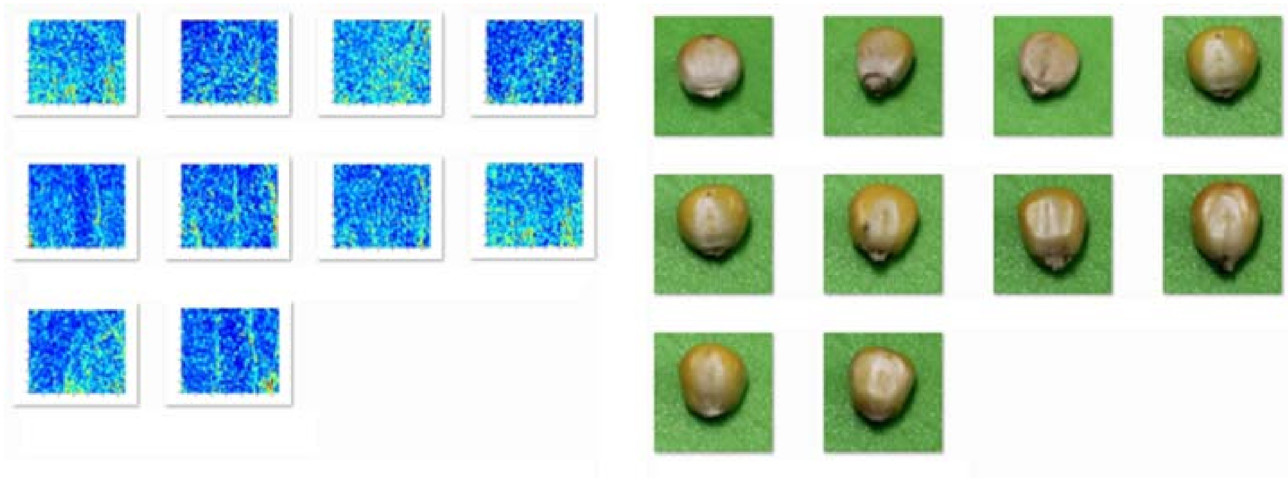

Fig. 8 T-a samples.
Left image: after processing LASCA; right: RGB images.

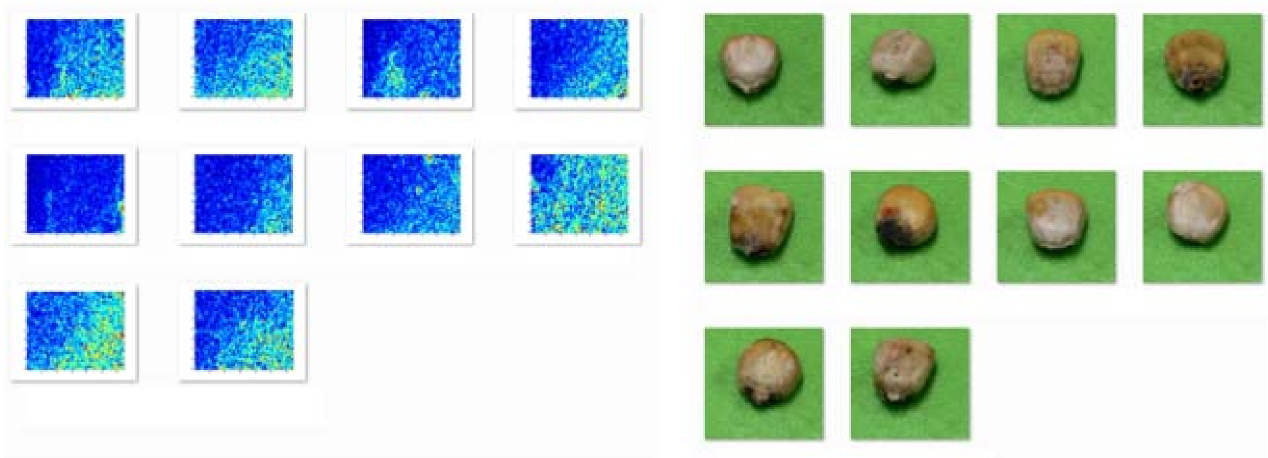

Fig. 9 T-b samples.
Left image: after processing LASCA; right: RGB images.

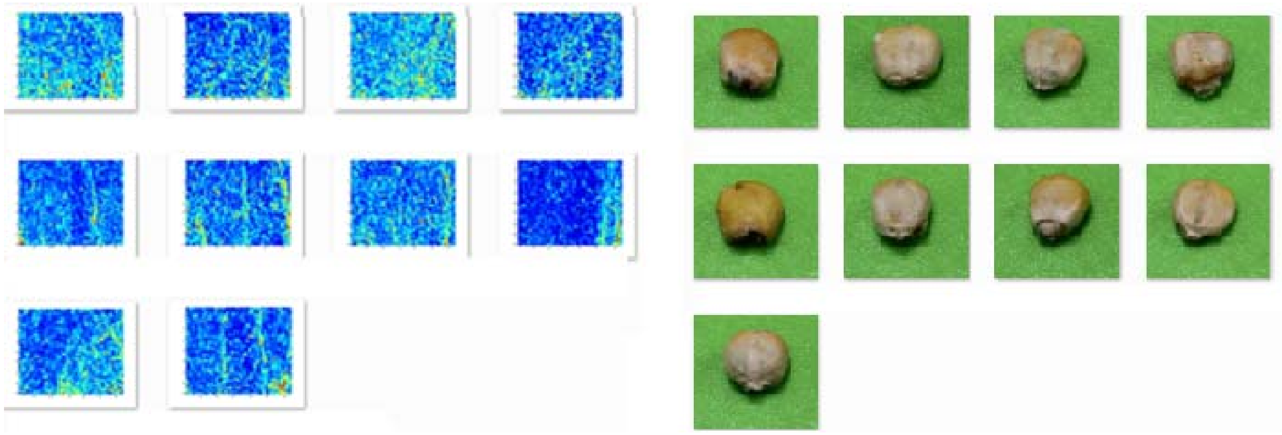

Fig. 10 T-c samples.
Left image: after processing LASCA; right: RGB images.

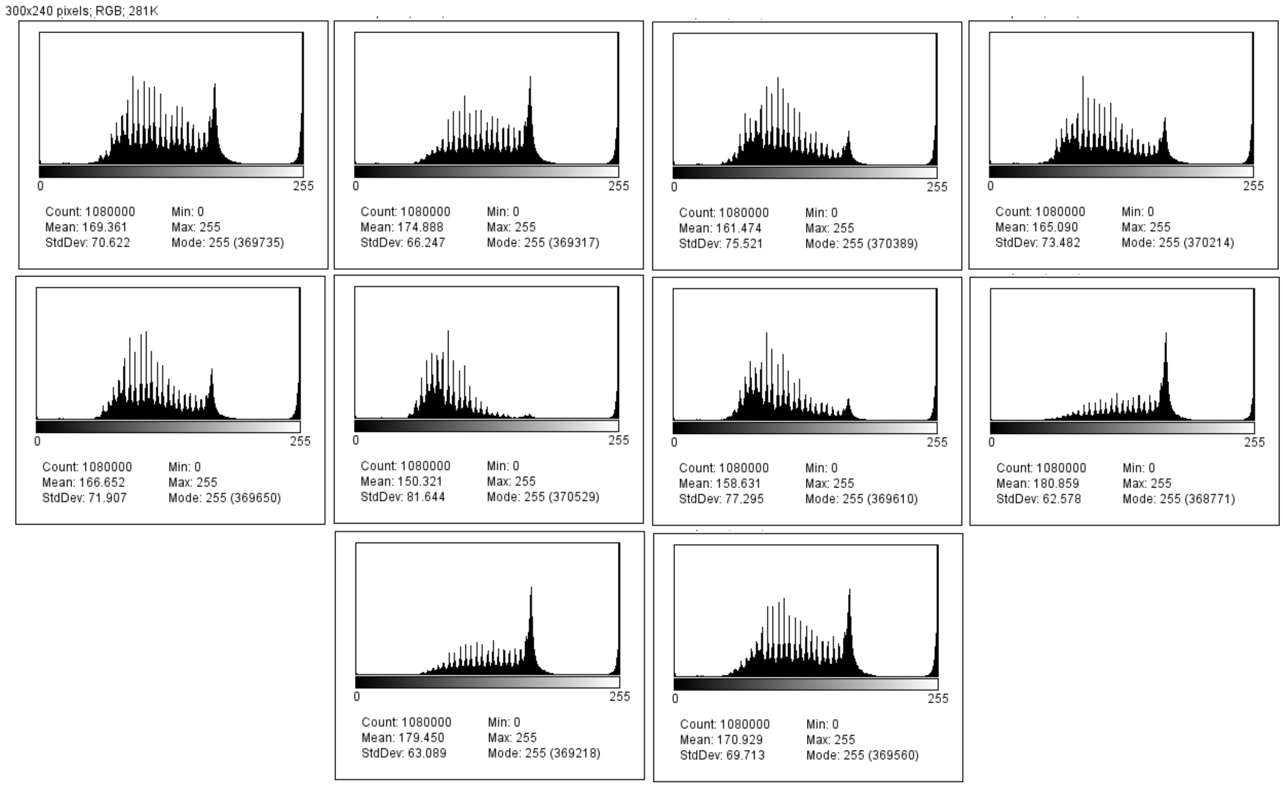

Fig. 11 Histograms of images processed through LASCA (NT samples).

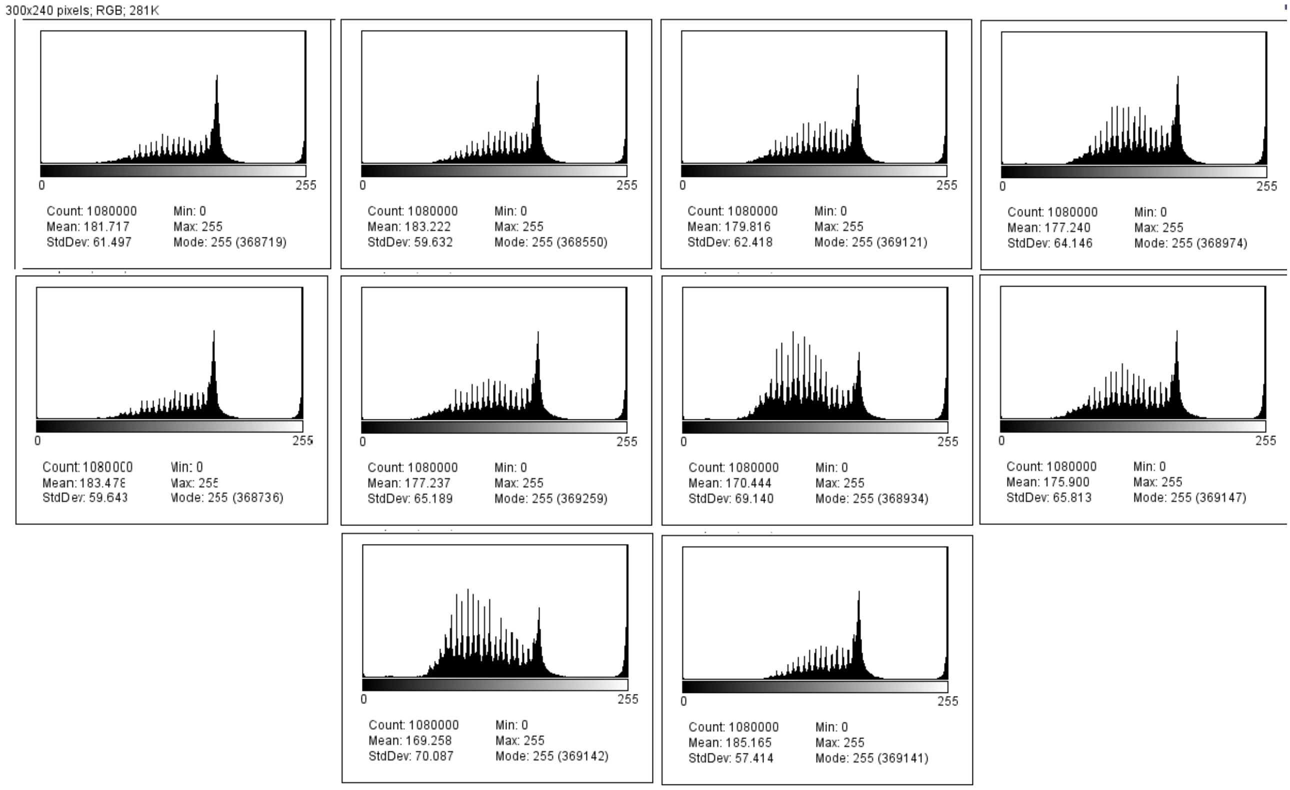

Fig. 12 Histograms of images processed through LASCA (T-a samples).

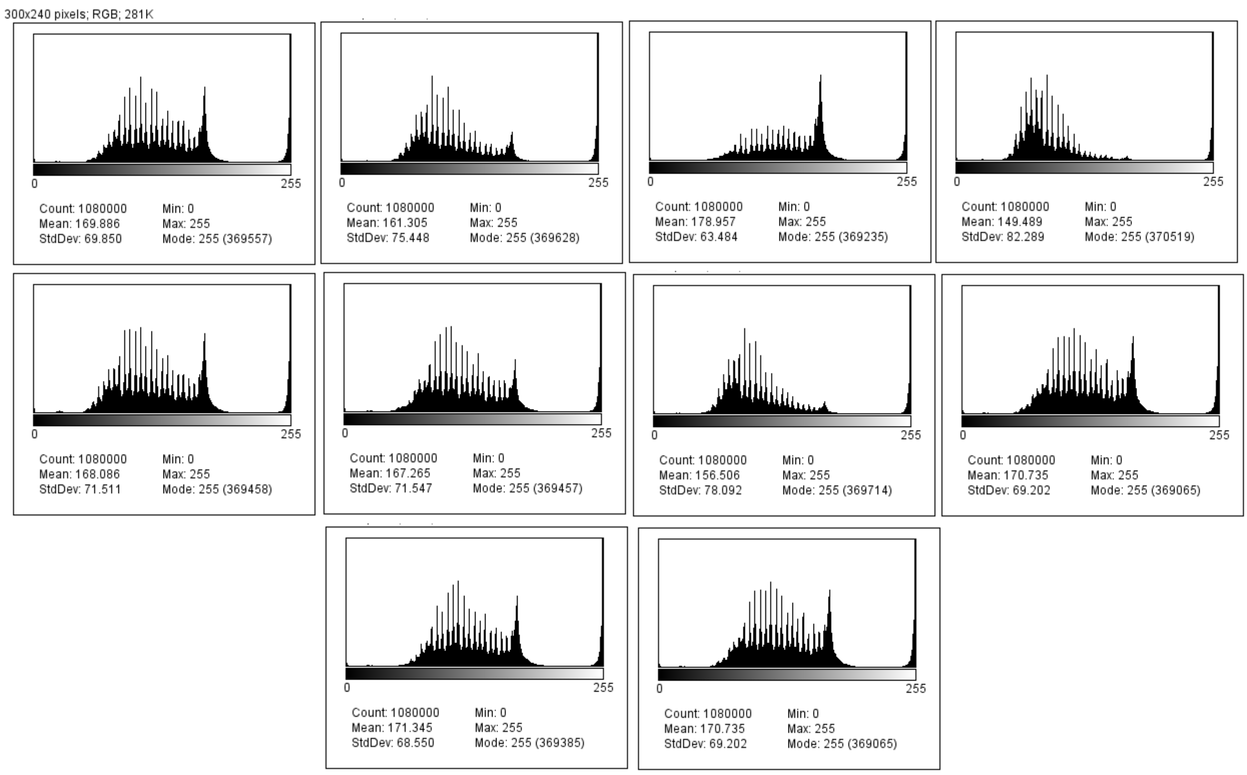

Fig. 13 Histograms of images processed through LASCA (T-b samples).

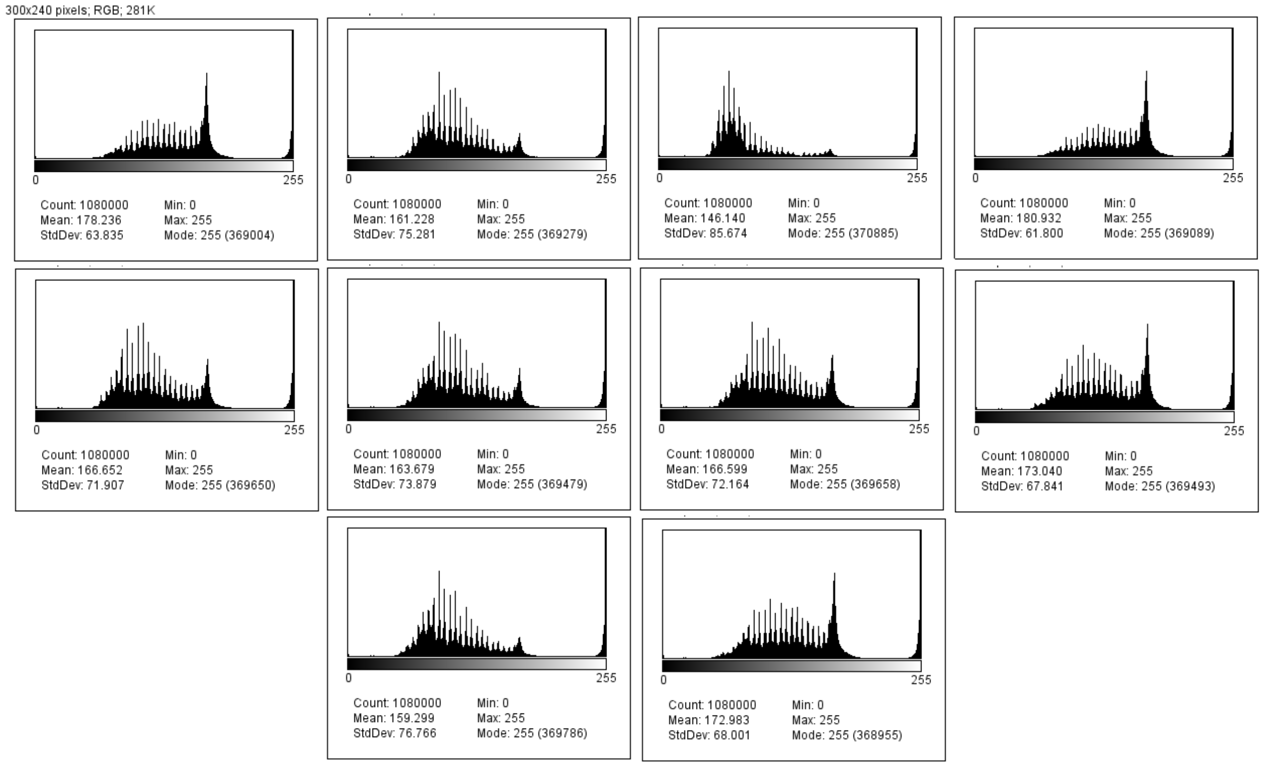

Fig. 14 Histograms of images processed through LASCA (T-c samples).

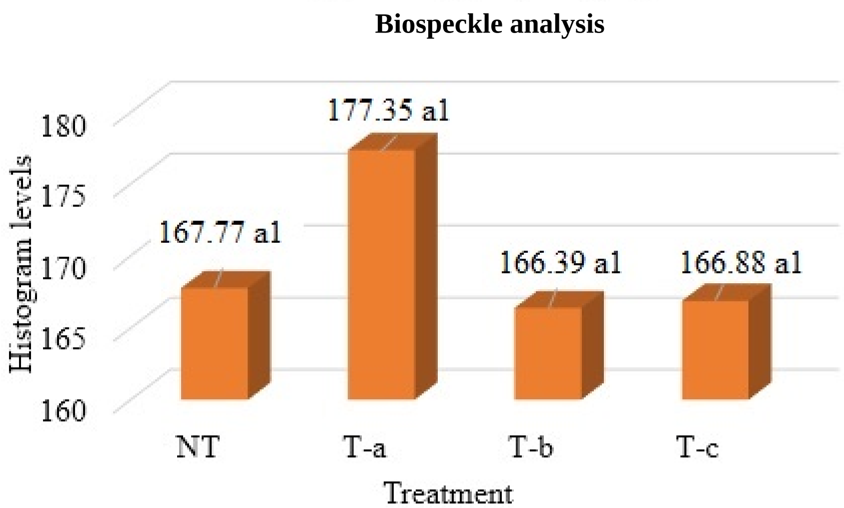

Fig. 15 Occurrence of levels from 0 to 255, for the four assessed treatments, submitted to biospeckle, statistically analyzed through Tukey test at 5% probability.

The histograms for the repetitions for each treatment represent on the *x* axis the range of pixel values. On the *y* axis, the counting of those intensities is represented.

3.7 Biospeckle

In the processed images, it was verified that, in the regions with higher biological activity, the tones of gray were darker. Thus, for the eight bit images (256 levels of gray), level 0 represents the highest biological activity, while 255 represents the lowest biological activity.

Figs. 7-10 are the LASCA images and Figs. 11-14 are the respective histograms.

The averages of each histogram were analyzed in order to obtain the graph in Fig. 15.

The treatment with the lowest occurrence of fungi presented the highest value of level of gray. The samples with NT indicated higher occurrence of fungi and also lower values of level of gray, and treatment T-a presented lower incidence of *Fusarium* spp. and higher value of level of gray. So, it is possible to use the biospeckle technique to compare microbial activity in different treatments, samples or lots of seeds. Through the images, it is possible to notice the relation among the images processed by LASCA with the red, green and blue (RGB) images. The seeds which are visually more infected presented an LASCA activity map with darker colors.

4. Conclusions

The treatment with the bioprotector, *T. harzianum*, is an alternative for the control of fungi associated with maize seeds, being efficient in reducing the fungi incidence.

Trichodermil besides reducing the incidence of *Fusarium*, also positively affects the length of the aerial part of the seedlings.

The biospeckle technique can be used to identify fungi activity and may be used in other studies as a way of evaluating or complementing the information of the other tests.

References

[1] Machado, J. C. 1994. "Pathogen Tolerance Patterns Associated with Seeds." *Annu. Plant Pathol. Rev.* 2: 229-63.

[2] Berjak, P. 1987. "Stored Seeds: The Problems Caused by Microorganisms." In *Seed Pathology—An International Advanced Course*. Passo Fundo: EMBRAPA; ABRATES, 93-112.

[3] Costa, R., and Kristbergsson, K., eds. 2009. *Predictive Modeling and Risk Assessment.* Boston, MA: Springer US: Springer e-books http://dx.doi.org/10.1007/978-0-387-68776-6 (Accessed November 26, 2017).

[4] Faria, A. Y. K., and Cassetari Neto, D. 2003. "Physiological Quality of Cottonseeds Submitted to Chemical and Biological Treatments." *J. Seed Sci.* 25: 121-7.

[5] Luz, W. C. 2001. "Effect of Bioprotectors on Seed Pathogens and Emergence and Yield of Corn Grains." *Fitopatol. Bras.* 26: 16-20. (in Portuguese)

[6] Filho, M. R. C., de Mello, S. C. M., dos Santos, R. P., and Menêzes, J. E. 2008. "Evaluation of *Trichoderma* Isolates in the Promotion of Growth, *in Vitro* Indole Acid Production and Endophytic Colonization of *Eucalyptus* Seedlings." *Bol. Pesqui. E Desenvolv.* 226: 1-16. (in Portuguese)

[7] Harman, G. E., Petzoldt, R., Comis, A., and Chen, J. 2004. "Interactions between *Trichoderma harzianum* Strain T22 and Maize Inbred Line Mo17 and Effects of These Interactions on Diseases Caused by *Pythium ultimum* and *Colletotrichum graminicola*." *Phytopathology* 94: 147-53. doi:10.1094/PHYTO.2004.94.2.147.

[8] Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., Mukherjee, P. K., Zeilinger, S., Grigoriev, I. V., and Kubicek, C. P. 2011. "Trichoderma: The Genomics of Opportunistic Success." *Nat. Rev. Microbiol.* 9: 749-59. doi:10.1038/nrmicro2637.

[9] Corrêa, B. O., Moura, A. B., d'Ávila Denardin, N., Soares, V. N., Schäfer, J. T., and Ludwig, J. 2008. "Influence of the Microbiolization of Bean Seeds on the Transmission of *Colletrichum lindemuthianum* (Saac and Magn)." *Rev. Bras. Sementes*. 30: 156-63. doi:10.1590/S0101-31222008000200019. (in Portuguese)

[10] Rabelo, G. F., Enes, A. M., Braga Junior, R. A., and Dal Fabbro, I. M. 2011. "Frequency Response of Biospeckle Laser Images of Bean Seeds Contaminated by Fungi." *Biosyst. Eng.* 110: 297-301. doi:10.1016/j.biosystemseng.2011.09.002.

[11] Briers, J. D. 1996. "Laser Speckle Contrast Analysis (LASCA): A Nonscanning, Full-Field Technique for Monitoring Capillary Blood Flow." *J. Biomed. Opt.* 1: 174. doi:10.1117/12.231359.

[12] Rabal, H. J., and Braga, R. A., eds. 2009. *Dynamic Laser*

Speckle and Applications. Boca Raton: CRC Press.

[13] Grassi, H. C., García, L. C., Lobo-Sulbarán, M. L., Velásquez, A., Andrades-Grassi, F. A., Cabrera, H., Andrades-Grassi, J. E., and Andrades, E. D. J. 2016. "Quantitative Laser Biospeckle Method for the Evaluation of the Activity of *Trypanosoma cruzi* Using VDRL Plates and Digital Analysis." *PLoS Negl. Trop. Dis.* 10: e0005169. doi:10.1371/journal.pntd.0005169.

[14] Sendra, H., Murialdo, S., and Passoni, L. 2007. "Dynamic Laser Speckle to Detect Motile Bacterial Response of *Pseudomonas aeruginosa*." *J. Phys. Conf. Ser*. 90: 012064. doi:10.1088/1742-6596/90/1/012064.

[15] Pieczywek, P. M., Cybulska, J., Szymańska-Chargot, M., Siedliska, A., Zdunek, A., Nosalewicz, A., Baranowski, P., and Kurenda, A. 2018. "Early Detection of Fungal Infection of Stored Apple Fruit with Optical Sensors—Comparison of Biospeckle, Hyperspectral Imaging and Chlorophyll Fluorescence." *Food Control.* 85: 327-38. doi:10.1016/j.foodcont.2017.10.013.

[16] Vivas, P. G., Resende, L. S., Braga, R. A., Guimarães, R. M., Azevedo, R., da Silva, E. A. A., and Toorop, P. E. 2017. "Biospeckle Activity in Coffee Seeds Is Associated Non-destructively with Seedling Quality: Biospeckle Activity in Coffee Seeds." *Ann. Appl. Biol.* 170: 141-9. doi:10.1111/aab.12314.

[17] Brand, S. C., Antonello, L. M., Muniz, M. F. B., Blume, E., Dos Santos, V. J., and Reiniger, L. R. S. 2009. "Sanitary and Physiological Quality of Soybean Seeds Submitted to Treatment with Bioprotector and Fungicide." *Rev. Bras. Sementes.* 31: 87-94. doi:10.1590/S0101-31222009000400010. (in Portuguese)

[18] Brazil. 2009. *Rules for Seed Analysis.* Brasília, DF: Ministry of Agriculture. (in Portuguese)

[19] Brazilian Ministry of Agriculture, Livestock and Supply (MAPA/ACS). 2009. *Secretariat of Agricultural Defense, Manual of Sanitary Seed Analysis*. Brasília, DF: MAPA/ACS. (in Portuguese)

[20] Krzyzanowski, F. C., Vieira, R. D., and França-Neto, J. B., eds. 1999. *Seed Vigor: Concepts and Tests*. Londrina: ABRATES.

[21] Schneider, C. A., Rasband, W. S., Eliceiri, K. W. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nat. Methods* 9: 671-5. doi:10.1038/nmeth.2089.

[22] Silva, E. R. 2007. "Study of the Properties of Biospeckle and Its Applications." M.Sc. thesis, University of São Paulo. (in Portuguese)

[23] Ferreira, D. F. 2014. "Sisvar: A Guide for Its Bootstrap Procedures in Multiple Comparisons." *Ciênc. E Agrotecnologia.* 38: 109-12. doi:10.1590/S1413-70542014000200001.