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# The Optical Absorption Coefficient of Barley Seeds Investigated by Photoacoustic Spectroscopy and Their Effects by Laser Biostimulation

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**Abstract** Laser light as a biostimulator has been applied in agriculture, and some scientific reports evidence its usefulness. A knowledge about seed optical parameters is of great relevance in the biostimulation process, because information can be provided about the light absorption of seeds. Thus, the objective of the present study was to determine the optical absorption coefficient ($\beta$) of barley (*Hordeum vulgare* L.) seeds by means of photoacoustic spectroscopy; these seeds were studied in two conditions: seeds in their natural color and seeds dyed with methylene blue. The seeds were biostimulated by a laser beam (650 nm wavelength) to evaluate the effects of pre-sowing biostimulation in natural mycobiota associated with different laser irradiation times (0 s, 60 s, 120 s, 240 s, and 480 s). The results of this research demonstrated changes in the optical parameters (absorption and penetration) that occur in the seeds by changing the natural condition to a dyed condition. The dyed seeds, by the methylene blue photosensitizer, become optically opaque, producing greater optical absorption at 650 nm which causes an increase in the effect of laser stimulation. The experimental results showed that the biggest mycobiota reduction (52 %) corresponded to dyed seeds irradiated with a laser for 120 s.

**Keywords** Barley seeds · Laser biostimulation · Photoacoustic spectroscopy

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## 1 Introduction

Photoacoustic spectroscopy (PAS) has been applied in different areas such as food and agriculture; in these areas, PAS has contributed in the study of the optical properties of cereal seeds [1–4], legumes [5], vegetables [6,7], and in the nutritional quality of several foods [8–10]. In the case of cereals, maize seeds (*Zea mays* L.) from different genotypes, with different natural pigments (white, blue, and yellow), have been characterized by their optical absorption coefficient ($\beta$) obtained from the photoacoustic (PA) signal, according to the methodology proposed by Poulet et al. [11]. Also, the PA signal has been mathematically analyzed, using the first derivative and mobile standard deviation, allowing one to distinguish better the maximum of the optical absorption peaks of maize grains with different pigmentations [3]. Other reports indicated that it is possible to obtain, by using PAS, the optical absorption coefficient of bean seeds (*Phaseolus vulgaris* L.), and also to define the optical range where the samples are optically opaque for different varieties and productive cycles [5]. A knowledge about the seed optical parameters and the wavelength range where the seeds are optically opaque or transparent could be relevant in the biostimulation process of agricultural seeds by laser irradiation. Scientific evidence presented by several authors provides the possibility of using laser biostimulation in agricultural seeds [12] for accelerating the maturity in plants, for causing a precocious growth; for causing an increase of their resistance to diseases; for influencing the activity of the alpha-amylase and the concentration of free radicals in the seeds of several plants that could activate seed dormancy; and for improving their germination rate and percentage. Also, laser stimulation on agricultural seeds increases the seed vigor; and causes an impact on the respiration process, photosynthesis activity, the content of chlorophyll and carotenoids of seedlings from seeds that were irradiated to level crop production, and could enhance the quality and quantity of plants [13–18]. Other research has applied laser irradiation in seeds and demonstrated that it could be potentially applied as a fungicide [19–24], but it is necessary to perform more experiments to determine its potential in controlling plant diseases and for seed applications especially related to fungi and mycobiota.

Barley is a crop that can be affected by pathogenic fungi during their formation in the field, and transport and storage; these fungi are capable of synthesizing secondary metabolites called mycotoxins, which could represent potential risk to human health [25]. The mycotoxigenic fungi correspond mainly to the genera *Fusarium, Alternaria, Aspergillus*, and *Penicillium*. Toxigenic species of *Fusarium* and *Alternaria* are commonly classified as field fungi, requiring high moisture content in the substrate for their development and mycotoxin synthesis ($>20$ %). The storage fungi are principally *Penicillium* and *Aspergillus* species which grow in low moisture contents [26]. The presence of fungi in the malt besides being a health risk causes undesirable colors and flavors in beer and effervescence phenomenon (gushing). Some species of these fungi can affect germination, the production of α-amylase, and subsequently the fermentation process [27].

Thus, the development of sustainable biophysical methods is of interest in potential practical applications, for example, the use of laser light for biostimulation. Bel'skii and Mazulenko [19] found that pre-sowing treatment with laser irradiation of barley seeds increased the resistance of the plant against infection by fungi. Other authors

have reported the effects of He-Ne laser irradiation, at 632.8 nm wavelength and 7.3 mW power, on the growth and development of seed-borne fungi in samples of soybean seeds, which showed that the samples with 1 min of irradiation increase the population of fungi in seeds even for the seeds pretreated with methylene blue, methylene red, and carmine dyes [20]. The influence of laser light has been also reported in winter rapeseed, irradiated with a He-Ne laser at 632 nm wavelength and 1 $mW \cdot cm^{-2}$ laser intensity. The best results of a positive influence of laser light on seedling resistant to *Phoma lingam* were obtained in combinations of 30 min to 90 min of irradiation time [28].

The fungicide effect of a diode laser (655 nm wavelength and 27.4 mW power) on maize seeds has been reported by Hernández-Aguilar et al. [23]. These authors found that the combination of two laser intensities, 16.3 $mW \cdot cm^{-2}$ and 4.6 $mW \cdot cm^{-2}$, with 5 min of irradiation time, reduces *Fusarium* genus by 61.11 % in infected seeds, and also the total fungi in irradiation treatments when compared to a control treatment. On the other hand, Yasemin et al. [29] found that SHG of Nd-YAG laser irradiation, at 532 nm wavelength, could be a better alternative method to control seed infection by fungi of hard wheat seeds, and also they found that the velocity of germination growth was modified when the seeds are treated in wet conditions. The effects of laser treatment on plant growth may be related to the result of bioenergetic structural excitement causing cell pumping and enzymatic stimulation.

Therefore, in the present research, the PAS technique was applied to determine the optical absorption coefficient ($\beta$) of malting barley (*Hordeum vulgare* L.) seeds under two conditions: natural color (BN) and methylene blue dyed (BD). After this, laser biostimulation treatments were applied to the seeds and mycobiota tests were established to evaluate the effects of laser irradiation on seed health quality.

## 2 Materials and Methods

### 2.1 Biological Material

In the present research, the Esperanza variety of malting barley seeds from Bajio, Mexico were studied, which were provided by the "Industry Malts of Mexico." For optical characterization, from the lot of seed, prior homogenization of the same, the thicknesses of nine seeds (natural and dyed) were measured using a Vernier caliper, and their average values were $(2.87 \pm 0.182)$ mm and $(2.66 \pm 0.282)$ mm for the two conditions of seeds: natural color (BN) and methylene blue dyed (BD), respectively. Before the laser irradiation, the seeds were photosensitized by soaking them for one hour in methylene blue at a dilution of 0.060 mg of methylene blue in 300 ml of $H_2O$. Two seeds of each condition were randomly selected to obtain their optical absorption coefficient by PAS, and five hundred of each condition were selected to establish the mycobiota test.

### 2.2 PAS Experimental Setup

Figure 1a shows the PAS experimental setup which consists of an Oriel xenon lamp (Model 6271) as the light excitation source, an Oriel monochromator (Model 77250) to

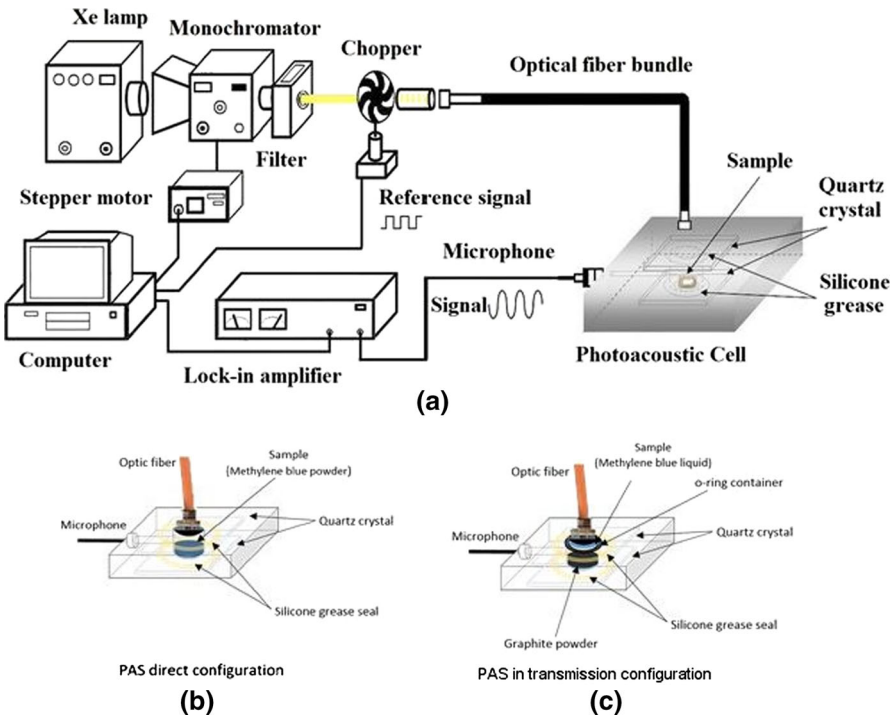


**Fig. 1** (a) PAS experimental setup, (b) PAS direct configuration, and (c) PAS in transmission configuration

obtain a monochromatic light beam, and a mechanical chopper signal recovery, Model 197 to modulate the light beam at a fixed frequency, $f = 17$ Hz. The modulated beam was focused onto an optical fiber in order to guide this beam to the photoacoustic (PA) cell. The acoustic signal generated in the PA cell was detected by an electret microphone through a fine channel (1 mm diameter and 1 cm length) between the PA cell and the microphone inlet. Then, the PA signal was amplified using a lock-in amplifier (EG&G, Model 5210), which is interfaced to a personal computer in order to record the PA signal as a function of beam wavelength. A PA signal was obtained in the wavelength range from 400 nm to 700 nm. From the PA signal was calculated, after checking that the sample is thermally thick, the optical absorption coefficient ($\beta$). The condition to be considered a thermally thick sample is $a_s l_s \gg 1$ (where $a_s = (\pi f/\alpha)^{\frac{1}{2}}$, with $a_s$ and $l_s$ the sample thermal diffusivity and sample thickness, respectively). The optical absorption coefficient ($\beta$) was determined from the PA signal amplitude using the equation proposed by Poulet et al. [11], putting in evidence $\beta$ according to Hernandez et al. [1]:

$$\beta = \frac{a_s \left[ q^2 + q \left(2 - q^2\right)^{\frac{1}{2}} \right]}{\left(1 - q^2\right)}, \tag{1}$$

where $q$ is the normalized photoacoustic signal amplitude (normalized by the emission spectrum of the Xe lamp, which is obtained by taking the photoacoustic signal of graphite powder). To evaluate the thermally thick sample condition, according to its definition above, it took into account the light modulation frequency $f = 17$ Hz and $\alpha = 4.44 \times 10^{-3}$ cm$^2$·s$^{-1}$ [30] ($\alpha$ for starch, which represents 57.1% to 59.5 % of dry weight for barley seed, according to Arendt and Zannini [31]). Having verified the necessary condition for thermally thick samples ($a_s l_s \gg 1$, see Table 1), the optical absorption coefficient ($\beta$) and the optical penetration length ($l_\beta = \beta^{-1}$) were obtained using Eq. 1 as a function of the incident wavelength. The value of $l_s$ for each sample corresponds to its average thickness. The optical absorption spectrum of the dye (methylene blue) was also obtained by PAS, in the wavelength range from 400 nm to 800 nm, for the dye in two conditions: diluted in water and in powder form.

### 2.3 Health Quality: Mycobiota Test

The health quality test was carried out at the Mycology Laboratory of Investigation Unit of Grain and Seeds-FESC, UNAM, establishing the mycobiota test. Two independent experiments were performed for each treatment: (1) natural seeds and (2) dyed seeds. For the establishment of the mycobiota test, 1000 seeds were randomly selected, half of them were dyed and the other half were in their natural color, placed in Petri dishes (15 cm × 1.5 cm) and then they were irradiated for the biostimulation process using a semiconductor diode laser (Tyson Technology Co), 27.4 mW power at 650 nm wavelength with 12 mm spot size. The samples were biostimulated during exposure times: 0 s, 60 s, 120 s, 240 s, and 480 s. Subsequently, the mycobiota tests were performed, according to the International Seed Test Association (ISTA) [32]. The tests were conducted to evaluate the health quality of the barley seeds. The sowing technique of an agar plate with a culture medium of potato dextrose agar with tergitol was used in this research. A randomized completely blocked experimental design with four replications was used, and 25 seeds were taken as the experimental unit. Data were subjected to analysis of the variance using the SAS GLM procedures (SAS, 2008 version). The least significant difference (LSD) test at the 5 % probability level was used for comparing treatments [33].

## 3 Results and Discussion

The seeds under the two conditions, natural color (BN) and methylene blue dyed (BD), were thermally thick, which means that $a_s l_s \gg 1$ (see Table 1). Using Eq. 1 and from the photoacoustic signal obtained by PAS (see Fig. 2), it was possible to calculate $\beta$ and $l_\beta = \beta^{-1}$ [2] for each condition of barley seeds as a function of the wavelength of the incident light (see Fig. 3). Both conditions of the studied malting barley seeds showed statistically significant differences in their length and width; the thickness did not show differences (see Table 1). The BN seed condition was bigger in size (length and width) with respect to the BD seed condition. Also in Table 1, we will focus our attention on the photoacoustic signal at 650 nm because it is the wavelength of the diode laser used for seed stimulation. On the other hand, it is also possible to observe

**Table 1** Mean comparisons of the physical parameters of barley seeds in two conditions: BN and BD

| Variety | Length (mm) | Width (mm) | Thickness ($l_s$) (mm) | PA signal at 650 nm (a.u.) | $a_s l_s \gg 1$ | $\beta_{650}$ ($cm^{-1}$) | $l_\beta$ (mm) | $l_\beta$ (650 nm) < $l_s$ |
|---|---|---|---|---|---|---|---|---|
| 1 (BN) | 9.63 ± 0.168a | 3.53 ± 0.186a | 2.87 ± 0.182a | 0.02b | 314 | 3.21 | 3.11 | Optically transparent |
| 2 (BD) | 9.03 ± 0.353b | 3.21 ± 0.297b | 2.66 ± 0.282a | 0.16a | 291 | 29.12 | 0.34 | Optically opaque |

*BN* barley seeds in their natural color, *BD* barley dyed by methylene blue. Means with the same letter in a column are statistically equal (LSD, 0.05); *LSD* lows significant differences; the different letters (a, b) indicate that there are significant statistical differences

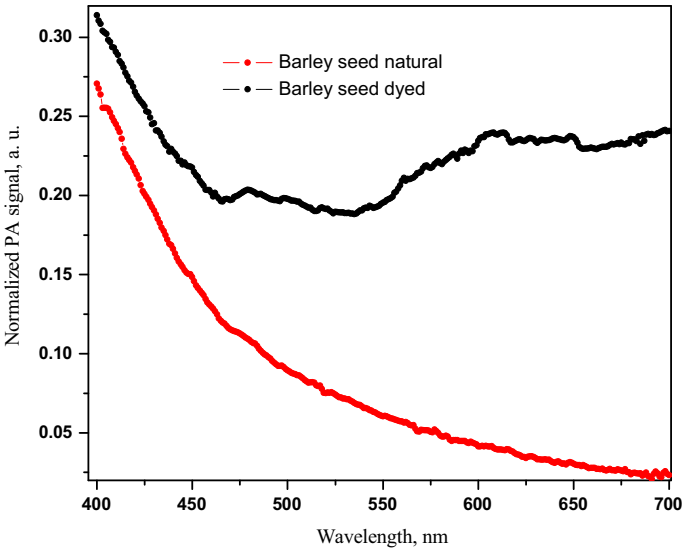


**Fig. 2** Normalized PA signal obtained for both conditions of seeds: natural color (BN) and dyed by methylene blue (BD)

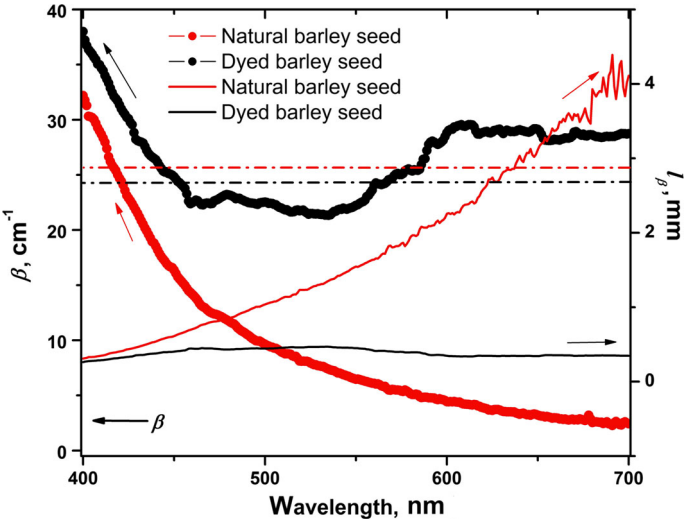


**Fig. 3** Optical absorption coefficient ($\beta$) and optical penetration length ($l_\beta$), as a function of wavelength for seed samples: barley seed natural color and dyed. *Horizontal dashed lines* represent the average sample thickness

that the PA signal amplitude of the barley seed in its dyed state (BD) is bigger when compared with the PA signal amplitude for the barley seed in their natural color (BN). Figure 2 shows the PA signal obtained for both conditions of seeds: natural color (BN) and methylene blue dyed (BD). The calculated optical absorption coefficient was directly proportional to the obtained PA signal, with the $\beta$ value from the dyed

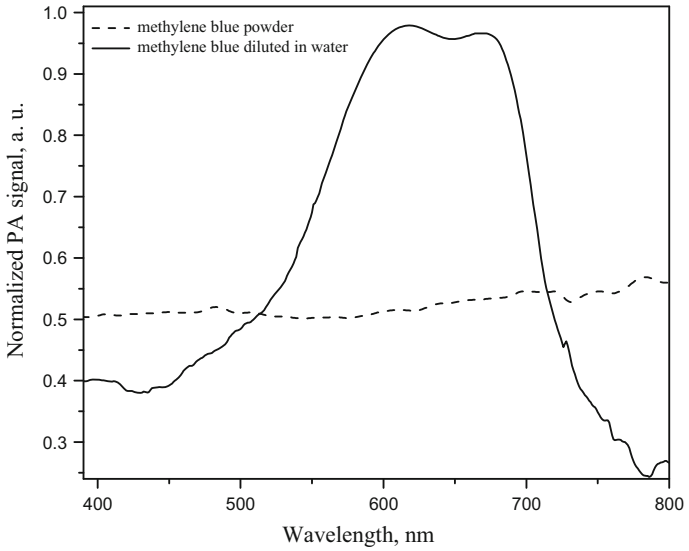


**Fig. 4** Normalized PA signal by PAS for the dye (methylene blue) in two conditions: diluted in water and in powder form

barley seed being 88 % bigger when compared with the $\beta$ value calculated from the barley seed in its natural color ($3.21\ \text{cm}^{-1} < 29.12\ \text{cm}^{-1}$).

Figure 3 shows the optical absorption coefficient ($\beta$) and optical penetration length $l_\beta$, as a function of the wavelength. The sample average thickness $l_s$ for each seed condition is denoted in this figure by horizontal lines. From Fig. 3, it is possible to observe that barley seeds, in the dyed condition, are optically opaque in the 400 nm to 700 nm range, which means that $l_\beta < l_s$. For barley seeds in their natural color condition, it is possible to observe that in the range of 400 nm to 640 nm, the samples are optically opaque, which means that $l_\beta < l_s$. For wavelengths ranging from 640 nm to 700 nm, the barley seeds in their natural color are optically transparent, which means that $l_\beta > l_s$. Also, the thermal diffusion length ($\mu_s = a_s^{-1}$) is smaller than the optical penetration length in both cases (BN and BD), i.e., $\mu_s = (\alpha/\pi f)^{\frac{1}{2}} < l_\beta$.

Figure 4 shows the normalized PA signal of 0.060 mg methylene blue powder diluted in 500 ml of water and the spectrum of the dye in powder form. The liquid sample was obtained through PAS by Transmit configuration (Fig. 1c) and by direct configuration for powder (Fig. 1b). It is possible to observe that, in the range of 610 nm to 675 nm, the diluted dye has a higher optical absorption. The experimental results of the mycobiota test indicated that there was a reduction of them by the interaction of the laser light treatment on the barley seeds. As is possible to see from Table 2, the barley seeds without pretreatment (natural color) showed significant statistical differences ($P \leq 0.05$) for different laser exposure times. The optimum laser irradiation time was 480 s, corresponding to the lowest percentage of mycobiota in the seeds, 41.6 % with respect to the control samples (without light treatment). Also, for the dyed seeds, significant statistical differences ($P \leq 0.05$) were found for different laser

**Table 2** Mean comparisons of mean total mycobiota percentage at various exposure times of laser treatment

| Irradiation times by laser (s) | MPT (BN) | MPT (BD) |
|---|---|---|
| 0 | 84.00a | 57.00a |
| 60 | 74.00ba | 43.00ba |
| 180 | 62.00bac | 28.00b |
| 240 | 60.00bc | 34.00b |
| 480 | 49.00c | 32.00b |
| LSD (0.05 %) | 23.39 | 16.17 |
| Means | 65.80 | 38.800 |
| Significance | 0.05 | 0.014 |
| VC | 23.08 | 27.060 |
| $R^2$ | 0.56 | 0.630 |

Means with the same letter in each column are statistically equal (LSD, $p \leq 0.05$). *LSD* Lows significant differences, *VC* variation coefficient, *MPT* percentage of total mycobiota, *BN* barley seeds in their natural color; *BD* barley dyed by methylene blue

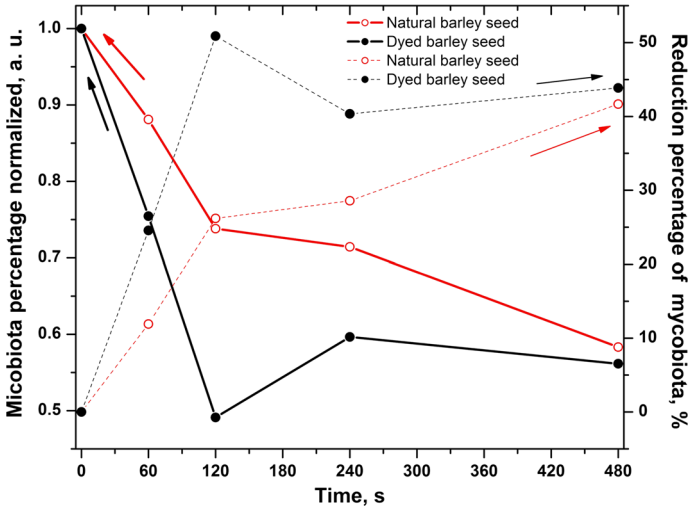


**Fig. 5** Normalized mycobiota percentage (time $t = 0$ s is the control sample) and treated seeds at four laser exposure times: 60 s, 120 s, 240 s, and 480 s

exposure times on the samples, the lowest percentage of mycobiota corresponds to a laser irradiation time of 180 s, 50.8 % with respect to the control samples (without light treatment). It is possible to observe that in the case of dyed seeds, exposure for shorter laser irradiation times had the lowest percentage of mycobiota when compared with natural seeds.

Figure 5 shows mycobiota percentage normalized at time $t = 0$ s (control) and treated seeds at four laser exposure times: 60 s, 120 s, 240 s, and 480 s. It is possible to observe that at all times of laser exposure, the barley seed mycobiota, naturally associated to the seed, show a bigger decrease in percentage for the dyed seed condition when compared with the seed in its natural condition. Both seed conditions had a tendency to improve the health quality of barley seeds, when they are compared with

the respective seed control. The biggest mycobiota reduction for the seed in its natural condition was found at 480 s (42 %). For the dyed barley seed, the biggest reduction was found at 120 s (52 %).

When the barley seeds are dyed with methylene blue, the optical absorption coefficient, at 650 nm wavelength, increases by a factor of nine when compared to the optical absorption coefficient obtained from undyed barley seed, at the same wavelength. Then it is possible to observe, according to the present study, that by modifying the seed optical absorption coefficient, the effects of reducing the percentage of seed mycobiota are increased. As can be seen from Table 2, in the case of the seeds with natural color, a laser irradiation time of 480 s was optimal, with the lowest percentage in obtained mycobiota, when compared to all treatments. For the dyed seeds, the lowest percentage of mycobiota occurs at 180 s of laser irradiation. It is possible to observe that the lowest percentage of mycobiota was found in the dyed seeds, with the highest optical absorption coefficient, as well as the lowest percentage of mycobiota was found at shorter exposure times, when compared with the seeds in their natural condition (undyed). In this research, laser irradiation was used in order to reduce the fungal contamination in barley seeds, particularly when the seeds were pretreated with methylene blue dye. This study showed that this treatment enhances the fungicidal effect of laser irradiation.

Other authors have reported the reduction of fungi such as Rhizoctonia solani, tenuissima Alternaria, Cercospora, and Colletotrichum truncatum kikuchii in soybean seeds using shorter times of laser irradiation; however, when soybean seeds were dyed and irradiated by longer laser exposure times, the fungi were completely eliminated [20]. In this research, the malt barley seeds were dyed with methylene blue and irradiated using a laser diode, with 27.4 mW of power at 650 nm wavelength. In addition, the optical characteristics, phenotypic, and genetic and thermal properties of the seeds were different from those of soybeans.

The results of this research demonstrate that the condition of seeds dyed with methylene blue has a higher optical absorption coefficient, a shorter optical penetration, and at 650 nm is an optically opaque sample, showing the major effects of biostimulation when compared with a seed in its natural state. It was evident that for a higher seed optical absorption coefficient, a bigger laser biostimulation effect is presented, decreasing the percentage of infected seed with fungi in less time. Thus, we can say that a knowledge of the optical parameters of the seeds is important for process biostimulation when it is necessary to apply the laser pre-sowing treatment as a fungicide. The basis of this mechanism could be the existence of phytochromes, not only in plants and seeds but also some viruses, bacteria, and fungi [23,34,35]. The absorption spectrum of these photoreceptors is in the red and infrared light [36]. In this way, fungi associated naturally in the barley seed could be affected with the red laser light treatment. The absorption spectrum of the barley seeds is modified when the seed is dyed and the effects of biostimulation are increased.

Therefore, future research will have to be carried out on a wider range of germplasm, irradiation parameters, and seed conditions to get applications to be used in practice.

## 4 Conclusions

1. By photoacoustic spectroscopy, it is possible to obtain the optical absorption coefficient of barley seeds at different conditions: in natural color and dyed with methylene blue. Also, it is possible to define the optical range where the samples are optically opaque or optically transparent.
2. The studied barley seeds, in the dyed condition, were optically opaque at 650 nm. This is the laser wavelength used in the pre-sowing biostimulation. The seeds in their natural color at 650 nm were optically transparent.
3. Both seed conditions had a tendency to improve the health quality of the barley seeds when they are compared with their respective seed control (without laser light treatment). The biggest mycobiota reduction for the seed in its natural condition was found at 480 s of laser irradiation. For the dyed barley seed, the biggest reduction was found at 120 s of laser irradiation. It was possible to observe that at all times of exposure to laser radiation of barley seeds, the mycobiota naturally associated to the seed decrease by a bigger percentage in the condition of a dyed seed when compared with a seed in its natural condition.

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