Investigating the effects of laser beams (532 nm and 660 nm) in annihilation of pistachio mould fungus using spectrophotometry analysis

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When moulds are illuminated by visible electromagnetic-EM radiations, several effects on nucleus materials and nucleotides can be detected. These effects have a significant influence on mould generation or destruction. This paper presents the effects and implications of a red diode laser beam (660 nm), a second-harmonics of a Nd:YAG laser emitting green beam (532 nm), or the combination of both, on the eradication of Pistachio mould fungus. Incident doses (ID) of both beams are kept identical throughout the experiment. The absorption spectrums of irradiated mouldy samples and the bright-greenish-yellow-fluorescence (BGYF) of fungus occurring in mould texture due to electronic excitation are investigated. We found that a combination of a green and a red laser beam with an ID of 0.5 J/cm^2 provides the optimal effects on Pistachio mould fungus eradication. [DOI: 10.2971/jeos.2010.10033s]

Keywords: photon, laser, illumination, fungus, eradication, absorption percentage

1 INTRODUCTION

Removing or, at least, reducing the number of contaminated units in food products, such as pistachio may have a significant impact on the world food industries. Pistachio is an important export item of many countries such as Iran, USA, Turkey, Syria, Greece and Italy [1, 2]. Its mould (*Aspergillus flavus*) (see Figure 1) can produce Aflatoxin B1 (AFB1) [3, 4], resulting in alterations of the digestive system and acute necrosis in humans, poultry and other livestock [5]–[7].

Virtually all sources of commercial pistachio contain minor amounts of Aflatoxin. However, in some of the exporting countries, a significant amount of pistachio products contain aflatoxin levels, which are usually far below the US Food and drug administration's (FDA) recommended safe level [3, 8]. In a number of fields, such as safely manufacturing food, occupational health, and the conservation of cultural objects and archives, existing methods for inactivating *Aspergillus flavus* are not always effective [9]–[11]. Hence, the issue of *Aspergillus flavus* and potential AFB1 contamination is a serious problem in the export of products such as Pistachio.

The germicidal effects of the ultraviolet (UV) portion of the solar spectrum have been documented since the early 18th century [12]. Since then, many advances have been made in developing technologies based on various wavelengths of the electromagnetic spectrum [12].

Since the invention of the laser by Maiman in 1960, the field of laser chemistry developed rapidly, and attention was focused on photochemical and photophysical processes, such as multiphoton excitation (MPE), vibrational spectroscopy, and electronic photochemistry [13]–[15]. Photochemistry has been shown to have a significant potential in various fields of medicine and industry [16, 17].

International committees, such as WHO, FAO and FDA, have concluded that the selective irradiation of food may reduce the risk of certain food borne diseases, including microbiological and parasitic infestations [17]. In particular, near-infrared (NIR), long wavelength soft-lasers ($<$ 200 mW), and their spectroscopy have been extensively used as a diagnostic technique to measure internal food quality attributes [18, 19]. On the other hand, short wavelength incoherent beams and LED's are now widely used for annihilating fungous spots, inactivating micro-organisms, or preventing bacterial growth in water and food purification systems [20]. Despite these significant

Received 31 January, 2010; published 01 September, 2010 ISSN 1990-2573

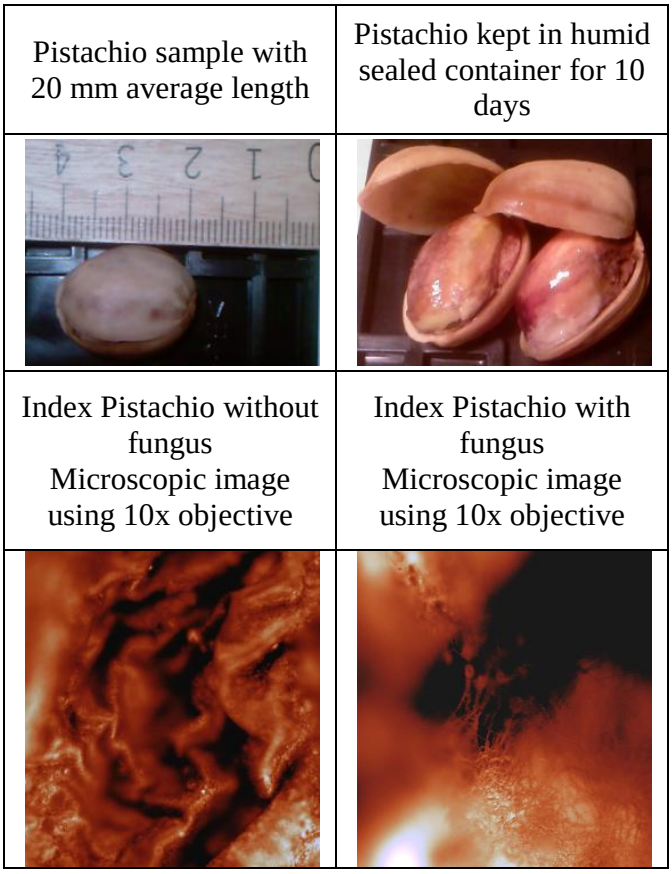

FIG. 1 Macroscopic and microscopic images of the "normal-index" and "mouldy-index" pistachio.

advances, developing new irradiation approaches to eliminate harmful fungus, such as pistachio mould fungus, without changing material characteristics in a cost-effective and environmentally neutral way is still a challenging task for researchers. To this purpose optimal wavelengths and radiation dosages have to be used. For example, long wavelength beams (near to mid-infrared) don't have the ability to kill the fungus. Conversely, short wavelength beams (gamma rays and UV) would increase food microbial control, but the texture, physical appearance and nutritional value of the food would be significantly changed [21, 22].

These techniques also do not render inert any pre-existing toxins. As Gamma rays and UV light have far less penetration depths than NIR, they can't neutralize fungus beneath the surface in some larger food products [21, 22].

Additionally, commercial lasers usually produce an output beam that has a bell-shaped intensity distribution (single mode or constructed bell-shaped beam that is not TEM_{00}, but looks like a Gaussian distribution), or an annular multimode beam. In this study, the output beams of the lasers are converted into the Flattened-Gaussian beams (FGB), using an appropriate optical setup that will be shown later. These outputs beam illuminate the samples uniformly, so that the mould receives a uniform *ID*.

The effects of visible flat-top 532 nm and 660 nm laser beams individually, and in combination, on the annihilation of *Aspergillus flavus* in pistachio are presented in this paper. Our results may have a significant impact on the industrial product quality of pistachio, without reducing or affecting nutritional value.

2 THEORY

Studying the effects of EM waves on biological specimens can be a useful guide toward a better understanding of developing new approach for the removal of microorganisms, such as fungus through illumination [14, 23]–[27].

As Rubinov indicated, biological processes can be influenced by a laser beam [27, 28]. Parameters, such as the local concentration, the spatial orientation of particles, and the composition of particles of different types can be altered or changed due to the laser beam irradiation [28]. Other biological processes, such as the selective increase of the partial temperature for larger particles, small reversible distortions of particles structure (cellular massage), and stimulation of conformational changes in enzymes and other structures are reported [15, 23]–[28].

Using uniform, coherent and linearly polarized laser beam in the visible range, an intense, oscillating electric field, E, is induced on the mouldy pistachio. If the fungi particles are exposed to the electric components of the electromagnetic waves, they can be polarized creating two Coulomb forces on separated charges in opposite directions [14, 27, 28]. In the non-uniform electric field that has a gradient along some direction, the Coulomb forces do not compensate each other and the resulting gradient force drives the particle towards the stronger field. The changes in spatial orientation of fungi particles caused by laser illumination (Green and Red) alter the mitochondrial membrane potential [14, 29, 30]. It is reported that Adenosine Triphosphate (ATP) synthesis can be enhanced by increased membrane potentials of mitochondria [14, 29, 30]. Contrastingly, because of changes in the charge distribution of the phosphate structure after illuminating by laser, the light-excited ATP can react faster with other substrates. These reactions increase Adenosine Diphosphate (ADP) and ATP synthesis [14]. Furthermore, the Reactive Oxygen Species (ROS) production that is cytotoxic can be increased due to this process and it can kill the fungi cells if the concentration becomes sufficiently high [29, 30].

3 MATERIALS AND METHODS

3.1 Cultivation of fungus and preparation the samples

Stock cultures of *Aspergillus flavus* were grown and stored on pistachios at 23°C. For the cultivation of fungi, 100 pistachios of the same size and weight were selected into two groups. The first group included 20 pistachios being placed in a dry, sealed container, the so-called "normal index" group. The pistachios in the second group were kept in a humid sealed container. Pistachios from both groups were kept in a biological incubator at 23°C. After ten days, mould appeared on the surface of pistachios in the second container. These mouldy samples were divided in four groups of twenty

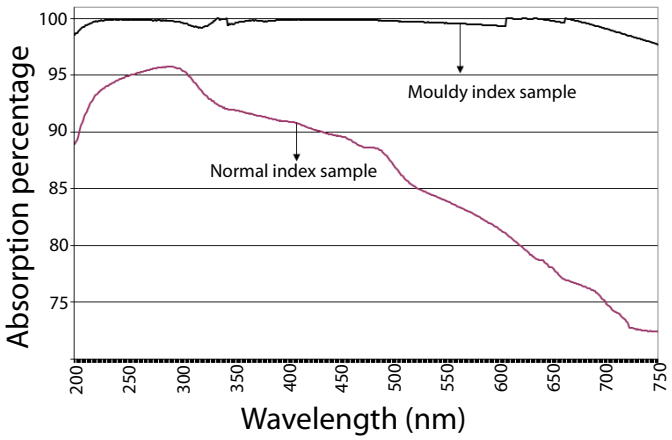

FIG. 2 Comparison between the average of the absorption percentages of the "mouldy-index" and "normal-index" pistachio in the wavelength range of 200 nm–750 nm.

pistachios. One of these groups that is not illuminated by laser beams is called the "mouldy-index" sample. The remaining three groups were labeled 1, 2, and 3 indicating the wavelengths of the lasers that would be chosen for illumination. Figure 2 represents absorption spectra of two index groups, mouldy and normal, using a Carry 500 Scan-spectrophotometer. These spectra show the average absorption obtained on randomly chosen pistachios from each index group ("normal" and "mouldy").

3.2 Lights sources and dosage of radiation

Considering the average of absorption percentage of index samples in Figure 2, the mouldy pistachio has the maximum absorption percentage in the wavelength range of 220 nm–300 nm (UV), 420 nm–540 nm (blue to green) and 610 nm–670 nm (red). The normal index pistachio has its maximum absorption percentage in the range of 200 nm–480 nm (UV to blue) [31]. Therefore, red and green laser lights were chosen for this study.

The output beams of our lasers (100 mW second harmonic of Nd:YAG emitting 532 nm and a 120 mW diode laser emitting 660 nm) are linearly polarized. These lasers normally have spatial intensity distributions that can be roughly approximated by a Gaussian function. Therefore, we have designed an optical beam homogenizer which produces a flattened Gaussian beam (see Figure 3).

During the irradiation process, mouldy samples were illuminated with these green and red beams, individually and in combination. The samples received 0.5 J/cm^2 incident doses (ID) for 7 days. Initially we have calculated the power density of our CW lasers as:

$$p = \frac{\bar{w}}{A} \tag{1}$$

where $\bar{w}$ (mW) and A (cm^2) are the average laser power and the area of exposure, respectively.

The ID is given by

$$ID = P \times t \tag{2}$$

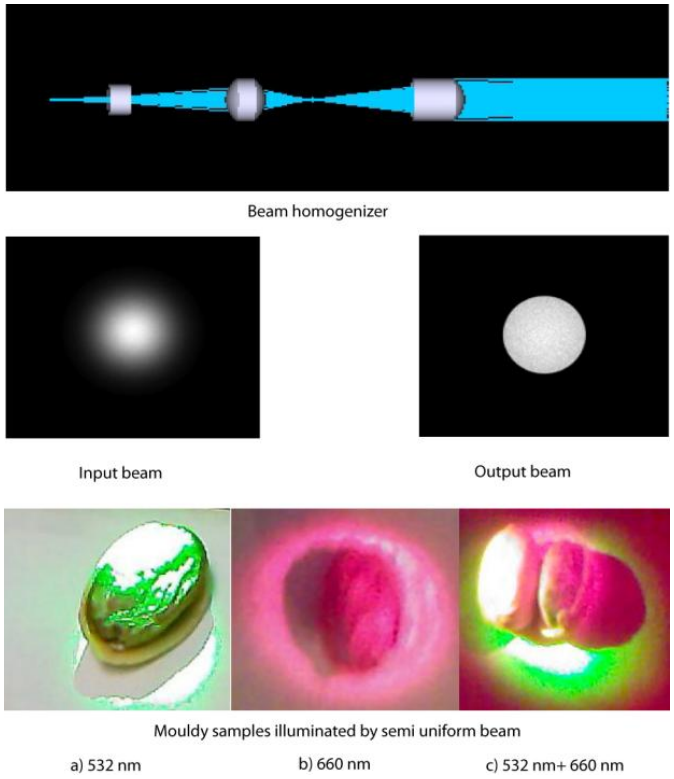

FIG. 3 Experiment set up and experimental samples.

where P is the power density, and t is the exposure time. In our experimental procedures, the light exposed areas were kept identical for all samples. Varying t for lasers having different power densities enables us to obtain identical ID for comparison.

3.3 Spectrophotometer apparatus

We have used a CARY 500 scans-spectrophotometer to study the optical properties of our samples. This spectrophotometer can measure the reflection/transmission spectrum of materials over a large spectral range (UV-VIS-IR / 175 nm–3300 nm).

When light transmission becomes nearly zero, diffraction is almost identical to diffuse reflection. It occurs, when incident light is scattered from our samples in all directions. Diffraction or diffuse reflection spectra are affected by sample shapes, particle size, packing density, and sample concentration. Due to contributions from transmission, internal reflection and specular components they can exhibit reflection and absorption features.

The incorporation of two 90° off-axis ellipsoids in the CARY 500 scans-spectrophotometer forms a highly efficient diffuse reflection collection system. One of the ellipsoids focuses the incident beam on the sample, while the second one collects the radiation diffusely reflected by the sample, and directs this reflected light to the instrument detector. Both ellipsoids are tilted forward, allowing the specular component to be deflected behind the collecting ellipsoid, thus minimizing spectral distortions.

Due to the pistachio's inner structure and thickness, the trans-

mission of light through the samples are approximately zero. Therefore, the absorption percentage can be calculated as

$$\text{Absorption percentage} + \text{Reflectiion percentage} = 100 \quad (3)$$

3.4 Microscope apparatus

One of the common ways of detecting the presence of fungus is the observation of bright-greenish-yellow fluorescence (BGYF) in mouldy samples after illumination by a short-wavelength laser [32, 33]. BGYF occurs via electronic excitation in mould textures, when it is exposed to UV-blue light of sufficient intensity.

In this study, all samples were illuminated by a laser emitting purple/blue (408 nm–488 nm) uniform output beam and the reflectance of the BGYFs from the mouldy surface were recorded and analyzed using a microscope, a CCD camera, and computer software. All images were evaluated for fungal decay by examining the interior and exterior of the images with a dissecting microscope (OLYMPUS U-EPA2) (4× and 10×) for the presence of fungal components.

3.5 Evaluation of treatment efficiency

Considering the large set of our data, for investigating the laser induced effects on the absorption spectra of the irradiated samples, the parametric Paired Samples t-Test (PSTT) and the Independent Samples t-Test (ISTT) were performed, using SPSS and Excel software [34].

The PSTT test reveals differences *within* groups, while the ISTT test reveals differences *between* groups. Using PSTT and ISTT, the difference between two variables can be evaluated and computed.

In order to determine whether our data can be accepted or rejected (acceptance or rejection of the null hypothesis H_0, the probability of the null-hypothesis between the pairs (P) was determined. If P value is less than 0.05, a significant difference between two variables can be expected, and if it is greater than 0.05, there is no significant difference between two variables [34].

Using frequency histograms, we are able to identify the case among our illuminated samples with the most similar characteristics compared with the "normal-index" sample. The frequency histogram is a way to summarize data that are measured on an interval scale. It is often employed in exploratory data analysis to illustrate the major features of the distribution of the data in a convenient form [35]. This method enables us to divide the absorption percentages set obtained in this research into particular groups or types. For each group, a rectangle is constructed. The width of this rectangle along the x direction shows a particular absorption percentage, and the F parameter along the y-axis demonstrates the number of wavelengths having that specific absorption percentage. Comparing these histograms, two histograms with the highest similarity are detected.

Performing PSTT, ISTT, and frequency histograms we determined the most effective illumination wavelength required for removal pistachio mould fungus.

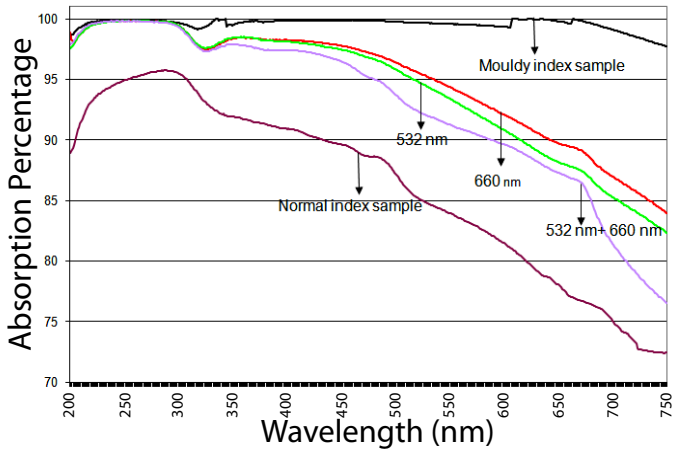

FIG. 4 Comparisons between the averages of the absorption percentages of irradiated mouldy pistachios with index pistachios in the wavelength range of 200 nm–750 nm.

4 RESULTS AND DISCUSSION

In this study, the interaction of coherent uniform laser radiation with pistachio mould fungus is investigated. The three groups that were labeled as 1, 2 and 3 in Section 3.1 are corresponded to 660, 532 and 532 nm + 633 nm indicating samples illuminated by diode laser emitting a 660 nm beam, the second harmonics of Nd:YAG laser emitting 532 nm beam and the combination of these two, respectively. Their spectrums showing the average absorption obtained on twenty pistachios of each group (normal as well as mouldy) are obtained and compared with the indices samples as shown in Figure 4.

Considering the absorption spectra of the "normal-index" pistachios, and "mouldy-index" pistachios in Figure 4, a direct relationship between the absorption percentage and the amount of fungus is obvious. The "normal-index" pistachio has the least absorption percentage, and the "mouldy-index" pistachio has the highest absorption percentage amongst all of the samples. Therefore, the absorption percentage is considered to be a fungi dependant factor.

The statistical analysis of Figure 4 is presented in Tables 1 and 2. It can be seen from Table 1 that there is a significant difference between the average absorption percentage of illuminated samples, and the "mouldy-index" pistachios. The pairs that have the biggest mean difference (difference between the averages of the absorption percentages of pairs) have the least similarity. The mean difference of pair 3 (mouldy sample and illuminated sample by the combination of 532 nm and 660 nm beams) has the largest value, and therefore has the least similarities with the "mouldy-index" pistachios.

The results of the statistical analysis of the observed differences between the illuminated mouldy samples and the "normal-index" pistachios are presented in Table 2. The mean difference of the "normal-index" sample and the sample illuminated by a combination of 532 nm and 660 nm beams, has the smallest value and thus, has the greatest similarity

Paired Samples Test								
		Paired Differences						
	t[a]	df[b]	P value[c]	Mean difference[d]	Std. error difference[e]	Standard deviation	95% Confidence Interval of the Difference	
							Lower	Upper
Pair 1 Mouldy index sample – Illuminated sample by 532 nm	25,009	550	9,141E-93	5,310	0.212	4,984	4,893	5,727
Pair 2 Mouldy index sample – Illuminated sample by 660 nm	25,145	550	1,867E-93	4,644	0.185	4,335	4,281	5,006
Pair 3 Mouldy index sample – Illuminated sample by 532 nm + 660 nm	25,530	550	2,042E-95	6,682	0.262	6,143	6,168	7,196

[a] Statistics is t-distributed with degrees of freedom equal to one less than the number of pairs
[b] Degrees of freedom
[c] It's a probability, with a value ranging from zero to one
[d] Difference between the average of absorption percentages of paired samples (Illuminated mouldy pistachio and mouldy index pistachio)
[e] Standard error of the mean difference

TABLE 1 Comparison between the averages of the absorption percentages of illuminated mouldy pistachio and mouldy index pistachio using 2-sided Paired Samples t-Test.

t-test for Equality of means							
		Paired Differences					
	t[a]	df[b]	P value[c]	Mean difference[d]	Std. error difference[e]	95% Confidence Interval of the Difference	
						Lower	Upper
1. Normal index sample – Illuminated sample by 532 nm	-21,078	1100	4.14E-83	-7,947	0,377	-8,686	-7,207
2. Normal index sample – Illuminated sample by 660 nm	-23,826	1100	1.66E-101	-8,613	0,361	-9,322	-7,904
3. Normal index sample – Illuminated sample by 532 nm + 660 nm	-16,073	1100	2.25E-52	-6,575	0,409	-7,378	- 5,772

[a] Statistics is t-distributed with degrees of freedom equal to one less than the number of pairs
[b] Degrees of freedom
[c] It's a probability, with a value ranging from zero to one
[d] Difference between the average of absorption percentages of paired samples (Illuminated mouldy pistachio and mouldy index pistachio)
[e] Standard error of the mean difference

TABLE 2 Comparison between the averages of the absorption percentages of illuminated mouldy pistachio and normal index pistachio using 2 sided independent samples t-Test method.

with the "normal-index" pistachios (see Table 2, number 3, last line). A comparison between the histograms of Figures 5 and 6 draws the same conclusion.

In Figure 5, the plot of the "mouldy-index" sample represents data with a pronounced peak in the absorption percentage in the range of 99.5%–100%. While there are "outliers", they are

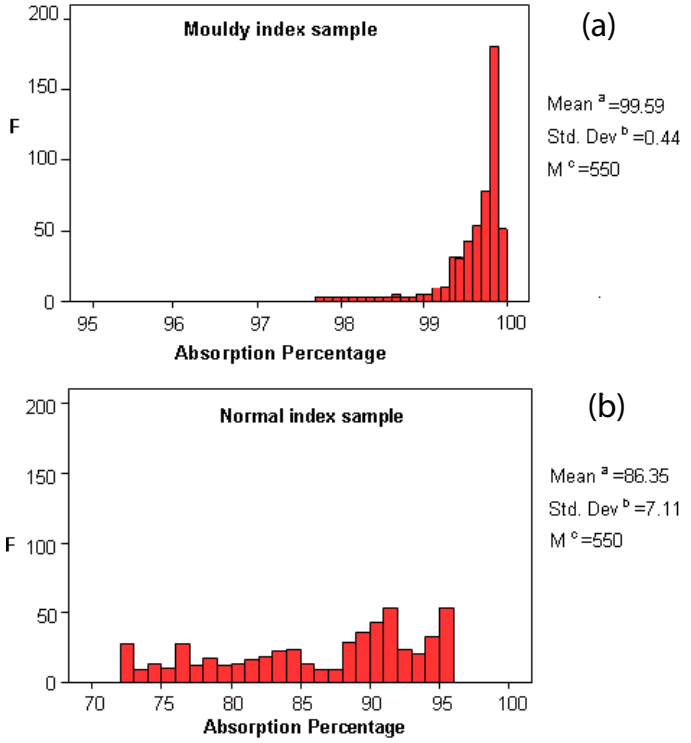

FIG. 5 The frequency histograms of index samples. The x-axis demonstrates the absorption percentage and F is a parameter representing the number of wavelength on the y-axis. (a) The absorption percentage of the mouldy samples, for all 550 wavelengths (200 nm-750 nm), is in the rage of 97.5%-100%. The reflection from the surface of these samples is about 3%. (b) For normal index-samples, the absorption percentage is in the range of 71.5%–96.5%. The reflection from the surface may reach to maximum 28.5%. [a] Mean: The average of absorption percentage of the "normal-index" sample for the wavelength range of 200 nm–750 nm. [b] Std.Dev: Standard Deviation. [c] $M = 550$, presenting the number of the wavelengths in absorption spectrum (200 nm-750 nm) provided by Carry-500 spectrophotometer.

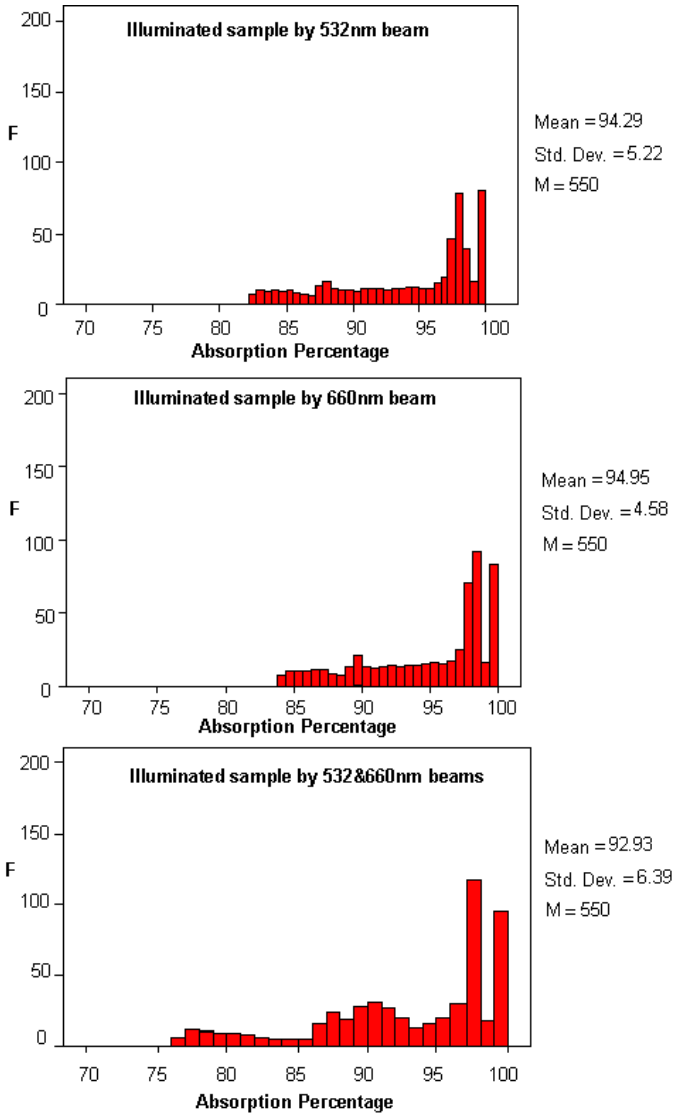

FIG. 6 The frequency histograms of illuminated mouldy samples. The x-axis demonstrates absorption percentage and F: the number of wavelength, respectively. The absorption percentage of the mouldy samples illuminated by 660 nm, 532 nm, and combination of 660 nm and 532 nm has been changed compared with the Figure 5(a). The standard deviation value is changed from 0.44 (the "index mouldy" sample) to 5.22, 4.58, and 6.39 for samples illuminated by 532 nm, 660 nm, and the combination of 532 nm and 660 nm, respectively. The absorption percentage for all 550 wavelengths (200 nm–750 nm), is in the rage of 70%–100%. Mean, Std.Dev, and M-number are as described in Figure 5.

of relatively low frequency. The mean value of absorption percentage is 99.59 indicating a high amount of light absorption by all the wavelengths in the range of 200 nm–750 nm. The reflection from the surface of the mouldy samples is the least, and it is less than 0.5%. Considering the frequency-histogram of the "normal-index" samples, there are numbers of peaks in the absorption percentage changing approximately in the range of 72-96%.

The frequency histograms of the mouldy samples illuminated by wavelengths of 532 nm, and 660 nm, or their combination are presented in Figure 6. It can be seen that the histogram obtained for the combination of 532 nm and 660 nm beams, is more similar to the frequency histograms of the "normal-index" sample presented in Figure 5. Three peaks, can clearly be detected in the histogram (two primary peaks in the range of 76%–83% and 86%–94% and a further peak in the range of 94%–100%.)

The mean value (average of the absorption percentage) and standard deviation of the "normal-index" sample has the closest value to the related parameters of the sample illuminated by the combination of the 532 nm and 660 nm beams.

From the spectrophotometers and statistical analysis, it can be seen that both red and green wavelengths can have major observable effects in the removal of fungus, however, the combination of these wavelengths has the most significant effects in the eradication of pistachio mould fungus.

To assess the results, the BGYF method was employed, and as elucidated in Section 3.4, electronic excitation in mould texture of the illuminated pistachios were recorded after each day of illumination. The decay of fungus in samples illuminated by the combination of 532 nm and 660 nm lasers on a particular marked area is shown in Figure 7(b) for a period of 7 days. It can be seen that, Figure 7 also confirms the same result as presented in Figure 4.

In conclusion, the combination of two illumination sources of different wavelengths (red and green), with ID of 0.5 J/cm^2

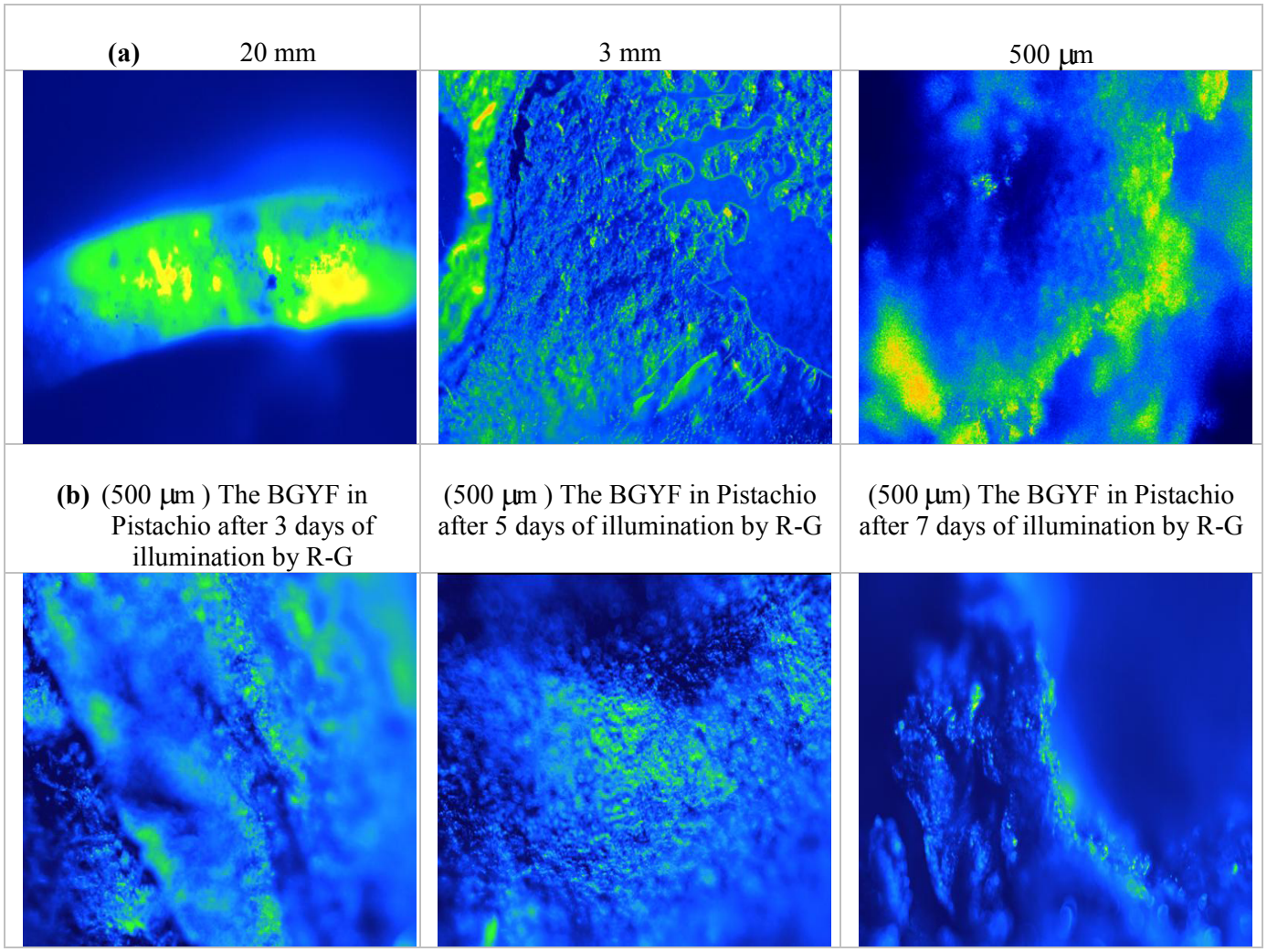

FIG. 7 BGYF in illuminated pistachios by 532 nm + 660 nm beam

has following benefits for suppression of the fungus in mouldy pistachios:

- the probability of two-wavelength absorption increases as a quadratic function with illumination intensity.
- a combination of a beam with a longer visible wavelength having higher penetration depth and less scattering (i.e. red/660 nm), and of a beam with higher energy/shorter wavelength having less penetration depth/high absorption (i.e. green/532 nm) eradicates fungi in the body as well as in the surface of pistachios.
- the used non-ionizing light sources does not cause toxicological hazards, and thus no special microbiological or nutritional problems in pistachio.

ACKNOWLEDGEMENTS

Authors would like to thank Mr. Thomas Keith Stackhouse for reading the paper, Dr. Hans G. Loew, and Mrs. Marashi for useful discussion.

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