In vitro study regarding the testing of treatments with inhibiting effect on the pathogenic fungi of *Alternaria alternata*

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Abstract

This research presents a series of in vitro tests regarding the antimicrobial effects on the A1 and A100 –Alternaria alternate colonies, induced by blue light irradiation, by growth in the presence of the Bengal Pink substance or by the combination of these treatment forms.

The in vitro investigations were performed in the laboratory using an experimental device consisted on blue light LED-s and A – alternate colonies were developed in the Petri plates. The Petri plates were incubated at 25-26ºC for 7 or 9 days and the diameter of A. alternata colonies was measured daily, starting with the third day after the inoculation moment, in order to monitor the inactivation and inhibition effect of the fungi colonies upon the application of different irradiation doses ($2.0J/cm^2$ and $2.7J/cm^2$).

The application of the physical treatment of blue light irradiation emitted by supra-luminescent LED-s has generated results of the inhibition and inactivation effects inferior to those generated by the application of the chemical treatment of growing in the presence of the Bengal Pink substance.

In order to establish the inhibitor effect determined by the simultaneous application of both treatments (blue light irradiation and inhibition with Bengal Pink) two concentrations of Bengal Pink were tested, added directly in the composition of the nutritive PDA (Potato Dextrose Agar - Fluka) medium. Subsequently, the samples cultivated in Petri boxes were irradiated with blue light.

Our experimental results demonstrate that the treatment performed in combined form, consisting of both blue light irradiation emitted by LED-s, and growth in the presence of the Bengal Pink substance, induced the best inhibition and inactivation effects on colonies of A. alternata.

Our future research will aim at the application of the combined treatment mentioned previously at the level of plants infected with microorganisms of A. alternate and cultivated in protected areas, in view of obtaining ecological harvests meant for the food industry.

Keywords: inhibition, blue LED-s , *Rose Bengal*, *Alternaria alternata*.

Introduction

Alternaria is the name which comprises a series of imperfect fungi frequently isolated from plants, soil, food and air in rooms. One of their main characteristics is the production of brownish pigments. The *Alternaria* genus comprises around 50 species. Among them, *Alternaria alternata* is the species most frequently isolated in human infections. Some authors suggest that *Alternaria alternata* is a complex of representative species and less a singular species, since it is made up of several species of heterogens. *Alternaria sp*. Grows quickly and the dimension of the colonies reaches a diameter of 3 to 9 cm, by incubation at 25 ℃, for 7 days on PGA (Potato Glucose Agar). The aspect of the germ colonies is plane, varying from fluffy to felt, being covered in time with short aerial hyphes, grey in colour.

The surface of the colony is grey-whitish in colour in the beginning, and subsequently becomes dark, passing to black-greenish or brown-olive, with a halo of light colour on the edge. On the inferior side, the colony is usually brown towards black, due to the production of pigment [1, 3].

The photodynamic inactivation of the pathogenic microorganisms is of interest for the microbiology of food products and, in this sense, research was performed in the field of disinfection and preservation systems, which proved very safe and with low energetic costs. Numerous researches had as goal the inactivation of a range of microorganisms, using the *Rose Bengal* inhibitor. The studies performed focused both on finding the minimum concentration of *Rose Bengal*, which causes quick death, and on investigating the inhibiting compounds L-histidine and crocetine, which protect, for example, the bacteria from photodynamic inactivation [2, 4 and 5].

A photosensitive element is represented by a molecule that absorbs light, in order to produce chemical reactions, which cannot take place otherwise. The majority of photosensitive elements in the biological systems imply the presence of molecular oxygen in the photo-oxidation processes. The wave length necessary for energizing the most reactions is longer than 320 nm, but every photosensitive element has specific maximum intensities, for example the xantanic colorant *Rose Bengal* has the maximum absorbance capacity at 540 nm [4 and 7].

Light absorption in living organisms by the endogenous and exogenous photosensitive elements, in the presence of oxygen generate chemical and biological effects that, generally, are to the disadvantage of cellular vitality. These applications might be used in obtaining a photodynamic conservation and increase the possibility to preserve perishable vegetal products by means of photosensitive elements incorporated into the packaging of products sensitive to natural light [4].

As a consequence, the experiments performed by us aimed at using supra-luminescent LED-s as physical treatment and the Rose Bengal substance as chemical treatment, for treating the microbiological cultures for the purpose of increasing the percentage of inactivation and inhibition of viability of *Alternaria alternata* colonies.

Material and Method

Biological material

Biological material consists on microbial ***Alternaria alternata*** cultures, **A1** and **A100** types, taken from the collection of the Genetics Laboratory of the Faculty of Biotechnologies (USAMV-Bucharest) and cultivated on the PDA (potato-dextrose - agar) type culture medium [3].

Manner of work used in establishing microbial cultures

- As materials were used: PDA type culture medium produced by BIOKAR DIAGNOSTICS FRANCE, sterile glass Petri disches (100 mm diameter), BIOHIT Proline micropipette 0,5-10µl with sterile tips and different capacities, test tubes with sterile distilled water, microbial cultures of *Alternaria alternata* from the **A1** and **A100** types [3]. For each experimental variant were executed 3 rehearsals (plates).

After sowing, the plates were thermal preserved at 25-26°C for 3 days, and then they were treated individually or simultaneously with blue light LED-s (LED - Light Emitting Diode) or *Rose Bengal* (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein disodium salt [6]). All 3 Petri plates considered rehearsals, prepared for each experimental variant, were irradiated for 5 days, and the measurements and observations were reported to the control

culture not irradiated, also prepared in 3 rehearsals for each of the two *Alternaria alternata* types.

Method. Experimental device

The experimental device consists on a laser head, a "mobile arm" and the power supply. The laser head has 12 LED-s emitting in the blue spectral range (430-470) nm. The main wavelength is 450 nm. The emitted power of the device is 60 mW. It works at 220V/50Hz.

The gripping system "mobile arm" type allows the adjustment of the distance between the LED-s system and the exposure surface (78.5 cm^2). The exposure surface is selected depending on the Petri bowl that must be irradiated and can be adjusted using the mobile arm.

Two irradiation doses were used in presented experiments. Table 1 presents the exposure times and the used doses for an irradiated surface of 78.5 cm^2.

Table 1. Irradiation doses versus exposure times for an irradiated surface of 78.5 cm^2.

T (min)	D (J/cm^2)
45	2.0
60	2.7

The dimensions of the used LED-s were of 5 mm in diameter and 8 mm in length. The entire **experimental device** was produced by the Romanian company **4R OPTICS SRL**.

The research was performed in the form of two categories of main tests.

I. Experimental tests, in which there were used microbial cultures of **A1** and **A100** *Alternaria alternata* types, cultivate on nutritive PDA (Potato Dextrose Agar produced by Biokar Diagnostics France) medium, on which the following treatments for inhibiting the growth of cultures were applied:

a. irradiation with blue light emitted by LED-s, observing two exposure variants (PI - 60' = 60 minutes and PI - 45' = 45 minutes), without cultivation in the presence of inhibitor Rose Bengal;

b. cultivation in the presence of inhibitor *Rose Bengal* added to the composition of the nutritive PDA medium in concentration of 0.002 mg or of 0.003 mg / 100 ml (PII – 0.002 mg Rose Bengal and PII – 0.003 mg Rose Bengal), without applying the irradiation with blue light emitted by LED-s. This experimental variant was included in the experimental diagram for determining an optimum Rose Bengal concentration with inhibiting effect, the results obtained going to be used in the application of the combined treatment (physical and chemical), with inhibiting effect;

c. irradiation with blue light emitted by LED-s, respecting two exposure variants (PIII - 60' = 60 minutes and PIII - 45' = 45 minutes), and the cultivation in the presence of inhibitor Rose Bengal 0.003 mg / 100 ml nutritive PDA medium. By selecting this experimental variant, the combining of the two methods (physical and chemical) for inhibiting the development of the *Alternaria alternata* colonies was achieved;

The experimental tests comprised the same experimental variants for the microbial cultures of **A1** and **A100** *Alternaria alternata* types, and the same working and incubation conditions were preserved (continuous dark outside the irradiation period and 25-26°C) throughout 5 days of treatment.

II. Control semples, represented by microbial cultures of **A1** and **A100** *Alternaria alternata* types which were cultivated on PDA culture medium and were not irradiated with blue light emitted by LED-s (PI – C for the experimental tests where only physical treatment was applied) and, respectively, witness test cultures, cultivated on nutritive PDA medium, where there were added 0.003 mg Rose Bengal /100 ml nutritive medium and not irradiated with blue LED-s (PII – C for chemical treatment and PIII – C for combined treatment). The control comprised for each type (**A1** and **A100**), of *Alternaria alternata* microbial cultures, 3 repetitions each / experimental variant, the Petri plates being maintained in conditions of continuous dark and temperature of 25 - 26°C throughout the 7 days of cultivation.

The determinings were made daily for 5 days (**Z1-Z5**) starting with day III (after 72 hours) after seeding the cultures, when the first individual treatment was performed (physical treatment with LED-s of blue light) or combined by simultaneously using physical irradiation treatments and the chemical inhibitor. In the determinations for the chemical treatment (with two concentrations of Rose Bengal) applied alone measurements were performed, daily, for a period of 7 days.

The five treatments, of irradiation with blue light emitted by LED-s, applied to the experimental samples a and c were performed individually, observing for each Petri plate 1 treatment / day, with duration of 60' or 45', according to the previously mentioned experimental diagram. The measurements and experimental observations were related to the control sample, not irradiated and also prepared in three repetitions for each of the two types (**A1** and **A100**) of *Alternaria alternata*.

Results and Discussions

The photo-biological response reactions constituted the purpose of the **physical treatment** experiment in view of testing *in vitro* the antimicrobial effect induced by **the irradiation with supra-luminescent blue light emitted by LED-s**.

The reaction capacity of the imperfect *Alternaria alternata* fungi under the action of the supra-luminescent LED-s was analyzed by using more executing several experimental variants, selecting the optimum variants and performing a number of three repetitions with the selected variants, for each of the samples. Several comparative laboratory tests were performed, in order to show the effect produced by the irradiation with supra-luminescent LED-s, on the two types (A1 and A100) of *Alternaria alternate*, our observations being focused on the determination of the percentage of inactivation and inhibition in their development.

In order to determine the irradiation conditions, the exposure dose is calculated, knowing the emission power of the device with LED-s and the surface on which the homogenous exposure is performed. Since the emission power of the 12 LED-s is of 60 mW, and the irradiation surface is established at 78.5 cm^2, then, the irradiation doses may be calculated. Also, the surface on which the light intensity is homogenously distributed was taken into account, such as each treated sample receives approximately the same dose of radiation. The calculation of the irradiation doses was done following certain experiments that lead to the selection of the range of doses that gave satisfying results, finally selecting the optimum dose following the experiments.

In case of using the blue light of supra-luminescent LED-s, the light radiation in the range of 400-500 nm was used, necessary to plants for optimizing the bio-physical and bio-chemical reactions. The material that is presently used for obtaining supra-luminescent LED-s with emission in blue is AlInGaN. The electrical and optical characteristics of the LED-s

used for executing the irradiation panel were measured. **Fig.1** shows the spectral distribution of the emission of the single LED. It has the maximum emission at approximately 450 nm.

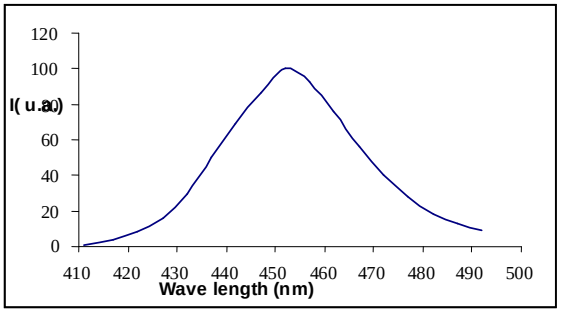
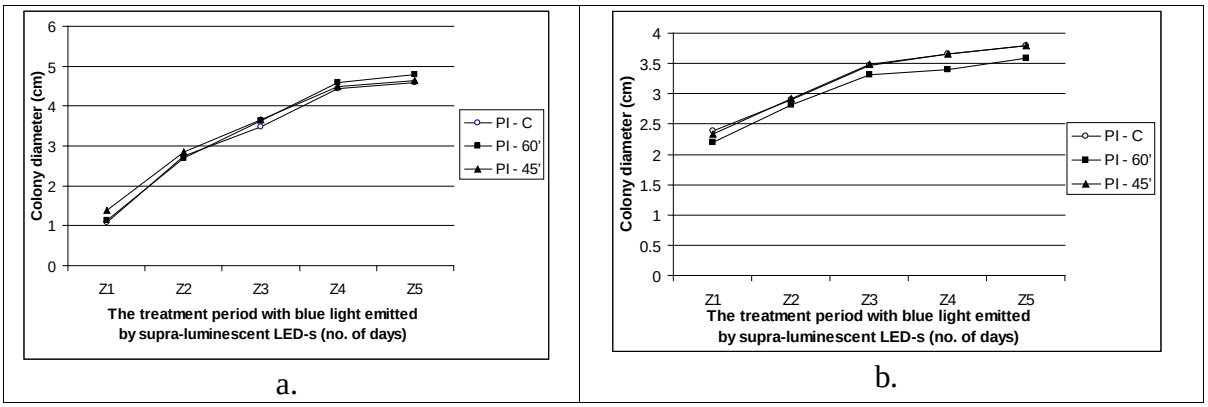


Figure 1. Intensity vs. wave length characteristic to the blue light of supra-luminescent LED-s

The experimental results obtained are graphically illustrated in the following.

Thus, in **Fig. 2a** presents the influence of treatments produced by the irradiation with blue light emitted by LED-s**,** on cultures of **A1** *Alternaria alternata* type applied for 60 min/day or 45 min/ day throughout 5 days, comparatively to the untreated control sample. The nutritive medium used for cultivating the experimental (**PI – 60 min; PI – 45 min.**) and control (**PI - C**) samples was PDA (Potato Dextrose Agar) type. Following the daily measurements (diameter of colonies), it was established that the values obtained in the experimental samples after irradiation were situated above the values recorded in the untreated colonies (**PI - C**), and, as a result, the desired inhibition effect was not registered.

In comparison with the results obtained in case of the **A1** *Alternaria alternata* type upon the application of treatments with blue light emitted by LED-s, in case of the microbial cultures of **A100** *Alternaria alternata* type it was established that throughout the 5 days of irradiation for 60 min / day, inhibition differences occur with respect to the development of the colonies, through the average increase values of the diameter (cm) measured in the 3 repetitions, comparative to the control sample (**PI - C**). The results of the average values recorded in case of the **PI – 60 min.** experimental variant were lower than the untreated control by more than 7.0% (Z1 with a difference of 7.95% and, respectively, Z4 with the difference of 7.10% with respect to control), compared to the **PI - 45'** experimental variant, where the inhibition of colonies was observed only after the application of the first treatment, the subsequent values exceeding or equaling the results of the untreated control (**Fig. 2b**).

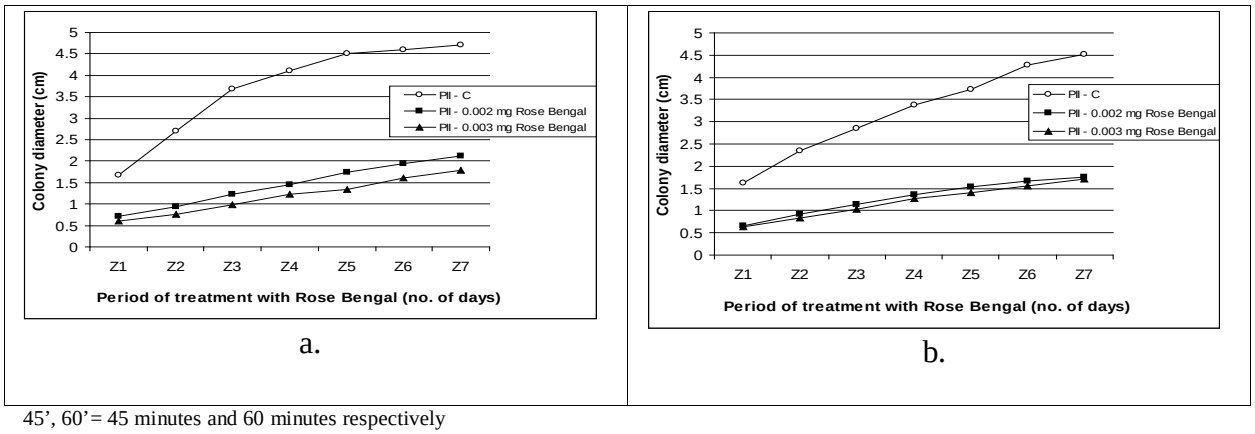


45', 60'= 45 minutes and respectively 60 minutes

Figure 2. Influence of treatments with supra-luminescent blue light emitted by LED-s for 60'/day (PI - 60') or 45'/day (PI - 45') applied on the colonies type A1 (a) or type A100 (b), comparative to the untreated control sample (PI - C).

Also in continuing our experiments, performed for researching the inactivation of colonies of ***Alternaria alternata,*** we **treated chemically,** by testing the **Rose Bengal inhibitor** added to the nutritive PDA medium in two concentration variants (0.002 mg or 0.003 mg / 100 ml). The measurements and observations were related to the control culture (nutritive PDA medium without Rose Bengal) prepared, as the experimental samples, in 3 repetitions for each of the 2 types of *Alternaria alternata*. The Petri plates were kept in continuous dark and temperature of 25- 26°C throughout the 7 days of treatment.

From the results graphically illustrated in **Fig.3a** for cultures of **A1** *Alternaria alternata* type and, respectively, in the graph in **Fig.3b** for cultures of **A100** *Alternaria alternata* type**,** there may be observed the effect of the *Rose Bengal* inhibitor added to the nutritive PDA medium, comparatively to the control sample (PII - C) cultivated on nutritive PDA medium without Rose Bengal. Both concentrations used (0.002 mg or 0.003 mg / 100 ml nutritive PDA medium) determined the inhibition of development of the colonies of *Alternaria alternata* with values of 200-300% with respect to the control cultivated only on nutritive PDA medium without Bengal Pink. The obtained inhibition differences between the two concentrations are not significant, but in the both case of the colonies the recorded values of the diameter (cm) were lower than in the control sample.



45', 60'= 45 minutes and 60 minutes respectively

Figure 3. Influence of treatments with Bengal Pink to cultures of **A1 (a)** or **A100 (b)** in comparison to the untreated control sample (PII - C).

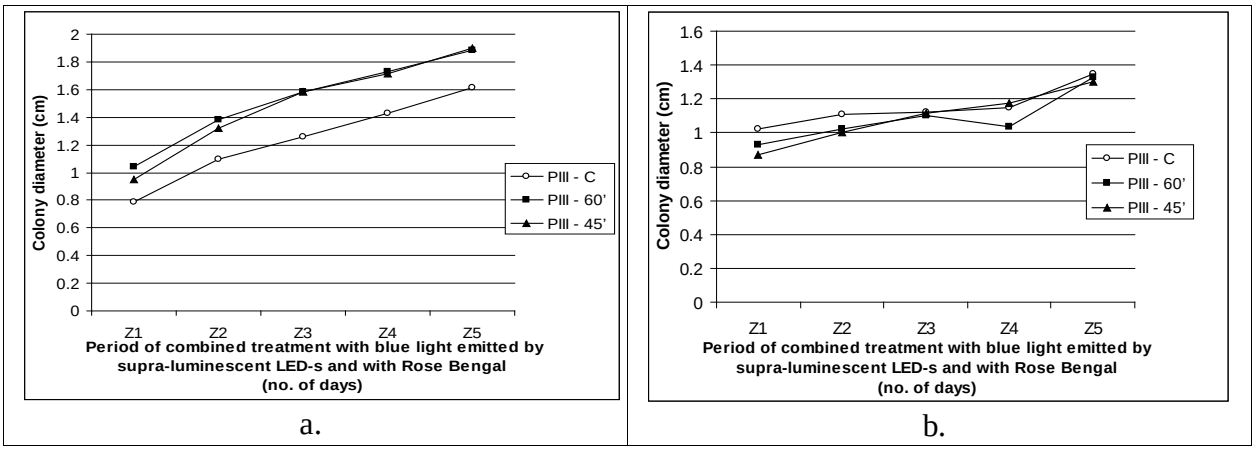
Since, on the basis of the results registered as a consequence of the experimental chemical treatment protocol it was established that the use of a chemical inhibitor of the Rose Bengal type may amplify very much the inhibition effect of the development of microbial cultures of *Alternaria alternata*, we continued to test the combined application of the two treatments**: physical** by irradiation with blue light emitted by supra-luminescent LED-s for 60'/ day or 45' / day, and **chemical** with Rose Bengal 0.003 mg / 100 ml nutritive PDA medium.

The measurements and observations were performed after 72 hours and were related to the control sample (PIII - C = Bengal Pink 0.003 mg / 100 ml nutritive PDA medium), in 3 repetitions for each of the 2 types of **A1** and **A100** *Alternaria alternata*.

Analyzing the results registered for the cultures of **A1** *Alternaria alternata* type it was established that they exceed the values obtained for the untreated control colonies (**Fig. 4a**) and, as a consequence, the colonies in the experimental sample were not inhibited.

In exchange, the effect generated by the combined application of the inhibition treatments determined the obtaining of good results (**Fig. 4b**), with respect to the microbial colonies of **A100** *Alternaria alternata* type for both exposure variants (PIII - 60' and PIII -

45'). At the same time, the dimensions of the colonies reduced by 50-60%, in comparison to the ones recorded in case of applying for five days the physical treatment alone (PI-60 = 3.58 cm ø cultures; PC = 3,80) compared to the case when it was applied simultaneously with adding Bengal Pink (PII-60 = 1.325 cm ø cultures; PC = 1,348 cm ø cultures).



45', 60'= 45 minutes and respectively 60 minutes

Figure 4. Influence of both treatments simultaneously applied on cultures of **A1 (a)** or **A100 (b)** ***Alternaria alternata*** type comparatively with the untreated control sample (PIII - C).

On the basis of the experimental data recorded, there can be considered the fact that in order to use blue-coloured supra-luminescent LED-s in treating microbiological cultures, for the purpose of increasing the percentage of inactivation and inhibition, it is important to establish the irradiation dose with the light produced by the LED-s (intensity of radiation and exposure time) for each separate experimental variant. Also, there may be appreciated the fact that for the colonies of **A100** *Alternaria alternata* type, the use of the combined treatment with blue light produced by LED-s and Rose Bengal may favour the inhibition of their viability.

Since the experimental data indicate the existence of an inter-dependency, between the parameters of the treatments applied and the types of *Alternaria alternata* used, we can appreciate that in the future there are necessary experiments which to regulate the inhibition of the colonies of *Alternaria alternata*, in order to obtain results which have value from the economic point of view, as well.

Conclusions

The experimental results obtained, by executing the working diagram presented in this paper, were mainly influenced by: the microbial type of *Alternaria alternata*, the individual or combined modality of applying the treatments and the exposure time. Thus, the inhibition of the development of the colonies of **A1** *Alternaria alternata* type was achieved only for the experimental variant that involved using the chemical inhibitor Rose Bengal 0.003 mg / 100 ml nutritive PDA medium. But, both the experimental variant that involved the individual application of irradiations with blue light emitted by supra-luminescent LED-s, as well as the one combined with a chemical inhibitor did not return results above the values obtained for the colonies of the experimental variants with the role of control.

In case of the **A100** *Alternaria alternata* type, the inhibition in the development of colonies was achieved, both in the individual application of the physical (irradiation with

blue light emitted by supra-luminescent LED-s) or chemical (cultivation in the presence of Rose Bengal administered in the nutritive PDA medium) treatment, and in their combined application.

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