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Representation of He-Ne laser irradiation effect on radish seeds with selected germination indices

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A b s t r a c t. In this paper the effect of He-Ne laser irradiation of radish seeds as a potential means to accelerate the germination rates have been investigated. We examined whether the change of final germination percentage (FGP) of irradiated seeds is accompanied by changes of other germination indices. Germination tests were carried out at the temperatures of 15 and 20ºC, and the selected germination indices were: mean germination rate, mean germination time, relative frequency, time for the first seed to germinate, time to reach 50% germination, time between 25 and 75% and time between 10 and 90% of final germination. The results showed that He-Ne laser light irradiation could significantly affect the FGP when compared to the control seeds, although these depended on the germination temperature. The irradiation improves the FGP only at 20ºC, although there were no differences detected in any other examined germination indices. The results led to the conclusion that irradiation did not affect any time-related properties describing the germination process. A simple comparison of the FGP of control and the irradiated seeds was found to be the only germination index which indicated the influence of He-Ne laser irradiation on germination.

K e y w o r d s: laser irradiation, radish seeds, germination

INTRODUCTION

Light, one of the most important environmental factors, plays a critical role in plants' photosynthesis. Also the non-photosynthetic processes involving action of light, like phototropism, photomorphogenesis, photobiosynthesis of carotenoids are well known and have been the subjects of investigations for decades. It is clear that also seeds respond to light with a complex variety of reactions that are affected both by the duration of exposition to and intensity of light as well as to light transmittance of seed coat which depends on the wavelength of applied light (Shinomura *et al.,* 1996; Hartmann and Mollwo, 2000).

It is generally accepted that germination process is sensitive to irradiation with various wavelengths of visible and infra-red light. Field experiments indicated that germination of buried seeds may be triggered by millisecond-exposures to sunlight and a five-seconds-long exposure to weak moonlight can saturate the germination of photosensitized seeds (Hartmann and Mollwo, 2000; Hartmann *et al*., 2005). Also small amounts (0.1-10 nmol m^{-2}) of monochromatic red light may induce germination of wet seeds (VanDer Woude, 1985), as red light, acting through the photo-receptor phytochrome, promotes germination (Duke, 1978; Orozco-Segovia *et al*., 1993; Scheuerlein and Braslavsky, 1985; Shichijo *et al*., 2001; Shinomura *et al*., 1994; Shinomura, 1997).

A large number of experimental studies carried out over the last years suggest that even a exposure of dry, dormant seeds to He-Ne laser irradiation (λ= 632.8 nm), also termed as laser stimulation, may trigger several biological reactions. Laser-inducted changes in electrochemical, biochemical and optical properties of seeds are well documented, and a majority of the ascribed biological effects of laser stimulations have been attributed to germination process and growth analysis (Chen *et al*., 2005a; 2005b; Gładyszewska and Koper, 2000; Han *et al*., 2002; Podleśny *et al*., 2001; Rubinov, 2003; Salyaev *et al*., 2003; Samuilov and Garifullina, 2007; Wu, 2007). Dormancy breaking and germination stimulating laser-based pre-treatments have essentially been focused on the cereal grains and vegetable seeds and experimental evidence suggests that there exist significant positive effects of laser irradiation in improving the quality of sowing material, like mustard seeds (Anghel *et al.*, 2000) and maize (Herandez *et al.*, 2006). It should be emphasized, that the He-Ne laser irradiation not always has a positive, or

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none, effect on seeds, but also may inhibit the germination and the emergence of seedlings (Koper and Dziwulska, 2003). Also He-Ne laser is not the optimal source of light to induce the phytochrome-mediated germination as the action spectrum of the light needed for phytochrome responses shows a peak in the red at about 660 nm and the absorption of photons by phytochrome particles upon actinic illumination with monochromatic light of λ = 632.8 nm corresponds to the value ≈ 0.55 of the maximum absorption peak (Sineshchekov *et al.,* 2000).

Our interest in laser light-germination responses stemmed from developing role of the laser irradiation methods in agricultural sciences (Paleg and Aspinall, 1970). With the increasing availability of laser sources and their potential to focus high amounts of energy of monochromatic radiation of visible or infra-red spectra to small spot sizes, research concentrated on their possible applications in other areas of agricultural production are widely carried out. One of the original applications of lasers in agricultural technology is the usage of laser irradiation to protect plants against fungal diseases (Bel'skii and Mazulenko 1984; Mathiassen *et al.*, 2006). Ouf and Abdel-Hady (1999) performed a study on soybean seeds and reported a clear reduction of seed-born fungi after the pre-sowing irradiation. Similar experiments were also carried out on alfalfa seeds (Wilczek *et al.*, 2004), but without explicit results. Laser irradiation could also reduce the negative responses of plant to hostile environmental conditions, as a protective effect of laser pretreatment to UV-B radiation was reported (Qi *et al.*, 2000; 2002). In this respect, laser irradiation seems to be promising to many areas of agricultural production.

The aim of the present study was not to use a He-Ne laser irradiation as a practical method to promote seeds germination, but to examine the effect of such pretreatment on the several indices associated with the germination process and find out indices which would give extra information about changes in seed germination dynamics after laser irradiation.

MATERIALS AND METHODS

Seeds material

Radish (*Raphanus dativus* L. cv. Pola) seeds used in this work were supplied by the local agricultural agent. Three thousands seeds of uniform size were hand-selected. The seeds were kept in dark for three weeks before the experiments to protect them from accidental influence of the red light, including sunset. No extra chemical or physiological photosensitization was applied before irradiation. Extraneous light sources, especially sunshine and white fluorescent bulbs, were carefully avoided during counts, irradiation and further preparation of seeds.

Laser source and irradiation procedure

Continuous wave radiation at λ = 632.8 nm was obtained from the He-Ne laser (Model LG-79-1, Russian Federation) with a total maximum output power of 5 mW cm^{-2} and beam diameter of 1.5 mm. The laser light intensity was measured with a power meter (Model CTL-2001, LaserInstruments, Poland). Irradiation was performed in dim green light at room temperature without thermostatic control. The output power of laser beam was set up to 4 or 2 mW cm^{-2}, and the corresponding irradiated samples were labeled as D4 and D2, respectively. In order to enlarge the irradiation area a short-focus positive lens was installed. During the irradiation, seeds were placed on a special conveying belt, as presented on Fig. 1. The laser and lens were mounted into a mechanical support, and the distance between lens and the conveying belt (about 30 mm) was optimized to enlarge a beam spot size up to the circle with the diameter of 20 mm. Thus, the resulted maximal optical power densities were 1.27 and 0.64 mW cm^{-2} for D4 and D2, respectively. The arrangement of the seeds filled the following requirements: seeds were placed in single monolayer in a straight line with 3 mm interval to eliminate light scattering and partial screening of seeds. The passing velocity through irradiation area was set to 0.02 m s^{-1}, so that each seed was subjected to irradiation for 1 s. Each seed was irradiated only once. It should be emphasized, that the exposure times and laser powers used in our experiments are insufficient to cause any thermal effects in seeds (McKenzie, 1990). The control samples, labeled as D0, were treated in exactly the same way, except the irradiation procedure.

Germination tests

After the treatments, irradiated and control seeds were placed in 9 cm glass Petri dishes lined with three layers of filter paper. Before the experiments, the Petri dishes were sterilized for three hours at 150°C. The germination tests were performed with samples of 100 seeds. Each treatment was repeated five times to confirm the repetitiveness of the results. Constant temperatures of 15 and 20ºC were assured

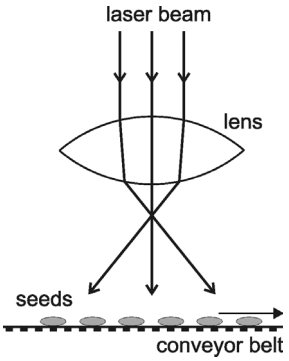

Fig. 1. The layout of laser irradiation system formed in laboratory.

by placing dishes inside ventilated thermal chambers. A soon as the temperature in the germination chamber had established, the dishes were moistened with 8 ml of distilled water and covered with the lid. All the seeds were moistened simultaneously. The relative humidity in the Petri dish was near 100% as evidenced by vapor concentration on the lid. During the experiment filter papers were kept at an optimal humidity level by adding the fresh distilled water to the Petri dish when needed. Petri dishes were set out in a completely randomized design and the positions of the dishes were randomly changed at every count to minimize positional effect in the chamber. Temperature readings were taken before each observation, and fluctuations of the mean temperature in each isothermal chamber ranged up to 1°C. The temperature measurements precision was 0.2°C. Seeds were germinated in dark, except when they were scored. Counts were made in 3 h intervals. Prior to the counting, the dishes were removed from the chamber. Seeds were examined under diffuse green light, provided by fluorescence lamp (Osram Luminux, 950 lm). Seeds were checked for presence of fungus and rotten seeds were systematically removed from Petri dishes. The germination criterion was the break of seed coat and emergence of the radicle, with the length equal to the length of the seed. The germinated seeds were withdrawn as soon as recorded. As soon as the counting procedure was finished (3-6 min), the dishes were returned to the chamber. Germination was judged to be complete when no further germination occurred for three successive counts.

Mathematical approach

Qualitative description of the results of laser irradiation was based on the analysis of several germination indices. The following indices were calculated for each treatment replication: final germination percentage (FGP); mean germination time (MGT); mean germination rate (MGR); relative germination rate (RGR); relative germination frequency (RF); the end of the lag phase *ie* time at which seeds began to germinate (T_1); time during the germination is finished for 50% of the all geminating seeds (T_{50}); numbers of hours during which cumulative germination changes from 25 to 75% of germinated seeds (T_{25-75}) and a spread of germination, defined as the time between 10 and 90% germination (T_{10-90}).

Using the counts, MGT was calculated in hours as follows:

$$MGT = \frac{\sum_{i=1}^{k} n_i t_i}{n}, \tag{1}$$

where: n_i – number of germinated seeds during a given time interval; t_i – number of hours counted from the beginning of the germination test; n – total number of seeds germinated at the end of the germination test. MGR index was computed as the reciprocal of MGT.

RGR expresses the increase of germination in terms of germination per unit of total germination in given time (Garcia-Huidobro *et al.*, 1982). RGR at the particular time t_i is defined as the number of seeds that germinated between two successive counts n_i, divided by the given time interval t and the number of seeds that germinated from the beginning of germination test to the time t_i:

$$RGB(t_i) = \frac{n_i}{\Delta t \sum_{i=1}^{i} n_l}. \tag{2}$$

The distributions of relative germination frequencies RF were calculated according to the formula (Labouriau and Pacheco, 1978):

$$RF(t_i) = \frac{n_i}{\sum_{i=1}^{k} n_l}. \tag{3}$$

The progress of germination was determined using the Gompertz curve from which the times to 1, 10, 25, 50, 75, and 90% of FGP for each experimental set were obtained. The Gompertz function is of the form (Windsor, 1932):

$$y(t) = y_{\max} \exp\left[-\exp\left(\frac{t - t_0}{b}\right)\right], \tag{4}$$

where: $y_{\max}$ is the proportion of seeds that finally germinated, t_0 is the curve inflection point, b is the slope parameter, and t is the number of hours since initial wetting. The Gompertz equation was fitted to the cumulative seeds germination data for each replication separately using SigmaPlot 9 (Systat Software Inc.). The final germination percentage (FGP) was taken as the value of calculated $y_{\max}$. The replicates were combined and analyzed using Excel software (Microsoft Inc.) to calculate the indices T_1, T_{50}, T_{25-75} and T_{10-90}.

Statistical analysis

To determine the effect of laser irradiation compared to the controls, statistical significance of all data was checked by the analysis of the standard deviations and the two sided Student's t-test. The significant difference at least at $P = 0.05$ was used to distinguish irradiation differences for the germination in the study. All the statistical analyses were performed with SAS 9.1 (SAS Institute) and SigmaStat 3.1 (Systat Software Inc.).

RESULTS

The overall stimulation effect on germination of laser irradiation is clearly seen by the plots of cumulative germination over time presented in Fig. 2. The cumulative germination curves represent the mean values of five replicated data. The fluctuation ranges (standard deviation) of each point on the plots were not indicated to maintain the

readability of the figures. The Gompertz's equation was fitted to the experimental data and a good correlation of the fitted curves with the recorded data at the temperature of 15ºC was found ($R^2 > 0.99$). At 20ºC however, the fit deteriorated. The poorest fit ($R^2 = 0.97$) was obtained for D0. This was caused by the increasing variation between repetition samples and poor convergence on the upper asymptote.

Table 1 shows the effect of laser irradiation on the germination of radish seeds, where mean values and their standard deviations for the studied indices are disclosed. At each temperature the sprouts began to emerge irrespective of the pretreatment (T_1 index), that is, no acceleration or delay of the germination process was observed. At both temperatures germination graphs in Fig. 2 shows little changes in germination rates for irradiated seeds, however, statistical analysis showed that differences between irradiated and control seeds during the whole germination process, expressed in the terms of T_{50}, $T_{25\text{-}75}$ and $T_{10\text{-}90}$, were not significant ($P<0.05$), as seen in Table 1. Positive and statistically significant effect was recorded only at the temperature of 20ºC, where irradiation improved the FGP index. Student's t-test applied to the data returned statistically significant differences when comparing control to irradiated seeds, with $P<0.05$ and $P<0.01$ for D2 and D4 irradiation doses, respectively.

Figure 3 shows the RGR and RF of control and irradiated seeds as kinetic functions. As seeds germination process is temperature dependant, the distributions of relative frequencies are different for different temperatures. No displacement of the relative position of mean germination time (t* symbol on graphs) in relation to the position on frequency plots was observed for irradiated seeds when comparing to the control at both germination temperatures. The data in Table 2 show that the increase of the temperature resulted in the increase of positive skewness for control

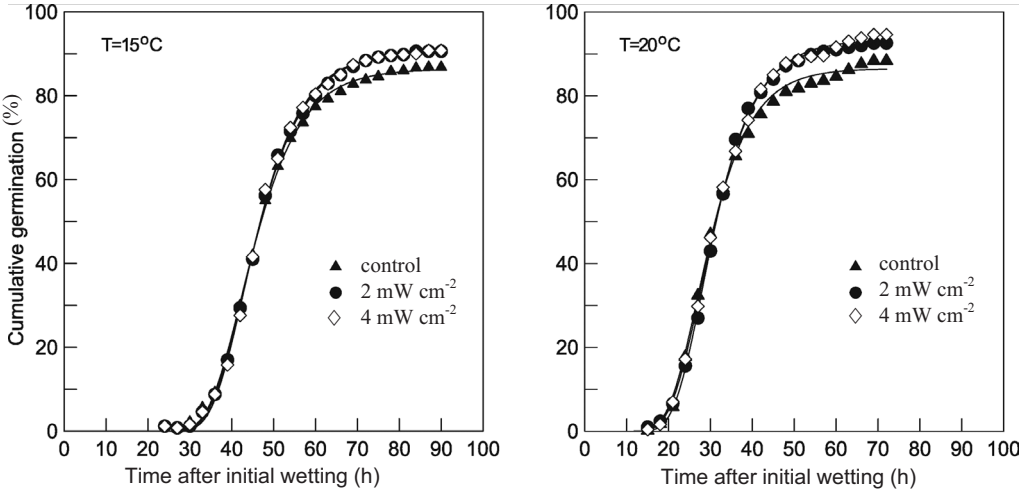

Fig. 2. The germination characteristics of radish seeds as a function of the germination time for various irradiations applied.

T a b l e 1. Overview of the most important data for the germination indices of radish seeds irradiated with laser and the corresponding control seeds germinating without irradiation with He-Ne laser

Temperature (°C)	Laser output power (mW cm^{-2})	FGP (%)	MGR (10^3h^{-1})	MGT (h)	T_1 (h)	T_{50} (h)	$T_{25\text{-}75}$ (h)	$T_{10\text{-}90}$ (h)
15	0	87.8 ± 1.6	20.4 ± 0.6	49.1 ± 1.9	29.1 ± 2.4	45.7 ± 2.4	13.8 ± 1.3	27.0 ± 2.6
	2	90.6 ± 2.5	20.5 ± 0.6	48.8 ± 1.5	31.0 ± 1.4	45.7 ± 1.7	12.2 ± 1.2	23.9 ± 2.6
	4	90.8 ± 3.5	20.5 ± 0.7	48.8 ± 1.6	31.2 ± 2.5	45.6 ± 1.1	11.9 ± 1.9	23.4 ± 3.8
20	0	86.8 ± 2.7	30.8 ± 1.1	32.5 ± 1.2	16.8 ± 1.4	29.7 ± 2.4	10.7 ± 1.8	21.0 ± 3.5
	2	92.6 ± 2.6*	29.8 ± 1.0	33.6 ± 1.1	17.7 ± 0.7	30.6 ± 0.9	10.7 ± 1.0	21.0 ± 2.1
	4	94.6 ± 2.7**	29.4 ± 1.1	34.1 ± 1.3	17.7 ± 0.7	30.6 ± 0.9	10.7 ± 1.0	21.0 ± 2.0

Data in table represent mean values of five replications and their standard deviations. Statistical significance level: * $P<0.05$, ** $P<0.01$, where P represents the probability level according to the Student's t-test relative to the comparison between each value and the corresponding control.

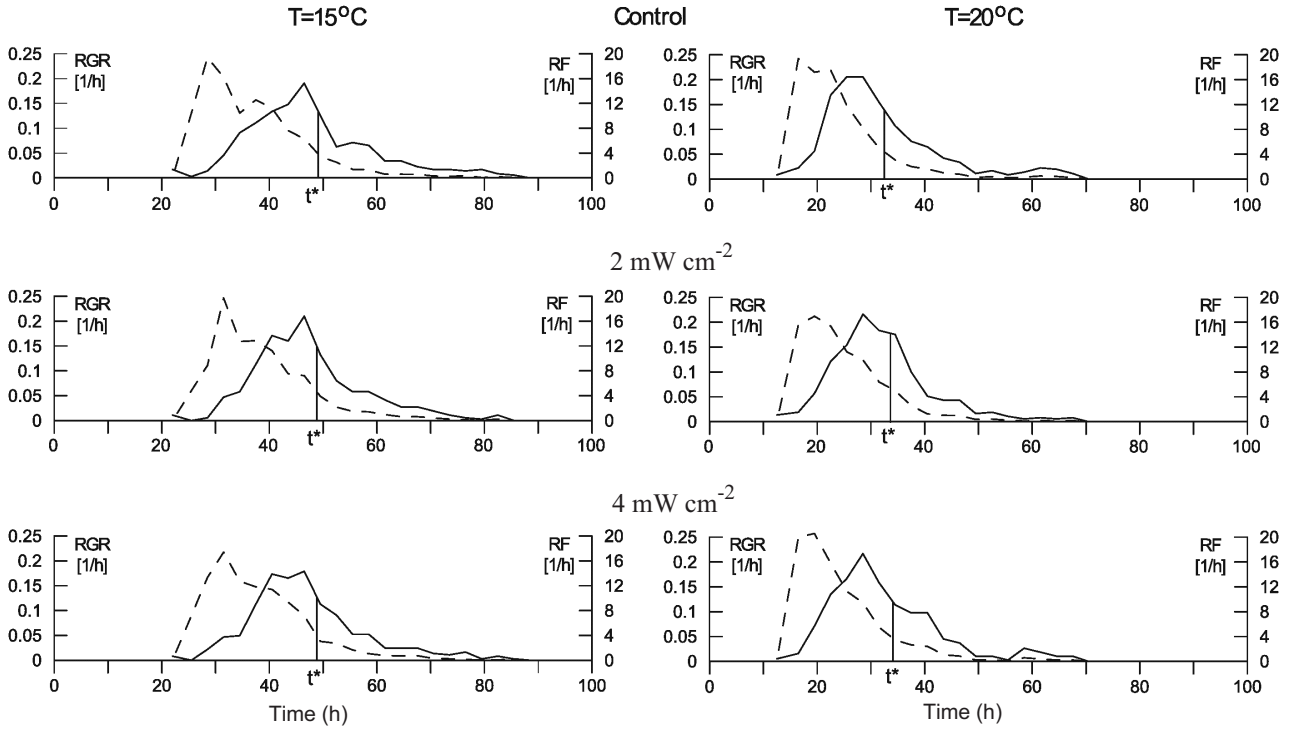

Fig. 3. Distribution of relative growth rates (dash) and relative frequencies (solid) of radish seeds along the germination time. The vertical lines denote the mean germination time (t*).

sample and decrease of the skewness for both irradiation doses. Moreover, the change of temperature caused the change of the sign of the kurtosis for irradiated samples. Thus, the observed differences in relative frequency distributions may be related to the laser irradiation. The non-zero values of skewness and kurtosis show also that none of the relative frequency distributions should not be approximated with a normal distribution, although the shape of RF functions are nearly symmetrical and the values of R^2 of corresponding Gauss distributions are all over 0.95, as shown in Table 2. In all cases the observed values of Kolmogorov-Smirnov test $D_{Observed}$ for the goodness of fit between the distribution of relative frequency and the adjusted Gaussian are much greater than expected values $D_{Expected}$ at significance level of $\alpha = 0.05$.

Concluding, laser irradiation increased FGP by 7 and 9% when comparing to the control seeds at the temperature 20°C for D2 and D4, respectively, which caused the change of the

T a b l e 2. The results of the fitting of Gauss distribution to the data of the relative frequency of germination for control and irradiated radish seeds

Temperature (°C)	Lasero utput power (mW cm^{-2})	R^2 of fitted Gaussian	$D_{Expected}$	$D_{Observed}$	Skewness	Kurtosis
	0	0.950	0.06448	0.2154	1.118	0.301
15	2	0.958	0.06381	0.1629	1.275	0.644
	4	0.968	0.06381	0.2322	1.235	0.293
	0	0.954	0.06519	0.2751	1.222	0.134
20	2	0.980	0.06305	0.3159	1.098	-0.198
	4	0.957	0.06244	0.2000	0.991	-0.126

sign of kurtosis of the germination frequency distribution. The other germination indices at both germination temperatures were not found to have been significantly changed.

DISSCUSION

It is clear, that data about behavior of seeds' population during germination process should not be combined into single value (Bewley and Black, 1985; Brown and Mayer, 1988). The purpose of this work was to compare several common germination indices in order to examine their abilities in description of the effect of laser irradiation on seeds germination process. It is important to use appropriate germination indices, because inappropriately selected indices might hinder interpretation of biological meaning of the obtained results. This paper described only a few indices concerning the germination, but it showed that analysis based on germination indices could be a useful tool for describing the effect of external stimulus to germination process.

Considering all calculated indices, it can be apprised that only FGP index, representing germination capacity, was found to indicate the influence of the laser irradiation. However, germination capacity is a quantitative index and is giving only global information about germination. Other indices, describing qualitatively the time, rate and homogeneity of germination process, did not reveal any influence of the applied pre-treatment. Unchanged values of those indices led to the conclusion that the laser irradiation had no effect on the course of germination, but only caused the increase in the number of germinated seeds. It confirms that He-Ne laser irradiation had it's the greatest effect on the slow-germinating viable seeds of the population by breaking their dormancy (Evenari, 1965). This effect can be illustrated in Fig. 1 by the increasing distance among plot curves of irradiated and control seeds. Moreover, analyzing obtained results, activation of viable seeds might be turned into increase of the number of germinated seeds only in specific temperature conditions. This effect is related to temperature dependence of phytochrome activation (Eisenstadt and Mancinelli, 1974; Łapko *et al.*, 1992; Mancinelli *et al.*, 1967; Pratt and Butler, 1970; Toole *et al.*, 1955).

CONCLUSIONS

1. The obtained data are still insufficient for the final conclusion about the effectiveness of He-Ne laser irradiation of dry seeds on germination kinetics. In our experiments, the irradiation by different fluence rates did not present obvious advantage or disadvantage of laser stimulation.

2. The temperature of 20°C provided the stimulatory effect of irradiation to germination, but temperatures close to 20°C may be unattainable for most producers to perform germination, especially in Central European countries. Although lower temperatures are more common, the temperature of 15°C used in our study indicated no statistically significant differences in germination of irradiated and control seeds.

3. This paper highlights the importance of succeeding studies in describing the influence of laser irradiation on seeds germination, as low germination activities of many seeds constitute significant problems in agriculture.

4. The germination capacities of irradiated seeds were improved by 7 and 9% when compared to the control, but the laboratory germination percentage cannot represent the percentage of seeds sprouting completely.

5. Further studies should be therefore carried out under field conditions where low emergence increases risk of fungal diseases, predation and low vigour.

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