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# Temperature dependent effect of difenoconazole on enzymatic activity from soil

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*Abstract*: The purpose of this study was to quantify the effect of difenoconazole (DFC) on the activity of a few enzymes commonly found in soil: dehydrogenase, urease, phosphatase and protease. Three experimental variants were established: under field conditions with variable temperature (10–21 °C, variants A1–A3), under laboratory conditions with constant temperature (30 °C, variants B1–B3) and untreated soil (C variant). The commercial product "Score 250EC" with 250 g DFC $L^{-1}$ was used at the following concentrations: 0.037 mg DFC $g^{-1}$ soil (variants A1 and B1), 0.075 mg DFC $g^{-1}$ soil (variants A2 and B2) and 0.150 mg DFC $g^{-1}$ soil (variants A3 and B3). The dehydrogenase, phosphatase and urease activities decreased significantly ($p < 0.05$) under both field (variants A1–A3) and laboratory (variants B1–B3) conditions compared to untreated soil (variant C). The protease activity was reduced in variants A1––A3 compared to variant C and increased at the dose of 0.150 mg DFC $g^{-1}$ soil in the variant B3.

*Keywords*: fungicide; soil; dehydrogenase; phosphatase; urease; protease.

## INTRODUCTION

Widespread and intense application of a large number of fungicides for controlling fungal pathogens of crops promotes high productivity in the modern agriculture. The fungicides used to inhibit the growth and developments of pathogenic fungi of crops have a negative effect on soil quality through quantitative and qualitative changes in the communities of microorganisms.[1–3]

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Soil microorganisms produce a variety of exo-enzymes: ureases, invertases, dehydrogenases, cellulases, amylases, phosphatases, proteases, *etc*. Enzyme activity can be used as a biomarker of soil fertility and an indicator of many biological processes manifested in the soil.[4] Fungicides applied directly to plants were also found in the soil.[5,6] Literature data concerning the effects of fungicides on soil reveal stimulation or inhibition of enzyme activities depending on the fungicide dose, incubation temperature and time of application,[7,8] and the inorganic and organic matter content of soil, the soil type, soil tillage, content of heavy metals and other environmental factors.[9–11]

The fungicides captan and trifloxystrobin applied for a short-term did not affect the phosphorous cycle in soil, but their application in large doses caused inhibition of enzymes involved in the nitrogen cycle.[12] Chen and Edwards[13] emphasized the toxic effect of benomyl, captan and chlorothalonil upon the microorganisms from soil and on the nitrogen cycle: reduction of fungi and nitrifying bacteria populations and inhibition of several enzyme found in soil (nitrogenase, dehydrogenase, cellulase, phosphatase urease and protease). Other fungicides, such as propiconazole and chlorothalonil, applied in the recommended doses to crops did not show inhibitory effects on urease and protease activities from soil.[14]

Published data concerning the effect of difenoconazole on communities of microorganisms in soil and on enzyme activities showed different aspects. Thus, depending on the soil type, short term application of difenoconazole had an inhibitory effect on microbial activity in unfertilized soils, but not in fertilized soils.[15] Another study concerning the effects of difenoconazole on the activity of microorganism community in soil based on enzymatic activities (urease, arylsulfatase, $\beta$-glucosidase, alkaline phosphatase and dehydrogenase) was conducted under laboratory conditions using different concentrations of fungicide. It reflected dose dependent effects of difenoconazole on the microbial population of the soil.[16]

The purpose of this study was to determine the effects of different doses of difenoconazole on soil quality during 21 days of contact with the fungicide, based on enzymatic activities (dehydrogenase, urease, phosphatase, protease), under variable (10–30 °C, in experimental fields) and constant (30 °C, in laboratory conditions) temperatures. To the best of our knowledge, this is the first study comparing the effects of difenoconazole upon some enzymes from soil at variable temperatures and a constant temperature, *i.e.*, under field and laboratory conditions.

## EXPERIMENTAL

### *Materials*

Difenoconazole (DFC, 1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-doxolan-2-yl]methyl]-1*H*-1,2,4-triazole) is a fungicide that inhibits sterol demethylation and is widely used against Ascomycetes, Basidiomycetes and Deuteromycetes. The experiments

were performed using a product sold on a local market under the trade name "Score 250 EC", that contains 250 g $L^{-1}$ DFC.

*Soil sampling*

The soil samples were collected from an experimental field located nearby Timisoara city, in an area where insecticides, fungicides, herbicides or chemical fertilizers were never used. Chernozem soil samples were collected from the top layer of soil (0–20 cm) from five different spots in quantities varying between 1 and 2 kg. The material was ground, sieved (2 mm) and spooned by random sampling, giving sub-samples of 20 g per polyethylene bag. The samples were preserved in a refrigerator and processed as soon as possible during the following 30 days.

*Treatment of soil samples with fungicide*

Three doses of DFC were prepared using distilled water: half dose HD – 0.037 mg DFC $g^{-1}$ soil, normal dose ND – 0.075 mg DFC $g^{-1}$ soil and double dose DD – 0.150 mg DFC $g^{-1}$ soil. The three DFC doses were prepared in distilled water and then applied to the soil samples to obtain 40 % humidity. The plastic bags containing the samples were homogenized on a rotary homogenizer for 2 h in order to achieve a uniform distribution in the sample.[17]

The following variants were obtained: Variant A with three sub-variants depending on the DFC concentration (A1 – HD, A2 – ND and A3 – DD), with soil pH 6.20, storage for 21 days under field conditions, at 10–21 °C (mean temperature 17.19 °C); Variant B (B1 – HD, B2 – ND and B3 – DD), with soil pH 6.44, incubated for 21 days at 30 °C (laboratory conditions); Variant C was untreated soil, with a soil pH of 6.14.

*Biochemical analyses*

The following enzymatic activities were assayed: dehydrogenase, urease, acid phosphatase and protease. The enzymatic activities were determined using a T90 UV/Vis spectrophotometer (PG Instruments, UK).

The dehydrogenase activity (*DA*) was measured using 2,3,5-triphenyltetrazolium chloride (TTC) as substrate, monitoring the reaction product (triphenylformazane, TPF) at 485 nm. The reaction mixture containing 3 g soil sample, 0.5 mL of 3% solution of TTC, 1.2 mL Tris buffer (0.1 M, pH 7.6) was kept at 37 °C for 48 h. TPF was extracted with 20 mL acetone and the absorbance of the supernatant was measured at 485 nm. The *DA* is expressed as mg TPF $g^{-1}$ soil during 48 h.[18]

The urease activity (*UA*) was determined in accordance with the method described by Alef and Nannipieri.[19] The reaction mixture consisted of 3 g soil, 5 mL phosphate buffer (0.6 M, pH 6.8) and 2 mL toluene. After homogenization (2 min on vortex), 5 mL 3 % urea was added and the mixture was vortexed for a further 2 min. Finally, the reaction mixture was incubated at 37 °C for 24 h. In the collected supernatant, the quantity of produced $NH_4^+$ was determined using Nessler's reagent. The absorbance was measured at 445 nm and the *UA* is expressed as mg $NH_4^+$ $g^{-1}$ soil during 24 h.

The phosphatase activity (*PhA*) was estimated measuring the phenol resulting from the hydrolytic separation of phenyl phosphate into disodium phosphate and phenol catalyzed by phosphomonoesterases. For each sample, about 3 g of soil were mixed into a test tube with 10 mL of 0.5 % phenyl phosphate and incubated for 48 h at 37 °C. Next, 50 mL of 0.3 % ammonium aluminum sulfate were added to each test tube and the mixture was then filtered through ash-free filter paper. From each test tube, 1 mL filtrate was transferred to an empty test tube together with 5 mL borax solution (0.1 M, pH 9.4). The mixture was brought to a

volume of 25 mL with distilled water and the absorbance was measured at 597 nm. *PhA* was defined[20] as mg phenol $g^{-1}$ soil during 48 h.

The protease activity (*PA*) was estimated by reaction of ninhydrin with the amino acids resulting from the hydrolysis of gelatin used as substrate. For each sample, about 3 g soil was mixed with 7 mL of 2 % gelatin and 0.5 mL toluene. The mixture was homogenized (2 min on vortex) and incubated at 37 °C for 24 h. Next, 25 mL of distilled water was added and the mixture was filtered through ash-free filter paper. From each test tube, 2 mL of filtrate was transferred to an empty test tube together with 5 mL of 0.2 % ninhydrin solution and the absorbance was measured at 578 nm. The *PA* was defined[20] as mg amino-N $g^{-1}$ soil during 24 h.

*Statistical data interpretation*

Statistical analysis of the recorded data was performed using variance analysis and the software MINITAB 17.[21] All data are presented as average values with standard deviation (*X*±*SD*). In order to establish the correlation coefficient, the Spearman test was used. Significant differences in variables were tested using Mann–Whitney at the 0.05 level of probability.

## RESULTS AND DISCUSSIONS

The enzymatic activities *DA*, *UA*, *PhA* and *PA* were assayed in 6 experimental variants during 21 days. The results revealed increases of enzymatic activities for some enzymes and decreases for other during the monitoring period, in relation with the incubation temperature and DFC concentration.

The average values and standard deviations for *DA* during the 21 days of experiment were determined (Fig. 1). The recorded values ranged between 0.462±0.375 mg TPF $g^{-1}$ soil in 48 h (variant B3) and 1.734±0.601 mg TPF $g^{-1}$ soil in48 h (variant A1). As the values of *DA* obtained in the soil samples containing DFC were lower than that registered for the control soil sample (5.847±0.501 mg TPF $g^{-1}$ soil in 48 h), it could be concluded that DFC had a toxic effect on the respiration process of microorganisms from soil. The higher was the DFC concentration, the higher was the percent of reduction of *DA* in the soil samples. For example, in variant B3, the *DA* activity was decreased with 90.16 % in comparison with the control sample (variant C).

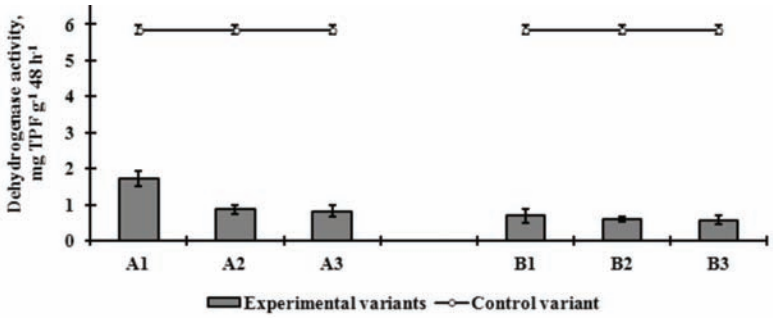


Fig. 1. Average values of the dehydrogenase activity in the soil samples.

The decrease of *DA* for the experimental variants A and B during the 21 days of the monitoring was roughly linear with the increase of the concentration of DFC. There are significant differences between variant A and B, during the 21 days of experiment ($p < 0.05$).

Among all the enzymatic activities assayed, *DA* was the most sensitive to the variation of the DFC concentration in the soil samples. *DA* is considered as an ecotoxicological test for an estimation of the toxicant effects on soil microorganisms as *DA* reflects the intensity of the respiration processes of these germs.[22]

The results of the present study were in good agreement with other published data. The studies conducted by Muñoz-Leoz *et al.*[23] revealed that small concentrations of DFC cannot show a clear effect on *DA*, but high concentrations of DFC applied on soil lead to significant decreases in the *DA*. Srinivasulu and Rangaswamy[24] reported inhibition of *DA* of soil microorganisms due to the treatment of soil with high doses of metalaxyl and mancozeb during a period of 35 days. There are reports mentioning that at low doses some fungicides increase *DA* when applied to soil,[7,25] but when large doses were applied, the *DA* of the soil microorganisms was reduced.[26,27]

Beside the concentration of DFC, other factors may also affect the enzymatic activities of soil microorganisms. There are reports mentioning that the water content from soil and the temperature influence the *DA* indirectly by interfering with the redox status of the soil.[28] In the present study under field conditions (variant A), *DA* presented a negative correlation with temperature ($r = –0.243$).

The values of urease activity (*UA*) registered during the 21 days of the monitoring ranged between 318.127±16.124 mg $NH_4^+$ $g^{-1}$ soil in 24 h (variant A2)[1] and 169.502±27.980 mg $NH_4^+$ $g^{-1}$ soil in 24 $h^{-1}$ (variant A3). The average values of *UA* are presented in Fig. 2.

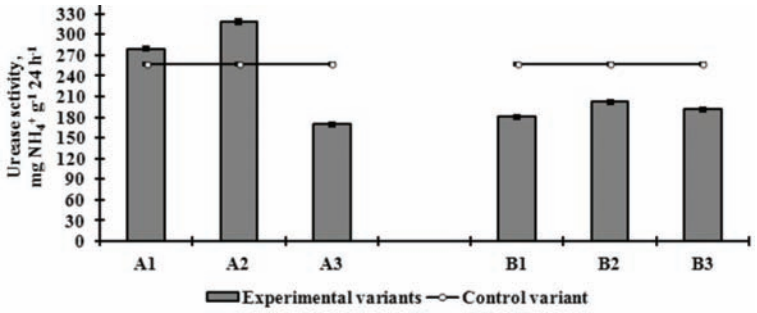


Fig. 2 Average values of the urease activity in the soil samples.

The highest values of *UA* were obtained in variants A1 and A2, an indication of the possible utilization of DFC as a carbon and nitrogen source by some microorganisms from the soil, at temperatures varying between 10 and 21 °C. Using

DFC as a substrate determines urease activity growth. Thus, the values of UA obtained in variants A1 and A2 were higher with 23.23 and 61.92 mg $NH_4^+$ $g^{-1}$ soil than the value of UA in the control soil (256.204±11.971 mg $NH_4^+$ $g^{-1}$ soil in 24 h).

Nevertheless, at high concentrations of DFC in the soil, the *UA* decreased significantly ($p < 0.05$): by 33.84 % in variant A3 and by 29.63 % in variant B3, in comparison to the value of *UA* in the untreated soil. Based on the differences of the values of *UA* in variant A and variant B during the 21-day period of monitoring, it could be concluded that temperature has a significant impact on the influence of DFC on the *UA* of microorganisms from the treated soil.

Other chemicals, such as profenofos, deltamethrin and thiram, seemed to increase *UA* in soil at low concentrations and to reduce it when applied at high doses.[29] At high concentrations, pyrimorph reduces significantly the *UA*.[7] Qian *et al*.[30] hypothesized that validamycin may be toxic for some species as several enzymatic activities were reduced, but the obtained higher values of *UA* and *PhA* may indicate the possible use of validamycin as a carbon source by some species of microorganisms. The biomass of the microbes that can use validamycin as a carbon source increased until this source was exhausted, subsequently, the number of microorganisms from the soil would return to the normal level. As the reduction of the *UA* by captan and trifloxystrobin can be as high as 70 % of that of control untreated soil, it was assumed that these chemicals could modify the nitrogen cycle in the soil. This kind of modification has to be considered as repeated applications of fungicides could lead to their accumulation in the treated soil.

As the negative effects on the populations of microorganism are stronger at high concentrations of DFC in soil, the importance of the optimal dose of fungicide that should be applied on soil becomes more obvious. The potential non-target side effects of pesticides against microbial communities from soil and the reduced rates of degradation of these chemicals should be considered principally when repeated treatment of soil is performed.[23]

As for the *DA*, the *UA* of organisms from soil treated with DFC was influenced by factors other than the DFC concentration. The correlation between *UA* and temperature (range 10–21 °C, variant A) was positive, although with a moderately low value for the correlation coefficient ($r = +0.439$). Similar studies confirmed a small increase in *UA* at moderate temperatures.[25] The time a pesticide acted on the microorganisms also affected the *UA* of germs from treated soils.[29]

The values of phosphatase activity (*PhA*) registered during the 21 days of the experiments ranged between 2.427±0.753 (variant A3) and 4.004±1.516 mg phenol $g^{-1}$ soil in 48 h (variant B3). DFC applied on soil caused a reduction of the *PhA* of microorganisms, as all the values of the *PhA* from the experimental variants were lower than the *PhA* found in the control untreated soil (4.828±

±0.751 mg phenol $g^{-1}$ soil in 48 h). For variant A, the decrease in *PhA* correlated almost linearly with the increase in the DFC concentration ($p < 0.05$). The average values of *PhA* are presented in Fig. 3.

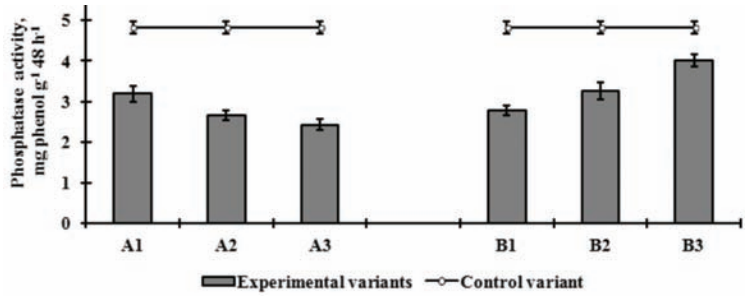


Fig. 3. Average values of the phosphatase activity in the soil samples.

In comparison to the control sample, the lowest value of *PhA* (49.73 %) was obtained for variant A3, when the highest DFC dose was used and the temperature ranged between 10–21 °C. The lower temperature (average = 17.19 °C) and higher dose of DFC provided the conditions for a significant decrease in the *PhA* of the microorganisms from the treated soil.

Under laboratory conditions at high and constant incubation temperature (30 °C), the *PhA* from variant B1 (the lowest applied concentration of DFC) decreased by 42.56 % compared with the control sample. In variant B3 (the highest concentration of DFC), although the *PhA* decreased, the extent of diminution was smaller.

Phosphatases are the enzymes responsible for releasing of orthophosphoric acid from organic combinations with metaphosphates and pyrophosphate. In soil ecosystems, phosphatases play a critical role in the phosphate cycle, being good indicators of soil fertility.[31] When phosphate is deficient in the soil, the amount of acidic phosphatase released by plant roots is increased to augment the solubilization and remobilization of phosphate, influencing the resistance of plants to stress conditions.[32,33]

In the short term, the phosphate cycle was not influenced by moderate doses of captan and trifloxystrobin applied on the soil.[12] In small concentrations, validamycin did not influence significantly *PhA* during the incubation period, but at high doses, an increase (29.8 %) of acidic *PhA* was observed.[30] For variant A (field conditions), a weak positive correlation ($r = +0.147$) between the variation of temperature and *PhA* was detected.

The values for the protease activity (*PA*) recorded during the 21 days of the experiments ranged between 5.948±3.843 (variant A3) and 19.824±7.354 mg amino-N $g^{-1}$ soil in 24 h (variant B3). In comparison with the control, untreated soil (13.289±1.751 mg amino-N $g^{-1}$ soil in 24 h), almost all other samples pre-

sented lower values for *PA*. The only exception was observed in variant B3, when the *PA* increased by 40.24 % comparing with the control sample. The most important decrease was observed in variant A3, when the *PA* was reduced by 56.98 %. The average values of *PA* are shown in Fig. 4.

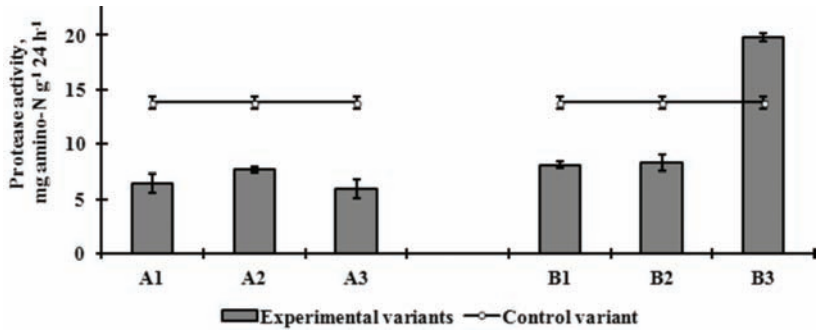


Fig. 4. Average values of the protease activity in the soil samples.

The values of *PA* obtained for variants A have similar values for all concentrations of DFC applied to the soil, but when the soil was incubated at a high temperature (variant B), there was a significant increase of *PA* at the highest dose of DFC. The fact that between variant A and B there was a difference of 5.38 mg amino-N $g^{-1}$ soil during the 21 days of the experiments, this could be considered as evidence for the influence of temperature on the *PA*. A constant incubation temperature of 30 °C seems to stimulate an increase of *PA*, perhaps by stimulating the overall metabolism. Under field conditions, the variation of temperature had a negative influence on *PA* ($r = –0.170$).

Proteases play an important role in the nitrogen cycle in soil, performing the hydrolysis of large peptides with production of amino acids and small peptides.[34] Fungicides, such as chlorothalonil and propiconazol, have a positive influence on urease and protease activities of soil microorganisms at low and moderate doses. Application of these chemicals in the recommended doses does not influence the metabolism of soil germs.[14] At high doses, chlorothalonil produces a reduction of *PA*, in comparison with untreated soil.[35] Similar results were obtained in case of mancozeb[10] and carbendazim.[36]

The increase in temperature has had a significant influence on the metabolic reactions of organisms from soil by promoting the development of communities of microorganisms resistant to DFC and supporting the use of the fungicide as a source of carbon and nitrogen. At high doses of DFC, these positive effects are overcome by the inhibition of the metabolism of organisms present in soil. As similar results were obtained in the case of other fungicides, it may be concluded that most of these types of chemicals at high and repeated doses may produce an inhibition of the metabolism of communities of microorganisms present in soil.[7]

The present study confirms that soil enzymes behave differently when exposed to DFC that can stimulate or inhibit some enzymatic activities depending on the dose and temperature. Several factors may influence the enzyme activity besides the concentration of DFC, such as temperature and time of exposure to the toxicant. The degree of absorbency of fungicide in soil could reduce the contact time between some pesticides and microorganisms. Biodegradation seems to be the most important mechanism for reduction the concentration of DFC in soil. At least at low doses, the microorganisms from soil can degrade chemicals from soil, including pesticides, progressively reducing their toxicity. Some degradation products may act as growth factors for certain microorganisms in the soil. Although the assay of the activities of enzymes from soil could be considered a good indication of soil quality and health, the levels of enzymes activities cannot be correlated with the quantities of pesticides in soil.

## CONCLUSIONS

The results indicated that DFC inhibited the enzymatic activities of dehydrogenase, urease, phosphatase and protease in the treated soil samples. Depending on the dose of DFC and incubation temperature, with rare exceptions, the recorded values of enzymatic activities were significantly lower ($p < 0.05$) than those in untreated soil. Above average increases were recorded for soil with urease at HD and ND of DFC applied to the soil sample and variable temperature (variants A1 and A2) and for protease at DD of DFC at constant temperature (variant B3). It could be concluded that the metabolism of the communities of microorganisms from DFC treated soil was affected by the DFC dose and incubation temperature.

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## ИЗВОД

## УТИЦАЈ ДИФЕНОКОНАЗОЛА НА АКТИВНОСТ ЕНЗИМА ИЗ ЗЕМЉИШТА

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Циљ овог рада је био да се квантификује ефекат фунгицида дифеноконазола (DFC) на активности ензима (дехидрогеназе, уреазе, фосфатазе и протеазе), који се налазе у земљишту. Успостављене су три експерименталне варијанте: у теренским условима са променљивом температуром (10–21 °C, варијанте А1–А3), у лабораторијским условима са константном температуром (30 °C, варијанте Б1–Б3) и контролна варијанта (нетретирано земљиште, Ц). Комерцијални производ „Score 250 EC" *са*. 250 g $L^{-1}$ DFC је коришћен у следећим концентрацијама: 0,037 mg $g^{-1}$ DFC/маса земљишта (променљиве

А1 и Б1), 0,075 mg $g^{-1}$ (променљиве А2 и Б2) и 0,150 mg $g^{-1}$ (променљиве А3 и Б3). Активности дехидрогеназе, фосфатазе и уреазе су биле значајно смањене у теренским и лабораторијским условима применом DFC ($p < 0,05$) у односу на нетретиране узорке. Протеазна активност је била смањена у варијантама А1–А3 у односу на варијанту Ц, а повећала се у варијанти Б3, када је примењена доза DFC 0,150 mg $g^{-1}$ земљишта.

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