Biocontrol of grapevine diseases

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Abstract. In 2018, work began on the project: Alternative protection of interregional plant production in the Pyrenees (PALVIP). A project whose main objective was the study of compounds of biological origin for the control of pests and diseases in vineyards and fruit trees. Participants in the project: Chambre d'agriculture Pyrénées orientales, INCAVI, University of Girona, Autonomous University of Barcelona, University of Perpinyà, and the companies Akinao and Futureco Bioscience. INCAVI focused on efficacy studies in the control of powdery mildew and downy mildew in field applications. Work has been done with microorganisms (bacteria) and plant extracts that have a biocidal or competitive action on the parasitic fungus and/or the ability to stimulate natural defenses. Interesting results have been obtained in the complementary use of the materials studied with those of reference: they allow to reduce the copper doses in the control of downy mildew or obtain good efficacy in the control of powdery mildew. PALVIP represents a further step in the progress towards organic growing vines with respect for the environment, improvement of working conditions and improvement of wines, basic objectives of current viticulture.

1 Introduction

The improvement of the quality and health of grape and wine production is one of the main challenges of current viticulture. The control of fungi, especially downy mildew and powdery mildew, is one of the main inputs in the cultivation of vines. In Catalonia, located in the north-east of the Iberian Peninsula, 40% of the wine production is organic following European rules, which limit the allowed substances for the control of pests and diseases. The problem is intensified by the widespread criterion of the necessary reduction of the application of copper (Cu) salts as an anti-mildew fungicide. INCAVI as an institution that works to improve viticulture in Catalonia, maintains different lines of work that try to provide solutions to the viticultural sector, the study of alternatives in the control of diseases and parasites is one of them. It works on strategies to reduce the use of Cu salts, both in the study of alternative products and in the reduction/optimization of treatments [1] and in the modeling of the development of fungi, the contaminating effect of Cu in the soils due to continuous treatments [2], work is being done on the study of resistant or less sensitive varieties and on the improvement of cultivation conditions to reduce risk conditions (vegetation management, soil management, etc.). The opportunity to carry out an Interreg collaborative project has meant an improvement in the knowledge of the different biocidal substances of natural origin and, especially, an improvement in the global approach to the investigation and technical assessment of these substances.

In 2018, the project: Alternative protection of the interregional vegetable productions of the Pyrenees (PALVIP) began. The project has been co-financed at 65% by the European Regional Development Fund (FEDER) within the framework of the Interreg V-A Spain-France-Andorra Program (POCTEFA 2014-2020).

The global objectives of PALVIP were three:

- Create an innovative cross-border research and experimentation network capable of tackling new problems.
- Allow the release of biocontrol products adapted to Mediterranean crops, effective and economically acceptable to users, in order to promote the development of sustainable agriculture.
- To favor, in Catalonia and Roussillon, the economic development of biological control companies and the development of local industries around the production of these alternative products, some of which are of local origin.

In order to meet the objectives, an ambitious project was proposed with an innovative approach in the study of biocontrol solutions for the management of vegetable crops in the Mediterranean environment. Thanks to a complementarity of skills between the partners and cross-border networking, a global approach was developed that went beyond the classic evaluation of effectiveness in the field, and that included:

- The formulation of new products
- The analysis of its mechanisms of action and the response induced in the plant.
- The study of its sensory, health, and environmental impact.

The project partners were:

- Chambre d'agriculture des Pyrénées-orientales
- Institut Català de la Vinya i el vi (INCAVI)
- Universitat autónoma de Barcelona (UAB)
- Universitat de Girona (UDG)
- Université de Perpignan Via Domitia (UPVD)
- Futureco Bioscience
- Akinao

The technical actions of the project were divided into three main blocks in order to achieve a global assessment of the subjects studied:

- Experimentation of alternative solutions for the protection of vegetable crops: included the

production of biocidal substances and field experimentation to assess their effectiveness in controlling parasitic fungi.

The assessment of fungicidal efficacy, in the case of the vine, was coordinated by INCAVI

- Work focused on fine-tuning methods and performing analyzes on the defense expression mechanism of the plant response was coordinated by the UDG and UAB with results such as those obtained by M. Ramos in the follow-up of the prunus persica trials [3].

Evaluation included the environmental and health impact of phytosanitary applications was coordinated by UPVD. The project included the study of the incidence of the treatments on the quality of the wines obtained from the vineyard trials.

2 Material and methods

2.1 Biocidal substances Products

The universities and companies that participated in the project provided the substances with biocidal capacity of natural origin.

2.2.1 Bacillus velezensis

Developed by the University of Girona. Microbial biopesticide under development. With antifungal and antibacterial activity based on the production of cyclolipopeptides and other secondary metabolites including surfactin, iturin, bacillomycin, fengycin, and antibiotic-type bacteriocins. It also acts by competition for space and nutrients with other microorganisms. It induces a defense response in the plant, activating genes of the jasmonic, salicylic, and ethylene pathways. There is no evidence of adverse effects of the strain in toxicity tests, nor of genotoxicity or pathogenicity. Therefore, the risk due to release of the product into the environment is considered low [4, 5].

2.2.2 Lactobacillus plantarum

Developed by the University of Girona. Microbial biopesticide under development. Antimicrobial activity (especially against bacteria) based on the production of the bacteriocin plantaricin and lactic acid. It also acts by competition for space and nutrients with other microorganisms. There is no evidence of adverse effects of the strain in toxicity tests, nor of genotoxicity or pathogenicity. Therefore, the risk due to release of the product into the environment is considered low. [6, 7]

2.2.3 EqUr

Developed by the Autonomous University of Barcelona. Its components are: Aqueous extract of Horsetail (Equisetum arvense L.) and Nettle (Urtica sp.). Active ingredients: silicon in the form of silicic acid and unknown secondary metabolites. It acts as a stimulator of natural defenses. Under development [8].

2.1.4 Akivi

Manufactured by AkiNaO/Greentech. Biocontrol product of plant origin with antifungal activity. Composed of *Inula viscosa* extract. AkiNaO and Greentech are working together for the market introduction of this product, in the 2025 horizon.

2.1.5 Bestcure

Product manufactured by Futureco Bioscience S.A. Composed of stabilized extract of Citrus × aurantium in cold press. Fungicidal action by contact and by induction of the immune response of plants [9]. Registered as a phytosanitary in South Africa, Colombia, Honduras, Peru, Guatemala, Mexico, Chile, Paraguay, Dominican Republic, Ecuador, and Ukraine. In the registration phase in the Philippines, Costa Rica, Morocco, USA, and Canada. BCS ÖKO Garantie organic certificate since 2014.

2.1.6 Pseudomonas putida B2017

Developed by Futureco Bioscience S.A. Fungicide and bactericide that acts by competition with the pathogen for nutrients and space [10, 11]. Active ingredient patented in Europe (EP3607048) and in patent phase in the USA. European phytosanitary registration dossier presented to the rapporteur country in March 2021, according to regulation (EC) 1107/2009, and completeness check passed.

2.1.7 Pseudomonas sp. B2021

Developed by Futureco Bioscience S.A. Biocontrol agent of microbial origin, acts by competition with the pathogen for resources and space and promotes plant growth. Registration dossier in the initial phases of construction.

2.1.8 Reference products

Reference products were used in each trial as a basis for comparison of the efficacy of the tested products and the possibility of alternative use.

The products used in the three trials are:

- Control of downy mildew: Copper oxychloride. It is applied at a full commercial dose or at a half dose mixed with other test products
- Powdery mildew control: wettable sulphur
- Botrytis Control: Serenade DOG. Bacillus subtilis strain QST 713.

2.2 Trial plots

- Efficacy trials were carried out in commercial vineyards and in full production, thanks to the temporary agreement with different winegrowers. Specifically, work was carried out on four plots of the Macabeu variety located in the DO Penedès, in the municipalities of:

- Gelida. Vineyard in which downy mildew control tests were carried out.
- La Granada. Vineyard where powdery mildew tests were carried out
- Vilafranca del Penedès y Sant Martí Sarroca in which the Botrytis cinerea control tests were carried out.

Cultivation conditions of these vineyards. Variety: Macabeu, cordon Royat formation, trellis in 1+2 wires, planting frame: 2.8 x 1.2 m. (approximately 3000 plants/ha.). The production is mainly intended for the production of base wine for cava. Organic cultivation.

2.3 Trial design

A random distribution was carried out according to a design of 4 repetitions and elementary plots of 10 vines (40 plants per treatment, surface per treatment: 130 m2). The data obtained in the different controls are analyzed by analysis of variance (ANOVA) of the data obtained and the separation of means by Tukey's method for multiple comparisons.

2.4 Carrying out treatments

The treatments were carried out with a backpack atomizer, which allows a good distribution of the product in the vegetation. The average volume of application in full vegetation of the vine was approximately 300 l/ha.

In the trials performed by INCAVI, fungus control was carried out in each plot on which the effectiveness of the studied products was studied. Treatments aimed at controlling other diseases were carried out by the vineyard manager. The control of the grape moth *Lobesia botrana* was carried out in all the plots with pheromones.

The treatment strategy was determined differently accordingly to the biology and infective capacity of the fungus to be controlled.

2.4.1 Downy mildew

To control downy mildew, a preventive strategy was carried out in order to avoid the germination of spores and the infection of plant organs. The necessary conditions for infection to occur are: presence of spores with infective capacity, sufficient vegetative development, temperature above 10ºC and the presence of liquid water (rain or dew) [12]. The application of phytosanitary products must be carried out before these conditions are met. The endoparasitic behavior of downy mildew makes it impossible to control it after infection if the applied substance does not have systemic capacity.

The study initially considered the treatment of each product individually to assess its control capacity. From the second year on, the combined use of some products with the reference product at half dose was proposed. The objective was to assess the complementary capacity of the products in the control of the disease and the compatibility of their mixed application. With these modifications of the initial approach, the treatments carried out are those listed in Table 1.

Table 1. Years and number of applications of the different treatments. Empty boxes mean that in the indicated year the treatment was not applied by agreement of the project technicians and according to the results obtained. Downy mildew control test:

	Years of application/applications made			
Treatments	**2018**	**2019**	**2020**	**2021**
L. plantarum	**4**			
B. velezensis	**4**	**3**	**5**	**1**
B. velezensis + Copper oxychloride (1/2 dose)		**3**	**5**	**1**
Bestcure	**4**	**3**	**5**	
P. putida B2017	**4**	**3**	**5**	**1**
Akivi	**4**	**3**	**5**	
EqUr	**4**			
EqUr + Copper oxychloride (1/2 dose)		**3**	**5**	**1**
Copper oxychloride ½ dose				**1**
Copper oxychloride	**4**	**3**	**5**	**1**
Control without treatment	**x**	**x**	**x**	**x**

2.4.2 Powdery mildew

A preventive strategy was carried out. The usual times in the Penedès area to carry out anti-powdery treatments are:

- 10-15 cm shoot growth of the vine
- Depending on the variety and/or the history of the vineyard to be treated, a treatment between sprouting and flowering of the vineyard is recommended.
- Start of flowering.
- Pea-sized berry.
- Treatment at bunch closure, to be carried out depending on the presence of the disease.
- Beginning of veraison.

As of the year 2019, it was determined to carry out the first treatment in general with the reference product and not to separate the anti-powdery mildew treatments by more than 15 days.

As in the case of mildew, the results obtained modify the work protocols. In the case of powdery mildew, Bestcure is replaced by Pseudomonas sp B2021. The treatments finally carried out are listed in Table 2.

Tabla 2. Years of application and number of applications. Empty boxes mean that in the indicated year the treatment was not applied by agreement of the project technicians and according to the results obtained.
Powdery mildew control trial.

	Years of application			
Treatments	**2018**	**2019**	**2020**	**2021**
L. Plantarum	3	4	4	4
B. velezensis	3	4	4	4
Bestcure	3	4		
P. putida B2017	3	4	4	4
Pseudomonas sp. B2021			4	4
Akivi	3	4	4	4
EqUr	3	4	4	4
Wettable sulfur	3	4	4	4
Witness	x	x	x	x

2.4.3 Bunch rot, Botrytis cinerea

The control of Botrytis in the vine is based on avoiding conditions of high sensitivity: poorly ventilated clusters, poorly lit, close to the ground, excessively compact, etc. The management of the vegetation as a whole has a decisive role in preventing the attacks of rotting fungi such as Botrytis. Phytosanitary treatments are complementary to this joint strategy and are based on preventively avoiding the development of infective forms and/or populations that can grow exponentially during grape ripening, a period of maximum sensitivity.

The times of treatment usually recommended are:

- Fall of the floral caps, at the end of fruit set, in order to prevent this organic matter present in the bunch from being the basis for infection and development of the fungus
- Pea-sized berry / cluster enclosure: with the aim of covering cluster surfaces that are difficult to access at a later time.
- Beginning of veraison.
- 21 days before the harvest.

As of 2019, it was agreed to skip the first treatment and not to separate the following treatments more than 15 days. The Botrytis trial was carried out in 2018, 2019, and 2020. The same treatments were maintained during the three years, incorporating Serenade ASO as the reference product for the last two. Four applications were made each year.

2.5 Controls

Periodic monitoring of the general condition of the vineyard was carried out throughout the vegetative period.

At the moment that symptoms of the targeted fungus were detected, a visual count was made, counting the following parameters based on the level of attack and the organs attacked:

- Leaves:
  - percentage of affected leaves (to determine disease incidence)
  - percentage of leaf surface affected (to determine severity)
- Bunches.
  - percentage of infected clusters.
  - percentage of cluster surface affected

If it was considered necessary, more than one count was performed to observe the evolution of the disease and the performance of the substances studied.

3 Environmental conditions

The weather conditions of the 4 years of work have been very variable, being a decisive factor in the development of the tests and in obtaining results.

Year 2018: very favorable conditions for the development of downy mildew, with the first symptoms being detected at the end of May. The mildew attack caused a very high impact: in the control plots without treatment on June 7. Downy mildew symptoms were observed in 78% of the leaves and 90% of the clusters. From this date, and having assessed the efficacy of all the products applied, it was decided to carry out a global treatment throughout the trial and end the efficacy assessment. These conditions so favorable to the development of downy mildew also favored its presence in the vineyard where the powdery mildew test was carried out, affecting many bunches.

Despite this, the effectiveness of anti-powdery treatments was correctly assessed. Summer weather conditions favored a slight attack of Botrytis. On the control date (with overripe grapes), symptoms were observed in 50% of the clusters, but with a degree of severity below 9%.

Year 2019: the conditions of little spring rain did not favor the development of downy mildew. In all the controls carried out in the mildew test, only one spot of mildew was detected on the leaf, without obtaining efficacy results. In the case of powdery mildew, the conditions of 2019 favored the presence of the fungus. At the beginning of August in the control plots, 75% of bunches were affected by powdery mildew, with an average severity of 26%. The presence of Botrytis was very low in 2019.

Year 2020: the affectation of mildew was very important in the entire Penedès area, affecting practically 30% of the global production of the area. Under these frequent rain conditions, the downy mildew test was carried out. It was only possible to assess the efficacy of the first treatments carried out, since the development of the fungus exceeded the control capacity even of the reference products used. Specifically, on June 4, the last control was carried out, in which 100% of the clusters and 72% of the untreated leaves were affected by mildew. The powdery mildew test was highly conditioned by the strong attack of downy mildew in the vineyard. The presence of Botrytis in the trial vineyard and in the region as a whole was very low.

Year 2021: only one application was made to control downy mildew and the presence of the fungus was not detected in the entire trial vineyard. The powdery mildew test was carried out under optimal conditions and with an average presence of the fungus that allowed to asses its effectiveness until the veraison of the grape. In the control carried out on July 9, the presence of powdery mildew with an average severity of 16% was detected in 75% of the clusters of the untreated control. On July 22, 98% of the bunches had symptoms of powdery mildew with an average severity of 37%. This increase in the presence of powdery mildew made it possible to assess the control capacity of the products in the medium term. No Botrytis test was performed during 2021.

4 Results

4.1 Downy mildew

In the trial carried out in 2018, none of the products studied obtained efficacy in the control of mildew. Only copper oxychloride (50% Cu) at an average dose of 2,250 kg/ha obtained an efficacy greater than 90%, statistically different from the control without treatment and from the rest of the treatments.

In the year 2020, in the controls carried out on May 22nd, the reference product, Cu oxychloride at an applied dose of 1.620 kg/ha, the EqUr mixed with oxychloride at a dose actually applied of 0.910 kg/ha, and mixed B. velezensis obtained good efficiencies. with Cu oxychloride at a dose of 0.980 kg/ha. The statistical analysis of the data clearly differentiates the group of EqUr + Cu oxychloride (1/2 dose), B. velezensis + Cu oxychloride (1/2 dose) and Cu oxychloride at full dose. The rest of the products did not differ from the untreated control.

The results obtained in the 2018 and 2020 trials in the control of mildew can be seen in Figures 1 and 2.

The data obtained confirm the good results of copper salts and open the door to further research on the complementary nature of biocidal products in controlling downy mildew.

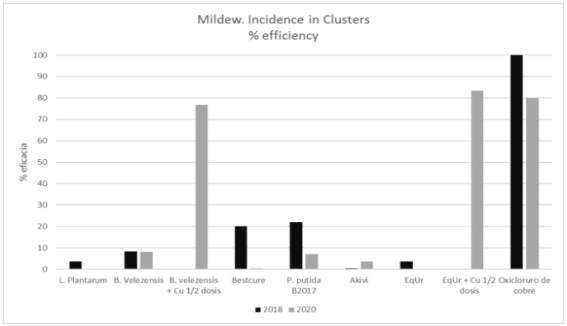

Figure 1. Percentage of efficacy in the downy mildew trials of the years 2018 and 2020. Incidence in clusters.

In 2022, INCAVI began a study to confirm these results by introducing the Cu oxychloride variable at a half dose in contrast to the mixtures already presented.

The conditions of 2022 in the Penedés did not favored the development of mildew and therefore no results have been obtained. Work will continue along this line.

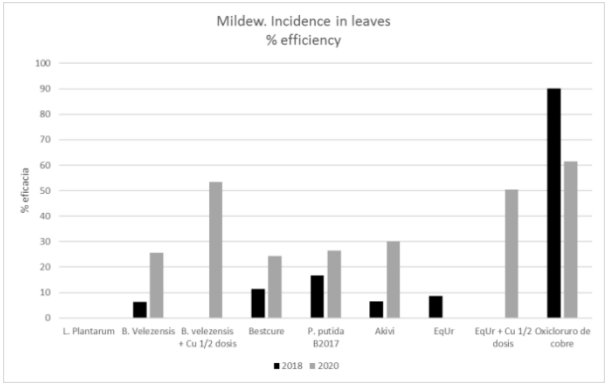

Figure 2. Percentage of efficacy in the mildew trials of the years 2018 and 2020. Incidence in leaves.

4.2 Powdery mildew

2019 and 2021 were the years in which powdery mildew developed in the vineyard and the trial was not conditioned by the development of downy mildew.

In 2019, the products that obtained the greatest efficacy were wettable sulfur (reference product), P. putida B2017, B. velezensis, and L. plantarum.

In the 2021 trial, the good efficacy of wettable sulfur as a reference substance continued, and of the products studied, P. putida was the one that maintained a more stable level of efficacy in the two controls carried out (July 9 and 22). Figure 3 shows the efficiencies obtained in controlling the incidence of the disease, counting the percentage of clusters with the presence of powdery mildew.

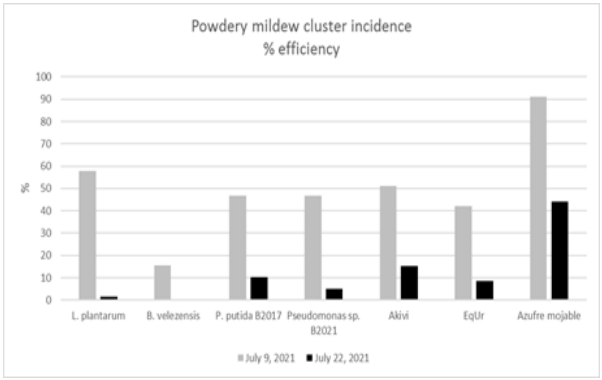

Figure 3. Percentage of efficacy in the powdery mildew trial in 2021. Evolution of the efficacy in the assessment of the incidence of powdery mildew in clusters in the counts carried out on July 9 and 22, 2021.

Efficacy levels in controlling the incidence of the fungus were reduced as pest pressure increased.

In the first control, the efficiencies obtained by the treatments carried out exceeded 40% (except in B. velezensis). In the second control, with 98% of the control clusters affected by powdery mildew, only sulfur maintained a correct control of the number of clusters with the presence of powdery mildew symptoms.

The observation of the severity, with the count of the surface of the cluster affected by the fungus, offers different results, as can be seen in Figure 4.

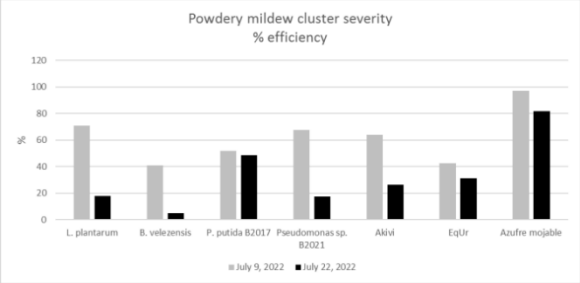

Figure 4. Percentage of efficacy in the 2021 powdery mildew trial. Evolution of the severity of powdery mildew in the cluster, in the assessment of the severity of the powdery mildew in the counts carried out on July 9 and 22, 2021.

In the first count, on July 9, with a degree of severity of powdery mildew in the untreated control of 16%, the efficacy of the products remained between 40 and 70%. Efficiencies that, from the point of view of agronomic control of the disease, are very interesting.

In the second control, with a control severity of 37%, a very good efficacy of wettable sulfur and a good result of P. putida B2017 were observed, which maintained the levels of efficacy despite the strong increase in the disease. These data show little capacity of the applied products to prevent new infections under conditions of strong disease pressure, but a certain capacity to prevent the growth of the fungus.

The results of the powdery mildew trials presented have not obtained statistically clear differences.

The behavior of the fungus that appears in heterogeneously located foci in the vineyard makes it difficult to obtain statistically solid and conclusive results in field trials.

However, the positive trend in the control capacity of the products studied with a medium degree of efficacy is confirmed, and it may be a very interesting complement to the products usually applied.

4.3 Botrytis

The tests carried out have not shown remarkable efficacy in the control of Botrytis cinerea. The weather conditions of the different years have not favored general infections in the trial plots.

With the grape from the Botrytis trials, wines have been made to observe possible effects caused by the treatments carried out. No incidence has been observed in the dynamics of fermentation or in the physical, chemical, and sensory analysis of the wines.

5 Conclusions

The PALVIP project has been carried out with permanent collaboration between all its participants. The assessment of biocidal products has been carried out in a triple approach: production of biocides and field trials, analysis of the plant response to the application of biocidal substances, and evaluation of the environmental and health impact of phytosanitary applications. This multiple work has involved a global assessment of the products based on the shared knowledge of the participating institutions [14].

In this global approach to work, INCAVI has carried out experimentation in the field to assess the efficacy of biocontrol substances in the phytosanitary fight against the main parasitic fungi of the vine. They have been applied maintaining the usual control strategies in the Penedès area, with the aim of assessing whether the proposed products can be a real alternative in the current agronomic conditions.

No significant results have been obtained in the control of downy mildew when biocidal products have been applied exclusively. In the 2020 trial, the applications of B. velezensis mixed with copper oxychloride at a half dose and EqUr mixed with copper oxychloride at a half dose, have obtained the same efficacy as copper oxychloride applied at the usual control dose (approximately 2 kg/ha). These results open the door to a very important reduction in the dose of copper salts to be applied and confirm the complementary and non-substitutive capacity of some biocidal products. However, it must be taken into account that these results were obtained in the last year of the project and its continuity in other conditions and years must be confirmed. INCAVI and the entities supplying the biocidal products will continue the tests to confirm these initial results

In the control of powdery mildew on grapevines, various biocidal products have obtained interesting efficacies, under medium pressure conditions of the disease. These good global results obtained are specified in the trial carried out in 2021 with efficiencies close to 40%. The results have been especially noteworthy in P. putida B2017, which has maintained efficacy levels under conditions of high disease pressure. Despite the variability of the results and the conditions in which it has been worked in the different years, the average control capacity of the biocidal products tested is confirmed and, consequently, the possibility of complementary use to the reference phytosanitary products in the field. powdery mildew control.

Botrytis control trials using the proposed biocide products have not obtained remarkable results.

INCAVI will maintain this line of work in the future, thanks to the knowledge acquired in the PALVIP project and the collaboration network established with the group of participating institutions.

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