***In-vitro* Evaluation of Biocontrol Agents on Juvenile Mortality of Root-Knot Nematode (*Meloidogyne incognita*) in Mulberry**

#  **Abstract**

Root-knot disease caused by *Meloidogyne incognita* is one of the major diseases affecting mulberry, which affects both quality and quantity of leaf. It causes around 10- 12 per-cent leaf yield loss in mulberry. *In-vitro* studies were undertaken in the Pathology laboratory, Department of Plant Pathology, College of Sericulture, Chintamani, to evaluate the efficacy of various bioagents *viz.*, *Trichoderma harzianum, Paecilomyces lilacinus*, *Lecanicillium lecanii, Bacillus subtilis,* *Pseudomonas fluorescens,* Microbial consortia 1 (*P. fluorescens+ B. subtilis+ L. lecanii)* and Microbial Consortia 2 (*T. harzianum* +*P. fluorescens*+ *L. lecanii*)*.* Different concentrations of culture filtrate of these bioagents (25, 50, 75, and 100%) were tested to assess their effects on larval mortality of the nematode after 24, 48 and 72 hours of exposure, compared to a control. Among the biocontrol agents tested, the highest juvenile mortality (64.33%) was observed at 100 per cent concentration of the culture filtrate of MC1 (*Pseudomonas fluorescens* + *Bacillus subtilis* + *Lecanicillium lecanii*), followed by MC2 (*Trichoderma harzianum* + *L. lecanii* + *P. fluorescens*), which recorded 59.33 per cent mortality at 72 hours after incubation.

**Keywords:** *Meloidogyne incognita*, mulberry, biocontrol agents, Microbial consortia, , juvenile mortality, culture filtrate

# **1.Introduction**

Mulberry (*Morus* spp.) is a hardy, deep-rooted, perennial tree species known for its fast growth and adaptability to diverse environmental conditions. It serves as the sole source of nourishment for the silkworm (*Bombyx mori*), and the quality and quantity of mulberry leaves directly influence cocoon yield and silk quality. However, leaf production and nutritional quality are often affected by several biotic and abiotic stresses, posing challenges to sustainable sericulture. India ranks as the second-largest producer of silk globally, following China. During 2022–23, the country produced 36,582 metric tonnes (MT) of raw silk against a target of 40,800 MT, marking a 4.8% increase over the previous year. Of this, the mulberry sector contributed 27,654 MT (BV: 8,904 MT and CB: 18,750 MT), cultivated over 2.53 lakh hectares.

One of the major constraints in mulberry cultivation is the incidence of pests and diseases, including plant-parasitic nematodes. Phytonematodes are considered a serious threat to the sericulture industry (Ramakrishnan and Senthilkumar, 2003). Globally, more than 42 species of nematodes belonging to 24 genera are known to cause root-knot disease in mulberry. Among them, *Meloidogyne incognita* (Kofoid & White) Chitwood is the most prevalent, affecting over 80% of mulberry plantations in major growing regions (Nandan *et al.,* 2022). This nematode alone is responsible for an estimated 10–12% reduction in mulberry leaf yield (Govindaiah *et al.,* 1991).

Management of root-knot nematodes is more challenging compared to above-ground pests, as these nematodes reside in the soil and primarily damage the root system. Although cultural, physical, chemical, and biological methods are available for nematode management, chemical control using nematicides remains a commonly adopted practice due to its high efficacy. However, the indiscriminate use of nematicides and soil fumigants can lead to soil health deterioration, environmental contamination, and toxicity to silkworms. As a result, regulatory restrictions have been placed on the use of these chemicals in agriculture.

Given these limitations, there is an increasing shift towards eco-friendly and sustainable management practices. Biological control using microbial bioagents has emerged as a promising alternative, offering cost-effective and environmentally safe solutions. In this context, the present study was undertaken to evaluate the efficacy of selected biocontrol agents under *in-vitro* conditions for the eco-friendly management of *M. incognita* in mulberry, thereby reducing reliance on chemical nematicides and promoting sustainable sericulture practices.

# **2.Material and methods**

 **Collection of cultures**

Root samples were collected from the infected mulberry garden in different places of Chintamani taluk based on the external symptoms. The mulberry plants showing stunted growth and yellowing symptoms were uprooted by using a scoop or spade. Root samples from 4 to 6 spots were collected randomly within the root zone of the standing mulberry crop. Root-knot infected roots were collected in polythene bags and brought to the pathology laboratory.

**Preparation of culture filtrates of fungal and bacterial bio agents**

Five different biocontrol agents were used in this study *viz., Trichoderma harzianum, Paecilomyces lilacinus, Pseudomonas fluorescens, Bacillus subtilis, Lecanicillium lecanii* along with two Microbial Consortia. Velume prime was used as standard check: different concentrations of culture filtrates were prepared and sterile distilled water served as a control.

The potato dextrose broth (PDB) for fungal agents and nutrient broth (NB) for bacterial agents were prepared, inoculated with respective bio agents in 1000 mL sterilized conical flask and incubated at 28°C in mechanical shaker at 100 rpm ensuring continuous agitation for 48 h. After incubation, the culture broth was centrifuged at 6000 rpm for 15-20 min at 4°C and supernatant was collected sterilized 1000 mL conical flask, which served as a stock filtrate of 100 per cent concentration. The stock filtrate was diluted to 25, 50, 75 per cent concentration using sterile distilled water.

**Collection of egg masses of *Meloidogyne incognita***

Root knot infected mulberry roots were collected from the sick plot and washed gently under running tap water to clear away all soil particles adhering to the roots. Egg masses were clearly seen attached to the surface of roots exactly above the galls developed. These egg masses were picked with the help of forceps under a stereo microscope and were transferred to a Petri plate containing sterile water.

**Extraction of juveniles (J2) from egg masses of *Meloidogyne incognita***

Egg masses from infected mulberry plants were carefully picked with the help of forceps under a stereo binocular microscope and transferred to a Petri plate containing sterile water and incubated at room temperature for 48-96 h to facilitate egg hatching and release of juveniles from the eggs. After 48-96 h, the hatched juveniles were harvested from the suspension. The juvenile population in the suspension was counted under a stereo binocular microscope by transferring 1 mL of suspension on a nematode counting dish and the population was made up to 100 juveniles per mL of suspension.

**Studies on juvenile mortality by cell-free culture filtrates of different biocontrol agents**

**Effects on juvenile mortality**

The freshly hatched juveniles from egg masses were collected and juvenile population was made up to 100 juveniles per mL of suspension as shown in 3.2.4. section.

Similar to the previous study, 1 mL of suspension with 100 juveniles was placed in 10 mL of culture filtrates of different concentrations (25, 50, 75 and 100 per cent) of all bacterial and fungal bioagents separately in sterilized Petri plates (5 cm) and the Petri plate with sterile water served as a control and were incubated at room temperature. The plates were observed for juvenile mobility after every 24 h interval for three days (24, 48 and 72 h). Based on the movement of juveniles on probing with a needle, the juvenile was considered as dead and number of dead larvae was counted in each treatment. Juvenile mortality was calculated according to the formula (Abbott, 1987)

$$JM (\%) =\frac{T}{C} X 100$$

where,

JM: Juvenile mortality

T: Number of dead J2 in treatment

C: Number of J2 used in control

**Experiment details:**

**Design:** Completely Randomized Design (CRD)

**Number of treatments:** 9

**Number of replications:** 3

**Number of units**: 27

**List 1-Treatment details**

|  |  |
| --- | --- |
| **Sl. No.** | **Treatments** |
| **T1** | Cell-free culture filtrates of *Trichoderma harzianum* @ 25, 50, 75,100 per cent dilutions |
| **T2** | Cell-free culture filtrates of *Paecilomyces lilacinus* @ 25, 50, 75,100 per cent dilutions |
| **T3** | Cell-free culture filtrates of *Lecanicillium lecanii* @ 25, 50, 75,100 per cent dilutions |
| **T4** | Cell-free culture filtrates of *Bacillus subtilis @* 25, 50, 75,100 per cent dilutions |
| **T5** | Cell-free culture filtrates of *Pseudomonas fluorescens* @ 25, 50, 75, 100 per cent dilutions |
| **T6** | Cell-free culture filtrates of Microbial Consortia 1 (*Pseudomonas fluorescens+ Bacillus subtilis+Lecanicillium lecanii)* @ 25, 50, 75,100 per cent dilutions |
| **T7** | Cell-free culture filtrates of Microbial Consortia 2 (*Trichoderma harzianum* + *Pseudomonas fluorescens* + *Lecanicillium lecanii*) @ 25, 50, 75,100 per cent dilutions |
| **T8** | Velume prime (Positive check) |
| **T9** | Distilled water (Negative check) |

**3. Results and discussion**

**Juvenile mortality of *Meloidogyne incognita***

The efficacy of four concentrations of culture filtrates (*viz.,* 25, 50, 75 and 100 %) of different bioagents were evaluated for their ability to cause juvenile mortality of *Meloidogyne incognita*. Observations were taken at 24, 48 and 72 h after treatment and are presented in Table 1, 2 and 3, respectively and Fig 1. It was observed that, under *in-vitro* condition, the culture filtrates of different bioagents had significant action on the mortality of juveniles (J2) in contrast to untreated control.

**After 24 hours of treatment**

Juvenile mortality was ranged between 8.00 to 28.67 per cent in the bioagents treated treatments at twenty-five per cent concentration. The maximum mortality of juveniles was recorded in positive check Velume prime. However, among bioagents tested the highest mortality was noticed in case of MC1 (*P. fluroscens + B. subtilis +L. lecanii)* (28.67%) and was minimum in *B. subtilis* (5.63%), they were significantly distinct from each other. The juvenile mortality recorded in rest of the bioagent treatment were in the order of *P. lilacinus* (18.33%), *L. lecanii* (15.33%), *P. fluorescens* (10.67%) and *T. harzianum* (8.00%), respectively. However, MC2 (*T. harzianum + P. fluorescens + L. lecanii*) exhibited juvenile mortality of 22.33 per cent and was the second highest performing bioagent at twenty-five per cent concentration.

The juvenile mortality at fifty per cent concentration was ranged from 9.90 to 35.67 per cent in the bioagents treated batches. The treatment MC1 (*P. fluroscens + B. subtilis +L. lecanii*) showed the maximum juvenile mortality of 35.67 per cent, followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (30.33%), *P. lilacinus* (26.63%), *L. lecanii* (19.30%), *P. fluorescens* (14.63%) and *T. harzianum* (12.07%), they were significantly distinct from each other. The minimum mortality of juveniles was recorded inthe bioagent *B. subtilis* (9.90%) treatment.

All the bioagents significantly caused juvenile mortality at seventy-five per cent concentration and was ranged from 12.63 to 40.30 per cent in the bioagents treated batches. The maximum juvenile mortality was recorded in the case of MC1 (*P. fluroscens + B. subtilis +L. lecanii*) (40.30%) followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (34.67%), *P. lilacinus* (28.90%), *L. lecanii* (26.63%)*, P. fluorescens* (18.63%) and *T. harzianum* (15.67%). The minimum juvenile mortality was observed in *B. subtilis* (12.63%). However, every treatment was significantly distinct from one another.

Juvenile mortality was ranged from 30.00 to 49.00 per cent at hundred per cent concentration of culture filtrate of bioagents. The treatment with MC1 (*P. fluroscens + B. subtilis + L. lecanii*) exhibited maximum juvenile mortality of 49.00 per cent, followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (44.67%), *P. lilacinus* (39.67%), *L. lecanii* (38.67%), *P. fluorescens* (37.33%) and *T*. *harzianum* (34.33%). However, among the bioagent treated batches the minimum juvenile mortality was recorded in the case of *B. subtilis* (30.00%).

**After 48 hours of treatment**

All the bioagents significantly caused the mortality of juveniles compared to control treatment under *in-vitro* experiments. Juvenile mortality was varied from 15.19 to 47.37 per cent at 25 per cent concentration of culture filtrate of bioagents. The highest juvenile mortality was observed with MC1 (*P. fluorescens + B. subtilis + L. lecanii*) (47.37%), followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (40.45%), *P. lilacinus* (30.38%), *L. lecanii* (25.61%), *P. fluorescens* (22.39%) and *T. harzianum* (19.34%). The lowest juvenile mortality among the bioagents treated treatments was recorded in the case of *B. subtilis* (15.19%).

The juvenile mortality was ranged between 28.63 to 49.57 per cent at fifty per cent concentration of culture filtrate of bioagents. The maximum mortality of juveniles was noticed in case of MC1 (P*. fluroscens + B. subtilis +L. lecanii*) (49.57%) followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (43.67%), *P. lilacinus* (39.90%), *L. lecanii* (37.97%), *P. fluorescens* (35.63%) and *T. harzianum* (29.97%). The minimum mortality was observed in the bioagent *B. subtilis* (28.63%). All the treatments were significantly distinct from each other.

Juvenile mortality significantly impacted by every bioagent, and was ranged between 31.97 to 52.63 per cent at seventy-five per cent concentration of culture filtrates. The maximum juvenile mortality was observed with the treatment MC1 (*P. fluroscens + B. subtilis +L. lecanii*) (52.63%), followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (47.63%). The juvenile mortality recorded in rest of the bioagent treatment were in the order of *P. lilacinus* (42.86%), *L. lecanii* (39.90%), *P. fluorescens* (36.93%) and *T. harzianum* (35.63%). The minimum juvenile mortality was observed in *B. subtilis* (31.97%). However, every treatment was significantly distinct from one another.

|  |  |
| --- | --- |
| **Treatments** | **Concentration of culture filtrates (%)** |
| **25%** | **50%** | **75%** | **100%** |
| T1 = *Trichoderma harzianum* | 8.00 | 12.07 | 15.67 | 34.33 |
| T2 = *Paecilomyces lilacinus* | 18.33 | 26.63 | 28.90 | 39.67 |
| T3 = *Lecanicillium lecanii* | 15.33 | 19.30 | 26.63 | 38.67 |
| T4 = *Bacillus subtilis* | 5.63 | 9.90 | 12.63 | 30.00 |
| T5 = *Pseudomonas fluorescens* | 10.67 | 14.63 | 18.63 | 37.33 |
| T6 = MC1 (*P. fluroscens + B. subtilis +L. lecanii)* | 28.67 | 35.67 | 40.30 | 49.00 |
| T7 =MC2 (*T. harzianum*+ *P. fluorescens* + *L. lecanii)* | 22.33 | 30.33 | 34.67 | 44.67 |
| T8 = Velume prime  | 43.66 | 53.00 | 60.33 | 68.67 |
| T9 =Distilled water | 0 | 0 | 0 | 0 |
| SEm ± | 0.43 | 0.31 | 0.34 | 0.47 |
| CD @ 1 % | 1.31 | 0.94 | 1.04 | 1.41 |

**Table 1. Per cent juvenile mortality of *Meloidogyne incognita* as influenced by culture filtrates of bioagents after 24 hours of treatment**

Note: M.C = Microbial Consortia

|  |  |
| --- | --- |
| **Treatments** | **Concentration of culture filtrates (%)** |
| **25%** | **50%** | **75%** | **100%** |
| T1 = *Trichoderma harzianum* | 19.34 | 29.97 | 35.63 | 37.63 |
| T2 = *Paecilomyces lilacinus* | 30.38 | 39.90 | 42.87 | 47.57 |
| T3 = *Lecanicillium lecanii* | 25.61 | 37.97 | 39.90 | 43.97 |
| T4 = *Bacillus subtilis* | 15.19 | 28.63 | 31.97 | 33.57 |
| T5 = *Pseudomonas fluorescens* | 22.39 | 35.63 | 36.93 | 40.97 |
| T6 = MC1 (*P. fluroscens + B. subtilis +L. lecanii)* | 47.37 | 49.57 | 52.63 | 56.63 |
| T7 =MC2 (*T. harzianum*+ *P. fluorescens* + *L. lecanii)* | 40.45 | 43.67 | 47.63 | 50.30 |
| T8 = Velume prime  | 56.33 | 65.33 | 73.33 | 84.33 |
| T9 =Distilled water | 0 | 0 | 0 | 0 |
| SEm ± | 0.38 | 0.39 | 0.27 | 0.31 |
| CD @ 1 % | 1.14 | 1.19 | 0.83 | 0.94 |

**Table 2. Per cent juvenile mortality of *Meloidogyne incognita* as influenced by culture filtrates of bioagents after 48 hours of treatment**

Note: M.C = Microbial Consortia

**Table 3. Per cent juvenile mortality of *Meloidogyne incognita* as influenced by culture filtrates of bioagents after 72 hours of treatment**

|  |  |
| --- | --- |
| **Treatments** | **Concentration of culture filtrates (%)** |
| **25%** | **50%** | **75%** | **100%** |
| T1 = *Trichoderma harzianum* | 29.50 | 31.00 | 35.47 | 38.53 |
| T2 = *Paecilomyces lilacinus* | 40.20 | 42.30 | 49.00 | 55.67 |
| T3 = *Lecanicillium lecanii* | 37.33 | 39.50 | 44.93 | 49.33 |
| T4 = *Bacillus subtilis* | 25.47 | 28.90 | 30.67 | 35.67 |
| T5 = *Pseudomonas fluorescens* | 32.53 | 35.97 | 39.47 | 40.00 |
| T6 = MC1(*P. fluroscens + B. subtilis +L. lecanii)* | 50.33 | 55.57 | 60.50 | 64.33 |
| T7 =MC2(*T. harzianum*+ *P. fluorescens* + *L. lecanii)* | 45.30 | 47.57 | 51.63 | 59.33 |
| T8 = Velume prime  | 64.33 | 76.67 | 85.33 | 93.33 |
| T9 =Distilled water | 0 | 0 | 0 | 0 |
| SEm ± | 0.30 | 0.34 | 0.42 | 0.43 |
| CD @ 1 % | 0.91 | 1.04 | 1.27 | 1.30 |

Note: M.C = Microbial Consortia

**Fig. 1: Per cent juvenile mortality of *Meloidogyne incognita* as influenced by the culture filtrates of bioagents after 72 hours of treatment**

The mortality of juveniles was varied between 33.57 to 56.63 per cent at 100 per cent concentration of cultural filtrate of bioagents. The treatment with MC1 (*P. fluroscens + B. subtilis + L. lecanii*) exhibited maximum juvenile mortality of 56.63 per cent followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (50.30%), *Paecilomyces lilacinus* (47.57%) and *L. lecanii* (43.97%), *P. fluorescens* (40.97%) and *T. harzianum* (37.63%). Minimum juvenile mortality of 33.57 per cent was recorded in *B. subtilis*. However, every treatment was significantly distinct from one another.

**After 72 hours of treatment**

The juvenile mortality was ranged from 25.47 to 50.33 per cent at twenty-five per cent concentration of culture filtrate of bioagents. The highest juvenile mortality was documented in MC1 (*P. fluroscens + B. subtilis + L. lecanii*) (50.33%), followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (45.30%), *P. lilacinus* (40.20%), *L. lecanii* (37.33%), *P. fluorescens* (32.53%) and *T. harzianum* (29.50%), which were significantly different from each other. Among the bioagents the lowest juvenile mortality was recorded in the case of *B. subtilis* (25.47%).

Juvenile mortality was varied between 28.90 to 55.57 per cent at fifty per cent concentration of culture filtrate of bioagents. The maximum mortality of juveniles was noticed in case of MC1 (*P. fluroscens + B. subtilis +L. lecanii*) (55.57%) followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) caused juvenile mortality of 47.57 per cent, making it the second highest-performing bioagent. Among the bioagents, the minimum juvenile mortality was observed in *B. subtilis* (28.90%). The juvenile mortality recorded in rest of the bioagent treatment were in the order of *P. lilacinus* (42.30%), *L. lecanii* (39.50%), *P. fluorescens* (35.97%) and *T. harzianum* (31.00%). However, every treatment was significantly distinct from one another.

The juvenile mortality was significantly varied among each bioagent, ranging from 30.67 to 60.50 per cent at 75 per cent concentration of culture filtrates of bioagents. The highest mortality (60.50%) was recorded in MC1 (*P. fluroscens + B. subtilis +L. lecanii*), followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (51.63%), *P. lilacinus* (49.00%), *L. lecanii* (44.93%), *P. fluorescens* (39.47%) and *T. harzianum* (35.47%), which were significantly distinct from each other. The lowest juvenile mortality (30.67%) was observed for *B. subtilis* among the bioagents.

Each bioagent substantially increased the death of juveniles at 100 per cent concentration of culture filtrate of bioagents. The mortality of juveniles was varied between 35.67 to 64.33 per cent in the bioagents treated batches. The highest mortality (64.33%) was observed in MC1 (*P. fluroscens + B. subtilis +L. lecanii*), followed by MC2 (*T. harzianum + P. fluorescens + L. lecanii*) (59.33%), with both treatments being significantly distinct from each other. The juvenile mortality recorded in rest of bioagent treatment were in the order of *P. lilacinus* (55.67%), *L. lecanii* (49.33%), *P. fluorescens* (40.00%) and *T. harzianum* (38.53%). However, among the bioagents *B. subtilis* exhibited lowest juvenile mortality of 35.67 per cent.

From the above data, it could be concluded that a similar pattern exists as seen with egg hatching inhibition. As concentration of culture filtrates and duration of treatment increases the Juvenile mortality of *M. incognita* was also increased compared to control.

Among the various concentrations of culture filtrates, the highest juvenile mortality was observed at 100 per cent concentration compared to 25, 50, and 75 per cent concentrations. The highest juvenile mortality was recorded at 72 hours compared with 24 and 48 h after treatment. However, when compared with all the treatments the positive control Velume prime resulted in significantly higher juvenile mortality. The recorded observations might be attributed to the production of lytic enzymes by certain PGPR, which break down the walls of *Meloidogyne* eggs. This breakdown leads to a delayed onset of juveniles (J2), ultimately resulting in the death of the nematodes (Turatto *et al*., 2018). The potent activity of extracted culture filtrates of *Pseudomonas* species on juvenile mortality of *M. javanica* infesting mungbean was studied by Nasima *et al.* (2002).

Present results are in conformity with the findings of Soliman *et al*. (2019), who reported higher mortality of juveniles (J2) (97.00%) of *Meloidogyne incognita* upon inoculation with culture filtrates of *Bacillus subtlis* bioagent. They showed that production of chitinase, chitosanase, and protease activities effectively inhibited egg hatching, and altered the eggshell structures. Moreover, eggs treated with the produced chitinase displayed large and more vacuoles in the chitin layer and increasing the mortality percentage of *M. incognita* J2 in *in-vitro* tests.

These observations also suggest that the suppressive efficacy of bacterial bioagents is due to the production of nematotoxic chemicals in the cell suspension, a finding consistent with Siddiqui *et al*. (2000). They investigated juvenile mortality of *Meloidogyne javanica* using various concentrations of hexane and ethyl acetate fractions.

Additionally, the results of the current study align with those of Sankaranarayanan *et al*. (2005), who found that the cell-free culture filtrate of *Pseudomonas fluorescens* was toxic to juveniles of *Heterodera cajani, M. incognita*, and *Rotylenchulus reniformis*. They concluded that increased exposure time to the cell-free culture filtrate resulted in higher juvenile mortality.

Hajji-Hedfi*et al*. **(**2023) reported that the culture filtrate (100%) of *Lecanicillium* spp. was highly effective against root-knot nematode, with 91 per cent rate of second-stage juvenile (J2) mortality. The 50 per cent and 75 per cent culture filtrate concentrations were almost as effective, in causing juvenile mortality. It might be due to the production of toxins, nematicidal metabolites, and lytic enzymes by fungal filtrates.

**4. Conclusion**

The percentage of juvenile mortality increased with prolonged exposure time, with all tested bioagents exhibiting maximum efficacy at 72 hours compared to 24 and 48 hours. The observed antagonistic effect against *Meloidogyne incognita* juveniles may be attributed to alterations in cuticular permeability, which disrupts the selective barrier function of the nematode cuticle. Additionally, the visible abnormalities in juveniles are likely a result of toxic secondary metabolites produced by the biocontrol agents, indicating their potential for effective biological suppression of root-knot nematodes under *in-vitro* conditions.

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