Arquivos do Instituto Biológico
ISSN 1808-1657
https://doi.org/10.1590/1808-1657000232021

SCIENTIFIC ARTICLE
Agricultural Entomology

Lime sulfur to control citrus flat mite and its interactions with the entomopathogenic fungus *Lecanicillium muscarium*

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ABSTRACT

Lime sulfur is one of the few products indicated to control *Brevipalpus yothersi* in Brazilian organic citrus orchards. Other strategies, such as the use of entomopathogenic fungi should be evaluated, and *Lecanicillium muscarium* is one of the basic choices for pest management. Knowledge of the interactions between lime sulfur and this entomopathogen is critical for developing control strategies. With this goal, it was conducted the toxicological characterization of lime sulfur to *B. yothersi* and the compatibility evaluation with *L. muscarium*. Finally, the effects of *L. muscarium* and lime sulfur mixtures on *B. yothersi* control were evaluated. Product evaluation for *B. yothersi* was done through direct and residual contact bioassay, and different concentrations of lime sulfur mixed in potato dextrose agar culture medium were used to evaluate compatibility with *L. muscarium*. Lime sulfur was effective against adults of *B. yothersi* and caused eggs unviability of up to 71.0%, at a dose of 80 L per 2,000 L of H_2O. The lethal concentration (LC_{50} and LC_{99}) of lime sulfur estimated for mite adults were 246.62 and 858.5 μg of sulfur per mL of H_2O (ppm a.i.). Lime sulfur concentrations of 180 to 560 ppm a.i. showed promise for use in combination with *L. muscarium*. However, concentrations of 1,000 and 5,600 ppm significantly reduced colony size and the number of spores/colony. The mixture of 100 and 180 ppm a.i. of lime sulfur with L. muscarium (10^8 conidia·mL^{-1}) was not able to reduce the lethal time of entomopathogen on *B. yothersi*.

Keywords: integrated pest management; *Brevipalpus yothersi*; organic citrus orchards; biological control; synergism.

INTRODUCTION

Mites of the genus *Brevipalpus* are the principal agent for transmitting the citrus leprosis virus, which can cause premature leaf and fruit drop and gradual drying out of branches, which may result in tree death (BASTIANEL et al., 2010; RODRIGUES; CHILDERS, 2013). *Brevipalpus yothersi* Baker is one of the most important citrus pests in Brazil and the predominant species in commercial orchards in São Paulo state, the principal citrus producer (BEARD et al., 2015; MINEIRO et al., 2015). In conventional citrus orchards, this mite species is controlled mainly with chemical acaricides, which reduce mite populations below the economic injury level (NEVES et al., 2002). However, the only product available to control this mite in organic citrus orchards has been lime sulfur (ANDRADE et al., 2010; KIM et al., 2010).

Lime sulfur can reduce the pest population density below the economic injury level but, probably due to the low persistence of this product, applications are more frequent compared to the use of conventional acaricides for controlling *B. yothersi* (CASARIN, 2010). In addition to lime sulfur, the use of entomopathogenic fungi for *B. yothersi* control could be implemented in organic citrus production programs (CONCESCHI, 2013; ROSSI-ZALAF; ALVES, 2006). Fungus of the genus *Lecanicillium* has a lower control efficiency of *B. yothersi* than the fungus *Hirsutella thompsonii*

Received: Sept 21, 2021. Accepted: Oct 06, 2022
Associate Editor: Silvia Galleti
Peer Review History: Double-blind Peer Review.

Fisher (ROSSI-ZALAF; ALVES, 2006). However, it would still be a viable option in managing this pest because it is easy to propagate using an artificial medium.

Another advantage of using *Lecanicillium* spp. would be their effect on other important pests, such as the citrus rust mite *Phyllocoptruta oleivora* (Ashmead), scale insects, such as *Coccus viridis*, and aphids (ALVES et al., 2000; KIM et al., 2010; ROSSI-ZALAF; ALVES, 2006). *Lecanicillium muscarium* Zare and Gams also shows promise in controlling whitefly (REN et al., 2010; SHAUKAT et al., 2017). However, the successful use of entomopathogenic fungi in organic citrus production depends on evaluating the interaction of these organisms with other products used to control pests and diseases (HIROSE et al., 2001).

Interactions between pesticides and entomopathogenic fungi have been studied with variable results. Sublethal concentrations of imidacloprid mixed with *Beauveria bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin had a synergistic effect on mortality of first instar of *Diaprepes abbreviatus* (L.) larvae (QUINTELA; MCCOY, 1997). *Lecanicillium muscarium* associated with matrine insecticide had a synergistic effect on the mortality and enzyme activities of *Bemisia tabaci* (Gennadius) (SHAUKAT et al., 2017). However, associations of fungi with organophosphates, carbamates and organochlorines showed no consistent interactions (FARGUES, 1973). Mixtures involving natural products, such as vegetable oil-based neem and biofertilizers, have also been studied for *B. bassiana* and *M. anisopliae* (HIROSE et al., 2001), when it was observed that neem oil reduced spore germination, colony diameter and conidial development. MOHAN et al. (2007) found isolates compatible with this oil and the mixtures had synergistic effects on the mortality of *Spodoptera litura* (Fabr.) larvae.

The objectives of this research were to evaluate the toxicological effect of lime sulfur on *B. yothersi* and *L. muscarium* and the effects of the mixture of these two products on *B. yothersi* control. Lime sulfur was tested on eggs and adults of *B. yothersi*, the toxicological characterization of lime sulfur was estimated, the compatibility of *L. muscarium* with different concentrations of lime sulfur was evaluated and *L. muscarium* was mixed with sublethal concentration of lime sulfur to evaluate control of *B. yothersi* under laboratory conditions.

MATERIAL AND METHODS

A population of *B. yothersi* collected from an unsprayed citrus orchard located in Piracicaba, São Paulo state, Brazil, was designated as one susceptible reference strain to acaricides and maintained under laboratory conditions on citrus fruits ('Valencia' and/or 'Pera Rio' varieties) collected in a pesticide-free citrus orchard. The lime sulfur, containing 20% of sulfur in its formulation was used. The fungus used for the control of *B. yothersi* was *L. muscarium* (= *Verticillium lecanii*) isolate 972, from the Esalq/USP Insect Pathology Laboratory.

Effect of lime sulfur on eggs and adults of *B. yothersi*

Doses of 20, 30, 40, 50, 60, and 80 L of lime sulfur per 2,000 L of water were tested on adult females of *B. yothersi*. The lime sulfur doses were sprayed on the upper surface of leaf discs, 2.6 cm in diameter, of the sweet orange 'Pera Rio', kept individually in 3.5 cm diameter acrylic plates, containing 2 mL of a solidified agar-water mixture (2.0%). In order to fix the leaf onto the agar and allow it to last longer, a border was made on the leaf using the same water-agar mixture to maintain leaf moisture and confine the mites (OMOTO et al., 2000).

The doses were sprayed with a Potter spray tower to the point of runoff. Each treatment consisted of five replicates and each one consisting of four-leaf disc with 10 mites each. The mortality of *B. yothersi* to lime sulfur was measured by direct contact and a residual contact bioassay. The residual contact bioassay was performed using leaf discs with residual lime sulfur 2 days old.

After treatment, bioassay dishes were kept in an environmental chamber at 25 ± 2 °C, 70 ± 10% relative humidity and a photophase of 14 h. Mortality was evaluated 24 h after exposing mites to the product and they were considered dead when there was no reaction to touching by a brush hair.

The effect of lime sulfur on *B. yothersi* eggs was evaluated on 'Pera Rio' orange fruits. The fruits were paraffin-treated with each one having an arena 4 to 6 cm in diameter, which was infested with 20 adult *B. yothersi* females, left to oviposit for 2 days. The mites were removed and the fruits containing *B. yothersi* eggs were sprayed with a Potter spray tower to the point of runoff. There were four treatments: a control, sprayed with distilled water, and concentrations of 40, 60 and 80 L of lime sulfur per 2,000 L of water. Each treatment was repeated four times over time and each replication consisted of 3 fruits.

The number of unhatched eggs was assessed 12 days after treatment. A total of 194, 293, 231, and 205 eggs were evaluated for the control and the concentrations of 40, 60 and 80 L of lime sulfur per 2,000 L of water, respectively.

All data were converted to percent mortality and subjected to a square-root (x + 0.5) transformation to improve the normality of the data prior to analysis. Analysis of variance was used to determine statistical differences among the treatments. Differences in means were then analyzed using Tukey's studentized range test at p = 0.05.

Toxicological characterization of lime sulfur

Initially, the toxicological characterization of lime sulfur on adults of *B. yothersi* were evaluated. Later, the estimated concentrations were used to evaluate the interaction of lime sulfur with *L. muscarium* in controlling *B. yothersi*.

Lime sulfur concentrations chosen for the bioassay were prepared by diluting the commercial product in distilled water. Five concentrations of lime sulfur logarithmically spaced and ranging from 100 to 560 µg of sulfur per mL of distilled water (ppm a.i. of lime sulfur) were used.

The bioassay method used was direct and residual contact, conducted in arenas of 2.6 cm diameter leaf discs of 'Pera Rio' orange following a similar methodology already described for assessing the efficiency of lime sulfur on adults of *B. yothersi*. The plates were sprayed after infesting the arenas with mites using a Potter spray tower calibrated to 10 psi. A volume of 2.0 mL of solution was used in each application, producing an aqueous residue of approximately 1.60 $mg{\cdot}cm^{-2}$.

Ten adult females were transferred to each arena from a mite stock colony. Each concentration was repeated four times over time and each replication consisted of approximately 40 mites. After spraying and drying, the plates were capped and kept in a climate chamber at 25 ± 1 °C, relative humidity of 70 ± 10% and photophase of 14 h. Mortality was evaluated 24 h after spraying with the aid of a brush with a single hair and a stereomicroscope. The mites were turned on their backs and those which managed to return to a normal position and walked were considered alive. The bioassays, which had over 15% mortality in the control were discarded, as well as those whose loss of mites in agar-water exceeded 15%.

Compatibility of *L. muscarium* with lime sulfur

The growth of colonies and the number of conidia per colony of *L. muscarium* were evaluated for six lime sulfur concentrations. The concentrations of 100, 180, 320, 560, 1,000, and 5,600 ppm a.i. of lime sulfur were compared with a control. The respective amounts of lime sulfur for each concentration were added to a potato dextrose agar culture medium at below 40 °C. After mixing the lime sulfur concentration in the medium, the solution was poured into 8 cm diameter acrylic plates. There were four plates for each concentration. With the aid of a stylus, each plate was inoculated with mycelium and conidia of *L. muscarium* in three regions. The plates were kept in a climate chamber at 26 ± 1 °C, a relative humidity 70 ± 10% and a photophase of 14 h. Six days after inoculation, the number of colonies, the colony diameter, and the number of conidia per colony were recorded.

The colony diameter and conidia number per colony were submitted to an analysis of variance and means were compared using the Tukey's test ($p \leq 0.05$).

The compatibility of lime sulfur concentrations on *L. muscarium* were calculated according to ALVES et al. (1998), as shown in Eq. 1.

$$T = 20VG + 80(ESP)/100 \tag{1}$$

In this model, values for vegetative growth and sporulation are given in relation to the control (100%). Where T = 0 to 30 = very toxic; 31 to 45 = toxic; 46 to 60 = moderately toxic; > 60 = compatible.

Effects of *L. muscarium* and lime sulfur when used in mixture and alone

Two concentrations corresponding to 100 and 180 ppm a.i. of lime sulfur were chosen to be used on *B. yothersi* in combination with the *L. muscarium* suspension containing 10^8 $conidia{\cdot}L^{-1}$. The concentrations of lime sulfur and *L. muscarium* were sprayed alone and in combination on *B. yothersi* following the methodology described for the toxicological characterization of lime sulfur. Each treatment was repeated twice over time and each replicate consisted of 60 adult mites. The mortality in each treatment was evaluated after 5 days. The data were submitted to analysis on Probit to estimate the lethal time (LT_{50}) for each treatment and to evaluate the hypothesis of parallelism between the lines of estimated concentration response (LEORA SOFTWARE, 1987).

RESULTS

Efficacy of lime sulfur on eggs and adults of *B. yothersi*

Lime sulfur was observed to be effective in controlling adult mites under laboratory conditions. In all cases, mortality of the lime sulfur treatments was greater than 90%, 24 h after mite contact with the product (Table 1). The doses evaluated did not differ significantly in direct contact (F = infinity; degrees of freedom [d.f.] = 5,109; $p < 0.0001$; coefficient of variation [CV%] = 0.0). However, the dose of 20 L of lime sulfur per 2,000 L caused mortality significantly lower than that of the other doses evaluated in the residual contact (F = 4,608.04; d.f. = 5,109; $p < 0.0001$; CV% = 2.9).

Table 1. Effects of different application rates of lime sulfur on the mortality of egg and adult stages of *Brevipalpus yothersi*.

Treatment	Application rate[1]	N[2]	eggs	n	Adult	
					Direct	Residual[3]
Lime Sulfur	80	205	71.0 ± 13.6a	180	100.0 ± 0.0a	100.0 ± 0.0a
	60	231	67.0 ± 4.9a	180	100.0 ± 0.0a	100.0 ± 0.0a
	40	293	51.3 ± 9.4a	180	100.0 ± 0.0a	99.4 ± 0.6a
	30		---	180	100.0 ± 0.0a	99.4 ± 0.6a
	20		---	180	100.0 ± 0.0a	93.9 ± 2.6b
Distilled water	---	194	5.6 ± 1.3b	180	0.0 ± 0.0b	0.0 ± 0.0 c

[1]L lime sulfur per 2,000 L of H_2O; [2]number of mites used; [3]exposition method.

Concentrations of 40, 60 and 80 L of lime sulfur per 2,000 of water when applied on *B. yothersi eggs* caused average mortalities of 51.3, 67.0 and 71.0% respectively. The difference in mortality between the concentrations studied was not significant (F = 10.62, d.f. = 3.15, p = 0.001, CV% = 25.7). It was observed that, after the product had dried, eggs that were in contact with the salts of the spray solution were dead, but when this did not occur, the efficacy was impaired, which may explain the low mortality rate for the three concentrations.

Toxicological characterization of lime sulfur

The mortality concentration-response data of *B. yothersi* to lime sulfur are shown in Fig. 1. The estimated LC_{50} for the susceptible *B. yothersi* population (n = 800) was 246.62 ppm a.i. of lime sulfur (95% FL, 212.49–281.25) and LC_{99} of 858.53 ppm a.i. (95% FL, 659.84–1,321.79) (corresponding values of 2.4 and 8.6 L of lime sulfur per 2,000 L of water, respectively), the slope (± SE) was 4.29 (± 0.259), and χ^2 was 10.50 (d.f. = 4, $p > 0.05$). The concentration that resulted in 100% control under laboratory conditions was 6 times less than the recommended field concentration.

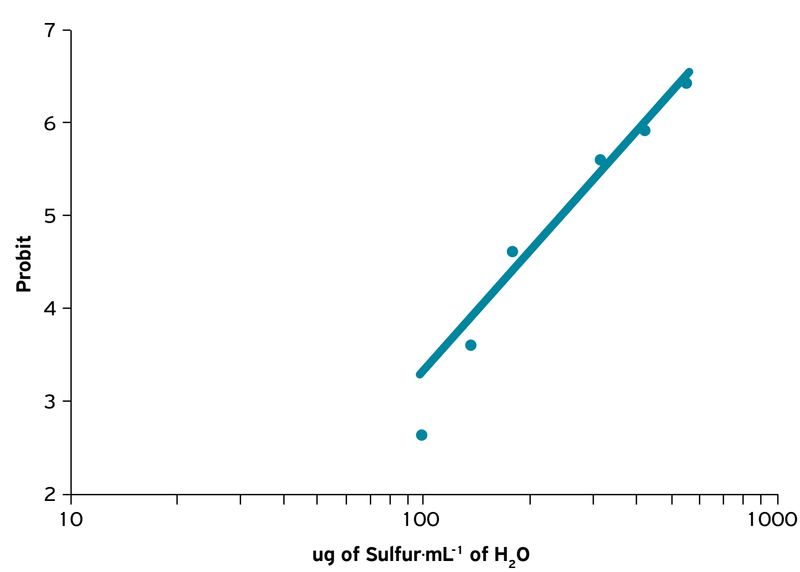

Figure 1. Probit analysis of *Brevipalpus yothersi* (Susceptible strain) to lime sulfur.

Compatibility of *L. muscarium* with lime sulfur

The results showed that the parameters of colony size ($F = 62.13$, $p < 0.05$) and number of spores per colony ($F = 125.89$, $p < 0.05$) behaved differently depending on the concentration. Concentrations of 100 and 180 ppm a.i. of lime sulfur were classified as compatible with *L. muscarium* (Table 2). However, these concentrations provided an initial delay in colony growth compared to the control, which was later suppressed and there was an increase in colony size and the number of spores per colony. Concentrations of 320 and 560 ppm a.i. were classified as moderately toxic and toxic respectively. Although colony growth was not affected, these concentrations significantly reduced the number of spores per colony. Concentrations of 1,000 and 5,600 ppm a.i. were classified as very toxic, greatly reducing colony size and the number of spores per colony. These concentrations inhibited the development of 1 and 6 points of the 12 *L. muscarium* inoculation points made for each concentration, respectively.

Table 2. Colony diameter (mean ± SD), conidia number (mean ± SD), and T values with the compatibility classification of lime sulfur concentrations on *Lecanicillium muscarium* – strain 972.

Concentration (ppm of sulfur)	Colony size (cm)	Number of conidia/colony × 10^7	Values of T
0	0.90 ± 0.06b1	9.64 ± 0.89b	
100	1.09 ± 0.05a	11.86 ± 0.35a	122.78 C
180	1.14 ± 0.05a	10.44 ± 0.51ab	112.16 C
320	0.75 ± 0.02b	4.27 ± 0.08c	52.19 MT
560	0.78 ± 0.02b	3.21 ± 0.23c	44.12 T
1.000	0.44 ± 0.02c	1.22 ± 1.13d	20.02 VT
5.700	0.25 ± 0.04d	0.16 ± 0.05d	6.91 VT
CV%	10.83	17.75	

C = compatible, MT = moderately toxic, T = toxic, VT = very toxic. 1 Means followed by different letters within each column are significantly different (P≤0.05) from the control treatment; Tukey's studentized range test. CV% = coefficient of variation. Source: Adapted from ALVES et al. (1998):

Effects of *L. muscarium* and lime sulfur when used in mixtures and alone

The lethal time (LT_{50}) estimated for the *L. muscarium* suspension containing 10^8 conidia·mL^{-1} was 88.7 h (95% FL, 85.9 to 91.4). Mixtures of *L. muscarium* with concentrations of 100 and 180 ppm a.i. of lime sulfur had an estimated LT_{50} of 95.6 h (95% FL, 92.6 to 98.6) and 82.9 h (95% FL, 74.7 to 91.8) respectively. The parallelism test showed that the concentration response curves were similar ($\chi^2 = 0.29$, d.f. = 2, $p = 0.86$). However, the hypothesis of equality of the intersections was rejected ($\chi^2 = 35.03$; d.f. = 2, $p = 0.00$) (Fig. 2).

The mixture of *L. muscarium* with 100 ppm a.i. of lime sulfur had a significantly higher LT_{50} than the LT_{50} of the *L. muscarium* alone, since the confidence intervals did not overlap. The LT_{50} of the mixture of *L. lecanii* with 180 ppm a.i. of lime sulfur was not significantly different from the LT_{50} of *L. muscarium* alone. Thus, there were no significant interactions with the concentrations of 100 and 180 ppm a.i. of lime sulfur.

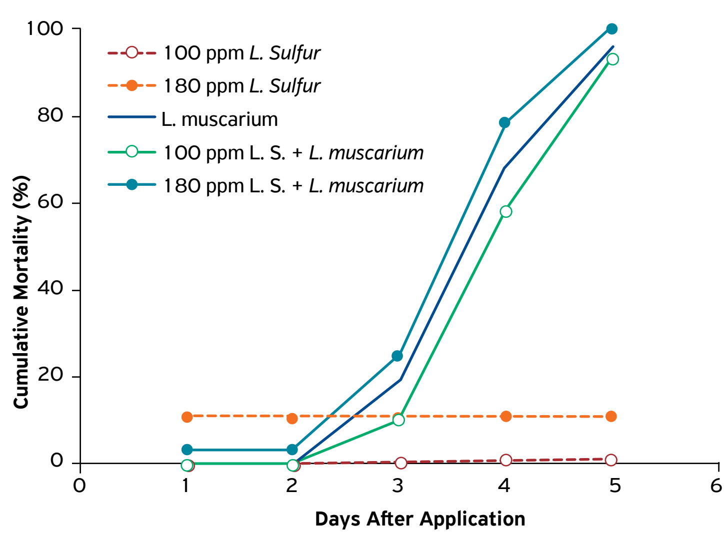

Figure 2. Mortality rate (%) of *Brevipalpus yothersi* over time, caused by a concentration of 10^8 conidia·mL^{-1} of *Lecanicillium muscarium* and 100 and 180 ppm a.i. of lime sulfur applied alone and in combination under laboratory conditions.

DISCUSSION

Lime sulfur plays an important role in controlling *B. yothersi* in Brazilian organic citrus orchards. It is the only acaricide with proven efficacy permitted for this agroecosystem, which can reduce the population density of this mite (ANDRADE et al., 2010). The laboratory studies prove the efficiency of this product with doses of 20 to 80 L of lime sulfur per 2,000 L of H_2O for adults of this pest. For 48-h-old eggs, lime sulfur reduces viability between 51.3 and 71.0% for doses of 40 to 80 L of lime sulfur per 2,000 L of H_2O.

Brevipalpus yothersi resistance to the main acaricides used in Brazilian citriculture, including lime sulfur, is well-known (CASARIN, 2010; OMOTO et al., 2008). The adoption of other control strategies for *B. yothersi* associated with the use of lime sulfur would be fundamental for maintaining product efficacy, besides reducing the biological disequilibrium caused by excessive use of this acaricide (ALVES et al., 2018).

Fungi of the genus *Lecanicillium* are widely known to cause mortality in different citrus pests. The association of an entomopathogen with these characteristics and lime sulfur could help the integrated management of *B. yothersi* and other pests in systems of organic citrus production. However, lime sulfur in concentrations/doses capable of controlling *B. yothersi* were not compatible with *L. muscarium*. Concentrations above 1,000 ppm a.i. of lime sulfur were classified as very toxic to *L. muscarium*, inhibiting points of inoculation and drastically reducing the formation of spores and colony size. Concentrations of 100 to 560 ppm a.i. of lime sulfur were more promising, ranging from compatible to toxic. For concentrations of 100 and 180 ppm a.i. of lime sulfur, a significant increase in the growth of *L. muscarium* colonies occurred. One explanation that supports the growth increase of *L. muscarium* exposed to these doses of lime sulfur could be that low concentrations serve as a source of nutrients for the fungus. ALVES et al. (2000), assessing the compatibility between entomopathogenic fungi and pesticides used in citrus, found that sulfur was also compatible to moderately toxic to the principal entomopathogenic fungi present in this agroecosystem.

However, the benefit of these lime sulfur subdoses to the growth of *L. muscarium* did not affect interactions, which increasing mortality and reducing lethal time of the entomopathogen on *B. yothersi*.

There is no possibility of lime sulfur association with the entomopathogenic fungus *L. muscarium*. Doses controlling the leprosis mite are very toxic to the fungus, while subdoses associated with the entomopathogen do not present synergistic interaction in the control of the pest.

AUTHORS' CONTRIBUTIONS

Conceptualization: Alves, E.B.; Omoto, C. **Data curation:** Casarin, N.F.B.; Gonçalves-Gervásio, R.C.R. **Formal analysis:** Alves, E.B. **Funding acquisition:** Omoto, C. **Investigation:** Alves, E.B. **Methodology:** Alves, E.B.; Omoto, C. **Project administration:** Alves, E.B. **Resources:** Omoto, C. **Supervision:** Alves, E.B. **Validation:** Omoto, C. **Visualization:** Alves, E.B. **Writing – original draft:** Alves, E.B. **Writing – review & editing:** Alves, E.B.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

FUNDING

This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

Not applicable.

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