SUBSTRATES FOR THE PRODUCTION OF ENTOMOPATHOGENIC ISOLATES OF *Fusarium caatingaense* TO CONTROL *Dactylopius opuntiae*

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Abstract: *Opuntia ficus-indica* (prickly pear) is used as a source of animal feed in Northeast Brazil. Its yield has been severely affected by insect pest *Dactylopius opuntiae* (cochineal scale). *Fusarium caatingaense* isolates have been reported to be effective in controlling this pest. Therefore, this study aimed to select substrates for the production of *F. caatingaense*. Conidia production and viability of five *F. caatingaense* isolates were evaluated on six substrates: rice, sugarcane bagasse, sugarcane bagasse + 0.66% w/v peptone, corn grains, corn grains + 0.66% w/v peptone, and sweet corn grains. Rice and corn were the best substrates for the production of most isolates. Isolate URM 6778 was selected for the analyses of sporulation, germination, and pathogenicity after storage in these substrates at 4 ºC and 28 ºC and periods of 0, 15, 30, and 60 days. Corn was the best substrate for this fungus and 4 ºC was the most suitable storage temperature. The highest conidia concentration was observed on the 30th day of storage, with a decrease on the 60th day. Conidia remained viable in all periods analyzed, except on the 60th day at 28 ºC. Pathogenicity against *D. opuntiae* was kept until 30 days of storage at 4 ºC and 28 ºC. With the accomplishment of this study, it was possible to indicate vegetable substrates for the production of *F. caatingaense* and the most suitable conditions for their storage, aiming at the application in the biological control of insects.
Index terms: Biological control, conidia production and viability, mass production, vegetable substrates.

SUBSTRATOS PARA A PRODUÇÃO DE ISOLADOS ENTOMOPATOGÊNICOS DE *Fusarium caatingaense* VISANDO CONTROLAR *Dactylopius opuntiae*

Resumo – *Opuntia ficus-indica* (palma forrageira) é utilizada como fonte de alimentação animal no Nordeste brasileiro. Sua produtividade tem sido afetada severamente pelo inseto *Dactylopius opuntiae* (cochonilha-do-carmim). Isolados de *Fusarium caatingaense* foram reportados como eficientes no controle dessa praga. Portanto, este estudo objetivou selecionar substratos para a produção de *F. caatingaense*. A produção e viabilidade de conídios de cinco isolados foram avaliadas em seis substratos: arroz, bagaço de cana-de-açúcar, bagaço de cana-de-açúcar + peptona 0,66% p/v, grãos de milho, grãos de milho + peptona 0,66% p/v, e grãos

de milho doce. Arroz e milho foram os melhores substratos para produzir a maioria dos isolados. O isolado URM 6778 foi selecionado para análises de esporulação, germinação e patogenicidade após o armazenamento nesses substratos nas temperaturas 4 e 28 ºC, por 0, 15, 30 e 60 dias. O milho foi o melhor substrato para esse fungo e 4 ºC foi a temperatura mais adequada para o armazenamento. A maior concentração de conídios foi observada no trigésimo dia de armazenamento, havendo um decréscimo no sexagésimo dia. Os conídios mantiveram-se viáveis em todos os períodos analisados, exceto no sexagésimo dia a 28 ºC. A patogenicidade contra *D. opuntiae* foi mantida até 30 dias de armazenamento em 4 ºC e 28 ºC. Com a realização deste estudo foi possível indicar substratos vegetais para a produção de *F. caatingaense* e as condições mais adequadas para o seu armazenamento, visando a aplicação no controle biológico de insetos.
Termos para indexação: Controle biológico, produção e viabilidade de conídios, produção massal, substratos vegetais.

INTRODUCTION

Prickly pear (*Opuntia ficus-indica* L. Miller) is a cactus widely grown in semi-arid regions of northeastern Brazil and it is used as the main food source for cattle, goats, and sheep. *Dactylopius opuntiae* (Cockerell, 1896) (Hemiptera: Dactylopiidae), known as cochineal scale, has a high harmful potential for *O. ficus-indica* culture. In many states in Northeast Brazil, the intense attack of cochineal scale has caused eradication of plantations, impairing livestock activity in the region (DINIZ *et al*., 2020; VELEZ *et al*., 2019).

The use of fungal agents for biological control of pests is important to minimize the application of synthetic chemical pesticides, which have negative effects on human, animals, and the environment (SANTOS *et al*., 2022). Therefore, conditions for the production of entomopathogenic fungi are needed, which include investigation of the most suitable substrate in terms of low cost, handling, and preparation, in addition to viability and virulence of the fungus (JARONSKI; MASCARIN, 2017). The selection of substrates for mass production of entomopathogenic fungi is essential for an effective and amenable biological control of insect pests (LIMA *et al*., 2020).

In Brazil, mycoinsecticides are mainly produced on solid substrates, such as whole rice, white rice, parboiled rice, broken rice and millet (MASCARIN *et al*., 2019). Rice is the solid substrate most commonly used for growth and production of aerial conidia of hypocrealean entomopathogenic fungi, such as *Beauveria*, *Metarhizium*, *Isaria* (= *Cordyceps*) and *Lecanicillium* (= *Akanthomyces*). However, some fungal strains are less or even unresponsive to rice, leading to poor yields (JARONSKI; MASCARIN, 2017). For this reason, other substrates have been investigated, such as corn (MAR *et al*., 2012) which is widely produced in Brazil and found in the Northeast region (CONAB, 2017), and sugarcane bagasse (PRASAD; PAL, 2014; SANTA *et al*., 2005; SHI *et al*., 2009), which is one of the most abundant agro-industrial waste in the northeast of Brazil and has high availability and low cost in this region (PAIVA-GUIMARÃES *et al*., 2020).

Entomopathogenic isolates of *Fusarium caatingaense* (= FIESC 20) Santos, Lima, Tiago & Oliveira have been reported to be effective for the control of *D. opuntiae* (DINIZ *et al*., 2020; SANTOS *et al*., 2016; VELEZ *et al*., 2019), host insect

of these fungi (CARNEIRO-LEÃO *et al*., 2017), and of *Nasutitermes corniger* (Blattodea: Termitidae) (DINIZ *et al*., 2021), besides not presenting ability to cause disease in beans and corn (MACIEL *et al*., 2021). Recent studies have shown that the *Fusarium* genus includes strong entomopathogenic agents, in contrast to earlier investigations that reported the *Fusarium*-insect relationships as mostly opportunistic. Many *Fusarium* strains have been reported to cause high mortality rates against insects and have shown fast action and abundant sporulation (SANTOS *et al*., 2020).

To use of *Fusarium* isolates for insect control requires tests to verify their efficacy under field conditions, as well as their specificity to the host insect, production of undesirable secondary metabolites, and effects on non-target organisms (SANTOS *et al*., 2020). The use of a substrate for mass production of these fungi facilitates these tests. Thus, this study aimed to select potential vegetable substrates for the production of conidia from isolates of *F. caatingaense* selected for the control of *D. opuntiae*. We also evaluated conidia production and viability of the isolates selected, as well as their virulence after storage in the substrates selected at different temperatures and storage times.

MATERIAL AND METHODS

Fungi and substrates

We used five *F. caatingaense* isolates from *D. opuntiae*: URM 6776, URM 6777, URM 6778, URM 6779, and URM 6782. These isolates were identified based on morphology, molecular phylogeny, and sexual compatibility (SANTOS *et al*., 2019) and were preliminarily selected as biocontrol agents of *D. opuntiae* (CARNEIRO-LEÃO *et al*., 2017). The isolates were deposited at the URM Micoteca Culture Collection of the Universidade Federal de Pernambuco (WFCC No. 604).

The following substrates were used: rice, sugarcane bagasse, sugarcane bagasse + 0.66% w/v peptone, corn grains, corn grains + 0.66% w/v peptone, and sweet corn grains. The substrates were prepared using 50 g of each substrate and 25 mL of distilled water arranged in Erlenmeyer flasks, which were autoclaved at 121 ºC for 20 min. In the supplemented substrates, 1g of peptone was added, equivalent to 0.66% w/v peptone (LIMA *et al*., 2020).

Conidial production and germination of *Fusarium caatingaense* isolates in vegetable substrates

To assess conidia production in substrates, 5 ml of a suspension of 1 x 10^7 conidia × ml^{-1} of each isolate were inoculated into different substrates in the Erlenmeyer flasks. After 15 days of incubation at 28 ºC in the dark, 1g of each substrate with fungal growth was suspended in 5 mL of 0.01% v/v Tween 80® solution, according to Lima *et al*. (2020). The number of conidia was estimated using a Neubauer chamber and expressed in conidia × g^{-1} of substrate.

To determine the germination rate, 70 µl suspensions of 10^6 spores × ml^{-1} were prepared from each substrate with fungal growth and spread on Petri dishes with culture medium Potato Dextrose Agar (PDA). After 14 hours of incubation at 28

ºC in the dark, 200 conidia germinated and non-germinated were counted under an optical microscope (100×) to verify germination percentage. The experimental design was completely randomized, with six treatments (substrates) and three replications, both for conidial production and germination tests.

The isolate selected for storage tests at different temperatures and storage times was grown at 28 ºC for 15 days on 50 g of each selected substrate and 25 mL of distilled water in polypropylene bags. After this period, the bags containing substrates with the fungi were stored at 4 and 28 ºC for 28 ºC for 15, 30, and 60 days. After each period, including time zero (15th day of cultivation), conidia production and germination were evaluated to verify sporulation and viability of the fungus over the storage period and at different temperatures, according to the methodology described above. The experiment design was completely randomized, in a factorial scheme (2 x 3), with two temperatures and three storage times, and three repetitions.

Virulence of isolates selected against *Dactylopius opuntiae* after different storage times at different temperatures

Pathogenicity tests were carried out using conidial suspensions grown in the substrates at 4 ºC and at 28 ºC, at 0, 15, 30, and 60 days. Specimens of *D. opuntiae* were reared on cladodes (rackets) of *O. ficus-indica* in a greenhouse. Thirty days after infestation, cladodes containing adult insects were sprayed with neutral detergent solution 0.02 % v/v to decrease the wax amount on insects. After drying, the cladodes were sprayed with 4 ml of suspension of 1 x 10^6 conidia × ml^{-1} in 0.01% v/v Tween 80® solution. For the control, insects were sprayed with 0.01% v/v Tween 80® solution. The cladodes were kept in a room with the temperature controlled at 28 ºC ± 2 ºC.

Insect mortality was assessed 10 days after inoculation of the fungi. Three areas of 8 x 8 cm were delimited on each cladode and 50 insects were evaluated per area, totaling 150 insects for each treatment. Total insect mortality was assessed according to Santos *et al*. (2016), using a stereomicroscope to observe features of insects, for example, dead insects were darker and less turgid than live insects. For mortality correction, data were submitted to the formula of Schneider-Orelli (PÜNTENER, 1981).

Statistical analysis

Data were subjected to the analysis of variance (ANOVA) and the means were compared by the Tukey test at 1% probability using the program SASM-Agri (CANTERI *et al*., 2001). For data on sporulation, germination, and pathogenicity of the isolates selected, differences between the means was determined using the polynomial regression analysis for the quantitative factors, temperatures, and storage times (days), when there was statistical significance. The analyses and graphs were prepared with the Microsoft Excel 2016® program in the statistical mode.

RESULTS AND DISCUSSION

Rice was the substrate with the greatest conidia concentration for the isolates studied, except for URM 6776, which had sugarcane bagasse as the best substrate (Table 1). Rice is the most commonly used vegetable substrate for conidia production of entomopathogenic fungi, such as *Beauveria, Metarhizium, Isaria* (= *Cordyceps*), and *Lecanicillium* (= *Akanthomyces*) (JARONSKI; MASCARIN, 2017). Sugarcane bagasse has been tested for the production of *Beauveria, Metarhizium*, and *Verticillium* (PRASAD; PAL, 2014; SANTA *et al*., 2005; SHI *et al*., 2009), demonstrating that its high porosity and water absorption provide an ideal environment for sporulation in solid-state fermentation, thus, leading to good spore production.

Corn (with or without peptone) was the second best substrate for most isolates. In some cases, sporulation in sweet corn was as high as in corn (with or without peptone) (Table 1). However, the use of corn is preferable as it is more economical, besides peptone makes production more costly and sweet corn has low production in Brazil. Studies have reported corn as a good substrate for the production of *Metarhizium* spp., *Paecilomyces lilacinus* (= *Purpureocillium lilacinum*) (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson and *Isaria tenuipes* (= *Cordyceps tenuipes*) (Peck) Kepler, B. Shrestha & Spatafora (MAR *et al*., 2012).

Table 1. Average values of conidia production (n x 10^6 conidia × g^{-1} of substrate) ± standard error from *Fusarium caatingaense* isolates grown in different vegetable substrates.

Isolate	Rice	Sugarcane bagasse	Sugarcane bagasse [a]	Corn grains	Corn grains [a]	Sweet corn grains
URM 6776	3.00 ± 13.23 bc	15.17 ± 24.66 a	1.00 ± 4.82 c	5.42 ± 24.83 b	5.55 ± 4.44 b	1.28 ± 2.36 c
URM 6777	762.5 ± 28.75 a	133.3 ± 2.89 b	68.3 ± 4.04 b	38.2 ± 2.19 b	28.3 ± 1.83 b	31.9 ± 1.56 b
URM 6778	147.67 ± 4.04 a	10.67 ± 1.76 cd	6.17 ± 4.86 d	45.00 ± 3.61 b	28.50 ± 15.12 bc	48.00 ± 21.63 b
URM 6779	290.80 ± 1.14 a	14.60 ± 0.14 d	29.60 ± 0.45 cd	49.40 ± 1.40 c	90.13 ± 0.16 b	111.93 ± 2.14 b
URM 6782	11.73 ± 5.86 a	0.56 ± 1.38 d	0.35 ± 1.51 d	2.65 ± 9.76 c	9.03 ± 7.09 b	2.46 ± 3.33 c

Means followed by the same letter in the same line are not significantly different by the Tukey test ($P \leq 0.05$). Original data. For the statistical analysis, data were transformed using the formula $\sqrt{x+k}$; with k = 0.1 for URM 6776 and URM 6782; k = 10 for URM 6777; and k = 1 for URM 6778 and URM 6779.
[a] Supplemented with peptone (0.66% w/v).

Substrate supplementation with peptone (0.66% w/v) did not increase conidia production in most cases, except for URM 6779 and URM 6782 with the addition of peptone to corn substrate. Lima *et al*. (2020) also observed that the addition of peptone as a nitrogen source increases the sporulation of some *F. caatingaense* isolates in certain substrates (i.e. cracked corn and wheat bran), while in other cases, sporulation is equal to or smaller in peptone-supplemented substrates. In these cases, it is indicated to choose the substrate without supplementation to reduce production costs.

The isolates showed 100% of conidia germination after growth on the substrates studied, a very important characteristic for their use in biological control. Lima *et al*. (2020) carried out a study with the same isolates on other substrates and found germination percentages from 0 to 100% among substrates. This variation in the germination

rate according to the substrate reinforces the importance of evaluating spore viability in the selection process of the substrate for production of entomopathogenic fungi.

Isolate URM 6778 caused high mortality rates against *D. opuntiae* in previous studies conducted under field conditions (CARNEIRO-LEÃO *et al*., 2017) and presented good conidia production and germination in the substrates tested. Therefore, this fungus was selected for the storage tests with rice and corn. The tests showed no interaction between substrate, temperature, and storage time for conidia production. The analysis of rice and corn as substrates showed that corn was the most suitable substrate for conidia production and storage of URM 6778, regardless of storage time (Table 2) and temperature (Table 3).

Table 2. Average values of conidia concentration (n x 10^6 conidia × g^{-1} of substrate) ± standard error of the isolate URM 6778 of *Fusarium caatingaense* produced on rice and corn grains at 4 ºC and 28 ºC, regardless of storage time (0, 15, 30, and 60 days).

Temperature	Rice	Corn grains	Average value
4º	0.99	2.07	1.53 ± 0.31 a
28º	0.24	0.65	0.45 ± 0.12 b
Average of substrates	0.61 ± 0.18 B	1.36 ± 0.30 A	

Means followed by the same letter (lowercase on column and uppercase on rows) are not significantly different.

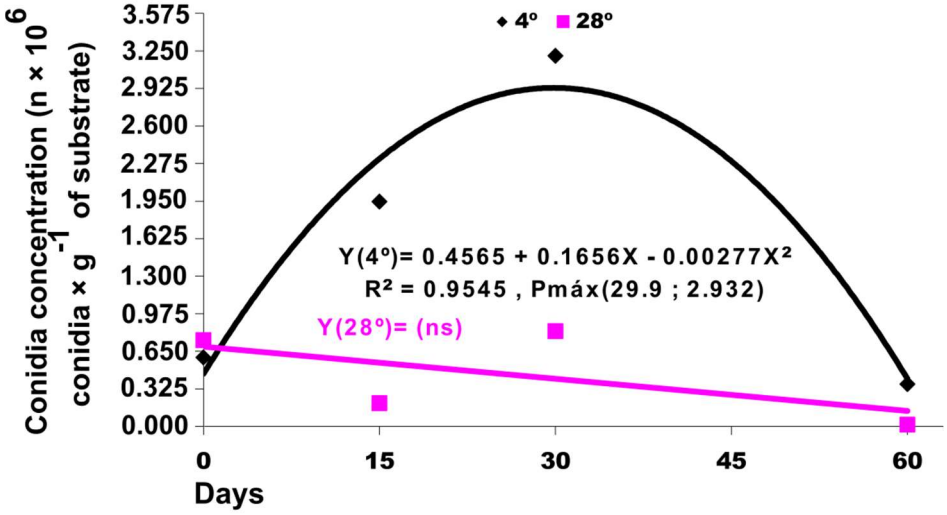
Table 3. Average values of conidia concentration (n x 10^6 conidia × g^{-1} of substrate) ± standard error of isolate URM 6778 of *Fusarium caatingaense* in rice and corn grains, in periods of 0, 15, 30, and 60 days, regardless of storage temperature (4 ºC and 28 ºC).

Time (days)	Rice	Corn grains	Average value
0	0.24	1.10	0.67 ± 0.19
15	0.75	1.40	1.07 ± 0.34
30	1.40	2.63	2.02 ± 0.50
60	0.07	0.31	0.19 ± 0.07
Average of substrates	0.61 ± 0.18 B	1.36 ± 0.30 A	

Means followed by the same letter on rows are not significantly different.

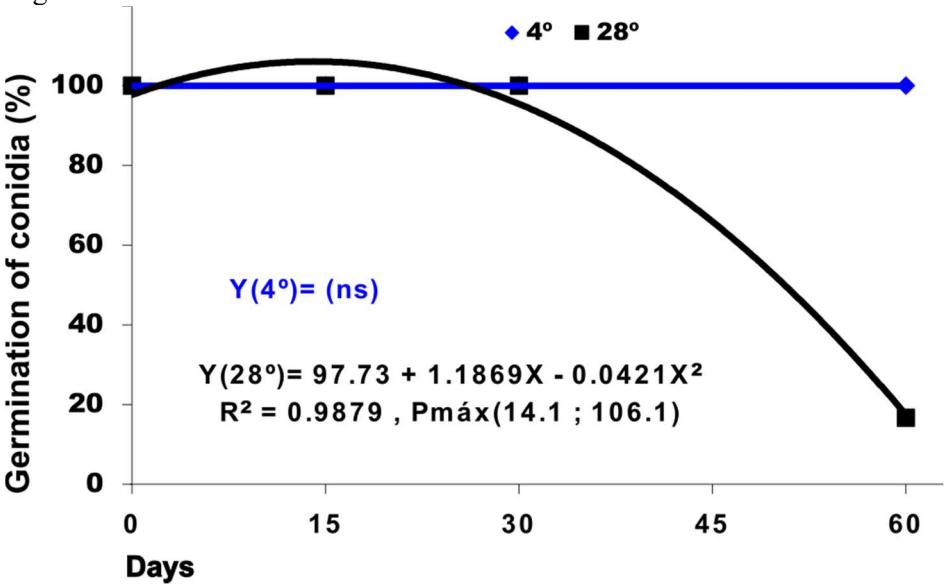
Temperature and storage time showed interaction and a greater number of conidia was observed at 4 ºC when compared to 28 ºC, regardless of the substrate used. More cycles of conidia production and germination must have occurred at 28 ºC, while at 4 ºC, the metabolic activity of most fungi was reduced, while conidia needed a longer time to germinate and therefore more spores are found. Conidia concentration showed a peak after 30 days stored at 4 ºC, indicating that this time is feasible for conidia storage. After this period, conidia concentration dropped, which shows that extending the storage time is not an effective procedure. Conidia concentration decreased at 28 ºC during the study period, possibly because substrate nutrients were depleted more quickly. Thus, storage at 28 ºC is not recommended (Figure 1).

Figure 1. Conidia concentration (n × 10^6 conidia × g^{-1} of substrate) of isolate URM 6778 of *Fusarium caatingaense* in rice and corn grains stored at different temperatures (4 ºC and 28 ºC) and time (0, 15, 30, and 60 days).



Isolate URM 6778 showed 100% germination stored at 4 ºC during the study time (Figure 2). The same was not observed at 28 ºC after 60 days, because most conidia were not viable, indicating that conidia viability of *F. caatingaense* is kept for a longer time at low temperatures. Loureiro *et al*. (2003) carried out tests at different temperatures (4 ºC and 26 ºC) and storage times with *Sporothrix insectorum* de Hoog & H.C. Evans grown in rice and observed that 4 ºC guaranteed longer viability of the conidia. The storage of *Beauveria bassiana* (Bals.-Criv.) Vuill., isolate ESALQ 447, grown in rice, in a freezer (-7 ± 1 ºC) ensured 100% viability of conidia for 80 months (MARQUES *et al*., 2000).

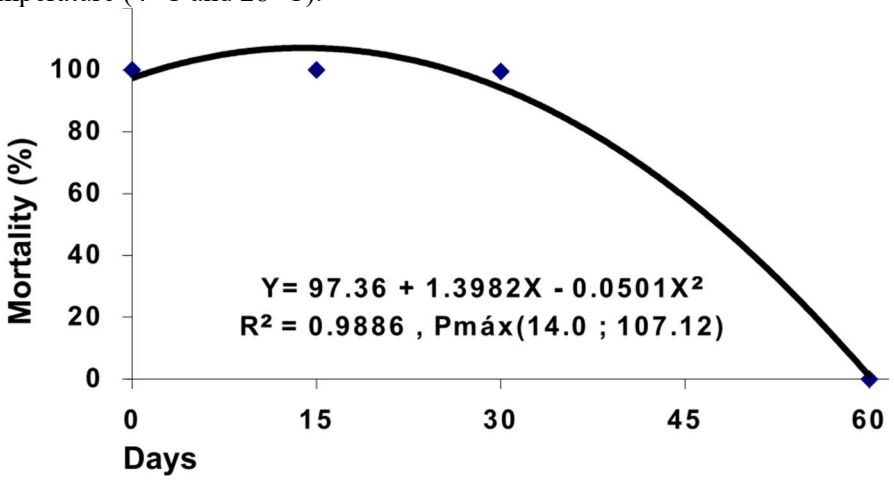
Figure 2. Conidia germination of isolate URM 6778 of *Fusarium caatingaense* in rice and corn grains stored at different temperatures (4 ºC and 28 ºC) and time (0, 15, 30, and 60 days), regardless of vegetable substrate.



The URM 6778 isolate maintained its virulence against *D. opuntiae* after 30 days of storage in the tested substrates and both temperatures (Figure 3). Since after 60 days, conidia concentration decreased and the number was not enough to perform the pathogenicity test. Marques *et al*. (2000) tested the virulence of *B. bassiana* against

Diatraea saccharalis Fabricius (Lepidoptera: Crambidae), grown in rice and stored in a freezer (-7 ± 1 ºC), and obtained 92% mortality initially and 94% mortality after 94 days.

Figure 3. Mortality percentages of *Dactylopius opuntiae* by isolate URM 6778 of *Fusarium caatingaense* during storage time (0, 15, 30, and 60 days), regardless of substrates (rice and corn) and temperature (4 ºC and 28 ºC).



CONCLUSIONS

This study found vegetable substrates suitable for the production of *F. caatingaense*. Rice and corn were the best substrates for mass production of most isolates tested. Corn storage at 4 ºC for 30 days is the most indicated condition to ensure conidial viability and virulence of isolate URM 6778 against *D. opuntiae*. As these conditions require electric energy use and the storage time was relatively short, future efforts to select applicable adjuvants in the formulation of the mycoinsecticide will be undertaken, for storage preserving fungal characteristics and product quality for a longer time under conditions with minimal costs.

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