Journal of Plant Diseases and Protection, **119** (2), 53–58, 2012, ISSN 1861-3829.

Effect of *Zataria multiflora* essential oil on *Alternaria alternata in vitro* and in an assay on tomato fruits

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Received 20 July 2011, accepted 22 February 2012

Abstract

The present study aimed to evaluate the effects of *Zataria multiflora* essential oil at different concentrations (0, 100, 200, 300 and 500 ppm) on the growth rate and morphogenesis of *Alternaria alternata* in *in vitro* and *in vivo* conditions. The antifungal activity of the oil was tested by the poisoned food technique (PF) and volatile activity assay (VA). The mycelial growth of the test fungus was completely inhibited at oil concentrations of 500 and 200 ppm in PF and VA assessments, respectively. Determination of conidial germination in the presence of the oil was also carried out and it was found that the oil caused 100% inhibition of conidial germination at the level of 300 ppm. Light Microscopy (LM) showed morphogenic changes in the test fungus with 300 ppm of the oil. These changes included a significant decrease and loss of conidiation, severely collapsed and squashed hyphae, a decreased hyphal diameter and granulation of cytoplasm. The treatment with the oil also resulted in highly lobate and bulbous anomalous structures at the hyphal tips. GC-MS evaluations of the essential oil revealed that the latter consisted of 14 compounds of which carvacrol was the major component. *In vivo Z. multiflora* oil at 500 ppm effectively reduced the percentage of decayed tomatoes. Findings of the current study suggest that essential oils could be used as an alternative for chemicals for control of postharvest phytopathogenic fungi on fruits or vegetables.

Key words: Morphogenesis change, postharvest disease

Introduction

Postharvest diseases account for substantial losses of fruits, vegetables, and other plant products during the transit and storage processes. *Alternaria alternata* is a fungal pathogen of tomato causing Alternaria stem canker and post harvest diseases at high frequency. During the development of the disease, the pathogen secretes host selective toxins (AAL toxins) eliciting cellular necrosis patterns characteristic of the disease (Abbas et al. 1995, Mahmoudi 2010). Application of synthetic fungicides has been considered as one of the cheapest and most common approaches for the control of plant diseases. A serious problem in the effective use of these chemicals is the development of resistance by the target microbes. Application of higher doses of chemicals to control the resistant strains, if possible, increases the hazard of high levels of toxic residues in products (Daferera et al. 2003). Thus, in the past few years, research has focused on the use of alternative control techniques (Amein et al. 2011) and natural products as plant protectants (Dayan et al. 2009). These include plant essential oils, a number of which have been reported to show antimicrobial activity against a wide array of plant pathogenic agents. Essential oils represents a potential source of antifungal compounds (Meepagala et al. 2002) with two key advantages; they are organic natural substances which denote both public health safety and environmental friendliness, and they have a low risk of resistance development by postharvest pathogens because they are often mixtures of oils with apparently different antifungal mechanisms (Sreenivasa et al. 2011).

The Lamiaceae is one of the largest plant families among the dicotyledons with global distribution, 200 genera and 2000-5000 aromatic species. *Zataria multiflora* (common name: Shiraz Oregano) is a member of this family that geographically grows only in Iran, Pakistan and Afghanistan (Ali et al. 2000, Hosseinzadeh et al. 2000) It is traditionally used for its antiseptic, anesthetic and antispasmodic properties (Hosseinzadeh et al. 2000) as well as a savor ingredient in a wide variety of food in Iran. The main constituents of its essential oil are phenolic compounds such as thymol, carvacrol (Mohagheghzadeh et al. 2004, Saleem et al. 2004), zatrinal, oleanolic acid, betulic acid, rosmarinic acid (Javidnia et al. 1999), monoterpenoids, sesquiterpenoids, p-cymene and γ-terpinene (Ali et al. 2000, Shaffiee & Javidnia 1997). In the past few decades, several studies have investigated the antimicrobial properties of *Z. multiflora* essential oil against human pathogens (Hosseinzadeh et al. 2000, Ramezani et al. 2004). However, relatively few studies have been conducted on the effects on phytopathogenic fungi (Gandomi et al. 2009, Naeini et al. 2010). Recently, the use of essential oils for biocontrol of postharvest diseases caused by *Penicillium digitutum* and *Aspergillus* sp. on stored citrus fruits has been reported (Abdollahi et al. 2011, Solaimani et al. 2009).

The aim of the present study was to evaluate the *in vitro* and *in vivo* antifungal efficacy of *Z. multiflora* essential oil to control the growth of *A. alternata*. The study also explored the effects of the essential oil on the morphogenesis of the tested fungus as well as the possible mode of action underlying the observed effects.

Materials & methods

Fungal strain and plant extraction

The strain of *A. alternata* used was obtained from the mycological herbarium of the Department of Plant Pathology, Isfahan University of Technology (Isfahan, Iran) and was maintained on Potato Dextrose Agar (PDA) medium at 25 ± 1°C. *Z. multiflora* was procured from Aren Co. (Shiraz, Iran) and had been collected from its natural habitat in Isfahan Province. For extraction, air-dried aerial plant parts were subjected to hydro-distillation for 2 h using a Clevenger-type apparatus. The extracted essential oil was analysed by gas chromatography mass spectrometry (GC–MS) (Termoques Finningan, UK). The gas chromatograph was equipped with DB5 capillary column (30 × 0.25 mm ID × 0.25 μm film thickness) and the data were acquired under the following conditions: initial temperature 50°C; program rate 2.5°C; final temperature 265°C and injector temperature 250°C. The carrier gas was helium and the split ratio was 120. The MS was run in the electron ionisation mode, using ionisation energy of 70 eV.

Poisoned food technique (PF)

The antifungal property of the essential oil against the test fungus was assessed using the poisoned food (PF) technique. PDA (20 ml) was poured into sterilised Petri dishes and different concentrations of oil (100, 200, 300, 500 ppm; 0 ppm = control) were added into the medium. After inoculation with 5 mm diameter mycelial discs cut from the periphery of a PDA culture, plates were incubated at 25 ± 1°C in the darkness and the average colony diameter was determined after 7 days. The lowest concentration which inhibited the growth of the fungus was considered as minimum inhibitory concentration (MIC). All treatments were carried out in triplicate. The fungitoxicity was expressed as percentage inhibition of mycelial growth and calculated by the formula of Pandey et al. (1982):

Percentage of mycelial growth inhibition = [(dc-dt)/dc × 100]

where dc is the average diameter of fungal colony in 0 ppm and dt denotes the average diameter of fungal colony in the treatment.

Volatile activity assay (VA)

Tests for the volatile activity (VA) of oil were performed using the inverted Petri plate method in 90 mm Petri plates containing 20 ml of solidified PDA. A 5 mm diameter disc of the test fungus was cut from the periphery of an actively growing culture, placed on the agar in each Petri plate, and the plates were kept in the inverted position. A sterilised cotton swab was placed in the upper lid of each inverted plate. Different concentrations (100, 200, 300 and 500 ppm) of oil were pipetted into the cotton swab and the plates were sealed immediately by parafilm to prevent loss of essential oil vapors. For each corresponding control, an equal amount of distilled water was pipetted into the sterilised cotton swab. The dose of essential oil was calculated as ppm volatile present in the air space in the Petri plate above the agar. The inverted Petri plates were incubated at 25 ± 1°C for 7 days. The inhibitory effect of the oil in the fumigant test was determined based on at least three replications.

Determination of mycelial weight

In order to assess the effect of essential oil on the production of mycelium by the test fungus, different volumes of the oil were added to Potato Dextrose Broth (PDB) in Erlenmeyer flasks, resulting in final concentrations of 100, 200, 300 and 500 ppm. The flasks were then inoculated with 10^6 spores ml^{-1} of *A. alternata* and incubated at 25 ± 1°C with shaking at 150 rpm. After 10 days, the cultures were filtered through Whatman filter no. 1. The mycelia were washed with distilled water and weighed after being dried at 60°C for 6 h, followed by drying at 40°C overnight. All treatments were carried out in three replications. Percent growth inhibition based on dry weight was calculated as follows:

[(Control weight − Sample weight)/Control weight] ×100

Spore germination assay

Conidial germination assessment of the test fungus was performed at five concentrations of oil (0, 100, 200, 300 and 500 ppm). Conidia from 10-day-old cultures were suspended in the different concentrations, and drops of the suspensions were placed in threefold on glass slides and incubated in a wet chamber at 25 ± 1°C for 24 h. For fixation and staining lacto-phenol-cotton blue was added to the suspensions, and the slides were observed under the microscope for conidial germination assessment. About 200 conidia were counted and the number of germinated conidia was scored under the microscope to calculate the percentage of conidial germination (Surender et al. 1987). A conidium was considered germinating if the length of the germ tube was apparently equal to that of the conidium.

Nature of toxicity of the essential oil

The fungitoxicity (fungistatic/fungicidal) of the essential oil was evaluated using the technique described by Thompson (1989). Different experiments were performed to demonstrate the toxic nature of the oil at its minimum inhibitory concentration (MIC), and hyper-MIC doses by the poisoned food technique and the volatile activity assay. After culturing on essential oil-containing plates for seven days, fungal discs were cut from the plates, washed with sterilised water and re-inoculated separately into Petri plates containing fresh medium. After seven days of incubation revival of growth was assessed based on the presence or absence of mycelial growth.

Mechanism of toxicity of essential oil: light microscopy

Following a seven-day incubation period, a sample of *A. alternata* mycelium was taken from the periphery of a colony growing on PDA amended with 300 ppm of essential oil. The samples were fixed in lactophenol-cotton blue stain and observed under the microscope (Nikon YS100, Japan) at 400 × magnification to examine the hyphal morphology. Samples from control plates without oil were also stained and observed. Photographs were taken with the help of a computer attached Canon Color Camera.

Effect of oil on decay of tomato fruits caused by *A. alternata*

In order to evaluate the effect of the oil on *A. alternata in vivo*, tomato fruits were washed, surface-disinfected with 90% ethanol, and after air drying, cuts were made on the fruits surfaces, measuring 5 mm in length and 0.5 mm in depth, using a sterile scalpel. Then, 50 µl aliquots of 100, 300 or 500 ppm of oil or sterile distilled water (as control) were pipetted into each wound. Four similar fruits were used in each inoculation assay. After 0.5 h, 20 µl of conidial suspension of *A. alternata* (5×10^4 spores ml^{-1}) were pipetted into each wound and the wounds were sealed with adhesive plastic tape. Treated tomatoes were then kept in the growth chamber at 20°C with 85% relative humidity. After five days fungal growth was assessed by measuring the diameter of the lesion.

Statistical analyses

Statistical analyses of the data were performed by variance analysis (ANOVA) using the SPSS commercial statistical package (SPSS, Version 11.5 for Windows) and differences among the means were analysed for significance at $P \leq 0.05$ using Least Significant Difference (LSD) with three replications for each treatments.

Results

Extraction of the essential oil from aerial parts of *Z. multiflora* by hydro-distillation yielded 1.9% essential oil. The oil consisted of 14 compounds of which carvacrol accounted for 76.1% of the total constituents. Out of 14 components, 13 were identified with a typical library search match exceeding 94%. Besides carvacrol, other significant components detected were γ-terpinene, eucalyptol, borneol, α-pinene and globulol (Table 1).

The effect of different concentrations of *Z. multiflora* oil on radial growth was studied in poisoned food (PF) and volatile activity fumigation tests. The results are presented in Table 2. The growth of the fungus was completely inhibited in PF at 500 ppm concentration of oil. In the direct contact test, the oil showed its fungicidal nature at 500 ppm by killing the fungus (Table 2). The VA assessment revealed that

Table 1: Components of *Zataria multiflora* essential oil identified by GC-MS analysis.

Peak no.	Components	Percentage in total oil
1	Borneol	2.78
2	α-Thujene	0.23
3	α-Pinene	2.44
4	β-Pinene	0.81
5	β-Myrcene	0.77
6	Eucalyptol	3.21
7	Linalool	1.56
8	γ-Terpinene	6.23
9	Thymol methyl ether	0.33
10	Carvacrol	76.12
11	β-Caryophyllene	1.09
12	Globulol	1.78
13	Thymol	0.89
14	Unidentified	1.28

Table 2: *In vitro* efficacy of different concentrations of *Z. multiflora* oil on mycelial growth and conidial germination of *A. alternata* after 7 days of incubation at 25 ± 1°C. Means of two experiments with four replications per treatment.

Conc. [ppm]	Inhibition [%]			Type of inhibition (S = fungistatic, C = fungitoxic)	
	Mycelial growth		Conidial germination		
	Poisened food technique	Volatile activity assay		Mycelial growth	Volatile activity
100	37.5	NT	31.4	NT*	NT
200	63	59.3	63	S	S
300	87.7	87.6	90.2	S	C
500	100	100	100	C	C

* Not tested

the essential oil at the level of 200 ppm completely prevented the fungal development after 7 days of incubation. The experiments on the toxic nature of the oil demonstrated that the oil was fungicidal at 500 ppm. In the PF test, the MIC was 500 ppm whereas in VA it was 200 ppm. Results revealed that the oil caused 100% inhibition of conidial germination at the 500 ppm concentration (Table 2).

In the mycelial dry weight assay it was found that concentrations of 300, 200 and 100 ppm caused a clear reduction of mycelial weight. Treatment with 500 ppm essential oil resulted in complete inhibition of mycelial growth (Table 3). The percent inhibition of dry weight formation in liquid culture (Table 3) and of mycelial growth on plates (Table 2) caused by the tested concentrations of the oil were similar.

The results of the observations under the light microscope (400x magnification), following treatment of *A. alternata* with 300 ppm of *Z. multiflora* essential oil are presented in Fig. 1. In addition to inhibited growth, mycelial colonies grown in the presence of essential oil showed distinct morphological changes as compared to the control. These variations included a significant decrease and loss of conidiation, and visible loss of pigmentation in the medium. Microscopic examination of untreated mycelium showed normal hyphae with homogenous, profuse conidiation and healthy conidia with long germ tubes (Fig. 1A, B). Treatment with 300 ppm of essential oil caused a significant reduction of conidiation, inhibition of conidial germination and a shortening of germ tubes (Fig. 1C). Furthermore, in some replications, there was a complete absence of conidiation. Mycelium treated with 300 ppm essential oil showed anomalies in the hyphae such as reduction in hyphal diameter, collapse, wrinkling and a granulation in the cytoplasm (Fig. 1D). One of the interesting findings of this study was that an extreme amount of chlamydoconidia was produced in old hyphae (Fig. 1E). Occasionally, the hyphae were very thin with sub-apical germina-

Table 3: Effect of different concentrations of *Z. multiflora* oil on dry weight of *A. alternata* produced after 10 days of cultivation in Erlenmeyer flasks at 25 ± 1°C. Means and standard error of four replications.

Conc. of oil [ppm]	Dry weight [mg ± SE]	Growth inhibition [%]
0	287.7 ± 2.3	0
100	162.5 ± 1.2	41.3
200	108.1 ± 2.0	62.4
300	72.3 ± 1.0	74.9
500	0	100.0

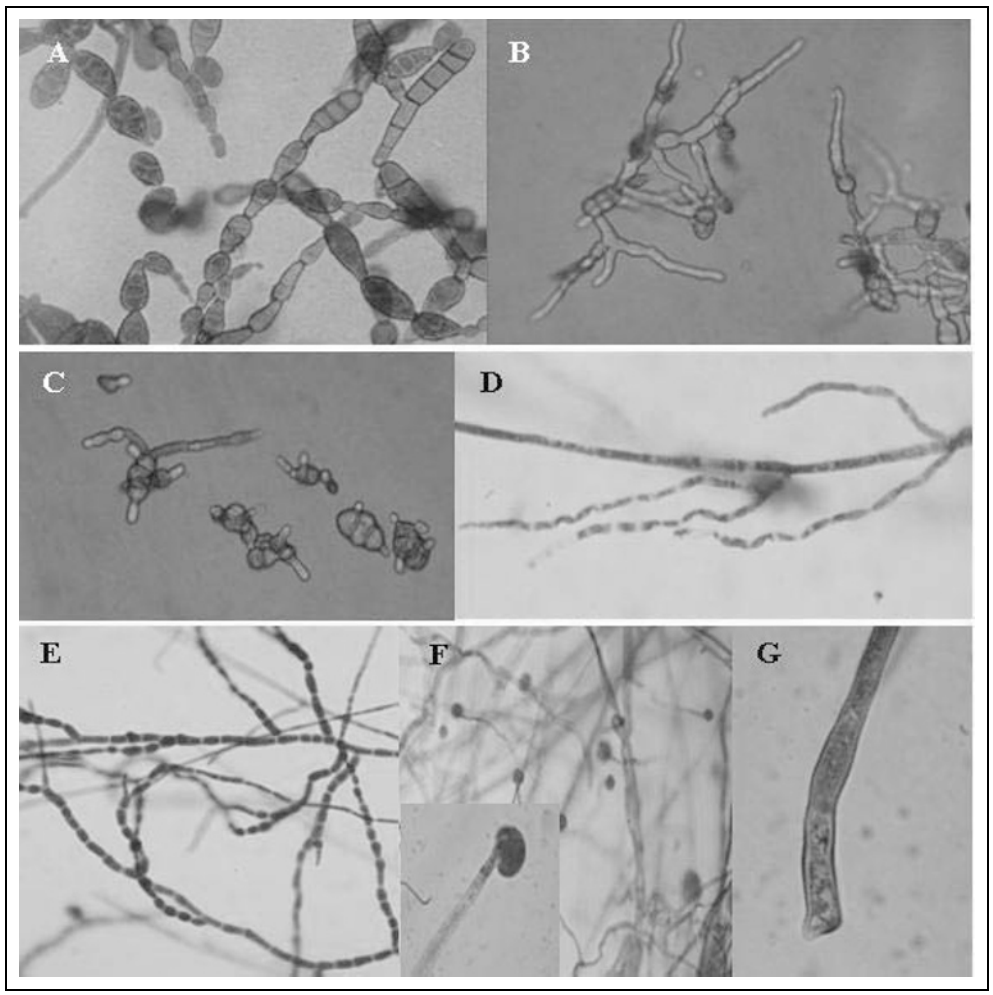

Fig. 1: Light microphotographs of *A. alternata* hyphae growing on PDA with or without *Z. multiflora* essential oil (300 ppm) after 7 days of incubation at 25 ± 1°C. *A. alternata* control conidiation and conidia germination, respectively (A, B). Treatment with 300 ppm of essential oil severely inhibited conidia germination (C); hyphae became wrinkled and cytoplasm became condensed and granulated (D); abundant formation of chlamydoconidia (E); bulbous anomalous structures at hyphal tip (F); inhibition of tip growth in hyphae (G).

tion and showed bulbous anomalous structures at their tips (Fig. 1F, G).

The results of the *in vivo* assay in Fig. 2 show that when wounded tomatoes were treated with *Z. multiflora* oil, all concentrations (except 100 ppm) significantly prevented damage by *A. alternata* in tomatoes stored at 20°C for 5 days. The percentage of decayed tomatoes treated by 500 ppm oil was reduced by 60%, as compared to the control. Treatment with Shiraz oregano oil did not cause any visible disorders and off-odor to the fruits after 5 days of storage.

Discussion

The present study showed that in addition to carvacrol as the major component, significant amounts of other compounds like γ-terpinene, β-myrcene, eucalyptol, borneol, β-caryophyllene and linalool were also present in *Z. multiflora* essential oil and it appears plausible that the antifungal activity of the oil arises from the synergistic activity of all components of the oil, with carvacrol as the major component. The observed findings suggest that *Z. multiflora* essential oil has mycotoxic potential and provide detailed information on the antifungal activity of this agent against *A. alternata*. The minimum inhibitory concentration determined by the poisoned food technique was 500 ppm, and volatile inhibitory activity was observed for all tested concentrations. The fungicidal nature of the oil indicates its potential economic exploitation as fungitoxicant which has not been reported before (Basti et al. 2007). Keeping in view the residual toxicity and the side effects of synthetic fungicides, the essential oil of *Z. multiflora* may be used in plant protection to control diseases caused by *A. alternata*, such as post harvest decay of tomato.

The fungicidal concentrations of essential oil of *Z. multiflora* in the poisoned food technique and volatile activity assay were 500 ppm and 200 ppm, respectively for *A. alternata*. Shiraz Oregano oil appeared to be more effective in the volatile phase, which is supported by the comparatively low MIC values in the volatile activity assay. Inhibitory effects of essential oils against mycelial growth (Sreenivasa et al. 2011) and sporulation of different fungi have been reported (Naeini et al. 2010, Tripathi et al. 2009). Tataoui-Elaraki et al. (1993) studied the effect of three Moroccan essential oils on fungal asexual reproduction stages including spore production. They reported that the partial inhibition of spore production could be attributed to mycelial destruction or inhibition of fungal growth. Mahanta et al. (2007) suggested that the impact of *Cymbopogon citratus* essential oil on sporulation may reflect the effects of volatile compounds emitted by the oil on the surface of developing mycelia. According to the results of the present study, it can be concluded that *Z. multiflora* essential oil is very effective in inhibiting spore production and mycelial growth. At 300 ppm concentration sporulation was completely repressed. Reduction of spore production could limit the spread of pathogens by lowering the spore load in the storage atmosphere and on surfaces. The level of 200 ppm appears to be sufficient to check the conidial germination of *A. alternata*. This concentration is lower than that of many other essential oils which were previously reported (Abdollahi et al. 2011, Gandomi et al. 2009). Soliman & Badeaa (2002) reported complete inhibition of the spore germination of *Aspergillus flavus*, *A. parasiticus* and *A. ochraceus* by the oils of thyme and cinnamon (< 500 ppm), marigold (< 2000 ppm), spearmint and basil (3000 ppm). Our light microscopic studies revealed that mycelium treated with oil was altered in the morphology of hyphae including severely squashed and collapsed hyphae, loss of conidiation and degradation of the cytoplasm. There are some reports indicating that the antifungal property of essential oils can lead to morphological and metabolic changes in the fungus such as cytoplasm granulation, cytoplasm membrane rupturing, cytoplasm hyperacidity, and breakdown of the electron transport chain (Gandomi et al. 2009, Tripathi et al. 2009). To our knowledge, no comparative studies have been carried out yet on growth, conidiation and morphology of *A. alternata* in an essential oil-containing medium. In this research *Z. multiflora* essential oil caused severe damage and alterations to vegetative hyphae which may result in complete destruction of cytoplasm and inhibition of tip growth in hyphae.

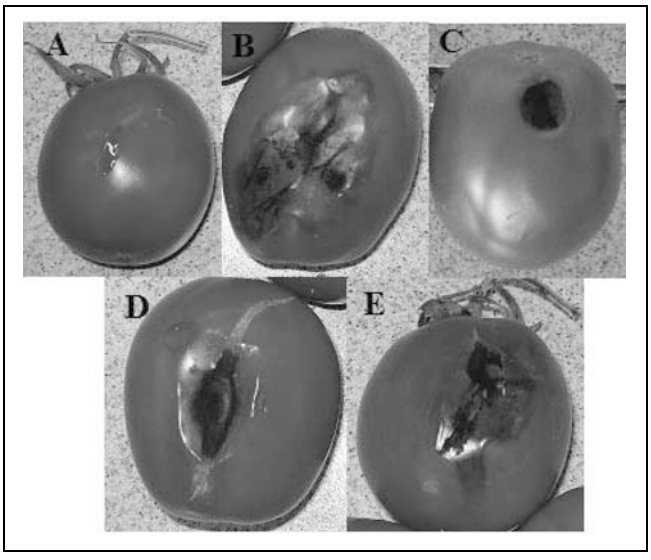

Fig. 2: Reduction of symptoms of *A. alternata* decay on wounded tomato fruits by different concentrations of *Zatari multiflora* essential oil: (A) un-inoculated fruit, (B) 0 ppm, (C) 500 ppm, (D) 300 ppm and (E) 100 ppm.

Most of the essential oils have been reported to inhibit postharvest fungi *in vitro* (Singh & Tripathi 1999). However, efficacy *in vivo* has only been shown for a few of the essential oils (Abdollahi et al. 2011). The results of this study showed that essential oil significantly reduced the rot in wounded tomato fruits. Whether the effective concentrations of *Z. multiflora* oil would affect the flavor of tomatoes is not known and requires more work on the quality parameters of treated tomatoes.

In conclusion, the results of the present study show that *Z. multiflora* essential oil possesses antifungal activity against *A. alternata* and the *in vivo* experiment shows that Shiraz oregano oil has the potential to reduce postharvest decay of tomatoes caused by *A. alternata*.

Acknowledgment

This research was financially supported by a grant (research project no. 362-1388/11/13) from the Research Council of the Khorasgan Branch, Islamic Azad University, Isfahan, Iran.

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