Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.8, No.4, 2018

Synergistic Activity of Leaves Extracts of *Mansoa alliacea* L. and *Allamanda cathartica* L. to Inhibit *Athelia rolfsii*, the Cause of Stem Rot Disease in Peanut Plants

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Abstract
Stem rot disease in peanut plants caused by *Athelia rolfsii* is an important disease because it can reduce peanut yield up to 13-59%. The study aimed to evaluate the synergistic activity of the leaves extracts of *Mansoa alliacea* L. and *Allamanda cathartica* as an alternative control of stem rot disease in peanut plants. Testing of Antifungal activity was carried out in the laboratory included: diameter of inhibition zone test with diffusion well method; colony growth test, sclerotia germination test, fungal biomass test, and ultrastructure observation using scanning electron microscope (SEM). The results showed that the crude extracts of the leaves of *M. alliacea* and *A. cathartica* were able synergistically to inhibit the growth of the fungus *A. rolfsii* with diameter of inhibition by 42 mm, while the diameter of inhibition zones for leaf extract of *M. alliacea* L. was only 30 mm and as for *A. cathartica* L. was only 9 mm. Minimum inhibitory concentration (MIC) of the mixture of leaves extracts of *Mansoa alliacea* L. and *A. cathartica* L. was 0.8% (w/v). Treatment with the extract's mixture significantly (P≤0,05) inhibited colony growth, germination rate of sclerotia, and fungal biomass of *A. rolfsii*. The result of mycelial observation of *A. rolfsii* by using SEM showed that surface of fungal mycelia treated with extract looked smaller in size and shrunk, and leakage of cell wall became visible; while at the control, the surface of mycelia remained intact and thrived perfectly.
Keywords: synergistic activity, leaves extract, stem rot disease

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important commodity that is widely cultivated as one source of the farmer's incomes, source of nutrition and raw materials for food processing industry (Parmer *et al*., 2015; Doley and Jite, 2013). Indonesia's peanut yield that averages 1.30 tons/ha is lower than the average peanut of 1.67 tons/ha. While China is the largest producer of peanuts in the world whose average yiled is 3.59 tons/ha (Center for Agricultural Information System and Data Ministry of Agriculture of Indonesia, 2016). Thiessen and Woodward (2012) stated one of the causes of low peanut yield is pests and diseases. Disease that often occurs on peanut plants is stem rot disease, which is caused by the soil pathogenic fungi *Athelia rolfsii* and *Sclerotium rolfsii*. Kator *et al*. (2015) stated the main pathogen of stem rot disease in tomato plants is *Athelia rolfsii*, which is also causes root rot and fruit rot on tomato plants. Rahayu (2015) stated that high economic losses due to stem rot disease is reported to occur in most countries including Indonesia. Remesal and Lucena (2010) stated that these two fungi are synonymous, *Athelia rolfsii* is a telemorphic (sexual) form of *Sclerotium rolfsii* which has a very close relationship, in which the DNA sequence similarity of the two was 99%.

Preliminary survei results of February 2016 at four peanut planting sites in two regencies in Bali, Klungkung and Badung, found a different percentage of stem rot disease incidences at each observation site. The highest percentage of stem rot disease occurred in Buduk Village of Badung Regency which was about 28%. Madhuri and Gayathri (2014) report that *Athelia rolfsii* and *Sclerotium rolfsii* have a sclerotia defense structure that can survive for years in the soil, when a suitable host plant and supported by environmental factors can infect the plant, making it difficult to control. While Elias *et al.* (2015) stated that *Athelia rolfsii* and *Sclerotium rolfsii* are capable of producing the extracellular enzymes used to attack and infect host plants.

One of the controls that can be applied to minimize the impacts of synthetic pesticides, ang thought to be environmentally friendly and safe for human is the use of botanical pesticides. Several researches on the utilization of plants as botanical pesticide have been done been done among others: Russo *et al.* (2013) reported that essential oils from *Thymus capitatus* plants can inhibit 100% of the mycelial growth of the fungus *Sclerotium cepivorum*. Rongai *et al.* (2012) reported that 500 extracts of sub-tropical plant species that have been tested against soil pathogenic fungus i.e. *Fusarium oxysporum*, indicated that only 3% showed strong inhibition and the rest were not able to inhibit the fungus. It is expected that only 10% of the Indonesian plant diversities

have been studied (Suprapta, 2014; Asmaliyah *et al.*, 2010). Ornamental plants are part of the biodiversity that holds the potential as a botanical fungicide, among others: *Mansoa alliacea* L. and *Allamanda cathartica* L. are an ornamental plant that grows crawling on the fences and trees. According to Guilhon *et al*. (2012) methanol and hexane extracts from *Mansoa alliacea* leaves show antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. While Essiett and Udo (2015) report that stems, leaves, and flowers of alamanda contain tannins, flavonoids, saponins, alkaloids, with the highest content found in leaves that have potential as antimicrobial. Therefore, this study was conducted to investigate the synergistic activity of the leaves extracts of *Mansoa alliacea* L. and *Allamanda cathartica* L. as a botanical fungicide to control the fungus *Athelia rolfsii*, the cause of stem rot diseases in peanut plants.

2. Research Methods

2.1. Preparation of plant extracts

The leaves of plants used as extracts, namely leaves of *Mansoa alliacea* L. were taken from Sanglah Hospital's garden Denpasar and Hotel Putri Ayu's garden Denpasar. While *Allamanda cathartica* L. leaves were taken from Gitgit Village Sukasada District Buleleng Regency Bali. The leaves of *M. alliacea* and *A. cathartica* plants used were mature leaves. The leaves that had been taken were cut into small pieces then chopped, and dried under room temperature for 5-6 days. The material was then blended until it became powder, then it was macerated with acetone solvent for 72 hours. Every 24 hours, it was filtered; the filtrate obtained from each filtering result was combined, then the solvent was evaporated with a vaccum rotary evaporator (Iwaki, Tokyo) at 40^0C. The crude extract obtained were used for further tests.

2.2. Isolate of Fungus

Isolate of fungus was isolated directly from peanut plants that showed symptoms of stem rot diseases and was confirmed as the causal agent of stem rot disease on peanut plant. The pure isolate obtained was identified macroscopically, microscopically and molecularly based on analysis of 16S rDNA. This work was done at Microbiology Laboratory of Biology Study Program, Faculty of Mathematics and Natural Sciences, Bogor Agricultural Institute, West Java, Indonesia. The isolate was identified as *Athelia rolfsii.*

2.3. Test of antifungal activity and minimum inhibitory concentration (MIC)

Antifungal activity testof crude extract of the leaves of *M. alliacea* and *A. cathartica* against the fungus *A. rolfsii* was done by the diffusion well method, singly on each extract and mixture on both extracts. The test for the mixture of both extracts was done with a ratio of 2: 1 (*M. alliacea* : *A. cathartica*). One milliliter of the fungal suspension of *A. rolsii* was poured into a sterile Petri dish, added with 10 ml of PDA medium (at temperatura of 45°C), mixed evenly and allowed to solidify. After becoming solid, each Petri dish was provided with two diffusion wells using cork borer (5 mm diameter). Each well was filled with 20 µl of crude extract, then incubated at room temperature (25°C). Observations were made daily by measuring the diameter of the inhibition zone formed around the diffusion well.

The test to determine minimum inhibitory concentration (MIC) was done by diffusion well method, by testing some concentrations of the mixture of the crude extracts of *M. alliacea* and *A. cathartica* leaves: 0.1%, 0.2%, 0.3%, 0, 4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5%, 2% (w/v) and control (the well was filled with sterile wáter).

2.4. Test of the antifungal activity against the growth of the fungal colony of Athelia rolfsii

Testing of antifungal activity against fungal colony growth used several concentrations of the leaves crude extract of *M. alliacea* and *A. cathartica* i.e.: 0% (control), 0.4%, 0.8%, 1.5%, 2%, and 2. 5%. The test was performed singly on each extract and mixture on both extracts. Each extract concentration was poured into sterile Petri dish, coupled with PDA medium (temperature around 45°C) according to the treatment concentration, wiggled for the extract to be mixed evenly with medium, then it was allowed to solidify. After being solid and the fungal colony of *A. rolfsii* of 5 days old that was then taken with cork borer (5 mm diameter) placed right in the bowl of Petri dish by using ose needle. Next it was incubated at room temperature (25°C), until the fungus on the control fully filled the Petri dish. The experimental design used was a complete randomized design (RAL) with 6 treatments and 4 replications. Observations were made by measuring the diameter of fungal colonies in each treatment. The formula to calculate the inhibitory activity (IA) of the extract treatments on the growth of fungal colonies is as follows:

$$\text{IA}(\%) = \frac{\text{Diameter of fungal colony of control} - \text{Diameter of fungal colony of treatment}}{\text{Diameter of fungal colony of control}} \text{ x } 100\%$$

2.5. Sclerotia germination inhibition test

Testing of sclerotia germination of the fungus *Athelia rolfsii* was done by using some concentrations of crude

leaves extract mixture of *M. alliacea* and *A. cathartica* i.e.: 0% (control), 0.1%, 0.2%, 0.3%, 0.4 %, 0.5%, 1%, 1.5%, 2% and 2.5%. Five milliliter potato dextrosa broth (PDB) medium was poured into steril Petri dish, then each extract was added according to the concentration, inoculated with 10 sclerotia. The experimental design used was completely randomized design (CRD) with 9 treatments and 3 replications.

Observation was done every day by counting the number of sclerotia germinated, if on the control of all sclerotia already germinated then the observation could be stopped. Inhibitory activity of extracts on germination of sclerotia could be calculated by comparing the amount of germinated sclerotia in the extracted and controlled medium. The formula for calculating the inhibitory activity (IA) of the extract treatment on sclerotia germination is as follows:

$$\text{IA}(\%) = \frac{\text{A - B}}{\text{A}} \text{ x } 100\%$$

Where A = Number of germinated sclerotia of control
B = NUmber of germinated sclerotia of treatment.

2.6. Examination of the extract effect on fungal biomass

Fungal biomass testing was performed using several concentrations of crude leaves extracts mixture of *M. alliacea* and *A. cathartica*, i.e : 0% (control), 0.1%, 0.2%, 0.3%, 0.4%, 0, 5%, 1%, 1.5%, 2% and 2.5%. Each extract concentration was put into an Erlenmeyer flask (100 ml) containing PDB medium according to the concentration of the extract, then each was added with 1 ml of the suspension of *A. rolfsii*, then incubated in the shaker for 8 days. Harvesting was done by filtering each fungal mycelia that grew, then dried in the oven and weighed. The design used was completely randomized design (CRD) with 6 treatments and 4 replications. The inhibitory activity (IA) of the extract on biomass was calculated using the following formula:

$$\text{IA}(\%) = \frac{\text{Fungal biomass of control - Fungal biomass of treatment}}{\text{Fungal biomass of control}} \text{ x } 100\%$$

2.7. Observation of the ultra-structural of hyphae of Athelia rolfsii

The preparation procedure for SEM (scanning electron microscope) is an modification of the method of Kawuri *et al.* (2012). Erlemeyer (100 ml) containing PDB medium was included in a leaves extract mixture of *M. alliacea* and *A. cathartica* at the oncentrations of 0.1%, 0.2%, 0.5%, 1% and 0% (control), then 1 ml of the *A. rolfsii* spore's suspension, and incubated in the shaker for a week. After a week the growing fungal mycelia was strained with gauze, then dried in the oven.

Fungal mycelia was then fixed with a 2% glutaraldehyde solution in 0.1 M sodium cacodylate (pH 7.2) buffer at 4°C for 4 hours, then it was kept at 28°C for an hour. The fixed sample was washed with sodium cacodylate buffer (pH 7.2) and fixation was continued with 1% osmium teroxide in 0.1 M cacodylate buffer and settled at room temperature (25^{o}C) for 5 hours. After that the sample was washed with distilled water, followed by dehydration using ethyl alcohol in series (40%, 60%, 80% and 100%). After the dehydration process the sample was cut with freeze cutting device (TF-2, Eiko, japan), then spilled with t-butylalcohol solution, and placed in vacuum freeze-drying (ID-2, Eiko, Japan). The dried sample was coated with osmium tetroxide (OPC 60A, Filgen, Japan) and platinum (JUC-5000, JEOL, Japan). The coated sample was observed with SEM using JSM-6510LA, JEOL, Japan with acceleration voltage 5 kV.

3. Results and Discussion

The results showed that the mixture of leaves extractd of *M. alliacea* and *A. cathartica* with 2: 1 ratio could inhibit the growth of *A. rolfsii* which was shown by the formation of a larger inhibitory zone diameter (42 mm) compared with the single test of each extract with the diameter respectively 30 mm and 9 mm for *M. alliacea* and *A. cathartica* (Figure 1). According to Paudel *et al.* (2014) if the diameter of the inhibition zone was formed ≥20 mm it meant very strong inhibitory activity, 10-20 mm strong inhibitory activity, 5-10 mm moderate inhibitory activity, and ≤5 mm inert inhibitory activity. Rivera *et al.* (2015) stated that the mixture of three plant extracts namely *Larrea tridentate, Flourensia cernua* and *Opuntia ficus-indica* with a ratio of 1: 1: 1 could inhibit the growth of pathogenic bacteria in food with a higher diameter of inhibition zone (30.13 mm) compared with treatment with signle extract. The results of this study were supported by Akinnibosum and Edionwe (2015) in which inhibitory activity of single leaf extract of *Bryophyllum pinnatum* and *Citrus aurantifolia* against *Staphylococcus aureus* bacteria resulted in the diameter of inhibition zone respectively 10.7 mm and 15.3 mm, but after both of the extracts were mixed, the diameter of the inhibition zone became higher, that was 22.7 mm.

In contrast to the results of research by Sneha *et al.* (2016) that a mixture of *Allium sativum* and *Zingiber officinale* extracts with a 2: 1 ratio tested against *Sclerotium rolfsii* became smaller by 23%, compared with a single test of higher inhibition zone respectively 100% for *Allium sativum* extract and 51.50% for *Zingiber*

officinale, while the treatment with a ratio of 1: 2 inhibitory activity became lost (0%). This proves that the active compounds present in the plant extract mixture may work synergistically against pathogenic fungi, which can further suppress the growth and development of the pathogen, but the active compounds in the extract mixture may also work antagonistically to push each other between one and the other so that the inhibitory activity becomes decreased or lost.

Figure 1. Inhibitory activity of (a) a mixture of leaves extracts of *M. alliacea* and *A. cathartica*, (b) leaf extract of *M. alliacea* and (c) leaf extract of *A. cathartica* against *Athelia rolfsii* on PDA médium. 1= Inhibition zone, 2= diffusion well containing extract

The minimum inhibitory concentration (MIC) test showed that the smallest concentration of the mixture of leaves extracts of *M. alliacea* and *A. cathartica* that could inhibit the growth of *A. rolfsii* was 0.8% (w/v). Guilhon *et al.* (2012) stated that methanolic extract of *Mansoa difficilis* could inhibit the growth of *Candida albicans* CCMB 266 with MIC by 0.125%.

Results of our present study showed that the mixture of the leaves extracts of *M. alliacea* and *A. cathartica* significantly (P≤0,05) could inhibit the growth of *A. rolfsii* i.e. the growth of mycelia, germination of sclerotia and biomass, as presented in Tables 1, 2, and 3. The higher the mixture concentration, the higher the inhibitory activity is. The mixture concentration of 0.4% extract (w/v) has been able to inhibit the growth of mycelia of *A. rolfsii* with an inhibitory activity of 34.20%. The concentration of extract of 1% (w/v) has been able to inhibit germination of sclerotia and fungal biomass with 100% inhibitory activity. Tables 1 and 2 show that at the same concentration of extract, there is a difference of inhibitory activity between the single test and the crude extract mixture of leaves of *M. alliacea* and *A. cathartica*. The mean inhibitory activity becomes larger in the test with two extracts compared with single extract.

Table 1. Inhibitory activity of the leaf extract of *M. alliacea* and the leaf extract of *A.cathartica* against the growth of colony of *A. rolfsii*

Extract concentration (%)(w/v)	Diameter of colony (mm)		Inhibitory activity (%)	
	Mansoa alliacea	*Allamanda cathartica*	*Mansoa alliacea*	*Allamanda cathartica*
0 (control)	84^{a*}	84^{a*}	-	-
0.4	74.12^{b}	81^{a}	11.76%	3.57%
0.8	63.87^{c}	76.60^{b}	23.96%	8.80%
1.5	57.12^{d}	68.75^{b}	32%	18.15%
2	48.62^{e}	62.25^{c}	42.11%	25.89%
2.5	29.87^{f}	47.25^{d}	64.44%	43.75%

*Values with the same letters in the same coloumn are not significantly different according to the Duncan's Multipple Range Test at 5% level.

Table 2. Inhibitory activity of the mixture of leaves extracts of *M. alliacea* and *A. cathartica* against the colony diameter of *A. rolfsii*

Extract consentrtion (%)(w/v)	Colony diameter (mm)	Inhibitory activity (%)
0 (control)	84^{a*}	-
0.4	55.25^{b}	34.20%
0.8	36.25^{c}	56.84 %
1.5	17.62^{d}	79.02%
2	0^{e}	100%
2.5	0^{e}	100%

*Values with the same letters in the same coloumn are not significantly different according to the Duncan's Multipple Range Test at 5% level.

According to Baljeet *et al.* (2015) the mixture of *Cuminum cyminum* and *Zingiber officinale* extracts inhibited *Salmonella typhi* bacteria to be higher with 18.1 mm inhibition zone diameter compared to the single

test of 13.8 mm and 11.1 mm, respectively. Meanwhile, Darvin (2013) stated that crude extract of *Ocinum sanctum* leaf and *Annona squamosa* could inhibit the growth of mycelia of *Athelia rolfsii* which causes stem rot in peanut plants with inhibitory activites of 23.70% and 29.30 %, besides it could also inhibit germination of sclerotia with inhibitory activities respectively 48.70% and 15.93%. Iqbal and Javaid (2015) found that the methanol leaves extract and roots of *Coronopus didymus* plant at 15 mg / ml concentrations significantly reduced the biomass of *Sclerotium rolfsii* with 67% and 58% inhibitory activities respectively for leaf root.

Table 3. Inhibitory activity of the mixture of leaves extracts of *M. alliacea* and *A. cathartica* against germination rate of sclerotia and biomass of *A. rolfsii*

Extract concentrtion (%)(w/v)	sclerotial germination (grain)	Inhibitory activity (%)	Biomass (g/100 ml)	Inhibitory activity (%)
0 (control)	10^{a*}	-	1.39^{a*}	-
0.1	10^{a}	0%	0.98^{b}	29.49%
0.2	10^{a}	0%	0.81^{c}	41.72%
0.3	9^{a}	10%	0.62^{d}	55.39%
0.4	6.67^{b}	33.33%	0.40^{e}	71.22%
0.5	6^{c}	40%	0.28^{e}	79.85%
1	0^{d}	100%	0^{f}	100%
1.5	0^{d}	100%	0^{f}	100%
2	0^{d}	100%	0^{f}	100%
2.5	0^{d}	100%	0^{f}	100%

*Values with the same letters in the same coloumn are not significantly different according to the Duncan's Multipple Range Test at 5% level.

Devi *et al.* (2017) reported the antifungal activity of *Duranta erecta* leaf extract against the growth of *Sclerotium rolfsii* with the inhibitory activity of 45.29%. Other study done by Sethi *et al.* (2015) found that *Alpinia allughas* leaf extract at 750 µg / ml could inhibit the mycelia of three soil fungi namely *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia sclerotium* with inhibitory activities of 87.5%, 54.16% and 86.66%, respectively. This is because the extract contains antifungal compounds with the highest content of phenol 43.70 µg / ml and also contains antioxidants. Suprapta and Khalimi (2009) stated that a formula containing 5% w/v extract of clove flower (*Eugenia aromatica*), 5% (w/v) *Piper betle* leaves, 5% (v / v) tween-80 and sterile water, were able to inhibit the growth of *Fusarium oxysporum* f.sp.*vanillae* on field scale with 92.3% inhibitory. Towne *et al.* (2015) reported that *Mansoa aliaceae* leaf water extract significantly reduced tumor cell growth in mice test. While Razu and Hossain (2015) reported that rice plants inoculated with *Curvularia lunata*, after being treated with *Allamanda cathartica* leaf extract could reduce the percentage of disease from 10.96% in control to 3.46% in treatment. The observation of the ultra structural of mycelia of *Athelia rolfsii* using SEM showed that there was a difference between the mycelial surface of control and the treatment (Figure 2).

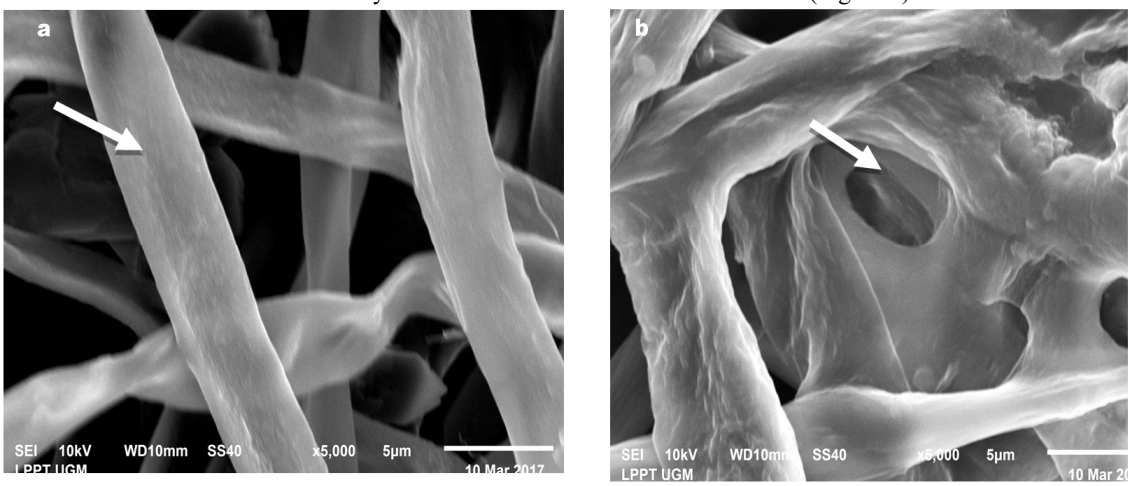

Figure 2. Scanning electron microscope photographs of the mycelia of *Athelia rolfsii* a. Control (without extract treatment), b. Treatment with extract (5000x magnification)

The size of fungal mycelia treated with extract was smaller, shrinking with visible leakage of the cell wall, whereas on the control of the mycelial surface remained intact and grew perfectly. Patel *et al.* (2013) reported that the leaf methanol extract, root and plant skin of *Mansoa alliaceae* contains active compound with the highest content of phenol (16.20 mg / g) found in root and plavonoid at root (3.27 mg / g). While Prabhadevi *et al*. (2012) reported that *Allamanda chatartica* ethanol extract contains active compound of plavonoid,

diterpenoid and triterpenoid which served as antifungal, pesticide, anticancer and antibacterial.

Upadhyay *et al.* (2014) reported that the active compounds of phenol and flavonoids effectively inhibited the growth of fungi and bacteria; and the mode of action of flavonoids damages the permeability of fungal or bacterial cell walls, and phenol capable of denaturing the cell wall building proteins that can damage the cell wall. Damage to the permeability of the cell wall causes the contents of the cell to go out, resulting in cell leakage. While the phenolic compounds can inhibit growth and disrupt the process of membrane formation of cell walls, so the cell wall membranes are not formed or formed imperfectly.

4. Conclusion

The crude leaves extract of *M. alliacea* and *A. cathartica* in a mixture synergistically inhibited the growth of *A. rolfsii* the cause of rot disease on peanut. Minimum inhibitory concentration of this extract mixture is 0.8% (w/v). Treatment with the extract mixture significantly could inhibit the growth of fungal colony, germination of sclerotia and fungal biomass. The size of fungal mycelia treated with the extract is smaller, shrank and leakage of cell walls is visible, while mycelia of control (without extract treatment) remained intact and thrived perfectly, suggested that this extract mixture is a promising agent to control stem rot disease on peanut.

Acknowledgement

Authors would like to thanks Laboratory of Biopesticide Faculty of Agriculture Udayana University for all the support and facilities that had been provided for during the research.

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