

ORIGINAL ARTICLE

Genetic identification of an invasive plant (*Parthenium hysterophorus*) and its crude extract phytotoxicity against palm oil weed (*Diodia ocimifolia*)

Noorul Jannah Zainuddin^{1*}, Norazlinaliza Salim^{3*}, Norhayu Asib², Siti Efliza Ashari³, Dzolkhifli Omar², Gwendoline Cheng Lian⁴

¹ Department of Chemical Technology and Food, Politeknik Tun Syed Nasir Syed Ismail, Johor, Malaysia

² Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia

³ Integrated Chemical Biophysics Research, Faculty of Science, Universiti Putra Malaysia, Selangor, Malaysia

⁴ Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, Selangor, Malaysia

Vol. 62, No. 2: 223–229, 2022

DOI: 10.24425/jppr.2022.141361

Received: January 13, 2022

Accepted: March 30, 2022

Online publication: June 29, 2022

*Corresponding address:
jannahzainuddin@ptsn.edu.my
azlinalizas@upm.edu.my

Responsible Editor:
Łukasz Sobiech

Abstract

This paper reports a study on genetic identification of *Parthenium hysterophorus* and the ability of *P. hysterophorus* crude extract (PHCE) to inhibit germination of weeds in palm oil plantations, particularly slender buttonweed (*Diodia ocimifolia*). *Parthenium hysterophorus* is a noxious plant with invasive properties. It has become one of the world's seven most devastating and hazardous weed-s due to its allelopathic properties. However, a comprehensive study on its genetic identification and its herbicidal activity against weeds in palm oil plantations had never been carried out. In the present study, internal transcribes spacer (ITS) region-based analysis was used as a molecular marker to ascertain the local Rumpit Miang Mexico (RMM) as a *P. hysterophorus* species. From the results, the DNA sequence obtained was 99% matched with *P. hysterophorus* species database. In the dose-response bioassay study, the phytotoxicity of PHCE on *D. ocimifolia* was simulated under laboratory conditions. Methanolic extract of *P. hysterophorus* significantly inhibited germination and growth of shoots and radicles of *D. ocimifolia* at low concentrations (1–5 g · l⁻¹). A study on the herbicidal activity of PHCE could be an additional management approach for this phytotoxic species and an alternative way to naturally control weeds on palm oil plantations.

Keywords: DNA profiling, green weed management, herbicidal activity

Introduction

Palm oil is an important crop in Southeast Asia especially in Malaysia, Indonesia and Thailand with the production of 36% of the total world vegetable oil production (Woittiez *et al.* 2017). However, strong competition from weeds has been reported and can decrease the productivity of palm oil in the range of 6 to 20% (Dilipkumar *et al.* 2020). The infestation of weeds on a palm oil plantation has been successfully managed by herbicides. However, due to weed resistance issues and herbicide toxicity, certain classes of synthetic herbicides such as class I herbicide (paraquat) need to be reduced or eliminated (Rutherford *et al.* 2011). Plant-

-derived bioactive compounds could be a good alternative to synthetic herbicides since they are known to be safer for the environment. Introduction of a natural compound with a new mode of action (MOA) would be beneficial to overcoming the problem of rapidly evolving resistance towards several classes of conventional herbicides.

Parthenium weed (*Parthenium hysterophorus* L.) is an herbaceous annual weed native to South and North America (Khan *et al.* 2013; Ojija *et al.* 2021). It was reported to be a product of the natural hybridization of *Parthenium confertum* and *P. bipinnatifidum*

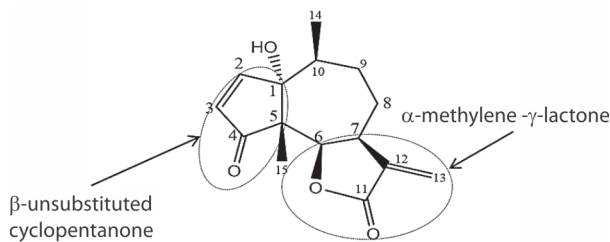


Fig. 1. The structure of parthenin

(Msafiri *et al.* 2013). Because of its high adaptability to diverse soils and climatic conditions, its high and rapid growth rate, strong competition (Khan *et al.* 2014), and allelochemical ability (Bajwa *et al.* 2020), the weed has a negative impact on plant diversity and ecosystems (Ojija *et al.* 2021). Previous researchers have reported the effects of *P. hysterophorus*' main allelochemical which is parthenin, on a broad range of plants including *Lactuca sativa* L., *Avena fatua* L., and *Triticum aestivum* (Marwat *et al.* 2008; Lim *et al.* 2013).

The plant contains a unique form of sesquiterpene lactone, parthenin (Fig. 1) which inhibits seed germination and growth of companion plants (Javaid *et al.* 2011). The presence of both α -methylene- γ -lactone functional groups as well as β -unsubstituted cyclopentanone could play a role in increasing biological nucleophile reactivity, and further give various properties to it (Reddy *et al.* 2011). Due to its aggressive characteristics, *P. hysterophorus* should be given special attention and studied for its herbicidal activity. Therefore, in this work, the genetic identification of *P. hysterophorus* was carried out and the crude extract of *P. hysterophorus* was tested for its herbicidal activity on *Diodia ocimifolia* which is one of the troublesome weeds in Malaysian palm oil plantations.

Materials and Methods

Genetic identification of *P. hysterophorus*

Fresh and mature leaves of *P. hysterophorus* were collected from Kampung Sentosa, Semenyih, Selangor (2°56'47.3"N 101°50'57.6"E), with permission and guidance from the Department of Agriculture Selangor, Malaysia. DNA extraction was carried out using FavorPrep™ Plant Genomic DNA Extraction Mini Kit (Taiwan), according to the manufacturer's protocol. DNA amplification was performed in a 25 μ l reaction volume with the following constituents: 2 μ l of DNA extract, 12.5 μ l of 2X DreamTaq Green PCR Master Mix (Thermo Scientific Inc., USA), 7.5 μ l of sterile distilled water and 1.5 μ l of each primer. Polymerase chain reaction (PCR) amplification was carried out in a C1000 Touch Thermal Cycler system (Bio-Rad, USA), based

Table 1. Cycling conditions for PCR program internal transcribes spacer (ITS)

Step	Cycle	Temperature [°C]	Time [min]	Time [s]
Pre denaturing	1	94	5	–
Denaturing	35	94	–	30
Annealing	–	60	–	30
Extension	–	72	–	30
Final extension	1	72	7	–
Keeping	–	4	∞	–

Table 2. List of internal transcribes spacer (ITS) primers and sequences (Sameem 2018)

Primer	Sequences
ITS 1	5'TCC GTA GGT GAA CCT GCG G3'
ITS 4	5'TCC TCC GCT TAT TGA TAT GC3'

on the protocols listed in Table 1. The double-stranded DNA of the ITS region from the sample was amplified with the universal primer pairs ITS1 and ITS4. The sequences of each primer used for the amplification are shown in Table 2. Purified PCR products were sent for sequencing to NHK Bioscience Solutions (Malaysia) and pairwise alignment was analyzed in BioEdit Sequence Alignment Editor Version 7.2.5. The DNA sequences were compared to the existing sequences from GenBank® database using Nucleotide Basic Local Alignment Search Tool (nBLAST), National Institute of Biotechnology Information (NCBI).

Solvent extraction of *P. hysterophorus*

Mature plants of *P. hysterophorus* were collected and left to dry at room temperature in a drying room (equipped with a drying rack) for 14 days to avoid photo-degradation of allelochemicals (Ojija *et al.* 2019). The dried plants were ground into a fine powder with a grinder and stored in a container at room temperature. A sufficient quantity of *P. hysterophorus* powder (100 g) was macerated with 600 ml of methanol (which was purchased from Merck, Germany) for 24 h. After filtration, the obtained solution was concentrated by using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) resulting in a green paste of crude extract. The crude extract was placed in a glass container wrapped with aluminum foil and stored at –20°C before further processes to avoid deterioration.

Preparation of the seeds of *D. ocimifolia*

Seeds of *D. ocimifolia* were collected from the Felda Raja Alias 4, Jempul, Negeri Sembilan (Malaysia) (2°54'11.3"N 102°25'15.1"E). The seeds were tested for their viability by immersing in water for 24 h, and

those which sank were preserved as viable seeds. To break the seed dormancy, viable seeds were soaked once again for 24 h in a gibberellic acid solution (0.25 g in 250 ml distilled water). Seeds were ready for use after being rinsed with distilled water a few times.

In vitro* phytotoxicity of *P. hysterophorus* crude extract (PHCE) against *D. ocimifolia

The PHCE solution with different concentrations (1, 2, 5, 10 and 15 g · l⁻¹) were prepared by dissolving PHCE in 1 l of water. The mixture was homogenized using a glass rod. Five Petri dishes (each 9 cm in diameter) containing one layer of Whatman filter paper No. 1 were impregnated with 10 ml of PHCE solutions (1, 2, 5, 10 and 15 g · l⁻¹). Then, 10 ml of distilled water was added to each Petri dish (as additional water to allow seed germination) before 20 seeds of *D. ocimifolia* (previously prepared as described in the previous section) were evenly arranged on the treated filter papers. A control Petri dish treated with 20 ml distilled water was set for comparison. The *in vitro* seed germination test was replicated three times. The Petri dishes were covered with lids and the seeds were allowed to germinate at 12 h light/12 h in the dark at 25°C for 12 days. To keep the filter paper moist, distilled water was added on a daily basis (Tobe *et al.* 2005; Lim *et al.* 2017). After 12 days, seed germination was visually counted in each of the replicated treatments and converted into seed germination (%). The lengths of the seedlings were measured with a measurement rod.

Statistical analysis

Data recorded for seed germination and seedling growth of PHCE at the different concentrations used were analyzed by the Analysis of Variance technique. Data means were separated by Tukey's test (at 95% confidence interval) using Minitab®, version 16 (Minitab Inc., USA). Data are presented with standard errors (SE).

Dose-response regression

The dose-response curves of the length of radicles and shoots were constructed by GraphPad Prism 7 software by fitting the equation provided:

$$Y = 100 / (1 + (10(\log ED_{50} - X) A)),$$

where: Y-axis – the percentage of the relative length of radicles and shoots, X-axis – log concentration of the prepared PHCE solution, ED_{50} – the half-maximal effective dose and A – the hill slope value.

The focus was made to get the experimental effective dose (ED_{50}) showing 50% inhibition of relative growth of radicles and shoots. The goodness of

dose-response model was evaluated by the coefficient of determination (R^2).

Results and Discussion

Genetic identification of *P. hysterophorus*

Our study found that Rumput Miang Mexico (RMM) DNA sequence gave 99% similarities to *P. hysterophorus* plant. To achieve the genus and species identification, the 633 bp of DNA sequence extracted from RMM plants was compared with the available DNA database in the National Center for Biotechnology Information (NCBI) Genbank® using the Basic Local Alignment Search Tool (BLAST) inquiry platform. The RMM DNA sequence was registered in GenBank® as *P. hysterophorus* species under accession number KY249559. Genetic identification is a study that allows individual identification of one species by using highly advanced DNA profiling technology. From the beginning, the collected plant (RMM) was only referred to as *P. hysterophorus* due to its similarities in morphological appearance. However, morphological similarities between the two plants are not necessarily ancestral and do not share the same family. The similarity may be the result of mimicry, environmental influence or convergence evolution (Poulin and Presswell 2016).

ITS refers to spacer found in the nuclear DNA. It is used for discrimination of different species and to identify contamination or adulteration (Baldwin 1992). ITS is one of the most extensively sequenced molecular markers (Won and Renner 2005) and more variable than other nuclear or mitochondrial DNA sequences due to its potentially high resolution of inter- and intra-specific relationships (Poulin and Presswell 2016). This conserved region can be used to design primers that are specific to a range of taxa due to the concerted evolution of inter- and intra-chromosomal loci. The divergence copies exist because of recombination among copies, pseudogenization of citrons, lineage sorting, and hybridization (Won and Renner 2005). Such techniques have been used to study the phylogenetic relationship by sequencing the nuclear ribosome DNA (rDNA) from ITS after DNA was extracted from 15 genus *Sorghum* and its related genera (Sun *et al.* 1994). In another study, the identification of medicinal plants and their closely related species was also carried out by using ITS region as a standard DNA barcode (Chen *et al.* 2010).

Growth experiment

In this study, the growth of *D. ocimifolia* was measured by observing the seed germination, and length of radicles and shoots of *D. ocimifolia*. Determining

Table 3. Effects of different concentrations of PHCE on germination rate, shoot height, and radicle length of *Diodia ocimifolia*

Crude extract concentration [g · l ⁻¹]	Germination rate [%]	Shoot height [mm]	Radicle length [mm]
Control set	75.00 ± 3.54 ab	29.05 ± 0.09 a	47.50 ± 0.25 a
1	83.75 ± 8.93 a	29.25 ± 0.19 a	38.25 ± 0.18 b
2	68.75 ± 6.50 b	27.25 ± 0.19 a	31.25 ± 0.22 c
5	8.75 ± 4.15 c	20.50 ± 0.22 b	7.00 ± 0.29 d
10	0.00	0.00	0.00
15	0.00	0.00	0.00

Data are means (±SE) of four replicates. Means with different superscript letters are significantly different at $p < 0.005$

seed germination, radicle and shoot elongation is a rapid and widely used phytotoxicity test with several advantages in terms of low cost, simplicity, sensitivity, and suitability for unstable samples (Munzuroglu and Geckil 2002). The overall growth development of *D. ocimifolia* seed demonstrated that PHCE exhibited allelopathic activities in all parameters measured (Table 3). In general, the rate of germination and length of shoot and radicle was reduced as the concentration of PHCE was increased. However, the result of seed germination showed an effect of hormesis where the toxicity acted as a stimulant at a low dose (1 g · l⁻¹) but as the extract concentration increased (2, 5, 10, 15 g · l⁻¹), the germination of *D. ocimifolia* was inhibited. Hormesis is a common observation in toxicological literature including studies on herbicides. The stimulatory response of the plant to low dose treatment was contributed to by the inner mechanism of the plant to optimize growth under unfavorable conditions (Cedergreen *et al.* 2007). Previously, parthenin from *P. hysterophorus* was shown to have a hormesis effect on other plants. A comprehensive study on the hormesis effect of parthenin from *P. hysterophorus* on *Lactuca sativa* was done by Belz and Cedergreen (2010). The study concluded that the effect of hormesis by parthenin could be due to phytochrome-induced auxin.

In contrast to seed germination, no stimulatory effects were displayed on radicle length and shoots. Maximum germination inhibition was displayed at a concentration of 5 where the germination of *D. ocimifolia* was suppressed almost nine times more than the seed germination of the control set. No sign of growth was observed for *D. ocimifolia* seed when the concentration of *P. hysterophorus* reached 10 and 15 g · l⁻¹ indicating a complete growth inhibition of the plant. Germination is a physiological process where seeds sprout after water imbibition by seeds and culminate in the emergence of rootlets (Belz and Cedergreen 2010). The phytotoxicity of *P. hysterophorus* contributed to the allelopathic nature of the plant. Various types of agronomic crops and weeds have been affected by this plant (Tefera 2002; Netsere and Mendesil 2011;

Patel 2011). The existence of an unsubstituted ring in the chemical structure of parthenin serves as a site for various chemical modifications and reactions (Singh *et al.* 2002).

Figure 2 shows the germination of *D. ocimifolia* seed 2 to 12 days after treatment (DAT) with *P. hysterophorus* crude extract (PHCE). According to the graph, the hormesis effect was seen at 6 and 12 of DAT where the germination percentage of *D. ocimifolia* was higher when treated with 1 g · l⁻¹ of PHCE than in the control set. Starting from 4 DAT, as the concentration of PHCE increased from 2 to 5 g · l⁻¹ a significant decrease in *D. ocimifolia* seed germination was displayed compared to the control where the germination percentage was 6.25 to 8.75%. This shows that less than 12 days were needed to inhibit 91.25% of *D. ocimifolia* seed germination by using 5 g · l⁻¹ of PHCE.

In terms of delay in seed germination, the germination index (*GI*) was calculated to measure the rapid germination ability (Kader 2005). Table 4 lists the germination index (*GI*) of *D. ocimifolia* seed after treatment with different concentrations of PHCE. Germination index represents the percentage rate of germination in a given time frame which was more concerned with germination speed. Some seeds may germinate slowly because of the inferior seed vigor caused by external

Table 4. Germination index (*GI*) for *Diodia ocimifolia* seed treated with different concentrations of PHCE

Concentration [g · l ⁻¹]	<i>GI</i>
0	13.12 ± 1.90 b
1	14.75 ± 2.00 a
2	11.32 ± 1.12 c
5	0.86 ± 0.62 d
10	0.00
15	0.00

PHCE – *Parthenium hysterophorus* crude extract

Data are means (±SE) of four replicates. Means with different superscript letters are significantly different at $p < 0.05$

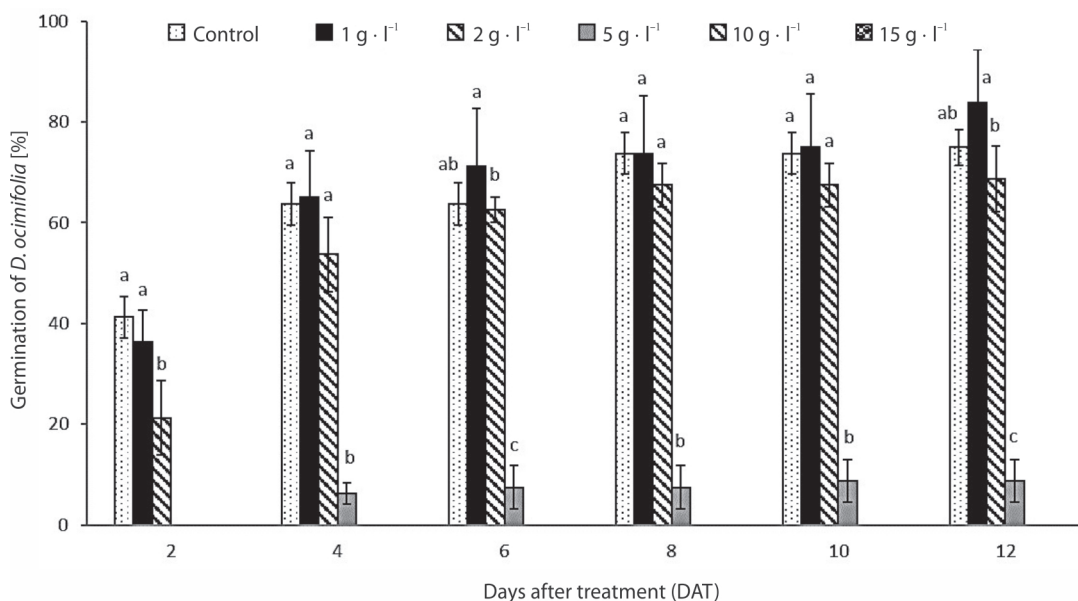


Fig. 2. Changes in the germination of *Diodia ocimifolia* for 12 days at different concentrations (1–15 g · l⁻¹). Means by different super-script letters are significantly different from the control at $p < 0.005$

factors (Tan *et al.* 2017). From the results, the *GI* values decreased as the concentration of PHCE increased which indicated that a high concentration of treatment caused a longer delay in the germination of *D. ocimifolia* seed than treatment at low concentrations.

Treatment of 5 g · l⁻¹ of PHCE had the lowest *GI* value (0.86) of all treatments which indicated a low germination rate. This was supported by having a delay in the germination of *D. ocimifolia* seed for 3 days before germination started at 4 DAT (Fig. 2) after being treated with 5 g · l⁻¹ of PHCE. The highest *GI* value (14.75) was shown with the treatment of 1 g · l⁻¹ PHCE which indicated a high germination rate. This was again seen by a graph in Figure 2 where treatment of 1 g · l⁻¹ of PHCE gave no significant difference in seed germination of the control set from 2 to 12 DAT. In general, the delay in seed germination could be contributed to allelochemicals present in the formulation (Tan *et al.* 2017). Several allelochemicals have been reported to be in *P. hysterophorus* including parthenin and other classes of sesquiterpene lactones, and phenolic compounds (Adkins and Shabbir 2014). These allelochemicals might have disrupted various physiological processes including photosynthesis, respiration and hormonal balance (Soltys *et al.* 2013) which in turn delay the germination of *D. ocimifolia* seeds.

The radicle and shoot growth analysis

The dose-response curves for inhibition of radicle and shoot growth of *D. ocimifolia* as affected by different concentrations of PHCE are shown in Figure 3. Growth responses were presented as a function of absolute herbicide quantities inside the receiver plant.

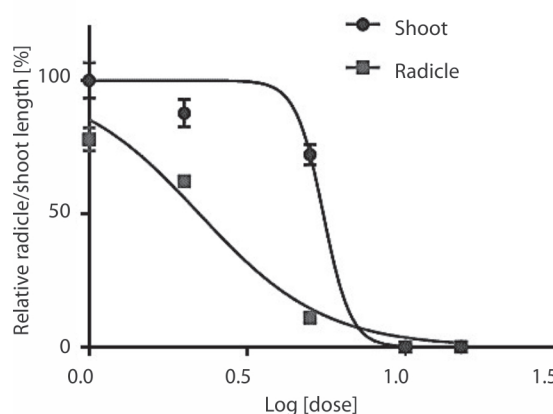


Fig. 3. Dose-response for relative growth of radicle and shoot of *Diodia ocimifolia* as affected by *Parthenium hysterophorus* crude extract (PHCE) in the concentration range 1–15 g · l⁻¹. The ED_{50} values of the crude extract were extrapolated: radicle ($ED_{50} = 2.23$ g · l⁻¹; $R^2 = 0.9759$) and shoot ($ED_{50} = 5.50$ g · l⁻¹; $R^2 = 0.9782$)

Therefore, ED_{50} would be a better indicative parameter for the affecting plant than concentration to prevent a dilution factor because of plant growth effects (Wagner *et al.* 2003). The ED_{50} values for radicle and shoot growth were extrapolated from the curves to determine the half-maximal effective dose. The coefficients of determination (R^2) values for both dose-response curves were in good fitting value. The radicle and shoot lengths were shortened with an increase in the concentration of PHCE treatments. The maximum inhibitory effect was at 5 g · l⁻¹ of PHCE with maximum radicle and shoot length being 7.00 mm and 20.50 mm, respectively, while the length of radicle and shoot for the control was 47.50 mm and 29.05 mm, respectively (Table 3).

In response to PHCE, the radicle appeared to be more sensitive to the crude extract than shoots with an ED_{50} value of $2.69 \text{ g} \cdot \text{l}^{-1}$ for radicles in comparison to $5.48 \text{ g} \cdot \text{l}^{-1}$ for shoots. Plant species are varied in their sensitivity to any treatment. Naturally, the common herbicide will be absorbed by the seed in the imbibition phase and later absorbed by the radicle in an overlapped second phase. The second phase generates most of the tissue damage (Wagner *et al.* 2003) where preparation for germination is inhibited and prevents radicle protrusion. With treatment by PHCE, radicle elongation was more inhibited than shoot growth which might be due to the lack of cuticle cover in the root and parthenin caused water loss as a result of root dysfunction (Singh *et al.* 2002; Hazrati *et al.* 2017). Although the reasons for such findings could not be determined in these experiments, it can indicate that *P. hysterophorus* interferes with normal cell division and leads to chromosomal aberrations since mitosis only occurs after radicle protrusion (De Castro *et al.* 2000). Treatment with PHCE proved to significantly inhibit radicle and shoot growth of *D. ocimifolia* seed. A meiotic study has demonstrated that *P. hysterophorus* allelotoxin effectively induced various chromosomal, mostly physiological, abnormalities of I type. Treatment with PHCE significantly inhibited radicle and shoot growth of *D. ocimifolia* at low concentrations (Kumar and Gautam 2008).

From the overall observation, our study revealed that the noxious and invasive weed locally known as RMM was successfully identified as *P. hysterophorus* L. by the DNA sequencing method. The methanolic extract of *P. hysterophorus* was found to have phytotoxic properties against *D. ocimifolia* at the minimum concentration ($1\text{--}5 \text{ g} \cdot \text{l}^{-1}$) in the laboratory growth experiment. Future research will be useful to investigate the bio-herbicidal potential of *P. hysterophorus* under natural field conditions where many other biotic and abiotic factors come into play.

Acknowledgements

The author would like to thank the Ministry of Higher Education Malaysia for my Ph.D. scholarship for Zainuddin N.J. and the Department of Agriculture Malaysia for giving permission and assistance during the collection of *P. hysterophorus* plant.

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