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NOTE

Hydrogen cyanide for treating wood against pine wood nematode (*Bursaphelenchus xylophilus*): results of a model study

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Abstract The pine wood nematode (*Bursaphelenchus xylophilus*) belongs to economically most important quarantine plant parasitic nematodes in Asia and Europe. As wood transported within the international trade plays major role in *B. xylophilus* spreading into new areas, attention should be given to wood treatment. With methyl bromide ban in the EU in 2000 new chemicals should be investigated. This work describes results obtained from model fumigation of hollow wooden blocks containing *B. xylophilus* nematodes in sawdust with gaseous hydrogen cyanide (HCN). Data considering HCN concentration in gas chamber and treated wooden blocks are also presented; HCN concentrations inside wooden blocks were recounted to ct product values which show irregular sorption of HCN by the wood. Total *B. xylophilus* mortality was observed in the variants treated with the initial HCN concentration of 12.30 g m^{-3} and exposure times from 8 to 20 h, 18.21 g m^{-3} and exposure times of 2, 4, 6, 10 and 16 to 20 h, 21.71 g m^{-3} and exposure times of 12, 18 and 20 h and 24.12 g m^{-3} and exposure times of 2, 6, 12 and 18 h. Results show overall good efficiency of HCN treatment on *B. xylophilus* mortality, however, research on naturally infested wood should be desirable.

Keywords *Bursaphelenchus xylophilus* · Methyl bromide alternative · Hydrogen cyanide · Fumigation · Wood treatment

Introduction

Goods exchanges play major roles in spreading quarantined pest organisms. This is especially true for wood-borne pests, which can also be transported within the packing wood. A major pest that can be transported this way is the pine wood nematode (*Bursaphelenchus xylophilus*). This nematode species currently presents a major threat to pine trees in large parts of Europe [1] and East Asia [2]. *B. xylophilus* originates from North America, where it lives in the local conifers from the genus *Pinus* without causing any substantial damage [3]. However, bringing the *B. xylophilus* nematode to Japan during the beginning of the 20th century has led to widespread mass devastation of the local pines. From the end of World War II to 2002, pine wilt disease caused the loss of 26 million m^3 of timber in Japan. The *B. xylophilus* nematode was first isolated and described as the cause of pine tree damage from the timber of Japanese black pine (*Pinus thunbergii*) as late as 1969 [1]. The presence of *B. xylophilus* in Japan is presumed to come from the United States, where this species was initially described in 1934 as one of many parasites of pines [4].

The presence of *B. xylophilus* was also identified in other Asian countries in the 1980s (Taiwan, 1985; China

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and South Korea, 1989) [5]. *B. xylophilus* occurrence was described in Europe in 1999 and in Portugal on *Pinus pinaster* [6]. The presence of *B. xylophilus* in Europe prompted the European Plant Pathology Organization (EPPO) to immediately include the species in its A1 list of pests recommended for regulation as quarantine pests.

The range of hosts for *B. xylophilus* include conifers from the genus *Pinus* (*nigra*, *silvestris*, *pinaster*, *thunbergii*, *bungeana*, *densiflora*, *luchuensis*) and conifers from other genera that could be inhabited, even in the absence of damage by *B. xylophilus*—e.g., *Picea* sp., *Pseudotsuga* sp. *Picea* sp., *Larix* sp., *Abies* sp., *Cedrus* sp. [1, 7, 8]. For susceptible *Pinus* species, the nematodes can destroy a fully grown tree within several weeks. The life cycle of *B. xylophilus* includes development through the phytophagous and mycophagous phases and their transport by beetle vectors—especially sawyer beetles from the genus *Monochamus* [7–12]. Passive transport of the nematodes in infested wood is crucially important when considering the spread of *B. xylophilus* on an international scale. Thus, wooden pallets, shipping crates and construction timber can easily transport *B. xylophilus* [13].

A triangular system that encompasses pine trees, nematodes and its vector is a perfect system for *B. xylophilus* survival and spreading and makes most pest management measures ineffective. Even if extensive research of the flight traps intended to capture *B. xylophilus* insect vectors is conducted [14–16] with promising results according to authors' opinion vector targeting management techniques could be used rather for *B. xylophilus* monitoring than direct tree protection. Chemical treatment aimed at protecting pine trees in affected countries is expensive and does not persist long. The only option is then the absolute decontamination of the affected area, which involves removing all of the host trees, including the roots, and establishing an extensive quarantine zone [17].

Thus, preventive measures are key to dealing with the possibility of *B. xylophilus* introduction into unaffected areas. Heat treatment of timber transported across borders was designed and described by EPPO standard no. PM 10/6 [18]. This type of treatment requires heating the core of the treated wood to at least 56 °C [19] for at least 30 min. However, this could be difficult to successfully perform with large logs. This technique also requires special facilities that are energetically expensive and complicated, especially when bulky construction timber has to be treated. The quality changes of the treated wood could also be problematic, in addition to the overall efficiency of the heat treatment technique [20, 21]. Therefore, alternative techniques for treating timber should be investigated.

Considering that chemical methyl bromide application is phased out by most industrial countries, no chemical is currently routinely used for *B. xylophilus* management. Experimental work now focuses on chemicals such as sulphuryl fluoride [22], methyl iodide [23] as fumigation agents or copper sulphate as liquid treatment [24]. Gaseous hydrogen cyanide (HCN) possesses certain advantages over other chemicals: it is naturally occurring, it is lighter than air gas and thus easy to ventilate after treatment, HCN's simple molecular structure makes it highly permeable in plant material and its considerable reactivity results in its rapid degradation without any undesirable residues. Currently, HCN is routinely used for fumigating food production and storage facilities [21, 25]. The main disadvantage of HCN in treating wood-borne organisms is its extreme acute toxicity to warm-blooded organisms, so treatment with HCN places a high demand on the staff. However, these drawbacks could be mitigated by ensuring that only trained workers interact and carefully handle HCN [26].

The main aim of this study was to evaluate the efficiency of the gaseous hydrogen cyanide treatment on pine wood nematode (*B. xylophilus*) mortality in a model experimental system. Because HCN affects the mortality of the free-living nematode *Caenorhabditis elegans* and of *B. xylophilus* was recently demonstrated [21, 27], this work focuses more precisely on different HCN concentrations and exposition times necessary to kill *B. xylophilus* in wood. Four HCN concentrations and ten exposition periods were tested. The experimental design enabled us to measure the HCN concentrations during treatment.

Materials and methods

Fumigation chamber, sample preparation and experimental design

All experiments were performed in a fumigation chamber localised within the Draslovka Kolín a.s.—the only European industrial manufacturer of gaseous HCN (trade name Uragan D2). The experiments were conducted in a stainless steel gas chamber (volume 650 l) equipped with forced ventilation, an air lock and glove manipulators, which enabled gas withdrawal from the samples and measurement of HCN concentrations using gas chromatography (Shimadzu GC-17A, RT-QPLOT, 30 m, ID 0.53 mm, GC Software Clarity DataApex). The GC method is based on comparing the detector response from the sample with an external standard with a known concentration. 0.5 vol.% HCN in nitrogen was used as the standard (Linde Gas). Four HCN concentrations in the gas chamber were tested: 12.30, 18.21, 21.71 and 24.12 g m^{-3} in exposition times 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h.

B. xylophilus individuals were obtained from in vitro cultures on *Botrytis cinerea* fungus maintained on MEA

medium. Two weeks after inoculation on *B. cinerea*, the nematodes were extracted from the fungal mycelium using Baermann's funnel extraction technique. Extracted nematodes were concentrated and transferred into fresh water to ensure their viability for subsequent experiments.

Pinewood sawdust was prepared by sawing *P. sylvestris* logs using a chainsaw. Two grams of sawdust (average moisture content 6.7 % with standard deviation 0.7) were inserted into Uhelon fabric bags with a mesh size of 18 μm to allow HCN free access to the sawdust and to restrict the possibility of spreading the nematodes from bags. 2 ml of tap water was pipetted into bags containing the sawdust to ensure that there was sufficient moisture in the bags for nematode survival. The moisture content of the sawdust was measured during sample preparation and evaluation using the data loggers. On average, moister content of sawdust containing nematodes was 32.30 % 2 h after inserting into gas chamber and 21.69 after 24 h lasting exposition (Table 1). Next, 500 μl of the nematode suspension containing approximately 1200 *B. xylophilus* individuals was pipetted into each bag. A magnetic stirrer was used during inoculation to ensure inoculum homogeneity. No water drops were observed on the bottom of the bags; whole volume of 2.5 ml of water was absorbed into sawdust as is apparent from its moisture content (Table 1). Next, the bags were sealed using steel wire and inserted into hollow spruce blocks (100 × 100 × 120 mm) covered by glass containing a silicon septum. The glass was fitted to the wood block using HCN impenetrable glue (Fig. 1). Five replicates were prepared for each exposition time and HCN concentration.

Sample treatment and evaluation

Prepared wood blocks containing the nematodes were transferred into a gas chamber and exposed to HCN at specific concentrations and exposure times at 24 °C. Because of the fumigation chamber, capacity samples were treated always in two separate batches, 1st from 2 to 10 h, 2nd from 12 to 20 h. HCN was introduced into the chamber in a liquid form, and vapours of the desired concentration were produced. HCN samples were withdrawn during treatment using the air lock and septum on the glass seal of the wood block, and the HCN concentrations were established using gas chromatography. The HCN concentration inside the gas chamber was also measured. After treatment, the HCN was ventilated from the blocks, the glass seals were crushed, the bags with sawdust and nematodes were removed and the nematodes were extracted from the sawdust for 24 h using Baermann's extraction technique. The obtained suspensions were surveyed using the stereomicroscope, and the nematodes were quantified. An untreated control variant was prepared in the same manner, and the control samples were placed next to the gas chamber during treatment of the experimental ones. The nematode numbers were expressed as the means of surviving nematodes. Cumulative Ct product values expressing effectiveness of a fumigant (concentration × time presented in g hm^{-3}) for the wooded blocks were calculated separately for both series of concentration measurements (2–10 h and 12–20 h) according to the following formula [28], where T_n is the time the first reading was taken in hours, T_{n+1} is the time the second reading was taken in hours, C_n is the concentration reading at T_n in g m^{-3}, T_{n+1} is the concentration reading at T_{n+1} in in g m^{-3} and $Ct_{n,n+1}$ is the calculated Ct product between T_n and T_{n+1}, in g hm^{-3}:

$$Ct_{n,n+1}\frac{(T_{n+1}-T_n)\times\ (C_n+C_{n+1})}{2.0}$$

Calculated cumulative Ct product values underwent basic statistical analysis (Statistica 12, StatSoft, Inc., Tulsa, OK, USA, 2013).

Results and discussion

The results demonstrated that HCN possesses an overall good efficiency as a nematode killing agent. All data considering nematode survival are presented in Table 1. The HCN treatment almost completely removed the nematode population; the overall mortality obtained was between 99.00 and 100 %. A complete nematode mortality was observed in the variants treated with the initial HCN concentration of 12.30 g m^{-3} and exposure times from 8 to 20 h, 18.21 g m^{-3} and exposure times of 2, 4, 6, 10 and 16–20 h, 21.71 g m^{-3} and exposure times of 12, 18 and 20 h and 24.12 g m^{-3} and exposure times of 2, 6, 12 and 18 h. In all cases where living nematodes were present at the time of extraction, no more than four individuals were identified thus indicating that no reproduction of nematodes occurred after treatment.

The HCN concentration inside the gas chamber decreased almost constantly (Fig. 2a, b) with gas sorption into wooden blocks. However, some HCN concentration variations occurred in the second half of the exposure periods with the HCN concentrations 18.21 and 21.71. The HCN concentration in the experimental wooden blocks varied substantially, though the observed variations did not considerably affect nematode mortality.

Considering the experiment design we can conclude that the methods used in this study are suitable for researching the nematicidal properties of HCN. The system used to maintain nematodes in sawdust worked well because nematodes in the untreated control survived the experiment period (Table 1). They appeared healthy and even multiplied if gravid females were present. Under no

Table 1 Number of surviving nematodes (mean from five replicates ± standard error, number of replicates with surviving nematodes in brackets) after HCN treatment in hermetic fumigation chamber after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h of fumigation, 4 HCN concentrations were tested; moisture content of sawdust (mean from five replicates ± standard error) is also included

Target HCN concentration in gas chamber (g m^{-3})	Nematode inoculum	Exposition time in hours									
		2	4	6	8	10	12	14	16	18	20
12.30	1200	0.25 ± 0.25 (1)[a]	0.2 ± 0.2 (1)	0.2 ± 0.2 (1)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
18.21	1200	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	0.50 ± 0.29 (2)	0 ± 0 (0)	0.20 ± 0.20 (1)	0.20 ± 0.20 (1)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)
21.71	1200	0.60 ± 0.40 (2)	2.20 ± 1.43 (2)	1.40 ± 1.17 (2)	1.60 ± 1.60 (1)	2.40 ± 1.91 (3)	0 ± 0 (0)	0.20 ± 0.20 (1)	0.20 ± 0.20 (1)	0 ± 0 (0)	0 ± 0 (0)
24.12	1200	0 ± 0 (0)	1.50 ± 0.96 (2)[a]	0 ± 0 (0)	0.25 ± 0.25 (1)[a]	0.25 ± 0.25 (1)[a]	0 ± 0 (0)	0.25 ± 0.25 (1)[a]	1.00 ± 1.00 (1)	0 ± 0 (0)	0.75 ± 0.48 (2)[a]
0 (control)	1200	1150 ± 103 (5)	1091 ± 66 (5)	1342 ± 49 (5)	1490 ± 227 (5)	1222 ± 121 (5)	1198 ± 129 (5)	1045 ± 64 (5)	1052 ± 59 (5)	1440 ± 159 (5)	1391 ± 305 (5)
Average moisture content of sawdust in percent		32.30 ± 1.10	30.99 ± 1.08	29.74 ± 1.06	28.48 ± 1.05	27.28 ± 1.02	26.06 ± 1.06	24.94 ± 0.99	23.84 ± 0.97	22.72 ± 0.94	21.69 ± 0.91

[a] Only four replicates tested

circumstances the moisture content of the sawdust was lower than 15 % during treatment—the level which correlates with nematode population decline [29].

Hydrogen cyanide possesses a strong potential as a nematode killing agent. The treatment was effective, even at the lowest concentration and shortest exposure times in this study. A fact that living nematodes were detected after treatment with highest HCN dose even if in minimal numbers could be connected with temperature in fumigation treatment and higher presence of eggs in samples at temperatures around 20 °C as was probably observed by [22] in the case of fumigation with sulfuryl fluoride. According to our results longer exposition periods would be desirable for *B. xylophilus* eggs treatment using the HCN. The high permeability of HCN, which was apparent from the concentrations measured in this study (Tables 2, 3), causes a relatively high sorption of the gas into the treated wood. This is advantageous when designing treatment protocols, especially if large volumes will need to be treated. Some varying HCN concentrations in wooden blocks during the experiment could be explained solely by the different wood structures of the separate wood blocks used in the experiments and fluctuation of the absorption of the gas in wood because the gas chamber is made of non-absorbing material and is perfectly sealed. The wood from younger trees with denser annual rings likely absorbs HCN less readily. This fact should be considered when planning model fumigation experiments. Ct product values (Tables 2, 3) show that establishment of the HCN concentration in sample wood blocks was rather fast, especially in the cases of higher initial HCN concentrations 21.71 and 24.12 g m^{-3}. From this reason no expected linear increase of nematode mortality in consequence of longer exposition and higher HCN concentration was observed. Hydrogen cyanide concentrations inside the fumigation chamber (Fig. 2a, b) show clearly sorption of the HCN into wooden blocks and decreasing of the HCN concentration in the chamber.

Earlier experiments have demonstrated the nematicidal effects of HCN against free-living *Caenorhabditis elegans* [27], where 100 % mortality was observed after 270 min of HCN exposure at 7 g m^{-3}. The nematicidal properties of gaseous HCN against *B. xylophilus* were observed earlier [21]. In this case, no living nematodes were observed after 18 h of HCN exposure at 20 g m^{-3}or after 40 h of exposure at 10 g m^{-3}. Similar results were obtained in this study with one substantial exception—in two cases, living *B. xylophilus* individuals were observed after 20 h of HCN exposure at 24.12 g m^{-3}. This exception could be caused by different nematode numbers used in the experiments. Only 450 nematodes were used in the inoculum in the previous trial [21], whereas we used an average of at least 2.5 fold more individuals. The number of nematodes used

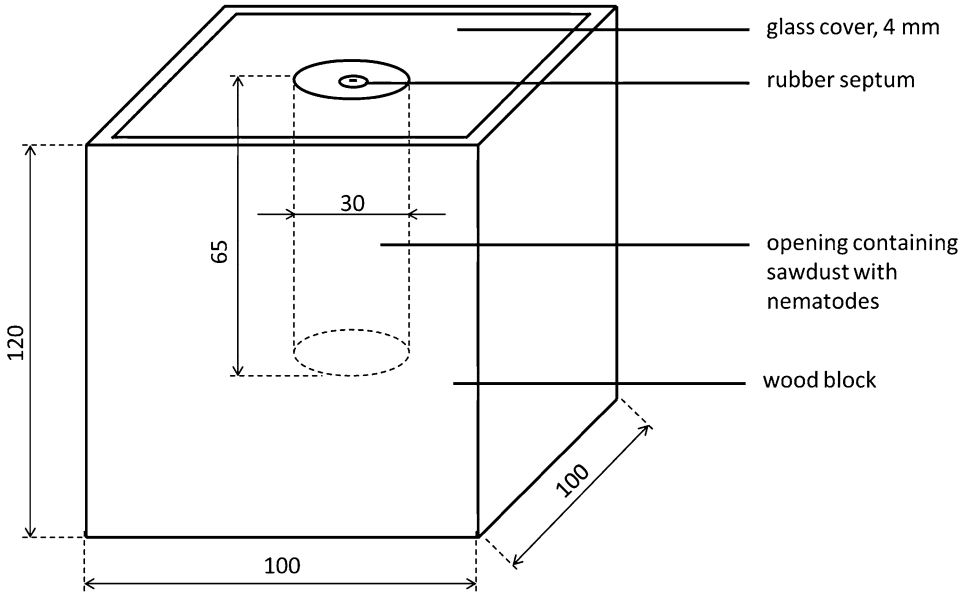

Fig. 1 Schematic figure of the experimental wooden block, dimensions in millimetres

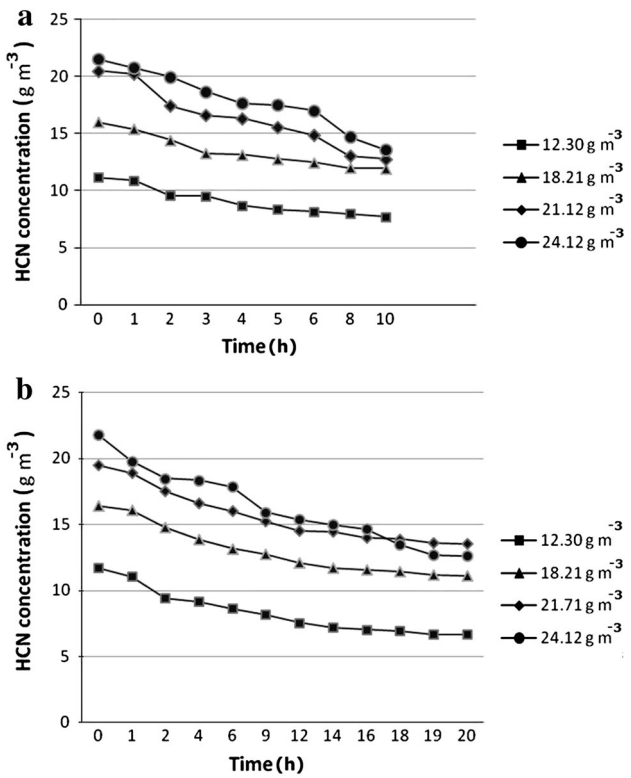

Fig. 2 **a** Mean changes of HCN concentrations in fumigation chamber for the initial concentrations 12.30, 18.21, 21.71 and 24.12 g m^{-3} during fumigation; exposition times 2–10 h. **b** Mean changes of HCN concentrations in fumigation chamber for the initial concentrations 12.30, 18.21, 21.71 and 24.12 g m^{-3} during fumigation; exposition times 10–20 h

in this study is more similar to the number observed in trees that are naturally infested with *B. xylophilus*, where the nematode population exceeds 1,000 individuals per gram of dry wood from 40 to 60 days after inoculation [5]. Nevertheless, the experimental treatment of naturally infested wood with HCN would be desirable, especially for treating construction timber.

Hydrogen cyanide efficiency as nematode killing agent is comparable to other methyl bromide alternatives that are currently being studied, such as sulphuryl fluoride and methyl isothiocyanate. However, comparison is informative only due to the different experimental designs used in several surveys. A fairly high number of *B. xylophilus* individuals were observed after 48 hours of treating naturally infested wooden blocks with sulphuryl fluoride [30]. However, in a subsequent study, 100 % of the *B. xylophilus* individuals were dead in naturally infested wood boards treated with sulphuryl fluoride after 24 h of fumigation at 15 and 30 °C [22], indicative of good efficiency. In the case of sulphuryl fluoride, its chemical properties must be closely monitored because sulphuryl fluoride is a powerful greenhouse gas and has a rather long atmospheric lifetime [31]. Even if it does not currently pose a problem because of the low amount of sulphuryl fluoride used globally for fumigation the rapid decomposition of HCN in the atmosphere represents an advantage when comparing the potential of those two fumigants. The effect of HCN on *B. xylophilus* appears to be comparable to methyl isothiocyanate [32], which is currently used as a soil fumigant and against soil-borne plant parasitic nematodes. However, its rather slow decomposition in soil [33] precludes its use in treating wood, especially package wood, although more research is needed to assess the properties of methyl isothiocyanate as a wood fumigant.

We conclude that the results of this study confirmed the nematicidal effects of gaseous HCN against *B. xylophilus*.

Table 2 Ct product of the HCN dose (mean from five replicates ± standard error) inside wooden blocks containing nematodes; exposition times 2–10 h

Initial HCN concentration in gas chamber (g m^{-3})	Ct product of the HCN treatment (average ± SE) Exposition time in hours				
	2	4	6	8	10
12.30	NA	0.835 ± 0.246	1.776 ± 0.372	4.698 ± 1.712	12.379 ± 4.628
18.21	NA	1.827 ± 0.555	7.344 ± 2.067	15.809 ± 3.157	19.600 ± 5.363
21.71	NA	3.057 ± 0.946	7.701 ± 2.563	15.645 ± 4.202	21.700 ± 1.503
24.12	NA	1.335 ± 0.383	12.601 ± 3.731	23.934 ± 3.869	30.641 ± 7.517

NA not assessed

Table 3 Ct product of the HCN dose (mean from five replicates ± standard error) inside wooden blocks containing nematodes; exposition times 12–20 h

Initial HCN concentration in gas chamber (g m^{-3})	Ct product of the HCN treatment (average ± SE) Exposition time in hours				
	12	14	16	18	20
12.30	5.631 ± 3.000	11.935 ± 1.656	13.213 ± 1.810	15.296 ± 1.165	18.665 ± 3.143
18.21	11.273 ± 2.383	13.162 ± 3.293	18.221 ± 3.727	22.852 ± 1.631	43.009 ± 7.116
21.71	22.732 ± 3.660	23.209 ± 2.188	37.032 ± 5.207	39.943 ± 3.479	44.566 ± 8.475
24.12	19.486 ± 4.794	41.652 ± 13.303	45.670 ± 9.873	57.868 ± 6.545	58.862 ± 0.006

The main issues in next testing are related with the size of the contaminated wood pieces submitted to fumigation. Thus further evaluation of the effects of HCN on *B. xylophilus* and a discussion about its possible commercial applications will require testing of large volumes of infested wood e.g. construction timber and package wood to see and understand behaving and mode of action of HCN in wood with intact wood structure and to meet numbers of nematodes exposed to each assay (HCN concentration/time) required by EPPO protocols [34, 35].

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