Efficacy of ethanedinitrile fumigant application against the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchidae), in pine logs

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Summary. Pine trees, *Pinus sylvestris*, were felled and the trunks were sawn into logs and infested with pinewood nematode (PWN) *Bursaphelenchus xylophilus*. The pine logs were fumigated with ethanedinitrile (EDN) in chambers at 20°C, with dosages of 25 g m^{-3}, 50 g m^{-3} and 75 g m^{-3} for 24 h, and 50 g m^{-3} for 12 h. The number of nematodes in wood samples was calculated 3 days before fumigation and then 24 h, 48 h, 10 days and 28 days after fumigation. The number of living nematodes in the logs before fumigation averaged from 245 to 3950 nematodes $(100\ g)^{-1}$ of fresh wood. In all fumigated logs the 100% death of PWN was observed. The most cost-effective doses for treatment were 25 g m^{-3} for fumigation for 24 h and 50 g m^{-3} for 12 h. Experimental results confirm the effectiveness of EDN against PWN, but further studies are needed to determine the dosage of this fumigant against nematodes for other temperatures and various types of wood commodities.

Key words: cost-effective fumigation doses, EDN, nematode mortality rate, pinewood disease, *Pinus sylvestris*, plant quarantine, PWD, PWN, timber treatment.

Trade between countries is increasing every year as well as the role of phytosanitary measures in relation to possible introduction of dangerous plant pests. Transported plants and plant products should be free of quarantine pests. These requirements also apply to exported timber, wood products, as well as to wood packaging materials that are used for moving commodities (FAO, 2017a, 2018).

Fumigation is one of the most commonly used phytosanitary measures for the disinfection of round wood, saw wood, wood chips or bark against pests (FAO, 2017a, b, 2018). The pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is a causal agent of pine wilt disease causing death of million pines in Japan, Korea, China and Portugal (Mota *et al.*, 2008; Zhao *et al.*, 2008; Soliman *et al.*, 2012; Vicente *et al.*, 2012; Futai, 2013). There is a risk of introduction of the PWN with wood products and wood packaging materials (pallets, containers, *etc.*) (Gu *et al.*, 2006; Sousa *et al.*, 2011; Bonifácio *et al.*, 2013; FAO, 2018). Various fumigants are applied to eradicate this harmful organisms in wood; these are sulfuryl fluoride (SF), phosphine (PH_3) and hydrocyanic acid (HCN) (Kairui *et al.*, 2009; Bonifácio *et al.*, 2014; Stejskal *et al.*, 2014; FAO, 2019) (Table 1). The death of the pinewood nematode *B. xylophilus* in wood is the main criterion for the effectiveness of fumigant, because high doses of fumigant used for nematodes usually kill both nematodes and insects (Bonifácio *et al.*, 2014; Lee *et al.*, 2014).

The pinewood nematode also poses a great threat to conifer forests in the Russian Federation (Kulinich & Kolossova, 1995; Kulinich *et al.*, 2010; Arbuzova *et al.*, 2016). The analysis of phytosanitary risk showed that if the PWN were to be introduced in Russia, possible damage could range from 1.6 to 3.2 billion $US a year (Kulinich *et al.*, 2013). Most countries of the world have included *B. xylophilus* in their lists of quarantine pests and require phytosanitary treatment of imported wood products (FAO, 2010; Armstrong *et al.*, 2014).

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Table 1. The main pesticide applied for wood fumigation at 25°C (FAO, 2019).

Fumigant active substance	Formula	Molecular weight (g mol^{-1})	Boiling point (°C) (1 atm)	Specific gravity (gas) (air = 1.0)	Solubility in water
Methyl bromide	CH_3Br	95	3.6	3.3	3.4 v/v %
Ethanedinitrile	C_2N_2	52	−21.2	1.82	Highly soluble
Sulfuryl fluoride	SO_2F_2	102	−55.2	3.72	Slight
Phosphine	PH_3	34	−87.7	1.2	0.26 v/v %
Methyl iodide	CH_3I	141.94	42.6	4.89	1.4 g (100 ml)$^{-1}$
Hydrogen cyanide	HCN	27	26	5.6-40	Miscible

Depending on the type of wood products, methyl bromide, sulfuryl fluoride or phosphine (Seabright *et al.*, 2020) are used for disinfection of wood against pests, but the effect of phosphine against the PWN is extremely limited (Soma *et al.*, 2001, 2005). Phosphine does not always cause 100% mortality of the PWN during fumigation of round wood, and sulfuryl fluoride is recommended mainly for application against PWN in sawn wood, including wood packaging materials (Bonifácio *et al.*, 2014, 2015; FAO, 2017b). The most effective and common fumigant for disinfection of any type of wood product, including round wood, is methyl bromide (CH_3Br), which can kill different insects and nematodes (Barak *et al.*, 2005; Ducom, 2012; Glassey, 2013). Banning of methyl bromide application forces scientists in many countries to study effective alternative, environmentally friendly, fumigants for decontamination of forest products (Ren *et al.*, 2011; Seabright *et al.*, 2020). The most promising alternative fumigant is ethanedinitrile (EDN) (Stejskal *et al.*, 2019). Being an effective analogue of methyl bromide it is safe for the ozone layer of the planet (Pranamornkith *et al.*, 2014a; Hnatek *et al.*, 2018). According to its chemical properties, EDN could replace methyl bromide, but its application against pests in wood products needs to be studied further (Stejskal *et al.*, 2017; Zakladnoy, 2018; Seabright *et al.*, 2020).

Ethandinitrile has a high penetration rate compared to methyl bromide, which makes it suitable for treating wood in order to eradicate wood insects and the PWN (Lee *et al.*, 2014; Hall *et al.*, 2015). Application of EDN was tested in the USA, New Zealand, the Republic of Korea and the Czech Republic. EDN is already used for log fumigation in Australia (Najar-Rodriguez *et al.*, 2015; Stejskal *et al.*, 2017). Russia exports about 20 million m^3 of round wood and replacing methyl bromide by other effective fumigant would be relevant for Russian timber exports. The aim of our research was to study the effect of various doses of EDN on the survival of the PWN in pine logs.

MATERIAL AND METHODS

Collection and preparation of wood material. In the experiment wood logs of 9-year-old Scots pine *Pinus sylvestris* L. were used. The pine tree was felled on July 19, 2019 and the tree trunk was sawn into logs of 20-25 cm long. The diameter of the logs ranged from 4.2 to 11.5 cm. The log weight ranged from 196 to 2467 g. Fifty wood logs were infested with the PWN, *B. xylophilus* (*US1-Bx* isolate), previously propagated on the fungus *Botrytis cinerea*. Two holes of 8 mm diam. were drilled in each log with the Makita drill and 100 µl of suspension with PWN introduced in each hole. The total inoculum volume was approximately 6,000 nematodes per log. Then logs were placed in the thermostat at 27°C for 5 weeks. Each log was checked for the presence of live nematodes 72 h before treatment with the fumigant. The ends of the wood logs were waxed prior to the PWN inoculation and the 5 mm waxed log ends were cut off just before EDN treatment.

Fumigation chambers and fumigant. STERIGAS™ ethanedinitrile (EDN) (C_2N_2), molecular weight 52.04, colourless caustic gas was applied as a fumigant (Table 1). Fumigation experiments were carried out in two fumigation chambers with a volume of 0.85 m^3. An EDN industrial fumigant store cylinder (STERIGAS 1000™ Fumigant: http://www.fumigaciya.ru/sites/default/files/public/page/2011-09/15/sterigas1000 fumigant.pdf) was placed on a digital electronic balance and connected to the fumigation chamber by tubes. The required amount of EDN was pumped into the chambers to achieve the required dosage. Ten wood logs were placed in each chamber. The fumigation was carried out on September 10 and 12, 2019, at room temperature (20°C), and the following doses of EDN were applied: 25 g m^{-3},

Table 2. Effect of ethanedinitrile treatments on the mortality of the pinewood nematode, *Bursaphelenchus xylophilus*, during fumigation of wood logs of Scots pine (*Pinus sylvestris* L.) at 20°C.

Dose	Ct* product (g h m^{-3})	The mean number of nematodes 72 h in the infested log before fumigation, (mean (100 g wood)$^{-1}$ ±SD)**	Nematode mortality after fumigation (%)			
			24 h	48 h	10 days	28 days
Fumigation, 12 h						
50 g m^{-3}	300	3950 ± 1389	100	100	100	100
Fumigation, 24 h						
75 g m^{-3}	900	1991 ± 461	100	100	100	100
50 g m^{-3}	600	245 ± 52	100	100	100	100
25 g m^{-3}	300	354 ± 91	100	100	100	100
Control sample***		849 ± 328	795 ± 265	806 ± 310	789 ± 340	831 ± 352

* Ct – Concentration × time.
** Data on the nematode extraction using the Baerman funnel method without a paper filter.
*** All nematodes in the non-fumigated PWN infested logs (control samples) were alive.

50 g m^{-3}, 75 g m^{-3} with 24 h exposure and 50 g m^{-3} with 12 h exposure. Ten wood logs infested with nematodes were kept without fumigation as control samples. The load factor of wood logs of one chamber in each experiment was about 2%. The Ct product was calculated taking into account the concentration and processing time (g h m^{-1}) and was used as a quantitative measure of fumigation efficiency. After fumigation, gas was pumped out of the chambers, up to five times, to create a vacuum.

The moisture content in the wood of each log was measured before fumigation using a Gann Hydromette HT 85 T.

Extraction and counting of nematodes. Wood samples were taken from the fumigated and control logs, three days before fumigation and 24 h, 48 h, 10 days and 28 days, after fumigation, to assess nematode populations.

Various modifications of the Baermann method are usually used for extracting nematodes from wood. The Baermann method is designed to extract the active stages of nematodes, which moving from wood tissue fall into the lower part of the funnel. This method is usually used to extract active juveniles and adults of pinewood nematodes. In our research, nematodes were extracted from wood in laboratory conditions using the Baermann funnel method at 24 h and a temperature of 22°C. A specific amount of chips (28 ± 2 g) was drilled from each log with a 20 mm diameter drill. The chips were previously weighed and then placed on a sieve in a funnel and the funnel was filled with water. It should be noted that nematodes were extracted from wood both using a paper filter (0.18 mm thick, 0.06% ash), which usually reduces the passage of nematodes during their extraction, and without a filter. The mesh size of the metal sieve was 50 µm. After taking chips from each wood log, the drill was thoroughly sterilised with alcohol and flamed to avoid accidental infection. The nematodes extracted from each sample were examined with a stereo microscope and their numbers in 100 g of fresh wood weight were calculated.

RESULTS

Fumigation toxicity of EDN to pine wilt nematode. All tests were carried out at 20°C, and EDN caused 100% of PWN mortality at all applied doses ($P < 0.001$) (Table 2). The most cost-effective treatment options were dosages of 25 g m^{-3} for 24 h fumigation and 50 g m^{-3} for 12 h. The Ct was 300 g h m^{-3} for both exposures.

The moisture content of wood, measured before treatment with the fumigant, was 69-78% and this parameter was not correlated with other fumigation parameters.

Infested wood logs were checked for the presence of nematodes 72 h before treatment with EDN. The number of nematodes compared to the initial inoculum in logs increased several times and reached to an average of 1723 nematodes (100 g)$^{-1}$ of wood log.

The number of nematodes before and after fumigation. The number of nematodes in wood samples was checked 3 days before fumigation and 24 h, 48 h, 10 days and 28 days after fumigation.

Table 3. Efficacy of the use of the ethanedinitrile for fumigation of pine logs infested with the pinewood nematode, *Bursaphelenchus xylophilus*.

Dose ($g\ m^{-3}$)	Ct ($g\ h\ m^{-3}$)*	The degree of filling the camera (%)**	Exposure time (h)	Temperature (°C)	Nematode mortality (%)	Source
48	–	68.2	24	–3.7-23.1	88.43	Chung *et al.*, 2007 Park *et al.*, 2014
69	–				77.94	
97	–				96.36	
148	–				98.02	
34 <	152.5	50	24	5.0 ± 0.5	99.99	Park *et al.*, 2012
	231.7				100	
	329.3				100	
100	398.68	46	24	21-33	100	Lee *et al.*, 2017a
120	547.22			6-12	100	
150	595.95	30		–1-3	100	
50	311.57	< 50	6	not given	100	Malkova *et al.*, 2016
			12		100	
			18		100	
40	678	< 50	24	20	100	Seabright *et al.*, 2020
60	1107		24	20	100	
100	1683		24	20	100	

* Ct – Concentration × time.

** filling the fumigation chamber with wood, (%).

In almost all variants of wood logs treatment with EDN numerous pinewood nematodes were extracted; however, all nematode stages (juvenile and adult), were dead. The average number varied from 11 to 188 nematodes $(100\ g)^{-1}$ of wood weight.

Additionally, nematodes from fumigated logs were also recovered by the Baermann funnel procedure with a paper filter. In this case, nematodes were not extracted. Based on these results, it was concluded that all nematodes in the logs died as a result of fumigation with EDN.

Comparison of the results of nematode extraction from PWN infested logs (control sample) using two Baermann funnel methods, with and without a paper filter, showed that the paper filter obstructs passage of nematodes by approximately 4-5%. All the extracted nematodes in both variants with and without paper filter, were alive and active, and their average number on 28th day after fumigation was 831 and 860 nematodes $(100\ g)^{-1}$ of wood, respectively. The average numbers of nematodes in the control logs remained almost unchanged during the entire study period (Table 2).

DISCUSSION

Ethanedinitrile is a new pesticide that can replace methyl bromide and is currently being tested in several countries to determine the doses for wood products. This fumigant can be used to eradicate various insects and nematodes associated with wood. In Table 3 the data from various publications on EDN fumigation of logs to eradicate the PWN are summarised. All results in Table 3 were obtained in experiments with pine logs. Application methods and parameters tested differ, but we tried to obtain a general comparison. According to published data, the lowest dose of EDN at which all pinewood nematodes in logs were killed is 34 $g\ m^{-3}$ for 24 h (Ct 231.7 $g\ h\ m^{-3}$) (Park *et al.*, 2012). Our results are most similar to the date of Malkova *et al.* (2016) with 100% death of nematodes at a dose of 50 $g\ m^{-3}$ (311.57) and 12 h exposure; however, the temperature was not indicated for this test.

Treatment of logs against *B. xylophilus* and their insect-vectors *Monochamus alternatus* carried out under similar conditions to the present study, showed the lethal minimum dose of EDN for nematodes at 20°C was 100 $g\ m^{-3}$, and for the eradication of beetles in the logs a 50 $g\ m^{-3}$ dose was required (Lee *et al.*, 2017a, b). Consequently, nematode eradication requires higher doses of fumigant then eradication of beetles, so we can assume that in the present study all beetles would have been killed.

The higher the temperature of the treatment lower dose and fumigation time are required to kill nematodes (Chung *et al.*, 2007; Park *et al.*, 2014; Pranamornkith *et al.*, 2014b; Hall *et al.*, 2015). The rate of sorption of EDN is considerably higher than that of methyl bromide (Hall *et al.*, 2017). To achieve 100% nematode mortality at low temperatures, the dose applied was 150 g m^{-3} (Lee *et al.*, 2017a). These data are especially valuable for round wood fumigation in the regions with low temperature conditions (Siberia, the Far East of the Russian Federation, and the northern regions of China). However, in this case, the consumption rates of the pesticide and its cost should be taken into account. Based on the analysis of published data and our experiments, it can be concluded that EDN may be an alternative to methyl bromide fumigant against PWN. However, additional tests are needed to study the efficacy of this pesticide in a range of temperatures and other types of forest products. In order to be included in ISPM standards for the eradication of the PWN from wood packaging materials, future tests must follow the requirements of the Technical Protocol (Magnusson & Schröder, 2009), namely the size of the wood pieces and the minimum of 100,000 nematodes to be tested in each combination (temperature – EDN dose).

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E.N. Arbuzova, O.A. Kulinich, A.A. Chalkin, V. Weis, R.K. Magomedov, Ya.B. Mordkovich, N.I. Kozyreva and A.Yu. Ryss. Эффективность применения динитрила щавелевой кислоты (ethanedinitrile) против сосновой стволовой нематоды *Burasphelenchus xylophilus* (Nematoda: Aphelenchidae) при фумигации бревен сосны обыкновенной (*Pinus sylvestris* L.).

Резюме. Фумигация относится к числу наиболее часто применяемых фитосанитарных мер при обеззараживании лесоматериалов от вредителей и болезней. Для этого обычно применяется бромистый метил (CH_3Br), однако рекомендации по применению этого препарата для сохранения озонового слоя Земли вынуждают ученых искать альтернативные средства обработки. К числу наиболее перспективных альтернативных фумигантов относится динитрил щавелевой кислоты (ДЩК). Исследования по его использованию ведутся в США, Новой Зеландии, Южной Корее, Чехии. Приведен аналитический обзор по применению данного препарата в этих странах. Целью наших исследований было изучение эффективности фумигации круглого леса препаратом ДЩК против сосновой стволовой нематоды *Bursaphelenchus xylophilus*. Древесные образцы (фрагменты ствола дерева сосны), зараженные нематодой *B. xylophilus* подверглись фумигации ДЩК в дозе 25 г/м^3, 50 г/м^3, 75 г/м^3 при экспозиции 24 ч и 50 г/м^3 при экспозиции 12 ч и температуре 20°C. Численность нематод в образцах была подсчитана за три дня до фумигации и составляла в среднем от 245 до 3950 особей на 100 г сырого веса древесины, и после фумигации через 24 ч, 48 ч, 10 дней и 28 дней. Во всех режимах обработки наблюдали 100% гибель нематод *B. xylophilus*. Наиболее экономически эффективными дозами обработки оказались 25 г/м^3 при фумигации в течение 24 ч и 50 г/м^3 в течение 12 ч. Опыты подтверждают эффективность препарата против нематод *B. xylophilus*, но необходимы дальнейшие исследования при других параметрах обработки ДЩК (температура, время экспозиции) и для иных видов лесопродукции.