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Evaluation of Different Applications of Ethanedinitrile (C_2N_2) in Various Fumigation Chambers for Control of *Monochamus alternatus* (Coleoptera: Cerambycidae) in Naturally Infested Logs

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Abstract

Monochamus alternatus Hopeis is an important vector of nematode pests of timber in Korea, particularly *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle. Previously, we reported that ethanedinitrile (C_2N_2) has the potential to replace methyl bromide and metam sodium to control *M. alternatus* larvae and *B. xylophilus* under low-temperature (<5 °C) conditions. Herein, we report on fumigation trials of C_2N_2 over a 3-yr period (February 2013–October 2015) conducted at higher temperatures. The trials were conducted under 24 different conditions that incorporated varying fumigation chamber types (plastic sheeting-enclosed chambers of differing construction or an ISO shipping container, interior size: 5.90 m length by 2.35 m width by 2.40 m height), log water content (24.1–43.5%), filling ratios (5, 20, and 40%), and temperatures (10.5–17.3 °C). Highest concentration × time (Ct) product values were obtained with the ISO shipping container followed (in order of decreasing Ct values) by a 0.1-mm-thick, low-density polyethylene tarpaulin enclosure, a 0.1-mm-thick polyvinyl chloride (PVC) tarpaulin enclosure, and a 0.05-mm-thick PVC tarpaulin enclosure. The correlation between Ct product value and mortality of *M. alternatus* larvae was calculated with all treatment combinations. From this, the $L(Ct)_{50}$ and $L(Ct)_{99}$ values for C_2N_2 were determined to be 73.19 and 194.90 g h m^{-3}, respectively. Ethanedinitrile showed promise as a practical alternative fumigant for use on fresh pine logs infested by *M. alternatus* larvae.

Key words: fumigation, ethanedinitrile, pine sawyer, *Monochamus alternatus*, timber

Pine wilt nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, commonly known as the pinewood nematode or pine wilt disease, is indigenous to North America (USA, Mexico, and Canada), but has been introduced to Japan, Korea, China, and Taiwan, South Africa, Portugal, and Spain (Dwinell 1997). Pine wilt disease is best known from Japan where it was first described in 1905 (Yano, 1913), although the pine wood nematode was not itself recognized as the causative agent until 1971, when, in association with an insect vector (the pine sawyer *Monochamus alternatus* Hopeis) it was found to be the cause of the wilt disease. The disease now causes massive economic damage to pines in Japan and increasingly in China, Korea, and Taiwan (Mamiya and Enda 1972, Morimoto and Iwasaki 1972, Yi et al. 1989, Kwon et al. 2005, Mota and Vieira 2008).

In South Korea, the pinewood nematode or the pine wilt disease has become a serious pest in pine forests since the first reports of its presence at Mt. Keumjong, Pusan, South Korea, in 1987 (Yi et al. 1989, Kwon et al. 2005). The worldwide spread of the nematode is thought to be largely due to increasing global trade of untreated wood products and logs infested with it and its vectors such as *Monchamus* spp. (Tomminen 1991, Li et al. 2009). *Monochamus alternatus* is the most important vector of *B. xylophilus* worldwide (Kwon et al. 2005).

Due to the seriousness of the pest problem, the European Union and China have banned importation of unseasoned wood from areas where *B. xylophilus* is known to occur (Mota and Vieira 2008). In Korea, the rate of spread of *B. xylophilus* has been decreasing annually since 2007 (e.g., the annual spread was 11,550 ha in 2013 and

9,644 ha in 2014). This is an apparent result of a national *B. xylophilus* management plan implemented by the Korea Forest Service (Korea Forest Service 2015).

There are several physical and chemical management practices used to address *M. alternatus* and *B. xylophilus* in pine trees in Korea, such as silvicultural control through preventative clear-cutting and the manual removal of logs, aerial spraying of insecticides, such as acetamiprid and thiacloprid, trapping of adult vectors with pheromone lures in infested areas during the adult emergence period, cutting and chipping of infested pine trees, trunk injection of nematocides such as abamectin benzoate and milbemectin, and fumigation of infested logs (Kwon et al. 2011, Park et al. 2014).

A major fumigant used previously for treating wood, methyl bromide, is now prohibited for use against pests in logs and other imported wood products in quarantine and preshipment treatments in Korea. Environmental and human safety concerns have prompted searches for alternative fumigants. Metam sodium, an organosulfur-based fumigant, is now commonly employed to eliminate potential forest pests from imported wood. It is applied at relatively high doses of l liter m^{-3} and spontaneously decomposes to methyl isothiocyanate by hydrolysis, and this has led to occupational health, safety, and environmental concerns (Woodrow et al. 2014). Another alternative fumigant, sulfuryl fluoride, showed efficacy against *B. xylophilus* in dry or low moisture content wood at high dose rates (>100 g m^{-3}), high temperatures (>15 °C), and long exposure times (>24 h; Bonifácio et al. 2014). However, sulfuryl fluoride is considered a dangerous compound and has been associated with cancer as well as reproductive, developmental, and neurological harm (Daily News Blog 2011). Phosphine is a fumigant that is widely used on grains and other commodities. It provides excellent penetration into the sapwood and efficacy in control of insect pests in logs and timber products (Ren et al. 2006, 2011). However, compared to methyl bromide and its aforementioned alternatives, fumigation time of phosphine is longer and efficacy is inconsistent when applied at low temperatures (Reichmuth 2002).

Ethanedinitrile (C_2N_2) has been examined as a timber fumigant (O'Brien et al. 1995, Viljoen and Ren 2001, Ren et al. 2005, Ren et al. 2011, Park et al. 2014). It penetrates quickly both with and across the wood grain, produces rapid suppression of insects and nematodes, and is not considered an ozone-depleting or greenhouse gas (Ren et al. 2005, 2006). It has a threshold limit value (TLV) of 10 ppm, which compares favorably with other fumigants (e.g., 1–5 ppm for methyl bromide and 0.3 ppm for phosphine). C_2N_2 has potential as an alternative to methyl bromide and metam sodium for control *B. xylophilus* and *M. alternatus*, specifically under low temperature conditions (Park et al. 2014). It was registered for timber treatment in Australia in 2014 and is being considered for registration for timber importation and log fumigation in Korea.

Here we report results of field trials of ethanedinitrile against *M. alternatus* larvae conducted from February 2013–October 2015, which included 24 different treatment combinations of different fumigation conditions. These consisted of different fumigation chamber types (various plastic-covered chambers and a metal shipping container), water content of logs (24.1–43.5%), filling ratios (5, 20, and 40%), and temperatures (10.5–17.3 °C).

Materials and Methods

Preparation of Wood Samples

The wood used for experiments was Japanese red pine (*Pinus desiflora* Sieb. & Zucc.) logs that were collected from forests in southern South Korea (34° 98′ N, 128° 24′ E), had been naturally attacked by *M. alternatus*, and were infested with mixed-aged larvae that had penetrated into the wood. In South Korea, *M. alternatus* has a two-year life cycle and cold winter temperatures terminate diapause; it overwinters as first- to fourth-instar larvae. Therefore, fumigation trials were conducted over a nearly 3-yr period (February 2013–October 2015). The logs were naturally infested also with *B. xylophilus*. The logs were from trees aged 15–20 yr, were 15–25 cm diameter, and cut into ~80–90-cm lengths. For each treatment, the moisture content and density of five randomly sampled logs was measured by standard methods (American Society for Testing and Materials, 1988). Wood samples (2.5 by 2.5 by 5 cm) were removed from the logs with the longest dimension cut parallel to the grain and at least 5 cm from the ends of the logs. For determination of the moisture content, the wood samples were heated at 103 °C for 7 h. The samples were weighed with a laboratory balance (3000g × 0.05g, ANYSCALES, Sunnybank, Queensland, Australia) before and after drying. Moisture content was the ratio of the weight of water (the difference between original and dried weight) to the dried weight expressed as a percentage. The moisture content of the logs over all the treatments ranged from 24.1 to 43.5%. For determination of wood density, the dimensions of the wood samples were measured (±0.2 mm) and weighed with the laboratory balance. Density was calculated as this weight divided by volume of the wood samples. The average number of logs used for each treatment was 28 (range of 25–30). The total number of logs used was >1,200: ~900 for fumigation treatments and 300 for controls.

Fumigant

Pressurized liquid ethanedinitrile (99% C_2N_2 and 1% air; BOC Australia, Sydney) was delivered into the chambers from a cylinder placed on a platform scale (A&D EM Scale, Sunnybank, Queensland, Australia). Liquid C_2N_2 was delivered directly through a 7.5 mm i.d. copper tube into the headspace of the chamber.

Fumigation of Pinewood Logs

Field fumigation trials with 24 distinct treatments were conducted at Jinju and Sacheon, Kyungnam Province, and Jeju Island, South Korea. Each treated and control chamber was loaded with 4–6, 15–18, and 30–35 wood logs (depending on size of logs) to achieve ~5, 20, and 40% loading ratios. The fumigations were conducted in a range of treatment chambers (lettering as in Table 1):

a. A box made from a stainless steel frame (100 by 100 by 100 cm) with five sides (four lateral sides and top) covered with a 0.10-mm-thick low-density polyethylene (LDPE) tarpaulin.
b. Same as "a" above but covered with a 0.05-mm-thick polyvinyl chloride (PVC) tarpaulin
c. A 20ft ISO standard shipping container (interior size: 5.90 m length by 2.35 m width by 2.40 m height) with a volume of 33.2 m^3 (Thermo King, Shanghai, China; pressure half-life < 1.5 min)
d. Same as "a" above but covered with a 0.10-mm-thick PVC tarpaulin
e. Same as "a" above but with all six sides covered with a 0.10-mm-thick LDPE tarpaulin.

The chambers a, b, d, and e were located on a concrete floor. The edges of the tarpaulin and joints between the tarpaulin and floor were sealed and held together with tape (Venture Tape, Metallized Cloth Duct Tape #1502, 3M, St. Paul, MN) to reduce gas escape from the fumigation enclosures.

Three loading ratios (5, 20, and 40%) calculated from equation 1 were tested. The dose range of C_2N_2 was from 20 to 100 g m^{-3}. During fumigation, the headspace temperature in the various

Table 1. Efficacy of C_2N_2 at controlling *M.alternatus* larvae in naturally infested pine logs under various fumigation conditions

Dose	Exposure time	Temp	Moisture content	No. of logs	Loading ratio	Fumigation	Ct product	No. of larvae			Mortality	Experiment date
(g m^{-3})	(h)	(°C)	(%)		(%)	Chamber	(g h m^{-3})	Alive	Dead	Total	(%)	Month and year
100	24	10.5 ± 1.5	25.3	20	40	a	230.9	0	29	29	100.0	Feb. 2013
100	24	10.5 ± 1.5	25.3	33	40	a	251.9	0	40	40	100.0	Feb. 2013
100	24	10.5 ± 1.5	25.3	20	40	a	269.5	0	32	32	100.0	Feb. 2013
100	24	10.5 ± 1.5	25.3	32	40	a	319.7	0	23	23	100.0	Feb. 2013
100	24	10.5 ± 1.5	25.3	43	40	a	350.7	0	33	33	100.0	Feb. 2013
40	24	15.1 ± 1.7	24.1	20	40	b	124.5	4	10	14	71.4	April 2013
40	24	15.1 ± 1.7	24.1	15	40	b	130.8	3	14	17	82.4	April 2013
50	24	15.1 ± 1.7	24.1	23	40	b	152.5	3	25	28	89.3	April 2013
30	24	10.9 ± 1.5	33.5	43	5	a	105.7	1	59	60	98.3	Sep. 2013
50	6	10.9 ± 1.5	33.5	28	5	a	145.7	3	57	60	95.0	Sep. 2013
50	24	10.9 ± 1.5	33.5	24	5	a	241.9	0	62	62	100.0	Sep. 2013
70	24	10.9 ± 1.5	33.5	23	5	a	297.4	0	55	55	100.0	Sep. 2013
20	6	11.5 ± 1.5	25.3	15	40	b	48.4	30	6	36	16.7	April 2014
40	6	11.5 ± 1.5	25.3	25	40	b	84.2	15	25	40	62.5	April 2014
50	6	11.5 ± 1.5	25.3	32	40	b	107.3	7	19	26	73.1	April 2014
50	24	13.0 ± 1.5	42.1	58	20	c	594.8	0	72	72	100.0	Oct. 2015
50	24	13.0 ± 1.5	42.1	39	20	c	606.7	0	99	99	100.0	Oct. 2015
100	24	15.0 ± 1.5	43.5	28	40	d	93.0	13	20	33	60.6	Oct. 2015
100	24	15.0 ± 1.5	43.5	27	40	d	154.6	0	13	13	100.0	Oct. 2015
100	24	15.0 ± 1.5	43.5	23	40	d	173.9	0	15	15	100.0	Oct. 2015
100	24	15.0 ± 1.5	43.5	17	40	d	191.7	0	14	14	100.0	Oct. 2015
100	24	15.0 ± 1.5	43.5	25	40	d	255.2	0	17	17	100.0	Oct. 2015
100	24	17.3 ± 1.5	41.2	38	40	a	245.1	0	48	48	100.0	Oct. 2015
100	24	17.3 ± 1.5	41.2	31	40	e	360.5	0	45	45	100.0	Oct. 2015

a A box made from a stainless steel frame (100 by 100 by 100 cm) with five sides (four sides and top) covered with a 0.10-mm-thick LDPE tarpaulin.

b Same as "a" above but covered with a 0.05-mm-thick PVC tarpaulin.

c A 20-ft ISO standard shipping container (interior size: 5.90 m length by 2.35 m width by 2.40 m height), with a volume of 33.2 m^3 (Thermo King, Shanghai, China; pressure half-life < 1.5 min).

d Same as "a" above but covered with a 0.10 mm thickness PVC tarpaulin.

e Same as "a" above but all six sides (four sides and top and ground) covered with 0.10-mm-thick LDPE tarpaulin.

chambers were automatically recorded (Thermo Recorder model TR-71U, T&D Corporation, Washington DC). Trials were conducted at seven different average temperatures that ranged from 10.5 to 17.3°C. Duplicate fumigation trials were carried out for either 6 or 24 h, and on completion, the chambers were opened and aerated for >24 h; then logs were split by hand for collection of target insects and nematodes. Loading ratios (Lr) as a percentage of total chamber volume were calculated as:

$$\mathrm{Lr} = \frac{\mathrm{Vt}}{\mathrm{Vf}} \times 100\% = \frac{\mathrm{Wt}}{\mathrm{Vf} \times \mathrm{Dt}} \times 100\% \qquad \text{Equation 1.}$$

Where: V_f = volume of fumigation chamber (m^3)
V_t = volume of wood samples (m^3)
W_t = weight of wood sample (kg)
D_t = density of wood sample (kg m^{-3})

Determination of C_2N_2 Concentrations

At timed intervals of 0.5, 1, 3, and 6 h during the 6-h fumigations and 0.5, 1, 3, 6, 12, and 24 h during the 24-h fumigations, a gas sample (400–500 ml) was withdrawn from the chamber using an electric pump (WOB-L model 2511, Welch, Mt. Prospect, IL) and stored in a 1 l capacity Tedlar gas sampling bag (SKC Inc., Sydney, Australia) for analysis, which occurred typically ≤ 1 h after sampling. The concentration of C_2N_2 was established with an Agilent Technology (Santa Clara, CA) model 7890N gas chromatograph (GC) with a flame ionization detector (FID) and a 30 m by 0.32 mm i.d. HP-5 (0.25 µm film) fused silica capillary column (Restek, Bellefonte, PA). The temperatures of the GC oven, injector, and detector were 150, 200, and 200 °C, respectively. The carrier gas used was helium at a flow rate of 2 ml min^{-1}. A 100-µl air tight syringe with valve (model 005279, SGE, Melbourne, Australia) was used for injection of gas samples into the GC. Injection volume of the gas sample was 60 µl. Periodically, the peak areas were calibrated relative to concentration of C_2N_2 by analysis of external standards. Standards were prepared by adding a known volume of C_2N_2 to a 1-liter Tedlar bag containing 500 ml air. The standards were analyzed by identical methods as the fumigation chamber samples, and integration areas were compared. The data reported are the means of replicate injections. The dose or volume of C_2N_2 (V_f in ml) at the experimental ambient temperature and pressure was calculated using the following equation (Ren et al. 2006).

$$V_f = \left(1 + \frac{T}{273}\right)\left(\frac{1.7 \times 10^6 \times C \times V}{P \times M \times N}\right) \qquad \text{Equation 2.}$$

Where: T = temperature (°C)
C = intended concentration (mg liter^{-1})
V = volume of fumigation chamber (liter)
P = atmospheric pressure (mm Hg)
M = molecular weight of fumigant
N = purity of fumigant (%)

Bioassays of *M. alternatus* Larvae

After 6- or 24-h exposure, the fumigation chambers were opened and aerated for 24 h. Mixed-age live and dead *M. alternatus* larvae

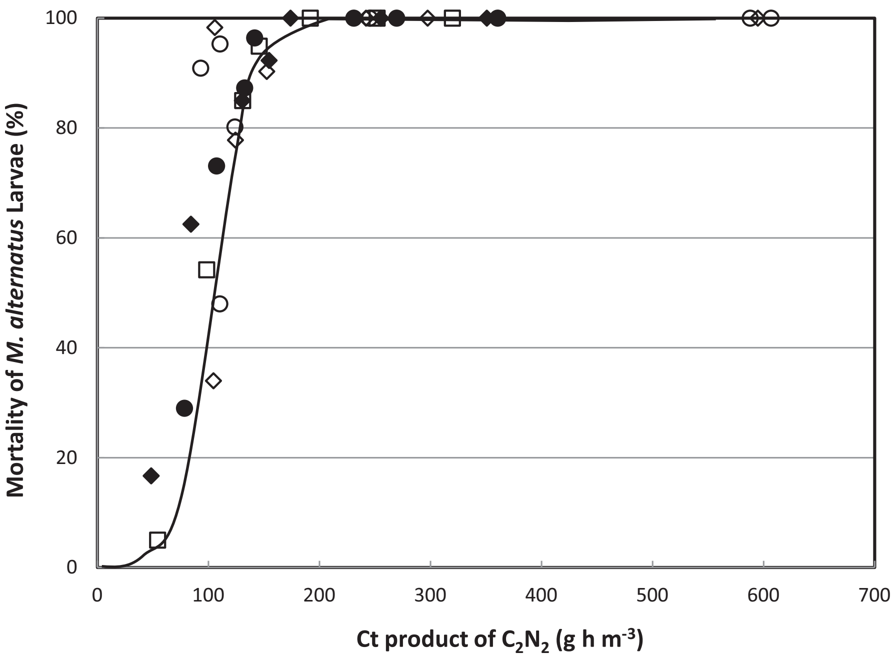

Fig. 1. The correlation between *Ct* product values of C_2N_2 and mortality of *M. alternatus* larvae in naturally infested pine logs. Chambers a (□), b (◆), c (◇), d (•), and e (○).

were collected by splitting all treated and unfumigated logs. All collected live and dead larvae were counted and then each five live and dead larvae were counted and placed into plastic petri dishes (9 cm diameter by 5 cm height; SPL Life Sciences, Korea) filled to 20% capacity with fresh red pine sawdust. The petri dishes were then transferred into an incubator (model i500, Steridium, Brendale, Queensland, Australia) for 72 h at 25 ± 2 °C and 70 ± 5% RH to measure end point of mortality. Unfumigated pine logs were used for calculating control mortality, and for these assays the control mortality was always zero.

Determination of Concentration × Time Products (Ct) of Fumigant in Fumigation Chambers

The concentrations of fumigants were monitored at time intervals over the exposure period (6 and 24 h) and used to calculate the concentration × time product (Ct as mg h liter^{-1}) from equation 3.

$$\mathrm{Ct} = \sum (\mathrm{C}_i + \mathrm{C}_{i+1})\ (\mathrm{t}_i - \mathrm{t}_{i-1})/2 \qquad \text{Equation 3.}$$

Where: C = fumigant concentration (mg liter^{-1})
t = time of exposure (hours)
i = the order of measurement

Statistical Analysis

The toxicological dose response to C_2N_2 by *M. alternatus* larvae was calculated by probit analysis using a computer program produced by P.C. Annis of the Commonwealth Scientific and Industrial Research Organization Entomology, Australia, based on Finney (1971). The indices of toxicity measurement derived from this analysis were $L(Ct)_{50} = 50\%$ and $L(Ct)_{99} = 99\%$ lethal concentration × time, or the doses required to cause 50 and 99% mortality in tested insects.

Results and Discussion

The results from 24 fumigation trials with C_2N_2 under various combinations of conditions are given in Table 1. Based on concentration of C_2N_2 measured at timed intervals, the cumulative Ct product values of the compound were calculated (Ren et al. 2011; Table 1).

Even though temperatures ranged from 10.5 to 17.3 °C, these resemble the normal temperature range in late spring and autumn in Korea. The correlation between Ct product value of C_2N_2 and mortality of *M. alternatus* larvae was calculated using the combined data from all trials. The observed and fitted data relating mortality to Ct product are shown in Fig. 1. The observed data covered mortality from 0–100% and adequately covered the intermediate range (Fig. 1). The results of probit analysis showed that there were highly significant ($P < 0.001$, $n = 23$) effects for fitting the slope parameter for each treatment. Based on this correlation, the slope was 5.49 (±0.58); the $L(Ct)_{50}$ and $L(Ct)_{99}$ values of C_2N_2 were 73.19 (95% confidence limits: 73.19–90.79) and 194.90 (177.01–211.55) g h m^{-3}, respectively. In our previous study of low-temperature fumigation (i.e., at 4.4 and 6.1 °C), $L(Ct)_{99}$ values of C_2N_2 for the larval stage of *M. alternatus* were estimated to be between 657 and 1,074 g h m^{-3} (Park et al. 2014). This result is typical for fumigants in that that fumigation at higher temperatures is usually more effective than at lower temperatures, although C_2N_2 offers good efficacy for controlling *M. alternatus* in green logs at low temperatures (Ren et al. 2011). The highest Ct products of C_2N_2 were obtained in the shipping container followed by the 0.1-mm-thick, low-density polyethylene tarpaulin, the 0.1-mm-thick PVC tarpaulin, and the 0.05-mm-thick PVC tarpaulin ($P > 0.05$). Tarpaulins are currently widely used in Korea and other countries to produce fumigation chambers. These trials confirmed that tarpaulins are a low-cost sealing material suitable for effective fumigation practices.

Based on the results of our field trials conducted under typical field application conditions at different fumigant dosages, environmental conditions, and fumigation chamber types, we conclude that—1) C_2N_2 is an effective fumigant under field conditions against *M. alternatus*, 2) C_2N_2 may offer better control of this pest in timber than metam sodium (Park et al. 2014), and 3) C_2N_2 may be an alternative to metam sodium for treatment of timber in a variety of fumigation chambers over a broad range of temperatures.

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