Mortality and Movement Behaviour of *Bursaphelenchus xylophilus* under Different Dosages of Copper Sulphate

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Abstract

Tan Q.Q., Wu H.Y., Jiang S.X., Ma H.B. (2013): **Mortality and movement behaviour of** *Bursaphelenchus xylophilus* **under different dosages of copper sulphate**. Plant Protect. Sci., **49**: 98–103.

The effects of *in vitro* exposure to different concentrations of copper sulphate for different durations on the mortality and locomotor behaviour of *Bursaphelenchus xylophilus* were evaluated. Copper sulphate showed strong effects against the nematode *B. xylophilus* at a low concentration (5 mg/l). The median lethal concentration (LC_{50}) at 8, 24, 48, and 72 h were 31.634, 7.353, 6.557, and 5.568 mg/l, respectively. Mortality rate of *B. xylophilus* after treatment with various concentrations of copper sulphate for 6, 8, 24, 48, and 72 h were significantly different compared with control animals exposed to distilled water ($P < 0.01$). Locomotor activity of the nematodes decreased with increased duration of exposure and was completely lost after 8 h of exposure to 100 mg/l copper sulphate. Our results indicate that copper sulphate is effective against *B. xylophilus* and restricts their harmful effects on plants at least in part by inhibiting their movement.

Keywords: LC_{50}; mortality; locomotor behaviour; pine wood nematode

The pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle causes a disastrous and uncontrollable disease – the pine wilt disease (PWD) in the East Asian countries of Japan, China, and Korea. The first reported occurrence of *Bursaphelenchus xylophilus* infection in China was in 1982 in the Sun-Yat-Sen Mauaoleum Landscapes of Nanjing (Cheng *et al*. 1983). Since then, *B. xylophilus* has spread widely in many regions of China, causing significant damage to pine ecosystems in 17 provinces, including Jiangsu, Zhejiang, Anhui, Fujian, Jiangxi, Taiwan, and Hong Kong (Zhang *et al*. 2010). Forest areas of approximately 300 000 ha have been destroyed and more than 5 billion pine trees have been killed by PWD. This disease causes economic losses of several billion dollars annually because of serious damage to pine forest resources and the natural landscape (China National Forestry Department Announcement, 2010; http://www.forestry.gov.cn//portal/main/s/196/content-340311.html).

Natural transmission of this nematode is by insect vectors, mainly sawyer beetles (*Monochamus* spp.), but long-distance transmission occurs via non kiln-dried wood products, such as logs, lumber, pallets, crates, wood chips, and furniture. Effective control of this nematode has been a challenging problem. Some compounds from plant sources and synthetic chemicals have been evaluated for their nematicidal activity against the pine wood nematode, such as trans-cinnamaldehyde and trans-cinnamic acids (Kong *et al*. 2007; Nguyen *et al*. 2009; Dang *et al*. 2010). However, those compounds are usually expensive, often limiting their practicality. Copper sulphate is a fungicide commonly used in agricultural production, and is more cost-effective and environment-friendly compared to other chemicals. Copper sulphate baths

Supported by the National Natural Science Foundation of China, Grant No. 31101420, the Special Fund for Agro-Scientific Research in the Public Interest, Grant No. 200903040, and the Technology Program of the Higher Education Institutions of Shandong Province, Project No. J11LC23.

have been shown to be effective for treatment of *Sparus aurata* fingerlings (Varo *et al.* 2007). High concentrations of copper in food (e.g., 320 mg/l) have been shown to significantly affect fly development and viability (Ding *et al.* 2006). Copper sulphate at 70, 33, and 65% has been reported to be effective against *Haemonchus*, *Ostertagia*, and *Chabertia* infestations, respectively, in sheep and goats (Kurtpinar *et al.* 1950). In another report, copper oxide wire particles were used to prevent gastrointestinal nematode infection in sheep and goats (Burke & Wang 2007, 2010). Jaworska and Gorczyca (2002) found that cadmium, copper, lead, and zinc increased the mortality of the invasive larvae of *Steinernema feltiae*, and decreased the pathogenicity of the insects; however, no systematic testing of locomotive behaviour following chemical exposure was carried out.

In 2009, a representative sample of the *B. xylophilus* population was collected from the Chengshan forest factory. We then studied changes in locomotion induced by exposure to copper sulphate in *B. xylophilus*. This study was aimed at evaluation of toxicity of copper sulfate against pine wood nematode (PWN) and the behavioural response of the nematode, which is manifested mainly as changes in locomotor behaviour, thus providing a quick and beneficial method to assess nematicidal toxicity incurred in *B. xylophilus*.

MATERIAL AND METHODS

Chemicals. Copper sulphate (99%) was obtained from the Tianjin Damao Chemical Reagent Factory (Tianjin, China). Five different concentrations of copper sulphate solution – 5, 25, 50, 75, and 100 mg/l – were used in this study.

Preparation of nematodes. *Bursaphelenchus xylophilus* was isolated and extracted by the Baermann funnel method from chips of infected pine wood collected from the Chengshan forest farm, in Rongcheng, Weihai City, Shandong Province, China, as described by Viglierchio and Shimit (1983). The fungus *Botryis cinerea* Pers. was cultured on potato dextrose agar medium in Petri dishes (90 mm in diameter) at 25 ± 1°C for 6 days. Petri dishes with fully grown fungi were inoculated with *B. xylophilus* and kept until fungal mycelia were completely consumed (~ 13 days). The cultured nematodes (mixed stage) were separated from the culture media by using the shallow dish technique (Takai *et al*. 2000), and then filtered through four layers of gauze on a 38 μm mesh screen and washed 3 times with sterile water before collecting. The suspension of nematodes was concentrated by centrifugation at 2500 rpm for 3 min and then placed into a 50 ml beaker and counted on a grid under a microscope. An aqueous suspension of the nematodes (800 nematodes/ml) was prepared by appropriate dilution for use as a working stock and stored at 4°C.

Bioassay. Five different concentrations of copper sulphate solution were tested, and distilled water was used as the control. A 50 μl sample of fresh nematode suspension was dispensed into a 96-well plate (approximately 40 nematodes in each well); 200 μl aliquots of different concentrations of copper sulphate were then added to

Table 1. Visual assessment assay (VAA) scores of nematode behaviour after exposure to copper sulphate

VAA scores	Activity condition of *B. xylophilus*
3.0	control, all nematodes active and vigorous, obviously sinusoidal in shape, amplitude is expressed as A
2.8	all nematodes motile, 80% of nematodes active and sinusoidal, but amplitude is less than A
2.5	all nematodes motile, 75% of them active and sinusoidal, but move more slowly than control
2.0	all nematodes motile, 30% of them no sinusoidal shape, body shrinkage, move slow, amplitude is expressed as B
1.5	all nematodes motile, 30% of them no sinusoidal shape, 60% body shrinkage, few head or tail swinging right and left, nematodes' shape '∞', '8' (Figures 2A–E), move frequency is slower than B
1.0	30% of nematodes motile, but no sinusoidal shape, head or tail move right and left, movement slow, other nematodes non-motile
0.5	less than 20% of nematodes motile, only head or tail weak activities, other nematodes non-motile
0.2	less than 5% of nematodes only head or tail slowly twitch every several seconds, other nematodes non-motile
0.0	all nematodes were not moving, body was straight or "J" shaped (Figure 2F)

each 96-well plate, with 4 replicate wells for each treatment concentration. The 96-well plate was covered with a lid, leaving a small gap for aeration, and the assay plates were incubated at 25 ± 1°C. Nematode behavior was observed under an inverted compound microscope at a magnification of 40× (AE31 Motic, Xiamen, China), and visual assessment assay value (Zasada *et al.* 2009) was recorded hourly from 0 to 8 h (Table 1). All experimental treatments and controls were carried out as 4 replicates, and repeated 5 times.

Mortality of *B. xylophilus* was evaluated under an inverted microscope at 0, 2, 4, 6, 8, 24, 48, and 72 h after exposure to different concentrations of copper sulphate. The nematodes were considered alive if they could move and retained an S-shaped curly and wavy body shape; they were considered to be dead if they did not move and had straightened body shape; J- and C-shaped nematodes could not recover upon immersion in water after treatment with different chemicals.

The behaviour of nematodes in each treatment group was observed under an inverted microscope and recorded (Motic SMZ-168). Survival ratio (SR) was determined using the formula: SR = $(Ta/Tb) \times 100$, where: Ta – number of live nematodes after chemical treatment and Tb – total number of nematodes; adjusted mortality (AM) was calculated using the formula: AM = $[(\text{TM} - \text{CM})/(1 - \text{CM})] \times 100$, where TM – mortality ratio after treatment of the nematode and CM – mortality ratio of the control.

Data analysis. Statistical analyses were performed using the SPSS Version 12.0 software and differences with $P < 0.05$ were considered statistically significant.

RESULTS

Nematicidal activity of copper sulphate

Nematicidal activity of copper sulphate was evaluated by comparing the median lethal concentrations (LC_{50}) for different concentrations on *B. xylophilus* under different exposure times. The results showed that copper sulphate had stronger toxic activity against *B. xylophilus* at low concentrations. There was no significant difference in mortality among 0-, 2-, and 4-h treatment times when different treatment concentrations were used ($P > 0.05$). Mortality of *B. xylophilus* after 6, 8, 24, 48, and 72 h of exposure at different treatment concentrations were significantly different compared with the control ($P < 0.05$). Adjusted mortality after 24, 48, and 72 h was 100% for copper sulphate concentrations of 50, 75, and 100 mg/l (Table 2). Nematicidal activities of copper sulphate at different treatment durations can be determined by measuring the median lethal concentration (LC_{50}). The LC_{50} values at 8, 24, 48, and 72 h were 31.6342, 7.3527, 6.5574, and 5.5678 mg/l, respectively (Table 3).

Locomotor behaviour of *B. xylophilus* after treatment with copper sulphate

The behavioral responses of *B. xylophilus* at different concentrations of copper sulphate within 8 h of exposure are shown in Figure 1. All nematodes were active and vigorous with an obvious sinusoidal shape at 0 h of treatment. With increase in the

Table 2. Mortality of *B. xylophilus* at different durations after exposure to different concentrations of copper sulphate

Concentration (µg/ml)	Mortality (%) at different times (h) after treatment							
	0	2	4	6*	8***	24***	48***	72***
5	0.00[a]	0.00[a]	0.58[a]	3.66[bc]	5.11[c]	21.64[b]	29.61[b]	35.12[b]
25	0.00[a]	0.00[a]	2.83[a]	11.57[abc]	47.92[b]	99.36[a]	99.59[a]	100.00[a]
50	0.00[a]	0.36[a]	3.31[a]	17.74[abc]	52.43[b]	100.00[a]	100.00[a]	100.00[a]
75	0.00[a]	0.98[a]	4.38[a]	20.31[ab]	80.46[a]	100.00[a]	100.00[a]	100.00[a]
100	0.00[a]	0.67[a]	5.85[a]	17.85[a]	89.53[a]	100.00[a]	100.00[a]	100.00[a]
Control	0.00[a]	0.00[a]	0.00[a]	0.00[c]	0.00[c]	0.00[b]	0.00[c]	0.00[c]

Means within the same column followed by different letters are significantly different ($P < 0.05$) according to the *LSD;* *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

Table 3. Toxicity of copper sulphate to *B. xylophilus* at different treatment durations

Exposure time (h)	Slope (± SE)	Correlation coefficient	LC_{50} (95% Cl)	LC_{90} (95% Cl)
8	0.83 (± 0.90)	0.9717	31.634 (10.636–390.048)	141.581 (31.491–4913.209)
24	0.63 (± 0.04)	1.0000	7.353 (7.352–7.354)	13.806 (13.804–13.808)
48	0.56 (± 0.04)	1.0000	6.557 (6.557–6.558)	12.545 (12.544–12.546)
72	0.52 (± 0.67)	1.0000	5.568 (5.568–5.568)	7.987 (7.987–7.987)

LC – lethal concentration expressed in mg/l copper sulphate with 95% confidence intervals (CI)

concentration of copper sulphate and treatment duration, locomotor behaviour of the nematodes started to change. After 1 h, the visual assessment assay (VAA) scores of treated juveniles were significantly lower than those for controls. The VAA score reached a low of 23.3% at the highest concentration of copper sulphate (100 mg/l) ($P < 0.05$). At the same concentration, *B. xylophilus* activity was significantly reduced relative to the activity of control nematodes in distilled water when observed after 8 h of treatment ($P < 0.05$). The '∞' and '8' shapes of nematodes were observed after 3 h at 75 mg/l, after 4 h at 50 mg/l, and after 5 h at 25 mg/l (Figure 2A–E). The activity of juveniles was reduced by 93.3% at the highest copper sulphate concentration after 8 hours. Although some juveniles remained alive after 8 h, minimal motility with only the head or the tail twitching slowly every several seconds was observed. For different durations of treatment, the activity of the nematodes changed significantly at 25, 50, 75, and 100 mg/l as compared with the control ($P < 0.05$). After 8 h, the activity of the nematodes changed significantly ($P < 0.05$) at different concentrations

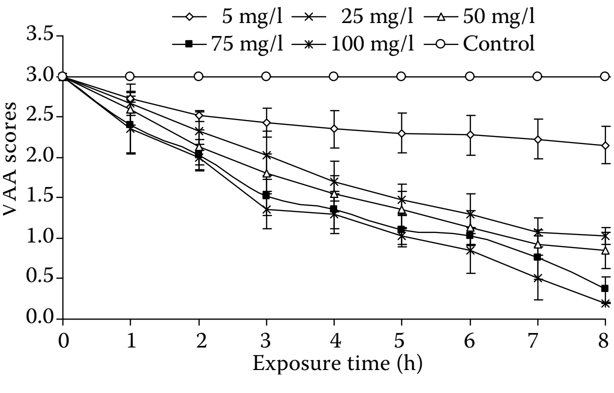

Figure 1. Visual assessment assay (VAA) scores over time after exposure of *B. xylophilus* to different concentrations of copper sulphate and untreated control

compared to the control. The highest concentration of copper sulphate (100 mg/l) produced an immediate effect on the nematodes' behaviour, resulting in decreased movement compared with the control; after 8 h, all nematodes were immobile with straight or 'J' shaped bodies (Figure 2F). In contrast, all nematodes in the distilled water control showed vigorous activity after 8 hours.

DISCUSSION

Previous studies have reported that copper sulphate and cupric chloride show nematicidal activity against PWN (Kobamoto & Izuma *et al.* 1984). However, the concentration of the nematicide and duration of treatment required for effective control of the nematodes are not well known. In the present study, a critical point for nematode mortality was determined: the adjusted mortality was 89.53% after exposure of the nematodes to 100 mg/l copper sulphate solution for 8 hours. Copper sulphate was found to have a pronounced effect on *B. xylophilus in vitro*, resulting in significant reduction of movement, head and tail swinging frequency, and body bending frequency. To our knowledge, this is the first report on the behavioural changes in *B. xylophilus* produced by a nematicide. In a previous study, Zasada *et al.* (2009) reported significant behavioural effect of benzyl isothiocyanate (BITC) on *Meloidogyne incognita* – significantly reduced infectivity was observed after treatment of second stage juveniles with 0.01 mmol/l BITC. In addition, egg production was almost completely eliminated (< 5% of control) by 0.03 mmol/l BITC. In a study of *Caenorhabditis elegans* with head thrash as an endpoint, toxicity from $CdCl_2$, $CrCl_2$, $HgCl_2$, and $Pb(NO_3)_2$ exposure at a low concentration

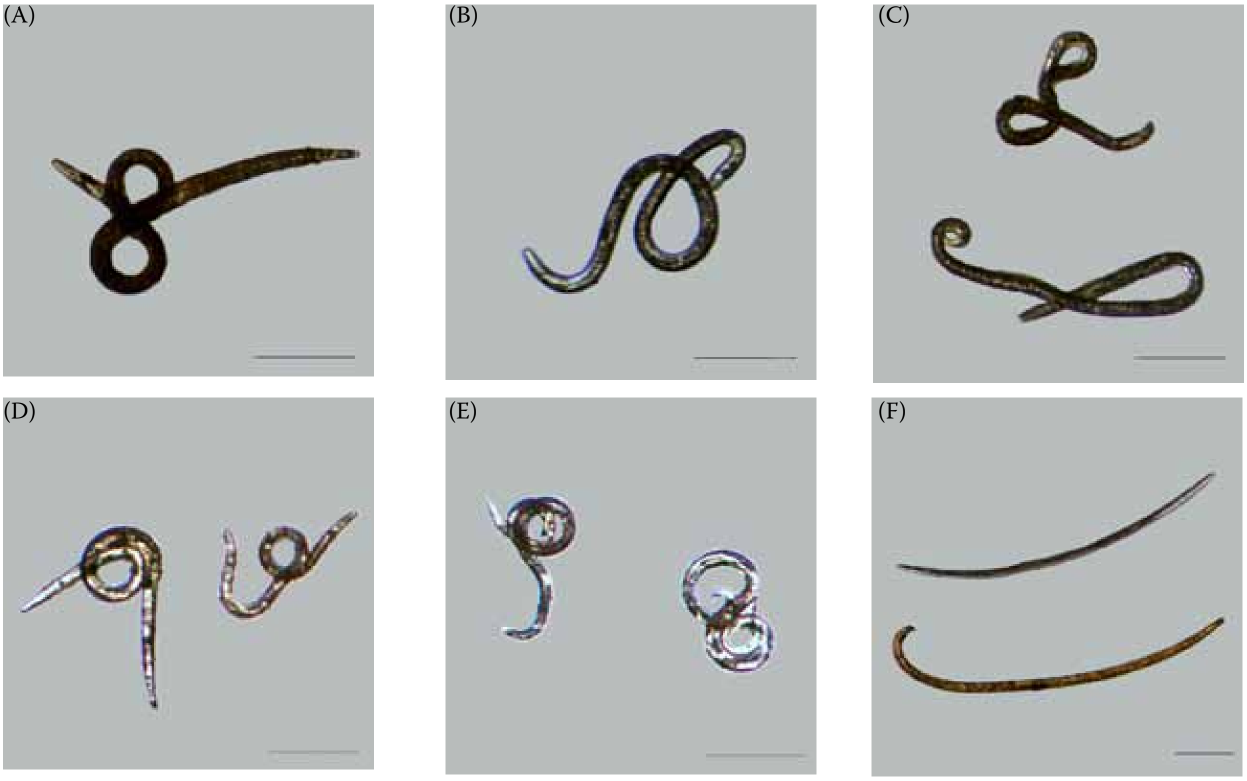

Figure 2. Shape of *B. xylophilus* following exposure to copper sulphate observed under an inverted microscope (bars = 100 μm)

(2.5 μmol/l) was detected and body bending was used as an assay to evaluate the toxicity from all assayed heavy metal exposures at different concentrations (Wang & Xing 2008). In the present study, movement-related behaviours, including body shrinking and head movement, of *B. xylophilus* exposed to chemicals for different durations was monitored. We observed that the nematodes slowed down abruptly when they were initially placed in different concentrations of copper sulphate, which may be due to sudden stimulation by copper sulphate, and this was followed by recovery within several minutes.

In a previous study, the effectiveness of control measures against PWN was evaluated on the basis of mortality, but their nematicidal effects were not evaluated. In the present study, exposure to 25 mg/l of copper sulphate for 72 h was found to have a significant effect on *B. xylophilus*, and the nematodes could not recover even after treatment with water. Our results indicate that screening of nematicidal chemicals for the control of PWD should adopt a combination of approaches involving assessment of nematode mortality and locomotor behaviour. Such an approach can provide a more reliable assessment of the efficacy of nematicides in controlling PWN infestation.

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Received for publication May 31, 2012
Accepted after corrections September 15, 2012

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