[Research Note]**Distinguishing between inactivated and dead second stage juveniles of *Meloidogyne incognita* using the NaOH method**Yuki Harada¹ and Toyoshi Yoshiga^{1,*}

The ability to distinguish inactivated nematodes from dead ones is sometimes difficult because of their immobile. We tested whether the NaOH method could be applied to distinguish second stage juveniles (J2s) of the root-knot nematode *Meloidogyne incognita* that were inactivated under different conditions. The J2s inactivated by abamectin, a GABA agonist, or under the hypoxic conditions caused by CO₂, reacted to the addition of NaOH. On the other hand, the J2s inactivated by fosthiazate, an organophosphate that causes muscle contraction, and those by NaN₃, which inhibits respiration, did not react well to the NaOH treatment. However, these J2s reacted to the NaOH treatment after subsequent removal of the chemicals. The J2s inactivated by low temperature showed no reaction to the NaOH treatment. The NaOH method was demonstrated to be applicable for naturally immobilized nematodes such as the entomopathogenic nematode *Steinernema litorale* and the plant parasite *Aphelenchoides besseyi*. These results suggest that the NaOH method is applicable to distinguish between inactivated and dead nematodes but caution is warranted with regard to treatment temperature and the modes of action of the chemicals that are used. Nematol. Res. 45(1), 51-55 (2015).

Key words: immobilization, inactivation, *Meloidogyne*, nematicide, sodium hydrate

INTRODUCTION

The root-knot nematodes (*Meloidogyne* spp.) are one of the major plant parasitic nematodes that reduce crop yield and cause economic losses all over the world (Sasser, 1980). Using chemicals, such as nematicides, to

prevent the invasion of the second stage juveniles (J2s), which is the nematode's only infective stage, is one of the most easy and efficient ways of control. Clarification of the effects and modes of action of the chemicals is important to evaluate their efficiencies. However, we are often faced with inactivation of J2s by chemical treatment, which makes it difficult to determine if the inactivated nematodes are dead or alive. For example, the nematicide abamectin is a mixture of avermectin B_{1a} and B_{1b}, and avermectins have been shown to rapidly and irreversibly impair the locomotor activity of *M. incognita* (Kofoed and White) Chitwood J2s (Wright *et al.*, 1983). In addition, J2s are often inactivated under different condition such as hypoxia or cold temperatures; however, it is not easy to determine if they are dead or alive.

Various methods have been employed to distinguish between inactivated and dead nematodes. Careful observation of the inner body structure of nematodes is one of the ways of determination because the destruction of cells and organs can often be observed in dead nematodes under a microscope. However, it can be subjective and difficult to distinguish if the evaluation is performed prior to significant cell destruction. Another commonly used method is probing with a fine needle, but the needle is difficult to manipulate and it is time-consuming to manage a large number of samples. Dead nematodes easily incorporate stains but live ones do not, which results in the application of staining methods (Cairns, 1960; Bird, 1979; Donald and Niblack, 1994). However, staining efficiency largely depends on the molecular permeability, and dead but undamaged nematodes do not incorporate staining and are not readily stained. Moreover, it is time-consuming to stain nematodes, particularly for the parasitic and phoretic stages of nematodes whose cuticles are less permeable.

Among these methods, the NaOH method has been shown to be better for evaluation of the viability of *Heterodera glycines* Ichinohe after exposure to fungal filtrates (Chen and Dickson, 2000; Chen *et al.*, 2000). The aim of the present study was to evaluate if the NaOH method could be used to distinguish between inactivated and dead *M. incognita* J2s under different conditions. In addition, we demonstrated whether this method could be applicable to other nematode species.

MATERIALS AND METHODS

Nematodes:

Meloidogyne incognita (Nishigoshi strain) originally obtained from Dr. Iwahori at NARO Kyushu Okinawa Agricultural Research Center, Kumamoto, Japan was

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maintained with mini-tomato *Solanum lycopersicum* (Pritz: Kaneko Seeds, Co., Ltd., Japan) grown in the growth chamber at 27°C (16-hr light/8-hr dark photoregime). Freshly hatched J2s from egg masses collected from roots 40-50 days after nematode inoculation were subjected to our experiments.

Aphelenchoides besseyi Christie was extracted from rice grains from Kagoshima Prefecture using the Baermann funnel method. *Steinernema litorale* Yoshida was maintained using *Galleria mellonella* (Linnaeus) larvae as a host. Infective juveniles (IJ) of *S. litorale* were used for the experiments.

Nematode treatment:

Abamectin standard (mixture of avermectin B_{1a} and B_{1b}, 016-20361, Wako Pure Chemical Industries, Ltd., Japan) and fosthiazate standard (066-03381, Wako Pure Chemical Industries, Ltd., Japan) were purchased. The chemicals were dissolved first in a small amount of acetone and then in deionized water to make a 200 µg/ml solution. An aliquot of 200 µl of 2 µg/ml solution prepared by serial dilutions was mixed with same volume of *M. incognita* J2 suspension in a well of a 24-well plate to prepare a final concentration of 1 µg/ml and the nematode suspension was incubated at 25°C for 24 h. As a control, nematodes were soaked in deionized water for 24 h. For NaN₃ treatment, J2s were soaked in a final concentration of 1 mM NaN₃ solution for 1 h. These chemically treated nematodes were used for the NaOH assay directly or after removal chemicals. To remove the chemicals, nematode suspension was centrifuged in a glass test tube at 3,000 rpm for 1 min and nematodes were washed with deionized water three times. The washed nematode suspension (400 µl) containing approximately 50 individual washed nematodes was added to a well of a 24-well plate for the evaluation of survival.

We obtained J2s inactivated by low temperatures by leaving J2s suspended in a 100 ml beaker at 4°C for 24 h. To obtain J2s inactivated under hypoxic conditions, approximately 3 ml of the J2 suspension was aerated with CO₂ gas for 30 min. A silicon tube attached to a bottle of compressed CO₂ was connected to a Pasteur pipette (5.75 inches in length; Iwaki, AGC Techno Glass Co., Ltd., Japan) and gentle bubbles were generated in the nematode suspension. To recover from the cold or hypoxic condition, J2s were left at 25°C for 1 h. Dead but less-damaged nematodes were prepared as a control by killing J2s in a 60°C water bath for 5 min.

Inactivated *A. besseyi* and *S. litorale* IJ were obtained after isolation by the Baermann funnel method without

any treatment or by leaving live IJ in water for 2 h, respectively.

Determination of viability:

Mortality was determined by following the methods described by Chen *et al.* (2000). The conditions we used were as follows. We added 40 µl of 1 N NaOH to a 400-µl nematode suspension with approximately 70 individual J2s per well of a 24-well plate and mixed by gentle shaking for several seconds. We observed nematode movement under a stereomicroscope immediately after the nematodes sank to the bottom of the well. The nematodes with a straight posture after NaOH treatment were judged as dead. The nematodes with movement were judged as alive. Those without movement but whose bodies were contracted and whose anterior parts were bent at the observation were also judged as alive because those were observed to move in prolonged observation in preliminary experiments. The experiment was performed at least twice, with three replicates per treatment.

RESULTS AND DISCUSSION

All the *M. incognita* J2s soaked in deionized water for 24 h immediately reacted to the addition of NaOH and showed clumsy undulatory movement (Figs. 1a, b). On the other hand, heat-killed J2s did not react to NaOH at all (Figs. 1c, d). These results indicate that live *M. incognita* J2s react to the NaOH treatment but the dead ones do not.

When J2s were soaked in abamectin solutions for 24 h, all of them were inactivated and most of them were immobilized in a straight posture (Fig. 1e). Immediately after the addition of NaOH, the inactivated J2s reacted and started slow but continuous movement (Fig. 1f). Within 10 min, some J2s stopped their movement with their bodies bent. After 20 min, some J2s started to take on a straight posture. Within 1 h, most J2s were immobilized in a straight posture. The immobilized J2s did not react to additional application of NaOH.

In contrast to the abamectin-treated nematodes, J2s treated for 24 h with fosthiazate, an organophosphate, showed strong contraction and the addition of NaOH resulted in little reaction (Figs. 1g, h). Thus, it was difficult to judge whether the J2s were alive or not by the direct addition of NaOH. However, 24 h after removal of fosthiazate, all of the J2s reacted to the addition of NaOH (Table 1). This indicates that the NaOH method can be used to determine the survival of inactivated J2s 24 h after removal of fosthiazate.

The J2s immobilized by hypoxic conditions using

CO₂ gas with a straight posture immediately reacted to the addition of NaOH (Table 1). Sodium azide, which inhibits respiration, is commonly used as an anesthetic agent in nematodes (Sulston and Hodgkin, 1988). Immediately after addition of NaN₃, J2s became immobilized in a bent posture that was kept at least for 1 h after soaking and gradually became straight over 24 h. The immobilized J2s did not exhibit any apparent reaction to NaOH addition in the presence of NaN₃ (Table 1). However, the immobilized J2s after soaking in NaN₃ for 1 h, followed by removal of NaN₃, reacted to the addition of NaOH (Table 1). These results suggest that the J2s that were alive but inactivated by NaN₃ were not able to react to NaOH in the presence of NaN₃ but could react after removal of NaN₃.

The J2s inactivated at 4°C for 24 h showed a straight

Table 1. Reaction of *Meloidogyne incognita* J2s after NaOH treatment

	Reaction to NaOH	
	In the presence of treatment	After removal of treatment
Abamectin ¹	+	nt
Fosthiazate ¹	—	+
NaN ₃ ²	—	+
Distilled water	+	nt
CO ₂	+	+
4°C	—	+
Heat-killed	—	nt

+: J2s reacted to the addition of NaOH solution.

—: J2s did not react to the addition of NaOH solution.

nt: not tested.

All experiments were performed at 25°C otherwise mentioned.

¹ Nematodes were treated for 24 h.

² Nematodes were treated for 1 h.

Fig. 1. Effects of NaOH treatment on *Meloidogyne incognita* J2s. J2s soaked in deionized water (a, b) and those inactivated in 1 µg/ml abamectin solution (e, f) for 24 h reacted to the addition of NaOH. In contrast, fosthiazate-treated J2s for 24 h showing body contraction (g, h) and heat-killed J2s (c, d) did not change their posture by the NaOH treatment.

posture and did not react to NaOH if it was added before the temperature increased. When the temperature reached room temperature before the addition of NaOH, inactivated J2s resumed their movement and reacted to the NaOH treatment, suggesting that the J2s inactivated at 4°C were alive.

Some nematode species become immobilized under certain conditions. For example, the plant parasitic nematodes *A. besseyi* often become inactive after extraction from rice grains (unpublished observation). Some species of steinernematid entomopathogenic nematodes become immobilized when they are maintained in water. To examine if the NaOH method could be applicable to endogenously inactivated nematodes, we performed the NaOH method on *A. besseyi* and *S. litorale*. We found that all immobilized nematodes of these two species reacted to the addition of NaOH (data not shown). Thus, the NaOH method could be widely applicable to distinguish inactivated nematodes from dead ones.

In addition, the NaOH method was useful to distinguish J2s inactivated by abamectin from those killed by it. On the other hand, J2s treated with fosthiazate or NaN₃ did not react to the NaOH treatment before the removal of the chemicals. We observed a difference in inactivated J2s between the two chemical treatments; the J2s inactivated by abamectin did not show any muscle contraction but those inactivated by fosthiazate or NaN₃.

To our knowledge, there is no available information on the mechanisms of the reaction of nematodes to NaOH (Chen and Dickson, 2000). However, the modes of action of the chemicals that inactivate nematodes appear to significantly affect the results of the NaOH method. Abamectin is a mixture of avermectin B_{1a} and B_{1b}, and the avermectins are a group of closely related natural products (macrocyclic lactones) isolated from the actinomycete, *Streptomyces avermitilis* (Wright *et al.*, 1983). The avermectins are believed to act at GABA-mediated synapses (Mellin *et al.*, 1983; Wright *et al.*, 1984) and inhibit transmission between interneurons and excitatory motoneurons in the ventral nerve cord as well as inhibit transmission between inhibitory motoneurons and muscle (Turner and Schaeffer, 1989). The fact that the movement of abamectin-inactivated nematodes was stimulated by the addition of NaOH suggests that this addition of NaOH may directly stimulate excitatory motoneurons in the nematodes whose neural transmission was partly inhibited. On the other hand, fosthiazate is an organophosphate that inhibits

degradation of acetylcholine (Opperman and Chang, 1990), which results in continuous neural stimulation and nematode contraction. If the assumption that NaOH acts at excitatory motoneurons is true, NaOH treatment may not be effective in the presence of fosthiazate, which is in accordance with our results. Sodium azide is an electron-transport chain inhibitor used as a chemical surrogate for hypoxia (Scott *et al.*, 2002). In yeast, NaN₃ inhibits both cytochrome c oxidase (Duncan and Mackler, 1966) and adenosine triphosphate (ATP) synthase (Herweijer *et al.*, 1985; Van der Bend *et al.*, 1985). Neural transmission of the NaN₃-treated nematodes may not be directly affected by NaN₃ because of its mode of action. However, energy supply for nematode movement could be completely blocked by NaN₃, and the addition of NaOH, which stimulates neurons, may not work in the presence of NaN₃. Taken together, the results of the NaOH method were largely influenced by the chemicals used for nematode inactivation. Thus, a possible mode of action of chemicals should be considered when using the NaOH method to determine the mortality of chemically treated nematodes.

The NaOH method is a simple and fast method to determine whether inactivated nematodes are alive or not, as long as the caveats are taken into consideration. Moreover, the differences in nematode reactions to NaOH suggest that the NaOH method could be used to investigate the unknown mode of action of chemicals and unknown reasons for inactivated nematodes.

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