AN *IN VITRO* TECHNIQUE FOR DISTINGUISHING BETWEEN LIVE AND DEAD *MELOIDOGYNE INCOGNITA* JUVENILES

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Abstract

Meloidogyne incognita (Root-knot Nematode, RKN) is one of the most important plant-parasitic nematodes which causes great damage to cotton in the U.S. The objective of this research project is to develop a quick method to distinguish between live and dead second stage juveniles (J2's) of *M. incognita*, which is the key point for any *in vitro* screening of chemical or biological control agents on paralysis of RKN J2's. The best irritant was selected from 1N sodium carbonate (Na_2CO_3), 1N sodium bicarbonate ($NaHCO_3$), and 1N sodium hydroxide (NaOH) at pH=10. *In vitro* 96 well plates were used and 30-40 RKN J2's were pipetted in each well. 1 µl, 10 µl, and 20 µl of each sodium solutions were tested to find the best concentration. Numbers of live J2's were counted and recorded before exposure and at 30 minutes after the addition of the sodium treatment. Results indicated that 1 µl 1N NaOH at pH=10 effectively ($P \leq 0.05$) determined live RKN J2's. The 1 µl 1N NaOH also caused the nematodes to display a rapid twisting movement and curling after a two minutes exposure which was visibly distinguishable. Dead J2's didn't move. The technique will be applied to high throughput *in vitro* screening of chemical or biological control agents on RKN J2's.

Introduction

Multiple methods have been tried to distinguish between live and dead nematodes such as probing nematodes with a fine needle (Cayrol, et al., 1986), staining eggs of nematodes with different dyes (Meyer, *et al*., 1988) or by fluorescein diacetate (FDA) solution (Bird, 1979), and using CellTracker Green labelling live organisms (Grego, *et al*., 2013). However, these methods are time-consuming or need expensive equipment or technical experiences which are not feasible for high throughput screening of chemicals or biological control agents. On the other hand, it has been reported that live nematodes responded to sodium hydroxide (NaOH) by changing their body shape to hook-shaped after about 30 seconds expose to NaOH (Chen, *et al*., 2000) and carbicarb (Na_2CO_3: 0.33 molar, $NaHCO_3$: 0.33 molar) were effective in treating animal acidosis of cardiopulmonary failure at lower sodium doses (Sun, *et al*., 1987). So, sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), and sodium hydroxide (NaOH) are promising candidates for quick distinguish between live and dead second stage juveniles (J2's) of RKN.

In this study, 1N sodium carbonate (Na_2CO_3), 1N sodium bicarbonate ($NaHCO_3$), and 1N sodium hydroxide (NaOH) at pH=10 were tested in 96-well plate *in vitro* to select the best irritant for determination of live RKN J2's. The future goal of this experiment is to apply the technique to high throughput *in vitro* screening of chemical or biological control agents on RKN J2's.

Materials and Methods

Preparation of RKN J2's

Fresh RKN eggs were extracted by 6.0% NaOCl and sieving method and hatched by using modified Baermann funnel (Xiang, *et al.,* 2014). RKN J2's were collected from the bottom of the sieve and poured into a 1.5 ml tube. Tube was centrifuged at 10,000 rpm for one minute. Then gently pipetted out water, added certain amount of 6.0% NaOCl to sterilize the J2's, centrifuged, and washed nematodes twice with distilled sterile water. Adjusted the nematode concentration to 30-40 J2's per 10 µl of nematode suspension for using.

Preparation of sodium solutions

1N Na_2CO_3 was made by dissolving 105.99 g Na_2CO_3 powder in 1,000 ml sterilized distilled water. 1N $NaHCO_3$ was prepared by dissolving 84.01 g $NaHCO_3$ powder in 1,000 ml of sterilized distilled water. 1N NaOH was made by dissolving 40 g NaOH powder in 1,000 ml of sterilized distilled water. All the solutions were adjusted pH values to 10 by using Acetic acid or NaOH solutions.

***In vitro* tests for irritant selection**

Thirty to forty RKN J2s were added in the wells of a 100 µl 96-well plate. 90 µl of distilled sterile water was transferred into each well of the 96-well plate. For each sodium solution, three concentrations 1 µl, 10 µl, and 20 µl were tested. Non-treated wells with water were used as control. Each concentration of each sodium solution had four replications. The experiment was repeated twice. Total numbers and live numbers of RKN J2's were counted before adding sodium solutions. Live numbers were continually counted and recorded at 2, 5, 10, 15, and 30 minutes after exposure to each sodium solutions. Percentage of live RKN J2's at each time point was calculated as the equation:
% live RKN J2's = (live RKN J2's / total RKN J2's) × 100. Increased percentages of live RKN J2's at 30 minutes were compared.

At the same time, the J2's were observed once exposed to the sodium solutions. Live J2's were stimulated into move in a curling fashion or hooked shape upon reacting to a certain concentration of sodium solutions. Dead J2's stayed immobile and didn't move or twitch.

Experimental design

The experimental design for *in vitro* assay was randomized complete block design with four replications. The trial was repeated twice. Data collected were analyzed in SAS 9.2 (SAS Institute, Inc.) using ANOVA and Dunnett's method with $P \leq 0.05$.

Results and Discussion

The average percentage of movement of RKN J2's for 1N NaOH 1µL/100µL at PH=10 ($P \leq 0.05$) was the most effective irritant to determine the number of live RK J2s at 30 minutes after application compared with water control (Table 1). As the concentration increased, the number of live RKN J2's were significantly decreased when applied 10µL and 20 µL of 1N Na_2CO_3, 10µL and 20 µL of 1N $NaHCO_3$, and 20µL 1N NaOH ($P \leq 0.05$) (Table 1). The 1µL of 1N NaOH caused rapid movement and curling of the live RKN J2's which is easily to be distinguished from the immobile dead J2's (Fig.1B).

Table 1 Percentage of increased live RKN J2's at 30 minutes after exposure to different concentrations of sodium solutions analyzed by ANOVA of Dunnett's method with $P \leq 0.05$.

Sodium	Treatment	Percentage of increased live RKN J2's at 30 minutes	Significance at 0.05 level
1N Na_2CO_3	1 µL	10.3	
	10 µL	-95.4	***
	20 µL	-93.9	***
1N $NaHCO_3$	1 µL	13.7	
	10 µL	-87.7	***
	20 µL	-89.1	***
1N NaOH	1 µL	21.8	***
	10 µL	-9.4	
	20 µL	-85.6	***
Water control	100 µL	0.0	

Figure1. RKN J2's in water with normal annulation at 30 minutes (A); RKN J2's exposed in 1µL of 1N NaOH with curling shape at 30 minutes (B).

Conclusions

In this study, applying 1µl 1N NaOH (pH=10) solution to 100 µl of RK J2's can successfully distinguish between live and dead RKN J2's in 30 minutes *in vitro*. Further experiment will be conducted to confirm that live J2's will be infective in plants in growth chamber tests.

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