Development of a Redox Dye-Based Rapid Colorimetric Assay for the Quantitation of Viability/Mortality of Pine Wilt Nematode

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Control of pine wilt disease, which is caused by pine wilt nematode *Bursaphelenchus xylophilus*, is heavily dependent on the use of chemicals such as abamectin. Although such chemicals are highly effective, demands for alternatives that are derived preferentially from natural sources, are increasing out of environmental concerns. One of the challenges to discovery of alternative control agents is lack of fast and efficient screening method that can be used in a high-throughput manner. Here we described the development of colorimetric assay for the rapid and accurate screening of candidate nematicidal compounds/biologics targeting *B. xylophilus*. Contrary to the conventional method, which relies on laborious visual inspection and counting of nematode population under microscope, our method utilizes a redox dye that changes its color in response to metabolic activity of nematode population in a given sample. In this work, we optimized parameters of our colorimetric assay including number of nematodes and amount of redox dye, and tested applicability of our assay for screening of chemicals and biologics. We demonstrated that our colorimetric assay can be applied to rapid and accurate quantification of nematode viability/mortality in a nematode population treated with candidate chemicals/biologics. Application of our method would facilitate high-throughput endeavors aiming at finding environment-friendly control agents for deadly disease of pine trees.

Keywords: Pine wilt disease, pine wilt nematode, *Bursaphelenchus xylophilus*, colorimetric assay, redox dye

Introduction

Pine trees, which belong to the pinaceae family, are mainly distributed in the northern hemisphere. Due to various factors, however, the area of pine forests has been decreasing. For example, in the mid-1990s, pine forests accounted for about 60% of the total forest area of South Korea, but by 2007, the figure had decreased to 23%. One major constraint that limited pine forests is pine wilt disease (PWD) caused by the insect-vector pathogen, pine wilt nematode (PWN), *Bursaphelenchus xylophilus* [1]. PWD was first reported in Japan in 1905 [2] and *B. xylophilus* was first reported later in the United States in 1934 [3]. Although pine trees in North America are resistant to PWD, the two-leaf pine species in Asia and

Europe are highly susceptible, leading to a major epidemic of the disease [4, 5]. PWD started in Japan, and in the 1980s spread to China, Taiwan and South Korea. In the 1990s, PWD landed in Central and South America and Europe, becoming the most devastating disease in pine trees [6, 7].

Such significant impact of PWD stems from the fact that infected trees show nearly 100% mortality and that there is no definitive control method [8, 9]. To date, control of the disease has involved control of insect vectors as well as use of chemicals having nematicidal activity. Use of chemicals such as abamectin, though highly effective against *B. xylophilus*, has potential risks to humans and animals [10], in addition to the high amount of labor and cost needed for trunk injection of chemicals.

In line with this, a number of studies have been aimed at

finding alternative, potential nematicidal compounds that are derived from natural sources including secondary metabolites of microorganisms [11–16]. These studies have gauged the nematicidal activity of candidates primarily by counting the number of live/dead nematodes under the microscope using behavioral changes as a main criterion. We argue that such approaches have two issues: 1) checking the effect of chemicals/biologics one-by-one using microscope is laborious and time-consuming; 2) behavioral changes of nematodes such as decrease/lack of movement or stiff/straightened body does not necessarily mean that the nematode is dead or metabolically inactive, possibly leading to over-estimation of nematicidal activity. In order to address such issues, methodologies employing fluorescent dyes were developed to evaluate the viability of nematodes, particularly for testing new anthelmintic compounds. However, no such easy, economic, and time-saving techniques have been reported yet for the pine wilt nematode [17].

Here we describe the development of viability/mortality assay based on the use of redox dye that changes its color in response to degree of metabolic activity within the *B. xylophilus* population tested. Our method for quantitation of cell viability/mortality is independent of inspection of individual nematodes under microscope, enabling visual assessment of whole metabolic activities in the population. We demonstrate that our approach is applicable to rapidly and accurately testing effectiveness of chemicals or biologics against these deadly pine pathogens.

Materials and Methods

Culture and Harvest of *Bursaphelenchus xylophilus*

Bursaphelenchus xylophilus was obtained from the National Institute of Forest Science (NIFS, Korea) and used throughout this work. *B. xylophilus* was multiplied by growing them on PDA (potato dextrose agar) plate cultures having *Botrytis cinerea*, which was also obtained from NIFS. For harvest of the nematode, plate cultures were flooded with 25 ml of sterile water and then were shaken 5 to 10 times to recover nematodes in a Falcon 50 ml conical centrifuge tube. Using 20 μ l of *B. xylophilus* suspension, the number of nematodes was counted under a microscope.

Setup of Colorimetric Assay Using Resazurin

To determine the optimal number of nematodes and optimal dose of resazurin dye (Sigma-Aldrich, USA), we tested different combinations of nematode numbers and resazurin doses in 96-well plates. The numbers of nematodes tested were 100, 200, and 500. The amount of resazurin tested was 10, 20, and 30 μ l. In all experiments, total volume was adjusted to 120 μ l in 96-well plates

and nematodes were incubated with resazurin at 25°C and 70 rpm for 16 h. Color change was monitored at intervals of 4 h for 2 days in order to determine the timing of color change. To test if the colorimetric assay actually exhibits a range of color spectrum depending on the survival rate of nematode, we checked out color changes in mixtures of live and dead nematodes ranging from 100% survival to 0% survival on a 96-well plate. The dead nematode was prepared by microwave for 1 min and mixed with live nematode proportions, following counting under microscope. Before incubation, absorbance was measured using a 96-well plate reader to determine if resazurin was actually reduced by metabolism of nematodes. Absorbance was measured at the wavelength of 630 nm, at which absorbance difference between resazurin and resorufin in absorbance is the largest.

Colorimetry-Based Assay on Drug Treatment

To test applicability of the assay for screening of nematicidal chemicals, we used abamectin dissolved in methanol. In order to rule out the effect of methanol on nematode viability, colorimetric assays were first performed at 100%, 50%, 20%, 10%, 7%, 5%, and 3% of methanol. Using information on solvent concentration, abamectin was added to the wells containing nematodes in amounts of 200, 100, 50, 25, 10, 5, and 1 μ M at final concentration.

Colorimetry-Based Assay on Biologics Treatment

In a previous study, a random insertional mutant library was prepared using EZ-Tn5 (Epicentre Technologies Corp., USA) mutagenesis kit in *Raoultella ornithinolytica*, which was isolated and shown to have strong nematicidal activity against *B. xylophilus* [18]. Mutant strains of *R. ornithinolytica* were cultured in 96-well plates using LB media. LB media were removed by spin-down of the plates, and then approximately 200 nematodes were added to each well along with 20 μ l of resazurin. A total volume of suspensions was set at 120 μ l and cultured for 16 h at 25°C and 70 rpm for monitoring color changes.

Results

Resazurin as a Redox Dye for Testing Cell Viability/Mortality

The assay we developed utilized a redox dye, resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide), which is used for measurement of metabolic activity and proliferation of living cells. In principle, the dye changes its color by bio-reduction of oxidized form (blue) and concomitant increase of fluorescent intermediate (red) (Fig. 1). When we added nematodes to suspensions containing resazurin, we were indeed able to see color change, which corresponds to conversion of resazurin into resorufin through metabolic activity of nematodes (Fig. 1, circles at the bottom), suggesting the potential of resazurin as an indicator for cell viability/mortality assay. Absorption spectrums of the two

Fig. 1. Schematic diagram showing conversion of resazurin into resorufin by metabolic activity of viable cells using NADH.

Metabolically active cells are able to change dye color from blue to pink depending on duration and intensity of cell metabolism.

forms show maximum difference in a range of wavelengths between 600 and 650 nm. Therefore, it should be possible to monitor the remaining amount of resazurin by measuring absorbance at 630 nm wavelength.

Correlation between Cell Viability/Mortality and Degree of Color Change

Given the potential of resazurin as an indicator, we then tested sensitivity of color changes in measuring viability/

mortality rate. If the degree of color changes is not proportional to relative composition of live and dead nematodes in a population, our dye-based method would not yield quantitative data but only binary outcome (either alive or dead). In order to examine this, we generated nematode populations having different proportions of dead nematodes (Fig. 2A). In our experiment, nematode suspension was divided into two tubes, and dead nematodes were prepared by microwaving one tube for 1 min to kill them. Our preliminary experiment showed that one minute is the duration required to kill all the nematodes in the tube, while leaving them morphologically intact. When we placed nematode-containing suspensions from the microwaved tube onto *Botrytis cinerea* plate culture, which is used to feed nematodes for multiplication, no reduction in hyphal growth of *B. cinerea* was observed even after prolonged incubation, confirming 100% mortality by microwave. By mixing dead nematode suspensions with live ones, we obtained nematode populations having varying proportion of dead nematodes. Incubation following additions of resazurin to these populations showed a gradient of color changes that seems to roughly correspond to the portion of live nematodes (Fig. 2B). Further confirmation of such correlation was obtained by gradual decrease in absorbance measurement in response to increment in viability, indicating that degree of color changes is a relatively accurate reflection of nematode viability/mortality in the sample.

Optimization of Colorimetric Assay

Although color change of the redox dye is a good indicator for nematode viability/mortality, the read-out of the dye-based assay can vary depending on a number of

Fig. 2. Correlation between cell viability/mortality and degree of color change.

(A) Schematic diagram describing how populations of nematodes with varying proportions of dead nematodes were prepared. (B) Color changes and absorbance value at 630 nm for different population of nematodes.

Fig. 3. Optimization of colorimetric assay parameters. (A) Effects of dye quantities for optimal colorimetric assay. (B) Effect of number of nematodes in the wells for optimal colorimetric assay. Unit indicates the number of nematodes tested in each well, while percentage values indicate the proportion of dead nematodes among the total number of nematodes in the well (20% interval). NC indicates negative control (no nematodes added to the well).

parameters including incubation time, amount of dye and number of nematodes used. To find the optimal parameter settings that can maximize the range of gradient in color change at read-out, we ran a series of experiments in which we changed the amount of dye and number of nematodes. We first tested effects of resazurin dose on assay outcome. Using different amounts of resazurin on a fixed number of nematodes (~ 200 nematodes), we found that using 20 μ l of

dye allows the largest contrast in color change after 16 h of incubation (Fig. 3A). Using less (10 μ l) or more (30 μ l) dye resulted in much smaller range of gradient.

Next, we examined the effect that the number of nematodes has on the assay by letting the number vary from 500 through 200 to 100 with fixed incubation time of 16 h. Our data showed that if we use 500 nematodes in each well, color change occurs rapidly regardless of proportion

Fig. 4. Application of colorimetric assay for testing efficacy of abamectin. (A) Color changes in samples treated with various concentration of abamectin. (B) Visual evaluation of nematode viability/mortality for comparison to color changes in (A).

of dead nematodes (Fig. 3B). On the contrary, it was difficult to differentiate among samples having different portion of dead nematodes, when only 100 nematodes were used for the assay. Under our experimental condition, use of ~200 nematodes and 20 μ l of dye with 16 h incubation provided us with the largest contrast among samples. It should be noted that we did not test incubation time independently, since optimal incubation time is dependent on other variables.

Test for Nematicidal Activity of Abamectin

Following optimization, we asked if our assay could be applied to evaluation of chemicals for their nematicidal activities. Since a library of chemicals is not available to us, we chose to use the well-known nematicidal compound, abamectin to test whether our assay is able to assess the nematicidal activity of abamectin with enough sensitivity. Our results showed that abamectin could kill nearly all the nematodes in our sample at 100 μ M or higher concentration. Interestingly, however, color change occurs as the dose of abamectin added to nematode suspensions increases, although it does not appear to be gradual (Fig. 4A). Rather, change seemed to occur only after particular concentration is reached. When we counted the number of live/dead nematodes in the same samples under the microscope, plotting the data indeed showed a staircase pattern, corroborating our observation on color changes (Fig. 4B). Again, these results indicate that our assay method using redox dye is able to accurately measure nematicidal activity of candidate compounds.

Test for Screening of Mutant Library

In a previous study, it was shown that an endophytic bacterium, *Raoultella ornithinolytica*, which was isolated from plants, possesses strong activity against *B. xylophilus* [18]. In an effort to identify genes or pathways responsible for such nematicidal activity, an insertional mutant library ($n = 1,343$) was generated for *R. ornithinolytica* in our lab using Tn5 mutagenesis method. Such resource allowed us to test if we could scale up our assay for high throughput screening. Using the wild-type strain as a positive control, we screened all the mutants. In our screening, we used 6 h of incubation instead of 16 h. This is because the bacterium itself would cause changes in dye color through its own metabolic activity. However, such color change will be accelerated by live nematode if the mutant loses the ability to kill them. Therefore, the rationale behind our screening scheme is that any mutants, in which key nematicidal genes are disrupted, would be impaired in biosynthesis of

Fig. 5. Representative image of plates showing color changes in response to treatment of nematodes with biologics derived from *R. ornithinolytica* Tn5 mutant library.

Wild-type strain of *R. ornithinolytica* (positive control) was marked with green circle. Mutant strains showing no/reduced nematicidal activity are marked with red circles.

nematicidal compound(s) and consequently result in color change of dye much faster than the wild-type strain. Through the screens using our redox dye-based assay, a single person was able to identify 27 candidate mutants warranting further genetic studies within three days, indicating the scalability of our assay method (Fig. 5).

Discussion

In this work, we have developed a colorimetric assay based on the use of redox dye resazurin for rapid and quantitative assessment of viability/mortality of PWN. In

efforts to discover alternatives to chemicals such as abamectin that have been heavily used for control of PWD, screening a large number of candidate chemicals/biologics can be one of the constraints. Conventional method for evaluating viability/mortality of nematodes relies on visual inspection and numeration of individual nematodes in the sample. Such a method is laborious and time-consuming. Moreover, judging viability of nematodes using visual and behavioral criteria can be mis-leading, resulting in over-estimation of mortality. One remedy for the problem is to use fluorescent dye, of which uptake into the cell is dependent on the functions and integrity of cellular membranes [17]. However, one still has to check individual nematodes one-by-one down the microscope, and determining viability/mortality based on fluorescence staining patterns can be ambiguous, making it impractical to use for high throughput screening.

On the contrary, methods using redox dye such as resazurin do not require the use of microscope and are able to easily quantify viability/mortality for a large number of samples just by monitoring color changes and/or measuring absorbance at a particular wavelength. Assumptions underlying the redox dye-mediated assay are mainly twofold: 1) the amount of color change should be proportional to the viability of nematodes in the sample; 2) viability/mortality of nematodes should be a single factor affecting redox dye chemistry. In our work, we showed that degree of color change (or absorbance measurements) correlates linearly to the proportion of live nematodes in the sample, indicating that our assay condition using resazurin meets the first assumption. The second assumption is related to the solvent used to dissolve candidate chemicals or media used to culture biologics. The LB medium we used in our assay affected neither viability/mortality of *B. xylophilus* nor chemistry of resazurin. However, we found that the methanol, which is used to dissolve abamectin, had some influence on color changes, although it did not significantly change the viability/mortality of nematodes at a low concentration (but high enough to dissolve abamectin), suggesting that choice of solvents and their concentration can be important factors. Therefore, if a number of candidate nematicides needs to be dissolved in different solvents, extreme caution is required to obtain correct read-out from the use of our assay.

In summary, our results demonstrated that the redox dye-based colorimetric assay developed here is easily scalable and applicable to screening of candidate chemicals and biologics. We anticipate that our assay would facilitate rapid discovery of chemicals and biologics from a large

number of candidates. It is also possible to apply our assay to studies aiming at providing information regarding the molecular and cellular basis for pathogenesis mechanisms of *B. xylophilus*. For example, our assay may serve as a platform technology for lifespan, biotic, and abiotic stress resistance assays. Screening of *B. xylophilus* mutants in particular would enable identification of key genes and their protein products that can be targeted for development of chemicals having potential to inhibit growth and development of this deadly pathogen.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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