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Regulation of the *C. elegans* NADPH Oxidase

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

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APPROVED:

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Abstract

Reactive oxygen species are derived from oxygen and produced during microbial infections of mammals. NADPH oxidases, such as DUOX, catalyze the first step in ROS synthesis, forming superoxide and subsequently hydrogen peroxide. *C. elegans* expresses a DUOX enzyme encoded by the gene *bli-3*. It is believed that DUOX may be involved in host defenses in *C. elegans* against *S. cerevisiae*. Its activity can be measured by hydrogen peroxide production. In this study, a hydrogen peroxide-measuring assay was tested for the future identification of genes that are required for ROS production in *C. elegans.* In the Amplex Red assay, hydrogen peroxide production increased when worms were infected with *S. cerevisiae*, suggesting that DUOX expression or activity were induced by infection.

Table of Contents

Acknowledgements

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Background

Reactive oxygen species (ROS) are highly unstable and reactive molecules that are derived from oxygen (Bedard & Krause, 2007). The phagocyte "respiratory burst" is the accompanying consumption of oxygen critical to the killing of certain pathogens. ROS are produced in large amounts by neutrophils and macrophages by the phagocyte NADPH oxidase in response to infection as a mammalian innate immune response (Bedard & Krause, 2007). For example, ROS are generated by *Caenorhabditis elegans* during yeast and bacterial infections (Chavez et al, 2009). NADPH oxidases create superoxides through electron transfer across biological membranes (Bedard & Krause, 2007). The enzyme NADPH oxidase catalyzes the first step in ROS synthesis by the reaction shown here, to produce superoxides:

$$NADPH + 2O_2 \rightarrow 2O_2^- + NADP^+ + 2H^+$$

Subsequent steps convert superoxide to hydrogen peroxide and other products, either through enzymatic activity or at low pH. These ROS forms lead to the killing of microorganisms (Bedard & Krause, 2007).

Different isoforms of the NADPH oxidase are known, which include two different forms of DUOX (DUOX1 and DUOX2) and the five NOX catalytic subunits (NOX1, NOX2, NOX3, NOX4, and NOX5). Together, these are considered to be a NOX family of oxidases because of their sequence and domain conservation (Bedard & Krause, 2007). For this study, the DUOX form is of greatest interest because it is part of the NOX family oxidases and it may be involved in host defenses in epithelial barrier tissues (Jain, et al, 2009, Chavez, et al, 2009). During infection of *C. elegans* by the yeast *S. cerevisiae*, DUOX activity is induced and can be measured by a chemical assay for hydrogen peroxide production (Jain et al, 2009).

The objective of this study is to understand how DUOX activity is controlled in response to an infection in *C. elegans* by the yeast *Saccharomyces cerevisiae*. In the innate immune response of *C. elegans* to bacterial and fungal pathogens, what is known about the activation of the pathways that may regulate DUOX begins with two G proteins (Figure 1). These two G proteins include GPA-1 and RACK-1 (Ziegler et al 2009). Downstream of these G proteins is the *C. elegans* homolog of the p38 MAP kinase pathway of mammals. The G proteins activate PLCγ and PLCβ, which catalyze PIP2 cleavage, leading to two different pathway branches. The first pathway is downstream of DAG, which activates PKCδ, which then phosphorylates TIR-1. TIR-1 then activates ASK1 (NSY-1) which phosphorylates MKK6 (SEK-1). MKK6 phosphorylates P38 (PMK-1) which by inference phosphorylates an unknown transcription factor (Ziegler, et al., 2009). The other pathway is downstream of inositol trisphosphate (IP3), which activates the IP3 receptor, a Ca^{2+} channel (Murphy, 2012). We hypothesize that the Ca^{2+} release mediated by the IP3 receptor activates DUOX, which contains EF hand domains that bind Ca^{2+} (Ziegler et al, 2009). Because PLCγ and PLCβ branch off into two different pathways, if these were mutated, these mutations would affect both pathways downstream of PIP2.

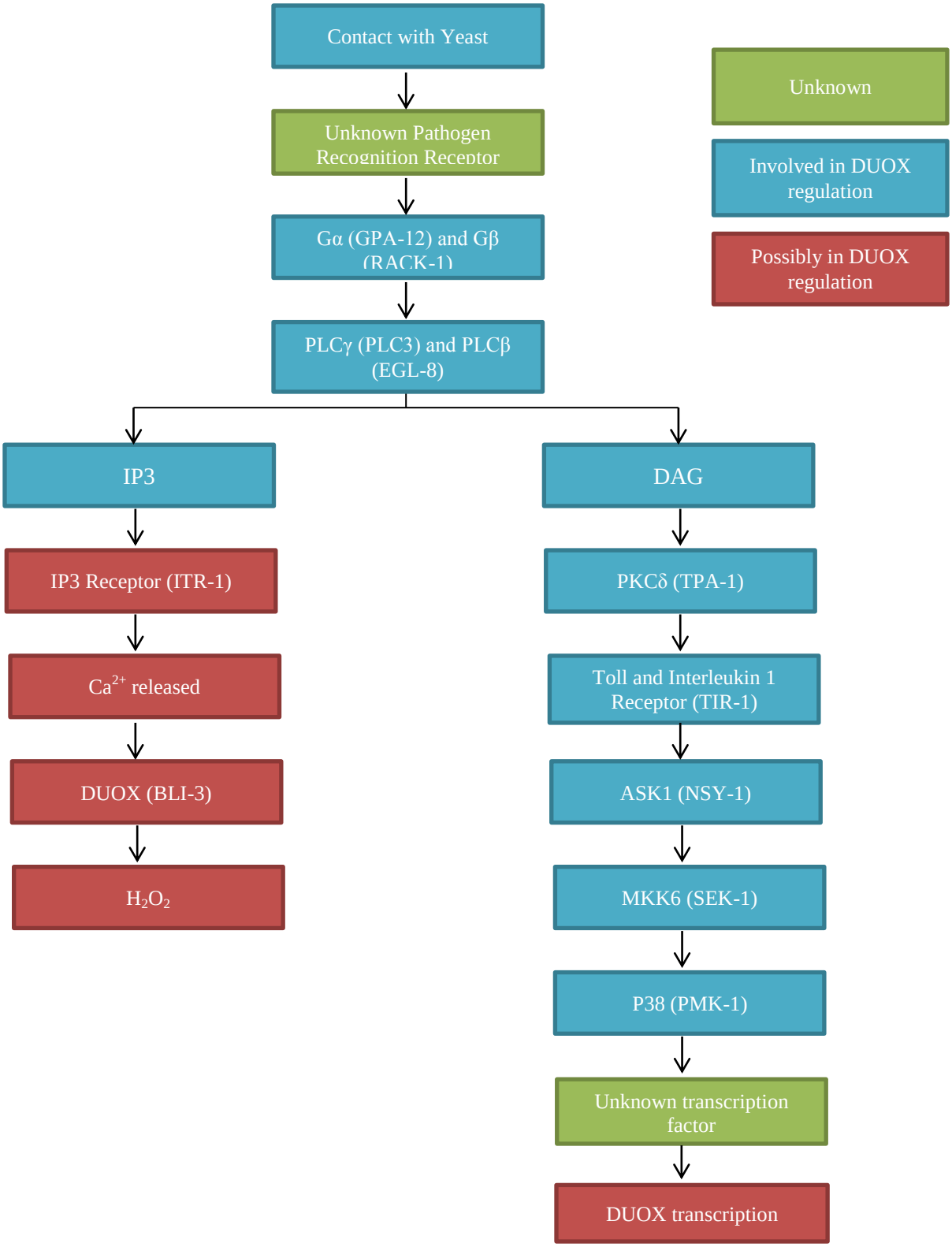

Figure 1: Pathways downstream of PIP2 in C. elegans. Pathway components shown in red are steps hypothesized to be important in DUOX regulation. The parts shown in blue are steps that have already been well-established in other studies (adapted from Ziegler, et al, 2009).

It is known that during a yeast infection, ROS is generated by *C. elegans* in an NADPH oxidase-dependent process (Jain et al, 2009). With this knowledge and our knowledge of the signaling pathways operating during microbial infections of *C. elegans*, we can focus on the downstream pathways that activate ROS production, because they are likely to activate DUOX activity in order to do so (Gravato-Nobre & Hodgkin, 2005).

The host model organism used for this study was *Caenorhabditis elegans*. These organisms have proved useful for studying innate immune responses for several reasons. Firstly, the genome sequence of *C. elegans* has already been determined (The C. elegans Sequencing Consortium, 1998). Many *C. elegans* genes are similar to those of humans (Kamath, et al., 2003). Their innate immune defenses are similar to those of mammalian innate immunity, making *C. elegans* a valuable host model (Gravato-Nobre & Hodgkin, 2005). Second, their morphology and unique characteristics allow for easy observation. These worms are microscopic but are also transparent which allows internal changes to be viewed in vivo (Gravato-Nobre & Hodgkin, 2005). Third, *C. elegans* reproduce by self-fertilization, therefore creating natural clones which provide genetically identical organisms which can be used in large numbers throughout the course of the study (Schulenburg, 2004). *C. elegans* have two major barriers to systemic infection. These include the intestinal epithelium and the cuticle or body covering, which covers the hypodermis (Couillault, et al., 2004). The hypodermis and intestine are prime candidates for immune tissues, based on proximity to potential pathogens (Breger, et al., 2007). Therefore, these worms have been used to study host-pathogen interactions (Gravato-Nobre & Hodgkin, 2005). Thus, for this experiment, the Amplex Red Assay will be used to test these interactions.

The Amplex Red Assay is a test that can be used to determine if worms produce hydrogen peroxide during microbial infections (Zhou, et al, 1997). This test uses N-acetyl-3, 7 dihydroxyphenoxazen, also known as Amplex Red, which is oxidized in the presence of hydrogen peroxide (Zhou, et al, 1997). In our application, worms that have been in contact with yeast are placed in microtiter wells, and the reagents of the Amplex Red assay are added to the wells. As seen in Figure 2, horseradish peroxidase (HRP) oxidizes the Amplex Red to form a colored compound, resorufin, whose red color optical absorbance can be measured. The absorbance in the microtiter well is proportional to the amount of hydrogen peroxide in the sample (Zhou, et al, 1997). DPI is a NADPH dependent oxidase-specific inhibitor that can control the DUOX specificity. It is important to do this in case there are other sources of ROS in *C. elegans*.

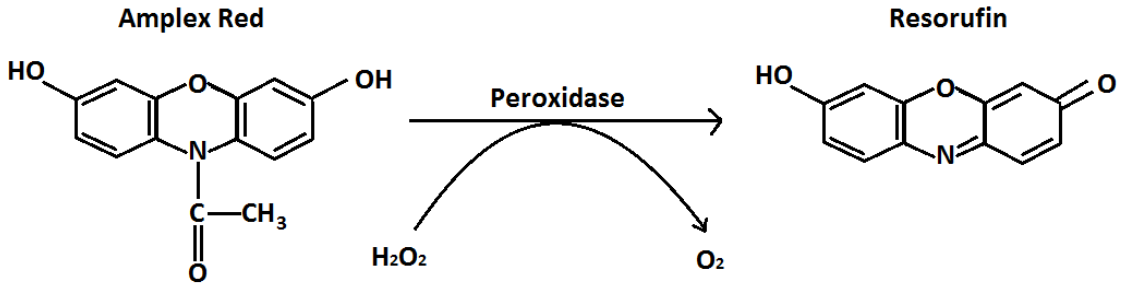

Figure 2: The reaction that forms the basis of the Amplex Red Assay. In the presence of hydrogen peroxide, HRP oxidizes the Amplex Red producing a red color resorufin (adapted from Life Technologies, 2012).

The Amplex Red assay will be used to measure NADPH oxidase-dependent production of hydrogen peroxide in *C. elegans* after yeast infection. Jain et al (2009) and Chavez et al (2009) showed that hydrogen peroxide production is induced upon yeast or bacterial infection of wild type *C. elegans*, respectively. The goal of this project is to use the Amplex Red assay to test if the genes hypothesized to induce *C. elegans* DUOX are indeed required for hydrogen peroxide production after infection. If a reduction in

hydrogen peroxide signal is observed in a loss of function mutant, it would suggest that the gene is required for hydrogen peroxide production by DUOX.

This would lead to the hypothesis that DUOX is regulated in some way by this gene. For example, if there is a reduction in the induction of hydrogen peroxide in the presence of MAP kinase pathway mutations, this would suggest that H_2O_2 production requires activity of the p38 MAP kinase pathway.

Materials and Methods

Stocks

The wild type strain of *C. elegans*, N2, was used for this experiment and was kept at 20°C on NGM agar plates supplied with lawns of *E. coli* OP50. Every day, three to four worms were transferred to new agar plates with fresh *E. coli* in order to maintain stocks.

Amplex Red Assay

The overall methods for this experiment are shown in Figure 3. More detailed steps follow.

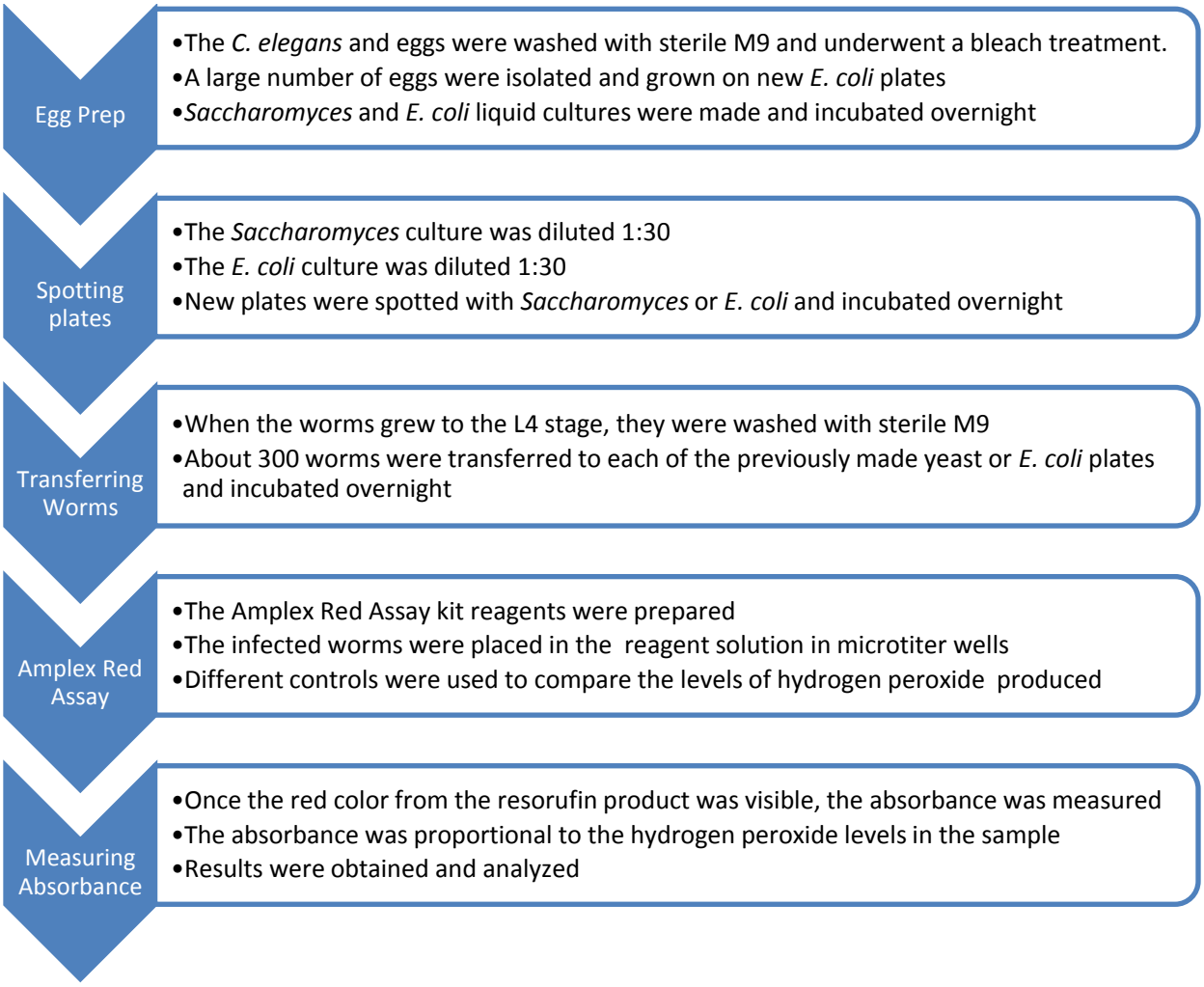

Figure 3: Basic flow chart of methods

Egg Preparation Protocol

For an "egg prep", at least four plates were used with as many eggs on them as possible, which usually occurred when most of the *E. coli* had been eaten. This stage took about four to five days from the date when the worms were plated.

Using a 5¾" sterile glass Pasteur pipet, a pipetful of sterile M9 was pipetted onto the plate, which was held at an angle, so that the liquid could collect at the bottom of the plate, while not spilling. The M9 was sucked back up with the pipet and released over the plate several times until all of the worms and eggs on the plate were collected. The liquid was added to a 15 mL disposable plastic centrifuge tube. Worms washed off different plates were combined into one or more 15 mL tubes as necessary.

The liquid levels for each conical tube were made equal by adding as much sterile M9 as necessary before centrifugation. The tubes were centrifuged at 1139 x g for two minutes. After centrifugation, a pellet of worms was seen at the bottom of each sample tube.

A Pasteur pipet attached to a vacuum system was used to remove as much of the M9 supernatant as possible, without disturbing the pellet. Sterile M9 was then added to the tubes to resuspend the worms so that they could be centrifuged and resuspended twice more, for a total of three centrifugations.

A solution containing 75mL of distilled water, 1g NaOH, and 25mL of 5% commercial household bleach was mixed until all of the NaOH had dissolved.

After the last centrifugation, as much M9 as possible was removed from the tubes without disturbing the worm pellets. A timer was set to 3:00 minutes to make sure the samples were not in the bleach solution for too long, as it could damage the eggs. The bleach solution was added to the sample tubes to the 10mL mark. The timer was started and the tube was capped and inverted a few times throughout the 3 minutes, to make sure the worms were in contact with the

bleach. The samples were quickly put back into the centrifuge for another two-minute cycle at 1139 x g. When the cycle was finished, the supernatants were quickly removed again, without disturbing the egg pellet but leaving as little bleach as possible. The samples were then washed quickly again three times with sterile M9 as described before.

For experiments, eggs were grown on NGM/*E.coli* OP50 plates. After the three washes with M9, the supernatants were removed to the 1mL mark. The egg pellet was resuspended by mixing, and using a glass 100 µL capillary pipet, 20µl of the egg suspension were quickly added to an *E. coli* plate. The number of eggs present were counted and multiplied by 50 to calculate the total number of eggs in the tube. The volume was adjusted as needed to obtain 5000 eggs/mL by adding more sterile M9, centrifuging at 1139 x g, and carefully vacuuming to the desired volume. No more than 50µl containing approximately 200 eggs were placed on each plate. After all the eggs were distributed to the plates, they were incubated at 20°C.

Preparation of Yeast and E. coli Test Plates

A single colony was picked from a YPD agar plate of *S. cerevisiae* (Y101), placed in a liquid culture containing 2mL of sterile YPD broth, and incubated overnight at 30°C in a rolling drum. The same was done for *E. coli* (OP50) grown on LB broth overnight in a 37°C incubator.

The yeast liquid culture was diluted 1:30 with YPD by combining 10 µL of the Y101 with 300 µL YPD in a sterile Eppendorf tube. This dilution was combined with YPD and Streptomycin sulfate (50 mg/mL stock, used to prevent contamination), in a 1:1:2 ratio. 20 µL of this mixture was spotted on NGM agar plates; the plates were then incubated overnight at 30°C. The overnight *E. coli* culture was diluted 1:30 with LB broth. From this dilution, 40µL were used to spot new plates. More *E. coli* was added than *Saccharomyces* because *Saccharomyces* grows faster than *E. coli*. The E. coli plates were incubated at 37°C overnight.

Transferring Worms to Yeast Plates

The yeast and *E. coli* plates were removed from the incubator and kept at room temperature to cool. After the eggs had grown into L4 worms (about 48 hours after plating the eggs), the worms were washed off the *E. coli* growth plates with sterile M9 as described previously. Worms were washed and centrifuged at 500 x g three times. After the last centrifugation, the supernatant was removed and the volume was adjusted to provide approximately 300 worms in 50 µL.

About 300 worms were added to each of 6-8 yeast plates in 50 µL or less. The same was done for the new *E. coli* plates, which were used as a control. The worms were left on the plates for about 24 hours in the 20°C incubator before performing the Amplex Red Assay.

Amplex Red Assay

The worms were washed off the *E. coli* and yeast plates with sterile M9 and centrifuged three times at 500 x g. Worms harvested from *E. coli* and yeast plates were kept in separate tubes. Between each centrifugation of the samples, the M9 was removed and more M9 was added to resuspend the worm and yeast pellet thoroughly, and the worms were allowed to settle on the benchtop. Once the worms had settled, the yeast was vacuumed off and M9 was added until it reached the top and centrifuged again. The worms were resuspended as many times needed in between centrifugations so that as much yeast as possible was removed from the supernatant so as to reduce sample turbidity during the Amplex Red Assay.

The volume of the samples incubated with yeast and *E. coli* was adjusted with sterile M9 to a concentration of 100 worms/50 µL.

The Amplex Red kit was purchased from Invitrogen (catalog # A22188). Amplex Red assays followed the instructions provided with the kit. All of the Amplex Red kit reagents were stored at -20° C when not in use. Before starting the assay, one tube of Amplex Red reagent and

a tube of Dimethylsulfoxide (DMSO) were allowed to warm to room temperature. The contents of the tube of Amplex Red reagent were dissolved in 60µL of DMSO. This was enough for approximately 100 assays. The Amplex Red reagent stock solution was used on the same day it was prepared. Because the Amplex Red reagent is light-sensitive, some experiments were performed in a dark room with minimal light.

The 1X Amplex Red Reaction Buffer was made by adding 4mL of 5X Reaction Buffer to 16mL of deionized water. This 20mL volume was sufficient for approximately 100 assays.

The contents of the tube of HRP (10 U) provided in the kit were dissolved in 1.0 mL of 1X Reaction Buffer. Any unused HRP stock solution was placed into single-use 100 µL aliquots and stored at -20°C.

The stock solutions were used to prepare a reaction mix by adding together 4.85 mL of 1X Reaction Buffer, 50 µL of Amplex Red reagent stock solution (10 mM), and 100 µL of 10 U/mL HRP stock solution. This 5mL volume was sufficient for approximately 100 assays.

A 96 well microtiter plate (Costar 3596) was prepared by filling wells with 200 µL of 0.05% Tween 20, to prevent sticking of worms to the bottoms of the wells. The Tween 20 solution was removed by inverting the plate and flicking it over a trash barrel. 50 µL of the worm suspension containing 100 worms from either the yeast or *E. coli* incubation plates were added to each well. In three out of the six wells for each of the two strains (yeast and *E. coli*), 3µl of the NADH oxidase inhibitor DPI, dissolved to a stock concentration of 3.3mM in DMSO, was added. 3 µL of DMSO was added to wells which did not contain DPI. Lastly, 50µL of the Amplex Red reagent/HRP working solution was added to each of the wells.

The plate was protected from light by wrapping it in aluminum foil and left at room temperature on a shaking platform. After about six hours on the platform, it was put in a 2°C fridge.

Using a microtiter plate reader, the absorbance at 540nm was measured for each well.

Results

An Amplex Red assay was performed on worm samples grown on yeast or *E. coli*, as well as samples containing the Amplex Red reagents without worms, to use as a control. Each experimental condition was performed in triplicate. Table 1 shows the results.

Table 1: Resorufin A_{540} readings for yeast and *E. coli* worms

Yeast	1.093	*E. coli*	0.229	No Worms	0.188
	1.083		0.348		0.202
	1.021		0.219		0.200
Mean	1.066	**Mean**	0.265	**Mean**	0.197

The mean absorbances from the wells without worms were subtracted from the mean absorbances of worm samples of yeast and *E. coli*. These corrected values are shown in Table 2.

Table 2: Corrected Absorbances of yeast and *E. coli* samples

	Corrected Mean Absorbances
Yeast	1.066 – 0.197 = 0.869
E. coli	0.265 – 0.197 = 0.069

In order to check if the absorbances found in yeast and *E. coli* samples were significantly different, pairwise ANOVA tests were done. The results were considered significant if the P-value was less than 0.05. These results are shown in Table 3.

Table 3: ANOVA results comparing yeast and *E. coli* worms

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Yeast	3	3.197248	1.065749	0.001549		
E. coli	3	0.865482	0.288494	0.004214		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.906189	1	0.906189	314.4882	5.94007E-05	7.708647
Within Groups	0.011526	4	0.002881			
TOTAL	0.917714	5				

Because the P-value was 0.000059, the differences measured are considered significant, and we can conclude that the absorbance difference was due to yeast infection.

After determining the significance of differences between samples, the uncorrected mean absorbances from Table 2 were used to construct a graph for a better visual comparison. This can be seen in Figure 4.

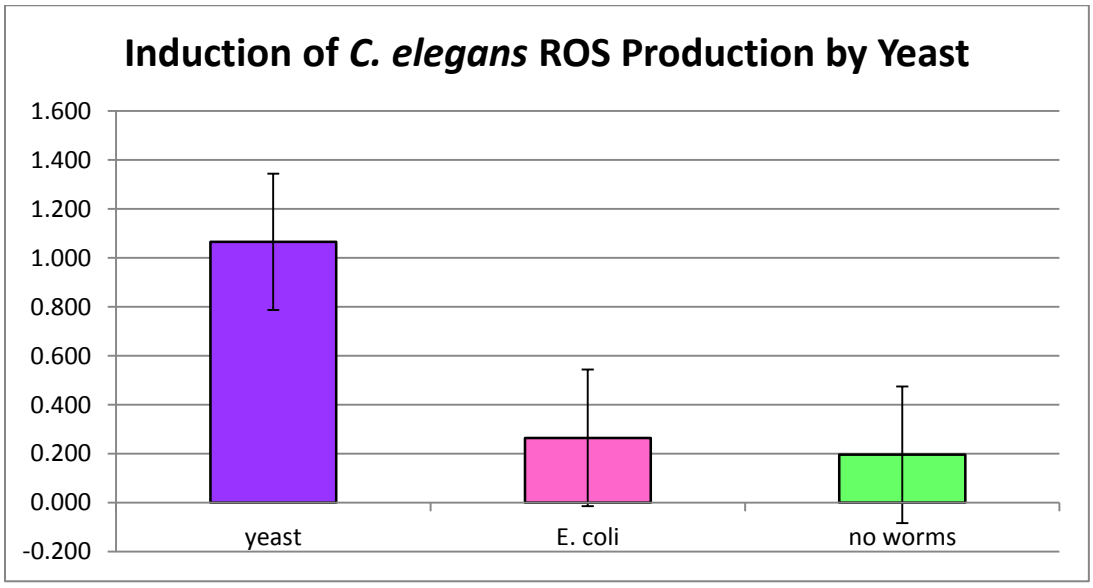

Figure 4: Graph of mean absorbances from Amplex Red assay. Error bars represent standard error.

As seen from Figure 4, the worm samples grown in yeast yielded significantly higher absorbances than those grown in *E. coli*, indicating that worms produced more hydrogen

peroxide when exposed to yeast. The difference between absorbances for the yeast samples and no worm samples were also highly significant (ANOVA $p = 0.000003$) indicating that the increased hydrogen peroxide production depended on the presence of worms. In contrast, the difference between absorbance for the *E. coli* and no yeast samples was not significant (ANOVA $p = 0.17$), indicating that contact with *E. coli* resulted in a minimal increase in hydrogen peroxide production.

Discussion

The purpose of this experiment was to understand how ROS production responds to a yeast infection in *C. elegans* through the measurement of hydrogen peroxide, using the Amplex Red Assay. When the Amplex Red reagent reacted with the worms and hydrogen peroxide was produced, a red color appeared and was measured for the absorbance. To check if the differences observed were significant, an ANOVA test was performed. The difference between the *S. cerevisiae* and *E. coli* samples was highly significant, as seen in Table 3 (ANOVA P = 0.000059). Once the results were graphed, as seen in Figure 4, it was clear that the samples of worms grown in yeast produced more hydrogen peroxide than the samples of worms in *E. coli*. An additional control was performed: adding the inhibitor DPI, which is specific for NADPH oxidase. However, this control did not work properly, possibly because of a problem with the DPI reagent (results not shown). Thus, although we would like to conclude from these results that a *Saccharomyces* infection induces the production of hydrogen peroxide by DUOX, we cannot eliminate the possibility that another cellular process created ROS under our conditions. In the study of Jain et al 2010, the Amplex Red assay did show diminished color in samples treated with DPI. However, the resorufin absorbance was not measured in their assays.

Thus the major contribution of this work has been to produce a quantitative version of the Amplex Red assay, which is now available for further experimentation to confirm the importance of DUOX under these experimental conditions. Moreover, the use of this assay to test loss of function mutants for effects on ROS production will be validated if the result can be demonstrated to be abolished by addition of DPI.

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