Development of a bioassay to quantify the ricin toxin content of castor bean (*Ricinus communis* L.) seeds

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ABSTRACT. In this study, we developed a bioassay to quantify the ricin toxin content of castor bean seeds. Existing quantification methods do not always reflect actual toxicity of the seeds analyzed, which may present lower ricin content even though they are more toxic than seeds presenting a higher content of ricin. This is because these methods actually measure the addition of ricin RCA, which is a compound less toxic than pure ricin. We decided to use in this study, the nematode *Caenorhabditis elegans*, which has been widely used by the pharmaceutical industry. We tested two strains of *C. elegans* using different methods in 8 experiments. We examined 4 methods of extracting the ricin complex and 3 methods of exposing the nematodes. Among the nematode strains and ricin extraction methods tested, we concluded that the best strain for testing ricin toxicity was the strain called N2 and that the best method for ricin extraction was a rotating bath followed by centrifugation and exposing the nematodes in 24 well plates with a solution of nematodes extracted from the media with destilated waterexposing the nematodes in 24-well plates This method is inexpensive, quick and adequate for the selection of offspring with lower RIP content.
Keywords: toxin, C. *elegans*, selection, *Ricinus*.

Desenvolvimento de um bioensaio para quantificar o teor da toxina ricina em sementes de mamona (*Ricinus communis* L.)

RESUMO. Foi desenvolvido um bioensaio para quantificar o teor de ricina nas sementes de mamona. Os métodos de quantificação existentes não refletem a toxidez real das sementes analizadas, que pode mostrar um resultado de menor teor de ricina e ainda assim ser mais toxico do que as sementes que apresentaram teores maiores. Isto ocorre por que estes métodos medem alem da ricina a RCA, que é um composto menos tóxico que a ricina pura. Foi decidido utilizar neste estudo o nematoide *Caenorhabditis elegans*, que tem sido amplamente utilizado na industria farmacêutica. Foram testadas duas estirpes de *C.elegans* usadas em diferentes métodos contando com 8 experimentos distintos. nós examinamos 4 métodos de extração de ricina e 3 métodos de exposição do nematóide à ricina. Foi concluído que a melhor estirpe para testar ricina foram as estirpes chamadas de N2 e o melhor método de extração da toxina foi o do banho maria seguido pela centrifugação e o melhor método de exposição dos nematóides foi o que conta com uma placa de 24 poços onde o nematóide suspenso em água destilada são expostos a toxina. Este é um método barato, rápido e adequado para seleção de materiais com baixo teor de RIP.
Palavras-chave: toxinas, C. *elegans*, seleção, *Ricinus*.

Introduction

The castor bean crop has an increased importance in today's world because of the quality of its oil. The United States buys castor bean oil and products made from it from mainly China, India and Brazil. Brazil currently has a low production of castor beans but in the past, produced a much larger crop.

The main problem with castor bean production is the presence of the toxin ricin, which is a threat to national security. However, ricin can be easily extracted.

Ricin is a ribosome inactivating protein (RIP) consisting of a heterodimeric polypeptide able to inactivate 1500 ribosome per minute, which interrupts protein synthesis and leads to cell death. Another dangerous protein in castor bean seeds is RCA, which is much less toxic than ricin. Texas Tech University is developing castor bean varieties with low toxin contents. It is necessary to have an inexpensive and safe way to test the toxicity of the castor bean seeds of the genotypes being developed. Many different tests have been developed to quantify ricin, but they do not show the toxicity level of the entire seed that contains ricin, RCA and other toxins. Current

methods are expensive because multiple tests are required when conducting selective plant breeding.

Nematodes have been used as an environmental thermometer around the world, especially free-living nematodes that show how well preserved the soil is or how many chemical contaminants are present. One of these nematodes is *Caenorhabditis elegans,* and it is expected to be a good model for testing ricin toxicity because it is a eukaryote and therefore has the same types of ribosomes as humans.

There are two major toxins found in mature castor seeds (MOSHKIN, 1986). The first of these is ricin, a two-chain polypeptide with an LD_{50} of 30 ppm, which is one of the most toxic compounds produced in nature. Ricin bond and blokthe function of ribosomes in eukaryotic cells. And RCA *Ricinus communis* aglutimin that is a protein that coagulates the blood.

Ricin is a member of the A-B toxin family of deadly plant and bacterial proteins that are able to enter and kill mammalian cells (LORD et al., 1984). Specifically, it depurinates 28S rRNA so that ribosomes are no longer able to synthesize protein, causing cell death (ENDO et al., 1987). Ricin is a 66-kilodalton (kDa) globular protein that makes up 1 to 15% of the weight of the bean of the castor plant, *Ricinus communis* (ROBERTUS, 1988). The toxin is stored in the matrix of the castor bean, together with a 120-kDa ricinus lecithin (YOULE; HUANG, 1976).

A Radial Immune Diffusion (RID) assay was adapted to facilitate screening for castor lines with reduced levels of ricin + RCA (PINKERTON et al., 1999). The RID assay uses a specific antibody against *Ricinus communis* lecithin incorporated into a thin layer of agar on which the antigens contained in the seed extract were placed. This method has a minimum sensitivity of 1.0 mg of total ricin + RCA/g of seed. The RID assay is relatively inexpensive but does require special equipment. According to Lubelli et al. (2006), the LOD of IPCR was more than 1 million times lower than that of ELISA. IPCR appears to be the most sensitive method for the detection of ricin and other RIPs.

With the use of this free-solution assay, the detection of 7.1 mol of ricin has been demonstrated. The presence of interfering proteins such as bovine serum albumin and casein do not inhibit this interaction at sub-nanomolar concentrations. When spiked with RNAse, ricin can still be detected down to a concentration of 1 nM, despite severe aptamer degradation. This approach offers a promising method for the rapid, selective and sensitive detection of bio-warfare agents (HAES et al., 2006).

An ELISA method has been shown to have a detection limit of 4 ng mL^{-1} ricin. However, the assay took several hours and required highly-trained personnel to perform. Ligler et al. (2003)

demonstrated a detection of 8 ng mL^{-1} ricin using an array-based immune sensor with analysis times of 15-30 min. as a result of considerable user intervention.

The traditional methods for ricin detection use antibody-based immunoassays (LIGLER et al., 2003; RUBINA et al., 2005) and Enzyme Linked immune-sorbent assays (ELISA) (POLI et al., 1994). However, these methods require the use of animals to produce antibodies, which has been more difficult with recent animal protection policies. Because of this problem, we looked for a method that does not require the use of higher animals, and decided to test whether nematodes could be used for ricin detection. Thompson and Pomerai (2005) used *C. elegans* to test the toxicity of different alcohols with different toxicity levels. This assay was used to determine the ecotoxicological point. This study also verified that *C. elegans* is a good alternative for testing the action of heavy metals and detergent contamination on the environment. *C. elegans* has been tested not only as a sentinel organism but also for examination of human reactions to *Vibrio cholerae* protease, fungicide, nicotine, various toxins, various drugs, *Ginkgo biloba* extract, and seven organic pollutants. *C. elegans* has also been used to examine host-pathogen interactions with *Salmonella typhimurium*, the aging process, xenobiotic resistance, and Alzheimer's disease.

Orgonie et al. (2003) verified that the nematode has the same reaction to ricin as humans; if consumed, a larger dose is needed than if it is injected. Mears et al. (2002) sequenced *C. elegans* rRNA and found the ricin-binding sequence. The purpose of our study was to develop an efficient and inexpensive bioassay using *C. elegans* to quantify the toxicity of ricin from different kinds of seeds and to select the best extraction method, the best nematode exposure method, and the best strain of nematodes. This was necessary because the RID and ELISA Lowery et al. (2007), methods are expensive and only quantify the ricin complex and do not evaluate its actual toxicity.

Material and methods

The assay was developed in the Soil and Plant Science Department of Texas Tech University in partnership with the Microbiology Department of the same university and the Agriculture Department of UNESP/FCA (São Paulo State University - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Ciências Agronômicas, Botucatu, São Paulo State, Brazil).

Castor bean cake cannot be used to feed cattle because of its toxicity. For breeding purposes, the only

methods that have been used to measure seed ricin content are ELISA, which is reliable but very expensive, and RID, which is too variable. Our goal was to develop a better method to measure ricin that is inexpensive, simple and precise. We performed sequential experiments, in which each experiment was conducted twice with 4 repetitions, leading to the next experiment. To determine the efficiency of each method, we used the same material as Pinkerton et al. (1999) and Lowery et al. (2007), who had quantified ricin using RID and ELISA The castor bean lines used were the following: PI25765401 VNIIMK N1651 from Russia, 2.4% ricin; PI176751 8838 from Turkey, 5,6% ricin; PI223013 Karchak from Iran, 6.5% ricin; PI170685 Acik Kahverengi from Turkey, 7.6% ricin; PI170684 Kahverengi from Turkey, 8.1% ricin; PI183468 Erari from India, 9.2% ricin; PI183347 Divela from India, 10.3% ricin; PI183076 Erari from India, 10.8% ricin; and Hale from EUA, 12.4% ricin. All of these varieties were obtained from the USDA Castor Bean Germplasm Bank.

To quantify ricin, we used different methods to expose the nematode *C. elegans* to ricin extracted by different methods.

To grow the nematodes for the test, we used plastic petri plates containing NGM agar (3 g of NaCl, 2,5 g of peptone, 17 g of agar and 975 mL of distilled water). The NGM agar solution was autoclaved for 45 minutes and allowed to cool to 55°C before the addition of 1 mL cholesterol solution (5 mg cholesterol mL ethanol^{-1}, which did not need to be sterilized), 1 mL of 1 M CaCl$_2$, 1 mL of 1 M MgSO$_4$, and 25 mL of 1 M potassium phosphate pH 6,0. After the agar had solidified in the plates, we spread a suspension of *E. coli,* waited 20 minutes, and then inoculated with the nematodes that would feed on the *E. coli*.

We grew *E. coli* OP50 in Luria broth/streptomycin overnight at 37°C. A 3-mL culture was centrifuged for 15 min. at 5000 rpm, the supernatant was removed, and the pellet was resuspended in 50 μL of M9 buffer. We spread this solution onto the NGM agar and allowed it to dry. After 20 minutes, 1 mL of a *C. elegans* suspension was spread onto the plates.

A 3-mL culture was centrifuged for 15 min. at 5000 rpm, the supernatant was removed, and the pellet was resuspended in 50 μL of M9 buffer. We spread this solution onto the NGM agar and allowed it to dry. After 20 minutes, 1 mL of a *C. elegans* suspension was spread onto the plates.

To begin ricin extraction, we used the method suggested by Pinkerton et al. (1999) using a chirurgical knife to cut the bottom of a castor seed in a manner that allowed the rest of the seed to germinate. Pinkerton et al. (1999) reported that most ricin was detected at this location (Figure 1). We placed this piece of seed in a micro-centrifuge tube with 1 mL sterilized water and pulverized it with a homogenizer.

The suspension we obtained was called the basic extraction and was used in different assays. Using the basic extraction as our starting material, we performed the following four additional ricin extraction methods:

(1) We left the tube to rest until it separated. Then we removed the oil with a syringe and inserted a small piece of filter paper into the ricin solution (Figure 1);

(2) Instead out of removing the oil and inserting a paper filter, we removed the ricin solution with a syringe by passing the needle though the oil and slowly aspirating the solution out of the centrifuge tube;

(3) Instead leaving the suspension to rest immediately after aspiration, the suspension was placed in a warm bath rotating at 70 rpm at 45°C. After 3 hours, the solution was removed as for method 2; and

(4) Instead leaving the suspension to rest after the warm bath described for method 3, we centrifuged the solution for 5 minutes at 4,000 rpm and then removed the solution as described for method 2.
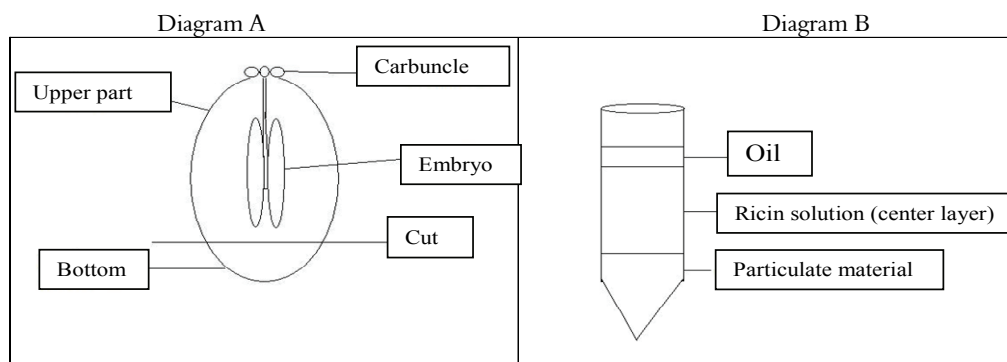

Figure 1. Diagrams (A) of the castor bean seed showing the part that was removed and (B) of the centrifuge tube showing the suspension separated into three layers.

To expose the nematodes to the ricin solutions, we used 3 different procedures:

(1) The piece of filter paper with the ricin solution was placed over the media containing the nematodes before returning the plate to the grown chamber;

(2) We made a hole in the center of the media containing the nematodes and pipetted 200 μL of the ricin solution into the hole before returning the plate to the grown chamber; and

(3) We washed the nematodes from the media with 200 μL water and placed this solution into the well of a 24-well plate, added 200 μL ricin solution, and placed these plates into the grown chamber.

For all assays, we used two strains of *C. elegans*, N2 and AB1. Each assay included 8 different sequential experiments using both strains. Each experiment was repeated two times.

Experiment 1

This experiment applied the method with the filter paper that had been immersed in the ricin solution (extraction method 1) and placed over the nematodes (exposure method 1). Water was used as a control for nematode exposure. We tested ricin solutions extracted from PI25765401 VNIIMK N1651 Russia, 2.4% ricin and Hale EUA, 12.4% ricin. We used a microscope to determine for dead nematodes 24, 48 and 72 hours after treatment.

Experiment 2

This experiment used a syringe to extract the rested solution (extraction method 2) that was pipetted into a hole in the center of the media (exposure method 2). We tested solutions extracted from PI25765401 VNIIMK N1651 Russia, 2,4% ricin and Hale EUA, 12,4% ricin. Results were recorded 24 hours after treatment by checking for dead nematodes and measuring halo size.

Experiment 3

This experiment was performed as for experiment 2, but dead nematodes were checked every 2 hours from 12 to 24 hours after treatment.

Experiment 4

This experiment used the same exposure method, varieties and controls as experiments 2 and 3, but compared the 3 different extraction methods described above. Results were recorded 18 hours after treatment.

Experiment 5

This experiment used the same exposure method as experiments 2, 3 and 4, and used extraction method 4, which used the warm bath with rotation followed by centrifugation for ricin extraction. For this experiment, all lines (PI25765401 VNIIMK N1651 Russia, 2.4% ricin; PI176751 8838 Turkey, 5.6% ricin; PI223013 Karchak Iran, 6.5% ricin; PI170685 Acik Kahverengi Turkey, 7, 6% ricin; PI170684 Kahverengi Turkey, 8.1% ricin; PI183468 Erari India, 9.2% ricin; PI183347 Divela India, 10.3% ricin; PI183076 Erari India, 10.8% ricin; and Hale EUA, 12.4% ricin) were used. We counted the number of dead nematodes on the plates 18 hours after treatment.

Experiment 6

This experiment used the same extraction method as experiment 5 but used nematode exposure method 4, which used water in the 24-well plates. We used the same varieties as for experiment 1. After 18 hours, we counted the number of dead and living nematodes in each well.

Experiment 7

This experiment used the same methodology as experiment 6 but analyzed solutions extracted from all of the lines.

Experiment 8

This experiment used the same methodology as experiment 7 but to count number of dead and living nematodes, 50 μL was extracted from each well and placed on a microscope slide that was counted under a microscope before calculating the percentage of dead nematodes.

The statistical design was totally randomized, with 4 repetitions performed 2 different times and analyzed by Test T LSD (SAS) followed by the Kruskal-Wallis method with a 5% significance level (using a Kruskal-Wallis statistical program modified by Professor João Corrente of ESALQ). We choose a non-parametric method because it is more accurate for the appraisal of the biology. The data did not show a normal distribution, which is common for assays involving microorganisms. We also examined the correlation between ricin content and dead nematodes.

Results and discussion

According to Lowery (2007), because the castor has a lot of variation in ricin content, all correlations greater than 0.75 between ricin content measured by a new method and by an old one were considered sufficient to validate the new method.

In experiment 1, we did not find differences between the treatments and the control. There were

was almost no dead nematodes because ricin and the other RIPs are large molecules that get caught by the paper and are not able to disperse onto the media.

In experiment 2, the suspensions differed from one another and from the control when analyzed by Test T SLD and Kruskal-Wallis. Because the CV% was high, we double-checked with the non-parametric method and found correlations between dead nematodes and ricin content of 0.855 and 0.927 for repeats with the N2 strain. The halos around the treatments were 0.945 and 0.916. There were also statistically significant differences between the standards and the control for the AB1 strain. However, the correlation was below 0.5, and there was no statistically significant difference, indicating that this strain is likely to have a mechanism to inactivate part of the ricin. Unfortunately, there is no other bioassay using *C. elegans* for RIP (Ribosome Inactivating Protein) to which we could compare. We began these assays because the nematodes have the same the kind of ribosome as superior animals.

In experiment 3, the data showed that approximately 18 hours after treatment was the best time to observe the results because there were a larger number of dead nematodes, and the difference between treatments was sufficient for statistical analysis.

In experiment 4, in which we compared the 3 extraction methods, the response of each strain to the different varieties were the same as in the previous experiments using extraction methods 2 and 4. We found correlations of 0.78 and 0.84 between the ricin content and the number of dead nematodes for the N2 strain. However, the last method was easier to determine the count of dead nematodes, and exposure method 2 showed a negative correlation as did the AB1 strain.

In experiment 5, AB1 showed no differences between the standards. N2 showed a statistically significant difference between the standards and the control and a correlation of 0.80 between the ricin content and the dead nematodes.

In experiment 6, we noticed that on the 24-well plates, the responses of both strains were the same as on petri plates. However, the results were much easier to read. The AB1 strain showed no correlation between the percentage of dead nematodes and the different levels of ricin. The N2 strain was different from the control and had a correlation of 0.80 between the ricin content and the percentage of dead nematodes.

In experiment 7, we confirmed the results with a correlation of 0.82 for the second time. The N2 and AB1 results were proportionally the same as in the other experiments.

In experiment 8, the correlation between dead nematodes and ricin content for the N2 strain was 0.78 and 0.87, showing a statistically significant difference among the treatments. There was a negative correlation for the AB1 strain.

The experiment showed that N2 is sensitive to ricin and that the methods used in experiment 8 were a good way to quantify differences in seed ricin content. Results could be analyzed more quickly with the use of coloring techniques with 5(6)-carboxyfluorescein diacetate as proposed by Gnoula (2007), especially if it is calibrated for analysis in a UV densitometer. The bioassay presented here is an important tool for the detoxification of castor bean by breeding for varieties with lower RIP.

If used to breed a less toxic castor bean, this method can help develop a castor cake that can be fed to cattle and manipulated with less danger.

This method is very efficient because each specific seed used to quantify ricin content is recovered and because the ricin toxicity is expressed genetically, and the genetic of the embryo determine the amount of the ricin on the seed.

Conclusion

The best bioassay to quantify the ricin toxin is the one used in experiment 8. Where the *C. elegans* strain N2 was used, ricin was extracted with a homogenizer followed by a warm bath at 45 Celsius degrees rotating at 70 rpm for 3 hours, and centrifugation for 5 minutes at 4,000 rpm. The center layer was removed with a syringe, and placed in a 24-well plate. The results showed an 87% correlation with the ELISA method. The high correlation indicates that this method can be used to select castor bean lines with levels of ricin.

Acknowledgements

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