

Detection of *Phakopsora pachyrhizi* by polymerase chain reaction (PCR) and use of germination test and DNA comet assay after e-beam processing in soybean

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Abstract

Soybean harvest is the main agribusiness in Brazil, which is the second largest exporter in the world and has a revenue of billions of dollars. Asian rust is caused by the fungus *Phakopsora pachyrhizi* and its dissemination is difficult to control, since it occurs through wind dispersion. Actually *P. pachyrhizi* is found in different parts of the world. Electron beam treatment could be an alternative process to minimize these losses, especially for the grains exportation industry. Besides the possibility of being disconnected when not in use, this source does not need to be reloaded, is easily available and, streamlines the process and reduces logistics costs. The present work aims to identify, by the polymerase chain reaction technique (PCR), the *P. pachyrhizi* fungus presence in the irradiated soybeans and the possibility to use radiation treatment as a sanitary alternative. Doses 0, 1.0, 2.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 kGy (IPEN-CNEN/SP Electron Accelerator) were applied and two fast-screening methods were performed: DNA comet assay (for the detection of DNA damage) and germination test (for the measurement of roots inhibition). These tests are very easy to carry out and measure damage response depending on radiation dose.

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1. Introduction

Soybean rust is a serious disease causing crop losses in many countries in the world. Soybean is the most important harvest in Brazil, the second largest exporter in the world, which has led to a significant amount of financial devise. The contamination consequence of Asian dust fungus, being harmed since the plantation up to the crop, with losses in Brazil only the harvest of 2003/2004 being near US\$2.28 billions (Carvalho and Figueiredo, 2000; Fanaro et al., 2004). The main factors that limit high profits are diseases caused by fungus, bacteria, nemathelminthes and virus. The most preoccuping soybean disease

at the moment is soybean rust. Soybean rust is caused by two fungal species, *Phakopsora pachyrhizi* (Asian soybean rust), an aggressive pathogen, and *Phakopsora meibione* (American soybean rust), a weak pathogen (Carvalho and Figueiredo, 2000).

The Asian soybean rust was introduced into the American continent in March 2001, in Paraguay, and the total yield losses in South America have been estimated to be from 10% to 80%. (EMBRAPA, 2005; Fanaro et al., 2004; Yorinori and Lazzarotto, 2004). The *P. pachyrhizi* is high-humidity dependent, and needs at least 6 h of free humidity to start contamination. Mild temperature conditions are ideal, but it is not a limitation, because this fungus can be developed between 15 and 30 °C (Yorinori and Lazzarotto, 2004). The dissemination has a hard control, because the uredospore dispersion is by the wind

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(EMBRAPA, 2005; Fanaro et al., 2004; Yorinori and Lazzarotto, 2004).

Irradiation with ionizing radiation is one of the most effective means disinfecting dry food ingredients (WHO, 1994; Hayashi et al., 1998). The treatment by irradiation can inhibit cellular life division, like microorganisms and promoting a molecular structural modification (Diehl, 1995). Vegetal cells are most sensitive on ionizing radiation than on fungi and bacterial spores (Diehl, 1995; Monk et al., 1995). The electron beam machine has several benefits compared to ^{60}Co irradiation to treat grains, for example, by easy handling and low degradation effect to the irradiated product, it can be built on ports and the soybean or any kind of grains can be irradiated few minutes before to get on board. Besides the possibility of being disconnected when not in use, this source does not need reloading, is easily available and has a high dose rate, streamlining the process and reducing logistics cost (Watanabe, 2000).

The polymerase chain reaction (PCR) is an in vitro method, which is used to enzymatically amplify a certain DNA sequence. Because of its high sensitivity, high specificity and rapidity, PCR is the method of choice for this purpose. PCR is widely employed in a tremendous variety of situations to produce high yields of specific DNA target sequences; however, it cannot be employed as a viability test (Candrian, 1995; Santos et al., 2001). In order to study fungal viability, a microbiological test should be performed. Germination test was carried out to observe the viability after radiation processing (Hammerton, 1996; Kawamura et al., 1996; Marin-Huachaca et al., 2004). Since DNA molecule is a target ionizing radiation, damage detection can be estimated by the comet assay method, a very fast and cheap screening test (Barros et al., 2002; Delincée, 2002; Villavicencio et al., 2004). The present work aims to detect *P. pachyrhizi* contamination by PCR, fungal viability by microbiological test, germination test and the DNA comet assay after e-beam processing, to observe possible modifications caused by the treatment.

2. Experimental

2.1. Addition of *P. pachyrhizi* spores

Once *P. pachyrhizi* uses soy leaves as a substrate for its growth and soybeans work as a vehicle for its transportation through cultures, the effect of ionizing radiation was also studied in decontaminated leaves. Decontamination was performed using a 0.4% sodium hypochlorite solution. After this, soy leaves were inoculated with *P. pachyrhizi* spores released by EMBRAPA.

2.2. Samples

The Brazilian soybean grains were donated by a food company in São Paulo, Brazil. Uncontaminated soy leaves were developed in our laboratory. Soybeans and soy leaves

artificially contaminated were packed in sterile polyethylene bags, labeled and identified with its respective irradiation doses.

2.3. Irradiation

Samples were irradiated at the IPEN-CNEN Electron Accelerator, a Dynamitron Machine (Radiation Dynamics Co. model JOB, New York, USA), with 0.550 MeV power, scan 100 cm and support speed 6.72 m/min with doses of 0, 1.0, 2.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 kGy. The applied dose rate was between 2.23 and 22.37 kGy/s and electrical current was between 0.3 and 3.2 mA. CTA dosimeters were used for the measurement of radiation dose.

2.4. *P. pachyrhizi* growth (viability test)

After irradiation process, contaminated soy leaves were placed in sterile Petri dishes (identified with its respective irradiation doses) with 10 mL of autoclaved distilled water and incubated at 25 °C for 5 days. As the irradiation process turned soy leaves dark, a new sterile fresh leaf was added into each Petri dish to confirm fungi growth. They were incubated at 25 °C for 5 days again.

2.5. *P. pachyrhizi* DNA extraction from soybeans (adapted CTAB method)

Four hundred microliters of extraction buffer (100 mM Tris-HCl, 1.4 M sodium chloride, 20 mM $\text{Na}_2\text{-EDTA}$, 2.0 g/L CTAB, pH 8.0) were used to suspend soaked spores. The samples were incubated for 60 min at 65 °C, shaking each 15 min. Once cooled, they were centrifuged at 6000 rpm for 10 min. Supernatants were transferred into a new 1.5 mL tube. The same chloroform volume was added and mixed slowly several times. They were centrifuged at 6000 rpm for 15 min. Supernatants were transferred into a new 1.5 mL tube. The last two steps were repeated again. Freeze isopropanol (0.6 volume of last supernatant) was added to the tubes, mixed by inversion five times and they were kept overnight at 4 °C. Next day, they were centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and 500 μL of 70% ethanol were added to the pellets and mixed. The precipitated DNA was pelleted by centrifugation at 12,000 rpm for 5 min, air dried by 30 min to 1 h and resuspended in 50 μL of bidistillate water. Those samples were directly used for PCR analysis (Greiner et al., 2005).

2.6. PCR conditions, oligonucleotide primers and analysis of PCR products

Amplification was performed in 0.2 mL tubes using 25 μL total reaction volumes containing 2.0 μL samples of extracted DNA. The reaction mixture consisted of 200 mM Tris-HCl pH 8.4, 500 mM KCl, 50 mM MgCl_2 , 10 μM of each primer, 2.5 mM of each deoxynucleotide triphosphate and 5 U/ μL units of Taq DNA-polymerase in an Eppendorf thermocycler. The following primers pairs were used:

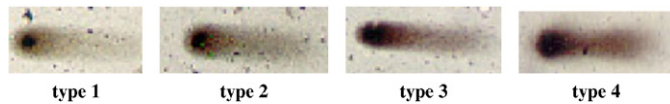


Fig. 1. DNA migration after irradiation treatment.

PPA1 (5'-TTA GAT CTT TGG GCA ATG GT-3') and PPA2 (5'-GCA ACA CTC AAA ATC CAA CAA T-3'). PCR conditions were 94 °C for 5 min (preincubation) and then 35 cycles at 94 °C for 1 min (denaturation), 55 °C for 1 min (primer annealing) and 72 °C for 2 min (primer extension) followed by a final extension at 72 °C for 7 min. The predicted size of the obtained PCR products is 332 bp. Amplified products were held at 4 °C until analyzed by standard agarose gel electrophoresis followed by ethidium bromide staining. The gel was photographed with a Vilber Lourmat Imager System.

2.6.1. Germination test

The germination test was carried out as an adaptation of Kawamura et al. (1996).

2.6.2. DNA comet assay

The DNA comet assay was carried out as described by Cerda et al. (1997). Comets were classified as shown in Fig. 1.

3. Results

Incubated Petri dishes containing soy leaves artificially contaminated, processed by e-beam with doses of 1.0, 2.0 and 5.0 kGy, did not inhibit *P. pachyrhizi* growth. Satisfactory effect of e-beam treatment to control *P. pachyrhizi* growth was reached with doses higher than 6.0 kGy. Using 6.0, 7.0, 8.0, 9.0 and 10.0 kGy, there was no evidence of fungi colony in the samples analyzed by the microbiological viability test. However, it was still possible to detect by PCR the presence of *P. pachyrhizi* DNA in the soybeans analyzed as observed in Figs. 2a and b; hence, artificial contamination really occurs in both soy leaves and beans.

Using the DNA comet assay, it was possible to detect DNA degradation due to the irradiation treatment. In addition, germination test determined the influence of ionizing radiation in roots elongation after incubation, as well as seeds viability. It was observed that the radiation damage increased with increasing radiation doses. Decreasing percentages of root growth were accompanied with increasing radiation doses. It was observed that radiation treatment with high doses had a negative influence on root growth (Fig. 3). Analyzing the comet assay results, we could observe that a slight DNA damage of soybeans appeared after radiation treatment with doses over 1.0 kGy. It was also observed that this degradation increased with the radiation dose applied, based on higher DNA migration found. Frequently non-irradiated soybeans exhibited comet types 1 and 2 only, with very slight

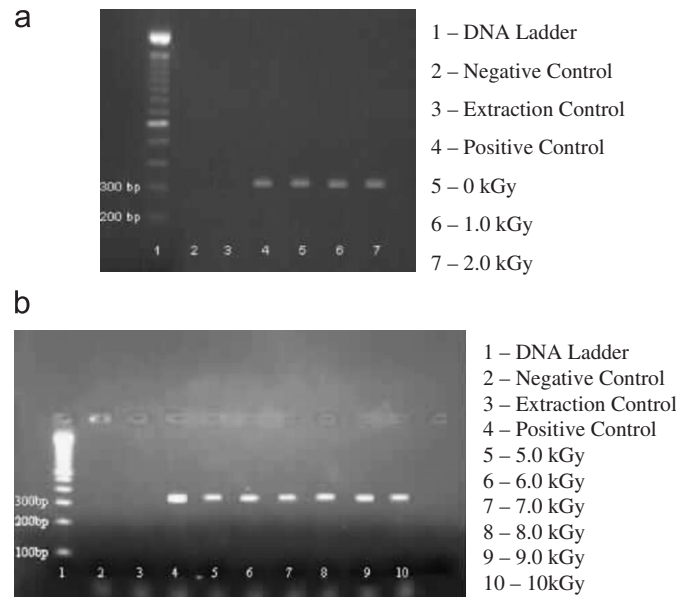


Fig. 2. (a) Agarose gel electrophoresis showing the positive presence of *P. pachyrhizi* DNA. (b) Agarose gel electrophoresis showing the positive presence of *P. pachyrhizi* DNA.

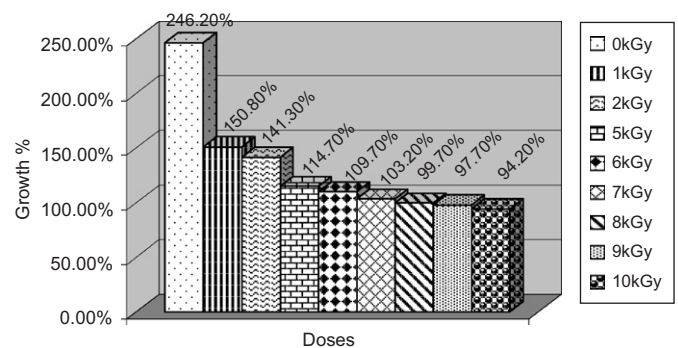


Fig. 3. Percentage of root growth with different radiation doses.

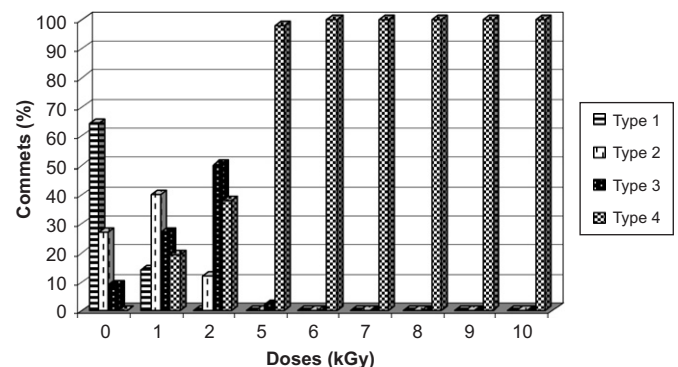


Fig. 4. Percentage of different comets types after e-beam treatment.

amounts of type 3 as observed in Fig. 4. With increasing doses, from 1.0 to 2.0 kGy, DNA fragmentation became obvious, increasing the amounts of type 3 comets. At higher doses, from 2.0 to 10.0 kGy, type 4 comets were also

found. The viability response to electron beam treatment was, in principle, not satisfactory in our machine for the soybeans grains tested here. Although the doses applied were effective in inhibiting fungi growth, the seeds lost viability with doses higher than 5.0 kGy. Additional experiments will be carried out to compare different machine potencies.

4. Conclusion

The aim to use e-beam in our experiment was to compare the superficial efficiency of that treatment. Only superficial effects were observed in the well-established dose (6.0 kGy), in the case of *P. pachyrhizi*, to eliminate any fungi contamination and with no nutritional damage or a minimal damage in DNA after the radiation treatment in the grains. We are working with more experiments and applying different energies to observe whether or not DNA degradation occurs after e-beam treatment in the grains.

Acknowledgments

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