Tropical Plant Pathology, vol. 36, 6, 414-418 (2011)

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SHORT COMMUNICATION / *COMUNICAÇÃO*

Development of a powder formulation of *Aspergillus parasiticus* for dry inoculation of peanut kernels

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ABSTRACT

The aim of this study was to develop a method for the dry inoculation of *Aspergillus parasiticus* on peanut kernels for post-harvest studies. Inoculum powder was prepared by adding sterile kaolin powder to *A. parasiticus* spores developed on filter papers soaked with spore suspension prepared with Czapek medium (5% agar), which were dried at room temperature. Concentrations were adjusted to 5 x 10^5 and 1 x 10^6 spores g^{-1} of kaolin. Varied amounts of the powder inoculum were then used to inoculate 1 kg of peanut kernels. Inoculation was performed by mixing the powder inoculum with the peanut kernels in plastic bags until the kernels were completely covered. The effectiveness of this inoculation method was compared with the common inoculation methods of immersion in aqueous spore suspensions and contamination through contact with fungal colonies growing on culture medium. Fungal growth assay was performed on PDA saline medium, and near-infrared radiation measurements were used to determine peanut kernel moisture content. The dry inoculation technique was effective in inoculating peanut kernels with *A. parasiticus*, especially at concentrations of 3 x 10^6 and 4 x 10^6 spores g^{-1} per 1 kg of peanut kernels. In contrast to usual methods of inoculation, the dry inoculation technique does not increase the moisture content of inoculated peanut kernels. Thus, the physical state of the peanut kernel remains intact.
Key words: *Arachis hypogaea*, post-harvest, powder inoculum.

RESUMO

Desenvolvimento de uma formulação em pó de *Aspergillus parasiticus* para inoculação seca de grãos de amendoim

O objetivo deste estudo foi desenvolver um método de inoculação seca de *Aspergillus parasiticus* em grãos de amendoim para estudos em pós-colheita. O inóculo em pó foi preparado adicionando-se caolim em pó esterilizado sobre os esporos do fungo desenvolvidos sobre papel de filtro embebido com uma suspensão de esporos, preparada em meio Czapek (5% de ágar), e secos em temperatura ambiente; concentrações foram ajustadas para 5 x 10^5 e 1 x 10^6 esporos g^{-1} de caolim em pó. Utilizaram-se as quantidades de 1; 2; 3 e 4 g da formulação em pó do inóculo para 1 kg de grãos, sendo misturado até a cobertura total dos grãos de amendoim. A eficácia desse método foi comparada com métodos tradicionais de inoculação – imersão em suspensão de esporos e contato dos grãos com a colônia fúngica. O teste de sanidade foi realizado em meio BDA salino e o de umidade por meio de radiação próximo ao infravermelho. A formulação em pó de *A. parasiticus* foi eficiente na inoculação de grãos de amendoim, principalmente quando se utilizou as concentrações de 3 x 10^6 e 4 x 10^6 esporos g^{-1} de caolim por 1 kg de grãos. Ao contrario dos métodos tradicionais de inoculação, o método de inoculação seca não aumentou o grau de umidade inicial dos grãos de amendoim, não interferindo nos estados físicos e fisiológicos dos mesmos, demonstrando ser viável em estudos pós-colheita.
Palavras-chave: *Arachis hypogaea*, inóculo em pó, pós-colheita.

Fungi are the most important microorganisms associated with stored peanuts (*Arachis hypogaea* L.). These microorganisms affect the color, odor, taste, and nutritional value of the peanut kernels and produce mycotoxins such as aflatoxins. The main species associated with peanut aflatoxin contamination are *Aspergillus parasiticus* Speare and *Aspergillus flavus* Link (Richard & Payne, 2003).

Methods to inoculate peanut kernels with toxigenic fungi, such as *A. parasiticus*, are useful in studies to evaluate aflatoxin contamination of peanut kernels during storage periods. Fungal inoculation of kernels and seeds is most commonly achieved by submersion in aqueous spore suspensions (Prado et al., 1996; Prado et al., 1999; Chiou et al., 1999) or through contact with fungal colonies growing on culture medium (Tanaka et al., 1989; Machado & Carvalho, 2001). However, these inoculation methods affect the physical and physiological state of the kernels or seeds due to the rapid absorption of water that occurs during the inoculation process. A dry inoculation method is desirable to maintain the initial levels of moisture, providing a better environmental control in post-harvest experiments.

Dried formulations of fungi have been used successfully at the biological control of insects (Knudsen et al., 1990), weeds (Walker & Connick Jr., 1983), nematodes (Salgado & Campos, 1993), and soil-borne

pathogens (Lewis et al., 1996; Moretini & Melo, 2007). The technique presented in this paper provides a method for the dry inoculation of peanut kernels with *A. parasiticus* that circumvents the drawbacks of the more common techniques.

Pods of BR-1, a Valencia-type peanut cultivar developed by the Brazilian Agricultural Research Corporation (Embrapa), were hand-shelled, and shriveled or damaged kernels were rejected. Intact kernels were irradiated at 25 kilogray (kGy) to reduce the mycoflora associated with the kernels. The irradiation was performed at the Nuclear Department of the Federal University of Pernambuco, Brazil. Irradiated kernels were maintained in plastic bags to avoid contamination after irradiation.

An isolate of *A. parasiticus* (#IMI242625) from the International Mycological Institute, England, provided by Dr. Guilherme Prado from Ezequiel Dias Foundation, Belo Horizonte, MG, Brazil, was used in this study. The fungus was grown on Petri dishes containing Czapek Agar in an incubator at 25 °C with a daily photoperiod of 12 h for seven days. Spore suspensions were prepared by adding 20 mL of sterile distilled water into each Petri dish; this suspension was then filtered using cotton gauze and incorporated into 100 mL of sterile molten Czapek Agar medium at 45 °C. Sterilized filter paper discs (30 mm diameter) were submersed in the mixture of Czapek Agar medium plus spores and placed in plastic Petri dishes 90 mm in diameter. The culture on the filter paper discs was then incubated at room temperature to allow the culture medium to dry; abundant sporulation of the fungus was observed after 10 days. Two filter papers with abundant and similar amounts of sporulation on their surfaces were selected. One was washed in 10 mL of distilled water to obtain a spore suspension. The second was used to prepare the dry inoculum; spores were removed and put into 10 g of sterilized kaolin powder using a brush. The number of spores mixed with kaolin powder was indirectly estimated using the number of spores counted in the spore suspension with a hemacytometer. The number of spores mixed with kaolin powder was adjusted to 5×10^5 and 1×10^6 spores g^{-1} of kaolin powder.

Amounts of 1 g, 2 g, 3 g and 4 g of inoculum powder containing either 5×10^5 or 1×10^6 *A. parasiticus* spores g^{-1} kaolin were used to inoculate 1 kg of peanut kernels. As a control treatment, 4 g of sterilized kaolin powder were mixed with 1 kg of peanut kernels. Inoculation was performed by mixing the powder inoculum with the kernels in plastic bags until the kernels were completely covered.

Inoculation using an aqueous spore suspension was performed by adding peanut kernels to an aqueous suspension of *A. parasiticus* spores in distilled water (1×10^6 spores mL^{-1}) for 30 minutes. Contamination with fungal colonies was performed by placing the kernels on *A. parasiticus* colonies growing in Petri dishes containing Czapek Agar. Kernels were maintained in contact with the colonies for 22 h.

Peanut kernels were evenly spaced on PDA saline medium (Potato – Dextrose – Agar + 6% of sodium chloride) in 90-mm diameter Petri dishes at the density of 10 kernels per dish. Kernels were incubated at 20 °C ± 2 °C with a daily photoperiod of 12 h for seven days. Fungal examination was done with a stereomicroscope at 80x magnification; a compound microscope was used whenever required.

Peanut kernel moisture content was determined using near-infrared (NIR) radiation, which was performed rapidly and nondestructively. NIR reflectance measurements were collected with a NIR spectrometer (XDS™ Masterlab, Foss Inc, Höganäs, Sweden) in the Analytical Chemistry laboratory at Embrapa Cotton. The spectrometer was calibrated using five samples of peanut kernels, which were immersed in distilled water for periods of 0, 5, 10, 20 and 40 minutes; samples were run in duplicate. The moisture levels obtained from the five different groups ranged from 6.4% to 18.5%. The reflectance spectra of the samples were collected in the wavelength range between 1100 and 2500 nm at 0.5 nm intervals. Calibration was established by collecting the reflectance spectra in the wavelength range between 1800 and 2050 nm using a technical standard normal variate (SNV) and partial least squares (PLS) regression with cross-validation. Values used for calibration were obtained with the standard AOCS (American Oil Chemists' Society) official method Ab 2-49. After collecting the reflectance spectra, the samples were placed in a metal container, and their wet weight was recorded. The containers were then placed in a hot air oven at 130 °C for 3 h. At the end of the heating period, the containers were weighed again to determine the dry weight of the samples. The moisture content of each sample was determined as the percentage ratio of weight loss to the original wet weight of the sample. The value obtained for each sample was considered the standard oven moisture value. The standard oven moisture content values of the five samples were used to obtain regression models. Moisture content estimation for each treatment was performed before and after inoculation with *A. parasiticus*.

The experimental design was completely randomized, and all experiments were performed in triplicate. The statistical analysis was performed by following the hierarchical classification model with the amount of powder inoculum applied (1, 2, 3 or 4 g kg^{-1} of peanut kernels) within the inoculum concentrations (5×10^5 or 1×10^6 spores g^{-1} kaolin) and three additional treatments: control (sterile kaolin powder), immersion in aqueous spore suspension and contamination through contact with fungal colonies developed on Czapek Agar for 22 h. The experimental unit was a Petri dish containing 10 kernels. Statistical analyses were conducted using SAS version 9.1.3 (SAS Institute Inc. Cary, NC, USA). This assay was repeated twice.

All inoculation methods evaluated in this study – powder inoculum, submersion in aqueous spore suspension, and fungal colony contact – were efficient to inoculate *A. parasiticus* on peanut kernels compared with the control treatment (Tables 1 and 2, Figure 1). Significant

TABLE 1 - BRS 101 peanut kernels contaminated with *Aspergillus parasiticus*, and moisture content before and after inoculation with *A. parasiticus* using different inoculation methods

Methods	Kernels contaminated with *A. parasiticus* (%)	Moisture content (%)	
		Before inoculation	After inoculation
Powder inoculum (5 x 10^5 spores g^{-1} kaolin – 1.0 g kg^{-1} kernels)	53.3	7.0	6.8
Powder inoculum (5 x 10^5 spores g^{-1} kaolin – 2.0 g kg^{-1} kernels)	66.7	7.0	6.3
Powder inoculum (5 x 10^5 spores g^{-1} kaolin – 3.0 g kg^{-1} kernels)	63.3	6.9	5.9
Powder inoculum (5 x 10^5 spores g^{-1} kaolin – 4.0 g kg^{-1} kernels)	73.3	7.0	5.6
Powder inoculum (1 x 10^6 spores g^{-1} kaolin – 1.0 g kg^{-1} kernels)	73.3	7.0	6.6
Powder inoculum (1 x 10^6 spores g^{-1} kaolin – 2.0 g kg^{-1} kernels)	90.0	7.1	6.2
Powder inoculum (1 x 10^6 spores g^{-1} kaolin – 3.0 g kg^{-1} kernels)	96.7	6.9	5.9
Powder inoculum (1 x 10^6 spores g^{-1} kaolin – 4.0 g kg^{-1} kernels)	93.3	6.9	5.5
Control (kaolin – 4.0 g kg^{-1} kernels)	13.3	7.1	5.5
Aqueous spore suspension (1 x 10^6 spores mL^{-1} distilled water) – usual method	100.0	7.0	12.0
Colony contact (22 h) – usual method	100.0	7.0	14.0

TABLE 2 - Statistical analysis of BRS 101 peanut kernels contaminated with *Aspergillus parasiticus* using different inoculation methods

Contrast	DF[1]	Chi-Square
Control vs. powder inoculum – spore concentration	1	52.74**
Usual methods vs. control	1	84.65**
Usual methods vs. powder inoculum – spore concentration	1	20.31**
Between spore concentrations – 5 x 10^5 vs. 1 x 10^6 spores g^{-1} kaolin	1	22.96**
Powder inoculum amount (5 x 10^5 spores g^{-1} kaolin)	3	2.72^{ns}
Powder inoculum amount (1 x 10^6 spores g^{-1} kaolin)	3	8.69**
Contrast 1.0 g vs. 2.0 g (1 x 10^6 spores g^{-1} kaolin)	1	2.87^{ns}
Contrast 3.0 g vs. 4.0 g (1 x 10^6 spores g^{-1} kaolin)	1	0.36^{ns}
Contrast 1.0 g and 2.0 g vs. 3.0 g and 4.0 g (1 x 10^6 spores g^{-1} kaolin)	1	4.35**

[1]Degrees of freedom.
**Significant at p≤0.01.
[ns]Not significant at p≤0.05

differences were found between powder inoculum at spore concentrations of 5 x 10^5 and 1 x 10^6 spores g^{-1} kaolin as well as within spore concentration at 1 x 10^6 spores g^{-1} kaolin. Peanut kernel inoculation with 1 x 10^6 spores g^{-1} kaolin was more efficient than with 5 x 10^5 spores g^{-1} kaolin, mainly when 3 g (3,000,000 spores) or 4 g (4,000,000 spores) of powder inoculum were used per kg of kernels. No significant differences were found among the amounts of powder inoculum at 5 x 10^5 spores g^{-1} kaolin: 1 g (500,000 spores), 2 g (1,000,000 spores), 3 g (1,500,000 spores) and 4 g (2,000,000 spores). These results show that the efficacy of the powder inoculum method was primarily influenced by the total number of spores used to inoculate the kernels.

In contrast with the usual methods of inoculation such as aqueous spore suspensions, in which peanut kernels moisture content increased from 7% before inoculation to 12% after inoculation, or contamination through contact with fungal colonies growing on culture medium, in which the moisture content increased from 7% before inoculation to 14% after inoculation, the dry inoculation method did not increase the moisture content of the peanut kernels after inoculation (Table 1). Using this method, the average moisture content of kernels was 7% before inoculation and 6.1% after inoculation. The moisture content decreased as the amount of inert product (kaolin) increased. This likely occurred due to moisture absorption by the kaolin in contact with the seed coat of the kernels.

Kaolin is a complex aluminum silicate that is nearly chemically inert and non-abrasive and can be powdered easily to desirable particle size (Bhattacharyya & Basu, 1982). This product has been used successfully as filler in dried formulations of alginate-encapsulated mycelia or spores. Populations of fungi *Alternaria macrospora* Zimm., *Alternaria cassiae* Jurair & Khan, *Fusarium lateritium* Nees ex Fr., *Colletotrichum malvarum* (A Braun & Casp.) Southworth (Walker & Connick Jr., 1983), *Arthrobotrys*

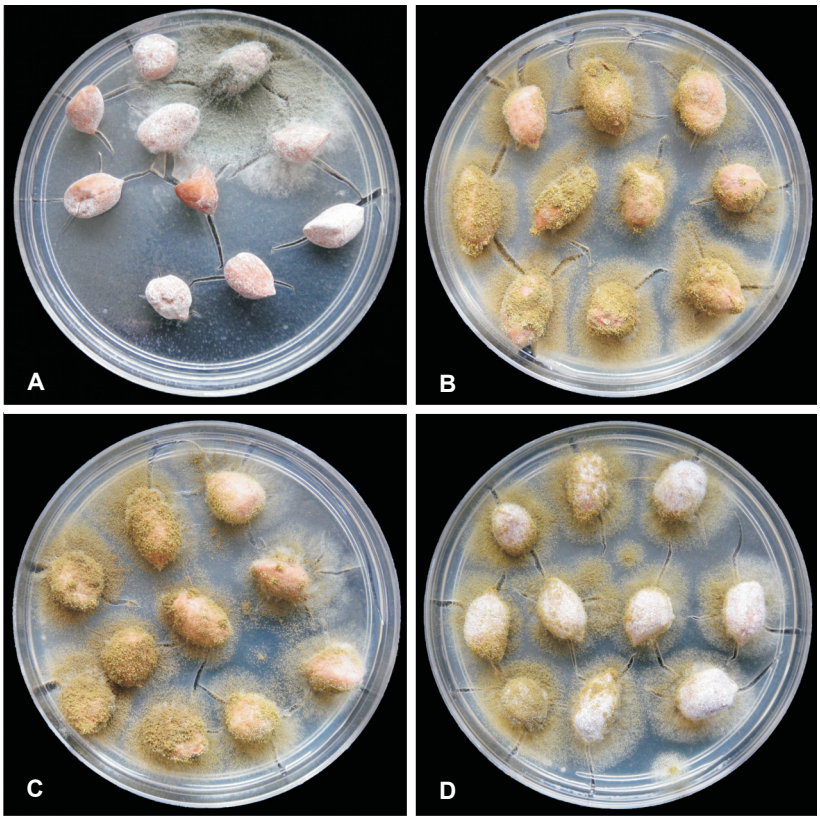

FIGURE 1 - BRS 101 peanut kernels not inoculated (A - control), inoculated with spore suspension of 1 x 10^6 spores mL^{-1} distilled water (B), inoculated through direct contact with colonies growing on culture medium for 22 h (C) and inoculated with dry spores of *Aspergillus parasiticus* mixed with kaolin at a concentration of 1 x 10^6 spores g^{-1} kaolin powder (D).

conoides Drechsler (Salgado & Campos, 1993), and *Coniothyrium minitans* W.A. Campb. (Moretini & Melo, 2007) were very stable in dried formulations that used kaolin as filler of alginate pellets for periods ranging from eight to 14 months stored at 4 °C. Viability of propagules of these fungi was 100% for up to 1 year.

Kaolin powder has also been used successfully as a carrier of different fungi and bacteria in dried formulations. Amer & Utkhede (2000) reported the efficacy of seed treatment with dried formulations made with kaolin as a carrier of biological agents such as *Bacillus subtilis* Cohn and *Pseudomonas putida* Trevisan for management of root rot of cucumber and lettuce. In these formulations, *B. subtilis* and *P. putida* survived up to 45 days stored at room temperature (about 22 °C). A dried formulation that used kaolin as carrier of *Aspergillus* sp. to inoculate barky jute was viable for at least 90 days stored at room temperature (31-33 °C) in the dark (Bhattacharyya & Basu, 1982).

In the present study, kaolin-formulated powder inoculum of *A. parasiticus* was efficient to inoculate kernels, and did not increase the moisture content of inoculated peanut kernels. Although there was a gradual decrease of viability of *A. parasiticus* with time of preservation, this fungus was viable up to 60 days stored at 4 °C temperature in this formulation (data not shown). In addition, kaolin failed to serve as a nutrient for any contaminants. As a result, in peanut kernels inoculated with kaolin-formulated powder, *A. parasiticus* grew predominantly, suppressing the growth of other organisms associated with the peanut kernels, which were not eliminated by irradiation.

Although inoculation using aqueous spore suspensions or through direct contact with fungal colonies are efficient techniques (Tanaka et al., 1989; Prado et al., 1996; Prado et al., 1999; Chiou et al., 1999; Machado & Carvalho, 2001), these techniques affect the physiological and physical states of the peanut kernels by increasing their moisture content (Table 1). Machado & Carvalho (2001) demonstrated that common bean seeds inoculated with *Colletotrichum lindemunthianum* (Sacc. & Magn.) Bri. & Cav. through direct contact with fungal colonies germinated 168 h after the start of the experiment due to the water absorption that occurred during the inoculation process. In contrast, the dry inoculation technique did not increase the moisture content of the peanut kernels, leaving the physiological and physical state of the peanut kernels intact. This demonstrates the viability of this technique for the inoculation of peanut kernels in post-harvest studies.

ACKNOWLEDGEMENTS

The authors thank Dr. Helen Jamil Khoury (Nuclear Energy Laboratory of the Federal University of Pernambuco) for the irradiation of the peanut kernels, and Dr. Guilherme Prado (Ezequiel Dias Foundation) for

providing *A. parasiticus* isolate. Thanks also to Dr. João Luís da Silva Filho (Embrapa Cotton) for advice on the statistical analyses.

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TPP 220 - Received 29 November 2010 - Accepted 11 December 2011
Section Editor: Wagner Bettiol