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Pathogenicity of *Colletotrichum truncatum* and its Influence on Soybean Seed Quality

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ABSTRACT

Pathogenicity of *Colletotrichum truncatum* and its influence on soybean seed quality were evaluated by artificial inoculation. *C. truncatum* enabled to establish as latent infection without showing any visible symptom in all seed components with maximum frequency values of 100% for seed coat, 43.0% for cotyledon and 30.0% for embryonic axes after 4 days of incubation period. The infection level remained the same in all seed components until the end of the incubation period. Fungal infection reduced seed germination by 29.2% and viability by 26.9% than un-inoculated seeds *in vitro*. Moreover, infection increased the electrolyte leakages compared with control. Under glasshouse conditions, pathogenicity of *C. truncatum* on seeds and seedlings was more virulent than that of controlled conditions. In the glass house, *C. truncatum* reduced seed germination and seedling survival by 46.4% and 75.8%, respectively and caused pre- and post-emergence damping-off of seedlings. However, fungal infection by *C. truncatum* increased protein and oleic acid content and reduced linoleic acid content, but did not change in extracted oil and other fatty acids when compared with un-inoculated seeds after 4 days of incubation.

Key Words: Soybean; Seed-borne infection; *Collotrichum truncatum*; Seed quality; Deterioration

INTRODUCTION

Soybean is an important oilseed crop in the world for its high constituent of protein and oil contents in the seed for animal and human consumption (Golbitz, 2003; Olguin *et al*., 2003; Belewu & Belewu, 2007). Seeds, when infected by microbial pathogens carried with, on, or in seeds, it cause a primary source of inoculums for damaging seeds and plants with varying degrees (Maude, 1996). Anthracnose caused by *Colletotrichum truncatum* [(Schw.) Andrus and W.D. Moore] is one of the most important seed-borne fungal pathogen of soybean (Sinclair & Backman, 1989). The disease causes a significant reduction of seed germination, seed quality and yield in the warm and humid subtropics. Yield losses up to 50% in Thailand and 100% in India have been reported due to anthracnose (Sinclair & Backman, 1989; Ploper & Backman, 1992; Manandhar & Hartman, 1999). Seed viability and vigor are the most important characteristics of seed quality for propagation of soybean. Higher physiological seed quality (seed viability & vigor) better ensures healthy seedlings establishment under wider range of environmental conditions (Copeland & McDonald, 2001).

Soya protein contains essential amino acids and its oil is also a rich source of plant fatty acids. These constituents are attractive to a wide range of fungal pathogens to survive and multiply themselves (Welbaum, 2006). Grain infections by fungi may cause chemical breakdown of protein, oil and fatty acids. Oil contains lower amount of saturated fat (palmitic & stearic acid) and higher amount of unsaturated fatty acids (oleic, linolenic & linoleic acid) which is highly desirable in human diets (Hagan & Higgins, 2006). Accordingly, fatty acid composition is an important determinant of oil quality. Free fatty acid (FFA) as oleic acid concentration in the oils or lipids of a seed lot can be used as a sensitive indicator of seed deterioration due to fungal infection (Dhingra *et al*., 1998). Severe seed infection by *C. truncatum* may be able to inflict considerable damage to seeds after harvest, consequently posing a serious problem to the economy in the world trade. Thus, the aim of the study was to evaluate the pathogenicity of *C. truncatum* on soybean and its effect on physiological and biochemical properties that determine seeds quality.

MATERIALS AND METHODS

The experiment was conducted at Plant Pathology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia.

Source of seed sample. Soybean seeds usually do not have

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unifungal infection and associated with a complex of seed-borne fungi (Kunwar *et al*., 1985; Sharma, 1992). Thus, healthy soybean seed lot of Palmetto variety was used in this study by artificial inoculation of *C. truncatum*. Seeds were collected from the department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, which were harvested in 2004 and stored for 1 year at 10ºC with 9.5% seed moisture content. Seed health status based on percentage germination was evaluated following the methods of International Seed Testing Association (ISTA, 2001) and Association of Official Seed Analysts (AOSA, 2002). The seed lot used had germination rate of 96.0% and free from pathogenic infection as tested using blotter and agar plate methods.

Fungal isolation and inoculation of seeds. Pathogenic *C. truncatum* was isolated from stored soybean seeds by agar plate method. *C. truncatum* was cultured on potato dextrose agar (PDA) for 20 days at room temperature to get a large amount of spores. The spores were washed off with sterile distilled water containing 1.5% sodium alginate solution. The spore suspension obtained was adjusted to a concentration of 1×10^7 spores mL^{-1}. Soybean seeds were surface sterilized with 10% Clorox® for 3 min and rinsed thrice with sterilized distilled water and dried for 1 h under laminar flow. Seeds were soaked in spore suspension (1:2 w/v) of *C. truncatum* and was shake by hand about 10 min and kept for 1 h. Afterwards, seeds were collected and surface re-dried about 3 h under laminar flow. The number of conidia for each seed were counted by a haemocytometer and approximated at 5×10^4 conidia $seed^{-1}$. Un-inoculated seeds were soaked in 1.5% sodium alginate solution for 1 h and served as control. Inoculated and un-inoculated seeds used for subsequent studies.

Infection level of *C. truncatum* on seed components. Seed components from the inoculated seeds namely seed coat, cotyledon and embryonic axes were separated and dissected carefully using sterilized sharp scalpel and plated separately on PDA. The plates were incubated for 6 days at room temperature (25±1ºC). The experiment was laid out in a completely randomized design with four replications and fifty seed components (seed coat, cotyledon & embryonic axes) were arranged for each replicate. Data on disease incidence were recorded every day after plating for each component and expressed as a percentage.

Physiological changes. Based on the above study, maximum infection level was recorded on PDA after 4 days of inoculation in all seed components. Thus seed lot incubated for 4 days after artificially inoculated by *C. truncatum* following previous procedure and considered as infected seeds for this study. Un-inoculated seeds were soaked in 1.5% sodium alginate solution for 1 h and incubated for 4 days served as control.

Seed germination. Inoculated and un-inoculated seed samples were used to determine the seed germination based on four replications with 100 seeds per replication. Both seed samples were taken randomly and placed separately on moist blotter paper and covered by a thin layer of moist sterilized sand on aluminum trays. Trays were incubated at room temperature (25±1ºC) with cool-white fluorescent light (~40 $\mu molm^{-2}s^{-1}$ & 400-700 nm) for 12 h during the day and with total darkness during the night. The seed germination was evaluated according to the rules of ISTA (2001) and AOSA (2002) and expressed as percentage.

Seed viability. Tetrazolium test (TZ) was used to assess the seed viability based on quantitative data in the laboratory. The test indicates the amount of viable seeds in a sample that are capable of producing normal plants under suitable germination conditions. Viable seeds were considered whereby seeds were completely stained, but not overly intense or minor unstained areas on cotyledons or extreme tip of the radicle unstained or stained but was darker than the cotyledons and tissue was firm. Non-viable seeds included those with one-third or more extremely dark or completely unstained of the embryonic axes and cotyledon with flaccid tissue (AOSA, 2000a). The test was replicated four times with each replicate consisting of 50 soybean seeds. Seeds were soaked in distilled water at room temperature for 12 h and dissected on moist filter paper longitudinally with a sharp sterile scalpel to expose the embryonic axes. Only half of the cotyledon of each seed attached with the embryonic axes was used in this test and immediately soaked in 1% solution of tetrazolium salt (2, 3, 5-triphenyl tetrazolium chloride) and kept in the dark at room temperature for 5 h. After 5 h soaking, seeds were rinsed with distilled water. The embryonic axes and cotyledon were examined using a magnifying lens and the viability of seeds was counted according to the staining pattern as described by AOSA (2000a).

Seed vigor. Seed vigor of inoculated and un-inoculated seed samples was measured by electrical conductivity (EC) test based on six replicates of 50 seeds per replicate (AOSA, 2000b). Seed samples were rinsed once through de-ionized water and surface-dried under laminar flow. Then samples were weighed (three decimal places) and placed in a vial containing 75 mL de-ionized water, and immediately kept at 25ºC for 24 h in an incubator. Seed leachate was transferred to a new vial for each seed sample and the conductivity of the solution was measured immediately by electrical conductivity meter (Sension 5™, Hach company, USA) as $\mu S\ g^{-1}\ cm^{-1}$ (Vieira & Krzyzanowski, 1999).

Pathogenicity testing. Inoculated and un-inoculated seed samples were sown to a depth of 2 cm in plastic tray (39 cm ×28 cm × 11 cm) containing sterilized soil mixture (top soil: peat soil: sand = 3: 2: 1 v/v/v). The experiment was repeated four times using 25 seeds at each repetition. Trays were arranged in a completely randomized design in the glasshouse with each tray considered as a repetition. After 14 days, data of seed germination, pre- and post-emergence damping off and survival of seedlings were recorded.

Bio-chemical changes. Inoculated and un-inoculated seed samples were milled to fine flour and 5 replicates of each sample were used for determination of protein, oil and fatty

acid composition. Reagents of recognized analytical grade were used. The nitrogen content of inoculated and un-inoculated seed samples was determined by the Kjeldahl method and it was converted to protein by multiplying with 6.25 as the protein conversion factor (AOAC, 1995). Oil content was determined according to Maestri *et al.* (1998). A weighed quantity (15 g) of milled seeds from each sample was extracted separately with 100 mL n -hexane in a soxhlet apparatus for 12 h at 60ºC. After drying the solution with anhydrous sodium sulphate, solvent was removed by rotary evaporator at 40ºC. The amount of oil was expressed as a percentage (g 100 g^{-1} dry weight).

Fatty Acid Analysis

Extraction of concentrated lipid. The concentrated lipid was extracted according to the method of Kinsella *et al*. (1997). The extraction was carried out at room temperature to eliminate the deteriorating effect of heat on the unsaturated fatty acids. Fifteen gram of milled seeds from each sample was extracted with each 100 mL of methanol and chloroform in Buchner funnel under vacuum section. Extracted lipids in chloroform were collected using separatory funnel. One mL of 1% butylated hydroxy toluene (BHT) solution (in chloroform 1:100 w/v) was added to prevent oxidation of fatty acids. The solution was filtered with anhydrous $NaSO_4$ and solvent was removed by rotary evaporator at 40ºC and transferred to a glass vial with a screw cap. The concentrated lipids were obtained stored at 0ºC until further use.

Preparation of fatty acid methyl ester (FAME) from concentrated lipids. The concentrated lipids obtained were converted to their constituent FAME. For each sample 0.5 g was converted to FAME by refluxing in 5 mL of reagent for 1 h at 60ºC. The reagent was prepared by mixing 1 mL of concentrated sulfuric acid, 10 mL of toluene and 20 mL of methanol (1: 10: 20 v/v/v). The solution was cooled for 5 min and then 3 mL of water and hexane was added separately in the solution. The solution was then transferred to a separatory funnel and shaken for 1 min and then left for 5 min to allow formation of two phases. The lower layer containing water soluble compound was discarded and the upper layer containing fats in hexane was taken and decanted through 2 g of Na_2SO_4 anhydrous to remove the water. The FAME was subsequently transferred into a glass vial with a screw cap and used for gas chromatographic analysis.

Analysis of fame by gas chromatography. Analysis of FAME was performed by gas chromatography, Model–GC-14B, Shimadzu, Japan. The gas chromatography was equipped with an oven, injector, flame ionization detector (FID), integrator and a fused silica capillary column (30 m × 0.32 mm id, 0.25 mm thickness), model Stabilwax, Supelco, USA. The analysis of FAME by gas chromatography was run thermodynamically with two different temperatures (100ºC & 240ºC). The program of oven was set to heat the capillary column to 100ºC for 2 min at the initial stage and increased to 240ºC at the rate of 6ºC per min and remained constant until the final stage. The injector and detector temperatures were 250ºC and 280ºC, respectively. When the oven temperature was increased to 100ºC and the status of gas chromatography was ready, 1 µL sample of FAME was injected. The peaks appeared on the chromatogram with carbon chain length of fatty acids increasing with retention time. Peaks according to ascending retention time were as follows: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3). The individual FAME was identified by comparison with a standard (Sigma®-Aldrich, Inc., USA). The total amount of fatty acids was calculated by the area of an individual peak calculated as a percentage of the total area recorded for all peaks in the chromatogram (Brodnjak-Voncina *et al*., 2005).

Statistical analysis. Data were analyzed by using statistical software SAS (SAS, 1999). Mean separation was carried out using Tukey's Studentized Range (HSD) of arcsine transformed values at the 5% level ($P \leq 0.05$) of probability.

RESULTS

Infection frequency of *C. truncatum* on seed components. Potential infections occurred in all seed components by C. *truncatum* (Fig. 1). The maximum infection frequency of *C. truncatum* was recorded on the seed coat (100%) that started just after the incubation and remained the same through the whole incubation period. The infection of *C. truncatum* on cotyledon and embryonic axes increased gradually with increasing incubation time. The highest level of infection on cotyledon and embryonic axes recorded was 43.0% and 30.0%, respectively 4 days after incubation and remained the same till the end of the incubation period (Fig. 1).

Seed quality. *C. truncatum* significantly reduced seed germination and viability by 29.2% and 26.8%, respectively (Table I). Significantly higher electrolyte leakage was found in inoculated seeds than those of un-inoculated seeds, which indicated the low seed vigor of soybean.

Pathogenicity testing. Under the glass house conditions, *C. truncatum* showed highly pathogenicity at seeds and seedling stages. The fungi reduced seed germination significantly (46.4%) as compared to the control (Table II). The highest frequency of pre-emergence damping-off (48.0%) was observed in *C. truncatum* inoculated seeds compared to 3.0% in control seeds. Where post-emergence damping–off occurred, 28.5% of seedling death within 14 days after sowing was recorded. Infected seeds and seedlings gave positive re-isolations of *C. truncatum* on PDA plates thus confirming its pathogenicity. Seedling survivability was also reduced by 75.8% in inoculated seeds than the control.

Bio-chemical changes. Effect of *C. truncatum* on biochemical changes of soybean seeds is summarized in Table III. Protein content of *C. truncatum* inoculated seeds was found to be significantly higher (40.6%) compared to un-inoculated seeds (38.2%). Similar level of the percentages of oil, palmitic acid, stearic acid and linolenic

Table I. Effect of *Colletotrichum truncatum* on soybean seed quality under the laboratory conditions

Treatment	Germination (%)	Viability(%)	Electrical Conductivity (EC) (μS cm^{-1} g^{-1})
Inoculated seeds	68.0 b (29.2)	69.5 b (26.8)	79.2 a
Un-inoculated seeds	96.0 a (0.0)	95.0 a (0.0)	58.6 b

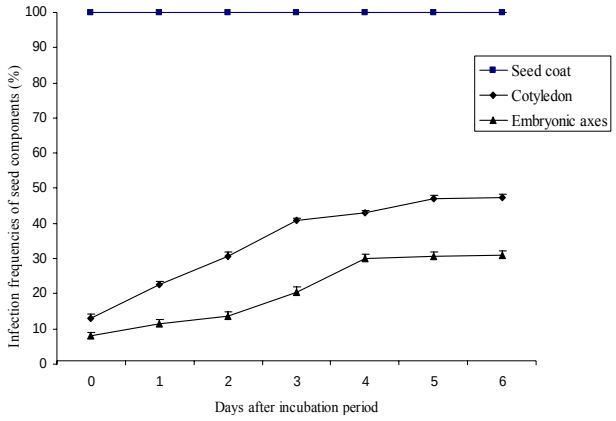
Data in parenthesis indicate the percent reduction over control
Means in the same column with different letters are significantly different at $P \leq 0.05$ according to Tukey's Studentized Range (HSD) test. The percentage data subjected to analyze using arcsine transformed values

Table II. Effect of *Colletotrichum truncatum* on the pathogenicity of soybean seed under the glass house conditions

Treatment	Germination (%)	Survival of seedlings (%)	Pre-emergence damping off (%)	Post-emergence damping off (%)
Inoculated seeds	52.0 b (46.4)	23.5 b (75.8)	48.0 a	28.5 a
Un-inoculated seeds	97.0 a (0.0)	97.0 a (0.0)	3.0 b	0.0 b

Data in parenthesis indicate the percent reduction over control
Means in same column with different letters are significantly different at $P \leq 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine transformed values

Fig. 1. Infection frequencies of *Colletotrichum truncatum* on seed components of inoculated seeds within 6 days of incubation period. Vertical bars represent the standard error of means



acid contents was found between inoculated and un-inoculated seeds. However, oleic acid was higher (26.3%) in inoculated seeds than un-inoculated seeds (24.7%). In contrast, linoleic acid (49.3%) was found to be lower in inoculated seeds compared to un-inoculated seeds (50.7%).

DISCUSSION

The present study confirmed that *C. truncatum* ascertained with seed tissues of soybean as latent infection resulting in the loss of physiological quality as germinability, viability and vigor (Table I & II). Moreover, this fungi deteriorated bio-chemical qualities of seeds due to change of nutritional profiles such as protein content and fatty acid compositions and decreased their value for sowing, as food or feed. The fungi have strong ability to infect all components of the seed and therefore, as reflected by the reduction of seed quality parameters. The incidence of *C. truncatum* infection was much more prevalent in seed coat followed by cotyledon and embryonic axes without any external symptoms during incubation period. This could be attributed to the latent infection of *C. trunvatum* into soybean seed coats (Sinclair, 1991). Germination was reduced (29.2%) by *C. truncatum* in soybean seeds compared to un-inoculated seeds (control). Additionally, TZ tests further confirmed the reduction of seed viability by 26.9% in inoculated seeds. Low viability refers to low germination which is a well known indicator of seed deterioration (Elias, 2006). *C. truncatum* decreased the seed viability and consequently reduced germination because of its infection in seed tissues and thus damaging the seed coat, cotyledon and embryonic axes (Hopperley *et al*., 1983; Srichuwong, 1992).

Vigor of *C. truncatum* inoculated seeds was determined by EC test. The EC value was found consistently higher in *C. truncatum* inoculated seeds compared to un-inoculated. When seeds were soaked in water, a greater electrolytes leakage (amino acids & organic acids) occurred during imbibitions and conductivity was increased in the water. The higher amount of conductivity in the water indicated the lower vigor, which was directly related to the poor integrity of cellular membranes (Bewley & Black, 1994). Diseased seeds having moderate to severe fissurse in the seed coat showed excessive electrolytes leakage and high conductivity levels (Loeffler *et al*., 1988). In this study, there was 100% seed coat infection by *C. truncatum* during incubation period, possibly related to loss of membrane integrity of the seed coat by fracturing tissues and increase electrolytes leakage of the seed coat. This increased leakage was contributed to seed deterioration by losing the seed viability and vigor (Harmon & Stasz, 1986; Khor, 2002). Results from glass house study demonstrated that *C. truncatum* exposed pathogenic to both seeds and seedlings stages of soybean. The seed germination and seedling survivability were found lower than that of un-inoculated seeds. Moreover, C. *truncatum* caused pre- and post-emergence damping off of seedlings. Similar observations were reported and confirmed by several researchers (Srichuwong, 1992; Mayonjo & Kapooria, 2003). The reduction of seed germination by *C. truncatum* was found higher in the glass house than in the laboratory conditions. This might be due to the crop growing conditions, soil type and moisture (Rush *et al*., 1992). Infected seeds of soybean are the primary source of inoculum and plants are susceptible to *C. truncatum* at all stages of development started from early seed and seedling stages (Sinclair, 1991). It was previously confirmed that *C. truncatum* was associated with tissues of seed coat, cotyledon and embryonic axes leading to local and systemic transmission during germination. Thus, infected seeds caused pre- and post-emergence damping off by progressive

Table III. Effect of *Colletotrichum truncatum* on biochemical change in soybean seeds under the laboratory conditions

Treatment	Protein[a] (%)	Oil[a] (%)	Fatty acid composition (% of total fatty acids)				
			Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Inoculated seeds	40.6 ± 0.28 a	19.3 ± 1.47 a	15.9 ± 0.53 a	3.0 ± 0.05 a	26.3 ± 0.71 a	49.3 ± 0.45b	5.5 ± 0.27 a
Un-inoculated seeds	38.2 ± 0.34 b	19.0 ±1.63 a	16.1 ± 0.70a	3.0 ± 0.12 a	24.7 ± 0.53 b	50.7 ± 0.26 a	5.4 ± 0.28 a

[a] Values are shown as percentage (g 100 g^{-1} dry weight)

Mean values with ± indicate the standard deviation

Means in the same column with different letters are significantly different at $P \leq 0.05$ according to Tukey's Studentized Range (HSD) test of arcsine

rotting of the hypocotyle-radicle axis (Siddique *et al*., 1983).

Soluble protein concentration of *C. truncatum* inoculated seeds was significantly higher than those in the un-inoculated seeds of soybean. Similar results were reported in *C. truncatum* infected soybean seeds (Srichuwong, 1992). A positive correlation was also found between protein concentration and fungal damage by *Fusarium* spp., *Cercospora* spp. and *Phomopsis* spp. in soybean seeds (Wilson *et al*., 1995). Protein serves as a primary source of carbon and nitrogen for growth and metabolism of the invading fungi in seeds (Robinson *et al*., 1974). The increase in enzymatic activity following fungal invasion in seeds may result in higher levels of total proteins (Farag *et al*., 1985; Hasan, 2000). Proteins were hydrolyzed through polypeptides to small peptides and further broken down to amino acids by increasing activity of protease enzymes, which raise the soluble nitrogen content in the fungal infected seeds. Consequently, the protein degradation was accelerated by hydrolysis to meet the requirements of pathogen development (Narayanasamy, 2006). Meriles *et al*. (2004) also detected a selective degradation of soluble proteins through hydrolysis in *Fusarium* spp. and *Diaporthe*/*Phomopsis* complex infected soybean seeds. Bhattacharya and Raha (2002) also found increase in protein content of maize, groundnut and soybean seeds by fungi during storage. They reported that increased protein content might represent the sum of seed protein plus the fungal protein, which could not be separated from seeds.

C. truncatum infection did not change the amount of extracted oil in inoculated seeds compared to un-inoculated seeds. However, *C. truncatum* influenced the fatty acid profiles of concentrated lipids. The content of oleic acid increased in *C. truncatum* inoculated seeds, whereas the linoleic acid content was decreased compared to un-inoculated seeds. There has not been previous information regarding the effect of *C. truncatum* on oil and fatty acid compositions. Bhattacharya and Raha (2002) found the increase of protein and FFA (oleic acid) content of maize, groundnut and soybean seeds infected by different fungi species including *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia*, *Alternaria* and *Rhizopus*. Meriles *et al*. (2002) also detected a high level of oleic acid and lower levels of linoleic and linolenic acids in *Fusarium* spp. and *Diaporthe*/*Phomopsis* complex infected soybean seeds. After processing, oxidation is the main problem to affect oil and lipid, leading to aldehyde production, which imparts strong disagreeable flavor and odor, referred to as rancidity (McKevith, 2005). Thus lipid oxidation may result in higher oleic acid and lower linoleic and linolenic acids concentrations in fungal damaged soybean seeds (Wilson *et al*., 1995). This lipid oxidation is responsible for rancidity of oil and consequently deteriorates the oil quality due to *C. truncatum* infection in seeds. The structural damage caused by fungal growth in the seed coat and the internal tissues of cotyledon will expose the lipid to oxidation, which is responsible for deteriorating oil quality (Dhingra *et al*., 1998). Protein and oil are stored as nutritional reserves mainly in the endosperm or cotyledon (Narayanasamy, 2006). The changes in oil, protein and fatty acid compositions may be attributed to infection of soybean seed tissues caused by *C. truncatum*.

C. truncatum remained with seed as latent infection without showing any visible symptoms and spoiled soybean seeds as planting materials by reducing germination and vigor and caused pre- and post emergence damping-off of seedlings. The fungus alleviated deleterious impact to raise protein and oleic acid and reduce linoleic acid, which may associate to reduce seed quality as food and feed.

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