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Phytotoxic Effects of Fumonisin B_1 on Cowpea Seed

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The cultivation of cowpea (*Vigna unguiculata*) plays a vital role in the livelihood of many subsistence farmers and rural communities in tropical and subtropical countries. The seeds are prone to fungal infestation and mycotoxin contamination during sub-optimal storage conditions. Fumonisin B_1 (FB_1), produced by *Fusarium proliferatum*, has been detected in cowpea seeds. Surface-disinfected seeds were imbibed in sterile distilled water amended with FB_1 at various concentrations. Percentage germination was determined according to the International Seed Testing Association rules. All the toxin concentrations significantly decreased seed germination and the two highest concentrations – 50 and 100 $\mu g\ ml^{-1}$ FB_1 – inhibited root and shoot elongation. FB_1-treated embryonic tissues evinced compaction of the protoplasm and separation of the plasmalemma from the cell wall. Lipid bodies accumulated, which seemed to be lining the cell wall. This is the first study to demonstrate the phytotoxic effects of FB_1 on cowpea seeds.

KEY WORDS: Cowpea; *Vigna unguiculata;* fumonisin B_1; *Fusarium* spp.; germination; ultrastructure.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.; also known as blackeyed pea) is a widely cultivated indigenous African legume crop that is of great importance in tropical and subtropical countries of Asia, Africa, Oceania, the Middle East, southern Europe, southern USA, and Central and South America (8). This crop has a variety of uses, which include providing excellent ground cover to prevent soil erosion, suppressing weed growth, improving soil nitrogen levels, and serving as a source of cash for rural communities through trade of the seed (22). Furthermore, many subsistence farmers and rural communities residing in less developed countries rely on the crop as a nutritious food, being a good source of dietary protein (22).

When the seeds are stored at high relative humidities and high ambient temperatures, fungal infestation may occur. Under these poor storage conditions some of the fungi may produce secondary toxic metabolites, namely, mycotoxins (21). Mycotoxins are well known to have a negative impact on the health of animals and humans (7), but some are also known to have toxic effects on plants (11,16). Previous studies have shown that aflatoxin B_1 and crude aflatoxins inhibited chlorophyll formation and seed germination in cowpea (5).

The fumonisins, the most recently characterized mycotoxins, are produced by certain *Fusarium* spp. including *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima)

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Nirenberg and *F. nygamai* Burgess and Trimboli (19,24). Some toxicological problems in animals, including leukoencephalomacia (LEM), a fatal brain disease in horses, and pulmonary edema syndrome (PES) in pigs (15,18), are known to be caused by fumonisin B_1 (FB_1). This toxin is linked statistically to the incidence of esophageal cancer in humans in Transkei, South Africa, and in China (18). Fumonisin B_1 is known to exhibit phytotoxic effects towards different plants, including economically important crops (1-4,9,14,16,26). Previous studies on other legume crops showed that soybeans (*Glycine max* L.) were severely damaged (necrosis and wilting) when sprayed with a 1000 μg ml^{-1} concentration of FB_1 (1).

Fumonisin B_1 has been found to be associated with cowpea seed from South Africa and Benin, West Africa (13). During that investigation, *F. proliferatum* was determined to be responsible for the production of the toxin on cowpea seed (13).

This paper reports the effect of the FB_1 toxin on (i) cowpea seed germination; (ii) root and shoot elongation; and (iii) the ultrastructure of the cotyledon and embryonic tissue of the seed.

MATERIALS AND METHODS

Seed material Cowpea seeds (cv. IT 85F-867-5) were obtained from Ecolink, Nelspruit, South Africa. Three replicates of 100 seeds were used for each treatment. Prior to the treatments, the seeds were surface disinfected with 1% sodium hypochlorite for 1 min and thereafter rinsed three times with sterile distilled water.

Toxin Dried FB_1 (batch A/01, 11.3 mg) was supplied by the PROMEC Unit, Medical Research Council (MRC), Tygerberg, South Africa. Methanol (20 ml) was added to FB_1 and 1-ml aliquots were placed in 20 vials. The aliquots were dried down under nitrogen gas and stored at $\pm$ 4°C until used.

Seed treatments For the toxin treatments, the required volume of fumonisin was added to 50 ml sterile distilled water to yield final concentrations of 10, 25, 50 and 100 μg ml^{-1}. The seeds were left to imbibe in the various solutions for 10 h. For the fungal inoculated treatments, sterile distilled water (50 ml) was added to 7-day-old cultures of fumonisin-producing strains of *F. verticillioides* (MRC 4315), *F. nygamai* (MRC 3997) and *F. proliferatum* (MRC 8278). The surface of each culture was scraped to free the conidia and the conidium suspensions were poured through muslin cloth into flasks and adjusted to a concentration of 1×10^6 ml^{-1} using a hemocytometer. The seeds were added to the flasks, mixed thoroughly and then left to dry for ~5 min. Slow-imbibed seeds (seeds placed in moist paper towels) were incubated at 25°C for 10 h (positive control). Seeds placed in sterile distilled water for the same period of time served as the negative control.

Seed germination Percentage germination was determined by placing the seeds between moist paper towels which were rolled up and placed individually in polythene bags, held upright in plastic buckets, and maintained at ~25°C in an incubator. Percent germination was determined after 5 and 9 days according to the International Seed Testing Association rules (12). Root and shoot elongation was determined after 9 days of growth.

Transmission electron microscopy Representative seeds from each treatment (as described above) were removed after the 10-h period of imbibition. The seeds were dissected and the embryonic axes and cotyledon tissue were removed. The tissues were fixed overnight in 2.5% glutaraldehyde in 0.075M phosphate buffer (pH 7.4). The samples

were rinsed three times in 0.075M phosphate buffer and post-fixed in 1% aqueous osmium tetroxide. Thereafter, the samples were rinsed and dehydrated in an ethanol series and embedded in Quetol 651 resin at 60°C for 48 h. Ultra-thin sections were prepared using a Reichert Ultracut E ultramicrotome (Vienna, Austria) and stained for viewing with a Philips EM301 transmission electron microscope (Eindhoven, the Netherlands). Sections were also stained for viewing with a Nikon Optiphot light microscope (Tokyo, Japan).

Statistical analysis Two-way analysis of variance (ANOVA) was performed on all the data and least significant differences (P=0.05) were determined according to Student's t-test.

RESULTS AND DISCUSSION

Effect on seed germination and root and shoot elongation All four FB_1 concentrations significantly decreased seed germination when compared with both the positive and negative controls (Fig. 1). The lowest percentage (6.67%) of seed germination was at the 100 μg ml^{-1} concentration. It is apparent from these results that the toxin may block various biochemical reactions that are necessary for normal germination to take place. Danielsen and Jensen (9) found a significant negative correlation between fumonisin content and corn (*Zea mays* L.) seed germination. However, it was not established whether the fumonisins had a direct effect on germination or not. On the other hand, Doehlert *et al.* (10) reported that FB_1 (100 μg ml^{-1}) had no effect on corn seed germination but the toxin did, however, inhibit radicle elongation in the seeds by up to 75% after 48 h of imbibition. Those authors found that amylase production in the endosperm was also inhibited, which could suggest that FB_1 interfered metabolically with germination (10).

TABLE 1. Effect of *Fusarium* spp. on cowpea seed germination, seedlings, and root and shoot elongation

Parameter	Positive control	Negative control	*F. verticillioides*	*F. nygamai*	*F. proliferatum*
Seed germination (%)	85.7*c	73.0b	61.7a	84.3c	64.3ab
Normal seedlings (%)	52.3b	45.3b	12.0a	50.3b	20.7a
Abnormal seedlings (%)	29.0b	32.0b	14.3a	33.0b	13.7a
Non-germinated seeds (%)	7.0b	16.3a	1.0a	0.7a	1.3a
Diseased seeds and seedlings (%)	9.3a	5.0a	75.0b	17.3a	64.3b
Root elongation (mm)	55.2ab	54.8ab	41.4a	68.6b	49.7a
Shoot elongation (mm)	115.5ab	99.9a	105.7a	133.7b	103.9a

*Each value is a mean of three replicates. Within rows, values followed by a common letter do not differ significantly (P=0.05) according to Student's t-test.

In this study, *F. verticillioides*- and *F. proliferatum*-inoculated seeds also showed significant reduction in germination (Table 1). It was not established whether the fungus alone affected germination or whether it was a combination between the production of a toxin and fungal infestation. Danielsen and Jensen (9) found no significant correlation between *F. verticillioides* infection and seed germination in corn. At 9 days, a significant increase in non-germinated seeds was noted in the toxin-treated seeds (Fig. 2). Correspondingly,

these treatments had the lowest number of normal seeds. With the exception of *F. nygamai*, the *Fusarium*-inoculated seeds and the 50 μg ml^{-1} and 100 μg ml^{-1} toxin-treated seeds revealed the highest amounts of diseased (fungi and bacteria infested) seeds (Table 1).

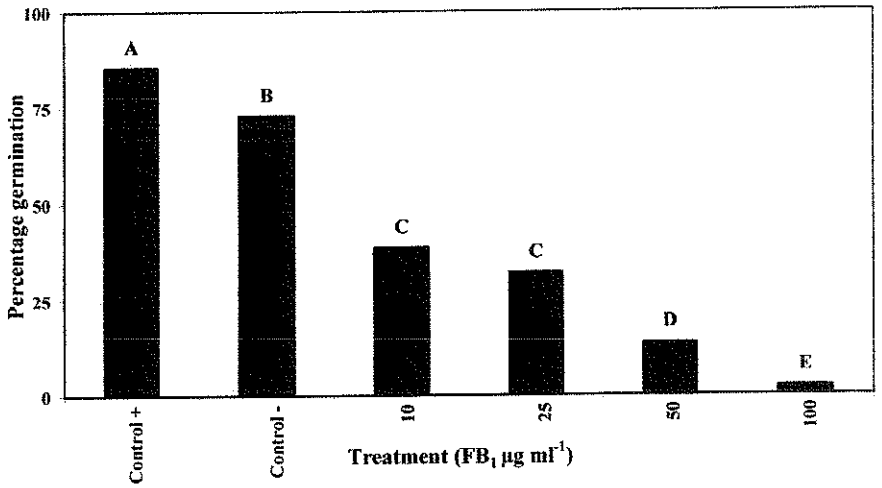

Fig. 1. Effect of FB_1 on cowpea seed germination. Each bar is a mean of three replicates. Values of the columns with a common letter do not differ significantly (P=0.05) according to Student's t- test.

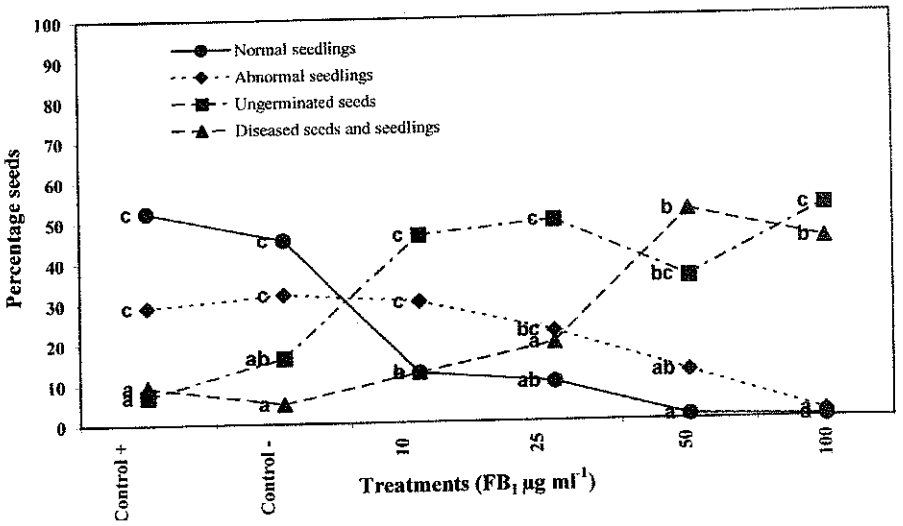

Fig. 2. Effect of FB_1 on cowpea seeds and seedlings. Values of the lines with the same symbol and marked with a common letter do not differ significantly (P=0.05) according to Student's t- test.

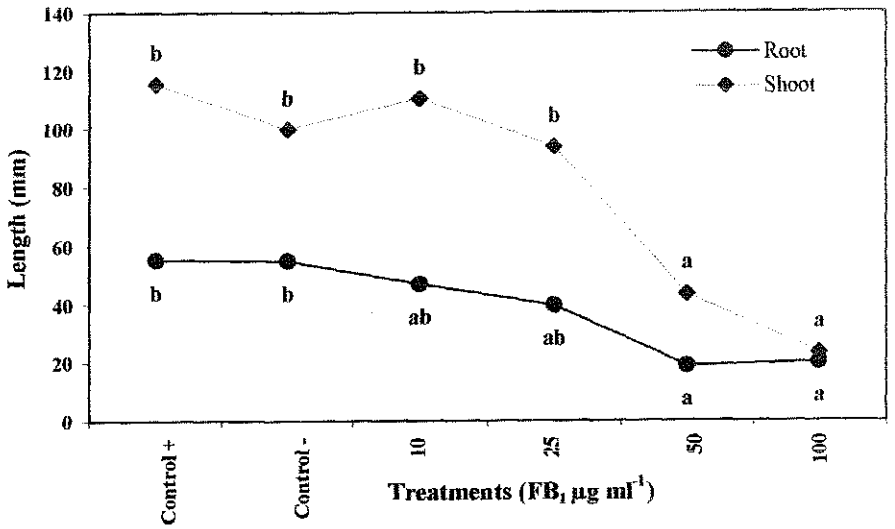

Fig. 3. Effect of FB_1 on root and shoot elongation. Values of the lines with the same symbol and marked with a common letter do not differ significantly (P=0.05) according to Student's t- test.

Only the 50 and 100 μg ml^{-1} toxin concentrations inhibited root and shoot elongation significantly (Fig. 3). In several cases, the toxin caused severe stunting of the roots. Lamprecht *et al.* (14) found that FB_1 and the FB_2 and FB_3 analogs caused dose-dependent reductions in root and shoot length and dry mass in corn seedlings. The three *Fusarium* spp. artificially inoculated onto the cowpea seeds showed no inhibitory effect on the growth of the roots and shoots of the seedlings (Table 1).

Effect on ultrastructure The ultrastructure of both controls of the untreated embryonic axes (Fig. 4a, b) and cotyledon tissues (Fig. 5a, b) revealed neat, intact cells with clearly defined nuclei and other organelles. Numerous lipid bodies, ribosomes and vacuoles can also be seen. When looking at the micrographs of the embryonic axis tissues, there seemed to be no noteworthy differences in the ultrastructure of the lower toxin-treated tissues (Fig. 4c, d) when compared with the control. The 25 μg ml^{-1}-treated tissue did, however, show an abundance of vacuoles containing protein bodies and lipid bodies throughout the protoplasm (Fig. 4d). The only distinctive destructive effects caused by the toxin are seen in the 100 μg ml^{-1}-treated embryonic tissues (Fig. 4f, g, h). The plasma membrane has separated from the cell wall and irregular sized vacuoles (Fig. 4f, g, h) have formed due to the contraction of the protoplasm. Some of the contents of the cytoplasm have passed through the plasma membrane as it separated away from the cell wall (Fig. 4f). The compacted protoplasm appears very dense and darker in color when compared with the control micrographs. An abundance of lipid bodies was noted next to the cell wall in the 50 and 100 μg ml^{-1}-treated seed tissues (Fig. 4e, h). The treated cotyledon tissue (25, 50 and 100 μg ml^{-1}) showed similar patterns with regard to the accumulation of lipid bodies (Fig. 5d, f). Baird *et al.* (6) found lipid bodies to be conspicuous at the margins of the cytoplasm, outlining the cell walls in dry radicle cells of soybean. Similarly, lipid

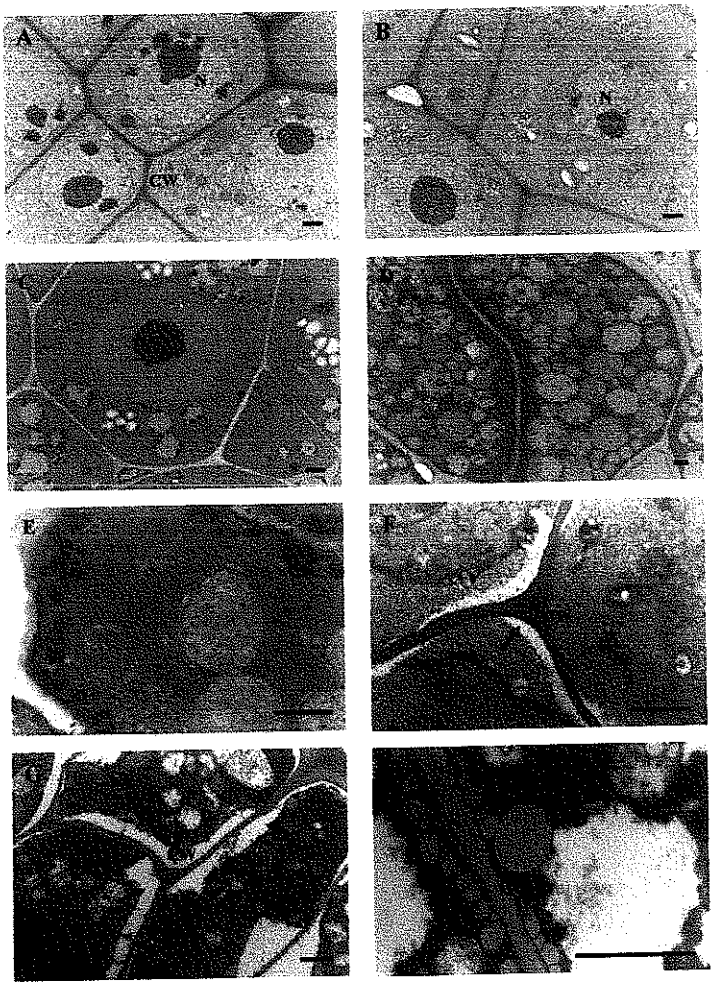

Fig. 4. Ultrastructural effects of FB_1 on cowpea seed tissue. TEM micrographs of the embryonic axes of cowpea seed (a) imbibed for 10 h in sterile distilled water; (b) imbibed for 10 h in moist paper towels; or imbibed for 10 h in sterile distilled water with the addition of FB_1 at (c) 10 μg ml^{-1}, (d) 25 μg ml^{-1}, (e) 50 μg ml^{-1}, (f, g and h) 100 μg ml^{-1}. CW = cell wall, L = lipid, N = nucleus, PM = plasma membrane, arrows – plasma membrane separated from cell wall. Bar = 1 μm.

droplets were closely appressed to the plasma membrane in dry cells of cowpea embryo tissue (25). During imbibition, lipid droplets decrease as they dissolve to become part of the membranous system of the cell. It is possible that FB_1 could prevent or reduce the normal metabolism of the cell so that the cell does not take up the lipids. The 100 μg ml^{-1}-treated cotyledon tissue did not reveal any noticeable effects caused by the toxin (Fig. 5f) as noted in the 100 μg ml^{-1}-treated embryonic axis tissue (Fig. 4f, g, h).

These destructive effects seen in the ultrastructure of the 100 μg ml^{-1}-treated embryonic axis tissue might play a role in the significant reduction in germination and

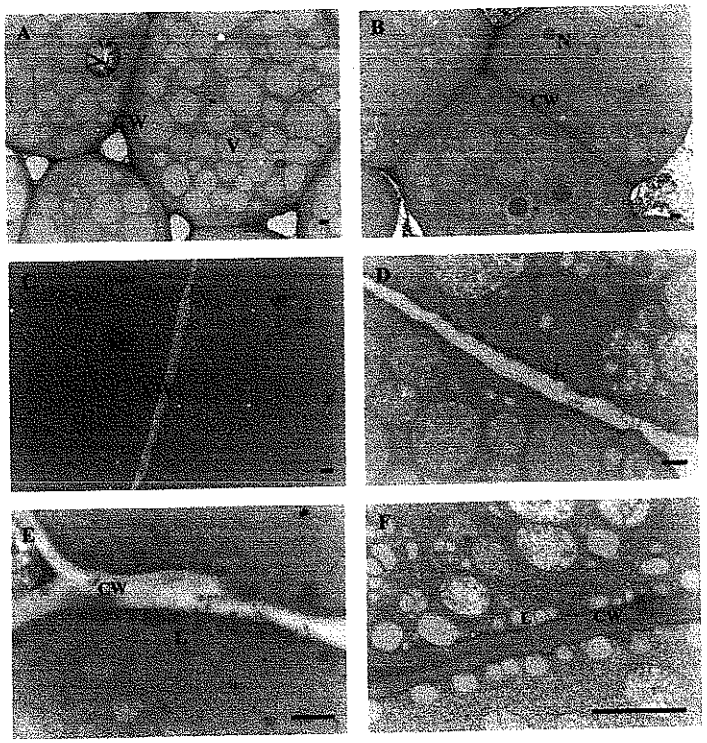

Fig. 5. Ultrastructural effects of FB_1 on cowpea seed tissue. TEM micrographs of cotyledon tissue of cowpea seed (a) slow imbibed for 10 h in moist paper towels; (b) imbibed for 10 h in sterile distilled water; or imbibed for 10 h in sterile distilled water with the addition of FB_1 at (c) 10 μg ml^{-1}, (d) 25 μg ml^{-1}, (e) 50 μg ml^{-1}, and (f) 100 μg ml^{-1}. CW = cell wall, L = lipid, N = nucleus, V = vacuole. Bar = 1 μm.

root and shoot length at the same concentration. M.A.J. Van Asch (Ph.D thesis, 1990, Univ. of Natal, Pietermaritzburg, South Africa) treated corn callus with different doses of FB_1, which produced deteriorative alterations in the cell ultrastructure, including cell wall thickening and the accumulation of large starch grains within a swollen plastid.

Fumonisin B_1 inhibits the enzyme ceramide synthase in plants, which leads to the reduced formation of sphingolipids and the accumulation of free sphingoid bases (3). Sphingolipids are highly bioactive compounds of cellular membranes that have profound effects on cell regulation (27). In plants, sphingolipids play a role in cell signaling, membrane stability, stress response, pathogenesis and apoptosis, but little is known about their precise functions (17,23). Studies of animal cells have also shown that fumonisins interfere with the synthesis of sphingolipids, which results in disturbances in cell growth, differentiation and morphology (20).

Although the mode of action of FB_1 in plant cells is uncertain, the interference of the metabolism of the sphingolipids by FB_1 could play a role in the alterations noted in the ultrastructure of the toxin-treated seeds. It is evident from this study that the toxin has interfered with the cell morphology in some of the treated tissues and that this interference

has had a negative impact on the germination of the seeds as well as on the growth of the seedlings. However, further research is necessary to determine the precise toxic effects of the toxin on the seed tissue.

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