CHAPTER 5

Phytotoxic Effects of Fumonisin B_1 on Cowpea Seed

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ABSTRACT

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The cultivation of cowpea plays a vital role in the livelihood of many subsistence farmers and rural communities in tropical and sub-tropical 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 for 10 h in 50 ml sterile distilled water amended with FB_1 to yield final concentrations of 10, 25, 50, 100 ppm (concentrations based on previous studies on maize). Slow imbibed seeds (placed in moist paper towels) incubated at 25˚C for 10 h and seeds placed in sterile distilled water for the same period of time served as the positive and negative control, respectively. Percentage germination was determined according to the International Seed Testing Association (ISTA) rules. Root and shoot length was measured after 9 days. Parts of the embryonic axes and cotyledon tissues were removed and prepared for transmission electron microscopy. All the toxin concentrations significantly decreased seed germination. The 50 and 100 ppm FB_1 concentrations inhibited root and shoot elongation. FB_1 treated embryonic tissues indicated 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.

Additional keywords: germination; Fusarium spp.; Vigna unguiculata; ultrastructure

Cowpea (*Vigna unguiculata* (L.) Walp) is a widely cultivated indigenous African legume crop that is of great importance in tropical and sub-tropical countries of Asia, Africa, Oceania, the Middle East, southern Europe, southern United States of America 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, its ability to improve soil nitrogen levels and is 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 greatly on the crop as a good source of nutritious food (22).

However, when the seeds are stored at high relative humidities and high ambient temperatures, fungal infestation usually occurs. It is under these conditions that some of these 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 (11,17). 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) Nirenberg and *F. nygamai* Burgess and Trimboli (19,24). Fumonisin B_1 (FB_1) (Fig. 1) has been known to cause various toxicological problems in animals. These include leukoencephalomacia (LEM), a fatal brain disease in horses, and pulmonary edema syndrome (PES) in pigs (15,18). There is evidence that suggests fumonisins are associated with birth defects i.e. neural tube defects in humans (16). This toxin is statistically linked to the incidence of esophageal cancer in humans in Transkei, South Africa and China (18). Fumonisin B_1 is known to exhibit phytotoxic effects towards different plants, including economically important crops (1,2,3,4,9,14,17,26,27). 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 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 this investigation, *F. proliferatum* was found to be responsible for the production of the toxin on cowpea seed. (13).

This paper reports on the effect of the FB_1 toxin on cowpea seed germination and on root and shoot elongation. The effect of the toxin on the ultrastructure of the cotyledon and embryonic tissue of the seed is also reported.

MATERIALS AND METHODS

Seed material. Cowpea seeds (cultivar 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 quantities were aliquoted into 20 vials. The aliquots were dried down under nitrogen gas and stored at ± 4°C until used.

Seed treatments. The required volume of fumonisin was added to 50 ml sterile distilled water to yield final concentrations of 10, 25, 50 and 100 ppm. The seeds were allowed to imbibe in the various solutions for a period of 10 h. Sterile distilled water (50 ml) was added to 7-day-old cultures of *F. verticillioides* (MRC 4315), *F. nygamai* (MRC 3997) and *F. proliferatum* (MRC 8278). The surface of each culture was scraped to free the spores and the spore suspensions were poured through muslin cloth into flasks. A spore concentration of 1×10^6 ml^{-1} was determined with the use of a haemacytometer. The seeds were added to the flasks and mixed thoroughly and thereafter allowed 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. Percentage germination was determined after 5 and 9 days according to the International Seed Testing Association (ISTA) rules (12). Root and shoot length 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 0.075 M phosphate buffer (pH 7.4). The samples were rinsed three times in 0.075 M 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, 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 the 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 to both the positive and negative controls (Fig. 2). The lowest percentage (6.67%) of seed germination was at the 100 ppm 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. It was, however, 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 had no effect on corn seed germination but the toxin did, however, inhibit radical elongation in the seeds by up to 75% after 48 h of imbibition. The authors found that amylase production in the endosperm was also inhibited, which could suggest that FB_1 interfered metabolically with germination (10).

In this study, *F. verticillioides* and *F. proliferatum* inoculated seeds also showed significant reduction in germination (Fig. 2). 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 nine days, a significant increase in ungerminated seeds was noted in the toxin treated seeds (Fig. 3). Correspondingly, these treatments had the lowest number of normal seeds. With the exception of *F. nygamai*, the *Fusarium* inoculated seeds and the 50 ppm and 100 ppm toxin treated seeds revealed the highest amounts of diseased seeds.

Only the 50 and 100 ppm toxin concentrations significantly inhibited root and shoot elongation (Fig. 4). 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 analogues caused dose dependant 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.

Effect on ultrastructure. The ultrastructure of both controls of the untreated embryonic axes (Fig. 5 a and b) and cotyledon tissues (Fig. 6 a and 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. 5 c and d) when compared to the control. The 25 ppm treated tissue did, however, show an abundance of vacuoles containing protein bodies and lipid bodies throughout the protoplasm (Fig. 5 d). The only distinctive destructive effects caused by the toxin are shown in the 100 ppm treated embryonic tissues (Fig. 5 f to h). The plasma membrane has separated from the cell wall and irregular sized vacuoles (Fig. 5 f to 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. 5 f). The compacted protoplasm appears very dense and darker in color when compared to the control micrographs. An abundance of lipid bodies was noted next to the cell wall in the 50 and 100 ppm treated seed tissues (Fig. 5 e and h). The treated cotyledon tissue (25, 50 and 100 ppm) showed similar patterns with regard to the accumulation of lipid bodies (Fig. 6 d to f). Baird et al. (6) found lipid bodies to be conspicuous at the margins of the cytoplasm, outlining the cell walls in dry radical cells of soybean. Similarly, lipid droplets were closely appressed to the plasma membrane in dry cells of cowpea embryo tissue (25). During imbibition, lipid droplets become less 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 ppm treated cotyledon tissue did not reveal any noticeable effects caused by the toxin (Fig. 6 f) as noted in the 100 ppm treated embryonic axis tissue (Fig 5 f to h).

These destructive effects seen in the ultrastructure of the 100 ppm treated embryonic axis tissue could possibly play a role in the significant reduction in germination and root and shoot length at the same concentration. Van Asch (26) treated corn callus with different doses of FB_1, which produced deteriorative alterations in the cell ultrastructure. These included 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 accumulation of free sphingoid bases (3). Sphingolipids are highly bioactive compounds of cellular membranes that have profound effects on cell regulation (28). In plants, sphingolipids play a role in cell signaling, membrane stability, stress response, pathogenesis and apoptosis, but little is known about their precise functions (23). Studies on 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 this interference has caused a negative impact on the germination of the

seeds as well as 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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Fig. 1. Chemical structure of fumonisin B_1.

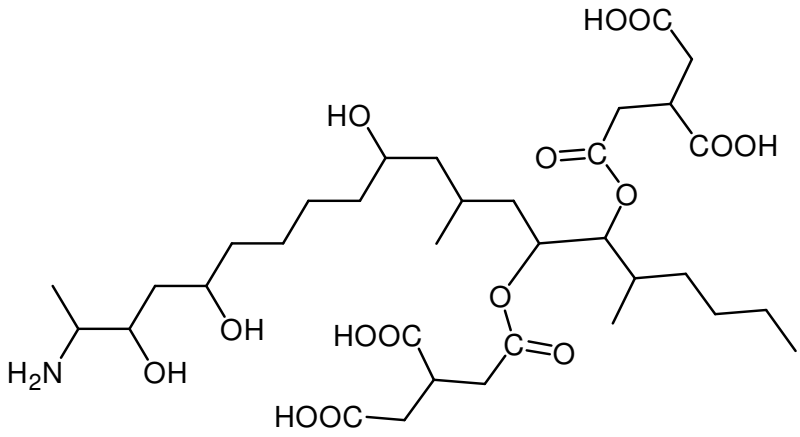
Fig. 2. Effect of FB_1 and *Fusarium* spp. on cowpea seed germination. Each bar is a mean of 3 replicates. Values of the bars not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 3. Effect of FB_1 and *Fusarium* spp. on cowpea seeds and seedlings. Values of the lines with the same symbol not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 4. Effect of FB_1 and *Fusarium* spp. on root and shoot elongation. Values of the lines with the same symbol not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 5. 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, c) imbibed for 10 h in sterile distilled water with the addition of FB_1 at 10 ppm, d) 25 ppm, e) 50 ppm, f, g and h) and 100 ppm.
(CW = cell wall, L = lipid, N = nucleus, PM = plasma membrane, V = vacuole, arrows – plasma membrane separated from cell wall). Bar = 1 µm.

Fig. 6 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, c) imbibed for 10 h in sterile distilled water with the addition of FB_1 at 10 ppm, d) 25 ppm, e) 50 ppm, f) and 100 ppm.
(CW = cell wall, L = lipid, N = nucleus, V = vacuole, arrows – plasma membrane separated from cell wall). Bar = 1 µm.



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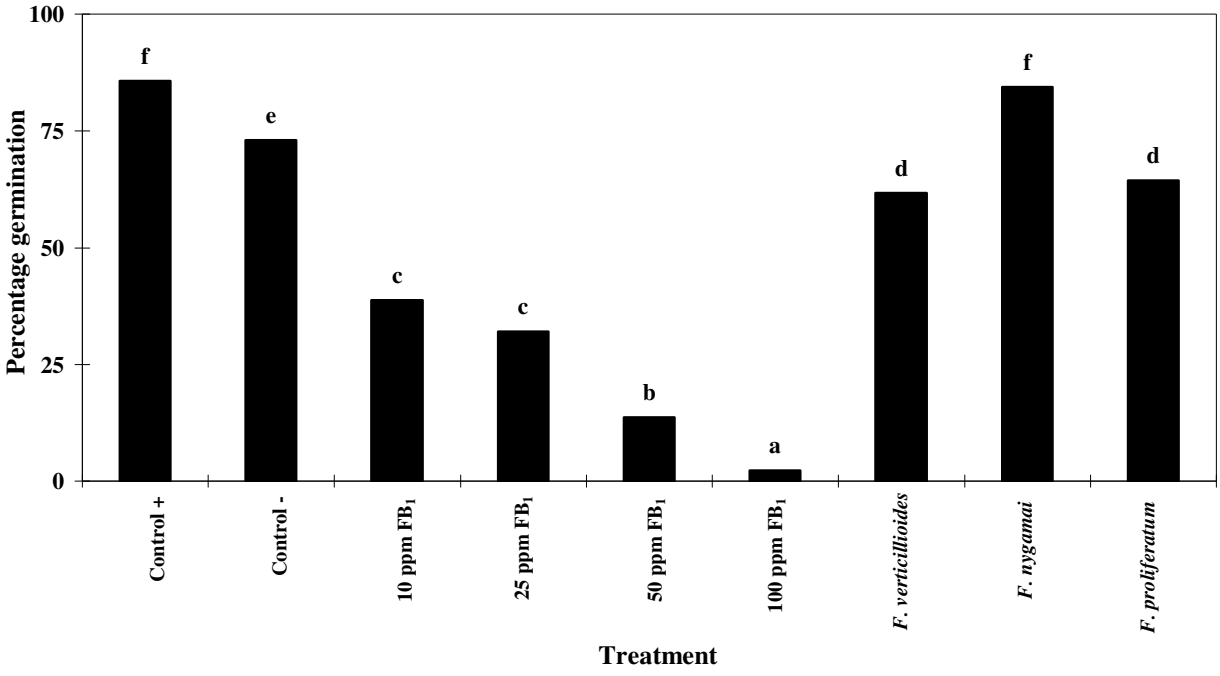

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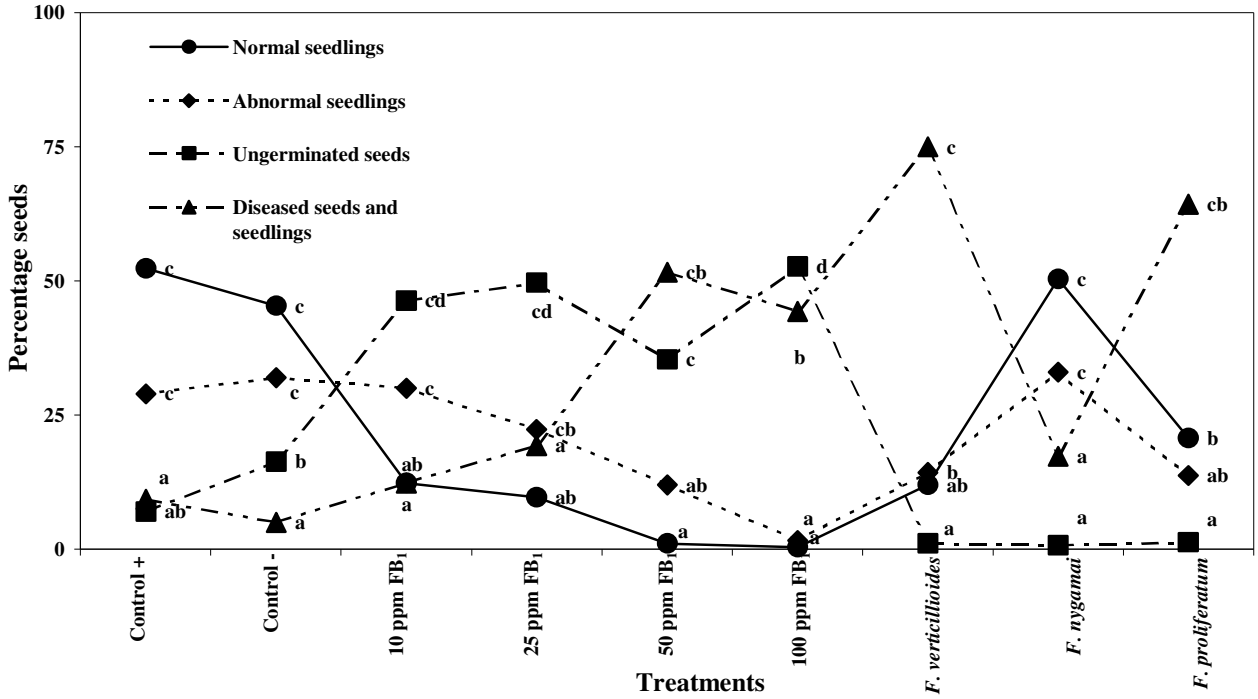

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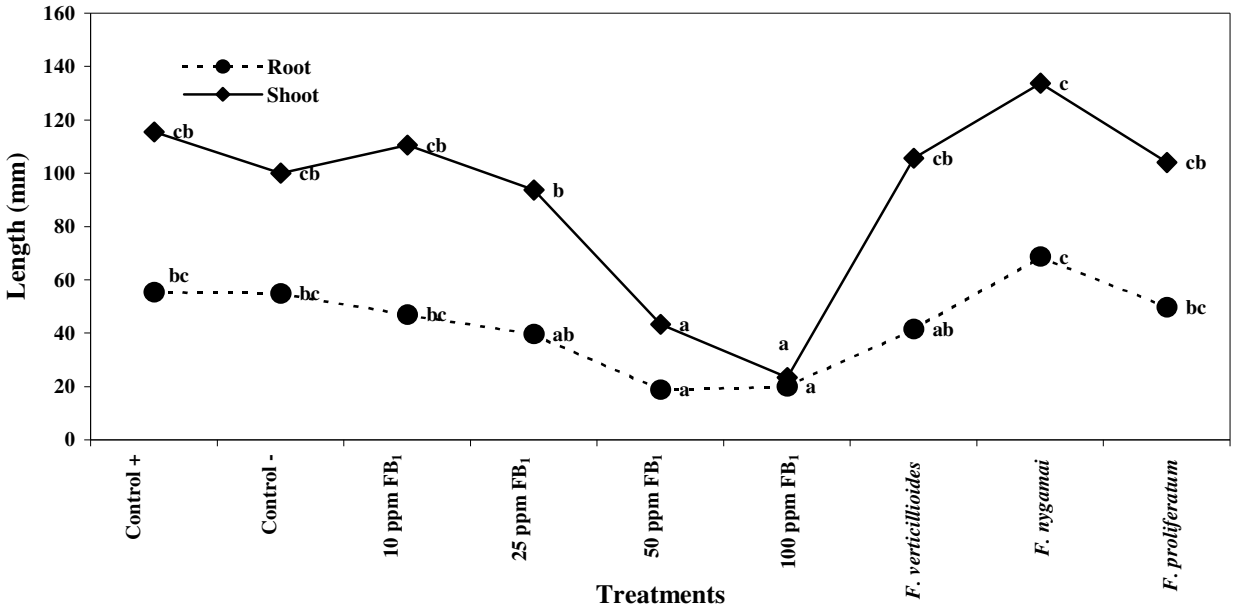

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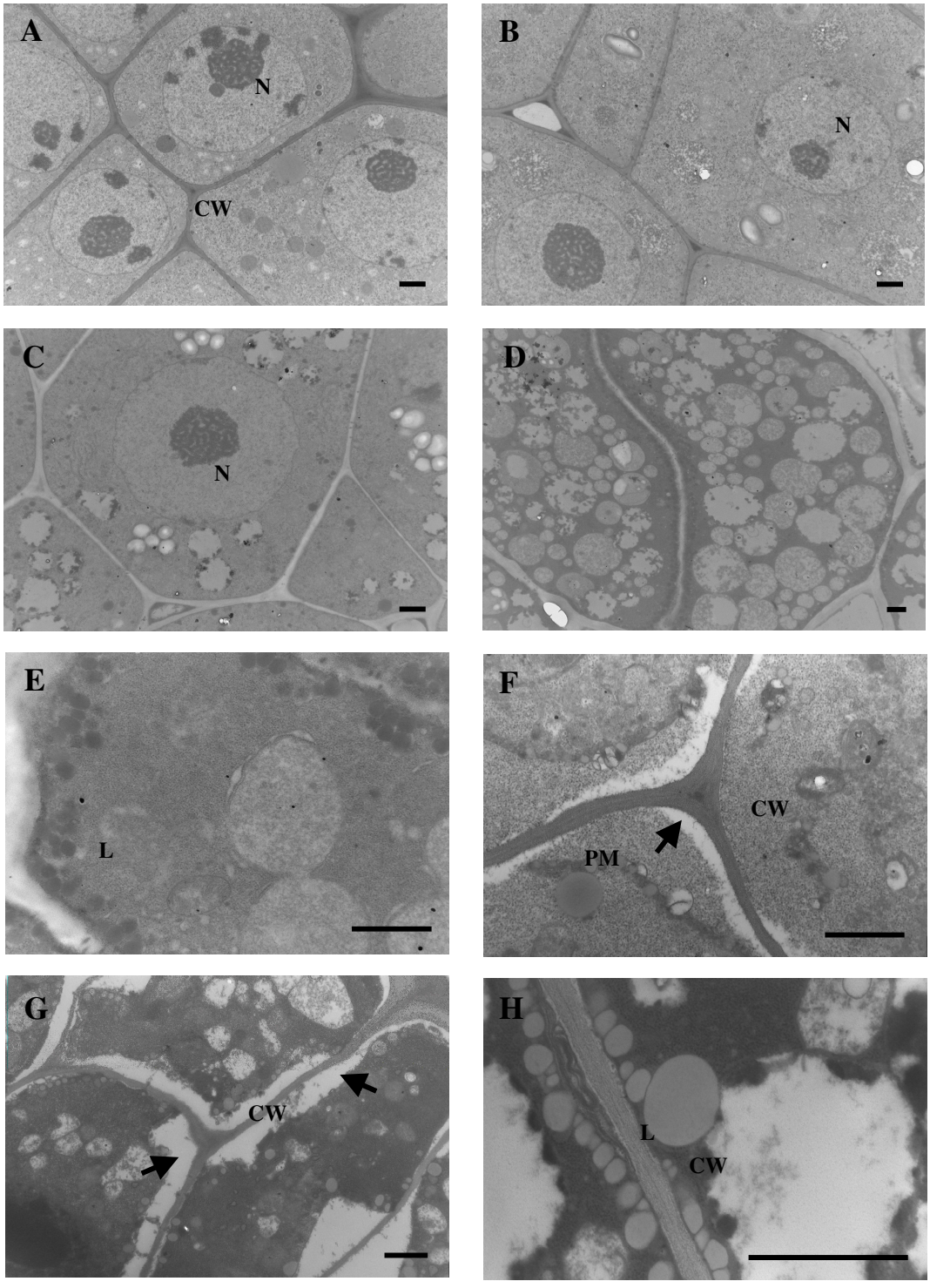

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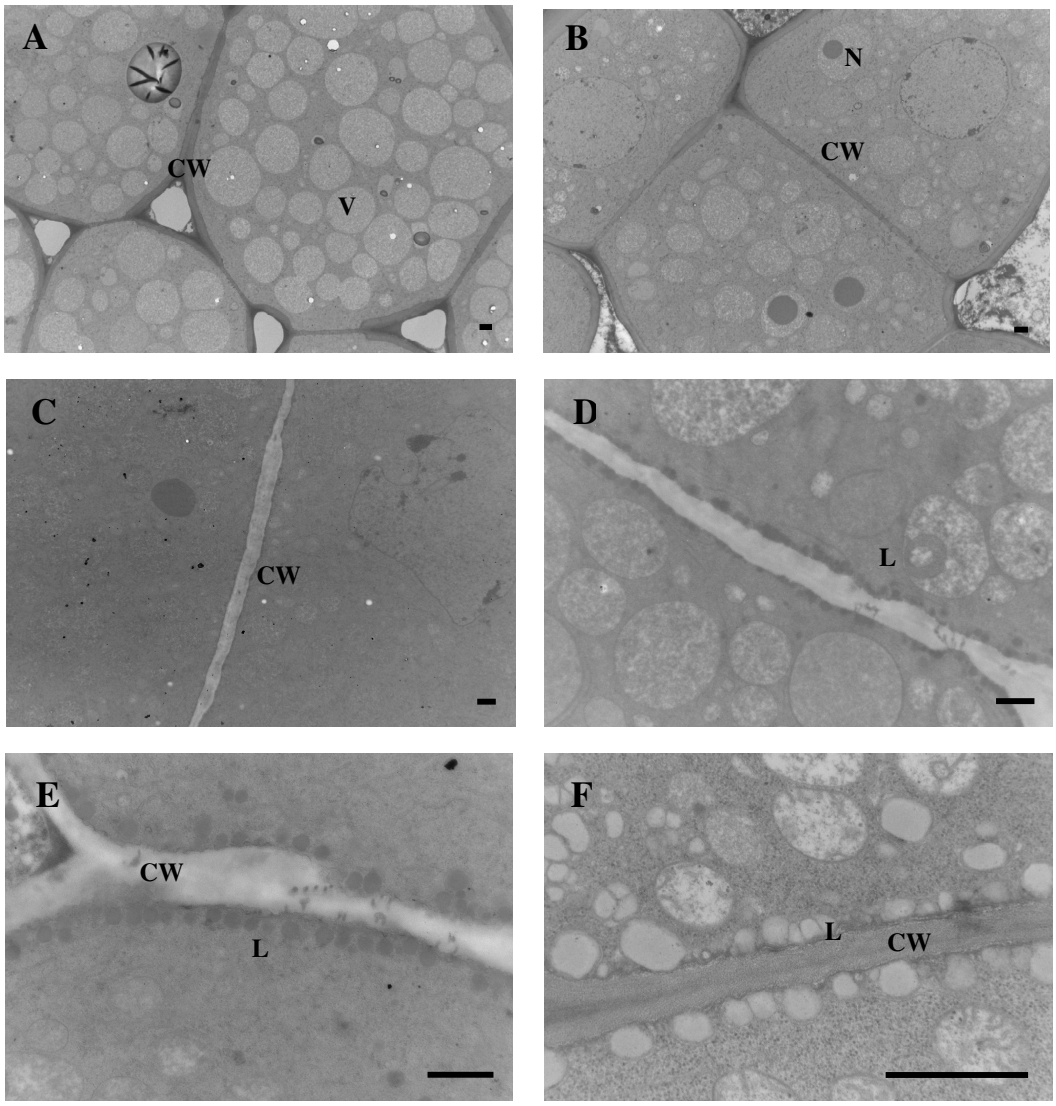

Fig. 6., Kritzinger, *Phytopathology*