Biological Control of *Fusarium moniliforme* in Maize

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Fusarium moniliforme Sheldon, a biological species of the mating populations within the *Gibberella fujikuroi* species complex, i.e., population A [= *G. moniliformis* (Sheld.) Wineland], is an example of a facultative fungal endophyte. During the biotrophic endophytic association with maize, as well as during saprophytic growth, *F. moniliforme* produces the fumonisins. The fungus is transmitted vertically and horizontally to the next generation of plants via clonal infection of seeds and plant debris. Horizontal infection is the manner by which this fungus is spread contagiously and through which infection occurs from the outside that can be reduced by application of certain fungicides. The endophytic phase is vertically transmitted. This type infection is important because it is not controlled by seed applications of fungicides, and it remains the reservoir from which infection and toxin biosynthesis takes place in each generation of plants. Thus, vertical transmission of this fungus is just as important as horizontal transmission. A biological control system using an endophytic bacterium, *Bacillus subtilis*, has been developed that shows great promise for reducing mycotoxin accumulation during the endophytic (vertical transmission) growth phase. Because this bacterium occupies the identical ecological niche within the plant, it is considered an ecological homologue to *F. moniliforme,* and the inhibitory mechanism, regardless of the mode of action, operates on the competitive exclusion principle. In addition to this bacterium, an isolate of a species of the fungus Trichoderma shows promise in the postharvest control of the growth and toxin accumulation from *F. moniliforme* on corn in storage. *Key words: Bacillus subtilis,* bacterial endophyte, biological control, corn, fumonisins, fungal endophyte, *Fusarium, Gibberella moniliformis,* mycotoxins, *Trichoderma, Zea mays*. — Environ Health Perspect 109(suppl 2):325–332 (2001).
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A biological species of the mating populations of *Gibberella fujikuroi* species complex, population A *Fusarium moniliforme* Sheldon (synonym *F. verticilliodes*; *G. moniliforme*), produces the fumonisin mycotoxins B_1 and B_2 (FB_1 and FB_2) in maize, *Zea mays*. The symptomless association of fungi of the *G. fujikuroi* species complex with maize as a descriptive feature was first described by Leonian (*1*), although its symptomless nature was depicted earlier (*2*). Numerous descriptions of the symptomless nature of this fungus have been made since these reports (*3–8*). These descriptions and others (*2,3,5*) were highly observational and did not indicate that endophytic hyphae were active physiologically. Indeed, most (*9,10*) considered these hyphae as latent, quiescent, or dormant and suggested that symptomless infected plants were nonhosts that served the purpose of overwintering the fungus, from which it produced conidia during its saprophytic stage.

The concept of symptomless (latent) infection was very narrowly defined as a quiescent or dormant parasitic relationship that after a time, can become an active parasitic one (*10*). The key to the latency concept is the use of *parasitic*, which was never defined (*9*) but was used as a synonym for *pathogenic*. Another aspect of the latent infection concept is that latent infections are different from dormant infections (*10*). This line of thinking persists and is the essence of recent reviews on the subject of symptomless infections (*9,10*). Gäumann (*11*), however, used latent infections to include all stages in the life cycle of a fungus during which no external symptoms can be seen. This wider definition would include actively growing hyphae of a large number of symptomless infections and currently serves as a descriptive basis for fungal endophytes. However, symptomless infections have been redefined and are considered biologically relevant (*8–15*). Before we focus on controls of endophytic fungi, we must first define endophytic microorganisms, which will justify the control strategies discussed in this review.

What are microbial endophytes? We refer to some fungi and bacteria as endophytes because they actively colonize host tissues and establish long-term associations—actually, lifelong symptomless associations—without doing substantive harm. Excluded from this characaterization of endophytes are localized cuticular infections and very localized epibiotic infections. The definition of endophytic fungi and bacteria used here is based on earlier concepts of microbial endophytes as symptomless infections, although there are also numerous exceptions. (*12*). We extend our definition of this habit from several well-established endophytic systems of grasses where the association is also used to include the mutualistic symbiotic associations, e.g., the tall fescue-endophytic fungus associations, which are not pathogenic, although exceptions occur here as well (*12–16*).

Thus, we extend our definition of *endophyte* to include those inconspicuous intercellular infections that are at least transiently symptomless but that are functionally relevant to the association as a viable, growing, and biochemically important component. This distinction indicates that endophytism is both a life habit and a biological association with ecological and physiological relevance. Further, latent or dormant infections are not characteristic features of endophytic hypha, although latent and dead hyphae and cells due to the aging process are undoubtedly necessary components of endophytic microorganisms. Microbial endophytes are associated with a large number of plants (*12,17–19*), and the occurrence of *F. moniliforme* as an endophyte is typical of several species within the genus *Fusarium;* for example, see the review by Kaldau and Yates (*20*).

Control, prevention, and detection of the endophytic infections by *F. moniliforme* in corn are difficult, especially because kernels appear to be of sound quality. The intercellular nature of this endophyte makes chemical control highly unlikely. Applications of systemic fungicides are impossible during later stages of plant growth, and because the fungus is a systemic seed-borne infection, conventional fungicides as seed treatments are also ineffective.

Because microbial endophytes can be important mutualistic components of plants (*12–19*), we propose that alternative

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endophytes might offer some control of the endophytic growth of *F. moniliforme* by competitive exclusionary principles. We review information on the endophytic fungus habit, which defines the requirements necessary for biological control and establishes production of fumonisins by endophytic hyphae of *F. moniliforme*. Second, we review biological control strategies that offer promise for both fungus reduction and toxin reduction. The first biocontrol uses an isolate of an endophytic bacterium, *Bacillus subtilis* (Ehrenberg) Cohn, that shows great promise in the control and reduction of mycotoxin accumulation during the growth of maize plants endophytically infected with *F. moniliforme*. Because *F. moniliforme* is also saprophytic, we review information on a second biocontrol that uses an isolate of the fungus *Trichoderma* sp. as a postharvest control for the growth of *F. moniliforme* and resulting toxin accumulation during storage.

Experimental Microorganisms and Approaches

Experiments designed to determine the production of the fumonisins by endophytic hyphae as well as biocontrols of fungal growth and toxin production have been reported. Experimental details for their use, laboratory manipulation, toxin analysis, and plant interaction are contained therein (*8,21–27*).

Microorganisms

All *F. moniliforme* isolates were selected for unique traits and have been described (*21,23,24*). Briefly, we used specific wild type (WT) strains of *F. moniliforme*: RRC410, RRCPAT, RRC408, and MRC 826. These WT strains were transformed with the *gusA* reporter gene, which encodes for β-glucoronidase (GUS), and the *hph* gene for hygromycin resistance (*21*). The transformed strains were used as ecological markers and were designated MRC 826gus, RRCPATgus, and RRC408gus. A positive reaction for GUS activity was detected by a specific histochemical stain, which confirmed that the specific strain recovered was the one inoculated into plant material initially (*21*). The isolate UPS101, a *Trichoderma* species, was recovered from root segments collected from Silver Queen plants grown in field plots (*22*). The maize cultivars used included yellow field maize Trucker's Favorite and Reid's Yellow Dent and sweet maize Silver Queen and Early Sunglow.

The endophytic bacterium was isolated from an unknown cultivar of maize from northern Italy (*23*). Using biochemical characteristics and specific fatty acids, this bacterium was initially identified as an aberrant type of *Enterobacter cloacae* (*23*); but using the 16S rRNA gene sequence similarity analysis, this bacterium matches the genetic distance format for strains within the *B. subtilis* group. However, the percent difference between this isolate and the closest match suggests a subclad, which indicates that we might be dealing with a subspecies or another closely related and/or unknown species within the diverse *B. subtilis* group. If so, this isolate would more than likely be a new species separated from the *B. subtilis* complex that is composed of two groups of closely related but different strains. The strains of this endophytic bacterium used in these studies included the WT *B. subtilis* RRC101 and its rifampicin-resistant mutants RRC26ss and RRC24wf. The mutants are identical in inhibitory activity and endophytic competence to the WT and were used as markers for field studies.

Plant Culture and Endophytic Studies

Experiments were conducted under gnotobiotic conditions (*24*) when the plants were young, vigorous, and showed less than 1% senescent leaves. The latter aspect was important because one could argue that plants might also be infected from outside sources, producing saprophytic production of fumonisin on senescent tissue. All experiments designed to study the fungus/bacterial/host plant relationship were performed on maize kernels subjected to double sterilization: The kernels were surfaced sterilized and then subjected to a mild heat treatment (*24*) to remove both external and internal bacteria and fungi. However, since gnotobiotic conditions can only be maintained for approximately 10 weeks, experiments to test for endophytic fumonisin production were conducted within this time period (*28,29*).

The Nature of the Endophytic State of *F. moniliforme*

As an endophyte of maize, *F. moniliforme* may remain a symptomless biotrophic parasite throughout the entire growing season (*4,7,8,30*). Some isolates enhanced the growth of maize seedlings (*12,31*). The fungus exists as an intercellular infection within the plant, although under certain circumstances it may become intracellular (*8*). The fungus endophytically infects seedling from its systemic infection of the seed, usually by the second day following germination (*8*), or within 10 days (Figures 1A–1E) (*32*). The systemic infection of maize seeds produces maternal-line vertical transmission from generation to generation. In addition to the vertical transmission phase, maize is subject to infection throughout the growing season (*33,34*). The fungus is also a saprophyte while residing in the soil, living off dead maize debris where spores are produced that can infect plants from the outside, e.g., maize silks. Thus, *F. moniliforme* is vertically transmitted to the next generation of plants via clonal infection of seeds, and horizontally transmitted to plants contagiously from saprophytic colonization of soil debris and insect vectors. The vertical dissemination phase of this fungus as an endophyte of maize kernels indicates that the relationship is not casual, but rather intimate and probably complex, since seed germination and seedling development usually are not affected (*1,7,8,30,35*). The horizontal infection phase can be reduced or eliminated by application of certain fungicides. The endophytic seed and plant infection, i.e., vertical transmission, is important because it remains the reservoir from which infection of each generation of plants takes place and from which renewed toxin synthesis can take place. Application of fungicides cannot control the fungus during this phase.

To understand the relationship of the host with the fungus, symptomless infections were studied at the light and ultrastructural levels (*8*). The two hygromycin-resistant mutants of *F. moniliforme,* RRC374H and MRC826H, used to examine the host–parasite relationships produced either symptoms of seedling blight or symptomless infections in two maize cultivars, Silver Queen and Truckers' Favorite. Intercellular hyphae were observed within asymptomatic tissue of leaves and roots of 1- to 8-week-old plants on both cultivars (Figures 1A–1E). Symptomless endophytic infections were observed in several cultivar–isolate combinations. Intercellular hyphae were in direct contact with host cell walls (*8*); at the ultrastructural level, the walls were not separated by an extracellular granular matrix reported for other intercellular (or epibiotic) fungal pathogens and mutualists (*36–38*). Throughout the entire period of host colonization by the symptomless fungal–maize combination, there were no effects from the intercellular hyphae on the integrity of host cellular structure and organelles. This suggests that during the symptomless state no toxins are produced by the fungus that are disruptive to the host cellular structure, and that no host reaction indicates no disease is produced. The symptomless infection occurred identically in both cultivars. Symptomless infections have been produced in 15 cultivar and inbred lines of maize, and identical ultrastructural responses were observed (data not shown).

Because *F. moniliforme* could be recovered from 2-day-old coleoptiles, infection must have occurred immediately before or during germination. The fungi were isolated from leaves, stems, hypocotyls, and roots during the 8- to 10-week observation period, and included both diseased and symptomless plants of the Silver Queen cultivar (Table 1). Identical results were obtained with the cultivar Trucker's Favorite. Symptomless

plants (95% of the treatment group) were positive for fungi throughout the observation period; diseased plants (3%–5%) died within 2 to 3 weeks, and 1% of the diseased plants recovered and grew symptomless. The distribution of *F. moniliforme* was the same in both diseased and symptomless fungal–seedling combinations. Thus, the lack of disease expression was not caused by the lack of fungi within specific organs of a maize genotype. We found that the fungus was not present within the vascular bundle tissue of the pedicel (*24*), nor did it invade the vascular bundle of plants, which supports the conclusion of Pennypacker (*39*) that *F. moniliforme* is not a vascular rot or wilt pathogen of maize.

The topical application (*8,24*) of the hygromycin-resistant mutants of *F. moniliforme*, RRC374H and MRC826H, to maize kernels and their recovery from gnotobiotic plants 6 weeks later (Table 1) indicates the importance of infected kernels both for dissemination and as a source of inoculum development. Further, surface-sterilized kernels known to be systemically infected naturally yielded *F. moniliforme*, which indicates that the systemically infected kernels are also important inoculum sources for the endophytic infections (data not shown).

Valleau (*2*), Thomas and Buddenhagen (*40*), and Kedera et al. (*7*) suggest that infection of maize can occur from kernel to kernel. To establish vertical transmission of this fungus, *F. moniliforme* was transformed with the GUS-reported gene (*21*). This isolate was applied to seed, which was planted under greenhouse conditions. The resulting kernels produced from plants under these conditions were surface-disinfected and the isolated fungus stained for specific GUS-specific products (*21*). The results indicated that approximately 35% of the *Fusarium*-infected kernels were infected with the transformed isolate. Further, when these kernels were planted and plants were grown under gnotobiotic conditions, the transformed fungus was isolated from seedling tissue. We interpret these data to mean that the fungus was transmitted from seed to plant and to seed again. This aspect of the life cycle of *F. moniliforme* indicates that it can be intimately associated with the maternal lineages of maize cultivars.

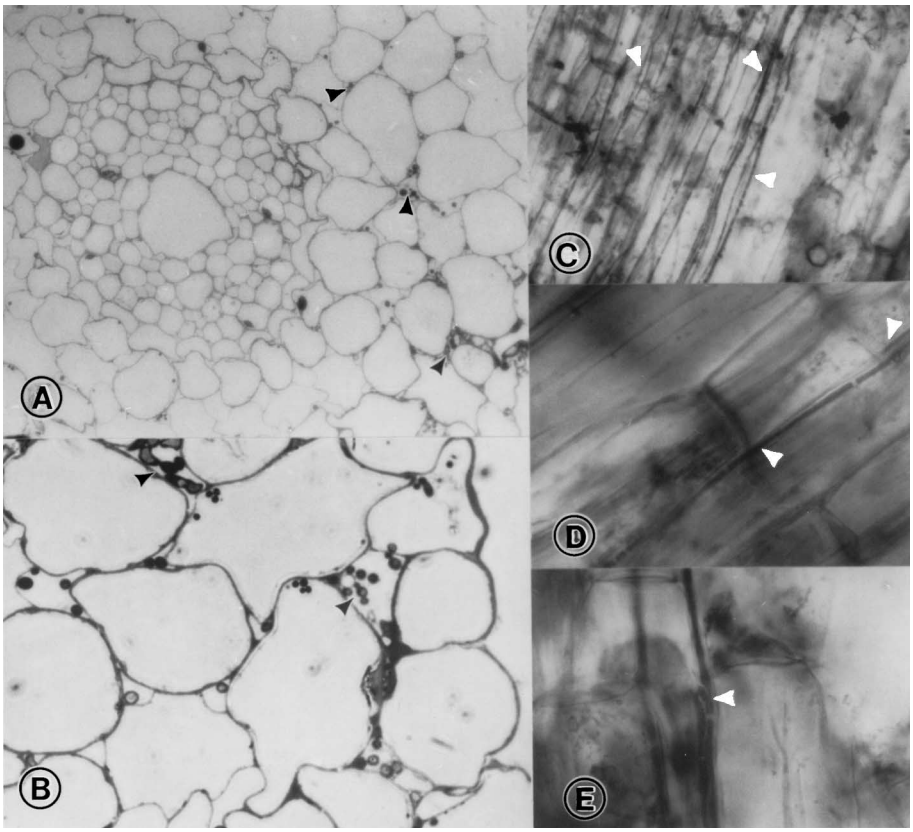

Figure 1. Light micrographs of the endophytic hyphae of *F. moniliforme* at the symptomless phase of infection, showing 1- and 2-week-old hyphae stained with analine blue (*8*). (*A*) Cross-section of a secondary root with hyphae (arrows) in the intercellular spaces (41×). (*B*) Cross-section through cortex of a primary root with groups of hyphae (arrows) in the intercellular spaces (82×, phase contrast). (*C*) Hyphae (arrows) running parallel within intercellular spaces of internodes of maize (41×). (*D*) Higher magnification of maize tissue showing a branching septate hypha (arrows) between two cells walls (204×, phase contrast). (*E*) Hyphae running parallel within intercellular spaces with a branching hypha at arrow (204×, phase contrast).

F. moniliforme infects maize very soon after germination, and, contrary to earlier views (*3,30,32*), this infection does not always develop into a disease. The initiation of the disease phase of the parasitic stage is not common, and may reflect plant selection against this phase (*41–43*), a physiological interaction of the fungus with phytoanticipins (*44*), or interactions with suitable environmental conditions, often late in the life of the plant (*43–46*).

Toxin Production by Endophytic Hyphae

It is assumed that the fumonisins are synthesized late in the plant–fungus interaction or during saprophytic fungal growth on damaged or dead tissue. Very little of this type of corn enters the human food and animal feed chains, although subsequent improper storage conditions can increase the fumonisin content. We do not know how early this occurs or what conditions affect the accumulation of the fumonisins by the endophytic symptomless phase. The presence of the fumonisins in sound-appearing, food-grade maize kernels (*47,48*) supports the hypothesis that low concentrations of the fumonisins are synthesized by symptomless endophytic hyphae. This hypothesis would also account for the production of the fumonisins in higher concentrations in sound corn when stored under poor postharvest conditions or in kernels and debris from cobs and in other plant parts not showing ear and stalk rot symptoms (*48,49*).

To test the hypothesis that fumonisins are produced by endophytic hyphae, corn seedlings were infected with *F. moniliforme* (*8*), grown under gnotobiotic conditions for

Table 1. Three- and 6-week isolation frequency of *F. moniliforme* from Silver Queen maize tissue inoculated with the fungus and grown for 6 weeks.[a]

Tissue type	Fungal isolation (%)	
	3 weeks	6 weeks
Primary root	100[b]	100
Lateral root	100	100
Mesocotyl	100	100
Crown root	100	100
Crown	100	100
Nodal root	100	100
Node #1	100	100
Node #2	100	100
Node #3[c]	ND	100
Node #4	ND	90
Leaves[d]	100	100

ND, not yet developed. [a]Data from Bacon and Hinton (8). [b]Results are mean values based on organs and tissues removed from 10 plants during each isolation period. Both symptomless and diseased plants gave identical percent isolation frequencies. Only symptomless infected plants were alive at the sampling period, and such plants remained infected for 10–12 weeks. The cultivar Trucker's Favorite gave identical results. [c]Nodes #3 and #4 were only on plants 6 weeks and older. [d]Leaves consisted of the oldest (lowest) green leaf during a sampling time and included both sheaths and blade tissue; senescent leaves were not included.

10 weeks, and harvested and separated into roots and shoots; this material was then analyzed for fumonisin content (*27*). This experiment showed not only that endophytic hyphae of *F. moniliforme* produced the fumonisin *in planta* but also that this mycotoxin is produced early in maize seedlings (Figure 2). Thus, this association is another example of toxin production by endophytic hyphae, and in this regard is similar to other fungal endophytes of grasses (*12,14*).

Additional experiments used GUS-transformed *F. moniliforme*-infected plants subjected to 2 weeks of drought and compared to normal watering conditions (*21*). The results indicated that endophytic hyphae of seedling are responsive to this abiotic stress (Figure 2). The fumonisins accumulated under drought stress, especially at –1.5 megapascals, which induced physiological wilt (Figure 2). A similar increase in fumonisin content was observed in plants inundated with water, simulating flooding (data not shown). Apparently it is stress and not necessarily drought that induces an increase in toxin synthesis. The content of fumonisins is significantly higher in roots than in shoots (data not shown), which probably reflects the preferential transport of translocates and apoplastic solutes to roots during this period (*50*).

These experiments prove that the symptomless endophytic stage of *F. moniliforme* is physiologically active. Further, endophytic hyphae isolated from treatment groups showed GUS activity following a specific staining procedure (*21*). Although we have no information dealing with plants grown for a longer time period, the accumulation and/or synthesis of toxin might resume as kernel development is initiated because products stored in the roots move upward and into development kernels at this time. We have grown plants under greenhouse conditions from kernels inoculated with *F. moniliforme* RRC408gus and recovered this strain from surface-disinfected matured kernels (an average of 40%) as well as all plant parts, establishing the vertical transmission of the fungus.

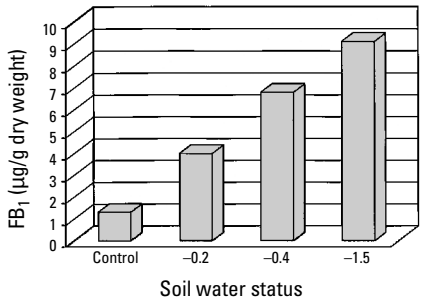

Figure 2. Production of fumonisin in gnotobiotic GUS-transformed (*21*) *F. moniliforme*-infected maize plants grown under drought stress (*25*).

Biological Control with *B. subtilis* Patent Number 5,994,117

Plant–Bacterium Interaction

Species of *Bacillus* are characterized as gram-positive, rod-shaped, aerobic, catalase-positive, motile cells that form endospores more resistant than vegetative cells to heat, drying, and other destructive environmental factors (*51*). *B. subtilis* is a large and diverse physiological group of nonpathogenic bacteria that is relatively easy to manipulate by genetics and simple to cultivate, which strengthens its suitability for industrial uses. Further, the presence of endospores is an advantage for most industrial applications and biotechnological uses. These bacteria and other members of the genus are used in fermentations for human food use, sources of extracellular enzymes for industrial and medicinal uses, and production of peptide antibiotics. The biocontrol bacterium *B. subtilis* RRC101 was shown to have highly desirable plant-enhancing characteristics as well as being an endophyte able to protect maize from pathogenic organisms (*23,50*). *B. subtilis* RRC101 is unique among other biocontrol isolates of this species in being endophytic, and this biocontrol isolate was patented for these properties: patent number 5,994,117 (*52*).

The biocontrol bacterium was obtained from seedling roots grown from endophytically infected seed following culture on agar media, soil, or filter paper. When sections of these roots were examined microscopically, bacterial cells were also observed internally (Figure 3A). On maize roots obtained from seeds germinated in culture dishes, the bacteria were distributed uniformly over the epidermis (Figure 3B) and randomly distributed intercellularly in several locations of the cortex (Figure 3B and 3C). No bacterial cells were observed within the endodermis, but bacteria were observed intercellularly within the outer margin of the pericycle, usually adjacent to phloem cells (Figure 3D). They were not observed among cells of the pith area. In no instance was there evidence of damage to host cells, and cells of this bacterium were completely intercellular (Figure 3C).

The isolates of *B. subtilis* used for *in planta* interactions were rifampicin mutants (*23*). There was no evidence at the ultrastructural level of pathological damage to cells of the endodermis and stele, nor were any bacteria observed within cells. A matrix-like capsule was observed surrounding the bacterial cells located on the primary root epidermis (*23*). The intercellular location of this bacterium was clearly demonstrated at the ultrastructural level. There is a proliferation of bacterial cells within the intercellular spaces, particularly those that were connected (Figure 3C and Figure 3D). The identical intercellular nature of this bacterium was observed in the field maize cultivar Trucker's Favorite as well as all other inbred lines and cultivars (data not shown).

Bacteria–Fungus Antagonism *in Vitro*

Inhibitory studies (*23*) between the bacterium and fungi were conducted on either nutrient agar or potato dextrose agar (PDA), and these zones of inhibition were used as bioassays for antagonisms. Besides the isolates of *F. moniliforme* listed above, 30 others were also tested (data not shown). All isolates were grown on PDA for 3–7 days, two mycelial plugs removed from the outer margins of each fungal species, and two plugs placed opposite each other on the outer margin of a fresh agar plate of either nutrient agar or PDA (*23*). The bacteria used for these experiments were grown on nutrient agar for 2–3 days, and one inoculation loop was streaked down the center of each plate, between each fungal agar plug of each plate, immediately after inoculation with the fungal plugs. Control plates consisted of fungi or bacteria placed on plates alone or fungi as described above but without the bacteria. All plates were incubated in the dark at 25–27°C, until the fungi on the control plates had grown together. The bacterium completely inhibited the growth of all isolates of *F. moniliforme* tested (*23*).

In addition to *F. moniliforme*, other fungi tested that were inhibited included *Alternaria alternata, Cladosporium herbarum, Colletotrichum graminicola, Diplodia zeae, Helminthosporium carbonum, Penicillium chrysogeum, Phythium* sp., and *Rhizoctonia solani.* Two mycotoxic but nonpathogenic species, *Aspergillus flavus* and *A. parasiticus,* were also inhibited by this bacterium.

B. subtilis in Planta Distribution

The distribution of bacteria within the aboveground portions of plants was determined by aseptically culturing maize seedlings produced from double sterilized kernels (*24*). The seedlings were grown for 1–6 weeks in the light room under gnotobiotic conditions as described above. Portions of plants were surface sterilized (*23*) and plated out on rifampicin-amended nutrient agar. The controls—uninoculated double-sterilized kernels germinated and grown as described for the treatment groups—did not yield bacterial colonies when their leaves and stems were surfaced sterilized and plated on rifampicin-amended medium. The bacterium was distributed in all organs of the plant at concentrations that varied during the entire season (Table 2). The highest concentration of the bacterium occurred in the roots [10^6 colony forming units (cfu)/g wet weight], and this concentration remained constant (data not shown). Further, the bacterium was

absent from maize kernels harvested at 14% moisture. Some cultivars yielded bacteria, but this was from less than 10% of kernels.

Bacteria-infected maize plants showed no evidence of disease in seedlings during a 6-week observation period under gnotobiotic conditions nor during the entire maize growth cycle under field conditions of approximately 120 days. The bacterium had positive effects on maize seedlings including increased root and shoot growth (Table 3) and an increase in the germination rate (data not shown) (*53*). Further, the bacterium has a protective action on maize seedlings when planted in soils infested with *F. moniliforme* (Table 4). The bacterium also caused an increase in herbage yield and leaf width when coinfected with *F. moniliforme* (Table 4). These data indicate that the bacterium protects the seedling and enhances growth when planted in *F. moniliforme*–infested soil. A comparison of *B. subtilis* RRC101 with other isolates of *B. subtilis* established that several strains can colonize roots (*54*). RRC101 prevented roots from being colonized by *F. moniliforme* in the two soil types tested (Figure 4) (*54*), indicating that these other isolates of *B. subtilis* cannot prevent the colonization of plants by the fungus (*54*). Further, the data show that the growth of the fungus was controlled in either a natural soil type (Cecil) or a synthetic soil (Figure 5). Although these nonbiocontrol isolates were able to colonize the roots, their duration of endophytic colonization declined during a 2-month observation period (*54*). Thus, *B. subtilis* RRC101 grows continuously *in planta* without negatively affecting the growth of maize and is found in relatively uniform concentrations ($10^{5–6}$ cfu/g tissue, fresh weight) throughout the season.

Maize inoculated with *B. subtilis* germinated and when the resulting seedlings were grown on sterile filter paper, distribution and location of bacteria were observed identical to those on the medium, suggesting that the initial observations on PDA were not artifacts. Similarly, the identical endophytic habit of this bacterium was established from roots of kernels germinated in soil. The presence of the bacterium only between cells and in young, healthy-appearing, developing seedling roots suggests that this relationship is not detrimental; thus, we do not interpret it as a stage in the decomposition of roots from the surface inward. We conclude from the microscopic data that this bacterium is intercellular,

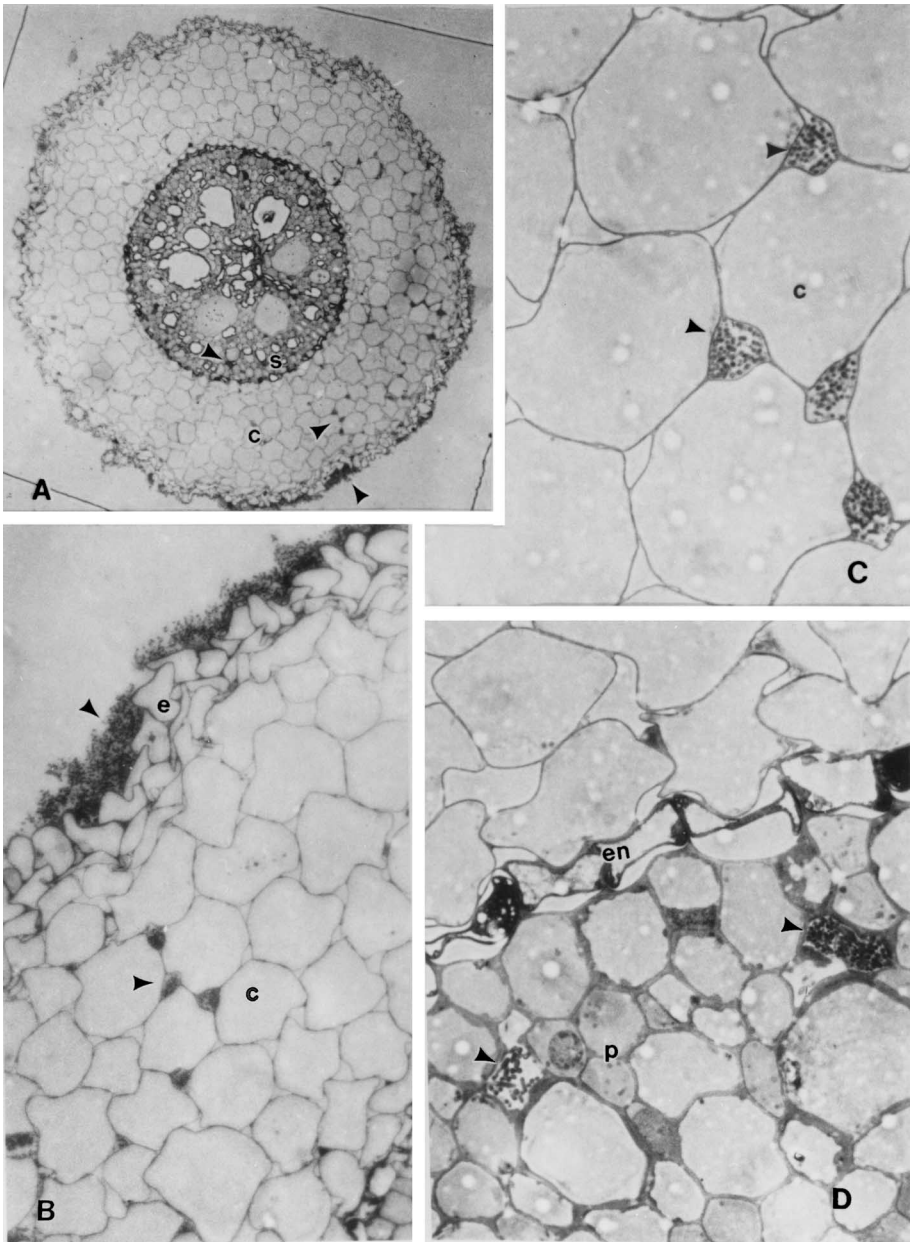

Figure 3. Phase-contrast light micrographs of a primary root of the Italian maize cultivar infected with *B. subtilis* (*23*). (*A*) Cross section of the root taken near the tip showing its organization into epidermis with bacteria at arrow, moving inward to the cortex (c) showing bacteria at arrow, and finally into the stele (s) with bacteria at arrow just within the endodermis. (*B*) Section of Figure 3A enlarged to show bacteria (arrow) located over the epidermis (e) and (*C*) bacteria located within the intercellular spaces of the cortex (c) showing the intercellular location of the bacterial cells, arrows. (*D*) Cells of the stele enlarged ×750 under oil showing the bacteria at arrow next to primary phloem cells (p) just below the endodermis (en).

Table 2. *In planta* distribution of *B. subtilis* cell in the maize cultivar Trucker's Favorite (cfu/g wet weight).[a]

Week	Blade	Sheath	Stem
4	1×10^3	1×10^1	1×10^3
8	1×10^3	1×10^2	1×10^3
12	1×10^4	1×10^4	1×10^3
16	1×10^2	1×10^2	1×10^4

[a]Roots were positive from week 1–16 with an average of 10^6 cfu/g wet weight.

Table 3. Effects of *F. moniliforme* and *B. subtilis*, WT and mutant, on maize seedling root and shoot growth.

	6-day-old seedlings	
Cultivar Treatment	Primary root length (cm)[a]	Shoot height (cm)
Silver Queen		
Control	15.4a	5.2a
B. subtilis RRC101	16.8b	6.6a
B. subtilis 26ss	14.5a	5.8a
B. subtilis 24wf	15.2a	5.8a
F. moniliforme 374	12.1b	5.8a
Reid Yellow Dent		
Control	13.0a	5.5a
B. subtilis	15.4b	6.4b
B. subtilis 26ss	16.1b	7.0b
F. moniliforme 374	7.2c	4.7a

[a]Values are means from at least four independent experiments; different letters for the wild type and rifampicin mutant indicate statistically significant ($p < 0.05$) differences from the controls.

whereas the symptomless and host-enhanced characteristics clearly define this bacterium as a true mutualistic endophyte as described above.

Fumonisin Reduction by *B. subtilis*

The effects of the WT *B. subtilis* Pat. No. 5,994,117 (RRC101) and its rifampicin mutants 26ss and 24wf on fumonisin production were determined during seedling growth. These plants were grown in pot culture in the light room, subjected to drought treatments as described above, and analyzed for fumonisin content. Not only did the bacterium prevent the fungus from colonizing the plants, but it also reduced the amounts of fumonisins produced *in planta* by an average of 50% when plants were grown under the normal watering (nonstressful) regiment (controls, Figure 6). Amounts of fumonisin were significantly reduced throughout the treatments but especially under drought stress, a condition conducive to high accumulation of fumonisin (Figure 6). The amount of fumonisin produced under physiological drought was reduced significantly compared to that in the noninfected *B. subtilis* group, to the level of the control nonstressed treatment (Figure 6). These experiments indicated that *B. subtilis* prevented the stress-induced increase of fumonisin produced by endophytic hyphae observed in the plants not protected by *B. subtilis*.

Table 4. Protective action of *B. subtilis* on the growth of maize seedlings Silver Queen.

Treatment	Seedling height (cm)	Blade width (cm)
Control	44a[a]	2a
B. subtilis	51b	2a
F. moniliformis	25c	1b
B. subtilis and *F. moniliformis*	49bd	2a

[a]Values are means from at least four independent experiments; different letters for the WT and rifampicin mutant indicate statistically significant ($p < 0.05$) differences from the nontreated control group.

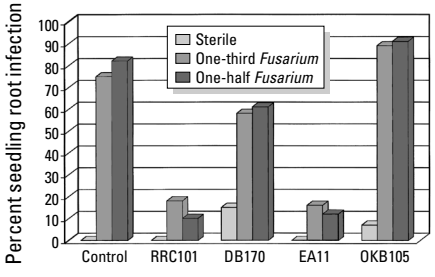

Figure 4. Comparison of *B. subtilis* RRC101 with two other *B. subtilis* strains, DB170 and QKB105, for protection against maize root infection by *F. moniliforme* in plants grown in soil infested with two concentrations of the fungus, based on soil dilutions (1/3 and 1/2) of the infested soil (*54*).

Transformed *F. moniliforme* RRC408gus was inoculated on bacteria-inoculated maize kernels of field and sweet cultivars cultured in pots located in either the greenhouse or outside plots. The plants were allowed to mature and set seed. Roots, stem, leaves, and kernels were collected and analyzed for bacteria, fungi, and fumonisin. The data (Figure 6) indicate that this bacterium-reduced fumonisin produced under stress *in planta* and suggest that this reduction will continue in matured plants at the kernel fill stage (*53*). The effects of this bacterium on fumonisin accumulation are continuing to be studied under field conditions in two locations. Preliminary data indicate that the bacterium remains within the plants during the entire season and can be recovered to a low extent in kernels, especially from one maize cultivar. Analysis of toxin production under field conditions is still in the preliminary stage, consisting of one replication for a single season.

Biological Control with *Trichoderma* sp.

Species of *Trichoderma* are common soil saprophytic hyphomycetes found in all climates throughout the world. The genus is complex and polyphyletic and is separated by morphological criteria into five sections or species aggregates, one of which is *Trichoderma* Bissett section *Trichoderma.* It is to this section that our biocontrol species belongs. Although the identity has not been conclusively established using molecular characters, the isolate has oblong conidia occurring on conspicuous whorled phialides with no yellow pigment produced on the reverse of a diagnostic medium. The appearance of whorled phialides and the absence of yellow pigment distinguishes this species from all others, indicating that this isolate is tentatively *T. koningii* Oudemans (= *Hypocrea koningii* Lieckfeldt, Samuels and Gams, the teleomorph) (*55*). However, we will refer to it as *Trichoderma* sp. until it has been subjected to molecular analysis (*56*). Members of this genus have been studied as antagonists in biocontrol systems against various plant pathogens and have been considered sources for numerous biotechnological applications

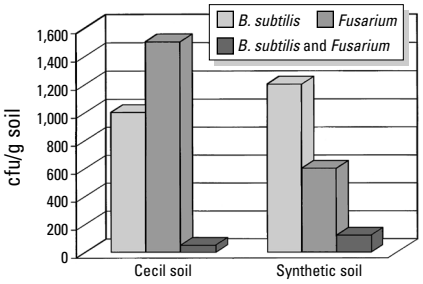

Figure 5. Protection of maize root from infection by *F. moniliforme* with *B. subtilis* RRC101 in plants grown in a Cecil soil and a synthetic soil.

for decades (*57*). These fungi are very effective as biocontrol agents because their powerful extracellular lytic enzymes produce necrotrophic action on fungi through lysis of cell walls. Much of the biocontrol potential of *Trichoderma* sp. has been reviewed (*58,59*) and most of the mode of action is probably caused by the lytic activity on fungal cell walls. Plant pathogens controlled by *Trichoderma* species include *Gaeumannomyces graminis, Phythium* sp., *Botrytis cinerea, Colletotrichum truncatum, Cylindrocladium floridanum, Phytophthora citrophthora, Sclerotinia sclerotiorum, Botrytis cinerea*, and numerous other fungal pathogens of economically important plants (*58*).

A few investigations have reported the potential of *Trichoderma* species as control organisms for phytopathogenic *Fusarium,* including competition of *Trichoderma* sp. and *F. moniliforme in vitro* (*60*). However, these investigators did not examine whether such competition occurs on natural substrates such as maize kernels nor whether mycotoxin production is reduced. *Trichoderma* species are applied mainly to the soil for biocontrols, and only a few reports deal with their application in the management of postharvest cereal and foliage diseases (*58*). Our objective was to investigate the influence of a strain of *Trichoderma* species, isolated from maize root segments, on the growth and production of FB_1 by *F. moniliforme* growing on maize kernels and its potential as a postharvest control for fumonisin production and/or reduction.

Effects of *Trichoderma* on Growth of *F. moniliforme*

In addition to reduction of fumonisin production, daily measurements of *F. moniliforme* colony diameter alone and in dual cultures with the isolate of the *Trichoderma* species showed antagonism to *F. moniliforme* (*22*). Radial colony extension of *F. moniliforme* cultured alone continued to increase throughout a 14-day measurement period on laboratory media (*22*). However, radial extension of *F. moniliforme*

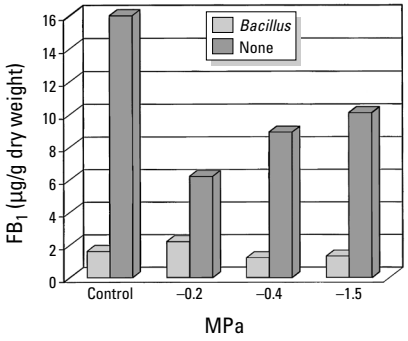

Figure 6. Fumonisin production in 10-week-old roots of *F. moniliforme*-infected maize plants of Trucker's Favorite, either coinfected with *B. subtilisor* or uninfected.

colonies in coculture with the *Trichoderma* sp. was decreased. The isolate of the *Trichoderma* sp. suppressed growth of *F. moniliforme* colonies with time, increasing from the 46% suppression observed on day 6 to a maximum of 91% by day 14 (*22*).

Control of Fumonisin Accumulation with *Trichoderma* sp.

Trichoderma species are common soil inhabitants. Their saprophytic nature and the absence of parasitism with plants suggest that this genus does not include biotrophic species; therefore an endophytic habit is impossible. However, no large-scale study has been conducted of endophytism in the *Trichoderma* species, so it is possible that our isolate and others may have this potential. Sumner (*61*) obtained *T. viride* from plants disinfected with sodium hypochlorite, which suggests an endophytic habit. However, the concentration of sodium hypochlorite was low, producing ambiguous data. Nevertheless, Kleifeld and Chet (*62*) proposed that *T. harzianum* can live in plants, where it enhances seed germination and promotes plant growth and flower production. Microscopic data indicate that this species was at least a localized systemic root infection (*62*), i.e., epibiotic. Thus, there is no documentation that *Trichoderma* species are endophytic. As an epibiont, *T. harzianum* enhanced germination and growth for bean, radish, tomato, pepper, and cucumber plants following inoculation with the fungus.

The interaction of *F. moniliforme* with *Trichoderma* sp. for fumonisin production on maize kernels was studied with *F. moniliforme* RRCPAT and RRCPATgus cultured on autoclaved yellow maize (454 g, moisture content 45%) incubated in the dark in 2.8-L Fernbach flasks (Fisher Scientific, Pittsburgh, PA) (*22*). Each flask was inoculated and incubated according to the following schedule: *F. moniliforme* RRCPAT for 12 weeks; *F. moniliforme* RRCPATgus for 12 weeks; *Trichoderma* sp. for 12 weeks; *F. moniliforme* RRCPATgus for 1 week; *F. moniliforme* RRCPAT and *Trichoderma* sp. for 12 weeks; *F. moniliforme* RRCPATgus and *Trichoderma* sp. for 12 weeks; and *F. moniliforme* RRCPATgus for 1 week and *Trichoderma* sp. for an additional 11 weeks (Table 5).

Samples of *F. moniliforme* RRCPATgus-inoculated maize kernels were harvested after only 1 week incubation to compare FB_1 production during this time with that in maize kernels to which *Trichoderma* sp. was added to 1-week-old cultures of *F. moniliforme*. The objective of incubating *F. moniliforme* for a week before adding *Trichoderma* sp. was to determine the efficacy of *Trichoderma* sp. in suppressing FB_1 production in maize kernels already contaminated with *F. moniliforme*.

The FB_1 concentration was 20 µg/g maize for kernels harvested after 7 days following *F. moniliforme* inoculation. Thus, the *Trichoderma* sp. did not immediately inhibit FB_1 production but did significantly reduce FB_1 production in comparison to cultures with *F. moniliforme* alone (Table 5). FB_1 production by the WT and GUS-transformed *F. moniliforme* incubated on maize kernels for 12 weeks was not significantly different. Furthermore, these strains did not differ in sensitivity to the isolate of the *Trichoderma* sp. (data not shown). The FB_1 produced during 12 weeks co-incubation of *Trichoderma* sp. with the fungi was reduced by > 80%. Thus, the insertion of the foreign genes for hygromycin resistance and GUS synthesis into *F. moniliforme* RRCPATgus did not influence FB_1 production or sensitivity to *Trichoderma* sp. in comparison to the parental WT, RRCPATwt.

FB_1 accumulation was reduced even in maize kernels on which *F. moniliforme* PATgus had been growing 7 days before the addition of the *Trichoderma* isolate (Table 5). FB_1 production was 64 µg/g of kernels with this inoculation procedure, a 72% reduction in fumonisin compared to that produced by *F. moniliforme* RRCPATgus growing alone (206 µg/g dry weight).

The current results provide the first evidence for activity of a species of *Trichoderma* as a suppressor of toxin synthesis. These results also support earlier reports that certain strains of *Trichoderma* sp. inhibit *F. moniliforme* growth (*60*). Our isolate of *Trichoderma* sp. suppressed *F. moniliforme* growth by 46% after 6 days (*22*) in comparison to the 10% suppression by strains described earlier (*53*). We have demonstrated that this *Trichoderma* sp. grew on maize kernels and in the process reduced FB_1 production. Even in the presence of *Trichoderma* sp., FB_1 content was above the maximum limit recommended by the American Association of Veterinary Laboratory Diagnosticians for fumonisins in feed for horses at 5 µg/g; pigs at 10 µg/g; and both beef cattle and poultry at 50 µg/g (*63*). However, under natural conditions, fumonisin content in the *F. moniliforme*-infected kernel would not be expected to reach the levels used for our experimental analyses.

Our studies demonstrated that this *Trichoderma* sp. meets several criteria essential for an effective biocontrol agent (*64*). One feature is that the biocontrol agent must colonize the substrate or plant part targeted by the pathogenic organism. The isolate of the *Trichoderma* sp. grew on maize kernels, the part of the maize plant most commonly associated with the fumonisins in causing harmful effects on animal and human health. The cocultivation of *F. moniliforme* and the isolate of *Trichoderma* sp. on maize kernels fulfilled another criterion in that the biocontrol agent must be active under environmental conditions such as pH and temperature, so that growth of the biocontrol agent and antagonist coincide. Another criterion is that the biocontrol agent must be compatible with other control procedures. The interactions of the isolate of this *Trichoderma* sp. with other pest control practices and ecosystem diversifications have not been analyzed. However, previous studies have indicated that species of *Trichoderma* may be more effective in certain ecological niches, such as specific soil types.

The primary purpose of this biocontrol organism is as a postharvest control in kernels in storage. Further, the biocontrol potential for this isolate is more suited for toxin reduction in maize kernels intended for animal feed. The successful activity of the fungus on kernels in storage will depend on air, moisture, and temperature requirements for this isolate. Thus, many more ecological parameters must be determined for this isolate of *Trichoderma* sp. before conclusions can be reached regarding the conditions under which this strain could function as an effective biocontrol agent for *F. moniliforme*.

Summary

The endophytic hyphae of *F. moniliforme* are neither latent nor dormant but are important for the vertical transmission of the fungus from generation to generation through seed, and it serves as a source of fumonisin *in planta*. The endophytic hyphae are neither latent nor dormant but instead physiologically

Table 5. Fumonisin B_1 production on maize kernels by WT and transformed *F. moniliforme* alone or with the *Trichoderma* sp.[a]

Inoculum	FB_1 (µg/g)[b]	FB_1 reduction by *Trichoderma* (%)[c]
Trichoderma sp.	0.0	NA[d]
F. moniliforme PATgus (7 days incubation)	20a (8)	NA
F. moniliforme PATwt	226b (44)	NA
F. moniliforme PATgus	206b (33)	NA
F. moniliforme PATwt and *Trichoderma* sp. (inoculated simultaneously)	42cd (17)	81
F. moniliforme PATgus and *Trichoderma* sp. (inoculated 7 days later)	64c (2)	72
F. moniliforme PATgus and *Trichoderma* sp. (inoculated simultaneously)	31ad (7)	85

Abbreviations: gus, transformed; NA, not applicable. [a]Data from Yates et al. (*22*). [b]Letters not common for FB_1 (µg/g) concentrations indicate the means are significantly different by ANOVA, means separation by LSD ($p < 0.05$), and numbers in parentheses are ± SD of the means. [c]Reduction expressed as the percentage FB_1 produced by either *F. moniliforme* PATwt or PATgus after 12-week incubation. [d]Harvested after 7 days incubation; all other treatments were harvested after 12 weeks.

active. Using transformed fungi, we demonstrated the maternal transmission of this fungus from seed to plant and back to seed under gnotobiotic conditions. The results indicated that during the life cycle of the fungus within its host tissue, the endophytic hyphae of the fungus responds to abiotic stresses imposed on the plant such as drought and flooding. Under these two stresses synthesis of the fumonisins increases. Plants maintained under uniform moisture—i.e., the controls—produce very little fumonisin. An endophytic bacterium and a fungus are being tested as pre- and postharvest biocontrols of fumonisin accumulation, respectively. The bacterium, a strain from a subgroup of *B. subtilis*, operates under the principle of competitive exclusion, reducing the fumonisin concentration *in planta*. The fungus *Trichoderma* sp. is being tested for postharvest control of fumonisin production in kernels during storage. The use of *Trichoderma* as a biocontrol is expected to have application for maize kernels especially for animal feed. We suggest that both organisms have high potential for controlling not only *F. moniliforme* but also several fungal diseases of maize. However, both biocontrol organisms still have ecological parameters that must be determined before their use in controlling growth and reducing fumonisin production by *F. moniliforme* can be commercialized.

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29. Seedling growth and inoculation procedures. Sterile kernels were inoculated with bacteria (10^4–10^6 cfu/mL) cultured on nutrient broth for 48 hr. The seedlings were grown during a 10-week period aseptically in culture tubes, 20 × 3 cm, containing sterile soil, in a plant growth room under 16 hr of light (an average of 256 μE • m^{-2} • s^{-1} at 21–26°C). All plants were fertilized with a sterile commercial nutrient solution (6-6-12) and/or watered with sterile water. Experiments designed to determine drought stress used aqueous solutions of polyethylene glycol (average molecular weight 20,000) in perlite (*25*). The matric potentials of this planting mix were adjusted to –0.2, –0.4, –1.5 MPa (*26*) to impose mild (–0.2, –0.4 MPa) and physiological drought (–1.5MPa) stress that lasted for 14 days. Plants in each treatment group were harvested for fumonisin analysis.
30. Toxin analysis. Fumonisin content in plant and kernel materials was based on dry weight of plant materials extracted (usually 5.0 g) with a solvent consisting of acetonitrile and water (1:1, vol/vol) in the ratio of 2–5 g plant material to 10 mL extraction solvent (*27*). Fumonisin B_1 was quantified by fluorescence detection after derivatization with *O*-phthalaldehyde and high-performance liquid gas chromatography separation following a previously described procedure (*27*). All experiments were run in duplicate with three replicates in a randomized complete block design. FB_1 was analyzed (*27*) and all concentrations were transformed to $\log_{10}$ and analyzed by analysis of variance and mean separation by LSD (*22*).
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