Effects of Deoxynivalenol on Content of Chloroplast Pigments in Barley Leaf Tissues


First author: Cereal Disease Laboratory, U.S. Department of Agriculture, Agricultural Research Service, St. Paul, MN 55108; second, third, and fourth authors: South Central Agricultural Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Lane, OK; and fifth author: Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108.

Accepted for publication 31 August 2009.

ABSTRACT


To understand further the role of deoxynivalenol (DON) in development of Fusarium head blight (FHB), we investigated effects of the toxin on uninfected barley tissues. Leaf segments, 1 to 1.2 cm long, partially stripped of epidermis were floated with exposed mesophyll in contact with DON solutions. In initial experiments with the leaf segments incubated in light, DON at 30 to 90 ppm turned portions of stripped tissues white after 48 to 96 h. The bleaching effect was greatly enhanced by addition of 1 to 10 mM Ca2+, so that DON at 10 to 30 ppm turned virtually all stripped tissues white within 48 h. Content of chlorophylls a and b and of total carotenoid pigment was reduced. Loss of electrolytes and uptake of Evans blue indicated that DON had a toxic effect, damaging plasmalemmas in treated tissues before chloroplasts began to lose pigment. When incubated in the dark, leaf segments also lost electrolytes, indicating DON was toxic although the tissues remained green. Thus, loss of chlorophyll in light was due to photobleaching and was a secondary effect of DON, not required for toxicity. In contrast to bleaching effects, some DON treatments that were not toxic kept tissues green without bleaching or other signs of injury, indicating senescence was delayed compared with slow yellowing of untreated leaf segments. Cycloheximide, which like DON, inhibits protein synthesis, also bleached some tissues and delayed senescence of others. Thus, the effects of DON probably relate to its ability to inhibit protein synthesis. With respect to FHB, the results suggest DON may have multiple roles in host cells of infected head tissues, including delayed senescence in early stages of infection and contributing to bleaching and death of cells in later stages.

Additional keywords: trichothecene toxin.

Fusarium graminearum, the principal cause of Fusarium head blight (FHB) in North America, is a necrotrophic pathogen, causing browning, bleeding, and premature death of barley and wheat heads (6,31,45). Grain yield and quality are reduced significantly (43,45). In the course of disease development, the fungus produces lesions in florets of the barley or wheat head. In barley, these may be entirely brown or have yellow or white centers surrounded by a brown ring (6,31). In heavily infected heads, lesions enlarge and coalesce to cover entire florets.

As FHB develops, F. graminearum produces deoxynivalenol (DON) and other trichothecene toxins that can accumulate to toxic levels, reducing suitability of grain for use by nonruminant animals and humans (11,15). DON is an inhibitor of protein synthesis in eukaryotic organisms and has been shown to induce programmed cell death (PCD) in mammalian cells (40,42). The toxicity of DON and other trichothecene toxins to plants has been shown by yellowing, browning, and necrosis of toxin-treated tissues (10,12,38). In addition, uptake of trypan blue (14) and electrolyte loss (9,29,30) indicate damage to plasmalemmas. DON also inhibits growth of wheat or Arabidopsis as measured in roots, coleoptiles, or callus cultures (5,10,34,35,44) and inhibits mitosis and cell division in roots (39).

The effects of DON in plants are often attributed to its ability to inhibit protein synthesis. DON inhibits protein synthesis in maize and wheat tissues (8,42). In mammalian leukocytes, DON induces signaling pathways leading to PCD and also to immune responses (40). In both cases, DON binds to target sites on ribosomes. In line with this, sensitivity to DON in protoplasts, cells, and tissues of tobacco was reduced when the target sites were modified in transgenic plants (16,25,37).

The toxic effects of DON suggest it has a role in FHB pathogenesis. Mutants of F. graminearum unable to produce DON or other trichothecene toxins initiated disease in wheat or barley heads, but had reduced ability to develop within heads (11,13,18), resulting in reduced necrosis and bleaching (4). In line with this, a mutant of F. graminearum, unable to produce toxin, was stopped by host wall thickenings, a defense response in the rachis of wheat heads (26). A toxin-producing strain inhibited formation of the wall thickenings, allowing the fungus to spread in the head. Overall, however, the cytological, physiological, and molecular effects of DON in either healthy or diseased tissue are not described sufficiently to reveal in what ways the toxin may promote disease development.

Toward a more complete understanding of the complex effects of DON in plant tissues and the roles the toxin plays in FHB, we observed changes induced by DON in detached barley leaf segments. In initial experiments, the toxin turned some tissues white as if it had a specific effect on chloroplast pigments. However, results were highly variable and DON sometimes had an opposite effect, preserving instead of reducing leaf pigmentation. Here we describe both of these effects of DON as well as experimental factors that influenced DON activity. We also investigated leakage of electrolytes and uptake of Evans blue as indicators of damage to the plasmalemma in relation to effects of DON on chlorophyll content.

doi:10.1094/PHYTO-100-1-0033
This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2010.
**MATERIALS AND METHODS**

**Preparation and treatment of leaf segments.** Experiments were done with primary leaves of *Hordeum vulgare*, cv. Robust, which is susceptible to FHB. The plants were grown from seed in a germination mix (Strong-Lite, Pine Bluff, AR) or a composted greenhouse soil in 7.5 or 10 cm plastic pots in a chamber with 16 h/day of HO and VHO cool white fluorescent light at 140 to 160 µmol m⁻²s⁻¹, as measured at the top of pots. Temperature was 21 ± 2°C. Seven days after planting, primary leaves were harvested and treated with toxin generally as described by Tada et al. (46). Epidermis was stripped with forceps from portions of the abaxial leaf surface, exposing mesophyll over 1/3 to 1/2 of leaf surface on each side of the midrib, which was not stripped. One to three segments 1.0 to 1.2 cm long were cut from each leaf, avoiding 1 cm at the leaf tip and any poorly stripped areas elsewhere. The segments were floated with the stripped surface down on 1 ml of test solution in glass dishes 2.5 cm in diameter and 0.8 cm high, or in some later experiments, in wells 2.5 cm in diameter and 2.1 cm high in 12-well polystyrene cell culture plates (Costar #3512 cell culture plate, Fisher, Chicago), which gave results similar to those in glass dishes. Three segments were placed in each dish or well. For incubation, the glass dishes (without covers) were placed in covered 9 cm petri dishes; lids on the 12-well plates were closed. The covered dishes and wells were incubated in a plant growth chamber (Model E54U; Percival, Boone, IA) under cool white fluorescent light at 150 to 170 µmol m⁻²s⁻¹ and incandescent light at 70 µmol m⁻²s⁻¹, totaling 220 to 250 µmol m⁻²s⁻¹. Lights were on for 16 h each day. Chamber temperature was 22 ± 1°C. Temperature within petri dishes was 25 ± 1°C when lights were on and 23 ± 1°C in dark periods, as measured with a shaded mercury bulb thermometer inserted horizontally through a hole in the side of a dish. A few experiments were done in a Model E15 plant growth chamber (Conviron, Winnipeg, Manitoba) with temperature and light adjusted to be the same as in the Percival chamber. DON was used at 10 to 90 ppm (µg/ml), equal to 33.7 to 303 µM. Cycloheximide was used at 30 to 90 ppm, equal to 107 to 320 µM. These and other components of test solutions were from Sigma-Aldrich (St. Louis, MO), except CaCl₂ was from Millipore Corp., Billerica, MA.

A typical experiment consisted of 96 leaf segments in 32 dishes or wells treated with four concentrations of DON. For each DON concentration, six leaf segments were collected for observation and analysis on each of 4 days after the start of treatment. For each day, segments representative of each treatment were photographed with a Minolta SRT 201 camera outfitted with a Rokker bellows. The photos were taken under incandescent light with Kodak T 35 mm color slide film. Photos were processed digitally for publication.

**Chloroplast pigments.** Relative chloroplast content was measured in intact stripped leaf segments with a chlorophyll content meter (Model CCM-200, OptiSciences, Tyngsboro, MA). This gave light absorbance at 660 nm, largely due to chlorophyll a, corrected for differences in leaf thickness and other factors affecting light absorbance, as determined by absorbance at 940 nm. The 10-mm-diameter circular orifice of the meter was covered with a disc of X-ray film, which had a 5 × 8 mm rectangular opening to delineate a zone used to measure chlorophyll content. The opening exposed portions of stripped and midrib areas of leaf segments for measurement. Except for the opening, the disc was covered with black adhesive shelf paper to block unwanted light.

For each experiment, relative chlorophyll content was calculated as the mean value for six leaf segments per treatment at each sampling time. Experiments usually were replicated two to eight times (as indicated) at intervals of one to several weeks. Results are given as mean ± standard error (S.E.) based on values among replicated experiments. Significance of differences among DON treatments was determined by two-way analysis of variance and Tukey multiple comparisons using R software (R Development Core Team, 2007: R Foundation for Statistical Computing, Vienna, Austria). Using data for all sampling times, each treatment (DON concentration) was paired with each of the other treatments within an experiment to find pairs with significant differences at P = 0.05.

Content of chlorophyll a, chlorophyll b, and carotenoid pigments was measured spectrophotometrically in leaf segment extracts. For this, leaf segments were prepared and treated with DON as described earlier, but 12 segments (instead of six) were prepared for each sampling time of a given treatment. The segments were removed from incubation dishes, blotted dry with filter paper, placed in 2 ml capped cryovial tubes, stored 1 to 11 days in a freezer at –80°C, and shipped frozen to the ARS-USDA South Central Agricultural Research Laboratory, Lane, OK, for spectrophotometric measurement of chlorophylls a and b and total carotenoids. Abbreviated methods of Lichtenenthaler and Buschmann (32) were used. For each measurement, two to three leaf segments (totaling 20 to 34 mg fresh weight) from each cryovial were weighed, combined with 2 ml of cold 100% acetone, and ground with mortar and pestle. The resulting slurry was transferred to a graduated centrifuge tube and brought to 5 ml with cold 100% acetone. The tubes were capped tightly and centrifuged for 5 min at 500 × g at room temperature. Supernatant, free of cellular debris, was transferred to 1 cm glass cuvettes and read on a scanning spectrophotometer (Shimadzu Model UV 160, Shimadzu, Columbia, MO). Absorbance was measured at 5 wavelengths: 750 nm for haze, 662 nm for chlorophyll a, 645 nm for chlorophyll b, 520 nm for phaeopigments (browning compounds), and 470 nm for carotenoids. Phaeopigments concentrations were below critical levels. The spectrophotometric separations of carotenoids were verified by high performance liquid chromatography. Amounts of chlorophylls a and b and total carotenoids were calculated using the formulas of Lichtenthaler and Buschmann (32). Two samples each with two to three segments were analyzed from a given cryovial. Reported results are based on the means for these duplicate samples.

**Conductivity.** To measure electrolyte loss from treated leaf segments, we measured electrical conductivity of solutions on which three segments per dish were incubated. Solution from each dish was diluted to 20 ml and conductivity measured as µmho/cm with a conductivity bridge (Industrial Instruments, Model RC 16B2) and a conductivity cell with S.I. constant 1.0 cm (Model 3403, YSI Hydrodata Ltd., Letchworth, UK). For each experiment, conductivity was measured for the solution in one dish for each DON concentration at each sampling time. The mean ± S.E. is reported for three to six replicated experiments, except for single values from an experiment with Ca(NO₃)₂ added to DON solutions. Differences among treatments in replicated experiments were analyzed for significance as described for relative chlorophyll content.

**Evans blue staining.** DON-treated leaf segments were stained with Evans blue, as used to detect dead or dying cells by uptake of the dye (20). Leaf segments were removed from DON solutions and incubated stripped side down for 20 to 30 min on 0.1% (wt/vol) aqueous Evans blue (Sigma-Aldrich) in 2.5 cm dishes. Leaf segments were washed briefly three times in water and mounted under a cover glass on a glass slide for observation. Mesophyll in stripped areas of leaf segments was observed with a Zeiss Standard bright field light microscope. A long working distance 40x objective lens (Nikon M Plan 40 0.5 ELWD) was used with 10x eyepieces, giving a field of view of 0.58 mm in diameter. Each leaf segment was examined at six sites in each of four rows within areas from which epidermis was removed. For each of the 24 sites, we counted the number of mesophyll cell lobes stained with Evans blue and total number of lobes. The use
of lobes instead of entire cells is explained in the Results section. For an individual experiment, the mean percentage of stained lobes was determined for two leaf segments. Reported values are for the means for three to seven such experiments. In addition, photos of representative fields of view were taken with a Nikon DXM 1200 digital camera.

**DON content in treated leaf segments.** Leaf segments were analyzed for DON using gas chromatography/mass spectrometry (GC/MS), as described by Mirocha et al. (36) for single kernel analysis. Each sample consisted of three partially stripped leaf segments which were incubated on DON solutions for 12 or 48 h. Segments were blotted dry, weighed, and frozen. After 1 to 3 weeks, segments were extracted in acetonitrile-water (84:16, vol/vol), cleaned, derivatized, and analyzed in a QP2010 GC/MS system (Shimadzu, Kyoto). The temperature program, which differed slightly from Mirocha et al. (36), was isothermal at

---

**Fig. 1.** Leaf segments floated 24 to 144 h on solutions of A, B, and D to G, deoxynivalenol (DON) or C, cycloheximide at indicated concentrations. A to E were incubated in light. F and G were incubated in dark. DON solutions were in A, B, and F, H2O. D and G, 10 mM Ca(NO3)2, or E, variously in H2O, 2 mM KNO3, 1 mM Ca(NO3)2, or 1 mM CaCl2. C, Cycloheximide solutions were in H2O.
150°C for 1 min, ramped at 30°C/min to 280°C, and held at that temperature for 4 min. Fragment ions at m/z 235.10, 259.10, 295.1, and 422.10 were used for selected ion monitoring. For quantification, a 10 point standard curve for 0.025 to 15 ng/µl DON was based on fragment ion 235.10. The DON standard was from Sigma-Aldrich. DON concentrations are given as ppm based on fresh weight of leaf segments. Mean values ± S.E. for each combination of applied DON and time of sampling are based on three replicated determinations from a single experiment.

RESULTS

To follow changes in relation to DON concentrations, we observed and photographed DON-treated leaf segments at 24 h intervals for 96 h. Presented first are experiments with segments incubated in light followed by results in darkness. Both were done with and without Ca(NO₃)₂, which had pronounced effects on results in light.

Incubation in light without Ca(NO₃)₂. Control leaf segments on 0 ppm DON without Ca(NO₃)₂ became chlorotic, losing virtually all visible chlorophyll by 96 h, as the segments became senescent after being detached from plants (Fig. 1A). DON at 10 ppm had no effect on this loss of chlorophyll in any of several trials. DON at 30 ppm induced scattered white spots and streaks at 48 to 72 h but also kept intervening areas greener than tissues in control segments through 72 h (Fig. 1A). DON at 90 ppm turned the entire stripped areas of some segments white at 72 to 96 h leaving tissues green in unstripped areas near the midrib and leaf edges (Fig. 1A). The white tissues appeared to lose virtually all chlorophyll and carotenoid pigment. However, other leaf segments at 90 ppm remained entirely green, often in the same incubation dish with white segments as shown for 72 and 96 h (Fig. 1A). These segments appeared to remain as green as segments were at 24 h. Thus, DON at 90 ppm had a twofold effect, causing loss of green and yellow pigments on one hand and retention of green on the other.

In several experiments with 20 to 90 ppm, DON gave white patches most consistently at 40 to 60 ppm (data not shown). The white patches were accompanied by water soaking in some segments, an indication that the treatment was toxic. However, DON concentrations of 70 to 90 ppm protected some segments against water soaking in addition to keeping segments green. In leaf segments incubated for extended periods (120 to 144 h) with DON at 40 to 50 ppm, tissues bordering white stripped areas sometimes turned reddish brown or dark brown (Fig. 1B). Unstripped tissues outside the border were chlorotic.

Leaves in most experiments were incubated at light intensity of 220 to 250 µmol m⁻²s⁻¹. Results were similar with 150 µmol m⁻²s⁻¹ but at 450 µmol m⁻²s⁻¹, white areas developed sooner and more abundantly than at lower intensities (data not shown). However, variation remained high at this high light intensity and temperature in incubating dishes could not be controlled adequately.

Cycloheximide, which like DON is an inhibitor of protein synthesis in eukaryotic cells (47), had effects on leaf segments much like those of DON. At 30 or 90 ppm, cycloheximide produced extensive white patches in segments incubated in light for 96 h (Fig. 1C). Again, nonwhite portions of leaf segments remained greener than water-incubated control segments which became chlorotic. Chloramphenicol, an inhibitor of protein synthesis in chloroplasts and prokaryotic organisms had little or no visible effect on leaf segments (data not shown).

Incubation in light with Ca(NO₃)₂. The bleaching effect of DON was markedly increased by addition of Ca(NO₃)₂ to DON solutions. Over a range of 0.1 to 100 mM, Ca(NO₃)₂ promoted loss of chlorophyll by 30 ppm DON (data not shown). The largest differences between DON-treated and control segments occurred

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Effect of deoxynivalenol (DON) on relative chlorophyll content of barley leaf segments incubated in **A and B**, light and **C and D**, dark. Chlorophyll content measured by light absorbance at 660 nm. Mean ± S.E. **A and C** were incubated on 0, 30, and 90 ppm DON in H₂O. **B and D** were incubated in 0, 10, and 30 ppm DON in 10 mM Ca(NO₃)₂. **A, 30 ppm DON differed significantly from 0 and 90 ppm DON at P = 0.05 (n = 3 to 8). B, 10 and 30 ppm DON differed significantly from 0 ppm DON at P = 0.05 (n = 6 to 8). C, 30 and 90 ppm DON differed significantly from 0 ppm DON at P = 0.05 (n = 3 to 8). D, 30 ppm DON differed significantly from 0 and 10 ppm DON at P = 0.05 (n = 6 to 8 except n = 2 for 10 ppm DON). Significant differences were determined by two-way analysis of variance-Tukey multiple comparisons.
most consistently at 10 mM, the concentration used in most experiments reported here. With 10 mM Ca(NO₃)₂, DON at both 10 and 30 ppm caused complete loss of color by 48 to 72 h as assessed visually in striped areas of leaf segments (Fig. 1D). Thus, DON at 10 ppm, which had no effect without Ca(NO₃)₂, was highly effective with Ca(NO₃)₂. Apart from promoting loss of chlorophyll pigments in response to DON, Ca(NO₃)₂ partially delayed chlorosis of control leaf segments incubated in light without DON (Fig. 1D).

CaCl₂ was as effective as Ca(NO₃)₂ in promoting DON-induced loss of leaf pigments as observed visually at 48 h (Fig. 1E). KNO₃ did not promote DON-induced loss of leaf pigments (Fig. 1E) but in the absence of DON, delayed yellowing (data not shown) much as described for Ca(NO₃)₂ earlier. The results indicate that Ca²⁺ promoted DON-induced pigment loss, whereas NO₃⁻ retarded chlorophyll loss in absence of DON.

**Incubation in the dark with Ca(NO₃)₂**. Control leaf segments incubated for 72 to 96 h in the dark on H₂O without either DON or Ca(NO₃)₂ became chlorotic (Fig. 1F), much as in light. On both 30 and 90 ppm DON, the loss of green pigmentation was prevented, as shown for 90 ppm (Fig. 1F). No white patches developed on either 30 or 90 ppm DON. However, some of the green leaf segments on 90 ppm DON became limp and showed water soaking by 96 h (Fig. 1F). As described later, electrolyte loss indicated tissues were damaged by DON in both light and dark, even though tissues in the dark retained most of their chlorophyll. Cycloheximide at 30 to 60 ppm likewise retarded loss of green pigment in the dark and caused some water soaking (data not shown).

**Incubation in the dark with Ca(NO₃)₂**. With Ca(NO₃)₂ added to 30 ppm DON, chlorophyll was usually retained in the dark (Fig. 1G) much as without Ca(NO₃)₂. Some tissues treated with DON again became water soaked and limp although without showing any bleached areas. In the dark, unstripped midrib portions of leaf segments seemed unaffected by DON as they became chlorotic without signs of injury, much like entire segments incubated without DON (Fig. 1G). Ca(NO₃)₂ without DON did not retard development of chlorosis in the dark as it had in light. Leaf segments yellowed by 72 to 96 h, as shown for 0 ppm DON (Fig. 1G).

**Relative chlorophyll content.** To confirm visual observations, we measured relative content of chlorophyll by absorbance of light in leaf segments. By 72 h, leaf segments incubated in light without DON or Ca(NO₃)₂ lost about 2/3 of the chlorophyll present at 24 h (Fig. 2A). DON at 90 ppm without Ca(NO₃)₂ had a negligible effect on this decline in chlorophyll content (Fig. 2A), although the treatment produced a mixture of white spots intermingled with tissue that appeared greener than controls as described earlier. On the other hand, DON at 30 ppm reduced chlorophyll content compared with treatments without DON (Fig. 2A), commensurate with presence of white spots and yellowing of other tissues.

Ca(NO₃)₂ accelerated DON-induced reduction in chlorophyll content in light (Fig. 2B). With DON at 30 ppm, most of the chlorophyll was gone by 48 h. With 10 ppm DON, most was gone by 72 h. As noted earlier, 10 ppm DON without Ca(NO₃)₂ had no visible effect in any of several trials. However, Ca(NO₃)₂ without DON tended to slow reduction in chlorophyll content (Fig. 2B) compared with controls on H₂O alone (Fig. 2A).

In darkness, leaf segments on 30 to 90 ppm DON without Ca(NO₃)₂ retained chlorophyll near initial values for 96 h (Fig. 2C), confirming visual assessments. With Ca(NO₃)₂, DON at 30 ppm was also effective in maintaining chlorophyll content (Fig. 2D). However, 10 ppm DON with Ca(NO₃)₂ was ineffective as was Ca(NO₃)₂ applied alone (Fig. 2D). The results confirm that DON can maintain chlorophyll content in darkness but that Ca(NO₃)₂ neither enhanced the effect of DON nor, by itself, affected chlorophyll content.
Electrolyte loss from DON-treated tissues. As an indicator of damage to DON-treated tissues, electrolyte leakage from leaf segments was determined by measuring the conductivity of solutions on which segments were floated. The solutions contained 0, 30, and 90 ppm DON, all without Ca(NO₃)₂. These incubating solutions initially had conductivity of $0.85 \pm 0.15$ µmho/cm. In light, conductivity increased slowly over 24 to 96 h for all treatments, but this increase was significantly greater for 90 ppm than for 0 or 30 ppm DON, reaching 13 µmho/cm (Fig. 3A). The segments on 30 to 90 ppm DON had patches of white tissue as shown earlier. In darkness, conductivity also increased without DON, but more rapidly with both 30 and 90 ppm DON, reaching 31 µmho/cm by 96 h (Fig. 3B). The leaf segments remained fully green, again as described earlier. Thus, the green leaf segments in darkness suffered more electrolyte leakage than partially white segments in light.

Conductivity was also measured in an experiment with 10 mM Ca(NO₃)₂ added to 10 ppm DON. This increased initial values of incubating solutions to 100 µmho/cm with or without DON. Without DON, tissues stayed mostly green as the incubating solution remained at 100 µmho/cm for 48 h, and then declined to 50 µmho/cm at 72 to 96 h (Fig. 3C), as if ions from Ca(NO₃)₂ were taken up by the leaf segments. With 10 ppm DON, tissues became white by 48 h as conductivity increased to 160 µmho/cm by 24 h and remained near that level through 96 h (Fig. 3C). Thus, these leaf segments released enough electrolytes to increase conductivity by 60 µmho/cm and possibly more if the segments took up ions from Ca(NO₃)₂ as apparently happened without DON. In either case, the net loss from leaf segments on 10 ppm DON in light at 24 to 48 h was greater and earlier in the presence of Ca(NO₃)₂ (Fig. 3C) than the loss on 90 ppm DON without it (Fig. 3A).

Uptake of Evans blue by DON-treated tissues. As a second way to evaluate damage in DON-treated leaf segments, we observed uptake of Evans blue microscopically. Leaf segments with portions of epidermis stripped away were floated on 10 and 30 ppm DON supplemented with 10 mM Ca(NO₃)₂. After 12 to 72 h, leaf segments were treated with Evans blue and examined for presence of the dye within mesophyll cells. In surface view, dye was visible in the lobes of the cells facing the exposed stripped surface of leaf segments. Each mesophyll cell usually had two or more lobes, but we could not discern which lobes belonged to a given cell. Consequently, dye uptake is presented for stained lobes as percentage of all lobes observed.

In control leaf segments not receiving DON, chloroplasts lined the walls of mesophyll lobes (Fig. 4A). A few lobes stained with Evans blue but always in less than 15% of all lobes (data not shown). After 12 h of treatment with 10 or 30 ppm DON, Evans blue stained 40 to 50% of lobes light blue, sometimes staining nuclei dark blue (as shown for 30 ppm DON, Fig. 4B). At this time, some chloroplasts were displaced from their usual position along the cell wall (not shown). After 18 h of treatment, 67 to 75% of lobes were stained and chloroplasts were frequently displaced from lobe walls, as shown for 10 ppm DON (Fig. 4C). After 48 to 72 h, 62 to 100% of lobes were stained and lobe contents appeared to be degenerate, exhibiting vacuolated vestiges of cytoplasm and chloroplasts as shown for 30 ppm DON at 48 h (Fig. 4D). However, a few darkly stained nuclei remained (not shown).

In darkness, results were similar to those in light. With 30 ppm DON, for example, 51% of lobes were stained after 12 h, 75%...
after 18 h, and 100% after 48 h. Thus, in both light and dark, DON rendered cells permeable to Evans blue. In both light and dark, the effect was detected within 12 to 18 h of treatment.

**DON concentration in treated leaf segments.** The concentration of DON within leaf segments treated with 30 ppm DON was measured after 12 to 48 h. When DON was applied with Ca(NO$_3$)$_2$, results were similar in light and dark. At 12 h, segments contained 8.8 ± 0.8 ppm DON in light and 8.7 ± 0.3 ppm in darkness. At 48 h, values increased to 14.7 ± 1.2 ppm in light and 13.4 ± 2.4 ppm in darkness. Without Ca(NO$_3$)$_2$, segments were again treated with 30 ppm DON but incubated only in light. At 12 h, they contained DON at 11.7 ± 1.8 ppm. At 48 h, they contained DON at 10.9 ± 1.0 ppm. Thus, DON content in light did not increase by 48 h as it did with Ca(NO$_3$)$_2$.

**DISCUSSION**

Against a background of gradual chlorophyll loss in detached leaf segments, we detected two opposing effects of DON that are relevant to potential roles in FHB. Treatments that were toxic, as judged by loss of electrolytes from leaf tissues and by Evans blue uptake, had a light-dependent bleaching effect expressed by loss of pigment in content of nearly all chlorophyll and carotenoid pigments. The effect was similar to bleaching in lesions produced in the palea and lemma of barley florets in FHB (6,31). Furthermore, the bleached areas sometimes had brown borders which resembled rings found around lesions on the palea and lemma in FHB (6,31). On the other hand, DON treatments that were not toxic, depending on DON concentration and other experimental factors, caused no injury but tended to prevent the gradual reduction in chlorophyll content of detached leaf segments. In these cases, DON delayed the senescence triggered by detachment.

When senescence was delayed in response to subtoxic concentrations of DON, such as 30 ppm applied without Ca$^{2+}$, chloroplasts, tonoplasts, and plasmalemmas remained intact as viewed by transmission electron microscopy (TEM) (7; W. Bushnell, D. Krueger, and T. Seeland, unpublished data). Ultrastructure of leaf cells was similar to that of freshly detached leaf segments. Cycloheximide, which like DON inhibits protein synthesis, also delayed senescence of barley leaf segments in our experiments, much as reported for detached oat leaves (33,47,50). With respect to FHB, we speculate that DON at subtoxic levels in the lemma and palea of infected floret tissues may have an effect similar to senescence delay, maintaining metabolic activity favorable for the initial biotrophic stage of disease development when the fungus interacts with living cells of the barley floret (6).

DON kept tissues green in two other experimental situations, although results in these cases are unlikely to be relevant to FHB. On one hand, leaf segments injured by toxic concentrations of DON remained green if the segments were incubated in darkness. Photooxidation of chlorophyll did not take place in the injured cells. This would not happen with respect to DON produced in FHB under daily periods of sunlight in the field. The second situation involved treatments in light with DON at much higher concentrations than normally required for toxicity, such as 90 ppm without Ca$^{2+}$. In this case, high DON concentrations seemed to override toxic effects, allowing expression of senescence delay. This anomalous effect seemingly could not occur in FHB, since DON would injure host tissues before reaching concentrations high enough to suppress toxicity.

The bleaching effect involving loss of chlorophyll and carotenoid pigments has not been reported for DON or other trichothecene toxins, except Desjardins et al. (12) who show a white leaf of *Arabidopsis* after treatment with an unidentified trichothecene toxin. However, reported symptoms of DON and other trichothecene toxins include yellowing, browning, and necrosis as noted earlier. In our experiments, tissues treated with toxic concentrations of DON lost chlorophyll a and b and carotenoid pigments in similar temporal patterns, except for negligible effects on carotenoid pigments at low DON concentrations (10 ppm). This loss of pigment was associated with degeneration of chloroplasts which was preceded by damage to tonoplasts and plasmalemmas (7), indicating that chloroplast degeneration was not a primary response to DON. Loss of electrolytes from DON-treated barley leaf tissues also indicated damage to the plasmalemma, much as reported for maize, muskmelon, and wheat (9,14,29,30). Membrane damage was also indicated by uptake of Evans blue in experiments with wheat (14). In darkness where DON-treated leaf segments remained fully green, tissues also leaked electrolytes and stained with Evans blue. Thus, damage to the plasmalemma was independent of light and pigment loss. Loss of pigment, on the other hand, was a secondary effect dependent on light but not required for toxicity.

The pronounced enhancement of DON toxicity by Ca(NO$_3$)$_2$ in our experiments can be attributed to Ca$^{2+}$ since CaCl$_2$ was effective and KNO$_3$ was not. Ca$^{2+}$ had no effect on DON uptake at 12 h when damage to the plasmalemma was first detected by Evans blue uptake, although Ca$^{2+}$ increased DON uptake at 48 h, possibly as a response to damage caused by the toxin. Otherwise, mechanisms of Ca$^{2+}$ action remain to be investigated. Nevertheless, our results with leaf tissues suggest that amounts of available Ca$^{2+}$ are likely to influence toxicity of DON produced in the palea, lemma, and caryopsis as FHB develops. Likewise, Ca$^{2+}$ may enhance toxicity in kernels, roots, leaves, and callus tissues used in bioassays to determine sensitivity of plants to DON.

Although not a trichothecene, the mycotoxin fumonisin B$_1$ produced by *F. verticillioides (=F. moniliforme*) caused photobleaching and electrolyte leakage in jimsonweed leaves (1). Results were remarkably similar to the effects of DON in barley leaves. However, fumonisin B$_1$ does not inhibit protein synthesis. Instead, it inhibits ceramide synthesis, disrupting sphingolipid metabolism (2). From examination of fumonisin-treated jimsonweed leaf tissues by TEM, Abbas et al. (1) concluded that membrane disruption may lead to loss of metabolic processes that normally protect chloroplast pigments, which is consistent with the sequence of events initiated by DON.

Cycloheximide, which inhibits protein synthesis in cytoplasmic ribosomes, had a dual effect much like DON in detached barley leaf segments. Low concentrations delayed senescence as discussed earlier; higher concentrations caused photobleaching, an effect not reported for cycloheximide. The similarity of their effects suggests that both DON and cycloheximide initiate events leading to senescence delay or photobleaching by inhibiting protein synthesis in cytoplasm. In contrast, chloramphenicol, an inhibitor of protein synthesis in chloroplasts (47), produced little or no bleaching in barley leaf segments in our experiments.

One emerging possibility is that inhibition of protein synthesis by DON initiates PCD. Cycloheximide and other inhibitors of protein synthesis have induced PCD (apoptosis) in animal cells (28,41,49). Laddering of genomic DNA indicated DON induced PCD in wheat leaves (14) as did induction of transcripts of genes involved in PCD (4). PCD, as a response of barley leaf tissue to DON, is suggested by the loss of the tonoplast as an initial event leading to cell death (7). Loss of this membrane is a key primary event in plant PCD (19,23,27). Furthermore, uptake of Ca$^{2+}$ into cytosol is a requirement for plant PCD (24,27), consistent with the enhanced toxicity of DON by addition of Ca$^{2+}$ in our experiments. Finally, fumonisin B$_1$, which parallels DON in effects on leaf tissues, induces PCD in plant and animal cells (3,22). PCD is postulated to be induced generally in plant diseases caused by necrotrophic pathogens (17,21,48).

The results here add to the list of ways that DON affects plant tissues. Bleaching provides visual evidence of degradation of chloroplasts and other components of cells, possibly as a result of PCD. Senescence delay indicates DON activates a response
separate from that leading to cell death. DON also has several other nontoxic effects, such as inhibition of cell division and growth as reviewed earlier, and inhibition of cell wall thickening formed as a defense response (26) and, conversely, activation of defense response genes (4). With potential for many varied effects, the roles of DON in development of FHB are likely to be multiple and complex. Regardless of this diversity of effects and of the precise sequence of events leading to loss of chloroplast pigments, bleaching is potentially useful as an indicator of the toxic effects of DON. For example, bleaching could be used for measuring differences in sensitivity to DON among barley genotypes. Any trials involving bleaching need to be done in light, preferably with attention to availability of Ca²⁺.

ACKNOWLEDGMENTS

This material is based upon work supported by the Wheat and Barley Scab Initiative of the Agricultural Research Service, U.S. Department of Agriculture. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the University of Minnesota and does not imply its approval to the exclusion of other products that also may be suitable. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture or the University of Minnesota. We thank Y. Dong and W. Xie for analysis of DON content in Fusarium head blight in spring wheat (Triticum aestivum). Can. J. Plant Pathol. 23:318-322.


Mitterauer, R., Poppenberger, B., Raditschung, A., Lucyszyn, D., Lemmens, M., Glössl, J., and Adam, G. 2004. Toxin-dependent utilization of...


Fusarium phytotoxin trichothecenes have an elicitor-like activity in Arabidopsis thaliana, but the activity differed significantly among their molecular species. Mol. Plant-Microbe Interact. 19:512-520.


