Chapter 10

Phytotoxicity of trichothecenes

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Many plant pathogenic species of the genus *Fusarium* produce trichothecenes, a large group of sesquiterpene epoxides that are inhibitors of eukaryotic protein synthesis. Although some *Fusarium* trichothecenes are virulence factors in plant disease, the phytotoxicities of many trichothecenes have only recently been investigated. Two test systems have been used to evaluate the structural features of trichothecenes that may impact phytotoxicity. Detached leaves of *Arabidopsis thaliana* plants and cultures of the unicellular plant *Chlamydomonas reinhardtii* were both treated with solutions of trichothecenes from a library of natural and synthetically modified trichothecenes. Results of these studies suggest that trichothecenes with a C-3 acetoxy group are generally less phytotoxic than those with a C-3 hydroxyl group but that other structural features may be important. Isotrichoderminol, the earliest trichothecene precursor of the mycotoxins T-2 toxin and deoxynivalenol, was toxic in both assays.
Trichothecenes are oxygenated sesquiterpene mycotoxins (Figure 1) produced by species of *Fusarium*, *Myrothecium*, *Trichothecium*, *Trichoderma*, and *Stachybotrys*. *Fusarium* trichothecenes all have an oxygen function at C-3 and can have additional oxygen functions at C-4, C-7, C-8 and C-15.

![General trichothecene structure. Numbers indicate carbons positions with oxygenation in Fusarium.](image)

**Figure 1.** General trichothecene structure. Numbers indicate carbons positions with oxygenation in *Fusarium*.

**Fusarium** Trichothecene Biosynthesis

The biosynthetic pathways of T-2 toxin in *Fusarium sporotrichioides* and deoxynivalenol or nivalenol in *F. graminearum* involve a sequence of oxygenation and esterification reactions controlled by up to 15 genes, most of which are localized in a 25 kB cluster (1, 2). Trichodiene, a hydrocarbon product of farnesyl pyrophosphate, is formed by the sesquiterpene cyclase, trichodiene synthase (3). Trichodiene (Figure 2) is oxygenated to form isotrichotriol (Figure 2) which then cyclizes to form isotrichodermol (3-hydroxytrichothecene) (Figure 2). The first four oxygenation steps are controlled by a single gene, *Tri4* (4). The pathway then proceeds through a series of oxygenation, esterification, and deacetylation steps to produce more complex trichothecenes such as 4,15-diacetoxyscirpenol (4,15 DAS), T-2 toxin, or nivalenol (Figure 2). One important feature of trichothecene biosynthesis in *Fusarium* is the early addition of an acetyl group at C-3. This acetyl group remains in place through the remaining oxygenations and esterifications (5).

Although a relatively large number of trichothecenes have been reported and structurally characterized, many of these compounds are minor products of large scale fermentations. A relatively small number of trichothecenes and related compounds have been examined for toxicity in animal or plant systems. Mutant strains of *F. sporotrichioides* and *F. graminearum*, generated during the characterization of trichothecene biosynthesis (5, 6, 7), have afforded an efficient means of generating a library of trichothecenes in quantities that are sufficient for phytotoxicity screens (8, 9). Additional derivatives have been prepared with biotransformation and synthetic modification.

**Trichothecene resistance**

Trichothecenes are inhibitors of protein synthesis in eukaryotes. Some trichothecene-producing fungi, such as *Myrothecium* and *Trichothecium*, have altered ribosomal proteins that render them less susceptible to their own toxins (10, 11). Yeast strains have also been identified that have an altered ribosomal protein L3 and are resistant to trichothecene mycotoxins (12). A modified L3
has been genetically engineered into tobacco to increase resistance to deoxynivalenol (13).

Research to determine how Fusarium protects itself from trichothecenes identified Tri101, an acetyltransferase gene that controls the addition of a C-3 acetyl group (14). Gene disruption of Tri101 resulted in the accumulation of isotrichodermol and indicated that the gene also controlled a key step in trichothecene biosynthesis (5). This acetyl group is removed by the Tri8 esterase as a final step in e.g. T-2 toxin biosynthesis (7). The acetyl group protects the fungus from its own toxin during biosynthesis and can be thought of as an off/on switch for toxicity.

Since trichothecenes have been identified as virulence factors in wheat head scab (15), a strategy for improving resistance to the fungal disease is to express genes for trichothecene resistance in plants. Tri101 has been engineered into tobacco (16), wheat (17), barley (18), and rice (19) in an attempt to introduce resistance to the toxin and thereby increase resistance to Fusarium. Engineering wheat and barley with Fusarium sporotrichioides Tri101 has been reviewed (20). Recent work has shown significant differences in substrate specificity for Tri101 proteins from F. sporotrichioides and F. graminearum. With deoxynivalenol as the substrate, the K_m values for F. graminearum TRI 101 and F. sporotrichioides TRI101 were 11.7 μM and 1463 μM, respectively (21). This suggests that trichothecene resistance could be greatly improved by expressing the F. graminearum Tri101 ortholog in plants.

![Structures of trichothecenes and the trichothecene precursors](image)

**Figure 2. Structures of trichothecenes and the trichothecene precursors trichodiene and isotrichotriol.**

**Phytotoxicity assays**

Toxicity studies on mycotoxins, including trichothecenes, have mostly focused on animals or animal cell lines. The identification of trichothecenes as virulence factors has increased interest in the phytotoxicity of these compounds. Trichothecenes are known to be inhibitors of protein synthesis but additional
effects have been noted including effects that include inhibition of mitochondrial function, electron transport, changes in membrane fluidity, reduced coleoptile growth, changes in seed germination, root and shoot growth, leaf chlorosis and necrosis, bleaching and degradation of chlorophyll (22). Bioassays have used whole plants, cell suspension culture and callus culture. Most bioassays have been limited by the availability of trichothecenes so often only a few compounds are tested. Many bioassays that were originally developed for work with environmental pollutants or herbicides should be adaptable for studies with mycotoxins.

**Chlamydomonas bioassay**

*Chlamydomonas reinhardtii* is a unicellular green alga that has been used as a model system for photosynthetic and other studies. It can be grown easily on a defined medium in the lab and has a rapid doubling time. It is amenable to transformation (23, 24) which may make it a viable system for screening genes for toxin resistance. *Chlamydomonas* sp. have been previously used in aquatic toxicology to assess the deleterious effects of metals and other pollutants (25). The algae have also been used to screen herbicides (26).

*C. reinhardtii* was used to assess the relative phytotoxicity of a group of trichothecenes (8) with either a C-3 hydroxy or C-3 acetoxy group. Cultures were initiated with 1 X 10^5 cells/ml on a high salt, high acetate medium (27) containing 80 µM of an individual trichothecene. Cells were counted after 8 days of growth and the number of doublings and doubling time were calculated. Of the fourteen trichothecenes tested, five compounds, isotrichodermol, 4,15-diacetoxyscirpenol (4,15-DAS), T-2 toxin, deoxynivalenol (DON), and 3-decalonectrin, inhibited growth and cell doubling of liquid cultures. One interesting finding from this survey was that the simple trichothecene, isotrichodermol, was as toxic as 4,15-DAS or T-2 toxin to *C. reinhardtii*. After 8 days, cultures grown in the presence of isotrichodermol had only 3 X 10^5 cells/ml compared to over 500 X 10^5 cells/ml in control cultures (8). Five C-3 acetoxy analogs, isotrichodermin, 3,4,15-TAS, 3-ADON, 3-acetyl T-2 toxin and calonectrin, were significantly less toxic. These data support the role that the C-3 acetyl group may have in controlling toxicity. Four other compounds tested, 8-hydroxy isotrichodermol, 8-hydroxy isotrichodermin, 3,15-didecalonectrin and 15-decalonectrin (8) were less phytotoxic (230-236 X 10^5 cells/ml after 8 days). This suggested that additional structural feature may lower toxicity.

**Effects of C-7 and C-8 oxygenation**

Two other sets of data comparisons suggest that additional hydroxylation decreases phytotoxicity. 8-hydroxyisotrichodermol and 3,15-didecalonectrin were less toxic than isotrichodermol. Similarly, 8-hydroxyisotrichodermin and 15-decalonectrin were less toxic than isotrichodermin (8).

In order to see if other trichothecene substitution patterns are correlated with phytotoxicity, some additional isotrichodermol derivatives were tested with
Chlamydomonas reinhardtii. C. reinhardtii cells were counted and cultures started with $1 \times 10^5$ cells/ml on a liquid high salt, high acetate medium as before (8, 27) but were supplemented with a higher concentration (100 μM final concentration) of the trichothecene to be tested and grown for a shorter time period – 5 or 6 days rather than 8 days. Table I shows the growth, number of doublings and doubling time for isotrichodermol and four related compounds. The 6 day incubation was adequate to see differences in toxicity. This screen identified three phytotoxic compounds, isotrichodermol (3OH), 7-hydroxy isotrichodermol (3,7-diOH) and 8-keto isotrichodermol (3OH 8keto), that strongly inhibited the growth of C. reinhardtii. 8-hydroxy isotrichodermol and 7,8-dihydroxyisotrichodermol (3,7,8 triOH) were less phytotoxic in this assay. The results indicated that addition of a C-7 hydroxyl or C-8 keto function did not affect toxicity but that C-8 hydroxylation decreased toxicity.

Table I. Effect of trichothecenes on C. reinhardtii growth after 6 days

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Cells (X 10^5)</th>
<th>Doublings</th>
<th>Doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75.6</td>
<td>6.2</td>
<td>23.1</td>
</tr>
<tr>
<td>3-OH</td>
<td>3.1</td>
<td>1.6</td>
<td>94.4</td>
</tr>
<tr>
<td>3,7 diOH</td>
<td>2.5</td>
<td>1.3</td>
<td>112.3</td>
</tr>
<tr>
<td>3,8 diOH</td>
<td>22.3</td>
<td>4.4</td>
<td>33.6</td>
</tr>
<tr>
<td>3,7,8 triOH</td>
<td>41.6</td>
<td>5.4</td>
<td>26.8</td>
</tr>
<tr>
<td>3OH 8keto</td>
<td>2.7</td>
<td>1.4</td>
<td>110.9</td>
</tr>
</tbody>
</table>

Phytotoxicity of Fusarium graminearum mycotoxins

The initial screen of fourteen trichothecenes with Chlamydomonas reinhardtii indicated that deoxynivalenol (DON) caused moderate growth inhibition ($79 \times 10^5$ cells/ml compared to $560 \times 10^5$ cells/ml for control cultures) (8). 3-ADON had growth rates similar to control cultures indicating that C-3 acetylation is an off/on switch for this toxicity.

Four additional deoxynivalenol and nivalenol compounds were tested against C. reinhardtii. The results of this study are shown in Table II and Table III. 15-ADON treated cultures had a growth rate similar to that of deoxynivalenol. Both compounds have a free C-3 hydroxyl group. Nivalenol (NIV) was the most toxic compound tested in this group, severely inhibiting growth to less than two doublings after six days. Acetylation of nivalenol to form 3,15-diANIV reduced this toxicity.
Table II. Effect of trichothecenes on *C. reinhardtii* growth after 5 days

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Cells (X 10^5)</th>
<th>Doublings</th>
<th>Doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.8</td>
<td>5.6</td>
<td>21.7</td>
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<tr>
<td>DON</td>
<td>28.2</td>
<td>4.6</td>
<td>27.1</td>
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<tr>
<td>3ADON</td>
<td>49.2</td>
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<td>21.4</td>
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<tr>
<td>15ADON</td>
<td>24.6</td>
<td>4.3</td>
<td>29.6</td>
</tr>
<tr>
<td>3,15diADON</td>
<td>38.3</td>
<td>5.4</td>
<td>22.1</td>
</tr>
</tbody>
</table>

Table III. Effect of trichothecenes on *C. reinhardtii* growth after 6 days

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Cells (X 10^5)</th>
<th>Doublings</th>
<th>Doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.9</td>
<td>6.2</td>
<td>23.4</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>3.2</td>
<td>1.6</td>
<td>100.6</td>
</tr>
<tr>
<td>3,15-diANIV</td>
<td>46.1</td>
<td>5.5</td>
<td>26.3</td>
</tr>
</tbody>
</table>

*Arabidopsis thaliana* model system

The second bioassay developed for evaluating trichothecene phytotoxicity used the small mustard plant *Arabidopsis thaliana*. *A. thaliana* is an attractive model system due to its short generation time as well as the availability of the complete genome sequence. In addition, *A. thaliana* is susceptible to infection by *Fusarium graminearum* (28, 29). A thorough study of the effects of three trichothecenes, 4,15-DAS, DON, and T-2 toxin on *A. thaliana* plants grown on toxin-amended agar indicated that both 4, 15-DAS and DON inhibited root growth (30). T-2 toxin caused stunted shoot growth and morphological changes to the leaves including reddening from anthocyanins. 4,15-DAS also inhibited seed germination, an effect also observed with tobacco seeds (16).

The *A. thaliana* detached leaf assay is a four week, labor-intensive process (9). Briefly, seeds of Columbia (Col-4) ecotype were surface disinfested with sodium hypochlorite and water. Seeds were then sown in a grid pattern on the surface of a Petri plates filled with Murashige-Skoog mineral medium adjusted to pH 5.9 in agar, and incubated 3 days in the dark at 4 °C. Plates and seeds were then incubated for an additional 11 days under fluorescent light at 25 °C. Agar blocks containing plants were cut from the Petri dish and transferred to 24 well plates and the plants were incubated for an additional week. Leaves with petioles were sliced from three-week old plants with a scalpel, floated in distilled water and then transferred individually to a well of 96 well microplates containing a solution of test solution in water. The treated leaves were scored for chlorosis after an additional week (9).

The study looked at 24 trichotheccenes ranging from trichodiene and isotrichotriol (Figure 2) to more complex oxygenated and acetylated compounds
Compounds were tested at five concentrations (0 to 100 μM) and dose response, LD$_{50}$ and ED$_{50}$ were calculated. This screen identified six relatively non-toxic compounds that showed little or not chlorosis at 100 μM and had an LD$_{50}$, the concentration at which 50% of the leaves were dead, over 100 μM: trichodiene, isotrichotriol, 3,15-didecalonectrin, 15-decalonectrin, scirpentriol and nivalenol (Figure 2). The most toxic compounds in this assay, with LD$_{50}$ under 10 μM, were isotrichodermol, calonectrin, 15-acetoxyiscirpenol, 4,15-DAS, HT-2 toxin, T-2 toxin, 3-acetyl T-2 toxin, 15-ADON and 3,15-diaceetynivalenol (9).

**Arabidopsis thaliana screen of toxin library**

Time and labor were a limiting factor in the number of trichothecenes originally tested with *A. thaliana* detached leaves. Additional compounds were screened in a similar manner but at a single concentration, 100 μM. Each leaf was scored after one week in a toxin solution as in the earlier study. The number of leaves that had more than 50% chlorosis (rating 3) or were dead were counted and this sum was used to calculate a %dead figure. This combined figure was previously used to calculate LD$_{50}$ (9). Two experiments with 48 leaves/compound were completed for each trichothecene or *Fusarium* metabolite. While this streamlined assay may miss subtle differences in toxicity, it was easy to distinguish between compounds that were toxic (>80% dead) and non-toxic (<10% dead) at 100 μM.

In addition to trichodiene and isotrichotriol, two other trichodiene derivatives, isotrichodiol and isotrichotetraol, and three non-trichothecene *Fusarium* metabolites, zearalenone, butenolide and culmorin were non-toxic to *A. thaliana* leaves.

T-2 toxin was the most phytotoxic trichothecene tested (LD$_{50}$ of 0.5 μM) in the first bioassay (9) and it caused 100% dead leaves in the second assay. Other neosolaniol-based trichothecenes (oxygenation at C-3, C-4, C-8, C-15), neosolaniol, 8-butyryl neosolaniol, 8-propionyl neosolaniol, HT-2 toxin, 3-acetyl T-2 toxin, as well as 4-deoxy T-2 toxin, caused substantial chlorosis and greater than 80% dead leaves.

4,15-DAS was also highly phytotoxic (LD$_{50}$ of 1.5 μM) in first assay (9) and killed all leaves tested at 100 μM in the second assay. 3,15-DAS caused more than 80% dead leaves in the second assay. Of the compounds tested with oxygenation at C-3, C-4, and C-15, only scirpentriol was relatively non-toxic (LD$_{50}$ > 100 μM) (9).

**Effect of additional hydroxylation**

The first screen of 24 trichotheccenes indicated that increased oxygenation was associated with lower phytotoxicity. Isotrichodermol was moderately toxic in the first bioassay with an LD$_{50}$ of 9 μM but 3,15-didecalonectrin and scirpentriol had reduced toxicity (9). In the second assay at 100 μM, isotrichodermol killed 60% of the leaves. Four additional hydroxylated
derivatives of isotrichodermol were tested at 100 μM, 7-hydroxyisotrichodermol, 8-hydroxyisotrichodermol, 7,8-dihydroxyisotrichodermol, and 4,8-dihydroxy-isotrichodermol, and each killed fewer than 10% of the leaves. 8-ketoisotrichodermol caused 14% dead leaves.

C-3 oxygenation

All Fusarium trichothecenes have a C-3 oxygen function as a result of the four oxygenations controlled by Tri4. Three Trichothecium roseum trichothecene metabolites that lack C-3 oxygenation were tested. Trichothecene (no extraskeletal oxygens) and trichothecolone (C4-OH, C-8 keto) were nontoxic (0% dead). The *T. roseum* mycotoxin trichothecin (C-4 butyryloxy, C-8 keto) however, was phytotoxic, causing 97.5% dead leaves. These results indicate that the trichothecene C-3 oxygen function is not required for phytotoxicity of Arabidopsis leaves.

Detoxification of trichothecenes

Acetylation of isotrichodermol to form isotrichodermin resulted in a decrease in phytotoxicity – from an LD50 of 9 μM to LD50 of 16 μM (9). Similar decreases in toxicity were observed between 4,15-DAS and 3,4,15-TAS, and 15-ADON and 3,15-diADON. In the second assay, isotrichodermin killed 40% of the leaves tested compared to 60% with isotrichodermol.

Both Chlamydomonas and Arabidopsis assays indicated that the C-3 acetyl group can reduce the phytotoxicity of some trichothecenes. However, this acetyl group can be removed by esterases in the fungus (7). There is ample evidence that plant esterases can remove C-3 and C-15 acetyl groups. For example, *Fusarium* strains can be characterized as 3-ADON producers or 15-ADON producers in culture, but *Fusarium*-infected grains are primarily contaminated with deoxynivalenol rather than one of the acetylated derivatives (31).

Two other derivatives of C-3 hydroxy trichothecenes were produced and tested with *A. thaliana* detached leaves. O-methyl derivatives of isotrichodermol and 4,15-DAS were prepared with methyl iodide. Neither 3-O-methylisotrichodermol nor 3-O-methyl 4,15 DAS caused any chlorosis of detached leaves at 100 μM (0% dead).

A soil bacterium has been isolated that can convert deoxynivalenol into 3-keto-deoxynivalenol (32). This compound had reduced immunosuppressive activity when compared to deoxynivalenol but the phytotoxicity of the 3-keto derivative was not determined. 3-keto derivatives of isotrichodermol and 15-ADON were prepared using a modification of the Swern oxidation method (33, 34). Both 3-keto products were completely non-toxic at 100 μM to *A. thaliana* leaves (0% dead).
Comparison of *Chlamydomonas* and *Arabidopsis* bioassays

There are some noticeable differences in the phytotoxicity of individual trichothecenes between the *Chlamydomonas* and *Arabidopsis* bioassays. C-3 acetylation significantly decreased the toxicity of five compounds in *Chlamydomonas* (8). Only C-3 acetylation of two of these compounds, 4,15-DAS and T-2 toxin, caused a large decrease in toxicity in *Arabidopsis* (9). These differences may be due to differences in uptake or in the relative metabolism of the compounds tested to less or to more toxic products. For example, glycosylation of a trichothecene C-3 hydroxyl group by a plant glycosyltransferase or removal of a C-3 acetyl group by a plant esterase could change which toxin was actually tested and at what concentration. Treated algal cultures and leaves were not examined for metabolism of the trichothecenes tested.

There was also a significant difference between the two assays for one pair of compounds, nivalenol and its acetylated derivative, 3,15-diacetylnivalenol. Nivalenol was not at all toxic to *A. thaliana* leaves (LD50 > 100) but 3,15-diacetylnivalenol was quite toxic (LD50 of 4.3 μM) (8). These compounds had the opposite effects on *C. reinhardtii* cultures. Nivalenol was quite toxic (Table III) but the 3,15-diacetyl derivative caused much less growth inhibition.

Targets for resistance

Although *Fusarium Tri101* has been the main focus for introducing trichothecene resistance into plants, other routes to trichothecene detoxification may lead to improved resistance. Glycosylation of the C-3 hydroxyl group has been correlated with moderate scab resistant lines derived from of Sumai-3 wheat and a glucosyltransferase has been isolated from *Arabidopsis thaliana* that can detoxify deoxynivalenol (35). Other possible detoxification proteins are C-3 oxidase (32), epoxide reductase (36), and epoxide hydrolase (37).

An alternate strategy for improving disease resistance is by chemically blocking, with plant metabolites or herbicides, trichothecene biosynthesis. The relatively simple trichothecene, isotrichodermol, was shown to be phytotoxic in both *Chlamydomonas* and *A. thaliana* bioassays. A single gene, *Tri4*, controls the conversion of trichodiene to form this toxic product (4). This suggests that inhibitors that block the TRI4 enzyme would effectively block the production of a phytotoxin and could decrease the virulence of the fungus. A screen of plant shikimates identified a number of compounds that block trichothecene T-2 toxin biosynthesis in *F. sporotrichoides* (38). To obtain the intermediate and TRI4 substrate trichodiene, inhibition of this enzyme by xanthotoxin was used (39). A yeast screening system for TRI4 inhibitors, using a synthetic trichodiene analog, identified flavone, xanthotoxin, and two other furanocoumarins as strong inhibitors of TRI4 activity (40).
Summary

This paper compared two bioassays for measuring the relative phytotoxicity of trichothecenes. Both Arabidopsis and Chlamydomonas are amenable to transformation and both phytotoxicity assays could be adapted to assess toxin resistance genes. Heterologous expression of genes in Chlamydomonas has been problematic due to its G-C rich codon bias (41). One goal of A. thaliana studies was to develop a model system for screening Arabidopsis ecotypes (28, 42) for new sources of resistance to trichothecenes, and to identify which toxin to use in such a screen. The detached leaf assay may not be the most efficient method for screening for phytotoxicity or trichothecene resistance in A. thaliana, but has the advantage of focusing on a single symptom of phytotoxicity, leaf chlorosis. Germinating and growing whole plants on toxin-containing media may be a more tractable assay if sufficient amounts of trichothecenes are available. Isotrichodermin (Figure 2) may be the best trichothecene for an initial screen of ecotypes. Using this compound will increase the likelihood that novel resistance genes can be found rather than acetyltransferase or glycosyltransferase genes.

The bioassays also indicated that the simple trichothecenes, isotrichodermol and isotrichodermin, are phytotoxic. Chemicals that target the TRI4 enzyme will block biosynthesis of the toxic trichothecenes and may increase plant resistance to Fusarium diseases in plants.

References


21. Garvey, G.S.; McCormick, S.P.; Rayment, I. Structural and functional characterization of the TRI101 trichotheccene 3-O-acetyltransferase from


