The Role of Deoxynivalenol and 15-Acetyldeoxynivalenol in Pathogenesis
by Gibberella zeae, as Elucidated Through Protoplast Fusions
Between Toxigenic and Nontoxigenic Strains

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ABSTRACT

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Deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) are
trichothecene mycotoxins produced by Gibberella zeae, a pathogen of
wheat, maize, and carnation. Low levels of DON inhibit protein synthesis
in maize and wheat. We tested the possible role of DON and 15-ADON as
virulence factors in inoculation experiments with regenerated products of
protoplast fusion of auxotrophs of a toxigenic, pathogenic strain (type A)
with auxotrophs of a nontoxicogenic, nonpathogenic strain (type B).
Nineteen independent fusion products and their parental strains were
evaluated for toxin formation and pathogenicity on four cultivars of maize
and one cultivar of carnation. Toxin production was analyzed by thin-layer
chromatography of extracts from infected maize and rice grains and by
axenic liquid cultures. Fourteen fusion products were nontoxic, but eight
of these were highly virulent ear mold pathogens of maize and stub dieback
pathogens of carnation. We conclude that DON and 15-ADON are not
pathogenicity or virulence factors for G. zeae on maize or carnation.

Gibberella zeae (Schw.) Petch (anamorph Fusarium graminearum
Schwabe) is a fungal pathogen of wheat, maize, and carnation. It causes root rot and seedling diseases (6,19), a head
and kernel blight of wheat called scab (13), stalk and ear rots of
maize (31), and a stub dieback of carnation (24). The pathogen
also produces several 12,13-epoxytrichothecenes mycotoxins, including
deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), and
the estrogenic mycotoxin zearalenone. The presence of these
compounds in grain is associated with swine feed refusal, vomiting,
and hyperoestrogenism (9,21,28,36). DON also causes skin
irritation, hemorrhaging, hematological changes, radiometric
effects, and severe immunosuppression in mammals (8,35). It is an
inhibitor of protein synthesis in animals (32). Similar
trichothecenes are toxic to many fungi (2,10,25,29) but not
bacteria (2,11).

G. zeae occurs in the north central United States as two
coeexisting populations (types A and B) in maize fields (7). The
populations differ in growth rate, cultural morphology, production of DON and 15-ADON, and pathogenicity on maize
ears and wheat heads. In nature, pathogenicity on maize ears and wheat heads appears to be correlated with the production of
DON and 15-ADON (7); only type A strains are pathogenic. Pathogenic
isolates produce DON and 15-ADON and sometimes low concen-
trations of zearalenone and are fast-growing. Type B strains are
not pathogenic on maize ears or wheat heads, do not produce DON
or 15-ADON, are slower-growing, and produce high levels of
zearalenone.

Recently, Casale (4) and Casale and Hart (5) showed that DON
inhibits protein synthesis in plants. This was determined by
monitoring isotope-labeled protein following incorporation of
3H-leucine in leaf disks and by cell-free translation systems from
maize and wheat. G. zeae produces DON beginning shortly after
infection of the maize ear and continuously throughout the
infection process (20).

The ability of DON to inhibit protein synthesis in plants suggests
a possible role in overcoming inducible plant defense mechanisms
during pathogenesis. We have hypothesized that DON or 15-ADON
is a toxin involved in plant pathogenesis, because of the association
between pathogenicity and DON production in many strains, and
because DON is toxic to plant cells. Arguing against a role in
disease is the fact that some strains of G. zeae that do not produce
DON or 15-ADON can cause seedling diseases and root rots of
many hosts (19). However, on developing grains of maize and wheat,
DON or 15-ADON could be required for disease; i.e., they
may be pathogenicity factors (30). Alternatively, DON or 15-ADON
may not be necessary to initiate disease but may increase disease
severity; i.e., they may be virulence factors (30).

Our objective was to test the role of DON and 15-ADON in
pathogenesis by the use of genetic manipulations of G. zeae. The
most straightforward approach is to induce strains of the pathogen
that produce no toxin, inoculate hosts, and observe for disease
development. G. zeae is a homothallic ascomycete, and,
unfortunately, type A strains do not outcross with type B strains
(1). Thus, sexual recombination was not available for selection of
toxinless progeny.

We have produced auxotrophic mutants of a toxigenic virulent
type A strain and a nontoxicogenic avirulent type B strain. Attempts
to form heterokaryons from types A and B by hyphal fusion and
complementation of auxotrophic mutants were prevented by
 genetic incompatibility systems (1). Therefore, protoplasts of the
auxotrophs were fused to form heterokaryons, and genetic
recombinants were selected from the regenerates (1).

We report herein the results of pathogenicity tests and analysis
for DON and 15-ADON production by wild-type parents, auxo-
	rophs, regenerated protoplast fusion products, and somatic
recombinants in vivo and in vitro.

MATERIALS AND METHODS

Fungal strains and protoplast fusion products. Two strains of
G. zeae and their derivatives were used for these studies: U-5373, a
type A strain from the Fusarium Research Center (16), and ATCC
20273, a type B strain from the American Type Culture Collection
(17). Auxotrophic mutants were induced with ultraviolet light
(1,17). Auxotrophs from the type A strain used in this study were
Lys and Try, requiring lysine and tryptophan, respectively, for
growth (1). The type B auxotrophs were Arg, requiring arginine
(17), and Nnu, a nitrate-nonutilizing mutant (18). Experimental
protocols for producing protoplasts and protoplast fusions have
been reported previously (1,17). All protoplast fusions were between an auxotroph of the type A strain and an auxotroph of the type B strain and were designated by combining the first initials of the two auxotrophs. For example, AT-la is a colony that regenerated following a fusion between the Arg- and Try- auxotrophs. The culture is from colony 1 on the regeneration plate and is an isolation from the fast-growing sector a, which later arose in a culture of AT-1 (1).

**Media.** The compositions of minimal medium, supplemented minimal medium (SPM), glucose-yeast extract-peptone medium (GYEP), and carboxymethyl cellulose (CMC) have been reported in detail elsewhere (1,3,27); 20 g of Bacto agar (Difco) per liter was added to solidify all media not stated to be liquid.

**Preparation of inoculum.** Macroconidia used for in vitro inoculations were produced by inoculating 50 ml of sterile liquid CMC in a 250-ml Erlenmeyer flask with a small amount of soil from a soil culture (33) of G. zeae. These flasks were incubated for 7 days at 25 C on a rotary shaker at 200 rpm. Macroconidia were harvested by pouring the CMC cultures through a 30-µm-mesh nylon screen (Tetko, Inc., Elmsford, NY) to separate the spores from the hyphal debris. The filtrate was then centrifuged at 5,000g for 10 min in sterile tubes. The pellet was resuspended in sterile water to a concentration of 10⁶ conidia per milliliter.

In vivo inoculations were made by inserting toothpicks colonized by the different strains of G. zeae through the husks and into the center of the ear (38). Toothpicks colonized by the auxotrophs were first autoclaved in SPM and then inoculated and incubated in petri plates containing SPM instead of minimal medium (15).

**Pathogenicity tests on maize ears and carnation stems.** Maize ears and carnation stems were inoculated with wild-type strains, auxotrophs, and protoplast fusion products of types A and B, to evaluate pathogenicity and measure the production of DON and 15-ADON. Four susceptible commercial sweet maize hybrids (Asgrow XP2547BC, Asgrow Aztec, Ferry Morse Bonanza, and Ferry Morse Style Pak) were planted for pathogenicity trials (15). These sweet maize lines have widely spaced differences in maturity and were used because of possible environmental influence on infection and production of DON and 15-ADON (12). Additionally, an inbred line, B79, which is especially susceptible to Fusarium ear rot (12), was included in some in vivo pathogenicity trials. Four ears, one per plant, were inoculated with each culture tested in each maize hybrid plot. The ears were inoculated as the addition neither 3-ADON, nivalenol, nor fusarenon-X were detected. The Arg- and Nnu- auxotrophs of type B also produced 15-ADON. Four susceptible commercial sweet maize hybrids of inoculation and analyzed for DON and 15-ADON. Extracts were determined by comparison to standards. Only the infected Karnals were collected around the point of inoculation and analyzed for DON and 15-ADON. Extracts were also compared to standards of nivalenol, fusarenon-X, and 3-acetyldiethylvalenol (3-ADON).

**Production of DON and 15-ADON in vitro.** The ability of strains to produce DON and 15-ADON was determined after growth of the fungi on cracked maize or rice grain. The procedure was as follows: 50 g (dry weight) of grain (35% moisture) was placed in each 250-ml flask, autoclaved for 30 min, inoculated with 1.0 ml of conidia (10⁶ conidia per flask), and incubated in the dark at 28 C for 14 days. The strains were also grown in stationary liquid GYEP, with 50 ml of GYEP per 250-ml flask, and analyzed for production of 15-ADON (16). DON is not produced in GYEP. Flasks were inoculated with 1.0 ml of conidia (10⁶ conidia per flask). The incubation time was 15 days at 28 C in the dark (16).

**Toxin analysis.** Methods used for analysis of DON and 15-ADON on grain from in vivo and in vitro experiments (14,16, 26,34,37) and from liquid cultures (27) have been reported in detail elsewhere. The minimum sensitivity of thin-layer chromatography (TLC) analysis of DON and 15-ADON was 100 ng. Gradations were determined by comparison to standards. Only the infected leaves in a maize ear were extracted for analysis. However, if there was no apparent infection, kernels were collected around the point of inoculation and analyzed for DON and 15-ADON. Extracts were also compared to standards of nivalenol, fusarenon-X, and 3-acetyldiethylvalenol (3-ADON).

**RESULTS**

**Characteristics of fungal strains and protoplast fusion products.** The type B parent (ATCC 20273) was avirulent on carnation and maize and never formed DON or 15-ADON in vitro (Table 1). In addition neither 3-ADON, nivalenol, nor fusarenon-X were detected. The Arg- and Nnu- auxotrophs of type B also produced no toxin and were not pathogenic. Other wild-type type B strains were weakly virulent on wounded carnation (data not shown); thus, ATCC 20273 might not be a typical representative of the type B group of G. zeae (7).

The type A parental strain (U-5373) was highly virulent on maize and 15-ADON production by the strain, thus, ATCC 20273 might not be a typical representative of the type

**TABLE 1. Pathogenicity of and production of the toxins deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) by parental strains and mutants of Gibberella zeae grown in vivo on maize and carnation and in vitro on maize, rice, and glucose-yeast extract-peptone medium (GYEP)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diebacka (cm)</th>
<th>Disease ratingb</th>
<th>DON (µg/g)</th>
<th>15-ADON (µg/g)</th>
<th>Disease ratingd</th>
<th>DON (µg/g)</th>
<th>15-ADON (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A parent</td>
<td>5.4</td>
<td>4.4</td>
<td>180</td>
<td>75</td>
<td>4.7</td>
<td>87.8</td>
<td>83.3</td>
</tr>
<tr>
<td>Lys</td>
<td>4.9</td>
<td>4.0</td>
<td>122</td>
<td>55</td>
<td>5.0</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Try</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Type B parent</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nnu</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

aLength of dieback of inoculated flower stem, in centimeters.
bPathogenicity ratings are the means for ears of maize hybrids (Asgrow XP2547BC, Asgrow Aztec, Ferry Morse Bonanza, and Ferry Morse Style Pak). Ears were inoculated in the field as the tassels turned brown. Not all isolates were tested on all four cultivars.
cMaize ears were rated for disease severity approximately 2-3 wk after inoculation. Disease ratings were made on a scale of 0-5, in which 0 = no infection, 1 = less than 10% of the ear moldy; 2 = 11-25%; 3 = 26-50%, 4 = 51-75%, and 5 = 76-100%.
dPathogenicity ratings are the means for susceptible maize ears (Asgrow Aztec, Ferry Morse Bonanza, inbred line B79, and an unknown cultivar). Not all isolates were tested on all four cultivars.

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and carnation and produced DON and 15-ADON in vivo and in vitro (Table 1) but did not form 3-ADON, nivalenol, or fusarenon-X. The two auxotrophs derived from this parent differed from one another. The lysine-requiring auxotroph (Lys-) was as virulent and toxigenic as the parent, even on maize, which is low in lysine. The tryptophan-requiring auxotroph (Try-) was avirulent, and thus no toxin could be found in vivo. However, the Try- mutant produced DON and 15-ADON in culture in vitro, in amounts similar to those produced by the wild-type parent (Table 1).

Pathogenicity and toxigenicity of protoplast fusion products. Table 2 summarizes the results of pathogenicity tests and TLC analysis for DON and 15-ADON production. Pathogenicity and virulence on carnation generally paralleled that on maize ears. Producing auxotrophs and avirulent, nonproducing auxotrophs did not consistently express virulence or toxin production as a dominant or recessive trait. Similarly, fusions between the virulent, nonproducing type B Arg- and the virulent, toxin-producing type A Lys- yielded three phenotypes: virulent toxin producers (more than 10 strains), virulent nonproducers (eight strains), and avirulent nonproducers (four strains); none were avirulent toxin producers. Fusions between avirulent strains, Arg- and Try-, yielded only virulent strains. Thus virulence was not a dominant or a recessive trait. A similar situation was true for toxin production.

Toxin analysis and production of DON and 15-ADON in vitro and in vivo. Production of DON or 15-ADON by a strain was generally greater on field-inoculated maize ears than in laboratory-inoculated maize ears, but strains caused more extensive infection in the laboratory than in the field (Table 2). Toxin production on

### Table 2. Pathogenicity of and production of the toxins deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) by selected products of protoplast fusions between auxotrophs of type A and type B strains of Gibberella zeae grown in vivo on maize and carnation and in vitro on maize and glucose-yeast-extract-peptone medium (GYEP)

<table>
<thead>
<tr>
<th>Strain</th>
<th>In vivo characteristics</th>
<th>In vitro characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carnation stub dieback&lt;sup&gt;a&lt;/sup&gt; (cm)</td>
<td>Maize ears in the field</td>
</tr>
<tr>
<td></td>
<td>Disease rating&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DON (μg/g)</td>
</tr>
<tr>
<td>AT-11</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>AT-9</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>AL-1e</td>
<td>5.3</td>
<td>2.5</td>
</tr>
<tr>
<td>AL-6a</td>
<td>4.3</td>
<td>3.5</td>
</tr>
<tr>
<td>AL-3i</td>
<td>4.1</td>
<td>3.5</td>
</tr>
<tr>
<td>AL-7</td>
<td>5.4</td>
<td>3.5</td>
</tr>
<tr>
<td>AL-3g</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>AL-1c</td>
<td>3.9</td>
<td>2.7</td>
</tr>
<tr>
<td>AL-4c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AL-5c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AL-6c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AL-2c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AL-5d</td>
<td>5.1</td>
<td>3.5</td>
</tr>
<tr>
<td>AL-1d</td>
<td>6.2</td>
<td>4.0</td>
</tr>
<tr>
<td>AL-2b</td>
<td>4.9</td>
<td>4.0</td>
</tr>
<tr>
<td>AL-3c</td>
<td>2.3</td>
<td>4.5</td>
</tr>
<tr>
<td>NL-3e</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NL-11f</td>
<td>6.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not shown are the more common products of protoplast fusions of type B Arg- × type A Lys- that were virulent and toxin-producing and the more common fusion of type B Nnu- × type A Lys- that were avirulent and nonproducing.

<sup>b</sup>Protoplast fusions designated AL-, AT-, and NL- were derived from fusions of the pairs of auxotrophs (type B) Arg- × (type A) Lys-, (type B) Arg- × (type A) Try-, and (type B) Nnu- × (type A) Lys-, respectively.

<sup>c</sup>Length of dieback of inoculated flower stem, in centimeters.

<sup>d</sup>Pathogenicity ratings are the means for ears of maize hybrids (Asgrow XP2547BC, Asgrow Aztec, Ferry Morse Bonanza, and Ferry Morse Style Pak). Ears were inoculated in the field as the tassels turned brown. Not all isolates were tested on all four cultivars.

<sup>e</sup>Maize ears were rated for disease severity approximately 2–3 wk after inoculation. Disease ratings were made on a scale of 0–5, where 0 = no infection, 1 = less than 10% of the ear moldy, 2 = 11–25%, 3 = 26–50%, 4 = 51–75%, and 5 = 76–100%.

<sup>f</sup>Pathogenicity ratings are the means for susceptible maize ears (Asgrow Aztec, Ferry Morse Bonanza, inbred line B79, and an unknown cultivar). Not all isolates were tested on all four cultivars.

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infected carnation stems in vivo could not be analyzed with available assays, because of insufficient plant material.

Generally, production of DON or 15-ADON was greater in vivo than in vitro. Occasionally in toxin analysis of inoculated cracked maize, faint and obscure spots appeared on TLC and made the interpretation of strains as non-toxigenic uncertain. Because cracked maize could have a natural trace contamination with DON or 15-ADON, in vitro toxin production of a fresh grain was added as a check test for specific isolates. Rice is a grain that is not susceptible to infection by G. zeae in nature. No nivalenol, 3-ADON, or fusarenon-X was detected in any in vivo or in vitro extraction.

**DISCUSSION**

We have described these fusion products as likely heterokaryons (1). The fusion products are prototrophic and easily reisolated from field-inoculated maize or carnation and are identifiable by their unusual colony morphology (1). Only one prototrophic nuclear phenotype is resolved when conidia or hyphal tips of the fusion products are isolated (1). The nuclear phenotype in all cases differs from the phenotypes of the parental auxotrophs, suggesting diploidy. However, each protoplast fusion product differs somewhat in cultural characteristics from other fusion products of the same two auxotrophs (1). Additionally, different sectors of faster-growing mycelium isolated from an initial fusion colony also differ in characteristics. The results of pathogenicity and toxin formation reported herein further demonstrate the variability among fusion products of the same two auxotrophs. Each of the protoplast fusion products differed in some aspects, such as toxin production, pathogenicity, virulence, colony characteristics, color, and formation of perithecial initials, regardless of whether or not they were produced from the same pair of auxotrophs. Such behavior is evidence that the fusion products are not diploids, because in a diploid a full complement of both genomes from the two fused auxotrophs would demonstrate a consistent phenotype. Virulence and toxin production would be expressed in diploids or heterokaryons if they were dominant.

Our data reveal an unusual pattern of inheritance or expression. This pattern of expression does not fit the phenotypes expected for a balanced heterokaryon or a diploid fusion product. The range of phenotypes in expression in fusion products would agree well with the genetic model of an unbalanced heterokaryon. However, single conidia and hyphal tips from these fusions are prototrophic (1), and this precludes an unbalanced heterokaryotic genotype. We observed that fusion products that expressed virulence or avirulence also shared cultural characteristics apparent in the virulent type A or the avirulent type B, respectively. Characteristics of type A included aerial red hyphae and perithecial initials. Characteristics of type B included apressed purple-red hyphae, pinnate, and the absence of initials. Thus, virulence was inherited with other parental characters of the virulent parent. Examination of the cultures of the fusion products led us to conclude that each product was dominated by one of the original nuclear types, with a few extra characteristics from the “subordinate” nuclear type. The data presented in Table 2 provide evidence to suggest that the fusion products are heterokaryons (parasexual recombinants with chromosome numbers ranging from nullisomics and aneuploids through the euploid series that are formed following hybridization). Normally, parasexual recombination is a rare event, but the heterokaryons formed in this study occurred with every fusion of type A protoplasts with type B protoplasts; all regenerated products were apparently heterokaryons. Genetic recombinations and formation of a hybrid strain following fusion of protoplasts has been documented similarly in *F. oxysporum* (22,23).

In vitro tests were essential in discovering whether lack of toxin production was innate or due to loss of virulence on a host. In vitro toxin production and analysis revealed that Try- was innately capable of producing high quantities of DON and 15-ADON, but the avirulent phenotype in vivo masked this ability, because it failed to grow. Production of low or undetectable quantities of DON and 15-ADON in vivo by AT-9 and AT-11 was evidently due to the low level of infection and colonization of maize ears, because in vitro tests revealed that these fusion products were innate high producers, approximating equivalent to the wild-type type A parent (Table 2). The loss of virulence in Try-, even though DON and 15-ADON were produced in vitro, was an indication that the toxins have no role in pathogenesis. Additionally, this mutant was valuable for determining whether virulence was dominant or recessive in a heterokaryon or protoplast fusion product. The fusion of Try- with Arg restored pathogenicity and toxin production on the host plant. This suggests that the loss of pathogenicity of the Try- auxotroph was most probably caused by auxotrophy and that virulence was expressed as dominant in fusion products derived from Try- and Arg but not in other fusions. The expression of virulence was controlled by the genetic background of the fusion product.

Of many protoplast fusion products screened for pathogenicity, 19 were selected for further study, and of these, 14 were found to be nonproducers of DON or 15-ADON. Eight of the 14 were also highly virulent; in addition, the Try- strain was nonpathogenic but produced a high level of 15-ADON in culture. The high virulence of nonproducers clearly proves that the nonspecific protein-synthesis-inhibiting toxins DON and 15-ADON are not required for pathogenicity. Additionally, protoplast fusion products with low virulence and high production of DON and 15-ADON (AT-9 and AT-11) are evidence that the toxins are not factors in virulence. Overall, it appears that the correlation of virulence with the production of toxicologically significant levels of DON or 15-ADON in natural populations of *G. zeae* and the correlation of virulence to maize ears with lack of toxin production is a phenomenon unrelated to mechanisms of pathogenesis (7). We suspect that the mechanisms for pathogenesis of *G. zeae* are the same for both maize and carnation, because strains were qualitatively and quantitatively similar in pathogenicity and virulence on the two hosts.

We conclude that DON and 15-ADON are not pathogenicity factors in these plant diseases, regardless of their effects on inhibiting plant protein synthesis and presumably any host mechanisms of defense activated after infection. The data also eliminate DON and 15-ADON as virulence factors, because ratings of the severity of disease on the host were not correlated with the amount of toxin produced. The role of trichothecene mycotoxins in the biology of *G. zeae* remains to be elucidated.

**LITERATURE CITED**