International Journal of Food Microbiology. 280: 24-29 (2019).

The final publication is available at www.elsevier.com via https://doi.org/10.1016/j.ijfoodmicro.2018.08.026

Short Communication

Inhibition of *Fusarium* trichothecene biosynthesis by yeast extract components extractable with ethyl acetate

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Abstract

While *Fusarium graminearum* readily produces trichothecenes in complex media containing sucrose as the carbon source (YS_60), the amount of the mycotoxin is quite limited when other sugars, such as glucose and fructose, are used. We found that autoclaving of media containing fructose and yeast extract (YF_60) results in the formation of inhibitors of trichothecene biosynthesis by *F. graminearum* JCM 9873, a strain that produces 15-acetyldeoxynivalenol (15-ADON) in liquid culture. Removal of the solvent fraction from the autoclaved media after ethyl acetate extraction attenuated the inhibitory activity against trichothecene production. In addition, extraction of the non-autoclaved complex media with ethyl acetate, followed by removal of the solvent fraction, similarly resulted in increased accumulation of the mycotoxin. Although the increase in trichothecene production differed considerably among fungal strains and yeast extract products, *F. graminearum* species complex generally responded to the medium treatments in the same way. These results suggest that some hydrophobic substances that arise during the drying and heating of yeast extract negatively affected trichothecene production in liquid culture. Modes of actions of inhibitory substances were partially characterized using strain JCM 9873, with focus on the transcriptional and functional analyses of *Tri6*, a key regulator gene in trichothecene biosynthesis. The presence of the ethyl acetate-extractable substances in autoclaved YF_60 media decreased the relative transcription level of *Tri6*, as well as that of a trichodiene synthase gene *Tri5*. Thus, the substances exerted their inhibitory action through suppression of *Tri6* expression. By using a yeast extract lot that completely prevented trichothecene production by the wild-type strain in autoclaved YS_60 medium, we prepared YF_60 media and cultured a constitutive *Tri6* overexpressor strain described by Maeda et al. (2018). Despite the high transcription level of *Tri6*, the presence of the ethyl acetate extractable-substances suppressed 15-ADON production. These results suggested that both Tri6p-independent initial activation of *Tri6* expression and

subsequent Tri6p-dependent activation of *Tri* expression were affected by the hydrophobic substances in the yeast extract products.

Keywords:
Complex medium; *Fusarium graminearum*; Inhibitor; Maillard reaction; Trichothecene mycotoxin; Yeast extract

Chemical compounds:
Chemical compounds studied in this article
15-Acetyldeoxynivalenol (PubChem CID: 10382483); Fructose (PubChem CID: 5984); Sucrose (PubChem CID: 5988)

Abbreviations:
15-ADON, 15-acetyldeoxynivalenol; NBP, 4-(*p*-nitrobenzyl)pyridine; TEPA, tetraethylenepentamine

Trichothecenes are mycotoxins produced by *Fusarium* and other fungal species, which are mostly plant pathogens (Desjardins et al., 1993; Kimura et al., 2007). The environmental factors that affect trichothecene biosynthesis vary depending on species and strain. For the *Fusarium graminearum* species complex (except the strain NBRC 4474 from our strain collection), sucrose is typically an inducing molecule for trichothecene biosynthesis (Nakajima et al., 2016). While most strains produce trichothecenes in YS_60 medium containing 6% (w/v) sucrose and 0.1% (w/v) Bacto™ yeast extract, trichothecene production is marginal when sucrose is substituted by other sugars. In addition to differences in the composition of the medium, the type of vessels in liquid shake culture also considerably influences the yield of trichothecenes (Nakajima et al., 2014). For example, 24-well plate culture with gyratory shaking imposes somewhat different physiological properties on the fungus and stimulates mycotoxin biosynthesis much stronger than culture in Erlenmeyer flasks (Nakajima et al., 2016).

In assessing the role of sugars in the activation of trichothecene biosynthesis, we found that yeast extract lots and brands considerably affect the activity of sucrose added to YG_60 medium, which contains glucose as the carbon source, that is required to induce trichothecene production in a 24-well plate culture (Nakajima et al., 2016; Sørensen et al., 2014). This suggests that regulation of mycotoxin biosynthesis is subject to subtle differences in medium composition, especially in those of nitrogen sources (Maeda, Nakajima, Tanahashi, et al., 2017). In addition, as autoclaving is usually used for preparation of axenic liquid culture, this denaturing process may also cause some changes in medium composition. Thus, to evaluate the influence of this sterilization method on trichothecene biosynthesis-inducing ability, we examined if such a heat treatment affects the level of mycotoxin accumulation in liquid culture. To this end, we compared the amount of trichothecenes that accumulated during culture in autoclaved and aseptically filtered media.

As the result, we found that yeast extract products contain inhibitory substances against trichothecene production. We attempted to characterize them using ethyl acetate, a solvent widely used to separate hydrophobic compounds from aqueous solutions. Some inhibitory activity could be eliminated by partitioning with the solvent, excluding the possibility of trichothecene-production promoting compounds being degraded by heating. To gain insight into the mode of actions of inhibitory substances, we focused on *Tri6*, a key transcription factor gene necessary for trichothecene biosynthesis (Proctor et al., 1995),.

2. Materials and methods

2.1. Chemicals and solvents

Ethyl acetate and high-performance liquid chromatography (HPLC)-grade solvents were purchased from Kanto Chemical Co. (Tokyo, Japan). Toluene, 4-(*p*-nitrobenzyl)pyridine (NBP), and tetraethylene pentamine (TEPA) were obtained from Wako Pure Chemicals (Osaka, Japan). Kieselgel F_{254} TLC plates (Silicagel 60 F_{254}) were purchased from Merck (Darmstadt, Germany). Bacto™ yeast extract was purchased from BD Diagnostics (Sparks, MD). Yeast extract BSP-B was from Oriental Yeast Co. (Tokyo, Japan). Minisart-Plus™ 0.22 μm sterile filter was purchased from Sartorius AG (Göttingen, Germany).

2.2. Strains, media, and culture conditions

F. graminearum JCM 9873 (Japan Collection of Microorganisms) used in this study substantially produces 15-acetyldeoxynivalenol (15-ADON) as the sole trichothecene product under the culture conditions (Nakajima et al., 2014). 3-Acetyldeoxynivalenol (3-ADON) and nivalenol (NIV) chemotype strains (NBRC and MAFF numbers) were obtained from NBRC (NITE Biological Resource Center, Kisarazu, Japan) and NARO (National Agriculture and Food Research Organization, Tsukuba, Japan) Genebank.

Media containing all the necessary ingredients (i.e., 6% sugar and 0.1% yeast extract) were sterilized by autoclaving for 15 min at 121 ^{o}C or aseptic filtration through Minisart-Plus™, and used for the main culture for trichothecene production. For preparation of inocula for the main culture, the spore suspension was added to 50 mL YG medium (2% glucose and 0.5% Bacto™ yeast extract) at a final density of 10^4 spores/mL in a 200-mL Erlenmeyer flask and incubated with reciprocal shaking at 125 strokes/min for 16 h. One milliliter of pre-culture was then inoculated into 100 mL of YS_60 (6% sucrose and 0.1% Bacto™ yeast extract) and into other main culture media, in which the sucrose of YS_60 medium was substituted by 6% (w/v) of glucose (YG_60) or fructose (YF_60). The main cultures thus prepared were incubated on a 1-mL scale using a 24-well plate at 25 °C with gyratory shaking at 135 rpm (Nakajima et al., 2014). Bacto™ yeast extract lot #1186275 was used for preparation of the media unless otherwise noted.

2.3. Analysis of 15-ADON

For semi-quantitative detection of trichothecenes, ethyl acetate extract from 0.6 mL liquid culture was developed on a TLC plate using ethyl acetate:toluene (3:1) as the solvent; 15-ADON was visualized with NBP/TEPA as previously described (Takahashi-Ando, Tokai, et al., 2008). For quantification of 15-ADON, the dried ethyl acetate extract dissolved in 25% (v/v) acetonitrile was analyzed by HPLC-UV as previously described (Nakajima et al., 2014). The limit of detections (LODs) of 15-ADON by TLC and HPLC-UV were 50 ng and 1 ng, respectively.

2.4. Real-time reverse transcription-quantitative PCR (RT-qPCR)

Total RNA isolation was performed as described previously (Etzerodt et al., 2015). cDNA was synthesized from the total RNA ($A_{260}/A_{280} > 1.77$) by using a reverse transcriptase (ReverTra Ace® qPCR RT Master Mix with gDNA Remover; TOYOBO,

Osaka, Japan) following the manufacturer's instruction. For each culture (YF_60 prepared by 5 different methods), 12 wells of a 24-well plate were combined and used to collect the necessary amount of mycelia for the RNA extraction. Real-time PCR was performed using the LightCycler 1.5 Instrument (Roche Diagnostics Japan, Tokyo, Japan) with THUNDERBIRD® SYBR® Master mix kit (TOYOBO) and specific primers for the reactions (Supplementary Table S1). The expression of the ubiquitin-conjugating enzyme gene (FGSG_10805; *Ubc*) was used as an endogenous reference. The crossing point values (*Cp*) and PCR efficiencies (E = 2.06, 2.04, and 2.06 for *Tri6*, *Tri5*, and *Ubc*, respectively) were experimentally determined and relative abundances of *Tri* mRNA to *Ubc* mRNA were determined as described previously (Pfaffl, 2001; Ponts et al., 2007). The reactions were carried out in triplicates from an RNA sample in LightCycler® Capillaries (20 µL) with 0.5 µL of the reverse transcription reaction mixture and 0.3 µM of specific primers for each gene. The parameters for the reaction consisted of an initial hot start PCR step at 95 ºC for 30 s, followed by 40 cycles of denaturation at 95 ºC for 5 s, annealing and extension at 60 ºC for 45 s, and fluorescence detection at the end of each cycle. The absence of non-specific PCR products was confirmed by melting curve analysis.

3. Results and discussion

F. graminearum JCM 9873 produced 15-ADON in the YS_60 medium, but not in YG_60 and YF_60 media (Fig. 1; TLC at upper panel), as previously reported (Nakajima et al., 2016). However, by aseptic filtering through a 0.22-µm sterile Minisart-plus™ filter instead of autoclaving for medium preparation, 15-ADON was detected in the YG_60 and YF_60 cultures as well (Fig. 1; TLC at lower panel). To determine whether such differences in trichothecene accumulation were correlated with

fungal growth on the media, mycelial dry weight (MDW) and toxin amount were measured over the 12-day time course. Although the growth of the fungus on the autoclaved medium tended to be slightly delayed, there were no statistically significant differences in the MDW between the autoclaved and aseptically filtered media (Fig. S1A). In contrast to such small differences in growth, the amounts of 15-ADON in aseptically filtered media were higher than those in the autoclaved media (Fig. S1B). These results suggest that glucose and fructose may also be used as the sole sugar source of the media for trichothecene production in a 24-well plate if thermal treatments are not applied to the media containing Bacto™ yeast extract.

Among the sugars of the above-mentioned media, only sucrose included in the YS_60 medium is a non-reducing sugar. It may be possible that products of the Maillard reaction (MR) , i.e., the condensation of amino groups of amino acids and peptides with carbonyl groups of reducing sugars (Hodge, 1953), suppressed trichothecene biosynthesis by *F. graminearum*. In addition to amino acids, Bacto™ yeast extract [16.3% (w/w); BD Nutrients Technical Manual] contains carbohydrates, where some reducing sugars are likely to be present. This could explain the slightly reduced amount of 15-ADON in the culture in autoclaved YS_60 medium than in filter-sterilized YS_60 medium (Fig. 1 and Fig. S1B). Indeed, trichothecenes were no longer detected from the culture in YF_60 medium prepared by mixing yeast extract and fructose solutions autoclaved separately (data not shown). To clarify the underlying mechanism responsible for the lack of trichothecene biosynthesis induction by heat sterilization of the media, we focused on the process of preparing the YF_60 medium containing fructose, a good reactant of the MR (Suarez et al., 1989).

3.1. Ethyl acetate-extractable substances that inhibit trichothecene biosynthesis are produced by heating YF_60 medium

As shown in Fig. 2A, the YF_60 medium was prepared by five different methods: (1) autoclaving for 15 min, (2) autoclaving for 15 min, followed by aseptic filtration, (3) autoclaving for 15 min, with elimination of hydrophobic substances by ethyl acetate extraction, followed by aseptic filtration, (4) autoclaving for 15 min, with recovery of hydrophobic substances by ethyl acetate extraction, reconstitution of the concentrated extract in the new YF_60 medium, followed by aseptic filtration, and (5) aseptic filtration. Autoclaving caused brownish yellowing of the medium, which was not observed by aseptic filtration (Fig. 2B). The YF_60 media prepared by these different methods were inoculated with the YG pre-cultures of strain JCM 9873, distributed onto a 24-well culture plate, and incubated as described under Fig. 1 for 5 days. Interestingly, the removal of some hydrophobic substances from the autoclaved medium using ethyl acetate [method (3); Fig. 2A] led to partial recovery of the trichothecene-inducing ability relative to the non-autoclaved YF_60 medium [Fig. 2A and C; compare methods (2), (3), and (5)]. In addition, jellied oil droplets firmly adhered to the surface of the tube [method (4)] during condensation of the ethyl acetate extract (Fig. 2A). If some of the inhibitory substances were deposited as insoluble particles [depicted as purple dots in a test tube in method (4)], then it follows that the concentrated ethyl acetate extract [depicted as light orange layer in a test tube in method (4)] contains only a part of the extractable substances with inhibitory activity against trichothecene production. The jellied oil droplets were insoluble in all of the tested solvents, including ethanol, methanol, acetonitrile, and ethyl acetate, and we were not able to fully reconstitute the inhibitory activity against trichothecene production originally occurring in the autoclaved medium [Fig. 2A and C; compare methods (2), (4), and (5)].

The MR is a series of sequential and parallel reactions accelerated by heating. In the early stage, sugar aldehydes react with amino groups to form Schiff base, leading to the formation of other early glycation products 1,2-enaminol and Amadori compounds. Then, through a series of chemical rearrangement, dehydration, and fragmentation

reactions, some early glycation products are further converted to the advanced glycation end products (AGEs), such as N^{ε}-carboxymethyl-lysine, pyrraline, pentosidine, and melanoidins (Bastos et al., 2015; Grandhee et al., 1991). As solvent partitioning of autoclaved medium did not clearly separate an active fraction containing inhibitory substances of trichothecene production (Fig. 2), the inhibitors may be comprised of mixtures of MR products (MRPs) with different polarities.

Some MRPs generate reactive oxygen species (ROS), while others, especially melanoidins, possess a strong activity of scavenging ROS, such as hydroxyl radicals, hydrogen peroxides, and superoxides (Hayase et al., 1989). To examine whether inhibition of trichothecene production is attributed to different degrees of ROS levels, we examined intracellular H_2O_2 and superoxide anions as described previously (Maeda et al., 2018). However, the amounts of these ROS were at similar levels in the cultures of both autoclaved and filter-sterilized YF_60 medium (data not shown). Thus, other properties of the heat-sterilized medium may be responsible for the inhibition of trichothecene biosynthesis.

3.2. Suppression of Tri6 expression caused reduced accumulation of 15-ADON in autoclaved YF_60 medium

In trichothecene-producing *Fusarium* species, the transcription of genes for trichothecene biosynthesis (*Tri* genes) is dependent on a Cys_2His_2 zinc finger transcription factor encoded by *Tri6* (Proctor et al., 1995). Tri6p activates the transcription of trichothecene pathway genes, such as *Tri5*, encoding trichodiene synthase (Hohn et al., 1989), and *Tri4,* encoding trichodiene oxygenase (McCormick et al., 2006; Tokai et al., 2007). To clarify whether the inability of the fungus to produce trichothecene in autoclaved YF_60 medium is mainly attributed to the suppression of *Tri6* transcription (Etzerodt et al., 2015; Maeda et al., 2016), rather than inhibition of trichothecene pathway enzymes (Maeda, Nakajima, Motoyama, et al., 2017;

Takahashi-Ando, Ochiai, et al., 2008), the effects of different methods of medium preparations were investigated. We used the aforementioned methods, (1) - (5), for preparation of the YF_60 medium (Fig. 2), and the transcription of *Tri6* and *Tri5* in each culture was quantified by RT-qPCR. Among the five cultures, the relative expression levels of both *Tri6* and *Tri5* at 60 h were highest in the mycelia grown in the YF_60 medium prepared by method (5), followed by method (3), and the transcript levels of the *Tri* genes were quite limited in the culture prepared using other methods (Fig. 3). These results indicate that ethyl acetate-extractable hydrophobic substances that arose by autoclaving fructose and yeast extract inhibited the transcription of *Tri6*, and hence caused the lack of transcriptional activation of *Tri5* and trichothecene accumulation in the medium.

3.3. Increased trichothecene production by eliminating inhibitory substances from the non-autoclaved complex media using ethyl acetate

Using different brands and lots of yeast extract products, we prepared YF_60 and YS_60 media for trichothecene production in three different ways: (i) autoclaving [method (1); Fig. 2A], (ii) aseptic filtration [method (5); Fig. 2A], and (iii) aseptic filtration after elimination of hydrophobic substances by ethyl acetate extraction [omitting the autoclave step from method (3); Fig. 2A]. When the YF_60 (Bacto™ lots #1186275 and #3189145) and YS_60 (BSP-B lot #031103050-3) media were used for the main culture, there were no differences in growth of the fungus. Although *F. graminearum* JCM 9873 did not produce trichothecenes in the media prepared by autoclave sterilization [Fig. 4 (i)], it did produce 15-ADON in the filter-sterilized YF_60 (Bacto™ lots #1186275 and #3189145) medium [Fig. 4 (ii)]. Compared to the use of the yeast extract Bacto™ lot #1186275 (average yield of 48 µg/mL 15-ADON), trichothecene production in the YF_60 medium was lower when the lot #3189145 was used (average yield of 12.1 µg/mL) [Fig. 4 (ii)]. With yeast extract BSP-B lot

#031103050-3, no trichothecenes were detected even in the YS_60 culture, which contained the inducer sugar sucrose as the carbon source. The amount of trichothecenes that accumulated in the fungal culture increased considerably by adding the ethyl acetate extraction step prior to inoculations into any medium, although the recovery of toxin production obtained with yeast extract BSP-B lot #031103050-3 was lower compared to the other products [Fig. 4 (iii)].

To examine whether the effects of different medium preparations on trichothecene production are also observed for other strains in the *F. graminearum* species complex, we analyzed the metabolites of NBRC 5269, NBRC 7520, MAFF 101551, and MAFF 240560, 3-ADON chemotype strains, and MAFF 240548, a NIV chemotype strain (Fig. S2). When the Bacto™ yeast extract products were used for YF_60, trichothecene production was higher in aseptically filtered medium than in autoclaved medium with all the strains examined; the ethyl acetate extraction process more or less led to an increase in trichothecene production. When using the yeast extract BSP-B that was not suitable for the toxin production even in the presence of sucrose (i.e., YS_60 medium), the ethyl acetate extraction step did not clearly improve trichothecene accumulation in these *Fusarium* cultures (Fig. S2) as did so in the JCM 9873 culture (Fig. 4). These results suggest that the removal of inhibitory substances originally present in the yeast extract products ameliorates the mycotoxin production-inducing ability of the complex media and that the presence or absence of such effects might be determined by the chemistry of yeast extracts and *Fusarium* strains. Drying and heating processes of yeast extracts during manufacturing may stimulate formation of hydrophobic substances that negatively affect trichothecene biosynthesis.

3.4. The inhibitory substances produced by autoclaving of media negatively affect Tri6p function in YF_60 medium

Although the inhibitory substances in the complex media suppressed *Tri6* expression (Fig. 3), it is not known whether they also suppressed the transcription of *Tri* genes by inhibiting Tri6p function. Indeed, dihydroartemisinin is known as an inhibitor that suppresses both Tri6p-independent initial activation of *Tri6* expression and subsequent Tri6p-dependent activation of *Tri* expression (Maeda et al., 2018). We thus compared the amount of 15-ADON that accumulated in the culture of *Tri6*$^{O/E}$ #2s1, a constitutive *Tri6* overexpressor strain derived from JCM 9873 (Maeda et al., 2018), by using the YF_60 (BSP-B lot #031103050-3) medium. When the medium was sterilized by autoclaving, use of BSP-B lot #031103050-3, the yeast extract product with extremely weak trichothecene production-inducing ability (Fig. 4), completely prevented accumulation of the mycotoxin even by the overexpressor strain [Fig. 5 (i)]. The overexpressor produced 2.1 µg/mL of 15-ADON when cultured on YF_60 prepared by filter sterilization [Fig. 5 (ii)], although the amount was quite limited compared to those when the wild-type JCM 9873 strain was grown in filter-sterilized YF_60 media with yeast extract Bacto™ lots #1186275 and #3189145 [Fig. 4 (ii)]. When the medium was sterilized by aseptic filtration after removing the ethyl acetate-extractable fraction, the amount of 15-ADON increased to 3.9 µg/mL [Fig. 5 (iii)]. These results suggest that some hydrophobic substances included in the yeast extract BSP-B lot #031103050-3 negatively affected Tri6p-dependent activation of *Tri* expression. The inhibitory activity against trichothecene production could not be detected using media with higher accumulation of trichothecenes such as YF_60 with Bacto™ lot #3189145 and YS_60 with BSP-B lot #031103050-3 (data not shown), indicating that the inhibition of Tri6p-dependent activation of *Tri* expression was rather weak and secondary to the inhibition of the Tri6p-independent initial activation of *Tri6* expression.

4. Conclusions

Yeast extract was found to contain ethyl acetate-extractable substances that negatively affect trichothecene production. Heating was suggested to increase the amount of inhibitory substances that suppressed the transcription of *Tri6* and, to a lesser extent, the function of the Tri6p transcription factor. Thus, the influence of such inhibitory substances should be considered when using the complex media for studying trichothecene biosynthesis regulation.

Acknowledgements

This work was supported by Grant-in-Aid from Kieikai Research Foundation (No. 2017S06).

Conflicts of interest

None.

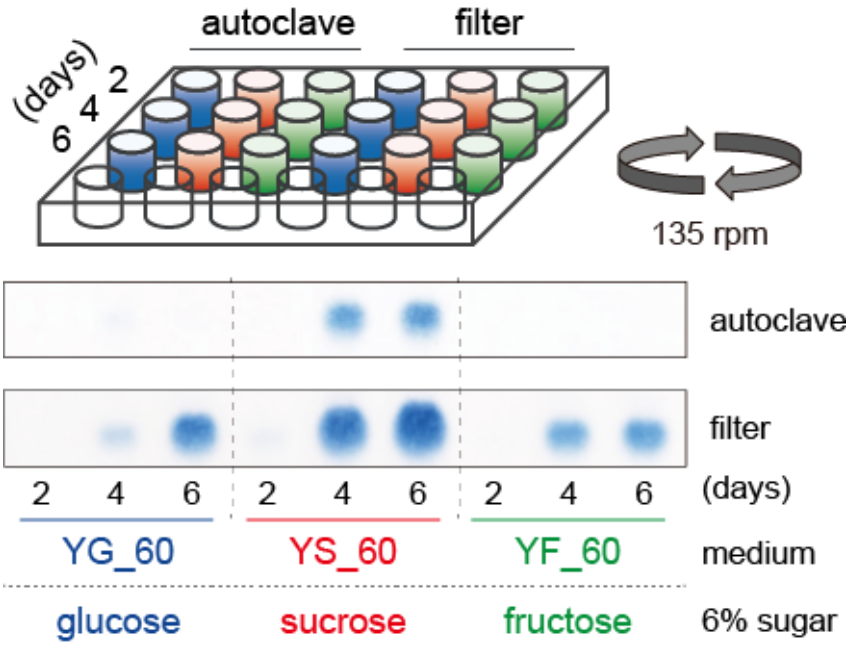
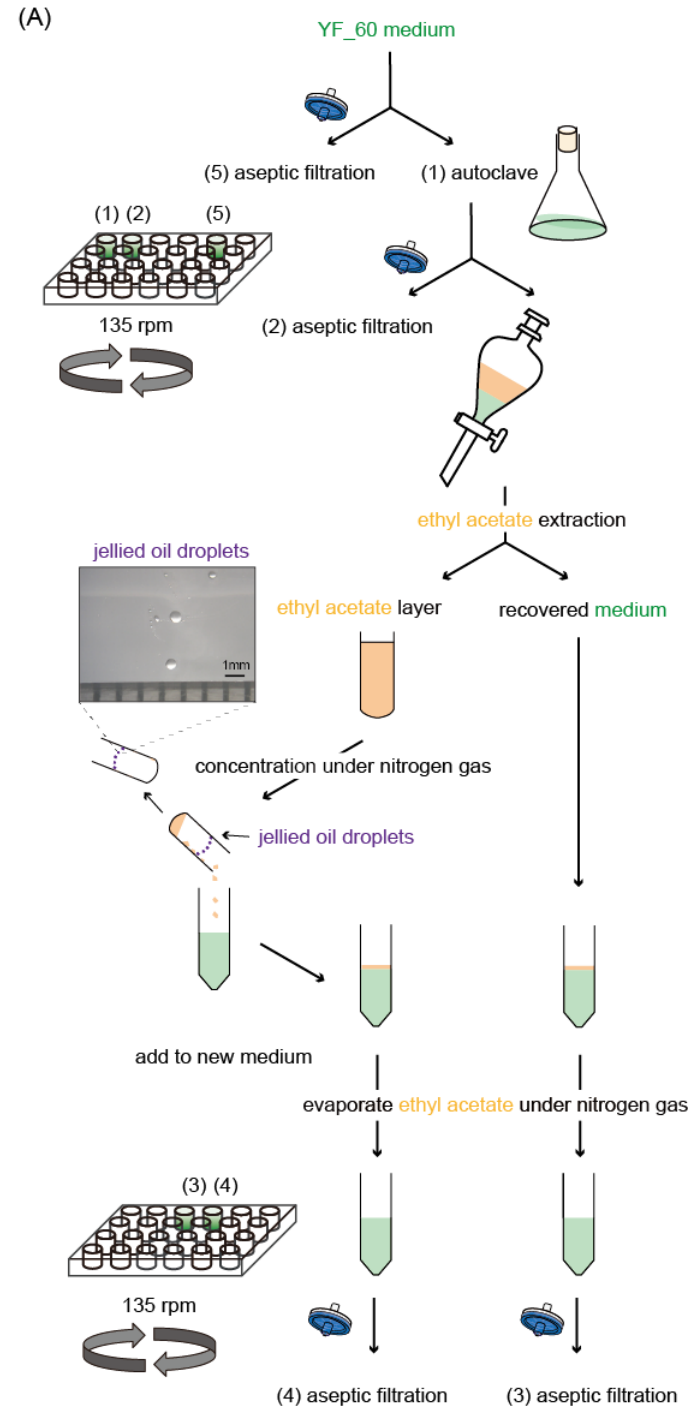
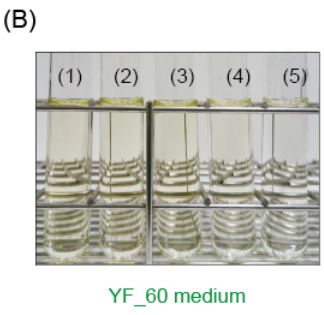


Fig. 1. Effects of autoclaving on trichothecene production inducing-ability of complex media containing Bacto™ yeast extract and a sugar, i.e., glucose (YG_60), sucrose (YS_60), or fructose (YF_60). The media were prepared either by autoclave or filter sterilization. After inoculation of each medium with *F. graminearum* JCM 9873, the fungal culture was distributed to wells in a 24-well plate and incubated for 2, 4, and 6 days with gyratory shaking at 135 rpm. Each lane of the TLC contains metabolites from 0.6 mL of culture.


(A)
YF_60 medium
(5) aseptic filtration
(1) autoclave
(1) (2)
(5)
135 rpm
(2) aseptic filtration
ethyl acetate extraction
jellied oil droplets
1mm
ethyl acetate layer
recovered medium
concentration under nitrogen gas
jellied oil droplets
add to new medium
evaporate ethyl acetate under nitrogen gas
(3) (4)
135 rpm
(4) aseptic filtration
(3) aseptic filtration


(B)
(1)
(2)
(3)
(4)
(5)
YF_60 medium

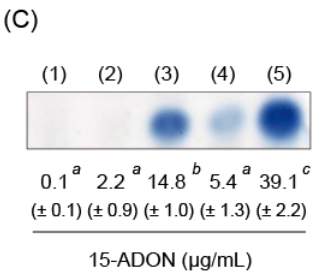

(C)
(1)
(2)
(3)
(4)
(5)
0.1 a
2.2 a
14.8 b
5.4 a
39.1 c
(± 0.1)
(± 0.9)
(± 1.0)
(± 1.3)
(± 2.2)
15-ADON (μg/mL)

Fig. 2. Effects of medium preparation methods on trichothecene production by *F. graminearum* JCM 9873 grown in a 24-well plate with gyratory shaking. (A) A flowchart showing the process of medium preparation. The YF_60 media were sterilized as follow: (1) autoclave, (2) autoclave and aseptic filtration, (3) autoclave, mixing with ethyl acetate, removal of ethyl acetate layer, evaporation of residual solvent, and aseptic filtration, (4) autoclave, extraction of hydrophobic substances with ethyl acetate, addition of concentrated ethyl acetate extract to a new non-sterilized YF_60 medium of the same volume (leaving the firmly adhered jellied oil droplets on the surface of the tube), evaporation of residual solvent, and aseptic filtration, and (5) aseptic filtration. The autoclaving was performed at 121 ^{o}C for 15 min. Jellied oil droplets that appeared during the solvent evaporation and adhered to the surface of test tubes were observed under an Olympus SZX16 stereomicroscope (Olympus, Tokyo, Japan). (B) YF_60 media with different degrees of brownish yellowing. Lane numbers correspond to the medium preparation methods listed in (A). (C) 15-ADON production in aseptic YF_60 media prepared using different methods. Strain JCM 9873 was grown in a 24-well plate at 25 ^{o}C with gyratory shaking for 5 days. Lane numbers correspond to the medium preparation methods listed in (A). A representative result of TLC that contains blue spots of 15-ADON is shown. For confirmation of reproducibility, preparations of the YF_60 media were repeated four more times (total $n = 5$), and the amounts of 15-ADON (μg/mL) in five different experiments were quantified. Different letters indicate significant differences analyzed by one-way ANOVA followed by Tukey-Kramer test ($P < 0.01$).

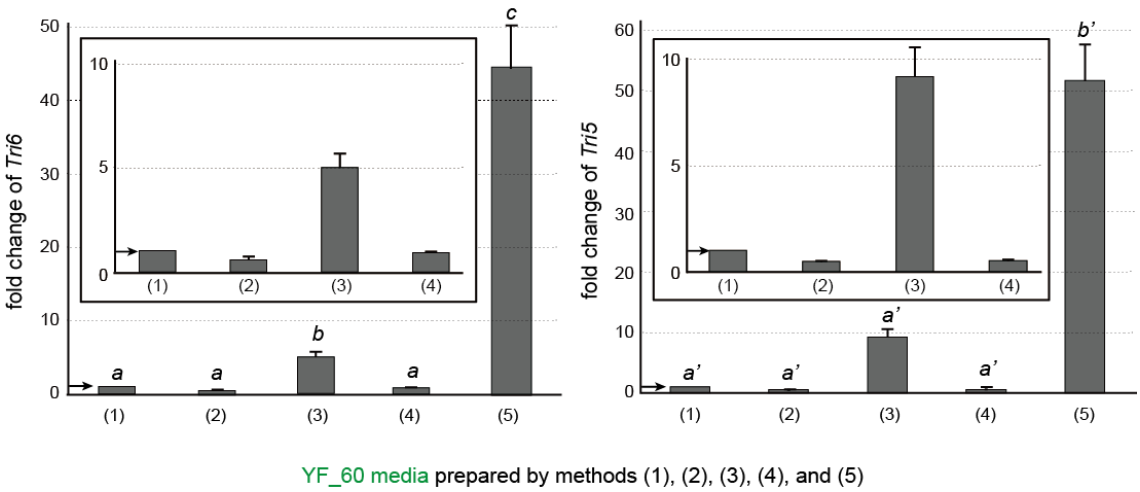
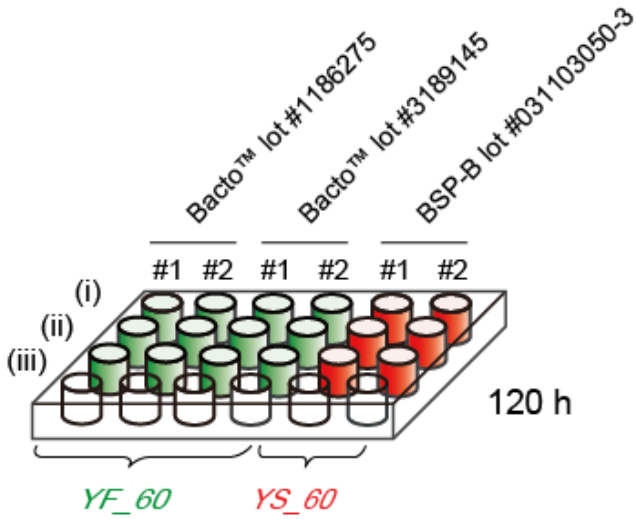


Fig. 3. RT-qPCR analyses of *Tri* genes of *F. graminearum* grown in YF_60 media prepared by different methods. The expression of *Tri6* and *Tri5* after 60 h of culturing in a 24-well plate was determined using the expression of *Ubc* as an endogenous reference in the same RNA samples. Different letters indicate significant differences analyzed by one-way ANOVA followed by Tukey-Kramer test ($P < 0.01$).


Bacto™ lot #1186275
Bacto™ lot #3189145
BSP-B lot #031103050-3
#1 #2 #1 #2 #1 #2
(i)
(ii)
(iii)
120 h
YF_60
YS_60

135 rpm

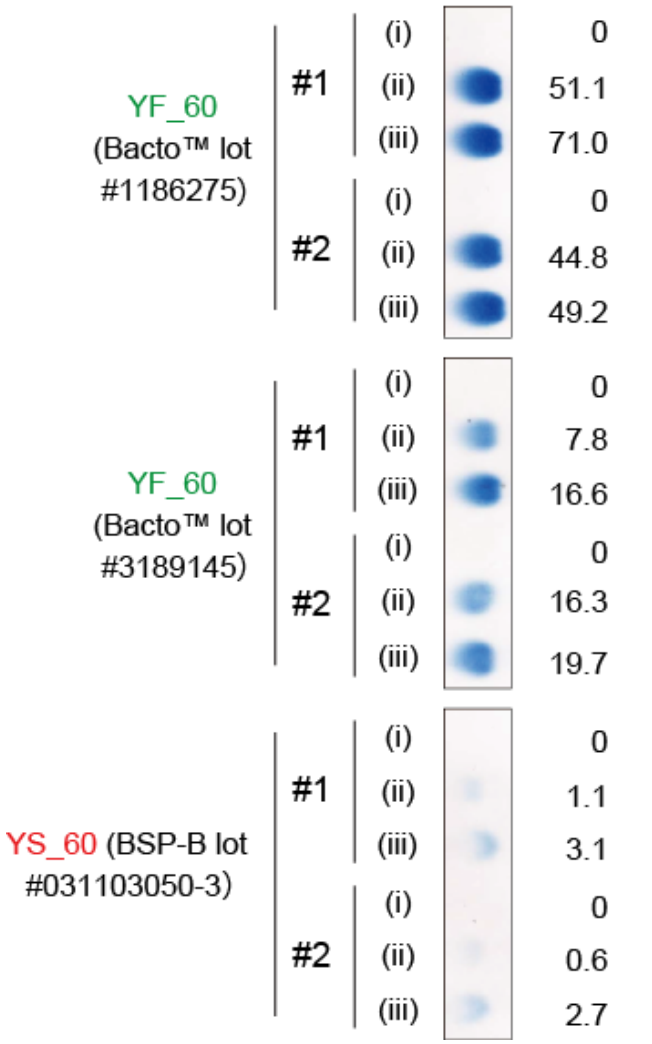

15-ADON (µg/mL)
YF_60 (Bacto™ lot #1186275)
#1
(i) 0
(ii) 51.1
(iii) 71.0
#2
(i) 0
(ii) 44.8
(iii) 49.2
YF_60 (Bacto™ lot #3189145)
#1
(i) 0
(ii) 7.8
(iii) 16.6
#2
(i) 0
(ii) 16.3
(iii) 19.7
YS_60 (BSP-B lot #031103050-3)
#1
(i) 0
(ii) 1.1
(iii) 3.1
#2
(i) 0
(ii) 0.6
(iii) 2.7

Fig. 4. Improvement of trichothecene production by removing ethyl acetate-extractable inhibitory substances from the yeast extract products. The complex media containing yeast extract were prepared by three different methods: (i) autoclaving at 121 ^{o}C for 15 min, (ii) aseptic filtration, and (iii) mixing with ethyl acetate, removal of ethyl acetate layer, evaporation of residual solvent, and aseptic filtration. The concentrations of 15-ADON in YF_60 (Bacto™ lots #1186275 and #3189145) and YS_60 (BSP-B lot #031103050-3) media were quantified after 5 days of culturing. Portions of a TLC (sample origin on far left and solvent front on far right) containing spots of 15-ADON are also shown. Each culture was performed in duplicate (#1 and #2).

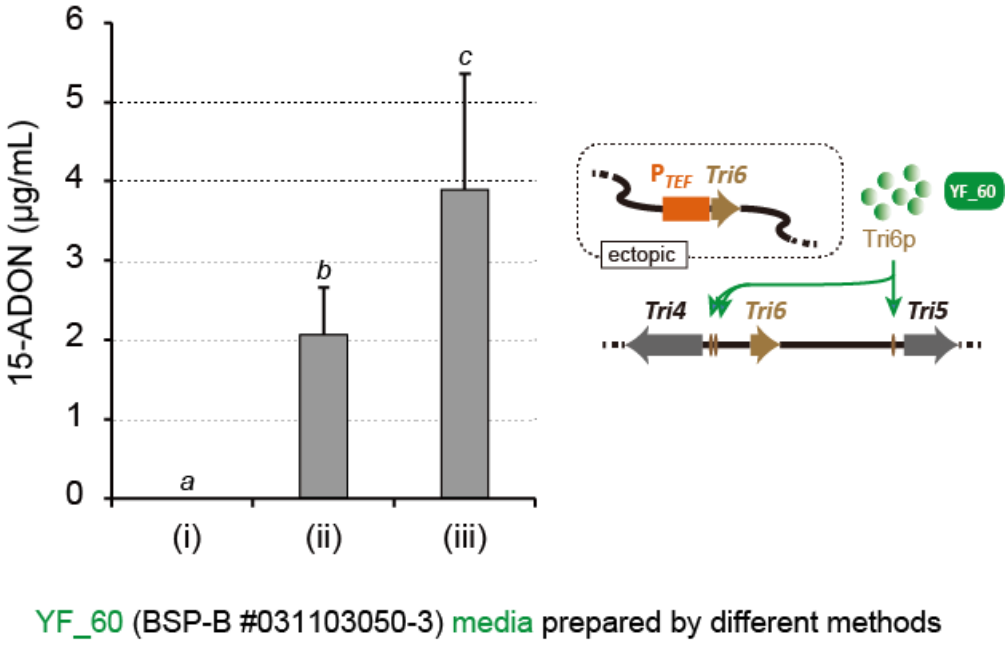


Fig. 5. Inhibitory activity of ethyl acetate-extractable substances on trichothecene production by a *Tri6* overexpressor strain in YF_60 (BSB lot #03110350-3) media. The media were prepared by methods (i)−(iii) as described in the legend of Fig. 4. The amount of 15-ADON was quantified after 120 h of incubation ($n = 4$). Different letters indicate significant differences analyzed by one-way ANOVA followed by Tukey-Kramer test ($P < 0.05$).

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Short Communication

Inhibition of *Fusarium* trichothecene biosynthesis by yeast extract components extractable with ethyl acetate

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Table S1. Primers used in this study.

Primer	Sequence (5'-3')	Description[a]	accession number
#01: JTri6syF	GGCCGAATCTCACTACGAATC	Primer for RT-qPCR of *Tri6* (position nt 15− nt 35 of *Tri6* cDNA)	LC114222.1
#02: JTri6syR	ACGCAACTCGATCAAAGAGG	Primer for RT-qPCR of *Tri6* (position nt 70− nt 51 of *Tri6* cDNA)	LC114222.1
#03: JTri5syF	ACGCCAAGAAGTTCTGCAAG	Primer for RT-qPCR of *Tri5* (position nt 962− nt 981 of *Tri5* cDNA)	FGSG_03537
#04: JTri5syR	ACTTGTGGATAAGCCCACTCC	Primer for RT-qPCR of *Tri5* (position nt 1043− nt 1023 of *Tri5* cDNA)	FGSG_03537
#05: ubc sybr F	GCGAGGATTTGTTTCACTGG	Primer for RT-qPCR of *Ubc* (position nt 80− nt 99 of *Ubc* cDNA)	FGSG_10805
#06: ubc sybr R	TGGATCGCAAGGAAGAAGAC	Primer for RT-qPCR of *Ubc* (position nt 164− nt 145 of *Ubc* cDNA)	FGSG_10805

[a]Primer positions (nt) indicate the base from the start codon.

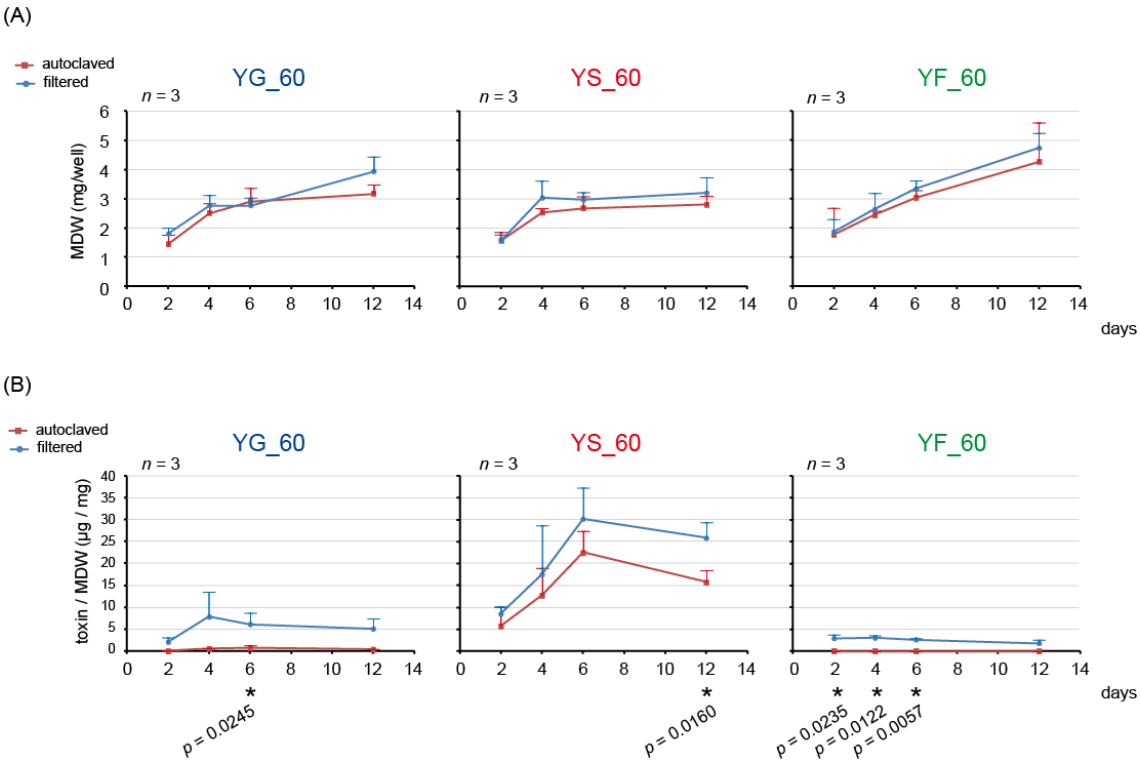

Fig. S1. Effects of autoclaving of complex media on *F. graminearum* mycelial dry weight (MDW) and trichothecene production inducing-ability over the 12-day time course. The fungal cultures (1 mL) in YG_60, YS_60, and YF_60 media were distributed to wells in a 24-well plate and incubated for 2, 4, 6, and 12 days with gyratory shaking at 135 rpm. The experiments were performed in triplicates. (A) Time course of MDW of culture in different complex media. At any time points (e.g., YF_ 60 after 12 days of culturing), no statistical significances were detected between the MDW obtained with the autoclaved and filter-sterilized media ($p > 0.05$; Student's *t* test). (B) Time course of toxin/MDW in different complex media. At the time points with asterisks (e.g., YG_ 60 after 6 days of culturing), statistical significances were detected between the amounts of 15-ADON (toxin/MDW) obtained with the autoclaved and filter-sterilized media (Student's *t* test).

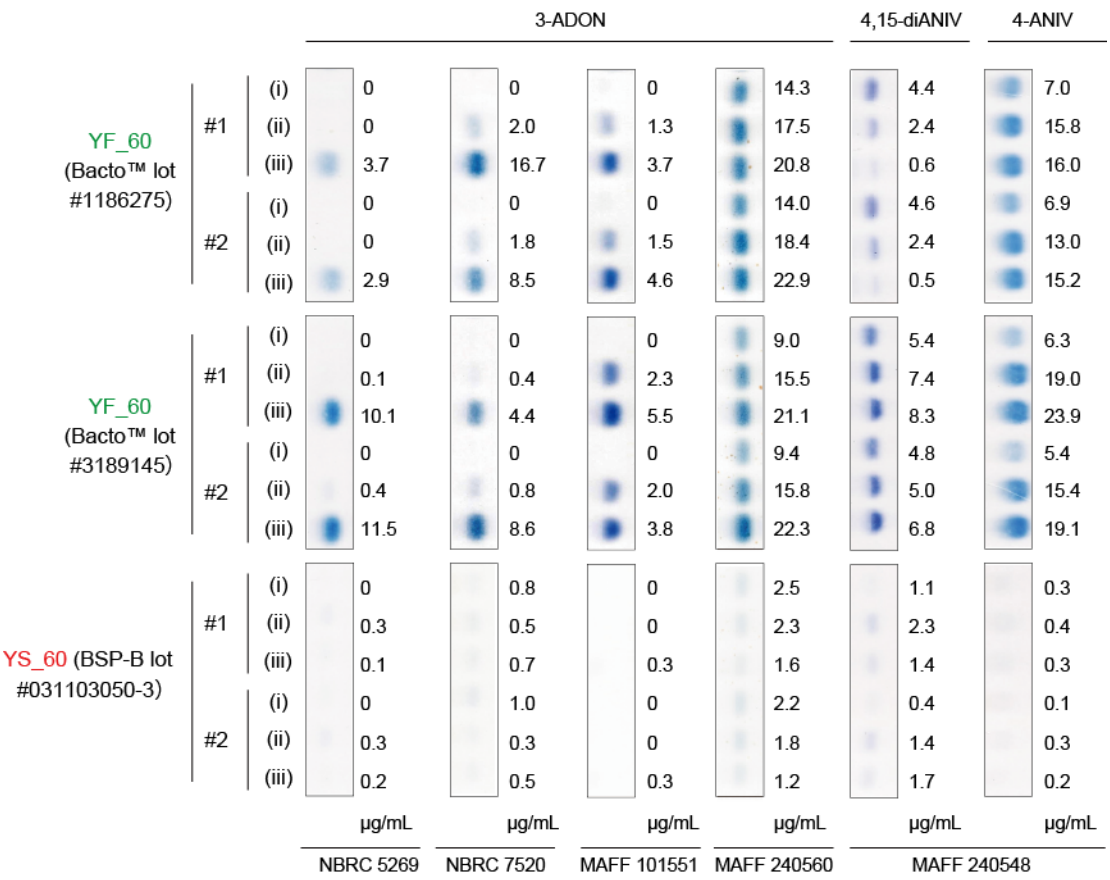

Fig. S2. Improvement of trichothecene production by removing ethyl acetate-extractable inhibitory substances from the yeast extract products. The complex media containing yeast extract were prepared by three different methods: (i) autoclaving at 121 ^{o}C for 15 min, (ii) aseptic filtration, and (iii) mixing with ethyl acetate, removal of ethyl acetate layer, evaporation of residual solvent, and aseptic filtration. The concentrations of 3-ADON, 4,15-diacetylnivalenol (4,15-diANIV), and 4-acetylnivalenol (4-ANIV) in YF_60 (Bacto™ lots #1186275 and #3189145) and YS_60 (BSP-B lot #031103050-3) media were quantified after 5 days of culturing. Portions of a TLC (sample origin on far left and solvent front on far right) containing spots of the trichothecene are also shown. Each culture was performed in duplicate (#1 and #2).