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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.

1. Introduction

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

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

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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 et al., 2017b). 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) GenBank. Media containing all the necessary ingredients (i.e., 6% sugar and 0.1% yeast extract) were sterilized by autoclaving for 15 min at 121 °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 et al., 2008b). 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 LightCycle 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 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C 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

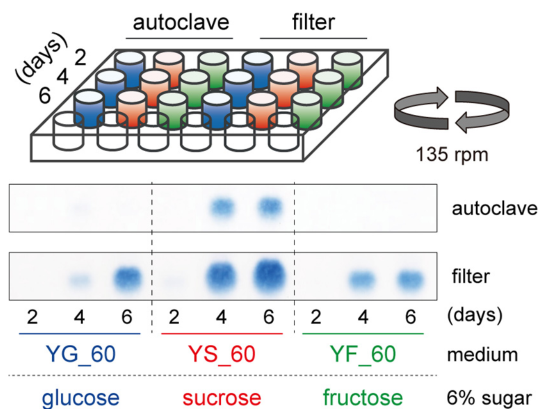

Fig. 1. Effects of autoclaving on trichothecene production inducing-ability of complex media containing Bacto™ yeast extract and a sugar, i.e., glucose (YG_60), sucrose (YS_60), or fructose (YF_60). The media were prepared either by autoclave or filter sterilization. After inoculation of each medium 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.

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