International Journal of Food Microbiology 214 (2015) 123–128

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) inhibits trichothecene production by *Fusarium graminearum* through suppression of *Tri6* expression

Thomas Etzerodt [a], Kazuyuki Maeda [b], Yuichi Nakajima [b], Bente Laursen [a], Inge S. Fomsgaard [a], Makoto Kimura [b,*]

[a] Department of Agroecology, Aarhus University, Forsøgsvej 1, 4200 Flakkebjerg, Denmark
[b] Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8601, Japan

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 22 June 2015
Accepted 10 July 2015
Available online 15 July 2015

Chemical compounds studied in this article:
15-Acetyldeoxynivalenol (PubChem CID 10382483)
Benzoxazolin-2(3*H*)-one (PubChem CID: 6043)
p-Coumaric acid (PubChem CID: 637542)
Deoxynivalenol (PubChem CID 40024)
2,4-Dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (PubChem CID: 28495)
2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (PubChem CID: 2358)
Homoorientin (PubChem CID: 114776)
Nivalenol (PubChem CID 31829)
6-Methoxy-benzoxazolin-2(3*H*)-one (PubChem CID: 10772)
trans-Ferulic acid (PubChem CID: 445858)

Keywords:
Fusarium head blight
Wheat
2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one
15-Acetyldeoxynivalenol
Tri gene expression

ABSTRACT

Fusarium head blight (FHB) is a devastating disease of wheat (*Triticum aestivum* L.) caused by a mycotoxigenic fungus *Fusarium graminearum* resulting in significantly decreased yields and accumulation of toxic trichothecenes in grains. We tested 7 major secondary metabolites from wheat for their effect on trichothecene production in liquid cultures of *F. graminearum* producing trichothecene 15-acetyldeoxynivalenol (15-ADON). 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) benzoxazinoid completely abolished toxin production without any apparent effect on fungal growth. DIMBOA strongly affected the expression of *Tri6*, encoding a major transcriptional regulator of several genes of the trichothecene biosynthesis pathway. DIMBOA also repressed expression of *Tri5*, encoding trichodiene synthase, the first enzyme in the trichothecene biosynthesis pathway. Thus, DIMBOA could play an important role against the accumulation of trichothecenes in wheat grain. Breeding or engineering of wheat with increased levels of benzoxazinoids could provide varieties with increased resistance against trichothecene contamination of grain and lower susceptibility to FHB.

1. Introduction

Fusarium head blight (FHB) is a devastating disease in wheat, mainly caused by *Fusarium graminearum* resulting in significant economic impact due to reduced harvest yields (McMullen et al., 1997). Furthermore, infection also leads to accumulation in grain of trichothecene mycotoxins. Trichothecenes are stable during food and feed processing (Lauren and Smith, 2001) and are thus a concern for both human and animal exposure if accumulation in wheat is not controlled.

The biosynthesis pathway for trichothecenes in *Fusarium sporotrichioides* and *F. graminearum* has been studied extensively and reviewed by several authors (Desjardins, 2009; Kimura et al., 2007). Trichothecene biosynthesis is regulated by the global transcription factor TRI6 (Proctor et al., 1995). Trichothecene biosynthesis begins with cyclization of farnesyl pyrophosphate to trichodiene catalyzed by

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; BOA, benzoxazolin-2(3*H*)-one; CA, *p*-coumaric acid; DIBOA, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DON, deoxynivalenol; FA, ferulic acid; NIV, nivalenol; MBOA, 6-methoxy-benzoxazolin-2(3*H*)-one.

* Corresponding author.
E-mail address: mkimura@agr.nagoya-u.ac.jp (M. Kimura).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.07.014
0168-1605/

trichodiene synthase (TRI5) (Hohn and Beremand, 1989), followed by four consecutive oxygenations catalyzed by a cytochrome P450 monooxygenase, TRI4, using molecular oxygen (McCormick et al., 2006; Tokai et al., 2007). These first steps in the trichothecene biosynthesis pathway are common for trichothecene-producing *Fusarium* species in general. Important trichothecenes that share these biosynthesis steps are deoxynivalenol (DON), nivalenol (NIV), their acetylated derivatives and T-2 toxin and HT-2 toxin.

Important tools to minimize FHB in wheat include taller cultivars, soil tillage, avoiding maize as a precrop to wheat and use of resistant wheat cultivars (*e.g.* Sumai-3). An important resistance mechanism is suppression of trichothecene accumulation by secondary metabolites in wheat (Boutigny et al., 2008), *e.g.* terpenoids (Chamarthi et al., 2014), lignans (Cho et al., 2007), phenolic acids (Bakan et al., 2003; Boutigny et al., 2010), flavonoids (Desjardins et al., 1988), and benzoxazinoids (Martyniuk et al., 2006; Miller et al., 1996).

Phenolic acids and flavonoids are ubiquitous and known for their antioxidant and health beneficial effects (Dykes and Rooney, 2007; Hollman, 2001). In wheat grains, the dominant phenolic acid is ferulic acid (FA) (Hernandez et al., 2011; Kim et al., 2006). Phenolics are well-studied for their correlation with *Fusarium* resistance in wheat (Engelharclt et al., 2002; Pani et al., 2014), maize (Atanasova-Penichon et al., 2012; Bakan et al., 2003) and barley (Bollina and Kushalappa, 2011).

Benzoxazinoids are a group of secondary metabolites with allelopathic properties, that have been extensively studied from young cereal plants (Carlsen et al., 2009; Etzerodt et al., 2008; Niemeyer, 2009). Benzoxazinoids occur mainly in rye, wheat and maize with 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) and the glucosylated derivative 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one-2-D-α-glucopyranoside (DIBOA-glc) dominating in rye, while in wheat and maize 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one-2-D-α-glucopyranoside (DIMBOA-glc) dominate (Niemeyer, 2009). Furthermore, benzoxazinoids have a series of putative health-protecting effects, although a few controversial results are reported (Adhikari et al., 2015; Prinz et al., 2010). Benzoxazinoids have also been correlated with FHB resistance in wheat heads (Søltoft et al., 2008). Benzoxazolinones benzoxazolin-2(3*H*)-one (BOA) and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) have been well-studied for their antimicrobial activity (Bravo et al., 1997; Martyniuk et al., 2006) and 4-acetyl-benzoxazolin-2-one (4-ABOA), from maize grain, efficiently inhibited 3-acetyldeoxynivalenol (3-ADON) production in liquid culture (IC_{50} of 4 μM) (Miller et al., 1996). This is the first study of the toxin suppressive effects of naturally occurring benzoxazinoids from wheat.

The purpose of this study was (1) to establish the activity of selected wheat secondary metabolites (phenolic acids, benzoxazinoids, and a flavonoid) on trichothecene accumulation in liquid culture of *F. graminearum* and (2) to identify the effect of DIMBOA on the expression of the genes within the trichothecene biosynthetic pathway.

2. Materials and methods

2.1. Chemicals and solvents

Sodium carboxymethylcellulose (CMC-Na), 4-(*p*-nitrobenzyl) pyridine (NBP), and tetraethylene pentamine (TEPA) were from Wako Pure Chemicals (Osaka, Japan). *p*-Coumaric acid (CA) (≥98.0% purity) and *trans*-FA (≥99% purity) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO). Kieselgel F_{254} TLC plates were from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO; biotechnology grade) was obtained from Nacalai Tesque (Kyoto). Bacto yeast extract (YE) was obtained from BD Diagnostics. Homoorientin, BOA (98%) and MBOA (98%) were obtained from Acros Organics (Geel, Belgium). DIBOA and DIMBOA were obtained as part of an ongoing patenting process (Fomsgaard et al., 2010). Millex 0.22 μm pore size PES membranes (for sterile filtration) and Miracloth, 22–25 μm pore size (for filtration of mycelium from CMC cultures) were purchased from Merck Millipore (Billerica, MA).

2.2. Fungal strain, media, and culture conditions

F. graminearum strain JCM 9873 [15-acetyldeoxynivalenol (15-ADON) chemotype] was maintained on V8 juice agar (20% V8 juice, 0.3% calcium carbonate, and 2% agar) at 25 °C in the dark. For sporulation, three mycelial plugs of JCM 9873 were transferred to carboxymethylcellulose (CMC) medium, composed of 1.5% CMC–Na, 0.1% NH_4NO_3, 0.1% KH_2PO_4, 0.1% YE and 0.05% $MgSO_4$ heptahydrate. After 3–4 days of incubation at 25 °C, conidia were harvested by filtration through Miracloth, followed by centrifugation (5000 ×*g* for 10 min at room temperature) and the pellet washed with sterile distilled water. The spore suspension (10^7 spores/ml) was added to YG medium, composed of 2% (w/v) glucose and 0.5% YE, to a final density of 10^4 spores/ml and incubated at 25 °C with shaking for 16–17 h. One milliliter YG pre-culture thus prepared was added to 100 ml main liquid culture, YG_60 [6% (w/v) glucose, 0.1% YE] (used only for test of FA and CA) or YS_60 [6% (w/v) sucrose, 0.1% YE] (Nakajima et al., 2014). YS_60 medium was used as a high toxin induction medium and YG_60 used as a low toxin induction medium to test the effect of different secondary metabolites from wheat.

2.3. Analysis of 15-ADON by TLC

Trichothecenes were analyzed in a semiquantitative manner by TLC using NBP and TEPA as color-developing reagents according to Takahashi-Ando et al. (2008). Briefly, dried ethyl acetate extracts from 0.6 ml liquid culture were dissolved in 12 μl of ethanol. Ten microliters of this solution was spotted onto a TLC plate (2 μl aliquots), allowing the spots to dry between applications and then developed with ethyl acetate:toluene (3:1) and visualized as described by Takahashi-Ando et al.

2.4. 15-ADON quantification from liquid cultures

One milliliter main cultures were grown in 24 well plates supplemented with 1 μl of inhibitor stock solution in DMSO (1000 times the final concentration of wheat secondary metabolites used in experiments). Cultures were incubated at 25 °C (gyratory shaking, 135 rpm) in an incubator hood for 48 h or 96 h. All experiments were performed in triplicates. Whole cultures were transferred to 1.5 ml microtubes and centrifugated at 15,000 ×*g* for 10 min at 4 °C. Five hundred microliter supernatant was transferred to new 1.5 ml microtubes and extracted with 500 μl ethyl acetate. The mixture was centrifuged at 15,000 ×*g* for 10 min at 4 °C and the ethyl acetate phase was evaporated at room temperature. The residue was dissolved in 40 μl 25% (v/v) acetonitrile and 10 μl analyzed by HPLC-UV on a Shimadzu LC-10A HPLC system. Column was Pegasil ODS SP100 (4.6 × 250 mm, 5 μm, 100 Å; Senshu Scientific Co., Ltd., Tokyo) maintained at 40 °C. Elution was done at 1 ml/min with 25% acetonitrile. 15-ADON was quantified from peak areas using an external calibration curve at 5–200 μg/ml concentrations (correlation coefficients ≥ 0.999). The results were expressed relative to the dried fungal biomass. Fungal biomass was measured by draining the mycelium in tubes, followed by drying overnight (16–20 h) at 65 °C.

2.5. Tri gene expression studies for DIMBOA

RNA was extracted from the mycelia grown in a 24-well titer plate using the RiboZol™ RNA Extraction Reagent (AMRESCO, Solon, OH). For each independent experiment ($n = 3$), YG pre-culture was freshly prepared each time and used for subsequent main YS_60 culture as described in Section 2.2. Three wells of a 24 well plate were allocated