



New tricks of an old enemy: *Fusarium graminearum* can also produce a type A trichothecene

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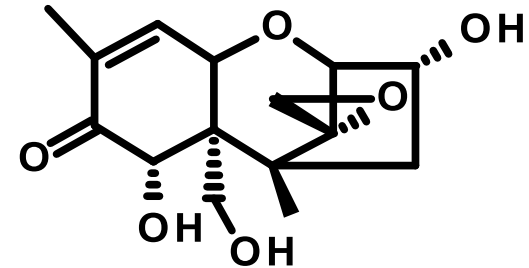
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Introduction

■ Fusarium Head Blight disease (FHB)

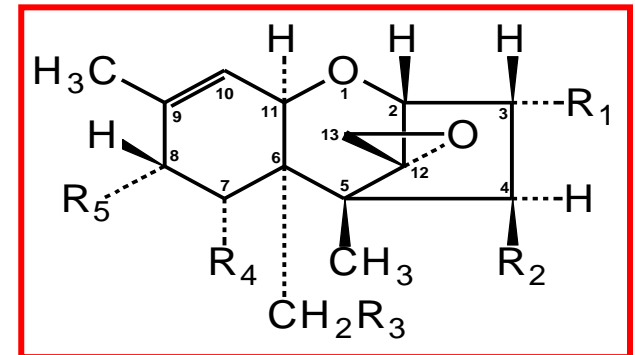
- caused by *Fusarium* spp.
predominantly *Fusarium graminearum*
- “the cereal killer” (wheat, barley, oats, ...)
- loss of grain yield and quality
- mycotoxin contamination: DON (vomitoxin)



Deoxynivalenol (DON)

■ Trichothecene backbone structure

- 12,13-epoxytrichothec-9-ene
- > 200 different subtypes
Grove J.F. (2007) Prog Chem Org Nat Prod. 88:63-130
- Type B: keto at C8
- Type A: either no oxygen at C8 (e.g. DAS) or oxygen function other than keto (T-2)

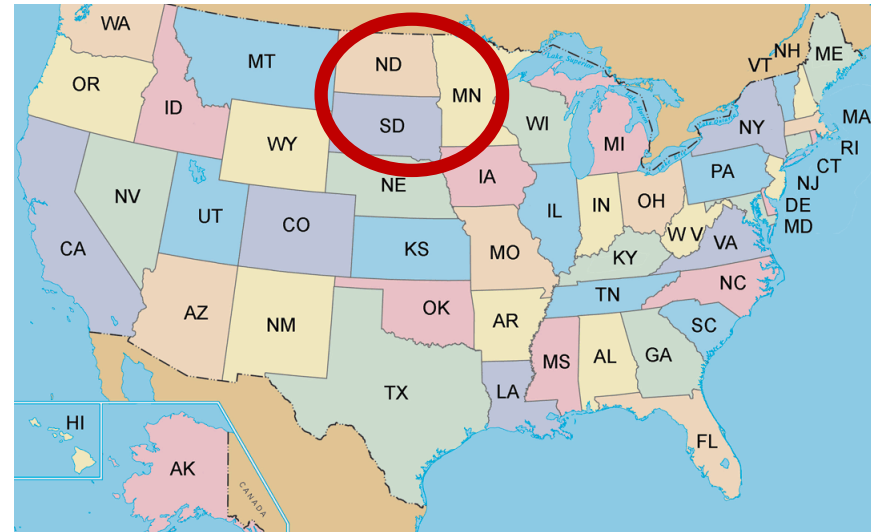


Background



US *Fusarium graminearum* population

- Large-scale population survey of *Fusarium graminearum* from the upper Midwestern United States (Corby Kistler group)
- **Aim:** determination of spatial and temporal dynamics
- **Three populations:**
 - „classical” 15-ADON genotype
 - „emergent” 3-ADON genotype
 - newly identified „Northland” population – contains „no-toxin” isolates: **N**



Gale et al. (2010) *Fusarium* head blight Forum

Session 2: Pathogen Biology & Genetics

A SUBSET OF THE NEWLY DISCOVERED NORTHLAND
POPULATION OF *FUSARIUM GRAMINEARUM* FROM
THE U.S. DOES NOT PRODUCE THE B-TYPE
TRICHOHECENES DON, 15ADON, 3ADON OR NIV

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No-toxin strains: useful as biocontrol organism?

Session 2: Pathogen Biology & Genetics

PREINOCULATION OF WHEAT HEADS WITH A NONTOXIGENIC
FUSARIUM ISOLATE INHIBITS DEOXYNIVALENOL
PRODUCTION BY A TOXIGENIC PATHOGEN

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.... disease symptoms by N-strains (2010 Forum)

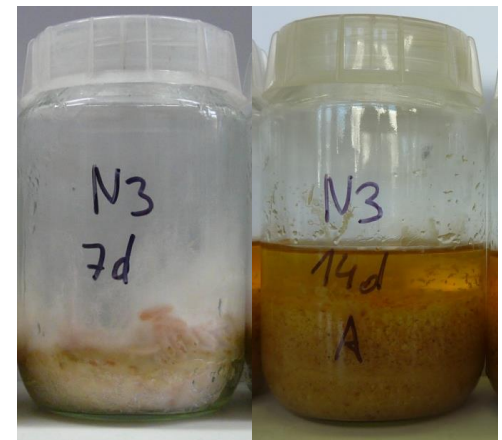
Hypothesis: N-strains produce a new toxin?

Analysis of N-strains

- 3-ADON-genotype
- Wheat ears (naturally contaminated)
 - normal aggressiveness and disease symptoms (premature bleaching)
 - no detectable levels of common trichothecenes (DON, ADONs, NIV)
- Rice culture extracts
 - Multi-mycotoxin method (LC-MS > 200 metabolites)
no known trichothecenes detectable
heavy ZEN-producer

Vishwanath V. *et al.* (2009) *Anal Bioanal Chem.* 395:1355-1372

- Volatiles analyzed by GC-MS (headspace)
trichodiene found → *TRI5* gene is active

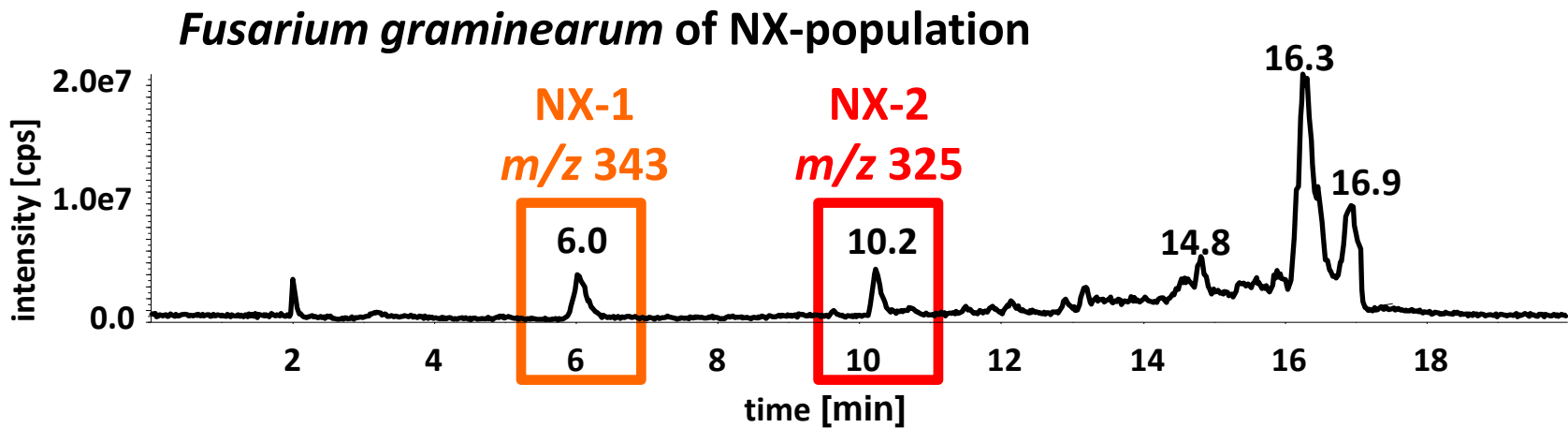
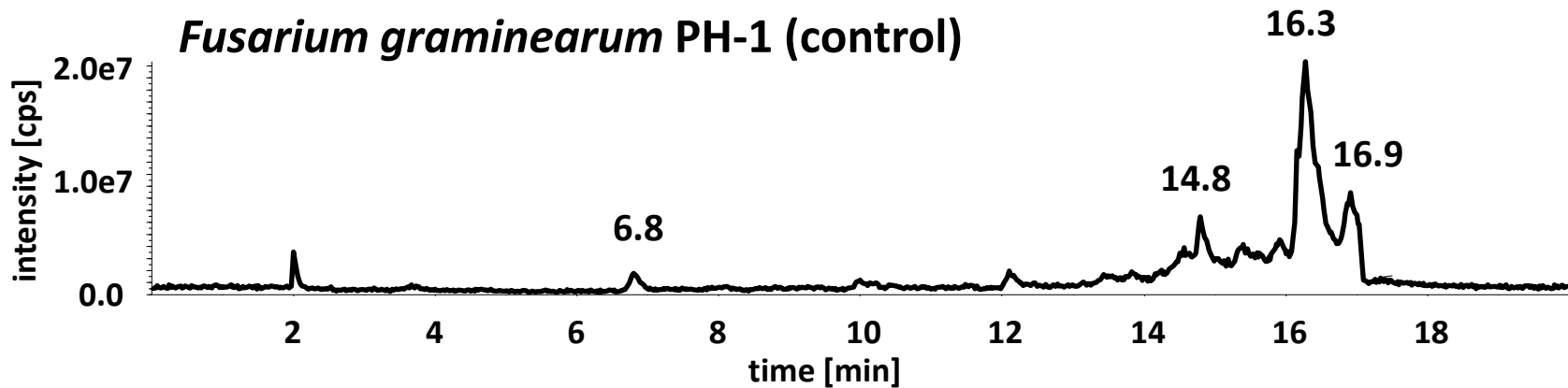


Screen for unknown metabolites

LC-MS full scan



- Rice inoculated with *Fusarium graminearum* for 3 weeks
 - total ion chromatograms (ESI pos)



Isolation and purification

NP and RP chromatography

- Cultivation on autoclaved rice
- Normal phase chromatography
 - silica gel (80 x 4 cm, 63-200 μm particle size)
 - elution with ethyl acetat/petroleum ether
- Preparative reversed phase HPLC
 - Phenomenex Gemini NX (150 x 21.2 mm, 5 μm)
 - water – methanol gradient
- Yield: 20-50 milligramms of substance

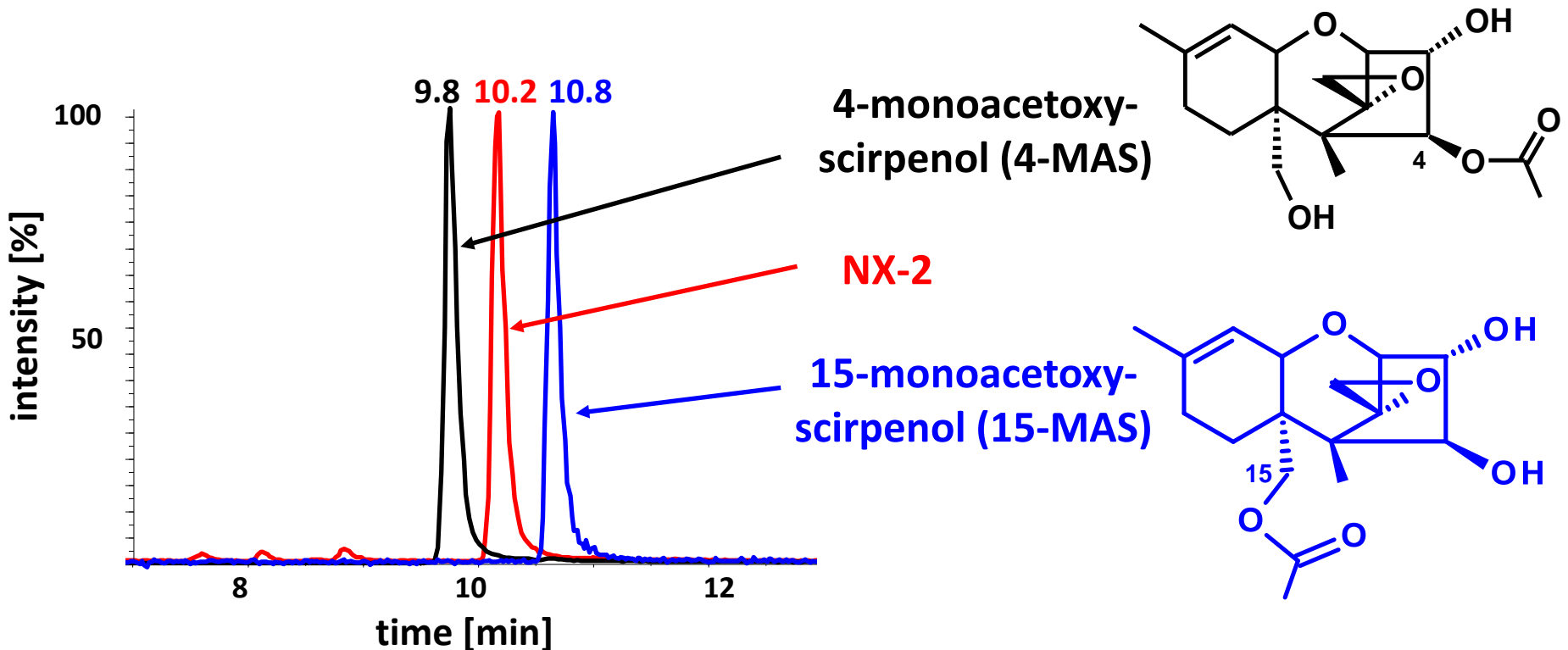


Compound characterisation

High resolution MS + comparison with known compounds



- **NX-2:** m/z 325.1643 \rightarrow $C_{17}H_{24}O_6$ ($\Delta m = 0.8$ ppm)
 \rightarrow same sum formula as monoacetoxyscirpenol

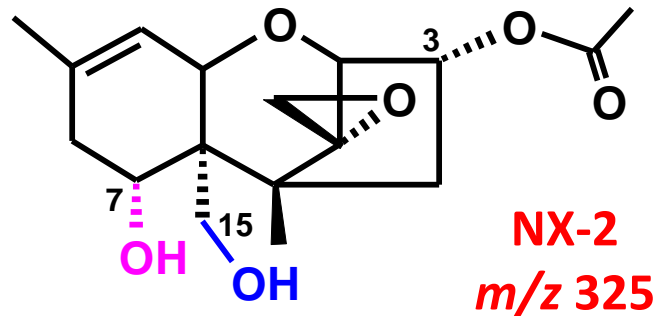


Purification/structure elucidation



Purification and NMR

- Trichothecene backbone with 3-OH groups, one acetylated
- 1D- and 2D-NMR
 - structure similar to 3-MAS
 - differ in the position of the hydroxyl-groups



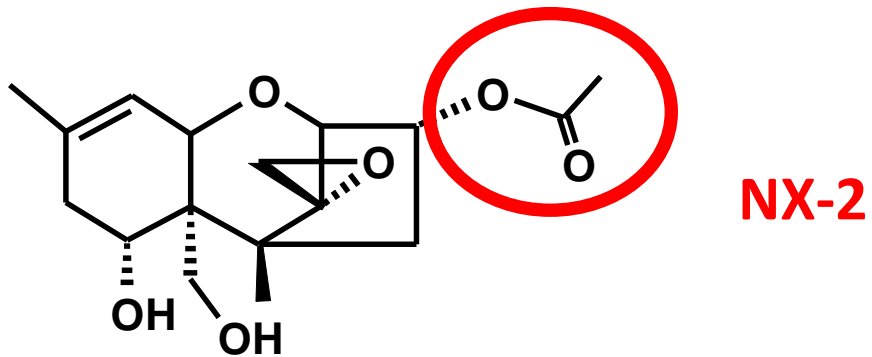
3 α -acetoxy, 7 α ,15-dihydroxy-12,13-epoxytrichothe-9-ene

(3-ADON lacking C8-keto group: type A)

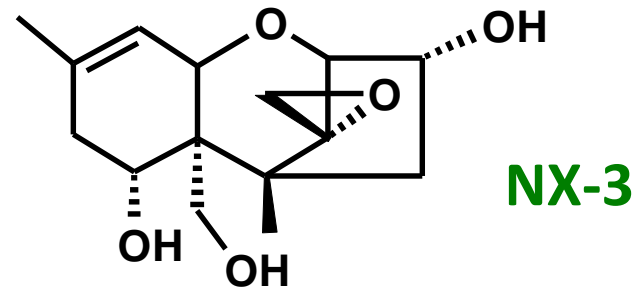
Hypothesis

Deacetylation *in planta*

- Plants might deacetylate NX-2
→ might be more toxic (e.g. 3-ADON vs. DON)



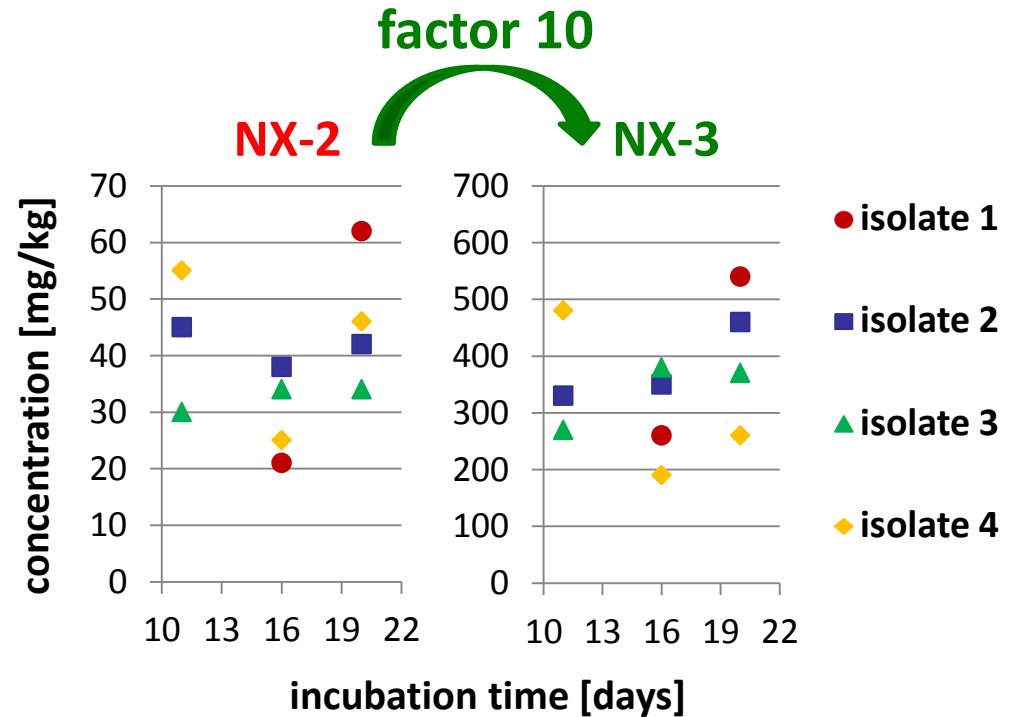
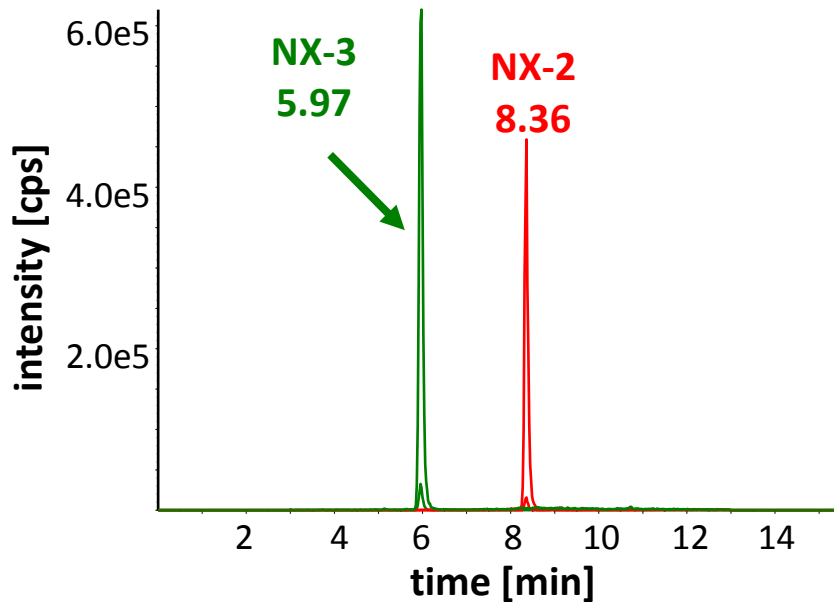
- Production of deacetylated form via basic hydrolysis
purification via preparative HPLC
 - structure confirmed by NMR



Wheat inoculation

Cultivar "Apogee"

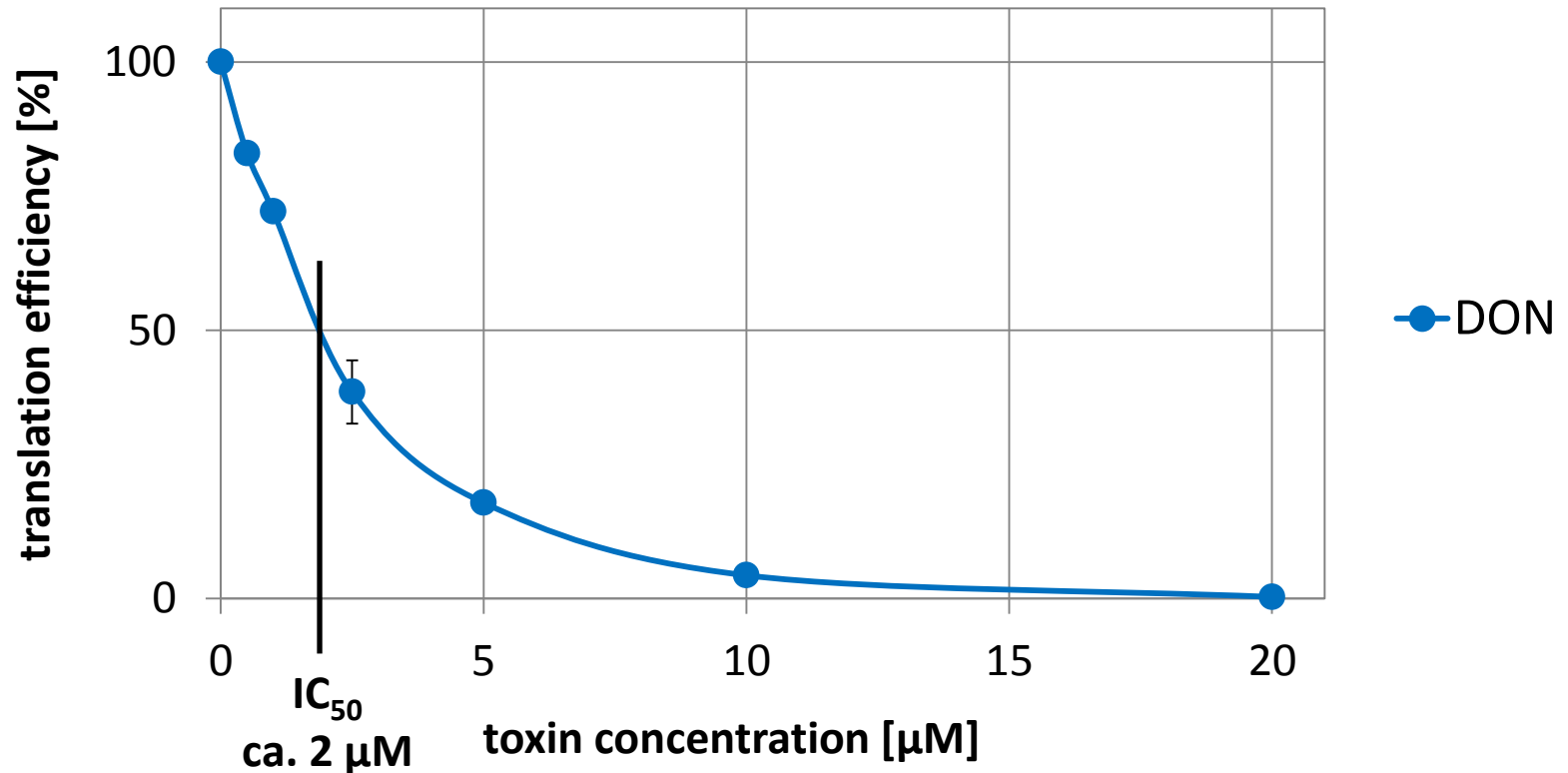
- Spray inoculated with spore suspension of
 - *F. graminearum* PH-1
 - four different *F. graminearum* NX-isolates



Toxicity tests

In vitro translation inhibition

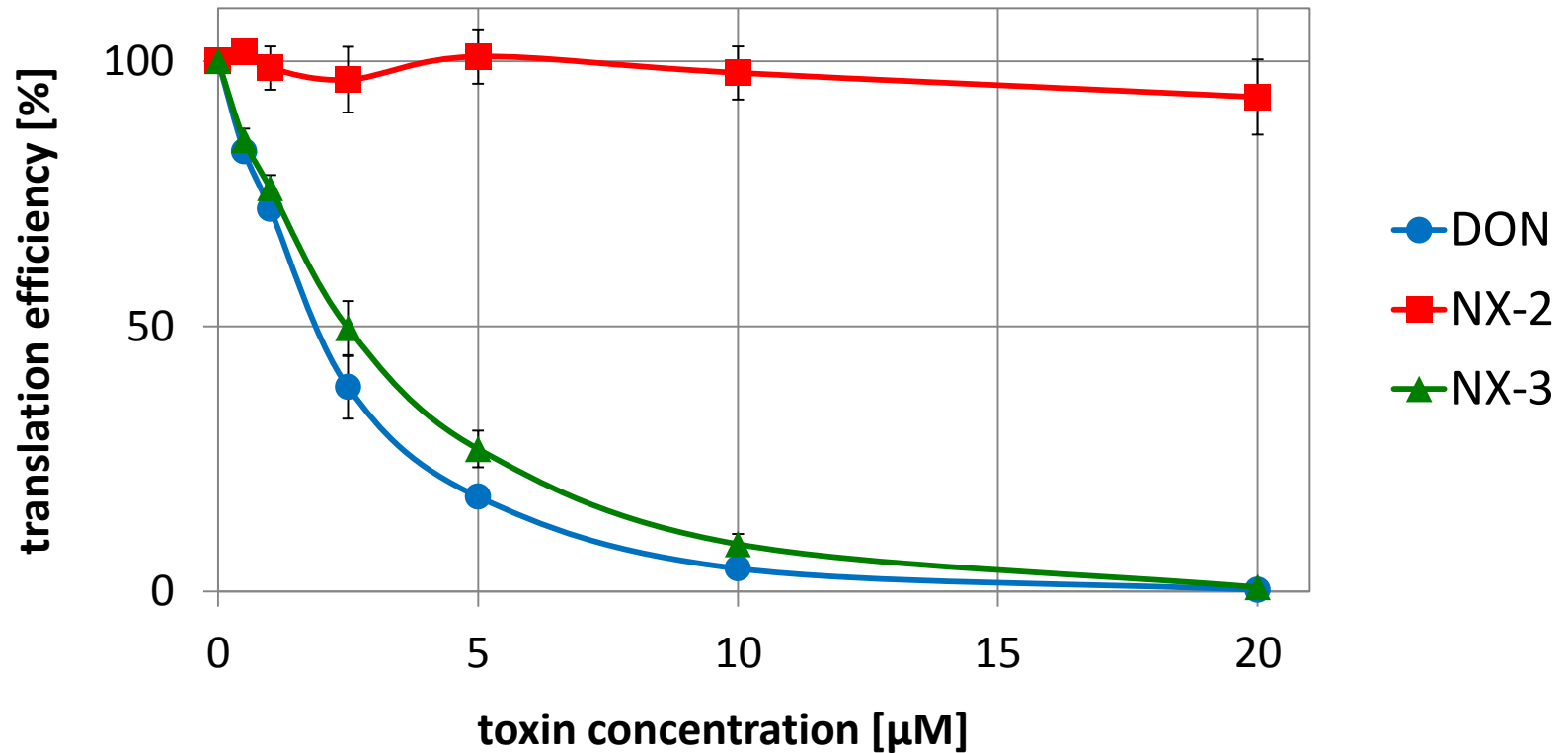
- Inhibition of protein synthesis
 - rabbit reticulocyte lysate
 - reporter gene: activity of fire fly luciferase



NX-3 has similar toxicity as DON



- Inhibition of protein synthesis
 - rabbit reticulocyte lysate
 - assessment: activity of fire fly luciferase

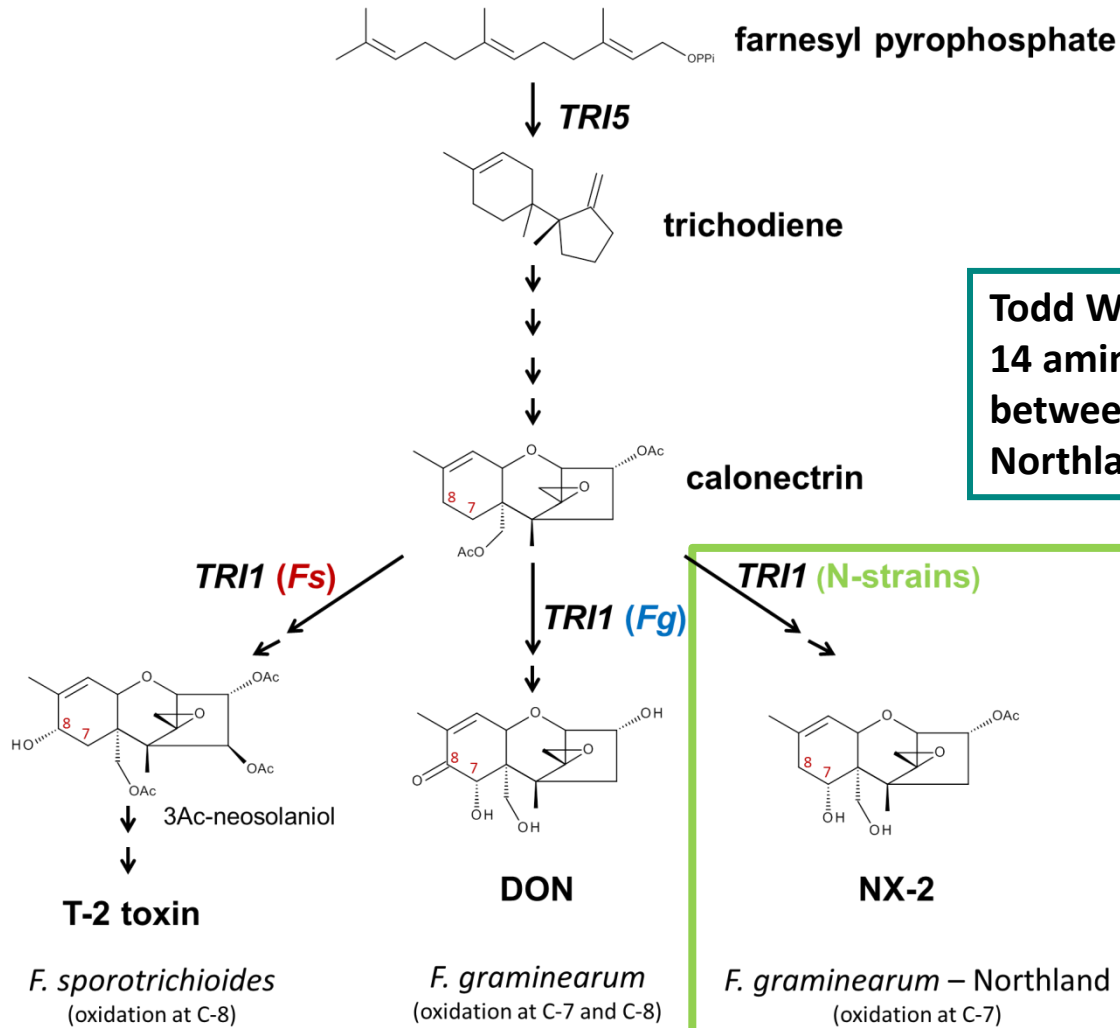


Relevance for plant breeding / Fusarium resistance management?

- **What is the molecular basis of NX-production?**
The relevant change can be used for molecular diagnostics ...
- **Does NX-production allow the fungus to adapt to resistant cultivars? (durability of resistance?)**

Biosynthetic pathway

proposed pathway to NX-2



Todd Ward:
14 amino acid changes in *TRI1*
between PH-1 and all
Northland strains

Hypothesis:
 altered *TRI1* in
 Northland strains
 is responsible for
 oxidation at C7

TRI1 swap experiment outline

PH-1

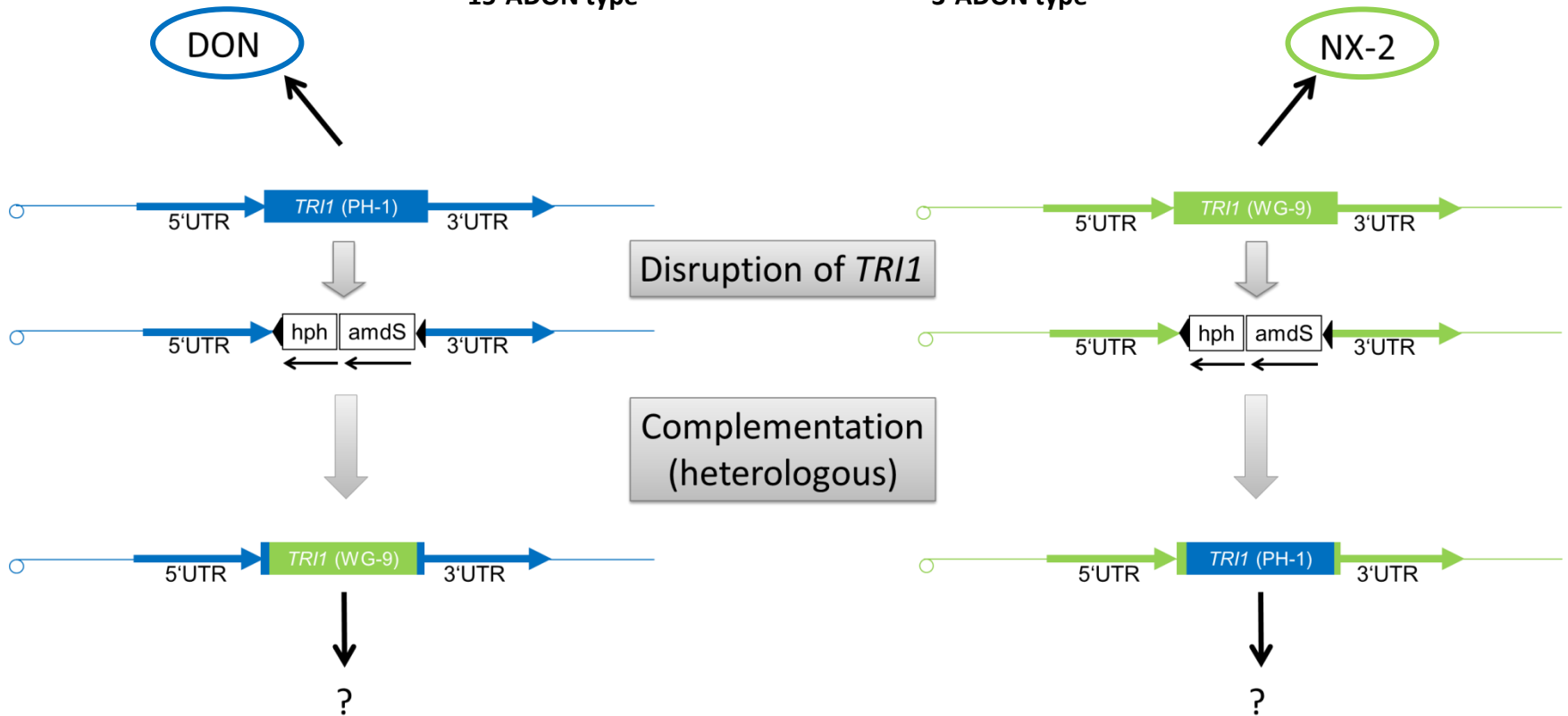
15-ADON type

WG-9

3-ADON type

DON

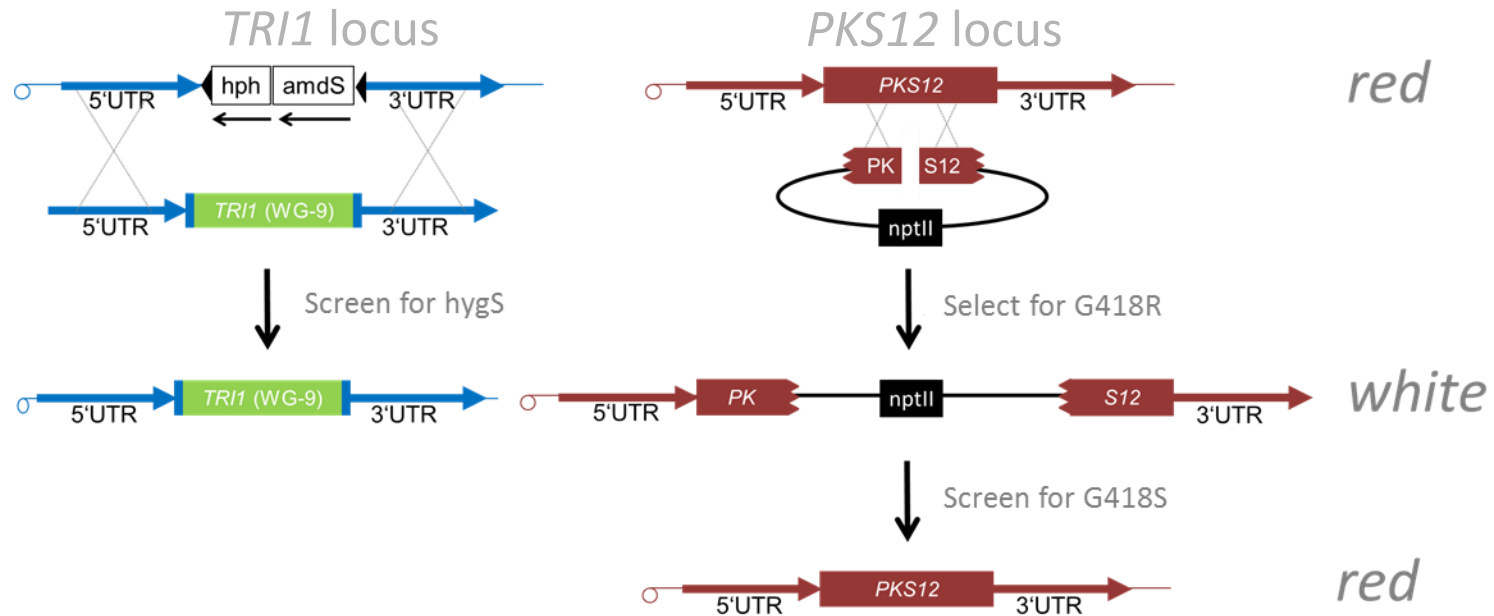
NX-2



TRI1 swap experiment transformation strategy



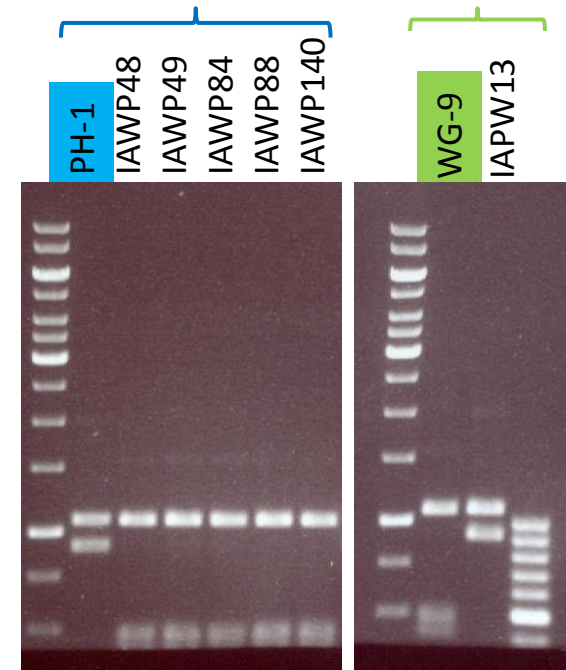
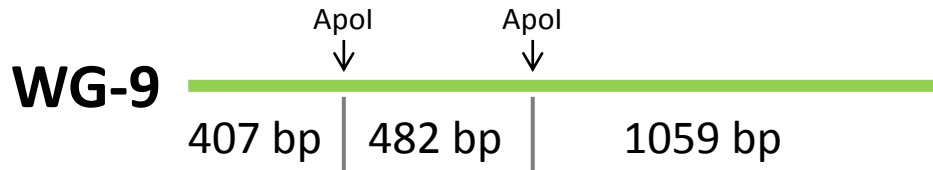
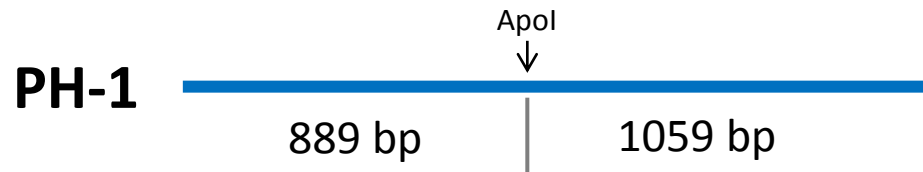
1. co-transform *tri1*Δ strain with *TRI1* construct and plasmid containing a truncated *PKS12* and *nptII* and select for G418 resistance
2. screen G418 resistant transformants for hygromycin sensitive strains
3. sporulate and screen for G418 sensitive/red offspring



Confirmation of swapped alleles



PCR/ Apol

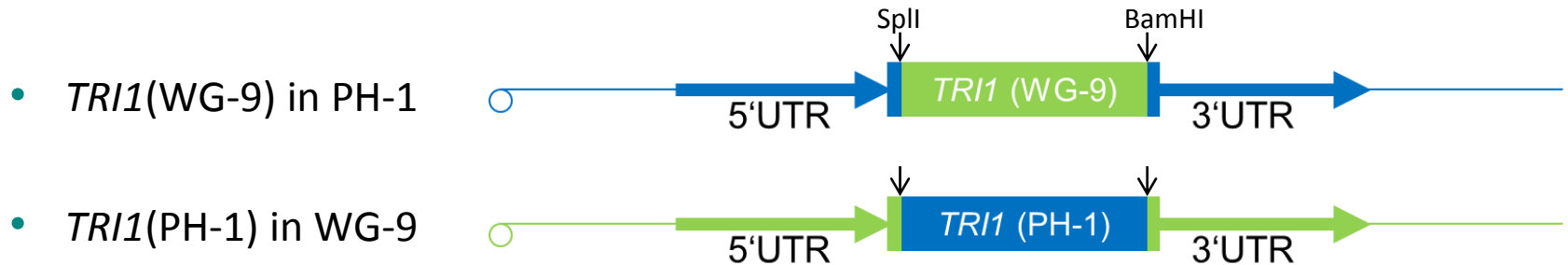


TRI1 swap experiment

isolation of „new“ metabolites



- newly constructed strains:

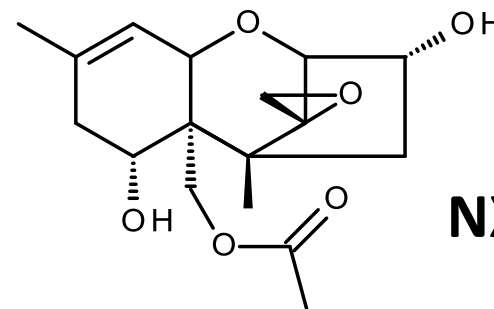


- inoculated autoclaved rice, grew cultures for 2 weeks
- purified main products of both strains by preparative reversed phase HPLC
- identified structures by NMR

TRI1 swap experiment

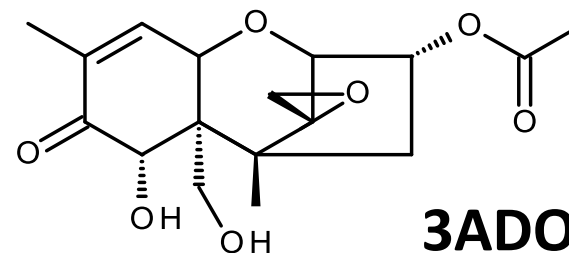
characterization of metabolites

TRI1(WG-9) in PH-1 - *15ADON* genotype



NX-4
new!

TRI1(PH-1) in WG-9 - *3ADON* genotype

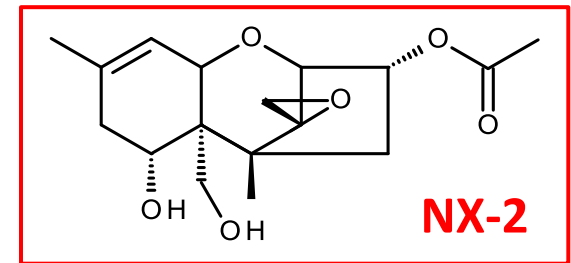


3ADON

Summary



- Novel *Fusarium graminearum* population? (3ADON genotype)
- Despite FHB symptoms
→ no known trichothecenes produced
- Novel mycotoxin discovered: **NX-2**
- Wheat inoculation: major metabolite **NX-3** concentrations up to 500 mg/kg
- Deacetylation also shown *in vitro* (wheat germ extract)
- Toxicity of **NX-3** comparable to that of DON/NIV (ribosome)
- Altered *TRI1* allele is responsible for specific oxidation at C7 in Northland metabolites



Publication:

in press

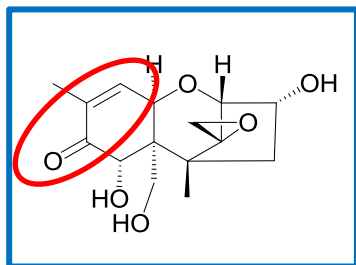
Environmental Microbiology (2014)

doi:10.1111/1462-2920.12718

New tricks of an old enemy: isolates of *Fusarium graminearum* produce a type A trichothecene mycotoxin

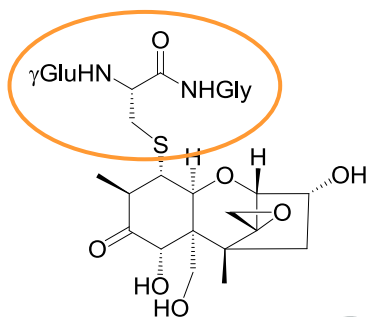


Selective advantage of NX-3 versus DON?



DON: **conjugated double bond + keto group**
Michael adduct with thiols possible!

Glutathione-mediated DON-detoxification
.... may be the basis of resistance QTL deployed
by breeders



Organic &
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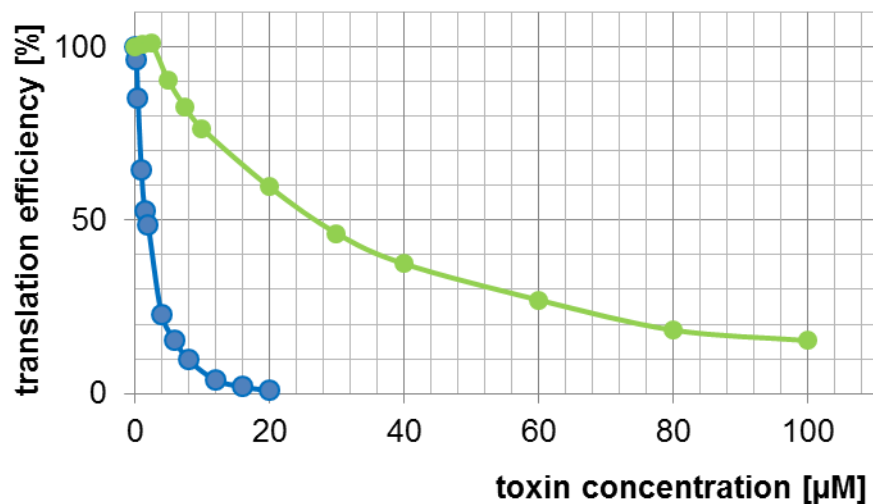
Cite this: *Org. Biomol. Chem.*, 2014,
12, 5144

Methylthiodeoxynivalenol (MTD): insight into the chemistry, structure and toxicity of *thia*-Michael adducts of trichothecenes†

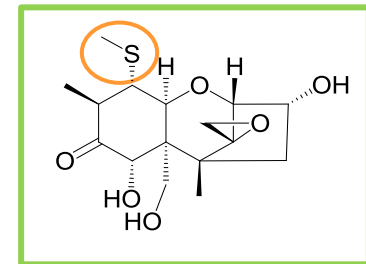
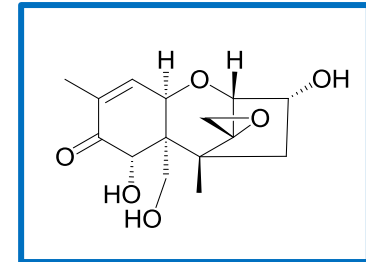
Philipp Fruhmann,^{‡a} Theresa Weigl-Pollack,^{‡a} Hannes Mikula,^{*a}
Gerlinde Wiesenberger,^b Gerhard Adam,^b Elisabeth Varga,^c Franz Berthiller,^c
Rudolf Krska,^c Christian Hametner^a and Johannes Fröhlich^a

Thiol-adduct formation is a detoxification reaction

Inhibition of *in vitro* translation by DON and SMD



● DON
● SMD



S-methyl-DON (Kushalappa, 2010 Fusarium head blight forum)
or methylthio-DON is less toxic – this should also be true for the bulky
DON-glutathione and DON-Cys adducts (found *in planta*)