

## A Sensitive and Inexpensive Yeast Bioassay for the Mycotoxin Zearalenone and Other Compounds with Estrogenic Activity

Rudolf Mitterbauer,<sup>1</sup>† Hanna Weindorfer,<sup>1</sup> Naser Safaie,<sup>2</sup> Rudolf Krska,<sup>3</sup>  
Marc Lemmens,<sup>4</sup> Peter Ruckenbauer,<sup>4</sup> Karl Kuchler,<sup>5</sup>  
and Gerhard Adam<sup>1\*</sup>

Center of Applied Genetics, University of Agricultural Sciences, A-1190 Vienna,<sup>1</sup> Center for Analytical Chemistry<sup>3</sup>  
and Department of Plant Production Biotechnology,<sup>4</sup> Institute for Agrobiotechnology, A-3430 Tulln, and  
Department of Molecular Genetics, University and BioCenter of Vienna, A-1030 Vienna,<sup>5</sup> Austria,  
and College of Agriculture, Tarbiat Modarres University, Tehran, Islamic Republic of Iran<sup>2</sup>

Received 17 July 2002/Accepted 12 November 2002

**Zearalenone (ZON) is a nonsteroidal estrogenic mycotoxin produced by plant-pathogenic species of *Fusarium*. As a consequence of infection with *Fusarium culmorum* and *Fusarium graminearum*, ZON can be found in cereals and derived food products. Since ZON is suspected to be a cause of human disease, including premature puberty syndrome, as well as hyperestrogenism in farm animals, several countries have established monitoring programs and guidelines for ZON levels in grain intended for human consumption and animal feed. We developed a low-cost method for monitoring ZON contamination in grain based on a sensitive yeast bioassay. The indicator *Saccharomyces cerevisiae* strain YZRM7 is unable to grow unless an engineered pyrimidine biosynthetic gene is activated by the expressed human estrogen receptor in the presence of exogenous estrogenic substances. Deletion of the genes encoding ATP-binding cassette (ABC) transporters Pdr5p and Snq2p increases net ZON uptake synergistically. Less than 1 µg of ZON per liter of medium is sufficient to allow growth of the indicator strain. To prevent interference with pyrimidines potentially present in biological samples, we also disrupted the genes *FURI* and *URK1*, blocking the pyrimidine salvage pathway. The bioassay strain YZRM7 allows qualitative detection and quantification of total estrogenic activity in cereal extracts without requiring further cleanup steps. Its high sensitivity makes this assay suitable for low-cost monitoring of contamination of maize and small grain cereals with estrogenic *Fusarium* mycotoxins.**

Zearalenone (ZON) (Fig. 1) is a resorcylic acid lactone (nonsteroidal) fungal metabolite with very high estrogenic activity. The mycotoxin ZON and derivatives of this compound are produced predominantly by *Fusarium graminearum* and *Fusarium culmorum* and also by several other species (7, 32). Under environmental conditions favorable for the fungus, high levels of ZON can be found in harvested maize and small grain cereals (for a review see reference 32).

ZON has a very high relative binding affinity for both estrogen receptor ER- $\alpha$  and estrogen receptor ER- $\beta$  and high transactivation activity (31, 37). Studies with various animal species showed that ZON and its metabolites have potent estrogenic and anabolic activities (32). ZON has been implicated in numerous mycotoxicoses in farm animals, and swine are the most sensitive and most frequently affected species (13, 14, 18). Typical complications associated with high-level ZON ingestion are severe morphological and functional disorders of reproductive organs (39). Mild forms of hyperestrogenism have been observed in pigs exposed to ZON levels as low as 250 µg/kg in their diet (5). The ZON levels in feed implicated in poisoning can be as high as 56 mg/kg (50). Certain *Fusarium*

strains can produce ZON in vitro in liquid cultures that yield up to 34 g/liter of medium (20).

ZON doses that are much greater than concentrations which have hormonal effects may have genotoxic and carcinogenic effects. A National Toxicology Program study found evidence of carcinogenicity in mice but not in rats (43). Likewise, species-specific formation of DNA adducts has been reported (46).

While ZON toxicoses in farm animals are widely recognized, the impact of this mycotoxin on humans is controversial (e.g., there have been proposed cases of precocious sexual development in Puerto Rico [17, 51, 53, 54]). However, high levels of ZON (up to 100 µg/liter) were reported in the sera of Hungarian children exhibiting early puberty symptoms (60, 61). Whether ZON promotes breast cancer (2, 54, 62) rather than reducing mammary tumorigenesis (21) is also controversial.

In a recent toxicological evaluation, the Scientific Committee on Food of the European Commission on Health & Consumer Protection arrived at a temporary tolerable daily intake for ZON of 0.2 µg/kg of body weight (15), while the Joint FAO/WHO Expert Committee on Food Additives recommended a provisional maximum tolerable daily intake of 0.5 µg/kg (23). No international harmonized maximum limits for ZON in grain exist yet, but several countries already have regulations, whose legal status differs (16). Austria, for instance, has had a guideline level for wheat, durum wheat, and rye intended for human consumption of 60 µg of ZON/kg (9) since 1993. A concentration of 200 µg/kg has been set as a guideline level for commodities in Brazil (maize), Uruguay

\* Corresponding author. Mailing address: Center of Applied Genetics, University of Agricultural Sciences, Muthgasse 18/05/66, A-1190 Vienna, Austria. Phone: 43/1/36006-6380. Fax: 43/1/36006-6392. E-mail: adam@edv2.boku.ac.at.

† Present address: Carlsberg Research Laboratory, DK-2500 Valby, Denmark.

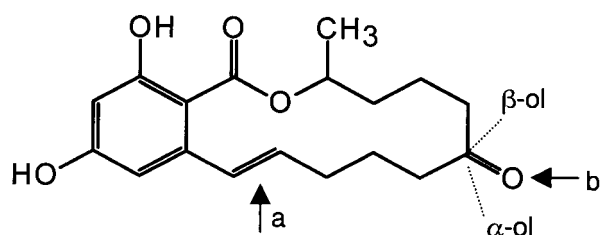


FIG. 1. Structure of ZON [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-beta-resorcylic acid lactone] and some important derivatives. Zearalanol (zearanol), which is used as an anabolic growth promoter, lacks a double bond (arrow a). In addition, the keto group (arrow b) is reduced in zearanol, as it is in  $\alpha$ -zearalanol (see text for details).

(maize and barley), and France (cereals and vegetable oil) (16). Several countries have also proposed guideline levels for animal feed; the suggested guideline level for breeding pigs in Austria (33) and Germany (8) is 50  $\mu\text{g}$  of ZON/kg of total feed.

In North America, contamination of grain with the *Fusarium* mycotoxin deoxynivalenol appears to be far more important than contamination with ZON (45, 56). In parts of Europe, delayed harvesting due to wet weather can lead to the occurrence of high ZON levels in wheat. Monitoring of data from southern Germany (42) indicated that in normal years the frequency of ZON-positive samples is less than 20%, and the observed ZON levels in most of the positive samples are well below 50  $\mu\text{g}/\text{kg}$ . However, in a year in which the weather conditions favored growth of the fungus 80% of the samples were positive, and the average level of ZON detected in wheat was  $178 \pm 994$   $\mu\text{g}/\text{kg}$ ; the highest levels were more than 8 mg/kg (42). Likewise, in 1998 in northern Germany 65% of the wheat samples were ZON positive (12), and the average level was 516  $\mu\text{g}/\text{kg}$  (maximum level, 2.2 mg/kg).

In industrialized countries most of the *Fusarium*-damaged grain is likely to be consumed by farm animals rather than by humans. However, the situation may be very different in other parts in the world. In Iran, for instance, the domestic wheat consumption is now about 16 million metric tons, but only about 10 million tons is produced. The main wheat production

areas are the hot and humid provinces of northern Iran (Caspian Sea littoral), where *Fusarium* head blight dramatically decreases yield (3). Furthermore, high levels of *Fusarium* toxins have been reported in Iranian wheat (63), with the ZON levels ranging from 1,266 to 5,487  $\mu\text{g}/\text{kg}$  (average, 3,464  $\mu\text{g}/\text{kg}$ ). Notably, the provinces of northern Iran are hotspots for esophageal cancer (52), and consumption of wheat flour is among the reported risk factors for this disease (22). Regular mycotoxin monitoring thus seems to be warranted, but the high cost of monitoring grain samples is prohibitive.

Usually, ZON levels in grains are determined by using tedious (liquid-liquid extraction) or very expensive (immunoaffinity column) cleanup procedures and subsequent separation and detection by high-performance liquid chromatography (HPLC), followed by fluorescence or UV detection (29). Our goal was to develop a sensitive and inexpensive bioassay for ZON in grain. We describe here construction of a yeast indicator strain and the first results of tests performed with this strain in a bioassay, which could become an attractive low-cost tool for monitoring ZON levels in agricultural commodities and for basic research applications.

#### MATERIALS AND METHODS

**Plasmid construction.** Genomic DNA of *S. cerevisiae* strain AB1380 (10) was employed as a template for all plasmid construction steps in which PCR was used. Construction of the  $\Delta urk1::TRP1$  plasmid pRM753 has been described previously (41). For construction of the  $\Delta fur1::LEU2$  deletion plasmid, oligonucleotides FUR-UP (5'-AATAACAATACCTCATTCTAC-3') and FUR-DW (5'-TACTCAAGATTAATCTCGAC-3') were used to amplify a 1,695-bp fragment containing the complete *FUR1* gene. This fragment was digested with *Hind*III plus *Eco*RV, and the resulting 1,529-bp fragment was inserted into pUC18 digested with *Hind*III plus *Sma*I. The resulting plasmid was digested with *Acc*I and *Xba*I to delete a 559-bp fragment located within the *FUR1* coding region and was religated after treatment with the Klenow enzyme (fill in) in order to reconstitute the unique *Xba*I site. A 1,990-bp blunt-end *LEU2* fragment (derived from pJJ252 [24] by *Sal*I digestion and filling in with the Klenow enzyme) was ligated into the filled-in *Xba*I site to create pRM765 ( $\Delta fur1::LEU2$ ).

Vector pCGS966 is a centromeric plasmid containing the *URA3* and *HIS3* genes (59).

**Yeast strains.** The *Saccharomyces cerevisiae* strains used in this study and their relevant genotypes are shown in Table 1. The strains were derived from the pyrimidine auxotrophic strain PL3 (*ura3- $\Delta$ 1*), in which transcription of an additional *URA3* gene can be induced by substances with estrogenic activity via three

TABLE 1. Genotypes of *S. cerevisiae* strains used in this study

Strain	Parental strain	Plasmid (relevant change)	Genotype and relevant alterations (episomal plasmids) <sup>a</sup>
PL3			<i>MAT<math>\alpha</math> ura3-<math>\Delta</math>1 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 trp1::P<sub>3xERE</sub>-URA3<sup>b</sup></i>
YYM6	PL3		PL3 $\Delta snq2::hisG^c$
YYM7	PL3		PL3 $\Delta prd5::hisG^c$
YYM8	YYM6		PL3 $\Delta snq2::hisG \Delta prd5::hisG^c$
YZGA304	PL3	YEp90	PL3 (2 $\mu\text{m}$ , <i>HIS3</i> )
YZGA305	PL3	YEp90-HEGO	PL3 (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZGA307	YYM6	YEp90-HEGO	PL3 $\Delta snq2::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZGA309	YYM7	YEp90-HEGO	PL3 $\Delta prd5::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZGA310	YYM8	YEp90	PL3 $\Delta snq2::hisG \Delta prd5::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> )
YZGA311	YYM8	YEp90-HEGO	PL3 $\Delta snq2::hisG \Delta prd5::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZGA439	YZGA311	pRM753/ <i>Cl</i> I+ <i>Not</i> I	PL3 $\Delta urk1::TRP1 \Delta snq2::hisG \Delta prd5::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZRM7	YZGA439	pRM765/ <i>Hind</i> III+ <i>Sph</i> I	PL3 $\Delta fur1::LEU2 \Delta urk1::TRP1 \Delta snq2::hisG \Delta prd5::hisG$ (2 $\mu\text{m}$ , <i>HIS3</i> -P <sub>PGK1</sub> -hER)
YZGA376	YYM8	pCGS966	PL3 $\Delta snq2::hisG \Delta prd5::hisG$ ( <i>CEN4 HIS3 TRP1 URA3</i> )

<sup>a</sup> For the strains derived from PL3 only the additional genetic markers present in the chromosome or on plasmids (in parentheses) are shown. ERE, estrogen responsive element; hER, human estrogen receptor cDNA; P<sub>PGK1</sub>, promoter of *PGK1* (phosphoglycerate kinase).

<sup>b</sup> See reference 48.

<sup>c</sup> See reference 35.

estrogen-responsive elements in the promoter (*trp1::3xERE-URA3*) (48). Derivatives of PL3 containing the vector YEp90-HEGO (expressing the human estrogen receptor cDNA under control of the constitutive *PGK1* promoter) or the empty vector YEp90 (2  $\mu$ m-*HIS3-P<sub>PGK1</sub>*) have been described previously (35), and particular transformants were renamed for reasons of clarity (Table 1). Strains harboring  $\Delta$ *urk1::TRP1* and  $\Delta$ *fur1::LEU2* deletions were constructed by transforming the corresponding parental strains with the *ClaI-NotI* fragment of pRM753 and the *HindIII-SphI* fragment of pRM765, respectively. Transformations of *S. cerevisiae* were performed by the LiAc method (19). Correct integration of the deletion constructs was confirmed by PCR.

**Yeast media.** Yeast strains were grown in rich medium (YPD medium) or in synthetic medium (SD medium) supplemented with auxotrophic components (57) but simplified by omitting methionine, tyrosine, phenylalanine, glutamic acid, aspartic acid, valine, and serine. Uracil (catalog no. 94220; Fluka), uridine (catalog no. U-6381; Sigma), cytosine (catalog no. C-3506; Sigma), and cytidine (catalog no. C-9505; Sigma) were added as 100 $\times$  aqueous stock solutions (2,000 mg/liter). Diethylstilbestrol (DES) and ZON were purchased from Sigma (catalog no. D-4628 and Z-2125).

YZRM7 ( $\Delta$ *urk1::TRP1*  $\Delta$ *fur1::LEU2*), which is unable to utilize exogenous pyrimidines and strictly requires estrogenic compounds in the medium, was propagated on SC-HIS medium plates supplemented with DES (1  $\mu$ g/liter) or ZON (10  $\mu$ g/liter). For spot tests YZRM7 was pregrown in liquid ZON-containing SC-HIS medium, harvested by centrifugation, and transferred to medium without an estrogenic supplement for 6 h.

**Extraction of naturally contaminated wheat and maize samples.** The ZON extraction procedure used was a down-scaled version of the previously described protocol (55). Briefly, 6 g of milled wheat kernels was homogenized and extracted in 24 ml of a mixture containing 75% (vol/vol) acetonitrile and 25% water by using an Ultra Turrax T25 mixer for 3 min at 24,500 rpm in a 50-ml Greiner disposable tube. After centrifugation (5,000  $\times$  g for 5 min), the supernatant was filtered through a Whatman GF/A filter. The acetonitrile extract (200  $\mu$ l) and appropriate dilutions of this extract were mixed with two 900- $\mu$ l portions of SC-URA-HIS medium (in which agar was replaced by 1% agarose) in 2.2-ml Eppendorf tubes and then poured into 35-mm-diameter petri dishes. After solidification of the medium, 6- $\mu$ l portions of diluted suspensions ( $A_{600} = 0.03$ ) of the indicator yeast strain and control strains were spotted on the plates and incubated at 30°C for 4 days. The yeast growth observed on control plates containing dilutions of a ZON calibrating solution was used as a reference to estimate the ZON contents of the extracts. Initially, a ZON calibrating solution was prepared by dissolving ZON (1 mg/ml; Sigma) in ethanol, but for further analysis a certified ZON calibrating solution, prepared at the IFA Tulln Center for Analytical Chemistry, was used. The standard and the reference maize flour samples were prepared as part of a project to obtain certified reference materials for determination of the mycotoxin ZON in maize. Immunoaffinity cleanup and HPLC with fluorescence detection were used for ZON determination as described previously (55).

## RESULTS

**Inactivation of the genes encoding the ABC transporters Pdr5p and Snq2p improves net uptake of ZON.** It was shown previously that two plasma membrane-localized ATP-binding cassette (ABC) transporter proteins encoded by the *PDR5* and *SNQ2* genes mediate efflux of estradiol in yeast (35), raising the question of whether deletion of these two genes affects uptake of the structurally unrelated, estrogenic mycotoxin ZON. The test system which we used is based on the estrogen-dependent growth of strains derived from PL3 on media without a pyrimidine supplement (48). These yeast strains express the human estrogen receptor cDNA under control of the yeast *PGK1* promoter (plasmid YEp90-HEGO). Binding of estrogenic substances to the receptor leads to activation of an engineered copy of the *URA3* gene having three estrogen-responsive elements in the promoter (*trp1::3ERE-URA3*), which allows de novo pyrimidine synthesis and growth of the otherwise pyrimidine auxotrophic (*ura3*) strain.

ABC transporter knockout strains derived from PL3 (Table 1), which had either a single mutation or the double mutation

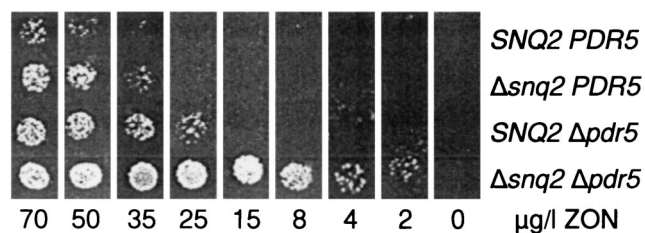


FIG. 2. Yeast strains were spotted on SC-HIS-URA plates containing different amounts of ZON. All of the PL3 derivatives contain the estrogen-dependent *URA3* gene integrated into the genome and the human estrogen receptor on the *HIS3* expression plasmid, but they differ with respect to the ABC transporter genes *SNQ2* and *PDR5*, as follows: YZGA305, wild type; YZGA307,  $\Delta$ *snq2*; YZGA309,  $\Delta$ *pdr5*; and YZGA311,  $\Delta$ *snq2*  $\Delta$ *pdr5*.

( $\Delta$ *snq2*  $\Delta$ *pdr5*), were spotted onto plates containing different amounts of ZON. As shown in Fig. 2, the *PDR5* *SNQ2* wild-type strain YZGA305 required about 70  $\mu$ g of ZON per liter for growth. Single disruptions of *SNQ2* or *PDR5* decreased the amount required about two- to threefold, whereas less than 2  $\mu$ g of ZON per liter was sufficient for growth of the PL3-derived strain lacking both ABC transporter Pdr5p and ABC transporter Snq2p (YZGA311).

**Construction of a mutant unable to utilize exogenous pyrimidines.** Although the  $\Delta$ *snq2*  $\Delta$ *pdr5* double mutant PL3 strain allowed detection of ZON at practically relevant concentrations, the presence of pyrimidines in complex natural extracts should also allow growth of this strain. To avoid false-positive results, we inactivated the *URK1* (27) and *FUR1* (28) genes, which encode pyrimidine salvage pathway enzymes (Fig. 3). First, the *URK1* gene was deleted by using the *Δurk1::TRP1* plasmid pRM753, resulting in strain YZGA439. In the second step, the *FUR1* gene was also destroyed, which yielded the final bioassay indicator strain, YZRM7. The different mutants were

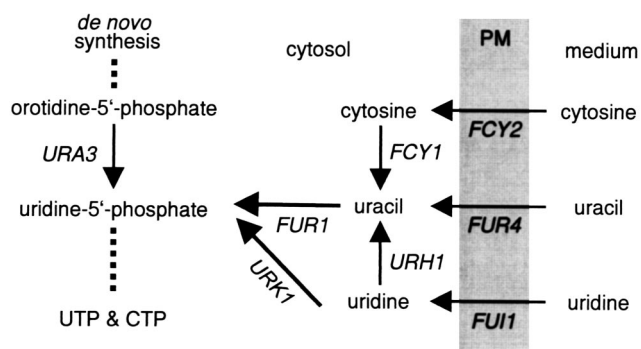


FIG. 3. Metabolism of external pyrimidines in *S. cerevisiae*. The genes encoding the proteins involved in the steps are indicated. External cytosine, uracil, and uridine are taken up by purine-cytosine permease (*FCY2*), uracil permease (*FUR4*), and uridine permease (*FUI1*), respectively. Cytosine can be converted into uracil by cytosine deaminase (*FCY1*), and uridine can be converted into uracil by uridine-cytidine *N*-ribohydrolase (*URH1*). Uridine 5-phosphate, the enzymatic product of the *URA3* step in pyrimidine de novo synthesis, can be formed either from uracil by uracil phosphoribosyltransferase (*FUR1*) or from uridine by uridine kinase (*URK1*). Blocking the latter two steps should therefore lead to an inability to utilize external pyrimidines. PM, plasma membrane.

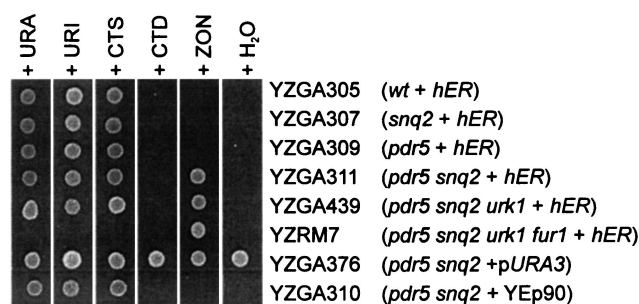


FIG. 4. Logarithmically growing cultures of strains (relevant genotypes are indicated in parentheses) were spotted onto SC-HIS-URA medium plates supplemented with (final concentrations) 20 mg of uracil (URA) per liter, 20 mg of uridine (URI) per liter, 20 mg of cytosine (CTS) per liter, 20 mg of cytidine (CTD) per liter, or 10  $\mu$ g of ZON per liter. Strain YZRM7 is unable to utilize external pyrimidines.

tested for the ability to grow on synthetic media with various pyrimidine supplements (Fig. 4). Only YZGA376 containing a *URA3* gene on plasmid pCGS966 could grow without supplements. The  $\Delta$ *snq2*  $\Delta$ *pdr5* double mutants, but not the single mutants, were rescued by 10  $\mu$ g of ZON per liter, a process dependent on the cDNA of the expressed human estrogen receptor (hER, present on plasmid YEp90-HEGO). In accordance with the pyrimidine pathway shown in Fig. 3, the constructed strain YZRM7 ( $\Delta$ *urk1::TRP1*  $\Delta$ *fur1::LEU2*) was unable to utilize exogenous pyrimidines but was rescued by ZON (and other estrogenic substances).

**Characterization of indicator strain YZRM7 and tests with maize and wheat.** To test whether ZON-dependent growth of bioassay strain YZRM7 can be monitored by simple turbidimetric measurement, this organism was grown in SC-HIS-URA liquid medium containing 0 to 10  $\mu$ g of ZON per liter. Growth curves were determined by measuring  $A_{600}$ , as shown in Fig. 5. The growth of the indicator strain was fairly slow, but it was strictly ZON dependent. The minimum amount of ZON sufficient to give detectable growth after 48 h was less than 1  $\mu$ g/liter, and it was more than 0.5  $\mu$ g/liter in liquid culture. Similarly, growth was observed on SC-HIS medium plates containing ZON concentrations higher than 0.8  $\mu$ g/liter (Fig. 5). The change from growth to no growth within a narrow concentration range thus allowed determination of ZON concentrations by simple titration and comparison with a ZON calibrating solution.

Since ZON is usually extracted from grain samples by using acetonitrile as the main solvent component, we determined the acetonitrile concentration necessary to inhibit growth of strain YZRM7. On solid media, growth was completely unaffected by final acetonitrile concentrations as high as 15%. In contrast, acetonitrile severely inhibited growth in liquid cultures, and the 50% inhibitory concentration was about 2.5% (data not shown). Therefore, we focused on development of the bioassay in which the agar plate format was used.

The bioassay was first tested with a reference maize flour (30) with a ZON concentration of 60  $\mu$ g/kg. Assuming that the ZON present in the maize flour was diluted fourfold during the extraction procedure and that the extract was diluted 10-fold in the yeast growth medium, the final ZON concentration should

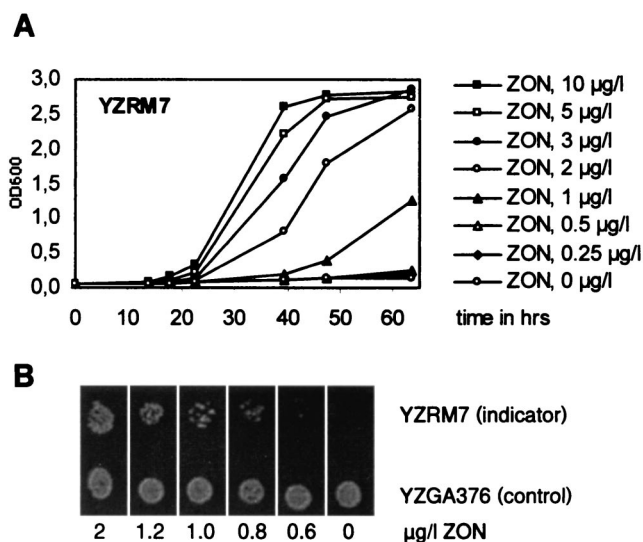


FIG. 5. ZON-dependent growth of strain YZRM7 at 30°C. (A) Turbidimetric measurement of growth in liquid SC-HIS-URA medium containing different amounts of ZON. OD<sub>600</sub>, optical density at 600 nm. (B) Growth observed on SC-HIS-URA medium plates after 4 days of incubation. The positive control strain YZGA376 contains a *URA3* gene (plasmid pCGS966).

have been 1.5  $\mu$ g/liter, which is about twice the concentration required to induce YZRM growth. We added 300, 200, 100, and 50  $\mu$ l of unpurified extract to 2 ml of agar and spotted indicator strain YZRM7 and several controls on the resulting plates. Growth of YZRM7 was clearly observed on the plates containing 200 and 300  $\mu$ l of maize flour extract, there was very slight growth on the plate containing 100  $\mu$ l, and there was no growth on the plate containing 50  $\mu$ l or the solvent controls (data not shown). We concluded that our indicator strain can detect the presence of ZON in crude extracts of maize without a loss of sensitivity due to matrix effects.

To test whether the bioassay can also detect ZON in wheat flour, we analyzed six grain samples derived from different wheat cultivars that were artificially inoculated at anthesis with the same *F. graminearum* strain. Extracts were prepared from the ground grains, and a series of twofold dilutions of the extracts was initially tested to determine the ZON concentration range. The highest dilution which still clearly gave a positive result in this test was defined as 100%, and further dilutions in 10% steps were then analyzed with the bioassay. For comparison, the extracts were also analyzed by conventional reversed-phase HPLC with fluorescence detection, as shown in Fig. 6. The calculated correlation coefficient for this experiment was 0.988, and the constant error as estimated by using the *y* intercept was  $-9.7$   $\mu$ g/kg. We subsequently analyzed more wheat and maize samples and confirmed that the bioassay is a very useful tool for detecting maize and wheat that is naturally contaminated with ZON and for evaluating the contents of grain samples. For instance, we analyzed kernels from wheat field trials performed in Iran, in which the concentration in only one of seven samples fell below the detection limit of 30  $\mu$ g of ZON equivalents per kg, as shown in Table 2.

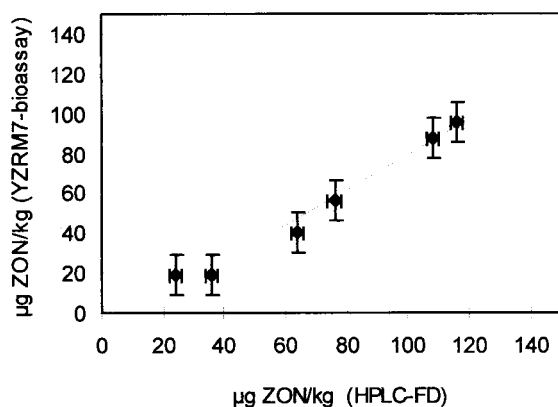


FIG. 6. Comparison of the ZON contents of wheat samples determined by the bioassay and by the HPLC method with fluorescence detection (HPLC-FD). The proportional error, determined by the slope, is about 11% and was primarily caused by overestimating the ZON concentration in the sample with the lowest content.

## DISCUSSION

We developed a sensitive and low-cost yeast bioassay for ZON and other estrogenic compounds, which is based on the estrogen receptor-dependent growth of an engineered yeast strain described previously (35, 48). The yeast growth response in this bioassay is very robust and reproducible, and the bioassay does not require preparation of cell extracts, chromogenic substrates, or expensive instrumentation, in contrast to conventional yeast two-plasmid systems, which rely on measurement of the expression of a reporter gene (for a review see reference 11), and especially in contrast to assays based on mammalian cells (36). Two findings were important for the development of the bioassay. The first was the observation that disruption of the *PDR5* and *SNQ2* genes, encoding two highly expressed ABC transporter proteins, improves intracellular retention of ZON. The second important observation was that inactivation of the yeast pyrimidine salvage pathway genes *URK1* and *FUR1* successfully prevents false-positive growth on pyrimidine substrates present in biological samples.

Disruption of *PDR5* and *SNQ2* leads to greatly improved uptake not only of 17- $\beta$  estradiol (35) and ZON but also of the ZON derivatives  $\alpha$ -zearalenol,  $\beta$ -zearalenol,  $\alpha$ -zearalanol, and

$\beta$ -zearalanol and several other estrogenic compounds (data not shown). By inactivating the two ABC transporters, the limit of detection could be lowered to the concentration range that is relevant with respect to existing or proposed national health and safety regulations.

A potential problem with  $\Delta snq2 \Delta pdr5$  mutants is that they are more sensitive to (accompanying) toxic substances that are also substrates of the broad-specificity transporters. We have previously shown that *PDR5* is responsible for the high trichothecene resistance of yeast (1), and more than 120 mg of the main *Fusarium* mycotoxin deoxynivalenol per liter (120 ppm) is tolerated by  $\Delta pdr5$  mutants. In view of the dilution steps involved in the assay procedure (40-fold), unrealistic amounts of deoxynivalenol would have to be present in a grain sample to produce an extract that inhibits the growth of the indicator strain. False-negative results are easily detected, since growth of the control strain YZGA376 is also inhibited.

As shown in Fig. 4, false-positive growth of the indicator strain is prevented by inactivating genes of the pyrimidine salvage pathway (*URK1* and *FUR1*). Since cytidine, which can be converted to uridine by cytidine deaminase (*CDD1*), does not support growth of the original PL3 strain (41), this pathway is not shown in Fig. 3. The inability of the  $\Delta urk1 \Delta fur1$  double mutant to grow on uracil, uridine, and cytosine shows that no other yeast gene products (e.g., YNL129w protein) have overlapping activity in the pyrimidine pathway.

The indicator strain YZRM7 can be propagated only on media containing estrogenic substances. However, if this strain is pregrown in the presence of high levels of potent estrogenic compounds (e.g., 1  $\mu$ g of DES per liter), several cell divisions can occur before growth arrest occurs. To avoid a high background level on the bioassay plates, it is advisable to inoculate the plates with highly diluted indicator yeast suspensions and to pregrow the strain for some time on a medium lacking an estrogenic supplement. Mutants of the indicator yeast strain able to grow without any estrogenic substance in the medium are observed after extended incubation periods. This phenotype could arise from mutations leading to expression of constitutively activating, truncated forms of the estrogen receptor (47).

The bioassay allows detection of estrogenic activity but not determination of the identity or concentration of a particular compound. *Fusarium* isolates produce not only ZON but also

TABLE 2. ZON contents of Iranian wheat samples

Location	Wheat cultivar	Growth responses <sup>a</sup>				ZON content ( $\mu$ g/kg of grain) <sup>b</sup>
		Undiluted	1/2 Dilution	1/4 Dilution	1/8 Dilution	
Karaj	Falat I <sup>c</sup>	—	—	—	—	25
Karaj	Falat II	++++	++++	++++	—	197
Parsabad Moghan	Sisonz	++++	++++	++++	(+)	219
Parsabad Moghan	Bacanoura	++++	++++	+++	+++	347
Parsabad Moghan	Kaspard	++++	++++	+++	—	190
Parsabad Moghan	Siren I	++++	++++	++	—	136
Parsabad Moghan	Siren II	+++	++	(—)	—	95

<sup>a</sup> Qualitative growth responses of the indicator strain were determined on plates containing 200- $\mu$ l portions of undiluted acetonitrile extract (undiluted) or twofold dilutions of the extract. (+), Slight growth above the titration point; (—), slight growth below the titration point. A positive result for the 1/4 dilution indicates the possible presence of estrogenic activity equivalent to twice the Austrian ZON guideline level (60  $\mu$ g/kg).

<sup>b</sup> ZON content as determined by HPLC with fluorescence detection.

<sup>c</sup> Most wheat samples were obtained in 2001; the only exception is the Falat I sample, which was obtained in 2000.

other derivatives (49). For instance,  $\alpha$ -zearalenol, which has 36-fold-higher estrogenic activity than ZON in mammalian cells (58), was found in wheat grain together with ZON (42), albeit at much lower concentrations. Our bioassay allows determination of only the total estrogenic activity in a sample, and the method is also limited to matrices without other interfering phytoestrogens. Soy flour samples, for instance, gave positive results. We also found that a honey-frosted corn flake sample was positive in the bioassay, but no ZON was detected in this sample by HPLC (40). One could speculate that the estrogenic activity originated from phytoestrogens present in clover honey. The presence of phytoestrogens in hops (38) also prevents use of the bioassay for beer.

Conventional ZON analysis procedures require sample cleanup before detection and quantification of ZON are possible (29). In our bioassay, the acetonitrile extracts can be used directly without further purification. If the ZON present in the sample is diluted 40-fold, the limit of detection for ZON in grain is about 30  $\mu\text{g}/\text{kg}$ , which is well below the Austrian guideline level approved for wheat and rye for human consumption (60  $\mu\text{g}/\text{kg}$ ). With wheat it is possible to increase the volume of acetonitrile extract added to the plates, and the detection limit can be lowered accordingly. The high fat content of maize extracts limits this approach.

The limit of detection of the bioassay compares favorably with the limits of detection of previously described antibody-based detection systems and commercially available kits (4, 6, 29, 34). A major disadvantage is the long time required for growth of the yeast indicator strain. In Austria, the bioassay would be a useful screening tool for identifying rare samples in which the concentration may exceed the ZON guideline level for grain intended for human consumption, which can then be confirmed by conventional analytical procedures. The few Iranian grain samples that we have analyzed so far confirm the presence of ZON, although the levels observed (Table 2) are much lower than the levels reported for the 1996 samples (63). The bioassay should be useful for obtaining a clearer picture of the situation in Iran.

The assay is very cost-effective, making it attractive for screening cheap grain commodities intended for feed use. In Europe, at least in theory, resorcylic acid lactones are illegal feed ingredients according to EC directive 96/22, appendix 1 (44). The import of meat from animals treated with zearalenol (zearanol) is banned, while feeding farm animals with naturally *Fusarium*-infected grain can also lead to the presence of zearalenol residues in pigs and cattle (26, 64). In view of increased public awareness of the problem of xenohormones or endocrine disrupters (25) and the need to monitor agricultural products for estrogenic *Fusarium* mycotoxins, we think that the bioassay which we developed may provide a valuable and economic screening tool. It is difficult to understand why the presence of estrogenic *Fusarium* mycotoxins in staple food should receive less attention than the presence of zearanol residues in hormone-treated beef or other environmental contaminants, which are orders of magnitude less potent than ZON.

In addition to grain testing, the bioassay is a valuable research tool, and we have used it to screen for ZON-degrading microorganisms and insertion mutants of *F. graminearum* which have lost the ability to produce ZON.

## ACKNOWLEDGMENTS

This work was supported by grants from the Austrian Federal Ministry of Agriculture and Forestry (BMLF ZI. 24.002/51-IIA/97) and from the Austrian Federal Ministry for Education, Science and Culture (bm:bwk GZ 309.007/3-VIII/B/8b/2000). The work on reference materials was supported by the European Community (grant EU SMT4-CT98-2228). We are grateful for a small grant from the City of Vienna (Hochschuljubiläumstiftung H-00199/97), which allowed G.A. to start the work on ZON. K.K. was supported by grant FWF P-1266 from the Austrian Science Foundation. N.S. is a recipient of fellowships from the Iranian Presidential Technology Cooperation Office and Ministry of Science, Research and Technology.

We thank A. Alizadeh (Cereal Research Department, Seed and Plant Improvement Institute, Karaj, Iran) for providing Iranian wheat samples and G. Wiesenberger and V. Cameron-Mills for critically reading the manuscript.

## REFERENCES

- Adam, G., R. Mitterbauer, A. Raditschnig, B. Poppenberger, T. Karl, S. Goritschnig, H. Weindorfer, and J. Glössl. 2001. Molecular mechanisms of deoxynivalenol resistance in the yeast *Saccharomyces cerevisiae*. *Mycotoxin Res.* **17**:19–23.
- Ahamed, S., J. S. Foster, A. Bukovsky, and J. Wimalasena. 2001. Signal transduction through the Ras/Erk pathway is essential for the mycoestrogen zearalenone-induced cell-cycle progression in MCF-7 cells. *Mol. Carcinog.* **30**:88–98.
- Alizadeh, A., and A. Saidi (ed.). 1999. *Fusarium* head blight of wheat and the possibility of its control in Iran. Progress Report of the National *Fusarium* Project. Seed and Plant Improvement Institute, Karaj, Iran. (In Farsi.)
- Barna-Vetro, L., A. Gyogyosi, and L. Solti. 1994. Monoclonal antibody-based enzyme-linked immunosorbent assay of *Fusarium* T-2 and zearalenone toxins in cereals. *Appl. Environ. Microbiol.* **60**:729–731.
- Bauer, J., K. Heinritz, M. Gareis, and B. Gedek. 1987. Changes in the genital tract of female swine after feeding with practice-relevant amounts of zearalenone. *Tieraerztl. Prax.* **15**:33–36.
- Bennett, G. A., T. C. Nelson, and B. M. Miller. 1994. Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study. *J. AOAC Int.* **77**:1500–1509.
- Botalico, A. 1998. *Fusarium* diseases of cereals: species complex and related mycotoxin profiles in Europe. *J. Plant Pathol.* **80**:85–103.
- Bundesministerium für Ernährung, Landwirtschaft und Forsten. 30 June 2000, posting date. Orientierungswerte für die Beurteilung der Gehalte an Deoxynivalenol und Zearalenon in Futtermitteln im Rahmen des §3 des Futtermittelgesetzes im Futter von Schwein, Rind und Huhn. BML Rdschr. 2000–324–3830/323. [Online.] <http://www.verbraucherministerium.de/landwirtschaft/futtermittel/orientierungswerte.htm>.
- Bundesministerium für Gesundheit, Sport und Konsumentenschutz. 1993. Richtwerte für Mykotoxine in Getreide für den menschlichen Genuß (GZ 32.110/1-III/B/1b/93). Mitt. Oesterr. Sanitätsverwalt. (Vienna) **94**:360.
- Burke, D. T., F. G. Carle, and M. V. Olsen. 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236**:806–812.
- Diel, P., K. Smolnikar, and H. Michna. 1999. *In vitro* test systems for the evaluation of the estrogenic activity of natural products. *Planta Med.* **65**:197–203.
- Ellner, F. M. 1999. Ein Jahr für *Fusarium*toxine, p. 1–4. In H. Rosner and P. Kielstein (ed.), Proceedings of the 21st Mycotoxin Workshop. German Federal Institute of Health Protection of Consumers and Veterinary Medicine, Berlin, Germany.
- Etienne, M., and J.-Y. Dourmad. 1994. Effects of zearalenone or glucosinolates in the diet in reproduction in sows: a review. *Livest. Prod. Sci.* **40**:99–113.
- Etienne, M., and M. Jemmali. 1982. Effects of zearalenone (F-2) on estrous activity and reproduction in gilts. *J. Anim. Sci.* **55**:1–10.
- European Commission Scientific Committee on Food. 22 July 2000, posting date. Opinion on *Fusarium* toxins. Part 2: Zearaleonone (ZEA). [Online.] Health and Consumer Directorate-General, European Commission, Brussels, Belgium. [http://europa.eu.int/comm/food/fs/sc/scf/out65\\_en.pdf](http://europa.eu.int/comm/food/fs/sc/scf/out65_en.pdf).
- Food and Agriculture Organization. 1997. Worldwide regulations for mycotoxins 1995. A compendium. *Food Nutr. Pap.* **64**:1–43.
- Freni-Titulaer, L. W., J. F. Cordero, L. Haddock, G. Lebron, R. Martinez, and J. L. Mills. 1986. Premature thelarche in Puerto Rico. A search for environmental factors. *Am. J. Dis. Child.* **140**:1263–1267.
- Gaumy, J. L., J. D. Bailly, G. Benard, and P. Guerre. 2001. Zearalenone: origin and effect on farm animals. *Rev. Med. Vet.* **152**:123–136.
- Gietz, R. D., R. H. Schiestl, A. R. Willems, and R. A. Woods. 1995. Studies on the transformation of intact yeast cells by the LiAc/ss-DNA/PEG procedure. *Yeast* **11**:355–360.
- Hidy, P. H., R. S. Baldwin, R. L. Greasham, C. L. Keith, and J. R. McMullen.

1977. Zearalenone and some derivatives: production and biological activities. *Adv. Appl. Microbiol.* **22**:59–82.
21. Hilakivi-Clarke, L., I. Onojafe, M. Raygada, E. Cho, T. Skaar, I. Russo, and R. Clarke. 1999. Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. *Br. J. Cancer* **80**:1682–1688.
  22. Hormozdiari, H., N. E. Day, B. Aramesh, and E. Mahboubi. 1975. Dietary factors and esophageal cancer in the Caspian littoral of Iran. *Cancer Res.* **35**:3493–3498.
  23. Joint FAO/WHO Expert Committee on Food Additives. January 2000, posting date. Joint FAO/WHO Expert Committee on Food Additives: position paper on zearalenone. Publication CX/FAC00/19. [Online.] Codex Alimentarius Commission, Rome, Italy. [http://www.who.int/fsf/Chemicalcontaminants/ZearalenonePP00\\_19.pdf](http://www.who.int/fsf/Chemicalcontaminants/ZearalenonePP00_19.pdf).
  24. Jones, J. S., and L. Prakash. 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast* **6**:363–366.
  25. Juberg, D. R. 2000. An evaluation of endocrine modulators: implications for human health. *Ecotoxicol. Environ. Saf.* **45**:93–105.
  26. Kennedy, D. G., J. D. G. McEvoy, S. A. Hewitt, A. Cannavan, W. J. Blanchflower, and C. T. Elliott. 1998. Zeranone is formed from *Fusarium* spp. toxins in cattle *in vivo*. *Food Addit. Contam.* **15**:393–400.
  27. Kern, L. 1990. The *URK1* gene of *Saccharomyces cerevisiae* encoding uridine kinase. *Nucleic Acids Res.* **18**:5279.
  28. Kern, L., J. de Montigny, R. Jund, and F. Lacroute. 1990. The *FUR1* gene of *Saccharomyces cerevisiae*: cloning, structure and expression of wild-type and mutant alleles. *Gene* **88**:149–157.
  29. Krska, R., and R. Josephs. 2001. The state-of-the-art in the analysis of estrogenic mycotoxins in cereals. *Fresenius J. Anal. Chem.* **369**:469–476.
  30. Krska, R., R. Josephs, H. Pettersson, and S. McDonald. 2001. ZONMAIZE: EU-SMT project for the production of certified reference materials (CRM) for the determination of zearalenone in maize. *Mycotoxin Res.* **17**:92–96.
  31. Kuiper, G. G. J. M., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, B. van der Burg, and J.-Å. Gustafsson. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor  $\beta$ . *Endocrinology* **139**:4252–4263.
  32. Kuiper-Goodman, T., P. M. Scott, and H. Watanabe. 1987. Risk assessment of the mycotoxin zearalenone. *Regul. Toxicol. Pharmacol.* **7**:253–306.
  33. Lew, H. 15 March 2000, revision date. Empfohlenes Richtwerteschema für Desoxygenivalenol (Vomitoxin) und Zearalenon im Futter. Foerderungsdienst 47:157. [Online.] <http://www.lebensministerium.at/download/dlpublik/FD0599.PDF>.
  34. Liu, M. T., B. P. Ram, L. P. Hart, and J. J. Pestka. 1985. Indirect enzyme-linked immunosorbent assay for the mycotoxin zearalenone. *Appl. Environ. Microbiol.* **50**:332–336.
  35. Mahe, Y., Y. Lemoine, and K. Kuchler. 1996. The ATP-binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids *in vivo*. *J. Biol. Chem.* **271**:25167–25172.
  36. Mayer, U., A. Butsch, and S. Schneider. 1992. Validation of two *in vitro* test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. *Toxicology* **74**:135–149.
  37. Miksicek, R. J. 1994. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J. Steroid Biochem. Mol. Biol.* **49**:153–160.
  38. Milligan, S., J. Kalita, V. Pocock, A. Heyerick, L. De Cooman, H. Rong, and D. De Keukeleire. 2002. Estrogenic activity of the hop-phytoestrogen 8-prenylnaringenin. *Reproduction* **123**:235–242.
  39. Mirocha, C. J., and C. M. Christensen. 1974. Estrogenic mycotoxins synthesized by *Fusarium*, p. 129–148. *In* I. F. H. Purchase (ed.), *Mycotoxins*. Elsevier, Amsterdam, The Netherlands.
  40. Mitterbauer, R. 2000. Molecular mechanisms of trichothecene resistance and development of a yeast-based bioassay for zearalenone. Ph.D. thesis. University of Agricultural Sciences, Vienna, Austria.
  41. Mitterbauer, R., T. Karl, and G. Adam. 2002. *Saccharomyces cerevisiae URH1* (encoding uridine-cytidine *N*-ribohydrolase): functional complementation by a nucleoside hydrolase from a protozoan parasite and by a mammalian uridine phosphorylase. *Appl. Environ. Microbiol.* **68**:1336–1343.
  42. Müller, H. M., J. Reimann, U. Schumacher, and K. Schwadorf. 1997. *Fusarium* toxins in wheat harvested during six years in an area of southwest Germany. *Nat. Toxins* **5**:24–30.
  43. National Toxicology Program. 16 December 2002, revision date. National Toxicology Program carcinogenesis bioassay of zearalenone in F344/N rats and F6C3F1 mice. National Toxicology Program Technical Report Series, vol. 235. [Online.] Department of Health and Human Services, Research Triangle Park, N.C. [http://ntp-server.niehs.nih.gov/htdocs/Results\\_status/ResstatZ/10770.-C.html](http://ntp-server.niehs.nih.gov/htdocs/Results_status/ResstatZ/10770.-C.html).
  44. Official Journal of the European Communities 1996. Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of  $\beta$ -agonists. *Off. J. Eur. Communities* **L125**:10–32.
  45. Park, D. L., and T. C. Troxell. 2002. U.S. perspective on mycotoxin regulatory issues. *Adv. Exp. Med. Biol.* **504**:277–285.
  46. Pfohl-Leschkowicz, A., L. Chekir-Ghedira, and H. Bacha. 1995. Genotoxicity of zearalenone, an estrogenic mycotoxin: DNA adduct formation in female mouse tissues. *Carcinogenesis* **16**:2315–2320.
  47. Pierrat, B., D. M. Heery, P. Chambon, and R. Losson. 1994. A highly conserved region in the hormone binding domain of the human estrogen receptor functions as an efficient transactivation domain in yeast. *Gene* **143**:193–200.
  48. Pierrat, B., D. M. Heery, Y. Lemoine, and R. Losson. 1992. Functional analysis of the human estrogen receptor using a phenotypic transactivation assay in yeast. *Gene* **119**:237–245.
  49. Richardson, K. E., W. M. Hagler, and C. J. Mirocha. 1985. Production of zearalenone,  $\alpha$ - and  $\beta$ -zearalenol, and  $\alpha$ - and  $\beta$ -zearalanol by *Fusarium* spp. in rice culture. *J. Agric. Food. Chem.* **33**:862–866.
  50. Rocco, J. A. 1988. Hyperestrogenism in swine due to natural poisoning with zearalenone. *Rev. Argent. Microbiol.* **20**:119–123.
  51. Saenz de Rodriguez, C. A., A. M. Bongiovanni, and L. Conde de Borrego. 1985. An epidemic of precocious development in Puerto Rican children. *J. Pediatr.* **107**:393–396.
  52. Saidi, F., A. Sepehr, S. Fahimi, M. J. Farahvash, P. Salehian, A. Esmailzadeh, M. Keshoofy, N. Pirmoazen, M. Yazdanbod, and M. K. Roshan. 2000. Oesophageal cancer among the Turkomans of northeast Iran. *Br. J. Cancer* **83**:1249–1254.
  53. Schoental, R. 1983. Precocious sexual development in Puerto Rico and estrogenic mycotoxins (zearalenone). *Lancet* **i**:573.
  54. Schoental, R. 1985. Trichothecenes, zearalenone and other carcinogenic metabolites of *Fusarium* and related microfungi. *Adv. Cancer Res.* **45**:217–290.
  55. Schuhmacher, R., R. Krska, M. Grasserbauer, W. Edinger, and H. Lew. 1998. Immuno-affinity columns versus conventional clean-up: a method-comparison study for the determination of zearalenone in corn. *Fresenius J. Anal. Chem.* **360**:241–245.
  56. Scott, P. M. 1997. Multi-year monitoring of Canadian grains and grain-based foods for trichothecenes and zearalenone. *Food Addit. Contam.* **14**:333–339.
  57. Sherman, F. 1991. Getting started with yeast. *Methods Enzymol.* **194**:3–21.
  58. Shier, W. T., A. C. Shier, W. Xie, and C. J. Mirocha. 2001. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicol.* **39**:1435–1438.
  59. Smith, D. R., A. P. Smyth, and D. T. Moir. 1990. Amplification of large artificial chromosomes. *Proc. Natl. Acad. Sci. USA* **87**:8242–8246.
  60. Szuets, P., A. Mesterhazy, T. Bartok, and G. Y. Falkay. 1998. Premature thelarche/mastopathy and *Fusarium* toxin contamination of foods: key to a mysterious disease. *Pediatr. Res.* **43**:86.
  61. Szuets, P., A. Mesterhazy, G. Y. Falkay, and T. Bartok. 1997. Early thelarche symptoms in children and their relations to zearalenone contamination in foodstuffs. *Cereal Res. Commun.* **25**:429–436.
  62. Tomaszewski, J., R. Miturski, A. Semczuk, J. Kotarski, and J. Jakowicki. 1998. Tissue zearalenone concentration in normal, hyperplastic and neoplastic human endometrium. *Ginek. Pol.* **69**:363–366.
  63. Yazdanpanah, H., M. J. Khoshnood-Mansourkhani, A. Shafaati, H. Rahimian, H. R. Rasekh, K. Gilani, and M. Moradkhani. 1997. Evaluation of natural occurrence of *Fusarium* toxins in wheat fields of northern Iran. *Cereal. Res. Commun.* **25**:337–341.
  64. Zöllner, P., J. Jodlbauer, M. Kleinova, H. Kahlbacher, T. Kuhn, W. Hochsteiner, and W. Lindner. 2002. Concentration levels of zearalenone and its metabolites in urine, muscle tissue, and liver samples of pigs fed with mycotoxin contaminated oats. *J. Agric. Food Chem.* **50**:2494–2501.