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Free radical-mediated lipid peroxidation induced by T-2 toxin in yeast (*Kluyveromyces marxianus*)

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Lipid peroxidation may be one of the main manifestations of cellular damage in the toxicity of several mycotoxins. A species of yeast, Kluyveromyces marxianus*, was used in this study to determine the oxidative damage induced by T-2 toxin (T-2). Malondialdehyde (MDA), produced from the decomposition of lipid peroxides, was monitored using the thiobarbituric acid reaction. The yeast showed a high sensitivity to T-2 because the addition of 25 ng T-2/mL medium greatly reduced the multiplication rate of yeast. In addition, the MDA content increased when the concentration of T-2 was increased in the growth medium. Preincubation of the yeast with linoleic and linolenic acids in the medium enhanced the effects of T-2. The addition of the antioxidant dl-α-tocopherol acetate completely quenched the effects of T-2 whereas ascorbic acid and reduced glutathione (GSH) acted as prooxidants in this system. Electron paramagnetic resonance (EPR) spin trapping technique using lipid soluble N-*tert*-butyl-α-phenylnitrone (PBN) or more water soluble α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN) as free radical spin traps showed that free radical production was promoted by T-2. Vitamin E effectively quenched the EPR signals of the spin adducts. The observed spin adduct hyperfine splitting constants were consistent with those of α-hydroxyethyl radicals. The spin trapping data strongly suggest that initially generated hydroxyl radicals react with ethanol that is present in the samples, and the α-hydroxyethyl radicals formed in this process are then trapped. These data demonstrate that T-2 stimulates lipid peroxidation in a biological system due to an increased generation of hydroxyl radicals.* (J. Nutr. Biochem. 9:370–379, 1998) © *Elsevier Science Inc. 1998*

Keywords: mycotoxins; T-2 toxin; yeast; malondialdehyde; lipid peroxidation; electron paramagnetic resonance

Introduction

A variety of substances promote oxidative stress in tissues. The targets of oxidative damage are usually critical biomolecules such as nucleic acids, proteins, and lipids.[1–3] The role of oxidation in the aging processes,[4] carcinogenesis,[5] and many metabolic diseases[6] has gained increasing attention. In recent years, it has been demonstrated that lipid peroxidation is one of the main manifestations of cellular damage in the toxicity of several mycotoxins that are found in a variety of substrates including food and feeds. Initially Rahimtula et al.[7] showed that ochratoxin A (OA), when added to rat liver microsomes, enhanced the rate of nicotinamide adenine dinucleotide phosphate (NADPH) or ascorbate-dependent lipid peroxidation as measured by malondialdehyde (MDA) formation. In vivo administration to rats also resulted in enhanced lipid peroxidation, measured by ethane exhalation. Studies by Hasinoff et al.[8] and Omar et al.[9,10] suggested that OA stimulated lipid peroxidation by mechanisms involving chelation and reduction of iron. The formation of reactive oxygen species by complex-

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ing with the NADPH-cytochrome P-450 reductase system was also proposed. More recently, Malaveille et al.[11] reported that the antioxidant Trolox C, a water soluble form of vitamin E, completely quenched the genotoxic effects of OA in *Escherichia coli*. A similar protective effect of the antioxidant vitamin C against OA genotoxicity in mice was demonstrated by Bose and Sinha.[12] Shen et al.[13] have shown that aflatoxin B_1 causes lipid peroxidation in rat liver. Pretreatment with vitamin E, selenium, or deferoxamine, an iron chelator, significantly inhibited lipid peroxidation as well as liver cell damage. Recently, Hoehler et al.[14,15] found that OA induces hydroxyl radical production in bacteria and microsomes as evidenced by electron paramagnetic resonance (EPR) spectroscopy and that OA enhances the permeability of the cellular membrane to Ca^{2+} in the bacterial system. According to Rizzo et al.[16] the trichothecenes deoxynivalenol (DON) and toxin T-2 (T-2) stimulate lipid peroxidation in rat liver along with a depletion of hepatic glutathione. (See *Figure 1* for the chemical structures of T-2, DON, and OA.) Dietary use of selenium, in combination with ascorbic acid and α-tocopherol, provided protection against acute toxicosis caused by the two mycotoxins. Taken together, these findings provide good evidence that lipid peroxidation is one of the mechanisms of mycotoxin-induced cell injury and that these effects can be quenched to a certain extent by free radical scavenging agents, especially vitamin E. It is not known, however, if these are the principal or the only effects of these mycotoxins. In addition, the precise nature of the radicals formed remains to be clarified. In the present study, a species of yeast, *Kluyveromyces marxianus*, was used as a model to determine if T-2 induced free radical production. Previous studies in our laboratory demonstrated that this organism was highly sensitive to the toxic effects of the trichothecenes, especially T-2.[17,18]

Materials and methods

Mycotoxins and chemicals

T-2 and DON were obtained from Sigma Chemical Co. (St. Louis, MO USA). OA was isolated from a culture of *Aspergillus alutaceus*.[19,20] The toxin standards were diluted with ethanol. The spin trapping agents N-*tert*-butyl-α-phenylnitrone (PBN), α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), as well as *dl*-α-tocopheryl acetate, reduced glutathione (GSH), ascorbic acid, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ferrous sulfate, tetrahydrofuran, and linoleic and linolenic acids were purchased from Sigma. Acetonitrile was obtained from Fisher (Winnipeg, MB, Canada). All other chemicals were of the highest grade commercially available.

Yeast, media, and growth conditions

K. marxianus (ATCC 34439) obtained from American Type Culture Collection (Rockville, MD USA) was maintained in culture tubes in a yeast extract-peptone-glucose broth (YPG; 1%-1%-2%) at 30°C in the absence or presence of 150 μg each of linoleic and linolenic acids per milliliter of culture medium. For the experiments YPG broth (0.1%-0.1%-4%, pH 7.5) was inoculated with a 2-day-old yeast culture and incubated in culture tubes for 24 hours at 37°C on an orbital shaker (Orbital Shaker, Bellco Glass Inc., Vineland, NJ USA) at 80 rpm. These procedures were similar to those previously described.[17,18] An amount of 2% ethanol (v/v) containing various concentrations of toxin, vitamin E, or other agents was added to all of the preparations.

Protein and malondialdehyde analyses

Protein concentration as a measure of cell growth was determined following the method of Bradford.[21] MDA produced by the decomposition of lipid peroxides following the thiobarbituric acid reaction was monitored by high performance liquid chromatography (HPLC).[22] For the MDA assay, 0.5 mL of yeast suspension was mixed with 0.85 mL of 5% trichloracetic acid, 0.1 mL of BHT (0.5 g/L in methanol), and 0.05 mL of 0.1 M $FeSO_4 \cdot 7H_2O$. BHT was used as an antioxidant and ferrous sulfate was added as a catalyst for decomposing hydroperoxides. The samples were homogenized for 20 seconds using an Ultra-Turrax homogenizer (Polytron, Kinematica GmbH, Luzern, Switzerland) and centrifuged at 13,000 · g for 15 minutes at 4°C to obtain a clear supernatant. One milliliter of 0.36% TBA and 1 mL of the supernatant were placed in a boiling water bath for 30 minutes in covered tubes. MDA standards were prepared as described by Kakuda et al.[23] HPLC was performed on a 4.6 by 250 mm ODS column (Beckman Instruments Inc., Allendale, NJ USA). The mobile phase consisted of 10% acetonitrile, 0.6% tertahydrofuran, and 5 mM phosphate buffer (pH 8) at a flow rate of 1.5 mL/min and 40°C. The peaks were detected at 532 nm.

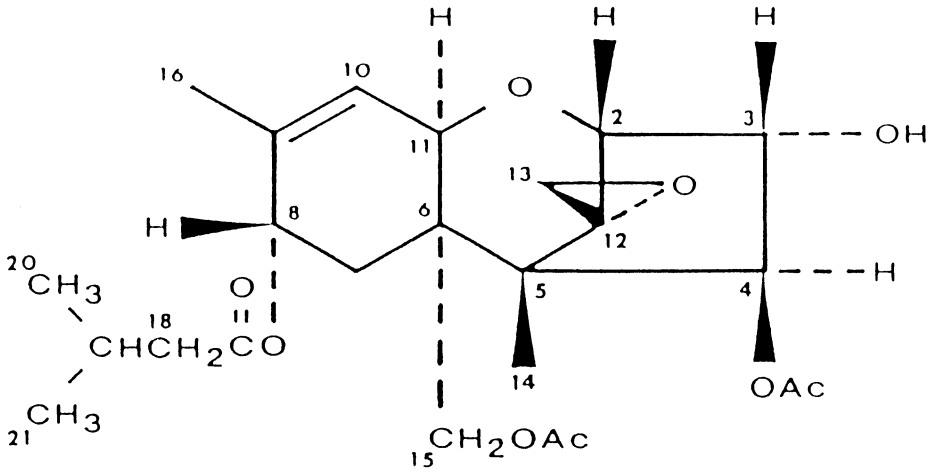
Analysis of free radicals

Late logarithmic cultures were used for the EPR spin trapping experiments. Yeast was grown for 48 hours in a YPG medium (0.1%-0.1%-4%, 150 μg each of linoleic and linolenic acids per milliliter) in Erlenmeyer flasks and the protein concentration was determined. Prior to the EPR experiments, the samples were centrifuged at 500 · g for 15 minutes, and the supernatant was discarded. Fresh medium (without unsaturated fatty acids) was added to the pellet to achieve a protein-concentration of 4 mg/mL. The samples were stored for up to 8 hours at 4°C and were incubated at room temperature 15 minutes prior to the EPR experiments. A small amount of ethanol (5% v/v) containing various concentrations of toxin or vitamin E was added to the preparations and each sample was scanned for up to 75 minutes (64 scans) at 37°C with either 75 mM lipid-soluble PBN or 75 mM water-soluble 4-POBN. All the reaction mixtures contained 5% v/v added ethanol. The spectra were measured with a Varian Associates Model E-12 EPR spectrometer operating at a microwave frequency of 9.02 GHz with 100 kHz modulation, which was interfaced with a Nicolet Instruments Model 1180 computer and Model 2090 transient recorder (Nicolet Instrument Corporation, Madison, WI USA). The computer program used was NTCESR (Nicolet Technology Corporation, Madison, WI USA). The instrumental parameters were: microwave power of 10 milliwatts, modulation amplitude of 0.8 G, and scan range of 100 G.

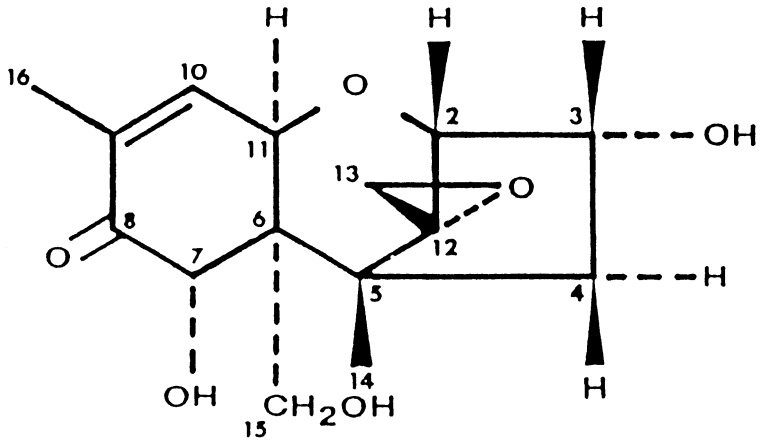
Spectra shown in *Figures 6* and *7* were determined on the same day from the same batch of cells and are representative of at least three independent experiments. In general the intensity of the signal for the controls were the same at the beginning and completion of the analysis.

Results

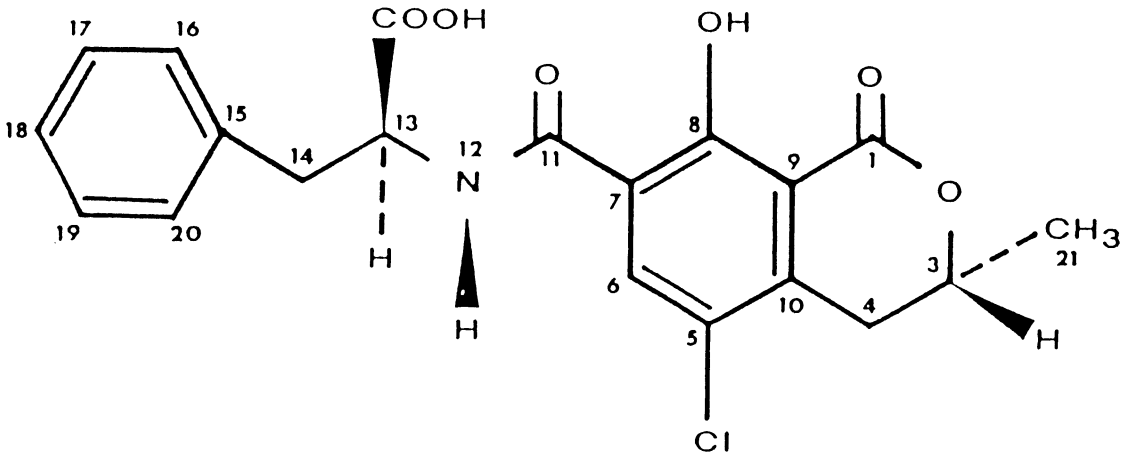
Yeast was highly sensitive to T-2 as the addition of as little as 25 ng T-2/mL medium reduced the protein concentration of the culture by 15% (from 128 ± 5 to 109 ± 2 μg/mL; *Figure 2A*). The addition of the high concentration of T-2



T-2



DON



OA

Figure 1 Structures of T-2 toxin (T-2), deoxynivalenol (DON), and ochratoxin A (OA).

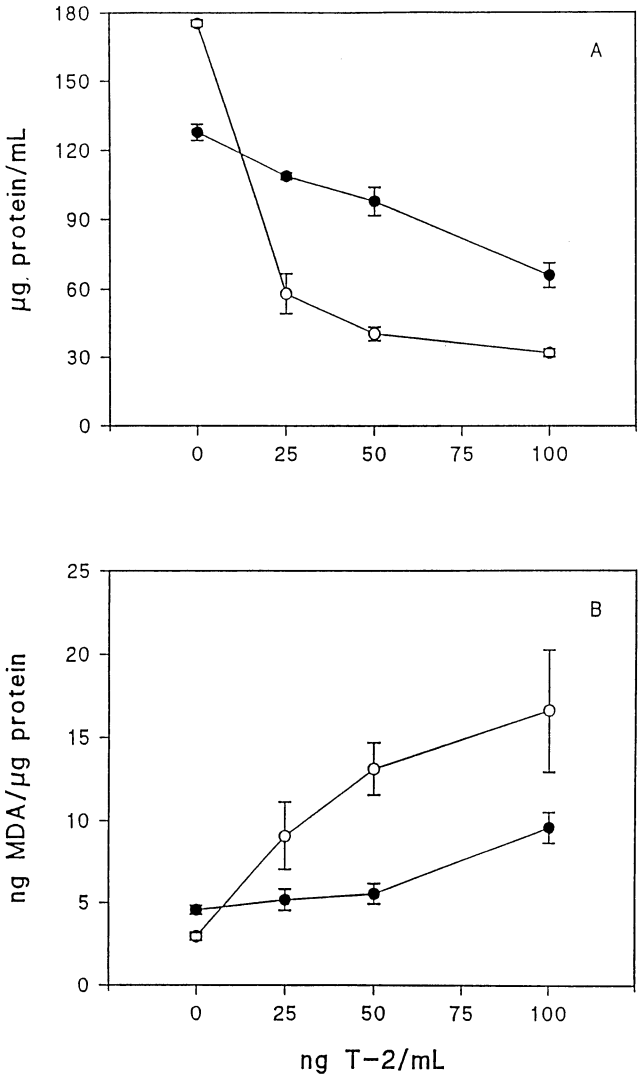


Figure 2 Effect of T-2 toxin (T-2) on growth (*Figure 2A*) and malondialdehyde (MDA; *Figure 2B*) concentration of *Kluyveromyces marxianus*. The yeast was cultured for 24 hours in liquid medium with various concentrations of T-2. Prior to the experiment the samples were cultured for 2 days in the absence (●) or presence (○) of 150 μg each of linoleic and linolenic acids per milliliter of culture medium. Protein and MDA were measured as described under Materials and methods. Each value is the mean and SD (n = 3) from a representative experiment.

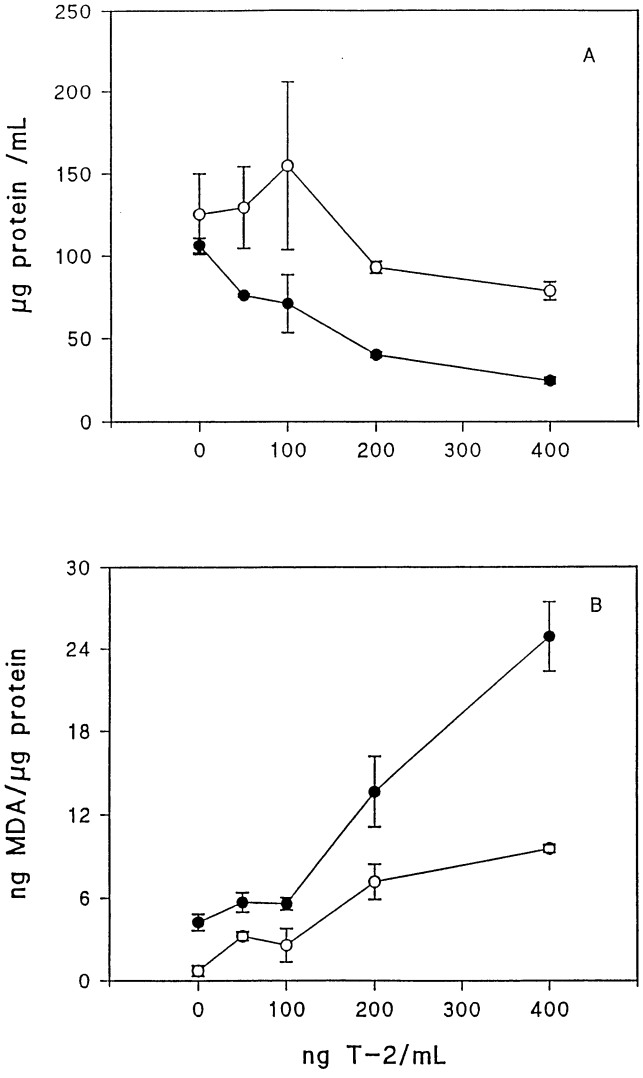


Figure 3 Effect of T-2 toxin (T-2) and vitamin E on growth (*Figure 3A*) and malondialdehyde (MDA; *Figure 3B*) concentration of *Kluyveromyces marxianus*. The yeast was cultured for 24 hours in liquid medium with various concentrations of T-2 either in the absence (●) or presence (○) of 25 mM vitamin E (*dl*-α-tocopherol acetate). Protein and MDA were measured as described under Materials and methods. Each value is the mean and SD (n = 3) from a representative experiment.

(100 ng/mL) resulted in an overall depression of the protein concentration by 51% (to 66 ± 5 μg/mL). Associated with this effect was a 100% increase in the concentration of MDA (from 4.6 ± 0.4 to 9.6 ± 0.9 ng/μg protein) with the addition of 100 ng T-2/mL of culture (*Figure 2B*). MDA was presumably produced by the decomposition of lipid peroxides. The addition of 150 μg each of linoleic and linolenic acids per milliliter of medium 2 days prior to the subsequent incubation of the yeast with the toxin for 24 hours resulted in an increase of the total protein content of the yeast cells in the control group from 128 ± 5 to 175 ± 1 μg/mL. In contrast, T-2 caused a much greater reduction in yeast growth when the medium contained the unsaturated fatty acids (67%, 175 ± 1 vs. 58 ± 9 μg protein/mL due to only 25 ng T-2/mL) compared with cells that were grown on the medium that did not contain these compounds (15%, 128 ± 5 vs. 109 ± 2 μg protein/mL). The corresponding MDA concentration in cultures obtained from the yeast stock that had been grown on the unsaturated fatty acids also increased dramatically from 2.9 ± 0.3 ng/μg protein for the controls to 16.6 ± 3.7 ng/μg protein for the culture containing 100 ng T-2/mL. Separation of the culture media from the cells by centrifugation followed by homogenization of the cells has demonstrated that more than 90% of the MDA was present in the medium with the balance being located in the yeast (data not shown).

In further studies, T-2 was added to the medium with or without vitamin E to determine if a free radical scavenging agent could reduce the toxicity of T-2. The addition of 25 mM α-tocopherol acetate to the growth medium greatly

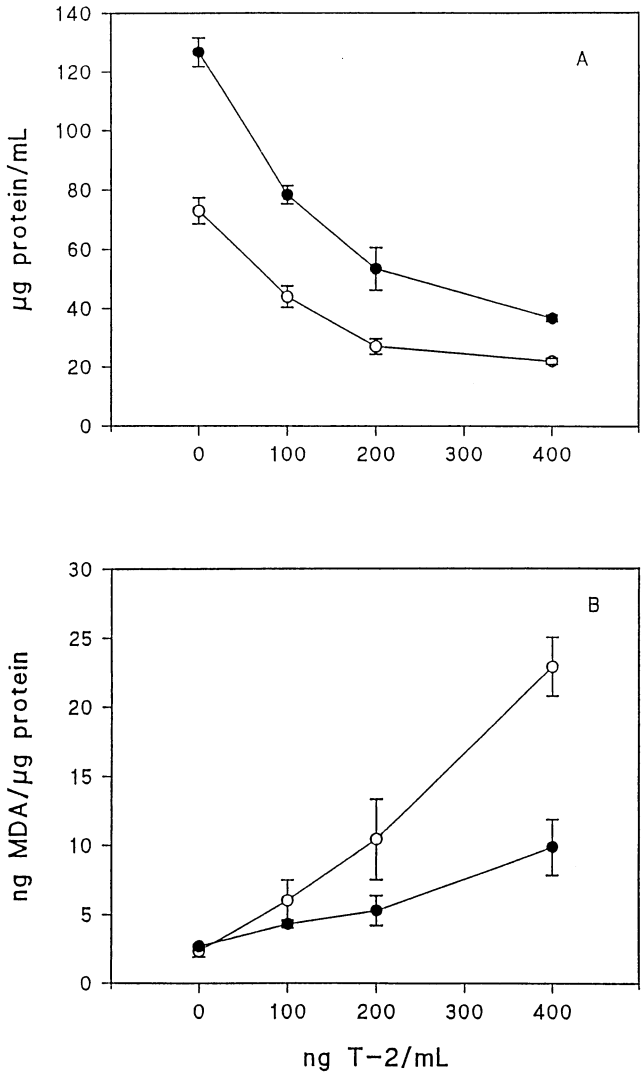


Figure 4 Effect of T-2 toxin (T-2) and ascorbic acid on growth (*Figure 4A*) and malondialdehyde (MDA; *Figure 4B*) concentration of *Kluyveromyces marxianus*. The yeast was cultured for 24 hours in liquid medium with various concentrations of T-2 either in the absence (●) or presence (○) of 25 mM vitamin C (ascorbic acid). Protein and MDA were measured as described under Materials and methods. Each value is the mean and SD (n = 3) from a representative experiment.

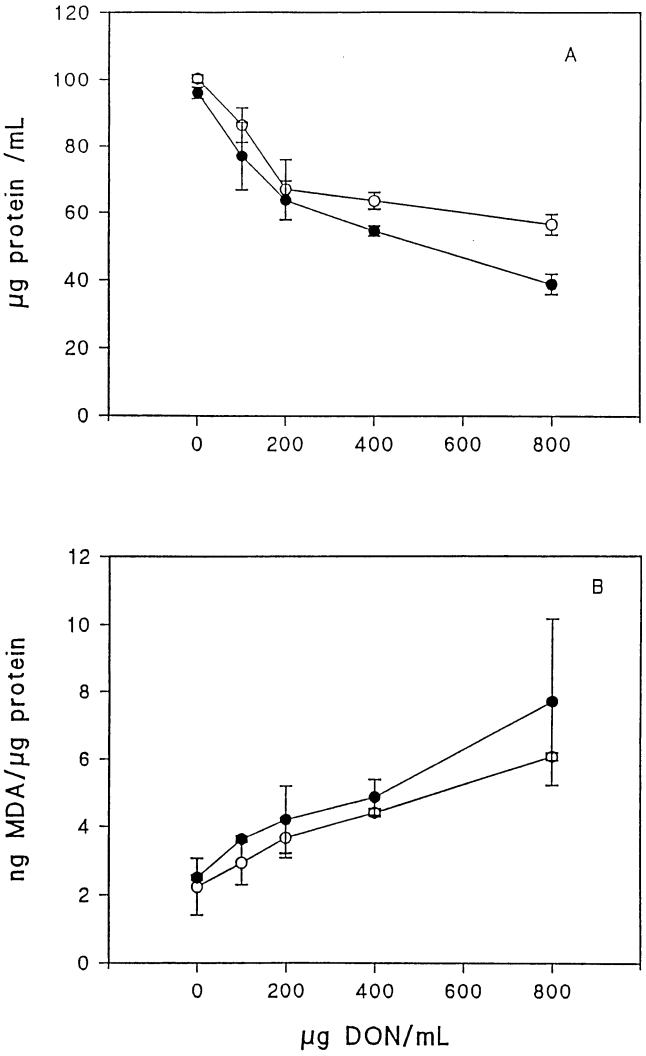


Figure 5 Effect of deoxynivalenol (DON) and vitamin E on growth (*Figure 5A*) and malondialdehyde (MDA; *Figure 5B*) concentration of *Kluyveromyces marxianus*. The yeast was cultured for 24 hours in liquid medium with various concentrations of DON either in the absence (●) or presence (○) of 25 mM vitamin E (*dl*-α-tocopherol acetate). Protein and MDA were measured as described under Materials and methods. Each value is the mean and SD (n = 3) from a representative experiment.

reduced the growth depressing effects of T-2 especially when the concentration of T-2 was less than 200 ng/mL (*Figure 3A*). Yeast growth in the presence of 400 ng T-2/mL of culture, for example, was only 23% of that of the controls (106 ± 5 vs. 24 ± 2 μg protein/mL) in the absence of vitamin E but 63% of the controls (106 ± 5 vs. 79 ± 6 μg protein/mL) in the presence of vitamin E. Corresponding studies with MDA demonstrated that vitamin E significantly reduced the level of lipid peroxidation at any of the selected T-2 concentrations (*Figure 3B*). For example, the concentration of MDA in the samples treated with 400 ng T-2/mL was approximately three-fold higher in cultures that did not contain added vitamin E compared with those that contained added vitamin E. The nonesterified form of vitamin E, in contrast to the results obtained with vitamin E acetate, did not protect yeast against T-2 toxicity (data not shown). This form of the vitamin may not have been taken up by the yeast. These observations demonstrate that the free radical scavenger vitamin E can protect yeast against the toxic effects of T-2 presumably by quenching free radical production and thereby reducing the degree of lipid peroxidation as assessed by MDA production.

In contrast to vitamin E, two other antioxidants (vitamin C and GSH) enhanced the toxicity of T-2. The addition of 25 mM versus no vitamin C to the cultures containing 0, 100, 200, and 400 ng/mL of T-2 reduced yeast growth by 43, 44, 49, and 41% (*Figure 4A*), respectively, and increased the corresponding MDA concentrations by 0, 39, 98, and 232% (*Figure 4B*). A similar pattern of response was also obtained with GSH (*Table 1*). These results suggest that both vitamin C and GSH greatly enhance the toxic effects of T-2 in yeast.

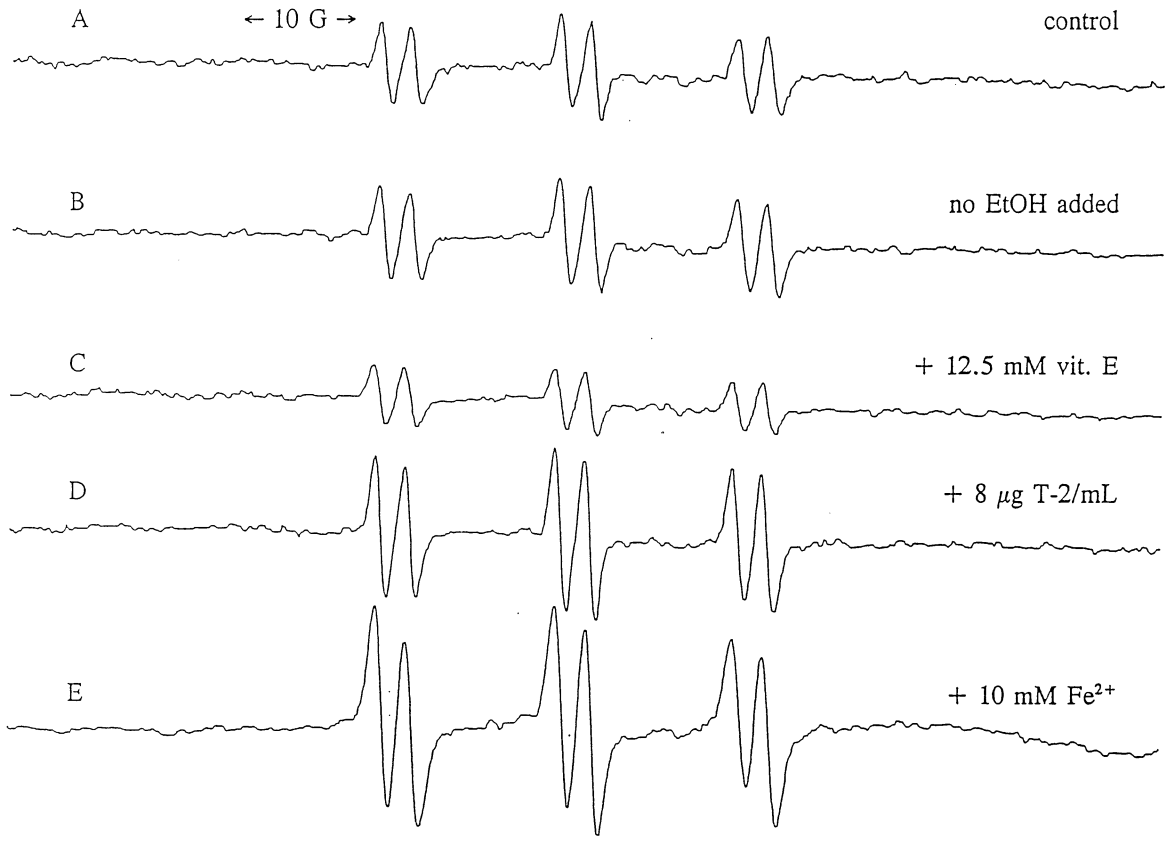


Figure 6 Electron paramagnetic resonance spectra of α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN) radical adducts following the incubation of *Kluyveromyces marxianus* with vitamin E, T-2 toxin, and $FeSO_4$. Yeast was cultured for 48 hours in liquid medium, centrifuged, new medium added to the pellets, and the protein concentration adjusted to 4 mg/mL. The spectra were recorded at 37°C immediately after adding 75 mM 4-POBN. In addition, the solutions contained 12.5 mM vitamin E (*dl*-α-tocopherol acetate; sample C), 8 μg T-2/mL (sample D), 10 mM $FeSO_4 \times 7\ H_2O$ (sample E). 5% v/v ethanol was added to samples A, C, D, and E. The spectra were accumulated 64 times.

The sensitivity of *K. marxianus* to another trichothecene, DON, was more than 1000-fold less than that of T-2, as 100 to 800 μg/mL of DON in the yeast culture caused growth reductions similar to those obtained with 25 to 100 ng/mL of T-2 (*Figure 5A*). These observations agree with the known LD_{50} values of DON and T-2 as obtained in animal studies[24] and are similar to those published by Madhyastha et al.[17] with the same organism. In the current study, a dose of 800 ng DON/mL reduced the protein content in the medium by 59% (from 96 ± 2 to 39 ± 3 μg protein/mL), whereas in the presence of vitamin E the reduction was 43% (100 ± 1 to 57 ± 3 μg protein/mL). Vitamin E appeared to have only a minor effect on MDA production in yeast that were treated with DON (*Figure 5B*). These results indicate that the protective effect of vitamin E against DON toxicity is considerably less than that obtained with T-2.

The sensitivity of *K. marxianus* to another mycotoxin, OA, was approximately 10,000 times less than to T-2 (*Table 2*). A significant reduction in the number of cells was achieved only when the concentration of OA was 1.0 mg/mL. At this concentration, OA also caused an increase of the production of MDA.

The second phase of the research was to determine if T-2 produced free radicals as monitored by EPR spectrometry and to determine if these effects were quenched by antioxidants. Incubation of yeast with up to 8 μg T-2/mL medium in the presence of either PBN or 4-POBN yielded characteristic six-line spectra for each spin trap (see below). The signals grew slowly and reached a maximum intensity after 64 to 128 accumulated scans (1 scan = 70 seconds). The signal was both stable and produced at a constant rate over a relatively long period of time (75 minutes). For routine analysis 64 scans were used. The spin adduct hyperfine splitting constants (SD) were a_N = 16.0 G, a_H = 3.6 G (0.03 G) for PBN (spectra not shown) and a_N = 15.4 G and a_H = 2.5 G (0.03 G) for 4-POBN. Finkelstein et al.[25] found hyperfine splitting constants for the 4-POBN α-hydroxyethyl adduct of a_N = 15.56 and a_H = 2.59 G as well as for a_N = 15.60 G and a_H = 2.65 G under different experimental conditions. Augusto et al.[26] reported hyperfine splitting constants for the same spin adduct of a_N = 15.50 G and a_H = 2.50 G. According to Pou et al.,[27] 4-POBN in conjunction with ethanol is the most efficient spin trap for the detection of hydroxyl radicals in biological systems. The initially generated hydroxyl radicals react with ethanol to yield α-hydroxyethyl radicals, which then are trapped by 4-POBN to form a very stable spin adduct. This spin trapping system yielded high efficiency, high sensitivity, and a marked stability of the resulting spin trapped adduct, even in the presence of superoxide. The current results

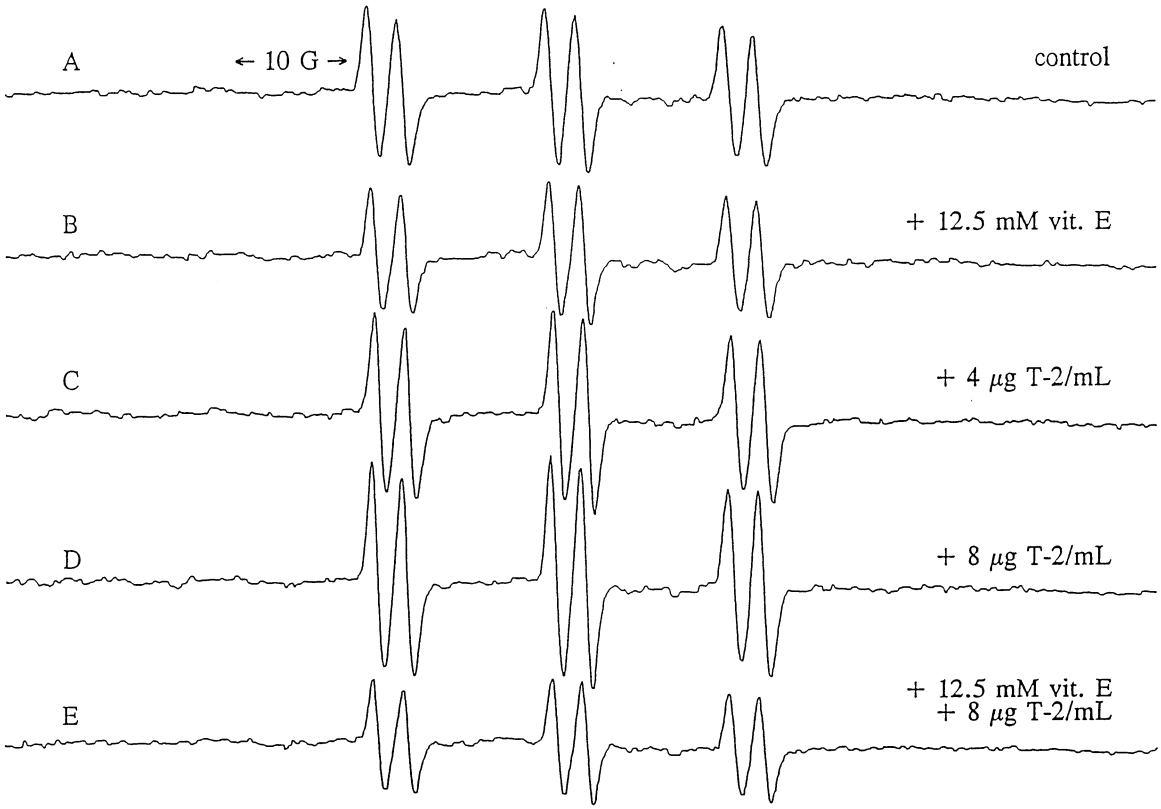


Figure 7 Electron paramagnetic resonance spectra of α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN) radical adducts following the incubation of *Kluyveromyces marxianus* with vitamin E and T-2 toxin. The yeast was cultured for 48 hours in liquid medium, centrifuged, new medium added to the pellets, and the protein concentration adjusted to 4 mg/mL. The spectra were recorded at 37°C immediately after adding 75 mM 4-POBN. In addition, the solutions contained 12.5 mM vitamin E (*dl*-α-tocopherol acetate; sample B), 4 μg T-2/mL (sample C), 8 μg T-2/mL (sample D), 12.5 mM vitamin E, and 8 μg T-2/mL (sample E). 5% v/v ethanol was added to all samples. The spectra were accumulated 64 times.

demonstrate that the spin adducts of each spin trap were α-hydroxyethyl radical adducts that presumably were produced due to the presence of hydroxyl radicals. Although the medium for the EPR experiments contained 4% glucose, it can be assumed that it would have been very difficult to produce glucose-derived free radicals in the present system, because it usually requires gamma irradiation to generate free radicals from sugars.[28]

In the current study, the use of the spin trap DMPO resulted in signals that were difficult to interpret (EPR spectra not shown). These findings agree with Pou et al.,[27] who pointed out that the instability of DMPO and many of its spin trapped adducts has limited the usefulness of DMPO in certain biological systems.

Figure 6 shows the 64 accumulated spectra of different samples. Ethanol (5% v/v) was added to all samples except sample B (*Figure 6*). The amplitude of the signal was approximately the same as in the control sample (*Figure 6A*). Therefore, the addition of ethanol would not have been necessary in order to generate α-hydroxyethyl radicals because glycolytic metabolism by yeast itself would have produced a sufficient amount of this compound. Nevertheless, ethanol was added to all samples as toxins and vitamin

Table 1 Effect of T-2 toxin (T-2) and glutathione (GSH, 10 mM) on growth and malondialdehyde (MDA) concentration of *Kluyveromyces marxianus*

T-2 (ng/mL)	GSH	Protein (μg/mL)	MDA (ng/μg protein)
0	−	145 ± 4	1.12 ± 0.44
100	−	112 ± 4	1.02 ± 0.36
0	+	99 ± 1	1.61 ± 0.51
100	+	67 ± 3	4.49 ± 0.89

Note: The yeast was cultured for 24 hours in liquid medium. Protein as a measure of cell growth and MDA was determined as described under Materials and Methods. Each value is a mean (±SD) of $n = 3$ from a representative experiment.

Table 2 Effect of ochratoxin A (OA) on growth and malondialdehyde (MDA) concentration of *Kluyveromyces marxianus*

OA (mg/mL)	Protein (μg/mL)	MDA (ng/μg protein)
0	83 ± 7	1.88 ± 0.48
0.5	72 ± 1	2.25 ± 0.29
1.0	58 ± 4	4.19 ± 0.51

Note: The yeast was cultured for 24 hours in liquid medium. Protein as a measure of cell growth and MDA was determined as described under Materials and Methods. Each value is a mean (±SD) of $n = 3$ from a representative experiment.

E were delivered to the yeast in ethanol. Both T-2 and $FeSO_4$, a strong prooxidant known to stimulate the formation of hydroxyl radicals, greatly increased the intensity of the EPR signal (61% increase with 8 μg T-2/mL, A vs. D; 121% increase with 10 mM Fe^{2+}, A vs. E) whereas the antioxidant vitamin E tended to reduce the signal (−31%, A vs. C). In addition, $FeSO_4$ generated spectra with the same hyperfine splitting constants as T-2.

Studies with different combinations of T-2 and vitamin E (*Figure 7*) demonstrated that the addition of vitamin E to the incubation mixture partially quenched the spectra relative to the controls (14% decrease in signal, A vs. B), that 4 and 8 μg T-2/mL enhanced the signal by 21% (C vs. A) and 44% (D vs. A), respectively, relative to the control, and that the addition of both vitamin E and T-2 to the culture yielded peak heights that were the same as those obtained with the sample containing only vitamin E. The intensity of the signal in the yeast culture with both T-2 and vitamin E (*Figure 7*, sample E) was also 31% less than the control sample (*Figure 7*, sample A) and 88% less than that obtained with the corresponding amount of T-2 in the absence of vitamin E (*Figure 7*, sample D).

The incubation of the yeast even with high amounts of OA (2 mg/mL), a known generator of free radicals, or high concentrations of DON (0.25 mg/mL) did not increase the EPR signals (data not shown). These results agree with the observation that the sensitivity of *K. marxianus*, as measured by growth and MDA production, was much lower when treated with DON or OA compared with that of T-2 (unpublished observations).

Discussion

The trichothecene mycotoxins are a class of secondary metabolites from different fungi that consist of more than 100 derivatives.[29] Madhyastha et al.[17] demonstrated that among the 16 trichothecenes studied, T-2 showed the highest relative toxicity. It was over 400-fold more toxic to yeast than DON, which is found most frequently in naturally contaminated food and feeds. Therefore, yeast was used in the current study to determine if there is an association between the toxicity of T-2 and its ability to induce lipid peroxidation as measured by MDA production and the generation of free radicals as assessed by the use of a spin trapping agent in EPR studies. Yeast is a particularly suitable model because it is highly sensitive to the effects of T-2,[17,18] it secretes MDA into the media, and it produces a strong sustained and reproducable signal, as shown in the current study when monitored in an EPR spectrometer. This is the first study that has demonstrated that T-2 is able to promote the production of hydroxyl radicals that may be mainly responsible for its toxic effects in animals.

Results from the literature regarding T-2 as a promoter of lipid peroxidation are rather contradictory. On the basis of both in vitro and in vivo studies, Schuster et al.[30] suggested that lipid peroxidation is not involved in T-2 toxicity. Segal et al.,[31] in contrast, reported that T-2 and DON stimulated lipid peroxidation in the livers of rats and that selenium in combination with ascorbic acid and α-tocopherol provided protection against acute toxicosis caused by these toxins. Some of these contradictions might be due, in part, to the limitation of the assay methods used to assess degree of lipid peroxidation, especially those assays based on the measurement of evolved hydrocarbon gases and the thiobarbituric acid reactive substances. These assays are not always accurate measures of lipid peroxidation because of their lack of specificity.[6,32] In the current study, MDA, as detected by HPLC rather than thiobarbituric acid reactive substances, was used as a measure of lipid peroxidation. This method, which was first developed by Bird et al.,[33] provides a more sensitive and specific measure of lipid peroxidation than the spectrophotometric methods.

The results from the current study demonstrated that when added to a yeast culture T-2 resulted in an increased concentration of MDA, particularly when the culture was preincubated with polyunsaturated fatty acids. These findings provide strong support that T-2 promotes peroxidative reactions because MDA has been identified as one of the important peroxidation products of polyunsaturated fatty acids.[34] According to the results obtained by other researchers, the polyunsaturated fatty acids, which are two of the fatty acids that are mainly responsible for MDA production,[6] were probably incorporated into the membranes of *K. marxianus*. Bossi and Martin[35] found in cultures of *Saccharomyces cerevisiae* that linoleic acid, which was not found in the cells grown under normal conditions, was preferentially internalized and incorporated into yeast microsomes when included in the growth medium. Furthermore, according to Pilkington and Rose,[36] linoleic and linolenic acids are incorporated into the membranes of *S. cerevisiae*. In the present study, yeast that was obtained from cultures that were preincubated with the fatty acids showed a higher sensitivity to T-2. Taken together, these results indicate that the fatty acids used in these studies were also incorporated into the membranes of *K. marxianus*. Yeast cultures enriched with unsaturated fatty acids seem to be a good model system for the study of peroxidative reactions.

Further strong evidence for T-2 mediated peroxidative reactions comes from the observed antioxidant effects of vitamin E. The free radical scavenging vitamin protected against T-2 toxicity, reduced the MDA concentration in T-2 treated samples, and quenched the magnitude of the EPR signal, which was attributed to an enhanced production of the final stable product from hydroxyl radicals. In similar EPR experiments, Kubow et al.[37] were able to completely inhibit the EPR signal following the addition of 500 mM vitamin E (*dl*-α-tocopheryl acetate) to lung microsomes. Due to the relatively low water solubility of tocopheryl acetate in our yeast culture system, only 12.5 mM of the antioxidant could be added to the incubation mixture. That may explain the incomplete quenching effects in our study. It is known that α-tocopherol is an efficient chain breaking antioxidant because it accepts single electrons to form stable intermediates, the α-tocopheryl radicals, and vitamin E can be regenerated from this radical following the single electron reduction by ascorbic acid.[6,38] Reducing compounds such as ascorbic acid and GSH therefore should enhance the biological efficacy of vitamin E. On the other hand, ascorbic acid sometimes may have prooxidant effects including the promotion of free radical formation particularly in the presence of transition metals.[39] It is well known that

ascorbate can reduce ferric iron as well as copper and promote the formation of hydroxyl radicals via the iron or copper catalyzed Fenton-type reaction.[40,41] For example, Ko et al.[42] reported that reducing agents such as ascorbic acid and GSH had a marked stimulatory effect on ferric iron induced lipid peroxidation in erythrocyte membrane lipids. Spear and Aust[43] reported similar prooxidant characteristics of GSH in copper dependent DNA damage. These results are consistent with our findings in yeast, especially the synergism between ascorbic acid or GSH and T-2 regarding toxicity and MDA generation. In contrast to mammals, yeast is known to be devoid of tocopherols and ascorbic acid;[44] therefore the observed effects may be exclusively related to the added amounts of ascorbic acid and GSH. Under such conditions these compounds may provide a source of electrons for the generation of oxygen radicals rather than being used for the reduction of the α-tocopheryl radicals.

In this study we observed very stable and steadily increasing EPR signals due to the reaction of the α-hydroxyethyl radical with the spin trap 4-POBN. This phenomenon seems to be unique in yeast cells due to their production of endogenous ethanol. Ethanol therefore may play an important role for yeast in preventing some of the damage associated with hydroxyl radicals because the same stable but weaker signals were also produced in control samples without prooxidative agents and in the absence of added ethanol. It also should be pointed out that free radicals presumably are formed inside the cells, because no EPR signal could be obtained when yeast cells were omitted from the samples (data not shown). The hydroxyl radical ($^{\cdot}OH$), which was produced within the cell, abstracted a hydrogen atom from ethanol to form the ethanol radical ($CH_3C^{\cdot}HOH$), which reacted with the spin trap agent to form the spin adduct. The exact mechanism (transport or diffusion of the ethanol radical out of the cells) cannot be proven based on the current data. The proposed sequence of reactions is:

$$^{\cdot}OH + CH_3CH_2OH \rightarrow H_2O + CH_3C^{\cdot}HOH +$$

$$\text{spin trap} \rightarrow \text{spin adduct}$$

Free radicals, particularly the hydroxyl radical, cause oxidative damage to a wide variety of cellular targets including nucleic acids and proteins and may well be responsible for some of the effects of mycotoxicoses, particularly those associated with T-2. However, the extent to which lipid peroxidation contributes to the overall toxicity of T-2 is not known. In addition, the mechanism by which T-2 catalyzes the production of the hydroxyl radical in yeast is not known. Yeast appears to be an excellent model for these studies because experiments can be carried out in the intact organisms and the results are highly reproducible and readily obtained.

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