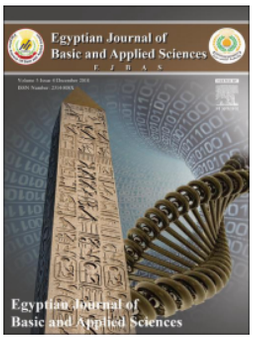

Egyptian Journal of Basic and Applied Sciences

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/teba20

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To cite this article: Berna Kavakcıoğlu Yardımcı, Zehra Mollaoğlu, Fatih Altıntaş & Ayşenur Güler (2023) Investigation of Dependency of Boric Acid and Lithium Metaborate Induced Yeast Toxicity on the Expressions of Antioxidative and Apoptotic Genes, Egyptian Journal of Basic and Applied Sciences, 10:1, 545-554, DOI: 10.1080/2314808X.2023.2238373

To link to this article: https://doi.org/10.1080/2314808X.2023.2238373

Published online: 21 Jul 2023.

EGYPTIAN JOURNAL OF BASIC AND APPLIED SCIENCES
2023, VOL. 10, NO. 1, 545–554
https://doi.org/10.1080/2314808X.2023.2238373

ORIGINAL ARTICLE

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Investigation of Dependency of Boric Acid and Lithium Metaborate Induced Yeast Toxicity on the Expressions of Antioxidative and Apoptotic Genes

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ABSTRACT

Boric acid and its less-known derivative lithium metaborate are inorganic acids used in a wide and diverse array of industrial and clinical applications. In particular, boric acid is accepted as a natural antimicrobial agent due to its activity on many different microbial species. We have examined the possible dependency of boric acid and lithium metaborate-induced yeast toxicity on the deletion of antioxidative CTT1, TRR2 and GSH2 genes and the expressions of certain important pro- and anti-apoptotic genes. While the investigated antioxidative genes have no function in the protection against boric acid mediated toxicity, the cell proliferation percentages of ▵GSH2 and ▵TRR2 mutants significantly decreased to approximately 57% and 75%, respectively, after 0.25% LMB treatment for 12 h compared to the wild yeast strain. The relative expressions of the AIF and NDI genes were generally induced for the highest concentrations of the drugs compared to the control. Similarly, anti-apoptotic BIR1 was also generally induced compared to the control, which indicates the importance of this gene in the resistance of yeast cells against BA and its derivatives mediated toxicity. These new findings are significant in terms of adding new data to the literature on understanding the toxic effect mechanisms of boron compounds.

ARTICLE HISTORY
Received 23 May 2023
Revised 10 July 2023
Accepted 14 July 2023

KEYWORDS
Boric acid; lithium metaborate; yeast; viability; antioxidant enzymes; apoptosis

Introduction

A metalloid boron (B) element in group 3A of the periodic table has the ability to form complexes with significant organic groups and is not found in pure form in nature, but as oxygenated compounds due to its high oxygen affinity [1]. Boric acid (BA) is the most important compound of B and is formulated as $B(OH)_3$ or H_3BO_3 [2–4]. This compound offers a wide range of industrial applications including fiberglass production, eyeglass lens making, ceramic works, fireproof and airtight fabric production, flame retardant in wood, detergent and soap production, nickel plating baths, hardening of steel, welding melting, brazing of copper, and production of borate [5]. It is also widely used as food preservative, fungal controller in citrus fruits, moderate antiseptic, and in pomades as a result of broad-spectrum toxic effects on microorganisms [6–10]. It is well known for years that BA inhibits proliferations of

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various bacteria such as *Brucella abortus*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella* spp., *Pseudomonas*, *Streptococcus*, *Staphylococcus* spp., *Enterobacter* and *Citrobacter* spp, *Morganella* and *Acinetobacter calcoaceticus* besides pathogenic fungal *Candida* species [11–15]. Nevertheless, the mechanism of action of BA, especially against fungi, is not fully known. In this regard, the unicellular eukaryotic yeast *Saccharomyces cerevisiae* has become a useful model organism with its high BA tolerance to investigate the detailed action mechanism of BA [16]. It was formerly stated that the membrane proteins BOR1, DUR3, and FPS1 involve in tolerance to BA and the maintenance of the protoplasmic B concentration [17]. We came across another literature that showed that yeast cells signal cell wall stress through the Slt2p pathway and increase chitin synthesis in response to BA treatment, most probably to repair cell wall damage [18]. The functioning of high osmolarity/glycerol signaling, the trehalose synthesis pathway and the superoxide dismutase enzyme are essential for BA resistance in *S. cerevisiae* [19]. Additionally, in our previous work, the toxic effects of BA and lithium metaborate (LMB), which is one of the industrially widely used salts of BA, were investigated besides their effects on superoxide dismutase and glutathione S-transferase enzyme activities, lipid peroxidation levels, and cell surface morphology [20].

In this study, we investigated the potential effects of the deletion of certain antioxidative genes encoding cytosolic catalase T (Ctt1p), thioredoxin disulfide reductase (Trr2p) and glutathione synthase (Gsh2p) on BA and LMB-based inhibition of cell viability in *S. cerevisiae* BY4741 cells for the first time. Also, the changes in the expressions of pro- and anti-apoptotic genes, namely YCA1, AIF1, NDI1, NUC1, BIR1 and NMA111, were evaluated depending on BA and LMB treatments. With these new findings, important and complementary information about the effects of BA and LMB on yeast cells has been added to the related literature.

Material and methods

Material

All the chemicals were of analytical grades, used without purification and obtained from Sigma-Aldrich, Inc. (St. Louis, MO) unless otherwise stated. A wild-type yeast strain *S. cerevisiae* BY4741 and its isogenic CTT1 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 CTT1:kanMX4), TRR2 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 TRR2:kanMX4) and GSH2 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 GSH2:kanMX4) deletion mutants were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Routine maintenance, growth and treatment conditions

The routine maintenance of wild and mutant *S. cerevisiae* BY4741 strains was carried out on yeast extract peptone dextrose (YPD) agar medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L bacto agar for 5 days at 30°C (pH: 5.6).

For the growth of all the strains, YPD broth consisting of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose (pH: 5.6) was used. Geneticin antibiotic (G418), 200 mg/L dH_2O, was also added to the growth medium of mutant strains. The inoculum was carried out in 250 mL Erlenmeyer flasks containing 95 mL broth plus 5 mL spore suspension whose optical density at 660 nm was 0.1. The yeast cells were grown until their early-exponential phase with 180 rpm agitation at 30°C. After the cells reached the desired density, the cells were treated with the agents as previously described [20]. Briefly, BA and LBM were added to the flasks at the final concentrations of 0.25–1% and 0.05–0.25%, respectively, and the cells were additionally incubated 12 and/or 36 h under the same conditions. At the end of the final incubation period, the cells were harvested with a centrifuge for 5 min at 3,000 g at 4°C and were kept at −80°C until further biochemical analysis.

Table 1. Primer sequences and amplification conditions.

Gene	NCBI reference sequence	Primer sequence (5′ → 3′)	Annealing temperature (°C)
MetaCaspase, YCA1	NC_001147.6	**F**: ATAATGGCTACCAACGGCCC	59
		R: TAAGCCATAGGGGGAGGACC	61
Apoptosis-Inducing Factor, AIF1	NC_001146.8	**F**: TACTGCCGGACTCTGGGTTA	59
		R: AATACGTTTCGGCGAGGTGT	57
NADH Dehydrogenase Internal, NDI1	NC_001145.3	**F**: AGCTCTGCCCATCGTTTTGA	57
		R: CTTCAACTTTGGCGACAGCC	59
Nuclease, NUC1	NC_001142.9	**F**: ATGATCGAGGCCATCAAGCC	59
		R: AGTACTCCAAATGCGCCCAA	57
Baculoviral IAP Repeat-containing protein, BIR1	NC_001142.9	**F**: GGCCTCACAGTGGTTCTCAA	59
		R: GCTGGAGTCGTATCGCATGA	59
Nuclear Mediator of Apoptosis, NMA111	NC_001146.8	**F**: TTTGGCTAAGGTCGGCTCAG	59
		R: AACCACTTGAACCGCCAGAA	57
Actin, ACT1	NC_001138.5	**F**: TCGCCTTGGACTTCGAACAA	57
		R: CAAAGCTTCTGGGGCTCTGA	59

Proliferation and metabolic activity assays

For the determination of cell proliferation percentages, the optical density values of the control and treated cells were measured at 660 nm. The value of control was accepted as 100% and the cell proliferation inhibition rate was calculated as the percentage of control.

Cell Counting Kit-8 (CCK-8) assay kit (Abbkine, Inc. China) was used to determine cell metabolic activity percentages. This assay is based on the reduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2 H-tetrazolium, monosodium salt (WST-8) by dehydrogenases and the formation of orange colored formazan product. In the test, briefly, 100 µL of 10^6 cell/mL suspensions were mixed with 10 µL CCK-8 solution included in the kit and incubated for 4 h at 30°C. At the end of the incubation period, the absorbance values were measured at 450 nm and cell metabolic activity was calculated as the percentage of control.

RNA isolation and quantitative real time PCR

RNA extraction from the control and treated yeast cells was carried out by using GeneAll Hybrid-RTM Total RNA Purification Kit (Seoul, Korea) following the manufacturer's protocol. The purity of the isolated RNAs was determined spectrophotometrically at 260 and 280 nm. VitaScript FirstStrand cDNA Synthesis Kit (Procomcure, Thalgau, Austria) was used for the synthesis of single-stranded cDNAs from total RNA samples, and the manufacturer's protocol was followed. The synthesized cDNAs were stored at −80°C for further use.

The qRT-PCR analysis was carried out by using PikoReal 96 RT PCR System (Thermo Fisher Scientific, USA) with 2X Magic SYBR Mix (Procomcure, Thalgau, Austria), and the manufacturer's protocol was followed. mRNA expression was normalized to control and Actin (ACT1) expression resulting in Mean Fold Change values or ΔΔCt. The primer sequences for the target genes and amplification conditions are given in Table 1.

Statistical analysis

The data are presented as the mean±S.E.M of three experiments. The differences in variance were analyzed statistically using ANOVA test by GraphPad prism 5.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a post hoc.

Results

The roles of catalase, thioredoxin disulfide reductase and glutathione synthase in BA and LMB treatment

We have investigated the possible protective roles of common antioxidant enzymes of *S. cerevisiae* against BA and LMB-induced inhibition of cell proliferation and metabolic activity. To understand whether Ctt1p, Trr2p and Gsh2p have any protective effects on BA and LMB-induced toxicity that we have investigated in our previous study [20], the viabilities of the knockout strains were compared with the wild-type yeast. Considering the solubility of the agents in the yeast cell medium, the cells were treated with BA and LMB in the concentration range of 0.25–1.0% and 0.05–0.25%, respectively. Statistically significant results were found only for 0.25% LMB, and the data are presented in Figure 1a,b.

As can be seen from Figure 1a, the cell proliferation percentages of ΔGSH2 and ΔTRR2 strains significantly decreased to approximately 57% and 75%, respectively, in the samples treated with 0.25% LMB for 12 h compared to the wild yeast strain. These data clearly indicate that both antioxidant enzymes have protective roles against LMB treatment in yeast cells. In addition, a statistically significant difference was determined between the cell proliferation percentages of the two deletion mutants, and it can be stated that the protective effect of Gsh2p is more dominant than Trr2p. On the other hand, the metabolic activity percentages of all three deletion mutants were found to be significantly different from those of wild type ($p < 0.0001$) with the value of about 85% but without statistically significant differences among themselves (Figure 1b). Therefore, it can be thought that these antioxidative enzymes affect the yeast metabolic activity in a similar rate as opposed to cell proliferation.

The roles of apoptosis related genes in BA and LMB treatment

The expression levels of YCA1, AIF1, NDI1, NUC1, BIR1 and NMA111 genes were determined in wild-type *S. cerevisiae* cells exposed to 0.25–1.0% BA and 0.05–0.25% LMB for 12 and 36 h, and the results are presented in Figures 2 and 3. According to the results, 0.25–1.0% BA treatments for 12 h gradually increased the relative expression levels of pro-apoptotic AIF1, NDI1 and NMA111 genes (Figure 2). By increasing the treatment period from 12 to 36 h, generally non-gradual increases in the relative expression levels of the same genes were determined with the increasing concentration of BA. But especially

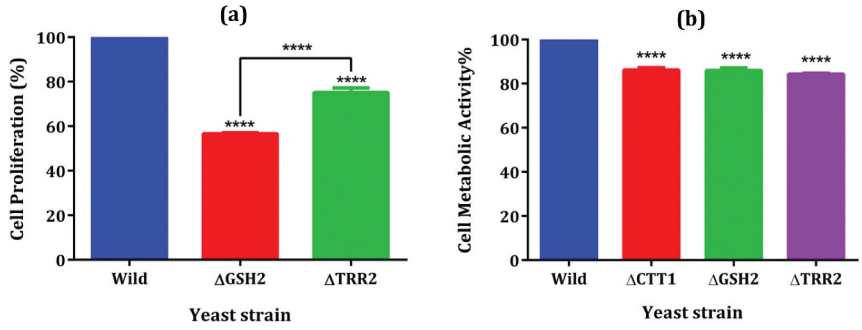

Figure 1. Cell proliferation (A) and metabolic activity (B) percentages of mutant strains compared to wild strain (Calculations were made by assuming the viability of the wild strain as 100%). Data with error bars are given as the mean ± S.E.M of three replicates. ****$p < 0.0001$ denotes significant differences between wild and other studied groups or indicated groups by Tukey's multiple range tests.

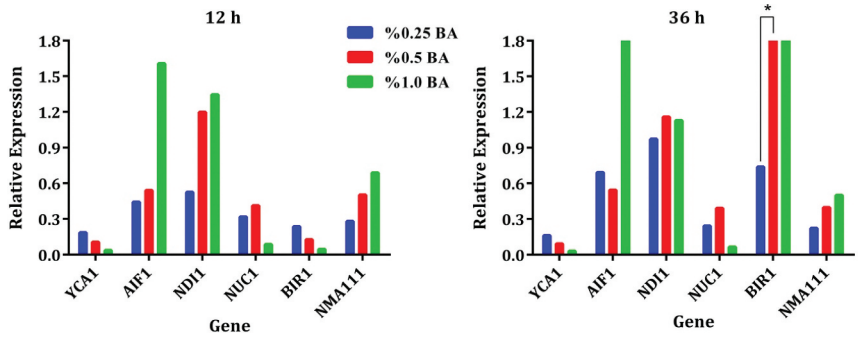

Figure 2. Relative mRNA expression levels of YCA1, AIF1, NDI1, NUC1, BIR1 and NMA111 genes in wild strain treated with 0.25–1.0% BA for 12 and 36 h compared to untreated control group. Data are given as the mean of three replicates. *$p < 0.05$ denotes significant differences between indicated groups by two-way ANOVA.

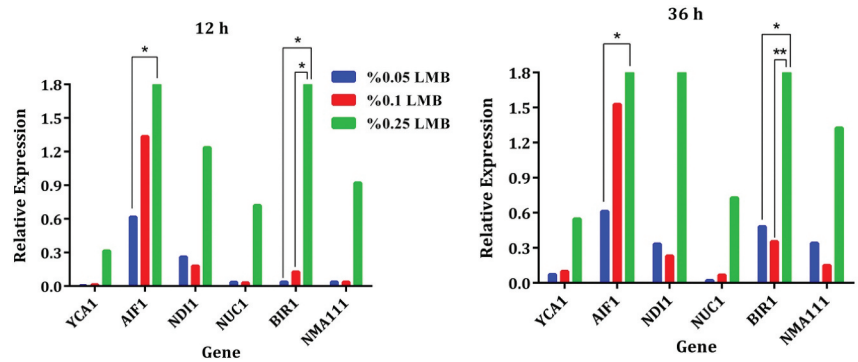

Figure 3. Relative mRNA expression levels of YCA1, AIF1, NDI1, NUC1, BIR1 and NMA111 genes in wild strain treated with 0.05–0.25% LMB for 12 and 36 h compared to untreated control group. Data are given as the mean of three replicates. *$p < 0.05$ and **$p < 0.01$ denote significant differences between indicated groups by two-way ANOVA.

the relative expression levels of AIF1 and NDI1 were found to higher than control in the cells exposed to higher concentrations of BA for both treatment periods. Additionally, while the expression of the anti-apoptotic BIR1 gene was suppressed depend on increased concentrations of BA in yeast cells treated with the agent for 12 h, it was observed that the expression of the so-called gene was induced in cells exposed for 36 h with a statistically significant difference between 0.25 and 0.5% BA treatments ($p < 0.05$). In yeast as in mammals, AIF1 gene encodes Aif1 protein (Aif1p) that translocate from mitochondria to the nucleus and cause large-scale DNA fragmentation upon an apoptotic stimulation [21]. NDI1, along with NDE1, is one of two AMID (AIF-homologous mitochondrion-associated inducer of death) related genes in yeast [22]. These genes are known to encode internal and external NADH dehydrogenase, respectively and it has been reported that overexpression of NDI1 only induces apoptotic cell death [23]. Although NDI1 seems almost identical to AIF1, there are studies showing that they lead to apoptosis in yeast cells in response to different conditions [24]. Nma111p, a product of NMA111, has a serine protease activity that regulates the nuclear mechanism of apoptotic death and the yeast IAP (Inhibitors of apoptosis proteins), Bir1p, was shown to be a substrate for Nma111p in

S. cerevisiae [23,25]. Considering the aforementioned mechanisms of action, it can be interpreted that BA induced toxicity in yeast cell culture is independent of caspase activity, which are confirmed by the suppression of YCA1 expression levels. Another important point is while the relative expression levels of BIR1 showed strong negative correlation with the levels of NMA111 after 12 h BA treatment ($r = -0.999$), strong positive correlation was found between the expression levels of these genes for 36 h ($r = 0.842$). As stated before by Carmona-Gutierrez et al. (2010), the reduction of apoptotic cell death by the overexpression of BIR1 can be antagonized by simultaneous overexpression of NMA111 [26]. However, in our study, the anti-apoptotic BIR1 gene was statistically significantly more expressed than NMA111. We have previously determined that the anti-proliferative effects of BA and LMB after 36 h treatment did not increase compared to 12 h treatment, on the contrary, statistically significant increases were found in the proliferation percentages of the samples treated with 0.25 and 0.5% BA [20; 27]. From this point of view, it can be certainly said that BIR1 is one of the factors contributing to the resistance of yeast cells to longer exposure of BA. In the case of LMB, remarkable increases were observed for all studied gene expressions when the concentration was increased to 0.25% for both 12 and 36 h treatment (Figure 3). Especially, statistically significant changes were recorded in the expression of AIF1 and BIR1 genes depending on the studied concentration. Previously we have reported that only 0.25% LMB caused significant decreases in *S. cerevisiae* cell growth and metabolic activity compared to untreated control ($p < 0.0001$) and lower concentration of the agent induced metabolic activity of these yeast cells [20]. This can therefore be considered an expected situation that while the relative expression levels of pro-apoptotic genes are generally above control at 0.25%, suppression rather than induction was observed for lower concentrations. On the other hand, it was found that the expression of the anti-apoptotic BIR1 gene induced at 0.25% LMB for both treatment periods compared to the control. Therefore, it can be said that this gene is important in the resistance of yeast cells against BA and its derivatives.

Discussion

The present study investigates the roles of some antioxidative and apoptotic genes on BA and its lithium salt LMB-induced toxicity in yeast. According to the literature view, although there are very limited number of studies on the antimicrobial effects of LMB, BA has been shown to be effective on many different species [6,28–31]. Our group has also previously shown that while 0.05–0.1% LMB did not cause statistically significant differences in *S. cerevisiae* growth compared to untreated control, 0.25–1% BA and 0.25% LMB significantly inhibited the growth of yeast cells [20]. Unfortunately, the mechanism of antimicrobial action of both agents is not yet fully known. Here, we firstly examined the possible protective roles of Ctt1p, Trr2p and Gsh2p activities using three separate mutant strains whose gene regions encoding these proteins have been deleted. The cytosolic Ctt1p along with Cta1p which is located in the peroxisomes is one of the two catalases found in *S. cerevisiae* cells [32–35]. It was formerly stated that while yeast cells may not be dependent on catalase activity for scavenging of endogenous H_2O_2 [36], the enzyme may play some part in the adaptive response to oxidative stress [37]. In this paper, we found that the deletion of CTT1 caused a statistically significant but unnoticed decrease in metabolic activity of yeast cells only treated with 0.25% LMB for 12 h. This mutant strain was not more sensitive to any other treatment compared to the wild strain. Many studies have reported that CTT1 deletion makes cells more susceptible to toxicity induced by various chemicals [38–40]. On the other hand, in a study conducted by Martins et al. (2019), it was

reported that while the antifungal azoles significantly stimulate catalase activity, the deletion of CTT1 makes cells more resistant to these azoles in part by the upregulation of SOD2 gene expression, which encodes mitochondrial manganese superoxide dismutase enzyme [41]. Therefore, although catalases are important for yeast cells to cope with stress, the expressions of other members of the antioxidant system may be more induced in conditions where the so-called enzyme activity is at negligible levels. Just like the ΔCTT1 strain, ΔTRR2 and ΔGSH2 mutants showed sensitivity only against LMB at the studied highest concentration. But differently, the growth of these two mutant strains was found to significantly decrease compared to wild yeast cells. TRR2 and GSH2 genes encode thioredoxin disulfide reductase (Trr2p) and glutathione synthase (Gsh2p) enzyme proteins, respectively. It is well known that both of these enzymes have vital roles in maintaining a reducing environment in the cell [42]. From this point of view, we thought that in particular BA does not cause significant induction of reactive oxygen species (ROS) in yeast cells. Supportingly, Ogando et al. (2011) stated that 30–300 μM BA supplementation for 1–12 h did not have any effect on ROS production in bovine corneal endothelial and human embryonic kidney 293 cells [43]. Additionally, we have previously determined that not LMB but BA significantly induced SOD activity [20], which indicates that the SOD is the dominant antioxidant enzyme in response to BA induced oxidative stress.

We also investigated the changes in the expression levels of certain pro- and anti-apoptotic genes namely YCA1, AIF1, NDI1, NUC1, BIR1 and NMA111 in wild strain treated with 0.25–1.0% BA and 0.05–0.25% LMB for 12 and 36 h. Apoptotic cell death is a genetically programmed mechanism which is of importance for both physiological and pathological conditions [44]. While this mechanism is mainly induced by extrinsic and intrinsic (mitochondrial) pathways in mammalians, yeast cells appear to only have homologs of the latest. Indeed, AIF1, NDI1, NUC1 and NMA111, the genes whose relative expression levels were investigated in this paper, are among the most important mitochondrial or mitochondria-associated pro-apoptotic factors. The anti-apoptotic Bir1p encoded by BIR1 gene was previously known for its role as a chromosomal passenger protein, but the latter appeared to be a substrate for Nma111p and a homologue of mammalian IAP in *S. cerevisiae*. Finally, the YCA1 gene encodes metacaspase, which is the yeast homolog of mammalian caspases not in biochemistry but in sequence and function [45]. The evaluation of RT-PCR results clearly showed that BM and LMB induced cell toxicity independent from yeast metacaspase. The relative expression levels of the YCA1 gene were below control levels for all the studied samples. Moreover, statistically insignificant reductions in the expression levels of YCA1 were recorded with the increasing concentration of BA. It has also been shown in previous studies that BA exerts its toxic effect independent from caspases on different cells including human prostate cancer cells, human breast cancer cells and mouse TM3 Leydig cells [46–48]. According to the obtained results, it seems that caspase-independent Aif1p and Ndi1p, which shows sequence homology with Aif1p, play more dominant roles in BA and LMB-induced toxicity [49] and BIR1 gene is one of the important factors which contribute to yeast resistance mechanism. However, a better understanding of the roles of these genes requires additional studies at the protein levels which can be regarded as a limitation of this study.

We conclude that the following results were reached about the toxic effects of BA and LMB on eukaryotic model organism *S. cerevisiae*:

- While ▵CTT1, ▵GSH2 and ▵TRR2 mutants do not show sensitivity to BA-induced toxicity, ▵GSH2 and ▵TRR2 mutant strains are

especially more sensitive to LMB-induced toxicity compared to wild-type yeast strain.
- Both agents induce toxicity in a metacaspase-independent manner.
- Inductions of the expression levels of the AIF1 and NDI1 genes were determined compared to control groups, particularly at the highest concentrations of BA and LMB, which suggest the roles of these gene products in the toxic effect mechanisms of the agents.
- BIR1 may play an important role in the mechanism of BA and LMB-induced stress tolerance in this yeast model.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The work was supported by the Pamukkale Üniversitesi [PAUBAP2021FEBE007].

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