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Resistance of *Saccharomyces cerevisiae* to Fungicide Chlorothalonil

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The toxicity of chlorothalonil on the growth of yeasts was investigated using several yeast strains. An alcohol tolerant yeast, *Saccharomyces cerevisiae* F38-1, was the most chlorothalonil-tolerant. The glutathione content and the glutathione S-transferase activity were related to the chlorothalonil-tolerant phenotype. Several thiol compounds affect the dissipation of chlorothalonil. However, there was no significant difference on the effects of chlorothalonil dissipation among the thiol compounds tested. The growth of yeast cells was arrested by chlorothalonil. It took about 13 h to dissipate 1 mg/l of chlorothalonil, and the growth was restored as the chlorothalonil content decreased. The glutathione content and glutathione S-transferase are suggested to be among the most important factors of yeast resistance to chlorothalonil.

***Key words*:** *Saccharomyces cereivisiae*, chlorothalonil, pesticide stress

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum chlorinated fungicide, which is highly efficient against the pathogens that infect mainly vegetables, fruits and other crops. In fungi, the chlorothalonil reacting with the sulfhydryl groups, present in proteins or in cofactors, has been proposed as the mechanism of its fungicidal activity (Roberts and Hutson, 1999). From yeasts to plants and mammals, the syntheses of a metal binding protein, metallothionein and heat shock proteins, as well as glutathione and glutathione S-transferase, in response to heavy metals, heat shocks, carbon starvation and various xenobiotics have been observed in most of the species examined. The syntheses of such stress-responsible proteins are very rapidly, and are induced to protect cells against the toxicity caused by stress thereby allowing organisms to survive in harmful environmental conditions (Hamer, 1986; Lindquist and Craig, 1988; Inouhe, 1989; Schlesinger, 1990; Blom *et al*., 1992; Huh *et al*., 1994). Glutathione is an especially abundant non-proteinous thiol compound found in most aerobic organisms, and has many physiological functions in cells (Meister and Anderson, 1983; Meister, 1985). Glutathione S-transferase (GST) catalyzed the conjugation of reduced glutathione to a wide variety of xenobiotics, resulting in detoxification and has been found in yeast stains (Kumagai *et al*., 1988; Choi *et al*., 1998).

In this study, five different yeast strains were used to investigate the resistance against the fungicide chlorothalonil. The effects of chlorothalonil treatment on the growth, and the budding ratio and proliferating populations of cells, were studied. Since chlorothalonil reacts with the sulfhydryl group in proteins or cofactors in its mechanism of fungicidal activity, the relationship between the resistance to chlorothalonil and the glutathione content and GST activity in the yeasts were also observed.

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Materials and Methods

Cells and chemicals

A yeast strain with alcohol tolerance, *Saccharomyces cerevisiae* F38-1, was obtained from Dr. Jin from the Department of Microbiology, Kyungpook National University, Daegu, Korea. The other yeast strains, *S. cerevisiae* ATCC24858, *S. cerevisiae* KCCM11215, *S. cerevisiae* S288C and *Schizosaccharomyces pombe* KCCM11527, were obtained from the Korean Culture Center of Microorganisms (KCCM). The yeast cells were grown in YPD medium (1% glucose, 2% Bacto-peptone and 1% yeast extract) at 30°C, under aerobic condition.

The chlorothalonil compound (97% pure) was provided by Dr. Kim in the Department of Agricultural Chemistry, Kyungpook National University, Daegu, Korea and was prepared by dissolving 1,000 mg/l in acetone, which was then added to the medium at the concentrations indicated. All other reagents were of reagent grade and purchased from commercial sources.

Growth assays

To estimate the effect of chlorothalonil on the growth of the yeasts, cells were grown overnight in 100 ml of YPD

medium at 30°C. The cells were harvested, centrifuged and washed, and then suspended in ice-cold YPD medium to give an OD of 1 at 600 nm. The yeast cell suspension was diluted 50-fold with fresh medium, containing various concentrations of chlorothalonil (0 to 1.5 mg/l), and incubated. The cell growth was monitored turbidometrically at 600 nm.

Calculation of IC_{50} values

The IC_{50} value represents the concentration of chlorothalonil that causes 50% inhibition of the growth rate, and was determined from the semi-log plot of the relative value of each parameter as a toxic function of the chlorothalonil. The growth rate was checked 10 h after the initial inoculation.

Glutathione content and GST activity assay

The yeast cells, grown in the presence or absence of chlorothalonil at its IC_{50} value, were harvested 16 h after the initial inoculation and used for glutathione content and GST activity assays. The glutathione content was measured by the method of Tietze (1969), using glutathione reductase. The sum of the reduced and oxidized forms of yeast glutathione was determined colorimetrically, based on the absorbance of the reaction product of glutathione and 5,5'-dithiobis (2-nitrobenzoic acid) [DTNB]. The glutathione content was determined by measuring the reduction of DTNB at 412 nm with $13,600 \times 10^5$ M^{-1} cm^{-1} used as the molar extinction coefficient.

The activities of the GST enzymes from the yeasts were evaluated by determining the kinetic parameters toward an artificial substrate (1-chloro-2,4-dinitrobenzene, CDNB), according to the procedure of Moron *et al.* (1979), modified by Ando *et al.* (1988). In the reaction mixture, 1 mM glutathione and CDNB were used as the substrate. A suitable volume of cell extract, equivalent to 100 mg of soluble protein, was added to the substrates, and the volume adjusted to 3 ml with 0.1 M phosphate buffer (pH 6.5), containing 2.5% ethyl alcohol to dissolve the CDNB. The reaction mixtures were incubated for 4 min at 30°C. The reaction was stopped by the addition of 0.5 ml of 1 M trichloroacetic acid. The GST activity was calculated by measuring the absorbance at 340 nm, utilizing 9.6 mM cm^{-1} as the extinction coefficient (Askelof *et al.*, 1975). One unit of enzyme activity was defined as the amount of enzyme that converts 1 mole of substrate to the corresponding product in one minute under the specified conditions. The total soluble protein of cell extracts was determined by the Bradford method (Bradford, 1976).

The dissipation rate of chlorothalonil by thiol compounds

To investigate the dissipation rate of the chlorothalonil by thiol compounds, various concentrations of thiol compounds were added to a reaction buffer (50 mM phosphate buffer, pH 7.0) containing 10 mg/l chlorothalonil. The reaction was performed for 1 h at 30°C. The mixture was then extracted with twice its volume of hexane. 0.5 ml of the extract was evaporated and then dissolved in methanol prior to analysis.

To detect the chlorothalonil, high performance liquid chromatography (HPLC) analysis was performed, using a Waters μBondapak C18 column (3.9×150 mm) with a buffer eluent of water:acetonitrile (1:1) for 10 min at a flow rate of 1 ml/min. The HPLC effluent was detected in series by UV monitoring at 235 nm.

Results and Discussion

Toxicity of chlorothalonil on yeast cell growth

The toxicity of chlorothalonil on the specific growth of yeast strains was investigated using *S. cerevisiae* F38-1, KCCM11215, ATCC24858, S288C and *Schizosacharomyces pombe* KCCM11527 cells grown in the YPD medium, containing 0 to 1.5 mg/l chlorothalonil. Table 1 shows the IC_{50} values for each yeast cell to chlorothalonil. An alcohol tolerant yeast, *S. cerevisiae* F38-1, was the most resistant to the chlorothalonil. Fig. 1 shows the growth inhibition of *S. cerevisiae* F38-1 in the medium containing various concentrations of chlorothalonil. There were no significant differences to the final growth, with the exception of the

Table 1. IC_{50} values of yeast strains to chlorothalonil

Yeast strain	IC_{50} (mg/l)
S. cerevisiae F38-1	0.475
S. cerevisiae ATCC24858	0.309
S. cerevisiae KCCM11215	0.289
S. cerevisiae S288C	0.217
S. pombe KCCM11527	0.190

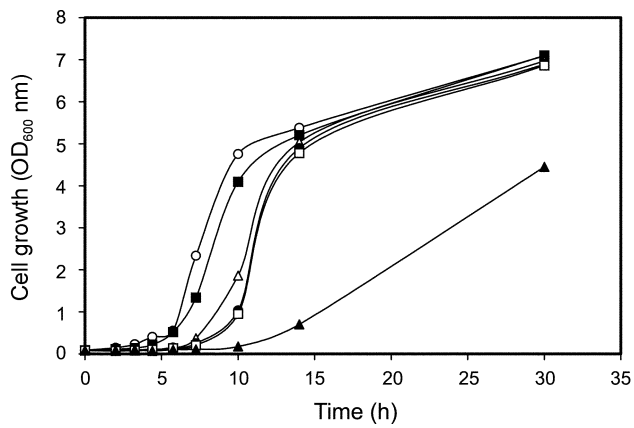

Fig. 1. The effect of chlorothalonil on the growth of *S. cerevisiae* F38-1. The experiment was performed in YPD medium containing various concentrations of chlorothalonil at 30°C under aerobic condition. The cell growth was measured by monitoring the optical density at 600 nm with aliquots harvested at the indicated times. ○-○, 0 mg/l chlorothalonil as control; ■-■, 0.25 mg/l; △-△, 0.5 mg/l; ●-●, 0.75 mg/l; □-□, 1.0 mg/l; ▲-▲, 1.5 mg/l.

highest concentration tested, although the initial growth of the F38-1 strain was delayed by increasing the concentration of chlorothalonil. Thus, the growth of *S. cerevisiae* F38-1, in the presence of chlorothalonil, was not effected by the final biomass production, but only by the growth rate when the fungicide used varied from 0 to 1 mg/l (Fig. 1). In the other strains, the same phenomena were observed, although the chlorothalonil was more toxic to these strains (data not shown). *S. cerevisiae* S288C, which was the most sensitive strain to the chlorothalonil of all the *S. cerevisiae* subspecies tested, did not grow at a chlorothalonil concentration of 1.5 mg/l (data not shown).

Determination of glutathione content

In the *S. cerevisiae*, normal glutathione levels are required for the detoxification process against cellular oxidative stress (Spector *et al*., 2001). Glutathione is also known to be one of the important factors in determining the resistance to oxidative stress and xenobiotics in yeasts (Izaa *et al*., 1995; Inoue *et al*., 1999). The glutathione content of the *S. cerevisiae* F38-1 and the chlorothalonil sensitive strain S288C, which was two times lower than that of F38-1 on the IC_{50} value (Table 1), was measured after chlorothanoil treatment, as described in Materials and Methods. The level of glutathione content in the F38-1 was higher than that in the S288C (Fig. 2). From this result, it was presumed that cellular glutathione may be involved in reducing or removing the toxicity caused by chlorothalonil. After chlorothalonil treatment, the cellular glutathione content of the F38-1 decreased significantly, and was less than that in the untreated F38-1 and S288C (Fig. 2). It has been reported that intracellular reactive oxygen species, generated by chlorothanoil, inhibit glutathione reductase (GR; Fujita *et al*., 1998). Considering the above report, it is suggested that the reduction of the cellular glutathione content may be due to a decrease in the GR activity caused by the chlorothalonil. In order to study this issue in more detail, the relationship between the GR activity and the cellular glutathione content need to be investigated before and after chlorothalonil treatment. The cellular glutathione content of the S288C also decreased after chlorothanoil treatment, but the ratio was less than that of the F38-1 (data not shown).

Determination of glutathione S-transferase (GST) activity

GST catalyzes the conjugation of reduced glutathione to a wide variety of xenobiotics, which results in detoxification. The GST activity is an important factor in plants for determining the resistance to various 2-chloroacetanilide herbicides (Scarponi *et al*., 1991). The conjugation with glutathione, to displace chlorine by the thiol group of glutathione, has been recognized as a major detoxification pathway in plants. This reaction may occur both nonenzymatically and enzymatically through the action of the GST (Lamoureaux *et al*., 1971; Leavitt and Penner, 1979; Breaux, 1987). Interestingly, the GST activity of the chlorothalonil-sensitive strain, S288C, was shown to be significantly lower than that of the chlorothalonil-resistant strain, F38-1 (Fig. 3). Therefore, the resistance of yeasts to chlorothalonil seems to be exclusively dependent on the GST activity. The GST activity of the chlorothalonil treated F38-1 was lower than that of the untreated one, but was similar to that of the untreated S288C (Fig. 3). The GST activity of the S288C also decreased like the F38-1, after chlorothanoil treatment (data not shown). As reported by Fujita *et al*. (1998), there is a possibility that the GST activity is also inhibited by the IC_{50} values of chlorothanoil, similarly to GR and superoxide dismutase. In order to study this issue in more detail, clone related gene(s) and an analysis of these gene expression levels caused by chlorothalonil, are required.

The effect of thiol compounds on the dissipation of chlorothalonil

In order to test our hypothesis, "glutathione is a major detoxification factor of chlorothalonil in yeast strains", the dissipation rate of chlorothalonil, caused by several thiol compounds, was investigated. Several thiol compounds

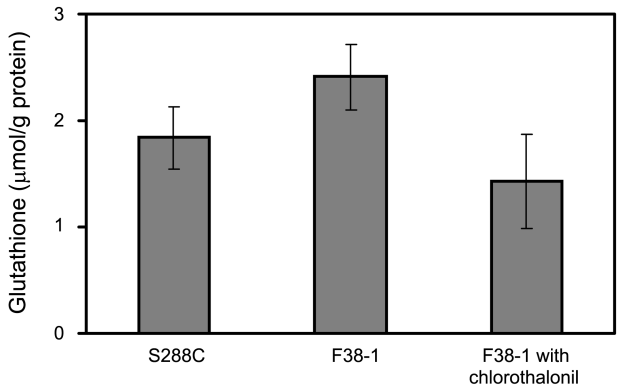

Fig. 2. Intracellular glutathione content of yeasts. The total glutathione content was measured as described in Materials and Methods. Data are the averages of triplicates.

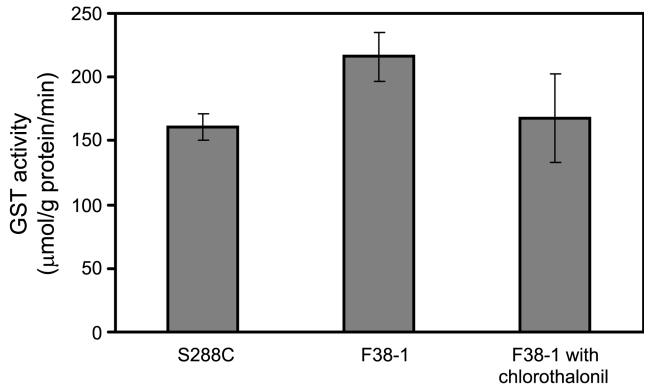

Fig. 3. Glutathione S-transferase (GST) activity of yeasts. The GST activity was measured as described in Materials and Methods. Data are the averages of triplicates.

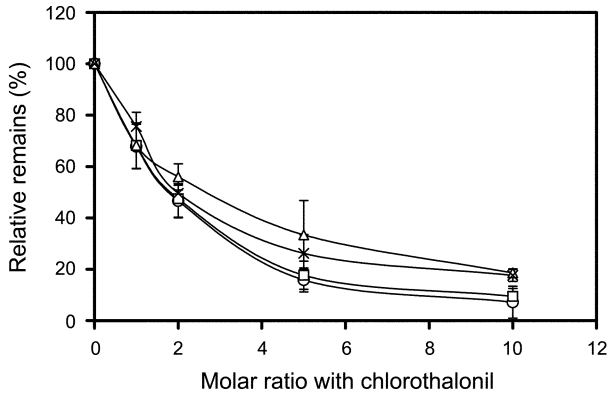

Fig. 4. Chlorothalonil dissipation by several thiol compounds. Various concentrations of thiol compounds were added to 50 mM phosphate buffer (pH 7.0) containing 10 mg/l chlorothalonil. The reaction was performed for 1 h at 30°C. The amount of chlorothalonil was analyzed by HPLC as described in Materials and Methods. ○-○, L-cysteine; □-□, glutathione; △-△, dithiothreitol; ×-×, β-mercaptoethanol

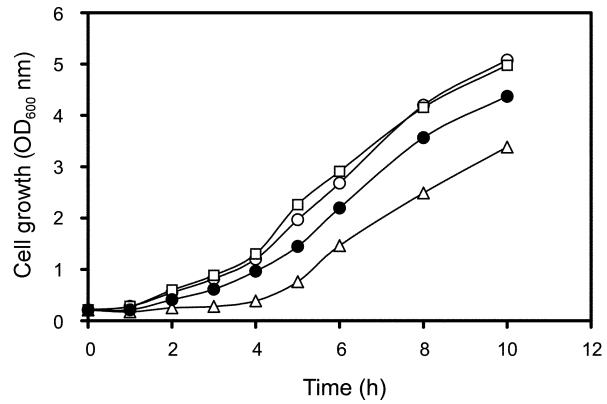

Fig. 5. The effects of L-cysteine on the growth of *S. cerevisiae* F38-1 in medium containing 0.5 mg/l chlorothalonil. The experiment was performed in YPD medium, both with and without 0.5 mg/l chlorothalonil and 10 mg/l L-cysteine, at 30°C under aerobic condition. The cell growth was measured by monitoring the optical density at 600 nm with aliquots harvested at the indicated times. ○-○, medium only; □-□, 10 mg/l L-cysteine; △-△, 0.5 mg/l chlorothalonil; ●-●, 0.5 mg/l chlorothalonil and 10 mg/l L-cysteine.

(cysteine, reduced glutathione, dithiothreitol, and β-mercaptoethanol) were added to a reaction buffer (50 mM phosphate buffer, pH 7.0) containing 10 mg/l chlorothalonil. The amount of each compound added to the reaction buffer was calculated from their molar ratios to chlorothalonil. Fig. 4 showed the positive effects of the thiol compounds on the dissipation of chlorothalonil. The chlorothalonil content in the reaction mixture decreased as the concentration of thiol compounds was increased. When a 10-fold ratio of the thiol compounds was added, at least 80% of the chlorothalonil was dissipated. However, there was no significant difference in the effect of the chlorothalonil dissipation among the tested thiol compounds.

An additional experiment was performed to determine whether the chlorothalonil dissipated by thiol compound

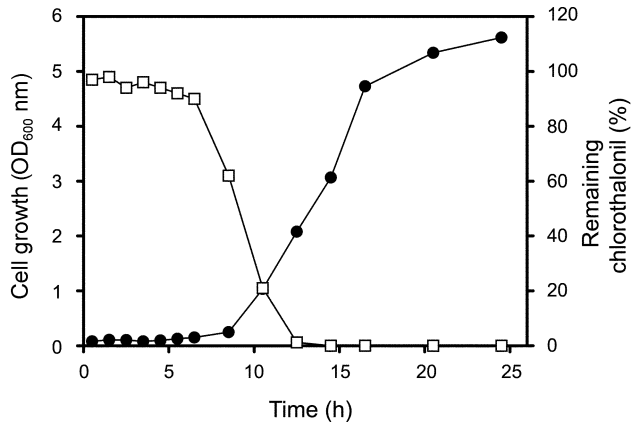

Fig. 6. The yeast cell growth and chlorothalonil dissipation. *S. cerevisiae* F38-1 cells were cultured in YPD medium containing 1.0 mg/l chlorothalonil. Aliquots were taken at the indicated times. The cell growth was measured, and the concentration of chlorothalonil was analyzed from the same aliquot. ●-●, cell growth; □-□, % of remaining chlorothalonil.

was toxic to the yeast cells. *S. cerevisiae* F38-1 was cultured in YPD medium in the presence or absence of 0.5 mg/l chlorothalonil and 10 mg/l cysteine. The cell growth was then monitored by the optical density at 600 nm. The cells growth was not affected by the addition of cysteine, but the toxicity of the chlorothalonil to the F38-1 was reduced (Fig. 5). The results presented in Fig. 4 and 5 support the idea that the cellular free thiol groups, mainly glutathione, are a major protective mechanism against toxicity due to chlorine compounds.

Arrest of yeast cell growth

When chlorothalonil was added to the medium, the growth of the yeast, *S. cerevisiae* F38-1, was arrested (Fig. 1). To study the changes that occurred when the yeast cells were arrested by chlorothalonil, *S. cerevisiae* F38-1 was grown in 100 ml of YPD medium in the presence or absence of 1 mg/l chlorothalonil. Then, 3 ml aliquots of each culture were taken at the indicated times. The morphology in the yeast budding was observed by a microscopy and the growth rate monitored by determining the optical density at 600 nm. Using the same culture aliquot, the chlorothalonil was extracted with the same volume of hexane, and the concentration of the remaining chlorothalonil analyzed by HPLC as described in Materials and Methods. The chlorothalonil concentration in the culture medium remained unchanged for 6 h, and the cell growth was also arrested for 6 h (Fig. 6 and Fig. 7). After 8 h the optical density of the yeast cells increased, and the cells division started. At the same time, the concentration of the chlorothalonil was reduced. It took 13 h to dissipate all the chlorothalonil, and cell growth was restored as the chlorothalonil content in the medium decreased. Chlorothalonil also transiently inhibited the proliferation of yeast cells. F38-1 cells can grow normally as the chlorothalonil

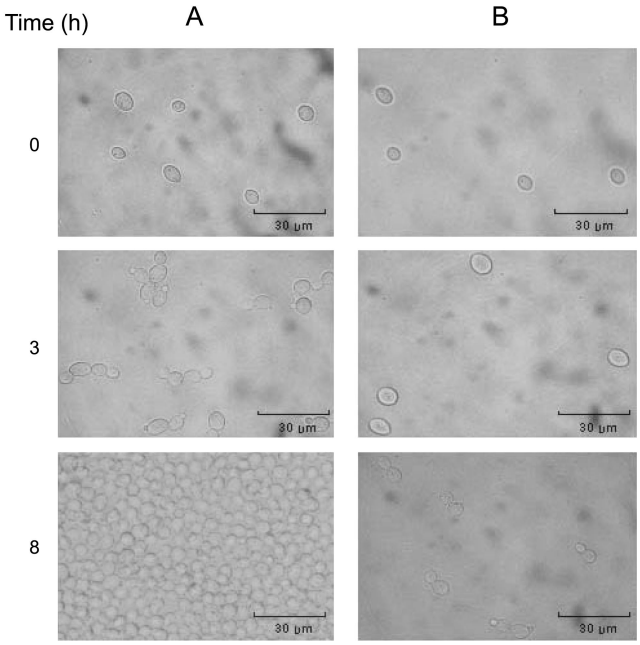

Fig. 7. Optical micrographs of the budding of *S. cerevisiae* F38-1. The cells were grown at 30°C under aerobic condition. Aliquots were taken at the indicated times. A, *S. cerevisiae* F38-1 in YPD medium; B, *S. cerevisiae* F38-1 in YPD medium containing 1 mg/l chlorothalonil.

is dissipated from the growth medium. This indicates that chlorothalonil is detoxified by conjugation with cellular glutathione. The conjugation of chlorothalonil may be catalyzed by GST in the presence of glutathione.

In conclusion, it is proposed that one of the most important factors of yeasts resistant to chlorothalonil is their endogenous thiol compound contents, mainly glutathione, and the catalytic efficiency of GST.

Acknowledgments

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