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2,4-DICHLORPHENOXICACETIC ACID AT LOW CONCENTRATIONS ENHANCES REPRODUCTIVE ABILITY AND OXIDATIVE STRESS RESISTANCE OF YEAST SACCHAROMYCES CEREVISIAE

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Abstract. 2,4-dichlorphenoxicacetic acid (2,4-D) is one of the most widely used herbicides with well documented toxic effects on non-target organisms. In this study, the effect of low concentrations of 2,4-D on reproductive activity and resistance of yeast *S. cerevisiae* to oxidative stress was evaluated. Supplementation of the cultivation medium with 0.1-100 μM 2,4-D did not affect the rate of yeast growth. In early stationary phase, yeast cultures grown with 0.1 and 1 μM 2,4-D had higher number of reproductively active cells than control ones (without 2,4-D). In exponential phase, *S. cerevisiae* cells grown in the presence of 1-100 μM 2,4-D were more resistant to hydrogen peroxide comparing to control ones. Thus, the herbicide increased reproductive potential and cross-resistance to oxidative stress in yeast but the effective concentrations of 2,4-D were different for these phenomena. In summary, the results suggest possible involvement of certain hormetic mechanisms in the influence of 2,4-D at low concentrations on yeast.

Keywords: colony forming unit, herbicide, hormesis, hydrogen peroxide, survival.

Abbreviations: CFU, colony forming unit; 2,4-D, dichlorphenoxicacetic acid; ROS, reactive oxygen species.

1. INTRODUCTION

Herbicides are agrochemicals that control the growth of undesired weeds, bringing about a significant overall increase in crop productivity. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most broadly used herbicides due to its relatively moderate toxicity to non-target organisms, when in concentrations resulting from adequate use in agriculture, and to its biodegradability in the soil [23]. Nevertheless, numerous scientific papers provide evidences of the significant toxicity of 2,4-D to non-target organisms in relevance to cancer risks, neurologic diseases, reproductive risks, hepatotoxicity and immunotoxicity [4, 10, 11, 15, 24]. Yeast *Saccharomyces cerevisiae* has been actively used as an eukaryotic experimental model to assess 2,4-D toxicity under different environmental and physiological conditions [5, 25, 29, 31], and to elucidate the mechanisms underlying the resistance to 2,4-D [9, 21, 25, 26, 28, 30]. It is supposed that the mechanisms underlying basic cellular processes and chemical stress resistance are apparently conserved among phylogenetically distant organisms, making it possible to extrapolate the knowledge gathered in yeast to higher eukaryotes. 2,4-D is a highly

lipophilic weak acid and it exists at low pH in its undissociated lipophilic toxic form (RCOOH), which can readily cross the plasmal membrane by passive diffusion. In the neutral cytosol, the molecular form of 2,4-D dissociates, leading to internal acidification [9, 21] and accumulation of the toxic anion (RCOO-), which cannot easily cross the plasmal membrane lipid bilayer due to electrically charged nature [5]. However, the acid ions may be actively exported through a specific inducible transporters [19, 27]. The induction of specific transporters such as PDR5 and TPO1, which are plasmal membrane multidrug resistance transporters of the ATP-binding cassette and major facilitator superfamilies [27], is postulated as effective adaptive defensive mechanisms against 2,4-D toxicity in yeast [25, 28]. On the other hand, oxidative stress development was demonstrated to accompany 2,4-D toxicity that being confirmed by increased production of reactive oxygen species (ROS) and enhanced levels of oxidative stress markers [1, 15, 23, 29]. The response to 2,4-D includes the upregulation of genes involved in peroxisomal beta-oxidation and mitochondrial oxidative phosphorylation, two metabolic processes leading to the endogenous generation of ROS [25]. Electron leakage from the mitochondrial respiratory chain might further increase production of ROS as a result of cell exposure to 2,4-D [23, 32]. Moreover, the transient increase in free radical generation and lipid peroxidation in the yeast cell exposed to 2,4-D correlated with stimulation of the activity of antioxidant enzymes, which were shown to be determinants of yeast resistance to 2,4-D [29]. While at high concentrations 2,4-D is lethal or possesses severe toxic effects, at low concentrations it can show beneficial effects on plants acting as an auxin analogue to promote plant growth [16]. This biphasic concentration-response can be a reflection of common hormesis phenomenon when generally favorable biological responses result from the action of low doses/concentrations of toxicants or other stressors, while exposure to high doses results in an inhibitory/detrimental outcome [6, 14]. ROS are considered as key mediators of low-dose beneficial events of different stressors [17]. Taking into account that toxicity of 2,4-D is connected with prooxidant mode of action and ROS generation, it can be hypothesized that low doses/concentrations of this herbicide can produce low levels of ROS which may function as signaling molecules that improve systemic defense mechanisms by inducing an adaptive response. To test this assumption, this work aimed to study the effect of 2,4-D low concentrations on S. *cerevisiae* reproductive ability and resistance to oxidative stress induced by hydrogen peroxide.

2. METHODS AND MATERIALS

2.1. STRAIN AND GROWTH CONDITIONS

The *S. cerevisiae* strain YPH250 (*MAT***a** *trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) used in this study was kindly provided by Dr. Youshiharu Inoue (Kyoto University, Japan). Yeast cells were grown at 28 °C with shaking at 175 rpm in YPD liquid medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, and 2,4-D in a range of concentrations from 0.1 μM to 100 μM. The addition of 2,4-D at indicated concentrations did not change pH of medium which was adjusted to 5.5 Although this pH does provide easy penetration of 2,4-D into yeast cells, it is used to study toxic effects of this herbicide [5]. Control cells were cultivated under the same conditions but without 2,4-D. The initial concentration of cells in the medium was about 0.3×10^6 cells ml^{-1}. Growth curves were followed by measuring culture optical density at OD_{620}. The parameter was monitored during 3 days of yeast cultivation.

2.2. EVALUATION OF REPRODUCTIVE CAPABILITY

Reproductive ability of yeast cells was analyzed by plating in triplicate on YPD agar after proper dilution of aliquots of the experimental cultures after 24 h growth (early stationary phase) under the conditions mentioned above. The plates were incubated at 28 °C for 72 h and the colony forming units (CFUs) counted [18]. Yeast colony growth was expressed as percentage of total amount of respective control cells plating on YPD agar.

2.3. Pre-Treatment with 2,4-D and Stress Induction

Yeast cells were harvested by centrifugation (5 min, 8000 g) after 16 h cultivation (exponential phase), re-suspended in fresh YPD medium, and incubated with different concentrations of 2,4-D (pH 5.5) at 28 °C for 2 h. Then the cells were harvested as described above, washed and re-suspended in equal volume of 50 mM potassium phosphate buffer (pH 7.0). Aliquots of the experimental cultures were exposed to 10 mM H_2O_2 at 28 °C for 1 h. Control cells were incubated under the same conditions but without hydrogen peroxide and 2,4-D. Cell survival after stress exposure was monitored by counting a number of CFUs on YPD agar plates as described above.

2.4. Statistical Analysis

Experimental data are expressed as the mean value of six independent experiments ± the standard error of the mean (SEM), and statistical analysis used Dunnett's t-test.

3. Results and Discussion

3.1. 2,4-D at Low Concentrations Does Not Affect Yeast Growth

Previously it was established that herbicide 2,4-D showed the toxicity on yeast *S. cerevisiae* in concentration-dependent and pH-dependent manner [5, 29, 30, 31]. Low values of pH (from 3.0 and lower) exacerbate toxic effects of the herbicide, in particular 100 μM 2,4-D being non-toxic at pH 4.5 becomes toxic for yeast grown at pH 3.5 [9]. In our experiments, YPD medium was supplemented with water solutions of 2,4-D in a range of concentrations from 0.1 to 100 μM. Adding of 2,4-D at indicated concentrations did not change pH of medium which was about 5.5. It can be conditioned by buffering properties of YPD medium [19]. Medium supplementation with 0.1-100 μM 2,4-D did not affect the growth pattern of *S. cerevisiae* YPH250 culture (Fig. 1). Thus, 2,4-D demonstrates no toxicity and does not stimulate yeast reproduction under conditions used in this study.

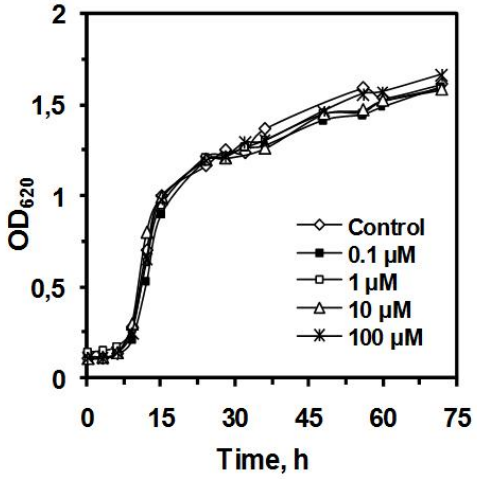

Fig. 1. Growth curves of S. cerevisiae in YPD medium without or in the presence of 2,4-D at different concentrations. The growth curves presented are representative of at least three independent experiments.

3.2. Growth with 2,4-D Increases Reproductive Capability of Stationary Phase Yeast Cells

It is known that any yeast population is heterogeneous and includes cells of different ages [22]. The ability of yeast cells to reproduce themselves reduces with age, and as a result in a population there are some alive cells unable to form colonies [3, 20]. Therefore next we studied the effect of growth with 2,4-D at low concentrations on reproductive potential of yeast cells entering early stationary phase. The reproductive activity of yeast was evaluated by monitoring cell ability to form colonies (CFUs) on complete medium. Fig. 2 demonstrates the colony-forming ability of YPH250 cells grown in the presence of 2,4-D at different concentrations. The percentage of cells capable to form colonies was

by ~19% higher in cultures cultivated with 0.1 and 1 μM 2,4-D compared to control cultures. At the same time, the number of reproductively active cells grown at 2,4-D higher concentrations, 10 and 100 μM, did not differ from that in control. Hence, 2,4-D can clearly affect a number of yeast cells in stationary-phase cultures capable to form colonies in dose-dependent manner. At very low concentrations 2,4-D increased a potential to reproduce and form colonies in stationary phase cells. In different models, the biphasic dose-response relationship for many toxic agents has also been demonstrated showing a stimulatory effect at low doses but inhibitory effect at high doses [2, 11, 18]. This phenomenon is well known as hormesis. In yeast, beneficial effects of low doses of toxicants are often connected with stimulation of colony growth which is experimentally evaluated as CFUs [7, 18]. Thus, one can conclude that hormesis might be responsible for enhanced CFUs.

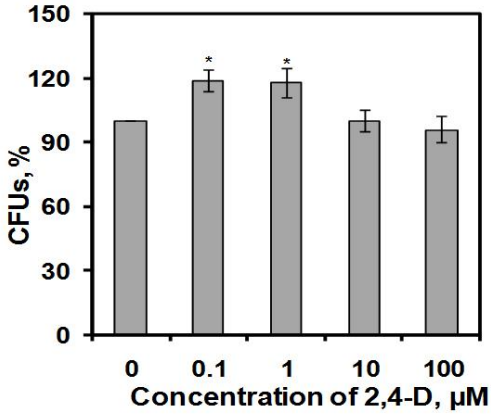

*Fig. 2. Colony forming units of S. cerevisiae after 24 h growth (early stationary phase) in YPD medium supplemented with different concentrations of 2,4-D. The number of reproductively active cells in control cultures is referred as 100%. *Significantly different from respective values in control cultures with $P < 0.05$. Results are shown as means ± SEM ($n = 6$).*

3.3. 2,4-D at Low Concentrations Increases Yeast Resistance to Hydrogen Peroxide

Hormetic action of many compounds was proposed to be connected with their direct or indirect pro-oxidant activity [17]. In this case, toxicants or/and ROS as byproducts of their metabolism are considered as mild stressors activating various defensive mechanisms resulting in the acquisition of resistance to further lethal stress [17, 18].

Since 2,4-D at low concentrations was found to enhance number of formed yeast colonies, next we evaluated the ability of this herbicide to induce cross-adaptation against hydrogen peroxide exposure. Growth with 0.1 μM 2,4-D did not influence yeast survival upon treatment with 10 mM H_2O_2 (Fig. 3). At the same time, yeast cells grown with higher concentrations of 2,4-D were more resistant to 10 mM H_2O_2 than control cells. Resistance to hydrogen peroxide enhances gradually with increasing of 2,4-D concentration accounting for 35%, 53% and 71% of survived cells after of H_2O_2 treatment in control cultures and cultures grown with 1 and 100 μM 2,4-D, respectively. Hence, 2,4-D at very low concentrations causes yeast cross-resistance to oxidative stress. Based on previous reports, it can be supposed that protective effects of 2,4-D may be attributed to its ability to stimulate antioxidant defense and enhance yeast survival under lethal oxidative stress. Analyzing the results, one can see that the concentrations of 2,4-D displaying growth stimulating and stress-protective effects are different. That is not surprising, since it is known that stimulating doses of stressors are usually lower than stress-protective ones, because under stimulating mild stress all resources are directed to ensure cell growth but not cellular protection. More severe stress may lead to redirecting resources, induction of defensive mechanisms and blocking of cell division [8]. In line with this, it was proposed that oxidative stress dependently on its intensity induces different signaling pathways providing an increase in adaptive potential or cell death [13].

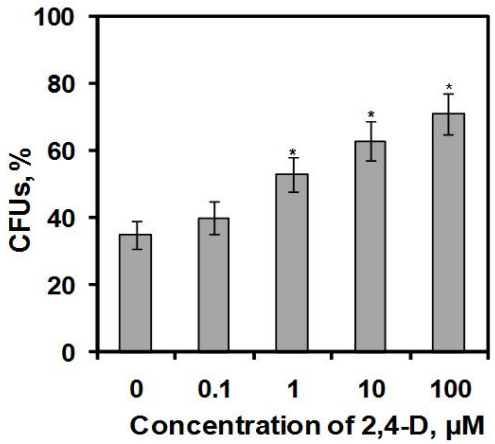

*Fig. 3. Effect of treatment with hydrogen peroxide on survival of exponentially growing S. cerevisiae after pre-incubation with 2,4-D at its indicated concentrations. Cells grown for 16 h in YPD medium were pre-incubated with 2,4-D at different concentrations for 2 h at 28 ºC were collected and re-suspended in 50 mM potassium phosphate buffer (pH 7.0), and then exposed to 10 mM H_2O_2 for 1 h at 28 ºC. *Significantly different from respective values without 2,4-D with $P < 0.05$. As 100% the cell survival in control cultures (without 2,4-D and H_2O_2) was accepted. Results are shown as means ± SEM (n = 6).*

4. CONCLUSIONS

Our results demonstrate that herbicide 2,4-D at low concentrations enhances reproductive potential of *S. cerevisiae* cells and yeast resistance to oxidative stress. It suggests that action of 2,4-D on yeast involves hormetic mechanism having beneficial effects at low concentrations and deleterious at high one. The effective concentrations of 2,4-D for yeast reproduction and cross-resistance to stress were different suggesting the dose-dependent induction of different signaling pathways in yeast cells.

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Байляк М.М., Бурдилюк Н.І. 2,4-дихлорфеноксиоцтова кислота за низьких концентрацій підвищує репродуктивну здатність і стійкість дріжджів *Saccharomyces cerevisiae* до оксидативного стресу. *Журнал Прикарпатського університету імені Василя Стефаника*, **2** (1) (2015), 93–99.

2,4-дихлорфеноксиоцтова кислота (2,4-*Д*) є одним з найбільш широко використовуваних гербіцидів, який проявляє водночас значний токсичний вплив на нецільові організми. У цьому дослідженні вивчали вплив низьких концентрацій 2,4-*Д* на репродуктивну активність та резистентність дріжджів *S. cerevisiae* до оксидативного стресу. Додавання до середовища культивування 2,4-*Д* у концентраціях 0,1-100 мкМ не впливало на швидкість росту культур дріжджів. На початку стаціонарної фази росту культури дріжджів, які вирощували у присутності 0,1 і 1 мкМ 2,4-*Д*, мали більшу кількість репродуктивно активних клітин, ніж контрольні культури (без 2,4-*Д*). Клітини *S. cerevisiae*, вирощені до середини експоненційної фази в присутності 1-100 мкМ 2,4-*Д*, були стійкішими до дії пероксиду водню, ніж контрольні клітини. Таким чином, гербіцид збільшував репродуктивний потенціал і перехресну стійкість до оксидативного стресу в дріжджів, проте ефективні концентрації 2,4-*Д* були різними для цих двох феноменів. Загалом, отримані результати свідчать про можливе залучення горметичного механізму до реалізації ефектів низьких концентрацій 2,4-*Д* на дріжджі.

Ключові слова: колоніє-утворююча одиниця, гербіцид, гормезис, пероксид водню, виживання.