matters

Synergistic toxicity between glyphosate and 2,4-dinitrophenol on budding yeast is not due to H_2O_2-mediated oxidative stress

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Abstract

Glyphosate is a widely-used herbicide that is frequently found as a pollutant of soil and water runoffs. Glyphosate toxicity is controversial but a toxic synergy with other molecules could result in deleterious consequences for living organisms and for the human health. Using budding yeast (*Saccharomyces cerevisiae*) as a eukaryotic model organism, we report here a strong toxic synergy between glyphosate and 2,4-dinitrophenol (DNP), a phenolic compound derived from diesel engine's combustion and industrial pollutant found frequently in surface water and rainfall. Glyphosate concentrations below 600 mg/L did not affect yeast growth but exhibit dose-dependent toxicity in the presence of non-toxic DNP concentrations (below 1 mM). This so-called 'cocktail effect' increases with DNP concentration. Yeast growth is totally abolished in the presence of the highest concentration of both molecules. We explored the implication of oxidative stress in this synergistic effect of glyphosate and DNP, by measuring H_2O_2 concentrations in the culture media, and by comparing cta1Δ/ctt1Δ catalase double-mutant with wild-type yeast. We did not find any glyphosate-DNP enhanced susceptibility for the catalase mutant and did not observe any clear increase of H_2O_2 in the presence of the pollutant mixture. All these data suggest that the redox homeostasis is not involved in this toxic synergy, that remains to be explained.

Introduction

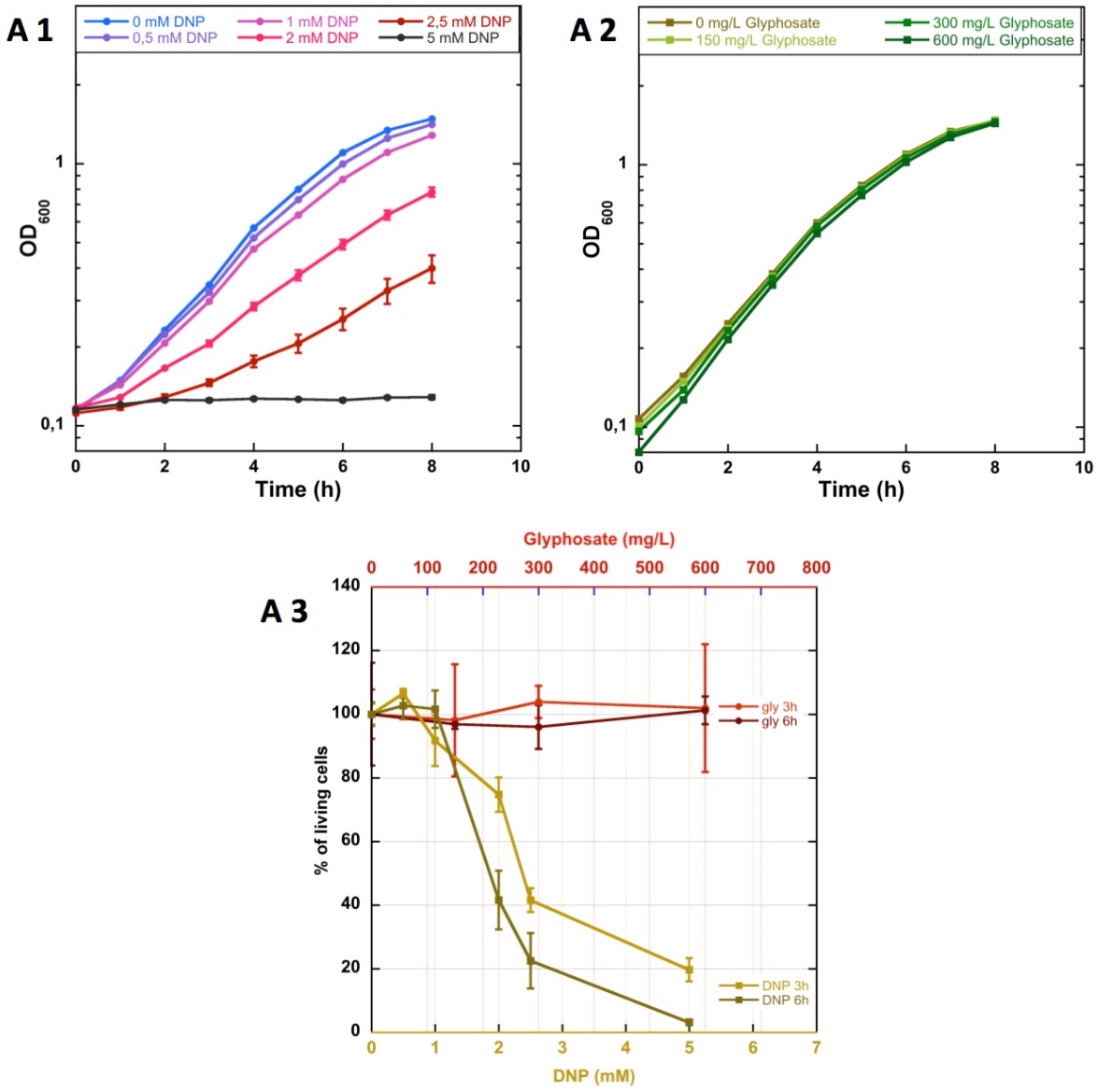
Glyphosate is the major worldwide used herbicide representing one-quarter of herbicide sales. Depending on the formulation of the commercial products, its recommended concentrations for agriculture range around 1 to 7 g/L, therefore, locally high concentrations after spraying can be expected. Because of its strong sorption and degradation by microorganisms glyphosate pollution in soils and in water runoffs remains low, up to 8.1 mg/kg and up to 0.7 mg/L respectively [1] [2]. The toxicity of glyphosate is enhanced in the presence of other pesticide pollutants like atrazine [3] and this so-called cocktail effect has to be taken into account to improve the toxicity assessment of glyphosate pollution. In our experiment, we analyzed the interaction between glyphosate and the highly toxic nitrophenol pollutant 2,4-dinitrophenol (DNP) found in surface water and in rainfalls [4]. DNP is also produced, like many other nitrophenols, by diesel engines, plastic and chemical industries and, indirectly, by photochemical reactions of phenolics. As a result, DNP is found in urban areas as in rural sites [5].

Saccharomyces cerevisiae has been proposed as a good eukaryotic model for assessing the toxicity of environmental pollutants given the extensive knowledge of its genome and metabolism, its easy culture in controlled conditions, and its fast growth [6] [7]. Moreover, when used at millimolar concentration, DNP is a mitochondrial respiratory chain uncoupling molecule that depletes energy-dependent processes in yeast, such as oxidative phosphorylation [8]. The DNP-triggered inhibition of oxidative phosphorylation in mitochondrion leads to high electron transport rates in the respiratory chain

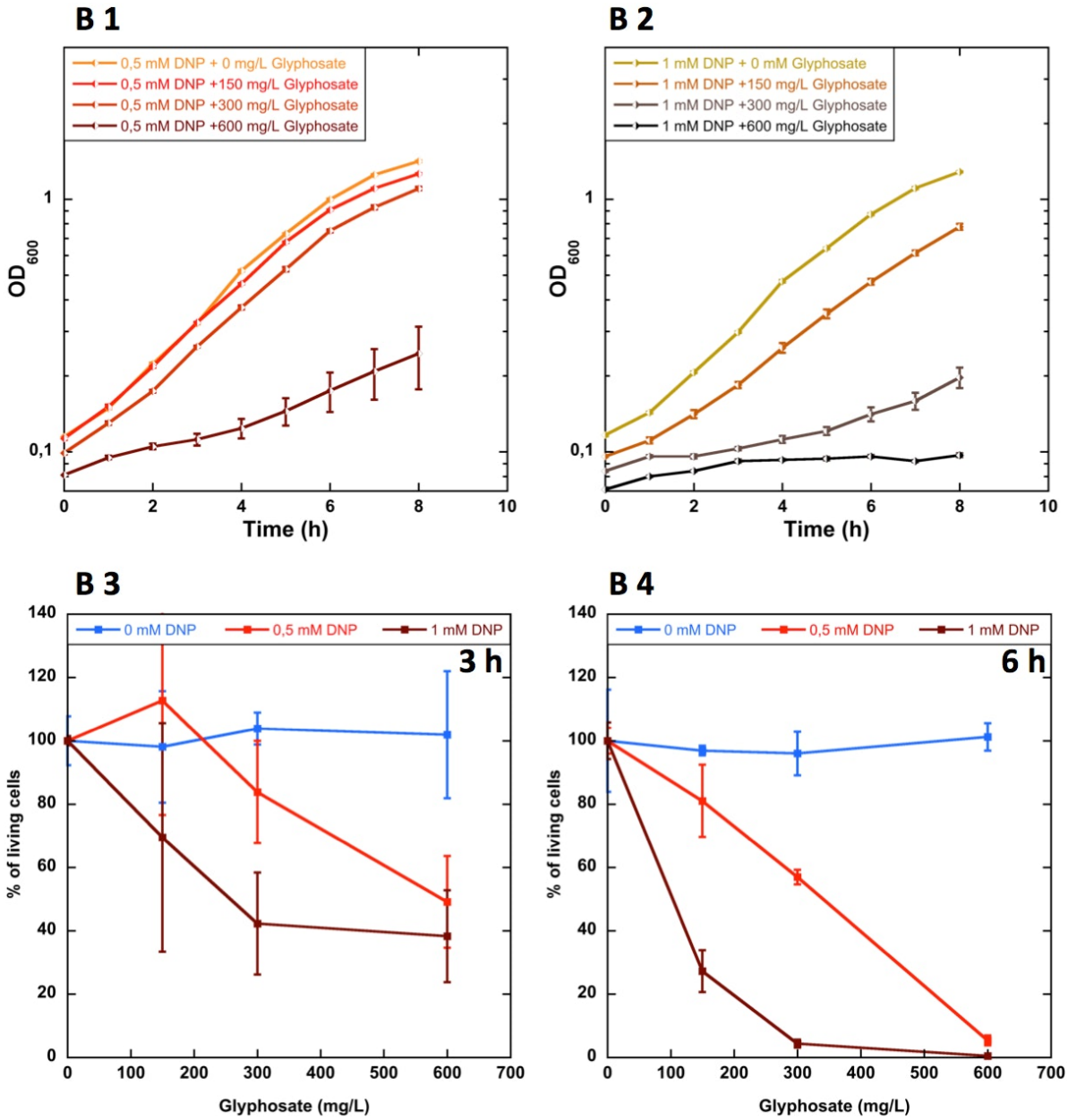
affecting the reactive oxygen species release from yeast mitochondrion [9]. Glyphosate is well known to induce oxidative stress in plants and mammals [10] [11] [12]. A few reports analyzed the effects of glyphosate in yeast. Among them, Braconi and coll. showed that, in its commercial formulation Silglif™, glyphosate promotes significant oxidative stress in *S. cerevisiae* [13] [14]. We thus investigated the involvement of the oxidative stress in the toxic interaction between glyphosate and DNP using the double knockout *ctt1*Δ/*cta1*Δ deficient in the H_2O_2-detoxifying catalase activity.

Objective

Our aim was to describe and quantify the putative synergistic toxic interaction between two major pollutants, the herbicide glyphosate, and the 2,4-dinitrophenol, on the eukaryotic microorganism, *Saccharomyces cerevisiae*.



a


B 1
0,5 mM DNP + 0 mg/L Glyphosate
0,5 mM DNP +150 mg/L Glyphosate
0,5 mM DNP +300 mg/L Glyphosate
0,5 mM DNP +600 mg/L Glyphosate
OD600
Time (h)
B 2
1 mM DNP + 0 mM Glyphosate
1 mM DNP +150 mg/L Glyphosate
1 mM DNP +300 mg/L Glyphosate
1 mM DNP +600 mg/L Glyphosate
OD600
Time (h)
B 3
0 mM DNP
0,5 mM DNP
1 mM DNP
3 h
% of living cells
Glyphosate (mg/L)
B 4
0 mM DNP
0,5 mM DNP
1 mM DNP
6 h
% of living cells
Glyphosate (mg/L)

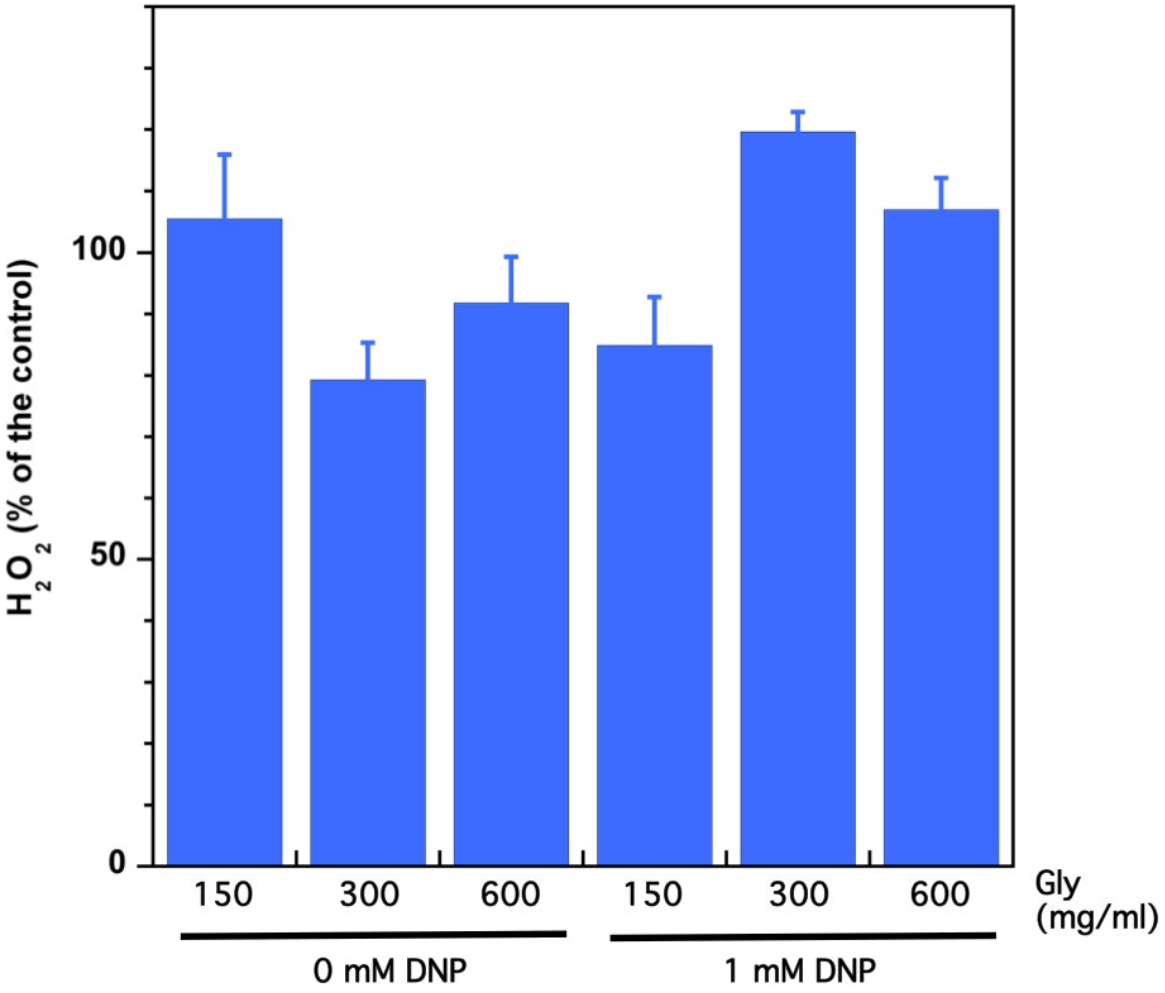
b

Table 1 : Generation time for the WT and ctt1/cta1 strains in presence of pollutants

	WT with 1 mM DNP			ctt1Δ/cta1Δ with 1 mM DNP		
	G (min)	Sdt Error	p[a]	G (min)	Sdt Error	p[a]
Gly (mg/L)						
0	**123,8**	± 1,67	NA[b]	**177,5**	± 6,03	NA[b]
150	**188,6**	± 6,82	0,025	**232,5**	± 10,35	0,025
300	**1105,2**	± 101,79	0,025	**1337,9**	± 167,23	0,025
600	**ND[c]**	NA[b]	NA[b]	**ND[c]**	NA[b]	NA[b]

[a] p corresponds to the p-value obtained when comparing the growth in presence of the indicated concentration of glyphosate to the same strain grown without glyphosate (Wilcoxon test). The generation times observed in YPD medium are 110.28±1.3 and 158.7±4.82 min for the WT and the ctt1Δ/cta1Δ strains respectively. [b] Non applicable. [c] No division.

c



d

Figure Legend

Figure 1. Effect of 2,4-dinitrophenol and glyphosate on yeast *S. cerevisiae* growth rate.

Effect of 2,4-dinitrophenol **(A1)** and the effect of glyphosate **(A2)** on *S. cerevisiae* growth. Cells were grown at 30°C in YPD medium supplemented with the indicated concentrations of DNP or glyphosate, and growth was monitored by measuring the absorbance at 600 nm (OD_{600}). **(A3)** Effect of DNP and glyphosate on yeast viability after 3 h and 6 h of growth. The percentage of living cells, relative to the untreated culture, at different concentrations of glyphosate and DNP is reported on a double X graph. All data are the means of three independent experiments with standard errors. Statistical analyses are presented in figure S1.

Figure 2. Synergistic effects of Glyphosate and 2,4 dinitrophenol on yeast *S. cerevisiae* growth rate.

Glyphosate effect in presence of 0.5 mM 2,4-dinitrophenol **(B1)** and 1 mM 2,4-dinitrophenol **(B2)**. Cells were grown at 30°C in YPD medium and growth was monitored by measuring the absorbance at 600 nm (OD_{600}). All data are the means of 3 independent experiments with standard errors. Yeast viability after 3 h **(B3)** and 6 h **(B4)** of growth for various combinations of the studied of 2,4-dinitrophenol and glyphosate concentrations. Data equate to the percentage of living cells relative to the untreated control. Statistical analyses are presented in figure S1.

Figure 3. Comparison of the generation time for the wild type (WT) and the double catalase knockout (ctt1Δ/cta1Δ) yeast strains in the presence of DNP-glyphosate mixtures.

Yeast strains were grown at 30°C in YPD medium in presence of 1 mM DNP supplemented with the indicated concentrations of glyphosate. Generation times for the reference strain and the ctt1Δ/cta1Δ mutant were calculated during the exponential growth phase (see Fig. 2) for up to 300 mg/L of glyphosate, as no growth can be observed for 600 mg/L of glyphosate.

Figure 4. H_2O_2 in the culture medium of yeast treated with glyphosate in the presence or in absence of 1 mM DNP.

Yeast strains were grown at 30°C in YPD medium in the absence or in presence of 1 mM DNP supplemented with the indicated concentrations of glyphosate. The concentration of H_2O_2 in the culture medium was determined after 6 h of culture. The percentages of H_2O_2 relative to the control (0 mM DNP or 1 mM DNP) are reported in the graph. All data are the means of 3 independent experiments with standard errors.

Results & Discussion

We investigated the toxicity of DNP and glyphosate on the growth of the BY4741 wild type strain of *S. cerevisiae* (Fig. 1, statistical analysis in Fig. S1). We used 3 concentrations of glyphosate (150; 300 and 600 mg/L) in the range of the sprayed commercial herbicides [7] and 5 concentrations of DNP (0.5; 1; 2; 2.5 and 5 mM) corresponding to the range of effective decoupling activity in yeast [9].

Growth curves in the presence of DNP revealed a clear dose-dependent toxicity of this pollutant from 2 to 5 mM which led to total inhibition of growth culture (Fig. 1A1). We confirmed these results studying the viability of yeast cells after 3 and 6 h of culture. No effect was observed below 1 mM DNP whereas a dramatic decrease in living cells was observed for the higher DNP concentrations (Fig. 1A3 and Fig. S1). The absence of effects at 0.5 and 1 mM was surprising given the strong decoupling effect of DNP observed on yeast mitochondrion at these concentrations [9]. Contrarily to readily accessible membrane of purified mitochondria, yeast cells could display a low permeability to this weak acidic molecule (pKa 4.08 [15]). DNP would cross the plasma membrane much more easily in its liposoluble acid form as described for other acid phenols as 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D) [16] [17]. We chose to use the non-toxic concentrations 0.5 and 1 mM DNP to further analyze the synergistic effect of DNP and glyphosate.

None of the glyphosate concentrations tested in this study exhibited a toxic effect on yeast growth, whether following the population growth or counting the living cells (Fig. 1A1 and A3). This is in agreement with the previous observations of Braconi *et al.* [7] showing an inhibitory effect of the commercial formulation Silglif™ on yeast growth and metabolism but not of its active compound glyphosate. These results led us to test the 3 concentrations of glyphosate (ie. 150, 300 and 600 mg/L) in interaction with the two non-toxic concentrations of DNP.

In the presence of 0.5 mM DNP, 600 mg/L glyphosate drastically slowed the growth of the yeast population (Fig. 2B1). The analysis of cell viability confirmed this effect. A significant decrease in living cells was observed after 3 h (Fig. 2B3) and 6 h (Fig. 2B4) treatment compared to the control treated only with DNP. After 6 h in presence of 0.5 mM DNP, the yeast growth with 600 mg/L glyphosate is also significantly lower compared to 150 and 300 mg/L (Fig. 2B3 and B4). This synergy of DNP and glyphosate observed with the highest concentration of the latter is even much more pronounced when the effect of glyphosate is tested in the presence of 1 mM DNP (Fig. 2B2–B4). In these conditions, all 3 concentrations of glyphosate used showed a toxic effect on yeast growth (Fig. 2B2). Cell viability decreased after 3 h of treatment with increasing concentration of glyphosate (except at 3 h when both 300 mg/L and 600 mg/L glyphosate gave a comparable result) (Fig. 2B3 and B4). We quantified this cocktail effect calculating the

generation time from the growth curves by comparing the detrimental effect of added glyphosate compared to conditions with DNP alone (Fig. 3). We observed a generation rate lowering by 1.5 times with added 150 mg/L glyphosate compared to the condition with 1 mM DNP alone. The generation rate lowered 6 times when the glyphosate concentration increases from 150 mg/L to 300 mg/L (Fig. 3). 100% growth inhibition was observed when applying 600 mg/L glyphosate in the presence of 1 mM DNP. Such a huge cocktail effect was previously observed on mammalian CHO-K1 cells treated with a combination of glyphosate, the herbicide atrazine, and their main breakdown products [3]. To our knowledge, the toxic synergy of glyphosate with another single pollutant has not been described before.

We tested if the toxic synergetic effect was a consequence of increased oxidative stress. Glyphosate was found to induce oxidative stress in *Arabidopsis thaliana* plants and in yeast [18] [14]. A mild mitochondrial decoupling activity observed with low concentrations of DNP (1 nM) is supposed to decrease the mitochondrion-generated oxidative stress and to increase yeast life span [9] but high concentrations could have the opposite effect as suggested with millimolar range concentrations on *Hordeum vulgare* plants [19]. Last but not least, the cocktail effect of glyphosate, atrazine and their breakdown products on CHO-K1 cells could result from ROS production [3].

As hydrogen peroxide is described as a major redox molecule playing a central role in the interconversions and detoxification of the different ROS species during oxidative stress [20] [21], we measured the H_2O_2 concentrations in the culture medium of yeast treated with glyphosate in the presence or in absence of 1 mM DNP (Fig. 4). No trend in variations can be evidenced with increasing glyphosate concentration, suggesting that H_2O_2 oxidative stress is not associated with the synergistic effect of glyphosate and DNP. However, we cannot totally exclude that other forms of oxidative stress specifically mediated by superoxide ion or hydroxyl-radical can be involved in this toxic synergy.

To confirm this observation, we also tested the synergistic effect of glyphosate and DNP on the double mutant *cta1Δ/ctt1Δ* defective for the H_2O_2 detoxification catalase activity. Both catalases Cta1p and Ctt1p are important for resistance to H_2O_2 stress [22] and pharmacological inhibition of catalase activity enhances the mitochondrial oxidative stress induced by Ca^{2+} [23]. Yeast *cta1Δ/ctt1Δ* double mutant display increased sensitivity to H_2O_2 and oxidative stress-generating conditions ([24], our observations with the COREPS students).

In order to compare the WT strain to the double mutant *cta1*Δ-*ctt1*Δ, we calculated the generation time (G) for the *cta1*Δ-*ctt1*Δ mutant. In normal growth conditions, we showed a weak increase of G (1.4 fold, see legend of Fig. 3) in the double mutant compared to the WT. Similarly, in the presence of 1 mM DNP, G is also 1.4 times higher, (see Fig. 3) in the *cta1*Δ/*ctt1*Δ genotype compared to the wild type, whatever the glyphosate concentration. These results indicate that the *cta1*Δ/*ctt1*Δ mutant is not more sensitive than the wild type to the glyphosate-DNP cocktail effect. Oxidative stress might not be involved in this observed glyphosate-DNP cocktail effect and that the mechanistic process of this toxic synergy remains to be elucidated.

Conclusions

Our results demonstrated that a mixture of glyphosate and 2,4-dinitrophenol, used at concentrations where neither is toxic alone, results in a dramatic decrease of *Saccharomyces cerevisiae* growth rate. In the presence of 1 mM DNP and 600 mg/L glyphosate (a concentration below that of the usual concentration of the sprayed commercial herbicides) yeast divisions are arrested. As both pollutants have been detected worldwide, one can postulate that this strong synergy might exert its toxic effects in conditions of, particularly highly contaminated environments. However, this requires relatively high DNP pollution with concentration close to millimolar [25]. We did not observe any increased sensitivity to the DNP-glyphosate synergistic effect on the double catalase knockout *ctt1*Δ/*cta1*Δ growth rate. We thus conclude that oxidative stress is unlikely to be responsible for the described cocktail effect. The mechanism underlying the synergy

between glyphosate and DNP on yeast remains to elucidate.

Limitations

2,4-dinitrophenol is a highly toxic pollutant found in many soils and in rainfalls. DNP concentration is around 200 nM/kg in soils and 50 nM in rainfalls. We used much higher concentrations in our experiments, up to 1 mM, described as triggering an uncoupling effect in yeast mitochondrion. Thus, the toxic synergy described here could only take place in an environment were DNP would be exceptionally concentrated. Interestingly, DNP is used as a drug in self-medicated slimming diets. Discarded DNP product in wastewater could lead to exceptional local contamination at high concentrations.

Alternative Explanations

Conjectures

We are currently investigating the mechanism underlying the synergy we described in this article.

Additional Information

Methods

Yeast strains, media, and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were as follow: reference strain (wild-type, WT) BY4741 (MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0), mutant strain ctt1Δ/cta1Δ (MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGR088w::kanMX4; YDR256c::kanMX4).

All strains were initially inoculated at OD_{600}=0.01 in liquid YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and incubated at 30°C with 225 rpm orbital shaking. Exponential phase cells were harvested inoculated at OD_{600}=0.1 in YPD medium supplemented or not with either 2,4-dinitrophenol, glyphosate or a combination of both. Cells were cultured further 8 h during which growth was monitored by measuring the absorbance at 600 nm (OD_{600}).

Reagents

Glyphosate was purchased from Sigma Aldrich (45521-250MG) and resuspended in distilled water at a final concentration of 6 mg/mL and then sterilized by filtration using a 0.2 µm syringe filter. 2,4-dinitrophenol was purchased from Sigma Aldrich (D198501-5G) and dissolved in Dimethyl sulfoxide (DMSO) at a final concentration of 2 M/L. All subsequent dilutions were done in YPD medium.

Viability

After 3 h or 6 h yeast cultures were washed twice in YPD medium then serially diluted, plated on YPD agar and incubated at 30°C for 2 days. Cell viability was determined by counting the total colony forming units (cfu) in each condition and expressed as a percentage of the control. Results are the average +/- standard deviation for 3 independent cultures (n=3).

H_2O_2 measurement

H_2O_2 concentration was determined as described in [26]. Briefly, 100 µL of reagent A (25 mM $FeSO_4$, 2.5 M H_2SO_4, 25 mM $(NH_4)_2SO_4$) was mix with 10 mL of reagent B (125 µM xylenol orange, 100 mM sorbitol). 900 µL of this solution was added to 100 µL of yeast

culture medium and incubated 30 min in the dark before measuring the absorbance at 560 nm.

Statistical analysis

All the differences observed in cfu counting between growth conditions were pairwise compared by the Wilcoxon test. A p-value of 0.05 was considered statistically significant. All statistical analyses are presented in figure S1.

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Ethics Statement

Not applicable.

Citations

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