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Optimization of detection methods for oxidative stress in *Saccharomyces* strains

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ABSTRACT

Oxidative stress is a change in the balance of the oxidation - reduction processes in the cell, which generates loss of cellular viability. This stress can be induced by numerous oxidants, including plumbagin, menadione sodium bisulfate, hydrogen peroxide and paraquat, which produce the formation of reactive oxygen species, causing disbalance at the cellular level. Melatonin, which is a hormone found in vertebrates, has been described as an antioxidant molecule in plants and vertebrates. The antioxidant role of melatonin on yeast has been recently described, but only against hydrogen peroxide. Thus, a wide study of its antioxidant capacity would need to be done, by using other oxidative stresses. The main objective of our work was the optimization of detection methods for oxidative stress in strains of *Saccharomyces* with different conditions. To carry out this study, we analyzed different media and concentrations of oxidants on solid-agar plates, as well as different techniques to apply the oxidant on the plate, and measure the inhibition halo caused by those oxidants. Then, we determined the possible protective effect of melatonin against this oxidative stress. Besides, we also analyzed the effect of the oxidants on cell viability using broth media, to compare the response of cells in solid and liquid media, against different oxidant concentrations. Our results showed that the best conditions to do these assays were using YPD medium and applying the oxidant directly in the plate (hole method). Moreover, the concentration of oxidant agent needed to see an effect on yeast growth on agar plate was much higher than the concentration needed in liquid medium. Finally, the antioxidant effect of melatonin was not evidenced in those conditions, due to the lower concentration tested.

Keywords: *Saccharomyces*, oxidative stress, plumbagin, menadione sodium bisulfate, hydrogen peroxide, paraquat. antioxidant, melatonin.

RESUMEN

El estrés oxidativo es un cambio en el equilibrio de los procesos de oxidación - reducción en la célula, lo que genera pérdida de viabilidad celular. Este estrés puede ser inducido por numerosos oxidantes, incluyendo plumbagina, menadiona sodio bisulfato, peróxido de hidrógeno y paraquat, que producen la formación de especies reactivas de oxígeno, causando desequilibrio a nivel celular. La melatonina, que es una hormona encontrada en vertebrados, ha sido descrita como una molécula antioxidante en plantas y vertebrados. El papel antioxidante de la melatonina en la levadura se ha descrito recientemente, pero sólo contra el peróxido de hidrógeno. Por lo tanto, un amplio estudio de su capacidad antioxidante se necesita hacer, mediante el uso de otras tensiones oxidativas. El objetivo principal de nuestro trabajo fue la optimización de métodos de detección del estrés oxidativo en cepas de *Saccharomyces* con diferentes condiciones. Para llevar a cabo este estudio, se analizaron diferentes medios y concentraciones de oxidantes en placas de agar sólido, así como diferentes técnicas para aplicar el oxidante en la placa, y medir el halo de inhibición causado por esos oxidantes. Luego, determinamos el posible efecto protector de la melatonina frente a este estrés oxidativo. Además, se analizó el efecto de los oxidantes sobre la viabilidad celular utilizando medios líquidos, para comparar la respuesta de las células en medios sólidos y líquidos frente a diferentes concentraciones de oxidantes. Nuestros resultados mostraron que las mejores condiciones para realizar estos ensayos fueron utilizando medio YPD y aplicando el oxidante directamente en la placa (método de agujeros). Además, la concentración del agente oxidante necesaria para ver un efecto sobre el crecimiento de levadura en placa de agar era mucho mayor que la concentración necesaria en medio líquido. Finalmente, el efecto antioxidante de la melatonina no se evidenció en esas condiciones, debido a la menor concentración ensayada.

Palabras claves: *Saccharomyces*, estrés oxidativo, plumbagina, menadiona sodio bisulfato, peróxido de hidrógeno, paraquat. antioxidante, melatonina.

1.INTRODUCTION

Yeast are defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body. In the latest edition of the monographic series ´´The Yeasts, A Taxonomic Study´´[1], there are described 700 species, 15 of them associated with winemaking[2].

The *Saccharomyces* group with its primary representative, *Saccharomyces cerevisiae*, is present on grape skins, winery equipment and fermenting must[3]. *S. cerevisiae* is the most important yeast for wine production and is responsible for the metabolism of grape sugar to alcohol and CO_2[4]. It has an equally important role to play in the formation of secondary metabolites important to wine[5], as well as in the conversion of grape aroma precursors to varietal aromas in wine[6]. The *non-Saccharomyces* yeasts contain numerous species, dominated numerically by the apiculate yeasts, e.g. *Kloeckera* spp. and *Candida* spp. that are found predominantly on grapes and in freshly processed must and in less numbers on winery equipment[7].

The yeasts responsible for beverage fermentation process are under strict microbiological control not only to avoid contaminations, but also to evade their loss during the fermentative process. This process can be very stressful, because it involves many stresses that can cause cellular damage, and therefore can impair fermentation. One of those is oxidative stress, which is caused by the toxic effects of reactive oxygen species (ROS). Those are formed from molecular oxygen, including superoxide anion (O_2·-), singlet oxygen (O_2), hydroxyl radical (OH-) or hydrogen peroxide (H_2O_2)[8]. Under normal physiological conditions, yeast cells can maintain a reduced intracellular redox environment but if it increases, it causes respiratory deficiencies that results in oxidative stress[9]. This oxidative stress is defined as a shift in the balance of oxidative and reductive processes in the cell toward oxidation that has deleterious consequences like the loss of cell viability[10]. Enzymatic systems, which include catalase, superoxide dismutase and glutathione peroxidase, are primary defenses that function to neutralize ROS. In contrast, non-enzymatic systems, such as the glutathione, glutaredoxin family or thioredoxins, are secondary defenses that repair or remove the products of oxidative damage[11].

In addition, oxidative stress causes can be endogenous (respiratory metabolism) or exogenous (compounds that can enter to cell and produced ROS by several mechanism). In this work, we are going to analyze the effect of four of these exogenous compounds (plumbagin, menadione, hydrogen peroxide and paraquat). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) undergoes one electron reduction by enzymes such as microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH ubiquinone oxidoreductase, yielding the corresponding semiquinone radicals. First, under aerobic conditions, the semiquinone radical participates in redox cycling to generate ROS. Second, quinones are potent electrophiles, capable of reacting with the thiol groups of proteins and glutathione (GSH). In fact, generation of GS-conjugates catalyzed by glutathione tranferase isoforms (GST) with depletion of GSH has been associated with oxidative stress[12].

Menadione (vitamin K-3) is a compound that increases the intracellular production of $O_{2.}$ One-electron reduction to the semiquinone radical by cellular flavoproteins is followed by reoxidation to the parent compound by molecular oxygen, coupled with the generation

of O_2. This process, called redox cycling, imposes a steady-state oxidative challenge within cells that can result in oxidative stress[13].

H_2O_2 damages cellular components by oxidizing lipids, proteins and nucleic acids[14]. H_2O_2 does not react directly with DNA. Instead, the OH., generated from H_2O_2 by the metal-catalyzed Fenton/Haber-Weiss reaction, induces DNA damage in yeast cells[15]. One of the major products of damage, 8-hydroxyguanine, is detectable in yeast cells exposed to lethal concentrations of H_2O_2[16]. Furthermore, H_2O_2 increases the frequency of intrachromosomal recombination in *Saccharomyces cerevisiae*, via the formation of hydroxyl radicals. It is therefore likely that oxidative DNA damage plays a role in DNA recombination in yeast[17].

Paraquat (or methyl viologen; N,N′-dimethyl-4,4′-bipyridinium dichloride) is a rapid-acting, nonselective herbicide that has been widely used for weed control[18]. It causes rapid membrane damage by accepting electrons from PSI and subsequently transferring them to molecular oxygen, resulting in the production of toxic ROS, which efficiently induce cell death[19]. Figure 1 shows how these compounds can compromise cellular integrity.

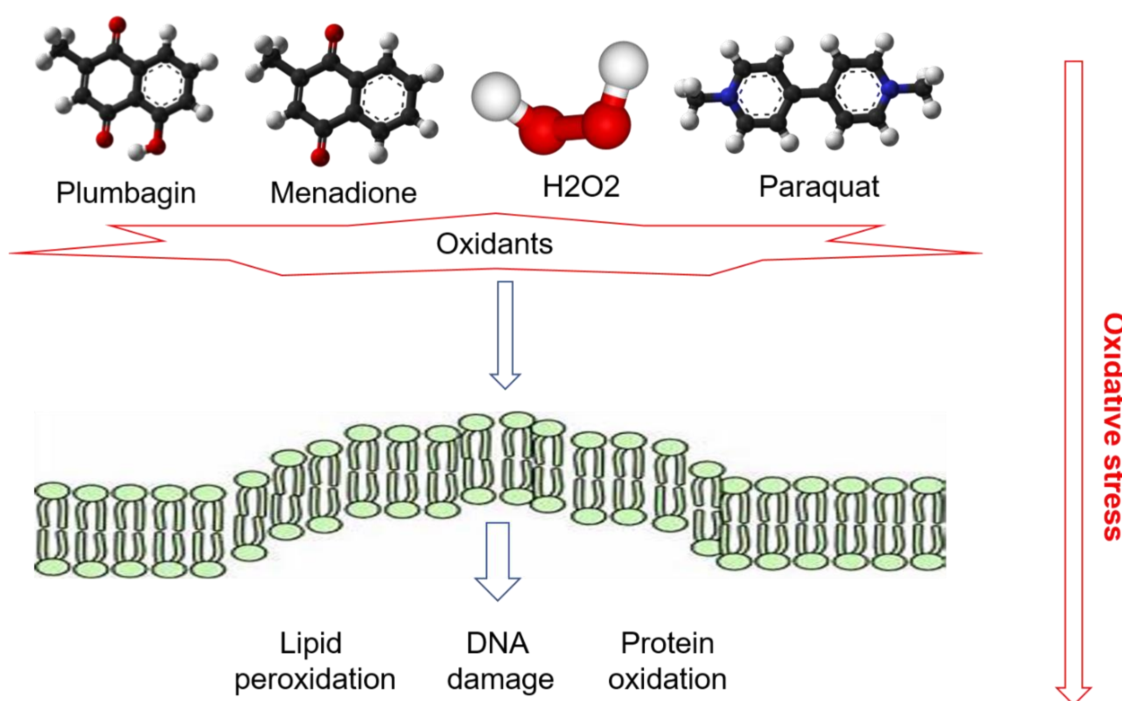

Figure 1. Oxidative stress scheme.

Although many compounds can generate oxidative stress, several molecules can offer chemical protection against it. The studies are frequently referred to them as antioxidants, and in the last decades they have become the focus of numerous investigations, like superoxide dismutase, catalase and glutathione peroxidase enzymes[20]. Also, among the molecules that offers chemical protection against oxidative stress, melatonin (Mel) stands out[21-23], been the main studies in vertebrates[24].

Mel (N-acetyl-5-methoxytryptamine) was first isolated and identified by Prof. A. B. Lerner et al., 1958[25]. At first, melatonin was identified as a hormone synthesized by the pineal gland in humans. Besides, it is also produced in plants, yeast and bacteria, as a secondary metabolite. The amino acid tryptophan is the precursor of all 5-methoxytryptamines (or indoleamines), and the biosynthetic pathway is via serotonin (5-hydroxytryptamine) in the case of plants and mammals[26, 27] as is shown in figure 2. The two functional groups present in Mel are essential for the proper functioning of the hormone[28]. There are several reasons why Mel has been proven to be particularly efficient as antioxidant. It has very low toxicity, even at rather large doses[29,30]. It can easily cross physiologic barriers because of its size, and has partial solubility in water and high solubility in lipids[31, 32]. After being metabolized, Mel protection against ROS does not decrease. In fact, it is maintained, or even increased, due to the antioxidant capacity of its metabolites[33, 34] on vertebrates[24].

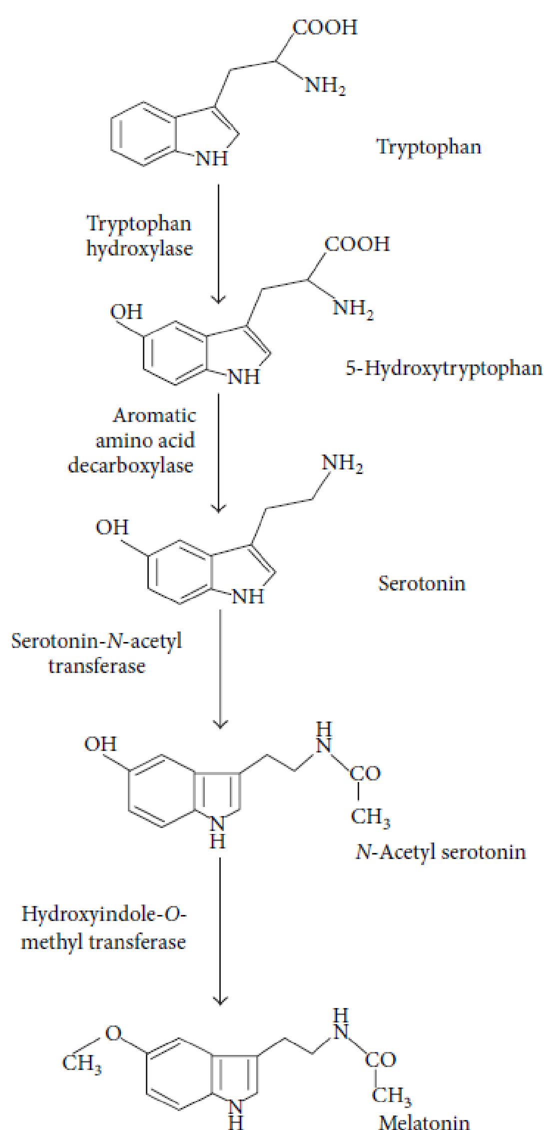

Figure 2. Synthesis of melatonin from tryptophan in yeast[35].

Moreover, Mel has direct and indirect antioxidant activity. Direct activity is due to its ability to scavenger free radicals. Its indirect activity is because Mel can stimulate some antioxidant enzymes[36]. In fact, previous studies described other antioxidant mechanisms of Mel as the stimulation of the synthesis of glutathione (an essential intracellular antioxidant)[37], the increase of the activities of other antioxidants[38] and the increase of the efficiency of mitochondrial electron transport chain (ETC), thereby lowering electron leakage and reducing free radical generation[39].

In fact, Mel can be found in small quantities in wine[40], not only because it is present in grapes[41], also because it is synthesized by yeast during alcoholic fermentation[42]. Also, several references analyzing the presence of Mel in wines and grapes, indicate the production of it during fermentation[43] The presence of Mel in wine has been linked to the yeast metabolism[44], although the number of references in this case is still rare, indicating the need to pursue further this subject[45].

The project in which we are involved, named "Synthesis of bioactive compounds in foods fermented by different strains of yeast", includes the determination of Mel production by wine yeast and its antioxidant effects against oxidative stress. Our study was focused in the optimization of detection methods for analyzing the effect of the oxidative stress on different *Saccharomyces* strains growth, at different conditions. To carry out this study, we analyzed different media and concentrations of oxidants on solid-agar plates, as well as different techniques to apply the oxidant on the plate, and measure the inhibition halo caused by those oxidants. Then, we determined the possible protective effect of melatonin (adding 5μM of Mel in the preculture) against this oxidative stress, reducing the inhibition growth. Besides, we also analyzed the effect of the oxidants on cell viability using broth media, to compare the response of cells in solid and liquid media, against different oxidant concentrations.

2. MATERIALS AND METHODS

2.1 Yeast strains and culture media

The study was conducted with six strains of *Saccharomyces*: Lalvin QA23 (*S. cerevisiae*, commercial wine yeast, Lallemand, Montreal, Canada), Diamond (*S. pastorianus*, used for producing lager beers, Lallemand, Montreal, Canada), Instaferm (*S. cerevisiae*, used for bakery, Lallemand, Montreal, Canada), Levucell SC20 (*S. cerevisiae*, used for ruminant feed, Lallemand, Montreal, Canada), Aroma white (*S. cerevisiae*, used for the production of aromatic wines, Enartis Ferm, USA) and BY4743 (laboratory strain of *S. cerevisiae*). All commercial strains were in active dry yeast form and were rehydrated according to the manufacturer's instructions.

The media used were YPD broth (2% glucose, 2% fructose, 1% yeast extract), YPD agar plates (YPD broth plus 1.7% agar), YNB Glu plates (5% glucose, 0.67% YNB, 1.7% agar) and YNB Gly plates (3% glycerol, 0.67% YNB, 1.7% agar).

2.2 Reagents

Four oxidants were used to generate oxidative stress on yeast. Plumbagin (from Plumbago indica) (Sigma-Aldrich, USA) was used at 0.5, 1, 1.5, 2, 4, 8, 16, 32, 64 mM. Menadione sodium bisulfate (Sigma-Aldrich, USA) concentrations were 2, 4, 8, 16, 20, 32, 50, 64, 100, 128, 256 mM. For hydrogen peroxide (30% stabilized pure, PanReac, Germany), the concentrations used were 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 mM. Paraquat (methyl viologen dichloride) (Aldrich, USA) was evaluated at 2, 4, 8, 16, 32, 64 mM. Plumbagin is dissolved with chloroform and the rest of oxidants with water.

Besides, Mel (Sigma-Aldrich, USA) was used at 5 µM and 50 µM. For obtaining a sterile solution, Mel solution was sterilized by filtration, with a filter of 0.45µM.

2.3 Inhibition halos in absence and presence of Mel

First, yeast cells were inoculated in YPD broth (about $5x10^5$ cell/mL) and incubated for 24h at 28°C with orbital shaking (120rpm). Then, yeast cells were inoculated (about $5x10^5$ cell/mL) in YPD broth with (5 or 50 µM) or without Mel and incubated until the optical density (OD) was 0.5 (exponential phase, $5x10^6$ cells)). At this moment, 100 µL of this culture were spread with glass beads on the different plates (YPD, YNB Glc or YNB Gly). Two methods to determine the inhibition halos were tested: disk technique and hole technique. In the disk technique, we soaked 6mm blank disks (done with Chromatography paper 3MM Chr (Whatman, Schleicher and Schuell) with 10 µL of the oxidant, and let it dry. Then, we put it on the agar surface seeded with the yeast to study. In the hole method, we performed holes of 6mm of diameter in the plate after yeast seeding, and put 10 µL of the oxidant inside. The plates were incubated at 28 °C for seven to nine days. The effect of oxidants was measured as the diameter of the inhibition halos around the disks or holes after 24, 48h and 7 or 9 days. Moreover, photos of the plates were taken with a ProtoCOL equipment. The experiment was done in triplicates and inhibition halos obtained with and without melatonin in the same conditions were compared. Moreover, disks or holes with only the dissolvent used for the preparation of each oxidant were also tested to determine which was the effect of the dissolvent on the growth of yeast cells.

2.4 Evaluation of yeast viability after H_2O_2 stress exposure

Yeast viability after 2 hours exposure to stress by H_2O_2 was evaluated by microplate bioassay. The methodology follows was as previous authors described[46] and the measured was made every 30 min using a microplate reader (Omega Polarstar, BMG Labtech Gmbh, Ortenberg, Germany).

2.5 Data analysis

Data were subjected to one-way analysis of variance (ANOVA) to evaluate the effect of each treatment. The results were considered statistically significant at a p-value less than 0.05.

3. RESULTS AND DISCUSION

3.1 Inhibition halos on YNB-Glu 5% and YNB-Glu 3%

The inhibition halos of each strain were measured on YNB-Glu and YNB-Gly, to determine the differences between the effect of oxidative stress in fermentative (YNB-Glu) and respiratory (YNB-Gly) metabolism. The measures were made at 24h, 48h and 7 or 9 days. For this experiment, the disk paper method was used. Plumbagin was the oxidant employed and the values obtained for QA23 and Diamond are represented on Figure 3 and 4, respectively.

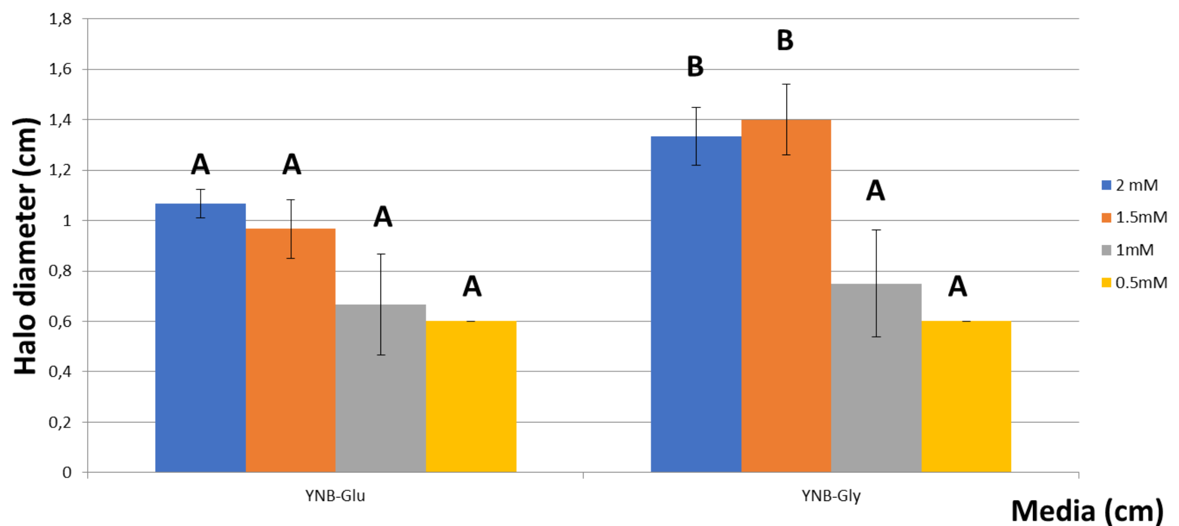

Figure 3. Inhibition halos for QA23 on YNB-Glu and YNB-Gly with plumbagin as oxidant. The letters indicate if there are significant differences between the halos in YNB-Glu and YNB-Gly for the same oxidant concentration (expressed on millimolar (mM)).

The figure 3 shows significant differences on halo diameters on 1.5 and 2 mM. In general, a bigger halo was generated on YNB-Gly compared to YNB-Glu. At 0.5 mM no inhibition halo was observed (0.6 cm is the diameter of the paper disk diameter). Moreover, it can be observed in both media that the increase on plumbagin concentration is directly related to the increase of halo diameter.

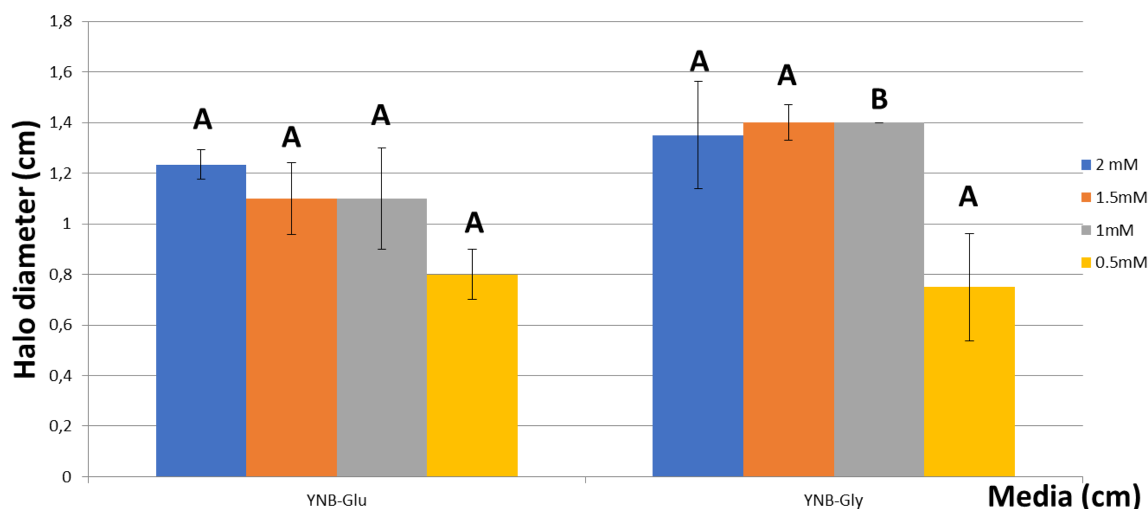

Figure 4. Inhibition halos for Diamond on YNB-Glu and YNB-Gly with plumbagin as oxidant. The letters indicate if there are significant differences between the halos in YNB-Glu and YNB-Gly for the same oxidant concentration (expressed on millimolar (mM)).

In figure 4, significant differences were only observed at 1 mM of oxidant. However, at higher oxidant concentrations, 4, 8 and 16 mM, significant differences were observed between the media (data not shown), and bigger halos on YNB-Gly compared to YNB-Glu. However, halos on YNB-Gly were asymmetric and sometimes overlapped, thus, they were difficult to be measured. For this reason, we decided to continue working with YNB-Glu.

Our results, with bigger halos obtained for YNB-Gly, which is a respiratory media, were in contradiction with previous studies. Those studies have reported that when yeasts go under aerobic metabolism, they are intrinsically more resistant to oxidizing agents, than when metabolizing anaerobically[47] .Besides, aerobic organisms have evolved inter-related enzymatic and non-enzymatic defense processes for protection against the effects of free radicals and their derived toxic species. Enzymatic mechanisms include superoxide dismutase (Sod), which catalyses the reduction of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen, and catalase (Cat) which converts H_2O_2 to water and molecular oxygen[48].

3.2 Inhibition halos on YNB-Glu and YPD

Then, we decided to compare the inhibition halos obtained on YNB-Glu with YPD medium. YPD is a rich media with the necessary components for having a good microorganism growth, so we want to evaluate if oxidative stress in yeast is affected by the nutrient limitation. For that reason, we measured the effect of different concentrations of plumbagin on the six strains in both media (Figure 5).

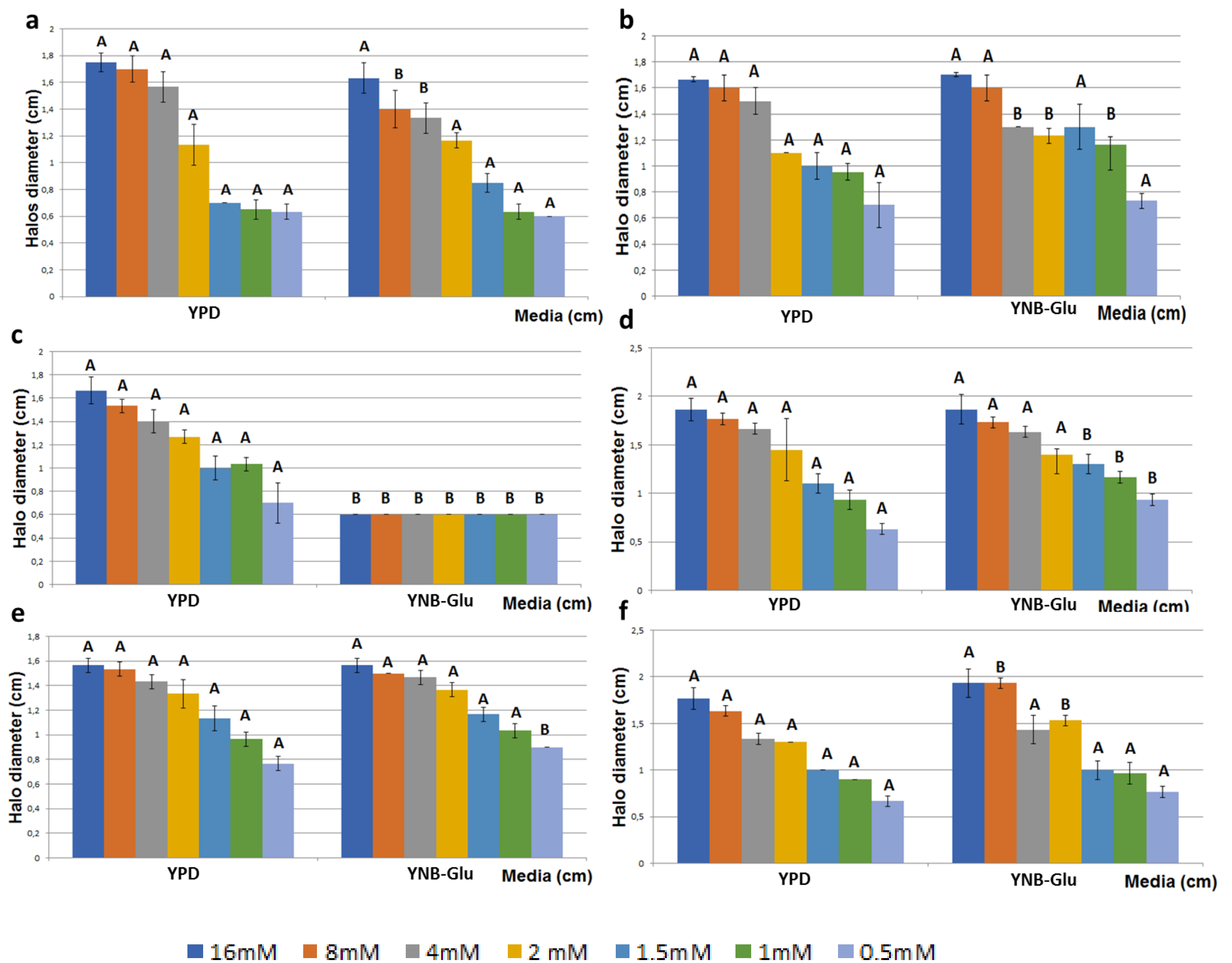

Figure 5. Inhibition halos on YPD and YNB-Glu for QA23 (a), Diamond (b), BY4743 (c), Levucell SC20 (d), Instaferm (e), Aroma white (f) with plumbagin as oxidant. The letters indicate if there are significant differences between the halos in YNB-Glu and YPD for the same oxidant concentration (expressed on millimolar (mM)).

In figure 5, we can observe that there are significant differences only in some concentrations of the oxidant, and this response is strain dependent, and very random. For QA23 the differences are at 4 and 8 mM, for Diamond at 1.5, 2 and 4 mM, for Levucell at 0.5, 1, 1.5 mM, for Instaferm at 0.5 mM and for Aroma white at 2 and 8 mM. BY4743 was not able to growon YNB-Glu because its autotrophies were not supplemented in the medium, so we cannot measure their inhibition halos.

Overall, halos were slightly bigger on YNB-Glu than on YPD. This results were a bit unexpected, since YNB-Glu is a more limited medium, and it's known that microorganism which growth on limited media are more prepared for stress because of the lack of required compounds[49], so when an oxidant is added to the culture they are supposed to be more prepared to resist it.

Finally, as the results were very similar in both media, we decided to continue our work with YPD, because YPD is a rich media, with a better growth for all strains, and more reproducibility on replicates (data not shown).

3.3 Inhibition halos on paper disc and holes

We evaluated which was the best method for measuring the oxidants effects, paper disks or holes. To accomplish this, we performed the experiment using QA23 strain, and four different oxidants agents: plumbagin, menadione, hydrogen peroxide and paraquat. Those results are shown in figures 6, 7, 8 and 9 respectively.

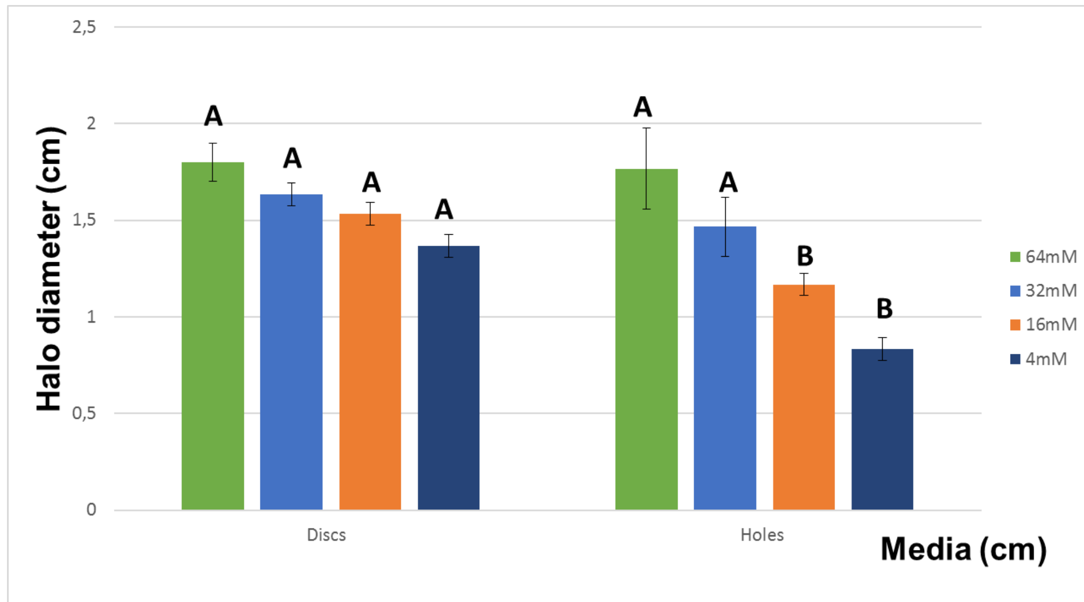

Figure 6. Inhibition halos for QA23 by disks and holes with plumbagin as oxidant. The letters indicate if there are significant differences between the halos in discs and holes for the same oxidant concentration (expressed on millimolar (mM)).

The figure 6 shows that the increase of oxidant concentration causes an increase of the inhibition halo. The differences between the different concentrations are more pronounced when the hole method is used. The main differences between both methods are at low concentrations.

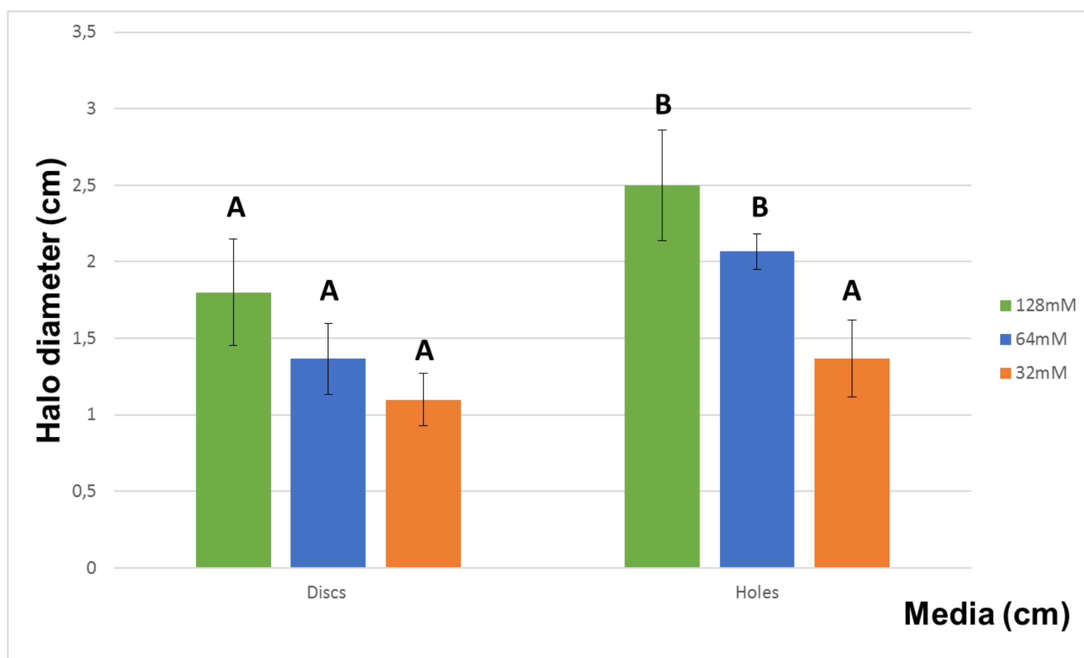

Figure 7. Inhibition halos for QA23 by disks and holes with menadione sodium bisulfate as oxidant. The letters indicate if there are significant differences between the halos in discs and holes for the same oxidant concentration (expressed on millimolar (mM)).

For the other oxidants, menadione (Figure 7), hydrogen peroxide (Figure 8), and paraquat (Figure 9) we can also observe significant differences between methods in some concentrations, in general presenting bigger inhibition halos with the hole method.

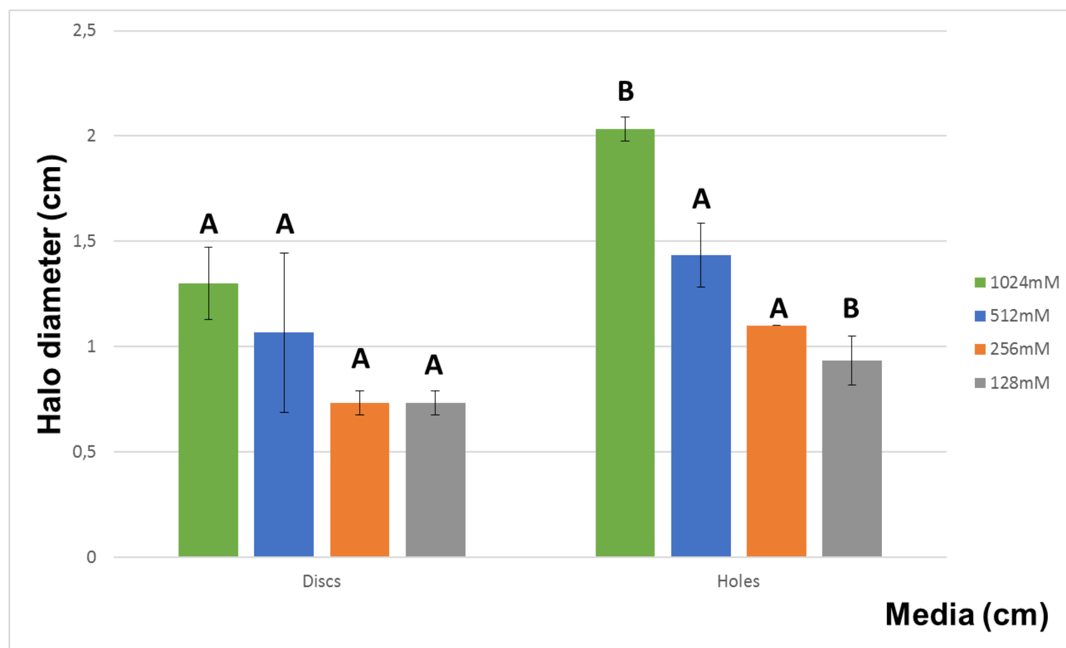

Figure 8. Inhibition halos for QA23 by disks and holes with hydrogen peroxide as oxidant. The letters indicate if there are significant differences between the halos in discs and holes for the same oxidant concentration (expressed on millimolar (mM)).

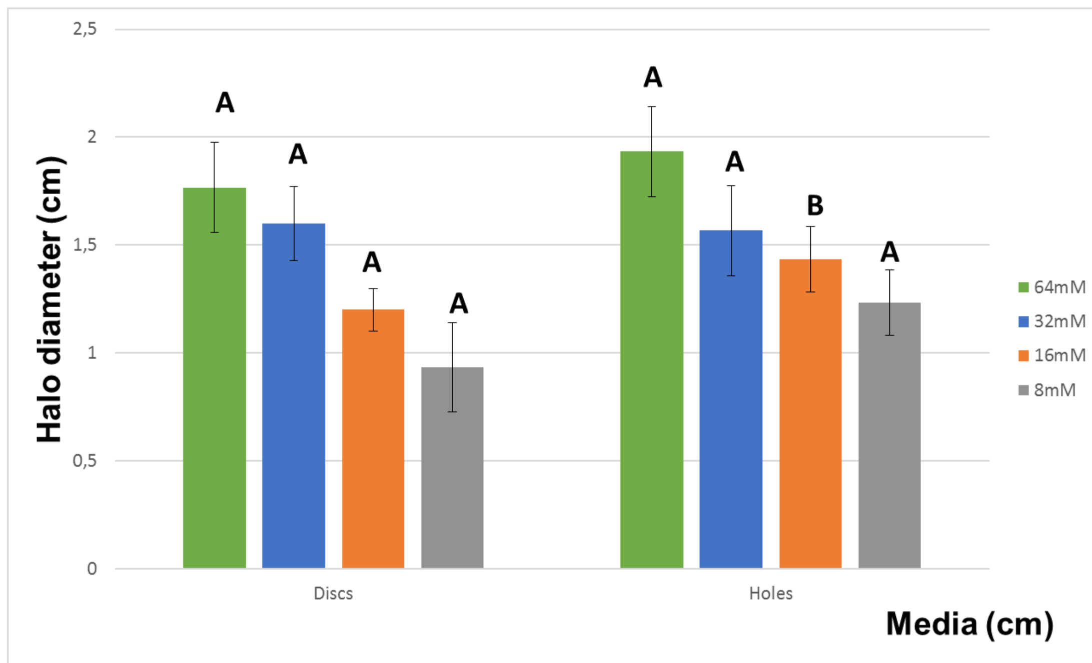

Figure 9. Inhibition halos for QA23 by disks and holes with paraquat as oxidant. The letters indicate if there are significant differences between the halos in discs and holes for the same oxidant concentration (expressed on millimolar (mM)).

As an example, we show in Figure 10 the effect of hydrogen peroxide on QA23 strain, with both methods.

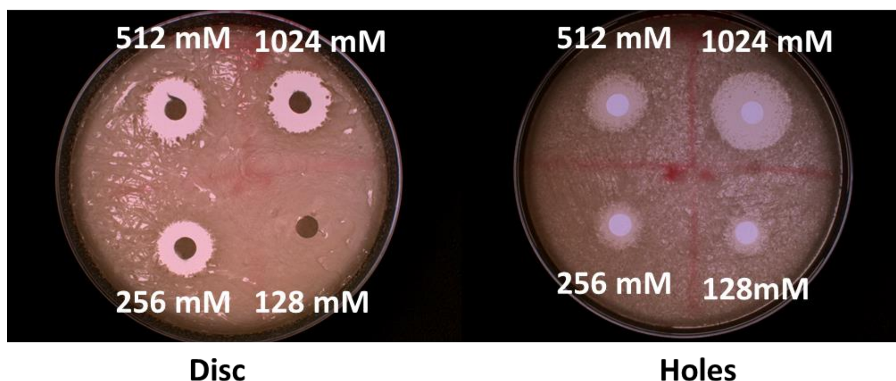

Figure 10. Inhibition halos for QA23 by disc and holes on YPD with hydrogen peroxide as oxidant.

Generally, if we evaluate the results of all oxidants we can say that for most of them the hole method shows better results. One hypothesis would be that when we put the oxidant into the hole, there is no loss by evaporation, and the diffusion of the oxidant is supposed to be better. Instead, on the disk method, we put the oxidant on the disk, and let it dry. This may cause the loss of part of the oxidant by evaporation, and also, the diffusion of the oxidant on the agar media could be limited due to the dried disk. This would explain why the halos are bigger on the hole method, and the reproducibility is higher.

3.4 Evaluation of yeast viability after H_2O_2 stress exposure in broth medium

To corroborate the effect of the H_2O_2 over yeast culture, we evaluated cell viability by growing the strain QA23 in YPD after inducing oxidative stress, with two concentrations of hydrogen peroxide (Figure 15). In this experiment, we observed the exposure to hydrogen peroxide mainly affected the lag phase, increasing with the concentration of H_2O_2. Surprisingly, a higher OD600 was obtained with the higher concentration of oxidant agent.

It has to be noticed that the concentration of oxidant agent needed to see an effect on yeast growth was different depending on the experimental conditions. As we can observe, for hydrogen peroxide, in broth media lower concentrations were enough to impair yeast growth, having an increase of the lag phase even at 2 mM (Figure 15), whereas, in agar media the concentrations to see inhibition halos should be much higher (Figure 8). Concentrations below 64 mM of H_2O_2 did not show any inhibition halo. This could be explained because of the different diffusion of the compound in the different matrix.

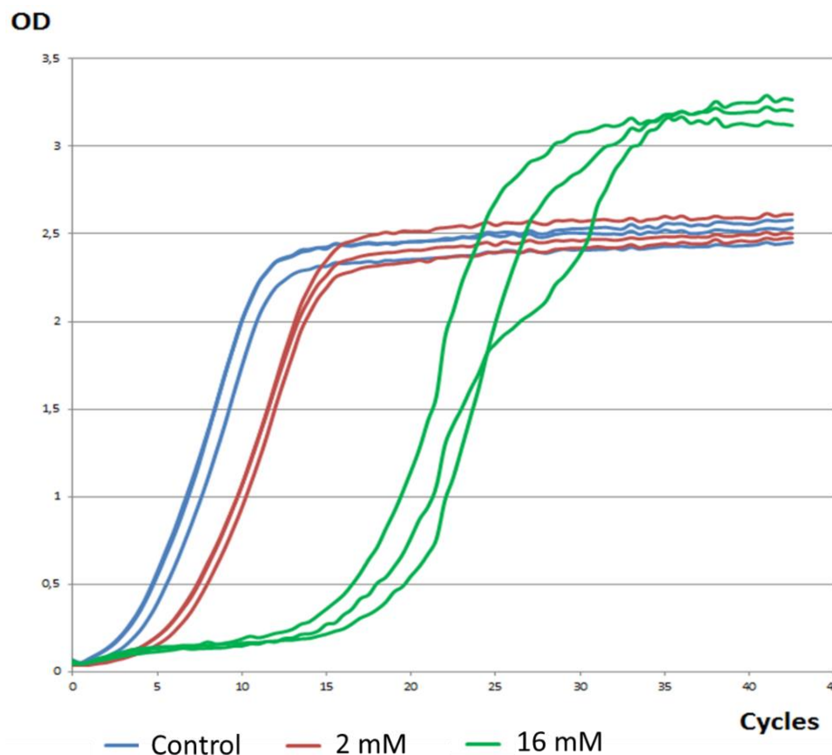

Figure 15. Growth curve of QA23 after 2 hours exposure to hydrogen peroxide. Lines with the same color represents the three replicates of the culture with the same oxidant concentration.

3.5 Melatonin effect on oxidative stress

Once we had optimized the method, we evaluated the effect that melatonin can have on oxidative stress, using different oxidant concentrations. In order to know this, we fixed the melatonin concentration at 5 μM, and different oxidant concentrations were added to the culture. The oxidants used were: plumbagin, menadione sodium bisulfate, hydrogen peroxide and paraquat. Those results are represented on figures 11, 12, 13 and 14, respectively.

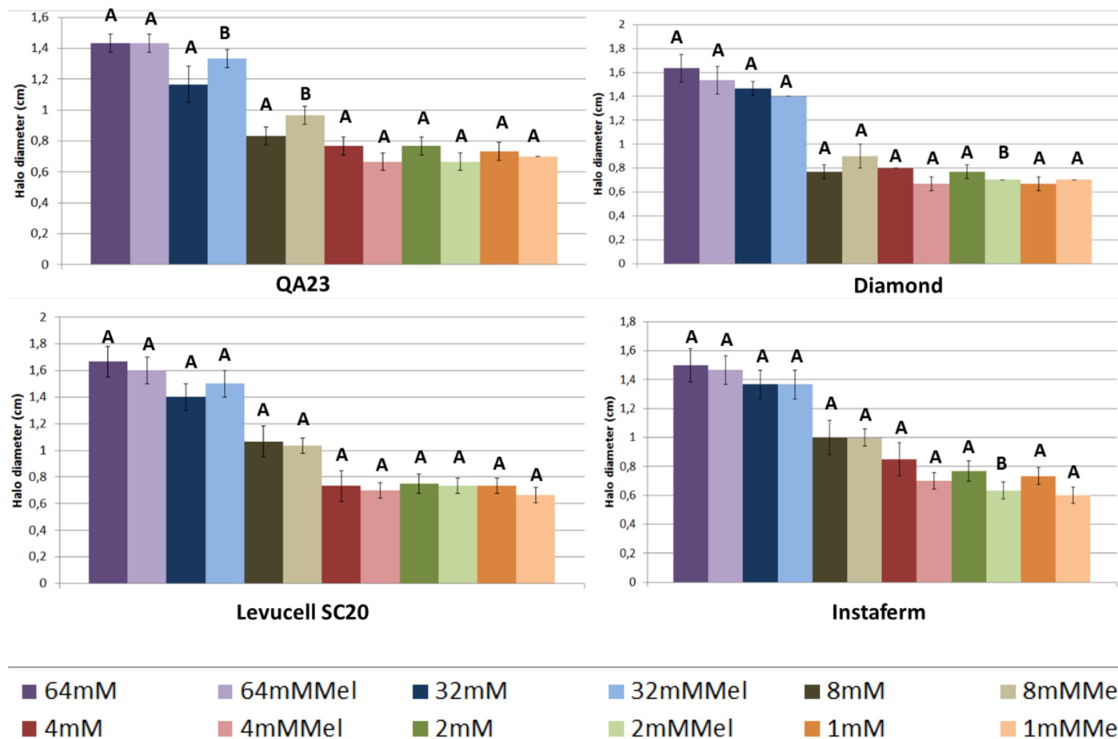

Figure 11. Inhibition halos with or without melatonin and plumbagin as oxidant. The letters indicate if there are significant differences between the halos obtained with and w/o Melatonin, for the same oxidant concentration (expressed on millimolar (mM)).

As we can see in figure 11, in general, the presence of Mel in the media does not protect the cell against plumbagin stress, as few significant differences were observed for the conditions and strains tested. QA23 only showed significant differences at 8 and 32 mM, being the inhibition halo even bigger in presence of Mel. On the other hand, Diamond and Instaferm showed onlysignificant differences at 2 mM. In those casesthe halos were slightly smaller with Mel.

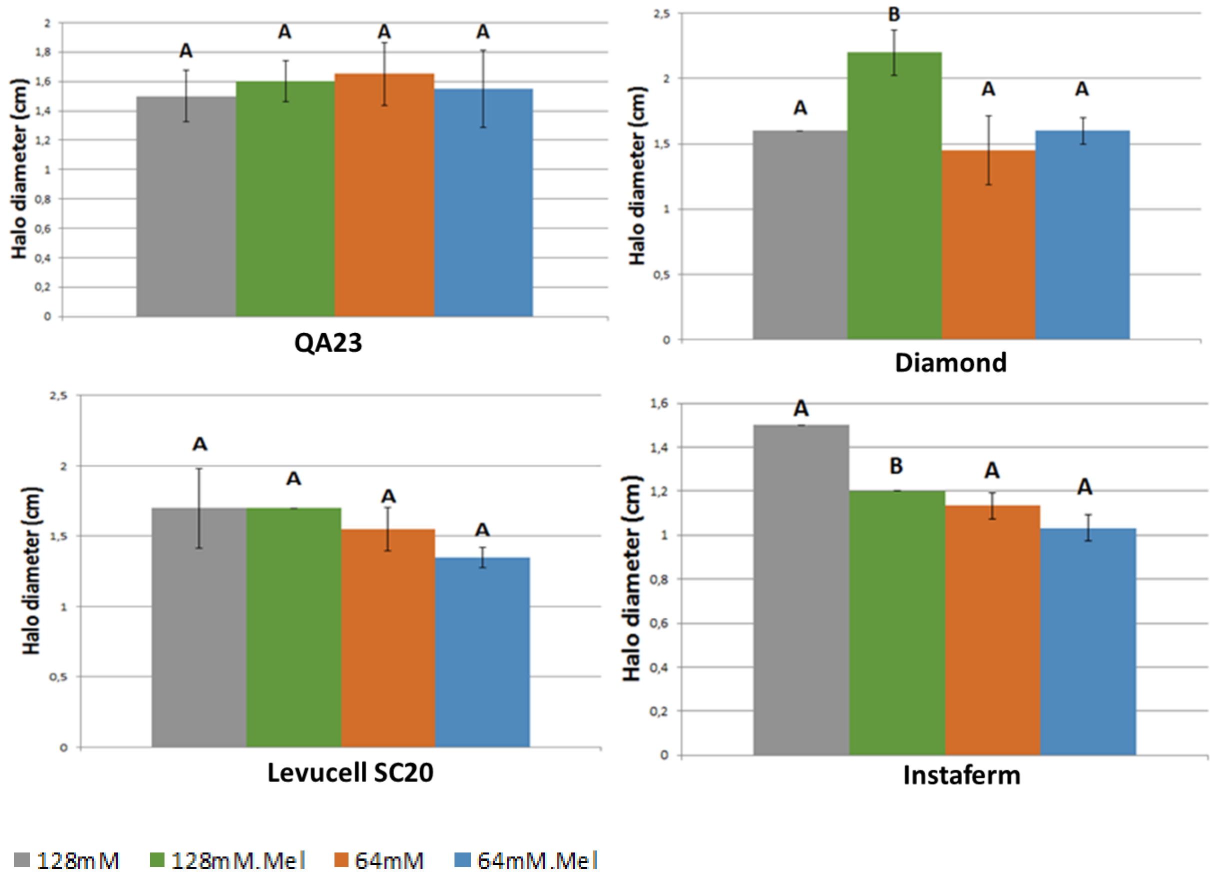

Figure 12. Inhibition halos with and without melatonin and menadione as oxidant. The letters indicate if there are significant differences between the halos obtained with and w/o Melatonin, for the same oxidant concentration (expressed on millimolar (mM)).

When using menadione as oxidant (Figure 12), again, no big differences were observed by the presence of melatonin in the media. Some protection was observed only in Instaferm strain, at 128 mM.

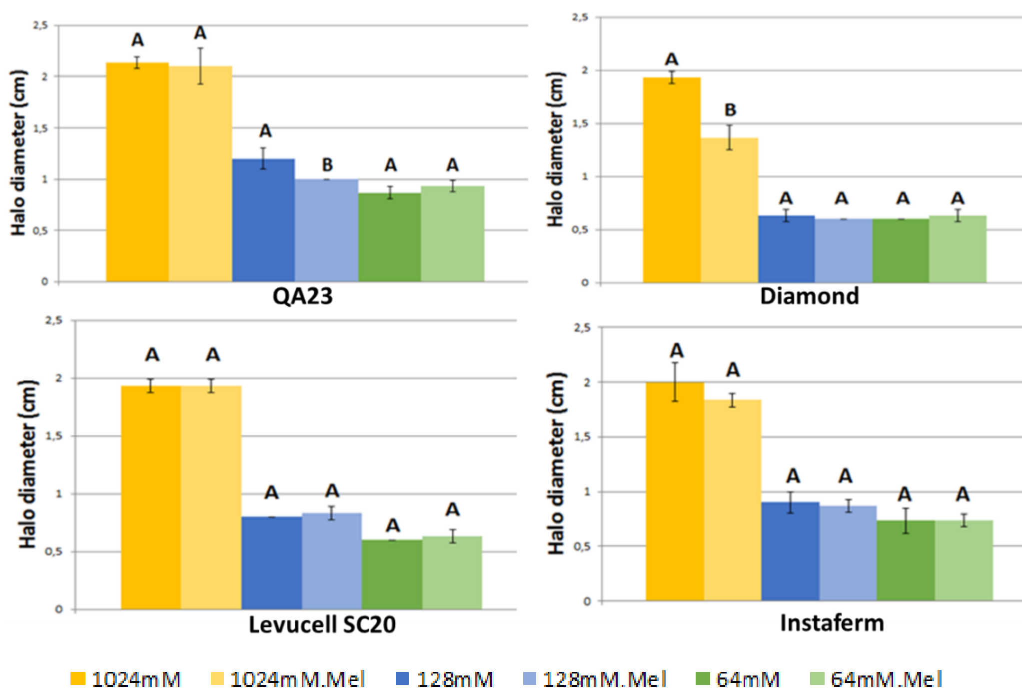

Figure 13. Inhibition halos with and without melatonin and hydrogen peroxide as oxidant. The letters indicate if there are significant differences between the halos obtained with and w/o Melatonin, for the same oxidant concentration (expressed on millimolar (mM)).

Melatonin did not present any effect neither when using hydrogen peroxide as oxidant agent (Figure 13), and significant differences were found only at 128 mM for QA23, and at 1024 mM for Diamond.

In paraquat (Figure 14), the only effect observed was with Diamond strains, but Mel seemed to even increase the stress, as the inhibition halo was bigger, contrary at what it was expected.

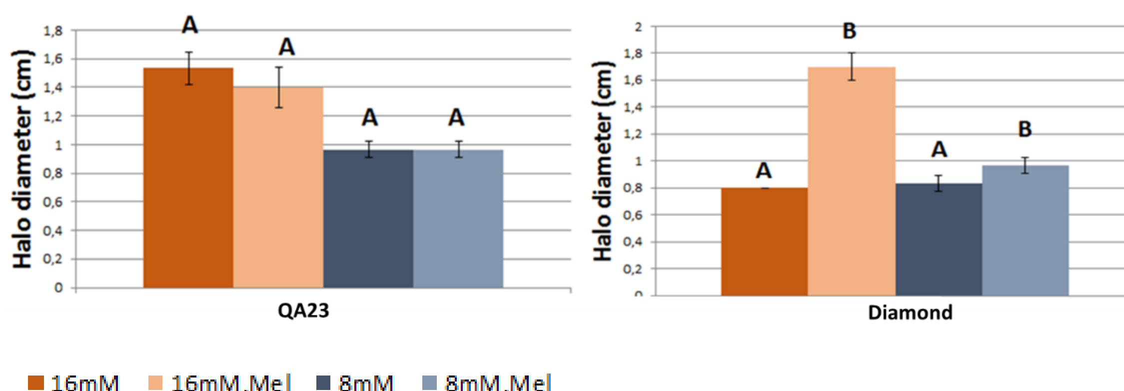

Figure 14. Inhibition halos with and without melatonin and paraquat as oxidant. The letters indicate if there are significant differences between the halos obtained with and w/o Melatonin, for the same oxidant concentration (expressed on millimolar (mM)).

The four oxidants used in this study generated inhibition halos in all the strains, which were higher with the increase of their concentrations. These results are in correspondence with previous studies, where it has been demonstrated that the presence of oxygen on yeast can produce reactive oxygen species (ROS) such as the superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH·). These ROS typically arise because of electron leakage from the electron transport chain onto dioxygen (O_2) during aerobic respiration. Failure of cell antioxidant defenses to impede ROS accumulation inevitably results in oxidative stress, a condition broadly defined as an imbalance between prooxidants and antioxidants, in favor of the former. This potentially leads to a situation where important cell biomolecules suffer severe oxidative damage, thus compromising the viability of cells[50] .

The use of antioxidants to encounter these stress conditions has been investigated before. Our results don't agree with previous research, where melatonin has been described for its antioxidant capacity, as a linchpin of the highly complex antioxidative defense system of microorganism, by a very wide number of essential molecular mechanisms. Melatonin has been designated as a refiner of physiology[51], a tranquilizing agent[52], a multitasking molecule and nature's most versatile signal[53]. A previous study in the group described that at 2 mM of H_2O_2 in a culture with melatonin, ROS presence decreased compared with a culture without melatonin, and this effect was independent of melatonin concentration, which was essayed at 5, 25 and 50 µM[46].

However, our results do not show melatonin effect, probably because the assays were made on solid-agar media, and thus, the concentration of oxidant needed to observe an effect on the growth of the strains was much higher than the one used in previous studies in broth media, due to the lower diffusion of the compounds. In this study, we used high concentrations of the oxidant but only a fixed melatonin concentration (5 μM), and perhaps this low concentration of Mel was not enough to encounter the high oxidant content. Thus, the melatonin concentration should also be increased to protect cells against these higher concentrations of oxidants. Therefore, further studies increasing melatonin concentration would need to be done to verify its antioxidant capacity.

4. SUMMARY AND CONCLUSIONS

In this study, we wanted to optimize a methodology to determine the effect of oxidative stress on yeast growth, and then, to evaluate the possible antioxidant role of melatonin in those conditions. Thus, it was required that all media and methodologies were optimized for this purpose.
The first step was to optimize the medium to analyze the growth inhibition due to oxidative stress, using different oxidant agents. We started comparing the inhibition growth in a respiratory (YNB-Gly) and fermentative (YNB-Glu) medium. Our results showed that even if the halos were slightly bigger in YNB-Gly, YNB-Glu medium allowed obtaining more symmetric halos than YNB-Gly. Then, we compared the inhibition growth by the oxidant agents in a nutrient limited medium (YNB-Glu) and a rich medium (YPD). Although we expected that in a nutrient limited medium the cells would be more prepared to face the oxidative stress, our results showed similar growth inhibition in YNB than in YPD. Thus, we had chosen to work with a rich medium, because of a better growth of the yeast strains.
Then, we tested two ways to apply the oxidant agent on the solid-agar plate: using paper disk soaked with the oxidant, or applying this oxidant directly in the plate, inside a hole. The hole method gave better and more reproducible results.
It is important to highlight that the concentration of oxidant agent needed to see an effect on yeast growth on agar plate was much higher than the concentration needed in liquid medium.
Finally, the antioxidant effect of melatonin was not evidenced in those conditions, due to the lower concentration tested. Further studies with higher concentrations of melatonin should be tested to prove its antioxidant role against the different agents.

Thus we propose to continue this study, first, increasing the concentration of melatonin used in the hole method (soli-agar media), to verify its antioxidant capacity, second, testing in broth media other oxidants than H_2O_2, and determine the concentration of oxidant needed to impair cell growth in this conditions, and then test the effect of melatonin also in these conditions.

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