Effect of Light and p-Coumaric Acid on the Growth and Expression of Genes Related to Oxidative Stress in Brettanomyces bruxellensis LAMAP2480

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**OPEN ACCESS**

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Specialty section:
This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 26 July 2021
Accepted: 01 November 2021
Published: 25 November 2021

Citation:
Catrileo D, Moreira S, Ganga MA and Godoy L (2021) Effect of Light and p-Coumaric Acid on the Growth and Expression of Genes Related to Oxidative Stress in Brettanomyces bruxellensis LAMAP2480. Front. Microbiol. 12:747868. doi: 10.3389/fmicb.2021.747868

**INTRODUCTION**

Brettanomyces bruxellensis is considered the most significant contaminant yeast in the wine industry since it causes a deterioration in the organoleptic properties of the wine and significant economic losses. This deterioration is due to the production of volatile phenols from hydroxycinnamic acids. These compounds possess antimicrobial properties; however, B. bruxellensis can resist this effect because it metabolizes them into less toxic ones. Recent studies have reported that B. bruxellensis grows under different stress conditions, including p-coumaric acid (pCA) but effective methods for its control have not been found yet. Since that in other yeasts, such as Saccharomyces cerevisiae, it has been described that light affects its growth, and we evaluated whether the light would have a similar effect on B. bruxellensis. The results show that at light intensities of 2,500 and 4,000 lux in the absence of pCA, B. bruxellensis LAMAP2480 does not grow in the culture medium; however, when the medium contains this acid, the yeast adapts to both factors of stress managing to grow. The expression of genes related to oxidative stress in B. bruxellensis LAMAP2480, such as SOD1, GCN4, and ESBP6, showed a higher relative expression when the yeast was exposed to 2,500 lux compared to 4,000 lux, agreeing with the growth curves. This suggests that a higher expression of the genes studied would be related to stress-protective effects by pCA.

**Keywords:** B. bruxellensis, light intensity, oxidative stress, ROS, p-coumaric acid
2007) and causing rejection by the consumer along with significant economic losses for the industry (Loureiro, 2003; Suárez et al., 2007; Oelofse et al., 2008; Wedral et al., 2010). Godoy et al. (2016) reported a comparative analysis of the transcriptome and genome profile of the strain B. bruxellensis LAMAP2480 grown in the presence of pCA, and an early resistance mechanism to this acid was observed, causing generalized stress in the cell, and therefore inducing the expression of genes that encode proton pumps and mechanisms related to the release of toxic compounds.

On the other hand, the presence of this acid activates the expression of different genes related to this response, such as SOD1, ESBP6, GCN4, and HSP12 (Godoy et al., 2016). Similar results have been described for Saccharomyces cerevisiae, where the presence of pCA also causes an increase in reactive oxygen species (ROS) and consequently an increase in the expression of genes related to oxidative stress (Piper, 1999; de Nobel et al., 2001; Mascarenhas et al., 2008; Welker et al., 2010; Sugiyama et al., 2016). SOD1 encodes a cytosolic superoxide dismutase, which is responsible for eliminating radicals produced by the cell and toxic to it (Steinman, 1980; Jamieson, 1998). Furthermore, it has been shown that this superoxide dismutase would be part of a resistance mechanism in the presence of sorbic acid (Piper, 1999; de Nobel et al., 2001). Another gene described is ESBP6, which codes for a protein similar to a monocarboxylate permease that promotes the exit of weak acids from the cell, allowing the regulation of intracellular pH and reducing stress levels (Sugiyama et al., 2016; Pereira et al., 2020). Likewise, it has been identified that GCN4 gene, which codes for a basic leucine zipper domain (b-ZIP) transcription factor and is related to a protection mechanism, inhibits the growth of mutants lacking this gene, and shows overexpression in the presence of hydrogen peroxide (H2O2; Jamieson, 1998; Mascarenhas et al., 2008). In addition, HSP12 gene encodes a membrane protein whose function is to stabilize it under stress conditions, including oxidative stress. This protein has been shown to have a protective role when S. cerevisiae is exposed to sorbic acid (de Nobel et al., 2001). In the case of mutant strains lacking HSP12, an inhibition in their growth was observed (Welker et al., 2010).

The effect of light on microorganisms has generated great interest for further research, considering that it can result in the control of cellular functions, which could not be achieved with diffusion processes (Kusen et al., 2017). In addition, it would allow the activation of biological processes in a non-invasive way. Therefore, studies have been focusing on knowing how this exogenous factor may trigger the expression of genes involved in metabolic pathways of cellular importance (Binder et al., 2016). Thus, for example, it has been described that visible light is harmful to cells, affecting cellular respiration by destroying cytochromes (Epel and Butler, 1969; Ninnemann et al., 1970; Woodward et al., 1978; Ulaszewski et al., 1979) or by the production of ROS, which can react with biomolecules, such as lipids, nucleic acids, and proteins and inactivate their function (Toledano et al., 2003; Perrone et al., 2008). This causes oxidative stress in the cell, as does the presence of pCA (Piper, 1999).

Oxidative stress is caused by an imbalance between the cell antioxidant mechanism and ROS production (Toledano et al., 2003). ROS includes different oxidation states of dioxygen (O2), such as singlet oxygen, superoxide anion (O2•−), H2O2, and hydroxyl radical (OH•). These species are invariably produced in aerobic environments by different mechanisms, such as the “leakage” of electrons during biological oxidations, the action of flavin dehydrogenases, and the physical activation of oxygen molecules by radiation energy (Toledano et al., 2003; Bergamini et al., 2004).

It has been reported that visible light in S. cerevisiae alters its metabolism through changes in respiratory oscillation and the expression of the YAP1 gene, which codes for yeast activator protein-1 and is related to oxidative stress (Robertson et al., 2013). Under this stress, the cell has different responses depending on how severe the exposure to ROS is. At low doses, cells would adapt, becoming more resistant to a subsequent lethal dose (Jamieson, 1992). At higher doses, the cell activates antioxidant defense mechanisms at the transcriptional level, mainly through transcription factors, such as the Yap1p mentioned above, Msn2p, and Msn4p (Jamieson, 1998; Gasch et al., 2000; Moradas-Ferreira and Costa, 2000), also causing a delay in cell division (Lee et al., 1996; Chiu et al., 2011). Even higher doses can cause the death of a part of the population initially due to apoptosis and finally in extreme doses due to necrosis (Zong, 2006; Perrone et al., 2008; Farrugia and Balzan, 2012).

Considering the importance of B. bruxellensis at the industrial level, it is interesting to research how this yeast responds to two stresses present in its natural habitat: pCA and light, allowing a deeper understanding of the metabolic ways of this spoilage yeast.

MATERIALS AND METHODS

Microorganism

B. bruxellensis LAMAP2480 strain was obtained from the collection at the Laboratory of Applied Microbiology and Biotechnology of the University of Santiago de Chile. The strain was grown in SD minimal medium (2% w/v glucose and 6.7 g/l yeast nitrogen base (YNB; Difco Laboratories, Detroit, United States) and kept in the dark until use).

Growth Curves

B. bruxellensis LAMAP2480 strain was grown in 5 ml of SD minimum medium (2% w/v glucose and 6.7 g/l yeast nitrogen base (YNB; Difco Laboratories, Detroit, United States)) at 28°C until saturation (stationary phase). Then, 1 x 10⁶ cells/ml were inoculated in 200 μl of the same medium in the absence and presence of 100 mg/l of pCA (Sigma-Aldrich, Inc.; United States) in triplicate on a Cell Culture Plate (SPL Life Sciences, Korea). The microplates were incubated at 28°C in the dark and at different light intensities using white fluorescent lamps at a light intensity of 2,500 lux and 4,000 lux. Varying light intensities were provided by adjusting the light with the help of Lux meter UNI-T UT 382 USB (Dongguan, China). Absorbance
measurements were made at 600 nm for 10 days in the Epoch™ equipment (BioTek, United States), coupled to the Gen5 program (BioTek, United States).

The specific growth rate was determined by the slope of the exponential growth phase according to the equation $x_t = x_0 + \mu t$, where $x_t$ and $x_0$ represent the biomass in optical density (OD) at time $t$ (h) and $t=0$, respectively (Barata et al., 2008). The lag phase was determined as described by Buchanan and Cygnarowicz (1990). All experiments were performed in triplicate.

RNA Extraction

*B. bruxellensis* LAMAP2480 strain was grown in SD minimal medium (2% w/v glucose and 6.7 g/l yeast nitrogen base (YNB; Difco Laboratories, Detroit, United States)) at 28°C until saturation. $1 \times 10^8$ cells/ml were inoculated in 100 ml of medium in the absence and presence of 100 mg/l of pCA (Sigma-Aldrich, Inc.; United States) at different light intensities (2,500, 4,000, 4,500 lux and in darkness). They were grown at 28°C until the end of lag phase (Godoy et al., 2016). Then, the culture was centrifuged at 1,370×g for 10 min. The pellet was resuspended in 200 μl of RNA Buffer (50 mm Tris–HCl pH 7.4; 100 mm NaCl; 10 mm EDTA), and 400 μl of acidic phenol and glass beads were added. Vortex 1 min, then ice 1 min, and repeat 3 min in vortex. 200 μl of RNA Buffer and 40 μl of 10% SDS were added. It was stirred for 6 min at 65°C. It was centrifuged at 16,000×g for 15 min at 4°C, and the upper phase was collected. 400 μl of acid phenol and 40 μl of 3 M sodium acetate were added, centrifuging at 16,000×g for 15 min at 4°C to finally collect the upper phase. Subsequently, 1 ml of 96% ethanol was added, and it was refrigerated for 2 h at −80°C. It was centrifuged at 16,000×g for 10 min at 4°C. Finally, the RNA Clean & Concentrator™ -5 protocol (Zymo Research, United States) was followed. RNA quantification was performed in the Epoch™ equipment (BioTek, United States). All experiments were performed in triplicate.

Quantification of Relative Expression

The RQ1 RNase-Free DNase protocol (Promega, USA) was used for the RT-PCR. q-PCR was performed in qStepOnePlus Real-Time PCR System Thermal Cycling Block equipment (Thermo Fisher Scientific, United States) coupled to StepOne Software (v2.0; Applied Biosystems, United States). All the primers used were designed to amplify fragments between 100 and 300 bp (Table 1). All reactions were performed in 20 μl according to the 5x HOT FIREpol EvaGreen qPCR Mix Plus (ROX) protocol (Solis Biodyne, Estonia). The program used was as follows: 15 min at 95°C, 35 amplification cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s.

The relative quantification of the expression of the candidate genes of *B. bruxellensis* was carried out using the mathematical method $2^{-\Delta \Delta CT}$ described by Livak and Schmittgen (2001), using actin 1 as the housekeeping gene. All experiments were performed in triplicate.

**Statistical Analysis**

The statistical analyses were carried out using ANOVA, and the mean values of the experiments were compared using the LSD test. The treatments were considered significant when the values of $p \leq 0.05$. The analyses were done using Statgraphics Plus, version 5.1 (StatPoint Technologies, United States).

### RESULTS

**Growth of B. bruxellensis in the Presence of Light and p-Coumaric Acid**

*B. bruxellensis* is the main contaminating yeast in the wine industry since it causes defects in the organoleptic properties of wine. Although SO₂ is the compound that manages to control the growth of this yeast, it has been described that this compound can cause allergy problems in consumers, hence the interest in finding new control methods. *B. bruxellensis* can grow in various environmental stress conditions, including low nitrogen, low vitamin, high SO₂ doses, low or high oxygen concentration, and high ethanol concentration. Considering this, innovative methods have been developed to reduce their contamination in wines, as are high hydrostatical pressure (HHP), pulse electric fields (PEF), ultrasound, UV light, and microwaves, among others (Pinto et al., 2020). These technologies are being studied to can be applied in the industry.

On the other hand, studies carried out in *S. cerevisiae* have shown that both the presence of hydroxycinnamic acids in the culture medium and light would affect its growth (Ulaszewski et al., 1979; Baranowski et al., 1980; Shu et al., 2009; Robertson et al., 2013). We evaluated how *B. bruxellensis* LAMAP2480 responds to these two factors (pCA and light). Growth curves were made in the absence and presence of pCA, either in darkness or exposed to 2,500 and 4,000 lux, calculating their kinetic parameters (Figure 1A; Table 2). It is possible to observe that when the yeast was grown in darkness, the duration of the lag phase was 26.25 h in the absence and in the presence of pCA (Table 2). This parameter, such as specific growth rate and generation time, did not show significant differences when comparing yeast behavior in the presence or absence of pCA.

In addition, when *B. bruxellensis* was exposed to a constant light intensity of 2,500 lux (Figure 1B; Table 2) in the presence of pCA, it was observed that the duration of the lag phase increased 3.75 times compared to control sample (darkness + pCA) and its specific growth rate increased 1.75 times.

### Table 1: List of primers used for RT-qPCR.

| Primer (5’-3’) | Sequence | Tm (°C) |
|---------------|----------|---------|
| ACT1 F        | GGT GAT GAC GCT CCA AGA | 64      |
| ACT1 R        | TTG ACC CAT ACC GAC CAT AA | 63      |
| SOD1 F        | GAG GAT AAC GAT CCA AA | 58      |
| SOD1 R        | CAA AGA ACC AGC ATC AC | 58      |
| GCN4 F        | CCA GGT GCT CCT TTC ATC | 58      |
| GCN4 R        | CTC AGT ACC TCT AGC TCT C | 58      |
| HSP12 F       | AAA CCA GCC ATC GAA AC | 60      |
| HSP12 R       | CTC AAA GAG AGG AAG ACA AG | 59      |
| ESSEP6 F      | GAC GCA TAC CTT TTA TC | 57      |
| ESSEP6 R      | GAG GAA CAA CCA AGA AG | 57      |
growth efficiency (calculated as area under the curve, considering 100% darkness + pCA) decreased by 55.8%.

When the yeast was exposed to 4,000 lux (Figure 1C; Table 2), there was an increase in the duration of the lag phase of 6.19 times compared to the sample control (darkness + pCA). The specific growth rate and generation time parameter did not show statistically significant differences with respect to the control. Additionally, the growth efficiency was 28.6%, 3.5 times lower compared to the control.

Also, by comparing the growth of the cells to 2,500 lux and 4,000 lux, it was observed that the lag phase was lower to 2,500 lux (Figures 1B,C).

Furthermore, it was observed that yeast growth was negatively affected when it was exposed to both light intensities and in the absence of pCA, where no growth was detected.

**Relative Gene Expression**

Light would induce gene expression related to oxidative stress (Toledano et al., 2003; Perrone et al., 2008). To evaluate the response of genes associated with this type of stress in B. bruxellensis LAMAP2480, different light intensities were tested. The relative expression at the end of the lag phase of some genes previously associated with oxidative stress in S. cerevisiae was quantified in B. bruxellensis in this study (Figure 2).

These results indicated that SOD1, GCN4, and ESBP6 genes were overexpressed (ER ≥ 2) at 2,500 and 4,000 lux. Particularly, the GCN4 and ESBP6 genes expression was 3.3 and 39.6 times higher respectively, compared to the 4,000 lux condition, being statistically significant.

For the HSP12 gene, no overexpression was observed in any condition.

**DISCUSSION**

The study of the growth control of B. bruxellensis is very important in winemaking, considering the need to reduce the use of SO2. This yeast has shown great resistance to different stress conditions during its growth in wine, being able to form spores (Dekkera species teleomorph) to survive. B. bruxellensis is resistant to different enological factors as high ethanol concentration, low pH, low oxygen transfer rate, and residual
ammonium scarcity (Curtin et al., 2015; Pinto et al., 2020). Hence, it is the importance of looking for new control methods to avoid contamination in winemaking.

Folch-Mallol et al. (2004) reported a mechanism called cross-protection in S. cerevisiae. This consists of adapting the microorganisms to a specific type of stress, which would allow it to resist other stress factors, although these are lethal in the absence of a previous induction. The molecular base would be to activate different stress genes involved in adaptive responses (Brown et al., 2014; Święciło, 2016). In this sense, the light stress to which B. bruxellensis LAMAP2480 was exposed in the absence of pCA affected yeast growth; however, the presence of another stressor, such as pCA, allowed B. bruxellensis LAMAP2480 to adapt to light stress, observing an increase in the expression of genes related to stress to oxidative, suggesting a possible stress-protective effect by pCA at cellular level.

Various environmental stress conditions, including high light intensities, extreme temperatures, and metals, among others, can induce oxidative stress and accelerated formation of ROS. In general, it has been described that visible light would produce ROS by endogenous photosensitizers, such as flavins and porphyrins (Molin et al., 2020). In this respect, hydroxycinnamic acids, such as pCA, and flavonoids, normally present in plants and fruits, exhibit radical scavenging ability (Scandalios, 2005; Yingbin et al., 2019).

In agreement with our results, Bayliak et al. (2016) evaluated the effect of quercetin, one flavonoid, on stress resistance of exponentially growing S. cerevisiae cell exposure to hydrogen peroxide, copper ions, and heat shock. Quercetin increased stress resistance in the yeast S. cerevisiae via antioxidants related at high concentrations providing partial protection to proteins against ROS-induced modification under heat shock and oxidative stress exposure.

There are no studies of the effect of light on the growth of B. bruxellensis, but few have been carried out on S. cerevisiae (Woodward et al., 1978; Ułaszewski et al., 1979; Edmunds, 1980; Robertson et al., 2013). The same occurs with studies at the genetic level on changes in gene expression in these yeasts when exposed to visible light.

Woodward et al. (1978) observed that at 1250 lux, the growth of S. cerevisiae is not affected; however, when increasing the intensity of the light (over 1,250 lux), the generation time of the yeast increases progressively. Studies carried out in S. cerevisiae indicate that this is due to the inhibition of the transport of sugars and amino acids. Furthermore, it was described that increasing growth temperature and light intensity also has a negative effect on yeast growth. Robertson et al. (2013) observed that S. cerevisiae expresses yeast activator protein-1 gene (YAP1) related to ROS response when exposed to visible light. This gene regulates several genes with antioxidant functions, such as thioredoxin reductase (TTR1), cytosolic thioredoxin (TRX2), and cytochrome-c peroxidase (CCP1). Yap1p would play an important role in the yeast's ability to tolerate the harmful effects of visible light. These same authors showed that in those cells with a deficiency in the ROS response, light would have a negative effect on their growth.

Like YAP1, GCN4 is part of the b-ZIP family of transcriptional factors (Rodrigues-Pousada et al., 2010) and has been shown to protect the cell under conditions of oxidative stress (Jamieson, 1998; Folch-Mallol et al., 2004; Mascarenhas et al., 2008). In S. cerevisiae, it has been reported that mutants lacking the GCN4 gene do not grow when exposed to different concentrations of H₂O₂. Our results show overexpression of GCN4 gene in both light intensities, which could be related to the antioxidant response induced by the presence of pCA in the medium.

SOD1 encodes a cytosolic copper-zinc superoxide dismutase which catalyzes the dismutation of the superoxide radical (O₂⁻) to oxygen (O₂) and H₂O₂ (Steinman, 1980). de Nobel et al. (2001) reported a comparative analysis between the proteome and transcriptome of S. cerevisiae when exposed to sorbic acid, which is a weak acid as pCA is. The results indicated that SOD1 is overexpressed in response to sorbic acid in S. cerevisiae, suggesting that it is part of the resistance mechanism to this acid. Some microorganisms can adapt quickly to a second stress type, having been previously exposed to another stress. For example, in Candida albicans, oxidative stress induces heat shock genes regulation, and S. cerevisiae has protection against oxidative stress if cells have been exposed to a mild heat shock. This protection stress would have unexpected responses to classical regulatory pathways to a specific stress due to combinatorial cross-talk (Brown et al., 2014).

Molin et al. (2020) have concluded that the protein kinase A plays a key role in yeast growth in the presence of light, which would be a circadian mechanism that is somehow conserved in yeast and mammals. These authors demonstrated that the presence of light reduces protein kinase A activity, which would be vital for cells when grown in the presence of light since mutants that were sensitive to light had a protein kinase A activity well above those of normal levels.

Sugiyama et al. (2016) reported the overexpression of ESBP6 gene in the presence of lactic acid. Through gene disruption and overexpression experiments, ESBP6 gene was shown to be involved in response to lactic acid adaptation in S. cerevisiae, although it does not appear to be involved in the transport of monocarboxylic acids. The overexpression of this gene suggests that it plays a fundamental role in the adaptation of S. cerevisiae to lactic acid. ESBP6 interacts with various proteins in response to stress (Hsp70, Hsp82, Hsp90, Ssa1, and Ssa2). These chaperones allow the refolding of denatured proteins, stabilize the interaction of proteins or transport, and degrade damaged proteins, which is crucial in adapting to weak acids since this causes an increase in intracellular pH, affecting the structure and function of the yeast proteins. In addition, Pereira et al. (2020) demonstrated that overexpression of ESBP6 gene generated greater resistance to aromatic acids, including pCA, reducing the stress level in the cell by promoting biomass yield. Our results suggest that pCA-induced overexpression of ESBP6 has a protective effect against light stress, allowing cell growth.

Cross-stress can show a low specify in defense/reparation mechanism, allowing alternatives mechanism with a similar biochemical response to assure important functions of a cell as a response to different types of stress can be translated.
into a stress adaptation highly relevant to the natural environment (Święciło, 2016).

It has been described that the incidence of light on yeast growth would depend on the initial concentration of the culture. It is possible that by increasing the cell biomass, there is protection of the external cells over the internal cells of the culture. The outer cells would absorb light energy, preventing the inner cells from being affected by the toxic effects of light, allowing their normal division (Molin et al., 2020). Also, we think the phase of growth is important. In the case of saturation phase, some of the principal structures to inhibit the effects of light should be actives, as are the cytochromes (Ułaszewski et al., 1979), or in this stage, the cellular wall should be totally formed, transformed into a barrier for the entry of light to the interior of the cell. Our experiments were carried out with the same initial concentration of cells, but it is an interesting point to consider for future trials.

Different environmental factors can activate the response to oxidative stress in microorganisms. Among them, we have the presence of hydroxycinnamic acids (a natural compound) and sunlight. In addition, it has been described that if a microorganism is exposed to more than one stress factor, it will acquire an overprotective quality. That is, the cell could trigger more than one defense response in such a way as to allow it to adapt to grow in the presence of various stressors. To our knowledge, our study is the first report on the effect of light on B. bruxellensis growth.

Our results showed that, in the case of B. bruxellensis, when exposed to a concentration of 100 mg/l of pCA and in the absence of light, the duration of the lag phase slightly increased, since after adapting to the culture medium, its growth reached similar optical density values as the control sample (absence of pCA). It was also observed that when yeast was exposed to pCA and light (2,500 and 4,000 lux), the duration of the lag phase was statistically longer compared to when the yeast is grown with pCA in the absence of light. This suggests that the response of B. bruxellensis LAMAP2480 would be related to cross-protection when exposed to both stressors. However, other effects of pCA and light on the cells should not be discarded.

Furthermore, expression levels of the genes indicated overexpression of the SOD1, GCN4, and ESBP6 genes for both light intensities in the presence of pCA, suggesting that the presence of pCA stimulates an antioxidant response allowing the growth of B. bruxellensis exposed at 2,500 and 4,000 lux. Although the effect of light causes an increase in the duration of lag phase, B. bruxellensis LAMAP2480 can adapt and, despite having a decrease in growth efficiency, can grow to a high concentration of cells.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

**AUTHOR CONTRIBUTIONS**

LG, DC, and MG: conceptualization and writing – preparation of original draft, review, and editing. LG and DC: formal analysis and methodology. MG: funding acquisition. MS: supervision. All authors have read and agreed to the published version of the manuscript.

**FUNDING**

This research was funded by ANID/CONICYT FONDECYT Iniciación 11180979 and Postdoc DICYT 081871GM POSTDOC Universidad de Santiago de Chile. Millennium Nucleus for Fungal Integrative and Synthetic Biology (NC120043) and Dicyt-USACH.

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