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REVIEW ARTICLE

The importance of strong inocula in fungal cultures

La importancia de inóculos fuertes en cultivos de hongos

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ABSTRACT

Inoculum quality as starters of industrial fermentations, as well as propagules (conidia) from entomopathogenic fungi, is an important parameter that could define the success of either fermentation or biocontrol agents. Strong quality inocula have the potential to overcome some disadvantages from industrial scale bioprocesses, compared to chemical processes, which in some cases are shared with biocontrol agents compared to chemical pesticides. Features of strong inocula include the ability to accumulate compatible solutes to withstand harsh conditions such as high temperature, and also high catalase activity to deal with reactive oxygen species generated by respiration. Some of these biochemical features are determined by the age of the inoculum, which in addition to the standardization of inoculum size, reduce the variability inherent to microbial processes, and also increase product yields. In some cases, the inoculum barriers from a single strain may be overcome by the co-culture of different microorganisms. Finally, novelty methodologies are discussed to obtain strong inocula by culturing fungi and yeast under sublethal stress conditions that induce cross-protection to resist other kind of stresses.

Keywords: conidia, inocula, compatible solutes, antioxidant responses, co-culture, cross-protection

RESUMEN

La calidad de inóculos como iniciadores de fermentaciones industriales, así como los propágulos (conidios) de hongos entomopatógenos, es un parámetro importante que puede definir el éxito de la fermentación o los agentes de biocontrol. Los inóculos con buena calidad tienen el potencial de superar algunas desventajas que tienen los bioprocesos de escala industrial, en comparación con los procesos químicos, que en muchos casos son compartidas con los agentes de control biológico cuando se comparan con pesticidas químicos. Las características de los inóculos fuertes incluyen la capacidad de acumular solutos compatibles para enfrentar condiciones difíciles como temperaturas altas, y una alta actividad catalasa para lidiar con especies reactivas de oxígeno generadas por la respiración. Algunas de estas características pueden ser determinadas por la edad del inóculo, que sumado a la estandarización del tamaño de inóculo reducen la variabilidad inherente a los procesos basados en microorganismos, y también incrementan el rendimiento de productos. En algunos casos las limitantes del inóculo de una sola cepa

pueden ser superadas mediante el co-cultivo de diferentes microorganismos. Finalmente, se discuten metodologías novedosas para obtener inóculos fuertes al cultivar hongos y levaduras en condiciones de estrés subletal que llevan a la inducción de protección cruzada.

Palabras clave: conidios, inóculos, solutos compatibles, respuestas antioxidantes, co-cultivo, protección cruzada.

1. INTRODUCTION

Bioprocesses are summited to an extensive monitoring and control and in some cases these assessments are difficult to achieve (Kreyenschulte *et al*., 2015). Monitoring and control are important since there might be high and undesirable variability when using microorganisms. Besides variability, the time required to obtain a product (biomass, enzymes or secondary metabolites), the possibility of losses by contamination and low yields of products, makes fermentation processes disadvantageous compared to the chemical processes counterparts (Rich *et al*., 2015). These disadvantages are frequently overcome by the constant improvement of microbial isolates, substrates and fermentation conditions. However, monitoring and improvements of bioprocesses are carried out when bioreactors or cultures already began, leaving out of evaluation the initial phase involving inocula quality.

The inoculum of the media culture triggers the fermentation process, and the quality of the inoculum is an important parameter that is frequently left apart (Cunha *et al*., 2002). A strong quality inoculum could be defined by a series of treats, including a fast germination and invasive growth over the substrate, decreasing the possibilities of contamination, in addition to enhanced tolerance to various stress conditions such as temperature and reactive oxygen species. Thus, a strong inoculum has the potential to avoid problems such as lack of reproducibility, long time fermentations, the growth of contaminating microorganisms; inoculum evaluation is particularly important when used as propagules for the production of fungal conidia for biological control (Jenkins & Grzywacz 2000). This review focuses mainly on fungal conidia, yeast, and edible mushroom spawns, and summarizes some of the biochemical (compatible solutes and antioxidant responses) and microbiological features (size, age and co-cultures vs monocultures) in strong inocula, as well as methodologies to obtain those propagules (sublethal stress conditions), and the effect in final products formation. Each culture should start with the preparation of strong inocula from a storage vial if possible, since inocula form continuous culturing of a fungal strain result in degeneration rendering variation in the culture (Ryan *et al*., 2002). Some features of strong fungal inocula are valid also for bacterial cultures.

2. COMPATIBLE SOLUTES

Small molecules as polyols and trehalose accumulate within the cell subjected to stressful conditions, and are frequently referred as compatible solutes or osmolytes (Jain & Roy 2009). Polyols include mannitol, glycerol, erythritol and arabitol, whereas trehalose is a disaccharide. These compounds have abundant hydroxyl groups that act as stabilizers of proteins by forming hydrogen bonds with proteins and other biomolecules (Sampedro &

Uribe 2004; Rangel, 2011). Water maintain protein structures by forming hydrogen bonds, however during harsh conditions when water activity can diminish abruptly, a stronger stabilization is needed, then polyols and trehalose can replace water molecules by interaction through hydroxyl groups which in turn protect proteins against denaturation (Liu & Brady 1996; Rangel, 2011). A similar mechanism is proposed for the protection of membranes by trehalose bonding to phospholipids head, which preserves membrane integrity under dryness conditions (Crowe *et al*., 1984).

The content of compatible solutes diminishes as culture ages, and conidia from some fungi frequently used as inoculum, tend to germinate slower if they are harvested from old cultures. In this sense, conidia mutants of *Aspergillus nidulans* lacking from trehalose-6-phosphate synthase, an enzyme of the biosynthetic pathway of trehalose, germinate slower than the wild type conidia (Fillinger *et al*., 2001). In the edible mushroom *Agaricus bisporus*, the storage under room temperature for five months results in a complete loss of basidiospore germination, along with a 10-fold decrease of trehalose content, in contrast to the storage at 2°C which increased conidia germination with a minimal decrease of trehalose of 1.5-fold (Tereshina *et al*., 2007). Furthermore, other reports correlated the trehalose and mannitol levels with the germination capacity of basidiospores of *A. bisporus* (Feofilova *et al*., 2004). Conidia germination in the entomopathogenic fungus *Metarhizium anisopliae* involves the increase of oxygen consumption and possibly the accumulation of reactive oxygen species (ROS) (Braga *et al*., 1999; Morales-Hernandez *et al*., 2010), even if antioxidant enzymes are produced, in addition trehalose also has direct scavenging activity towards ROS (Benaroudj *et al*., 2001; Luo *et al*., 2008). Rapid germination allows short lag stages, diminishes the necessary time of culture and avoids the growth of other microorganisms. In biological control germination of propagules is an important quality parameter of conidia, since is often associated with storage and formulation stability (Batta, 2007), and higher infectivity towards insect plagues (Morales-Hernandez *et al*., 2010; Miranda-Hernández *et al*., 2014).

During the field application of entomopathogenic fungi, some of the main problems include the exposure to high temperatures, the osmotic stress caused by desiccation of conidia and low water activity. Water activity (a_w) is an important parameter in submerged and solid-state fermentation, and it is inversely proportional to osmotic pressure of a culture. Depending of the bioprocess a_w can be adjusted in the initial stage of fermentation, but this parameter changes as culture evolves due to water loss from evaporation or release of protein or sugar monomers from hydrolase activities, also the production of water molecules by metabolic activity affects a_w in the cultures (Nagel *et al*., 2001). In other cases the modification of a_w is infeasible or difficult to achieve, therefore the selection of strains with tolerance to low a_w is desirable. For fungal entomopathogenic propagules, osmotic tolerance is a critic quality parameter during application, since conidia encounter desiccation in open fields and a low a_w in the insect cuticle. Kope *et al*. (2008) studied the germination of 17 isolates of *Lecanicillium* spp. under different water activities. At a_w value of 0.975, 16 of 17 isolates germinated, but at 0.85 a_w only 8 isolates were able to germinate. The accumulation of compatible solutes deals with these challenges by protecting proteins and by maintaining water within the cell. For these reasons, modification and quantification of compatible solutes is important to achieve strong inocula. To ensure high compatible

solutes in inocula, it is important to evaluate the age of inocula (Hallsworth & Magan 1996).

3. ANTIOXIDANT RESPONSES: CATALASES

The aerobic respiration uses oxygen to obtain ATP and many microorganisms are strictly forced to use oxygen to sustain life. However, reactive oxygen species (ROS) are generated as byproducts for the most part in mitochondria when aerobic respiration occurs. Main ROS produced are hydrogen peroxide (H_2O_2) and ion superoxide (O_2^-), which are not very reactive, however those compounds may act as signals for cell differentiation and as defense mechanism in fungi and other organisms (Hansberg *et al*., 1993, Egan *et al*., 2007). The risk of H_2O_2 accumulation comes from the reaction with iron and other metals inside cells; this leads to the formation of the extremely reactive hydroxide radical (HO·) and to a lesser extend to singlet oxygen ($^1O_2$). The HO· reacts and damages proteins, lipids and DNA. Since O_2^- is dismuted to H_2O_2 by superoxide dismutase (SOD), O_2^- accumulation is equally dangerous as H_2O_2 (Hansberg, 2002). Fungi, like many organisms, activate many antioxidant responses, including: a) enzymes as catalases, SOD and peroxidases; and b) non-enzymatic antioxidants as ascorbic acid, melanin, and carotenoids. In this review, attention will be focused in catalases as antioxidant response.

Catalases are a group of enzymes that catalyzes the H_2O_2 dismutation to water and diatomic oxygen. Catalases are involved in the resistance to adverse conditions as high temperatures, but also associated to high infectivity in entomopathogenic fungi; the resistance to harsh conditions, as well as increase in germination rate, has been related to the presence of catalases within fungal conidia which represent an advantage when used as inoculants (Morales-Hernandez *et al*., 2010; Miranda-Hernández *et al*., 2014).

High temperature stress is especially important in biocontrol with entomopathogenic fungi, since conidia are exposed to high temperatures and other stress conditions when used in field as mentioned above. Furthermore some insects raise body temperature or expose to heat sources in order to halt fungal infection (Kalsbeek *et al*., 2001; Anderson *et al*., 2013). In *Beauveria bassiana*, mutants lacking from *catA* gene encoding a catalase, lost up to 40% thermotolerance compared to wild type strain. This gene codes for a spore specific catalase, but similar thermotolerance reductions were observed in other knockout mutants for catalase genes (Wang *et al*., 2013).

As mention previously, conidia germination triggers oxygen consumption and consequently the production of ROS. Morales-Hernandez *et al*. (2010) proposed the overexpression of the catalase *cat1* gene in *M. anisopliae* to improve germination speed; in fact overexpressing mutant strain could easily remove H_2O_2 and germinate faster than wild type strain. The former results were reinforced by a faster germination and increased catalase activity in conidia harvested from cultures growing with 26% oxygen enriched pulses compared to normal oxygen atmosphere in two *Isaria fumosorosea* strains (Miranda-Hernández *et al*., 2014).

4. INOCULUM SIZE AND AGE

In many bioprocesses, the quantity of inoculum is an important parameter that should be optimized for proper yield of products (Pham *et al*., 2010; Zahangir *et al*., 2011). For example in *Penicillium cyclopium*, inoculum size is one of the most important factors in

lipase production, and further optimization of pH and initial substrate concentration resulted in 9-fold increase compared to non-optimized design (Vanot *et al*., 2001). For solid-state fermentation of *B. bassiana* it is recommended to inoculate substrate with 10^6 conidia per gram of initial substrate, since higher inoculum size caused a decrease in conidia production, and also implies greater amounts of seed cultures (Pham *et al*., 2010). Some fungi inhibit germination when conidia are in high densities, by means of volatile or nonvolatile compounds (Chitarra *et al*., 2004). Thus optimization of inoculum size is a complex parameter in fungal cultures. In edible mushroom production, inocula or spawn are obtained from mycelial growth in cereal grains. For the milky white mushroom *Calocybe indica* a 2% spawning is recommended since higher inoculum only shows minimal improvements in mushroom yield (Krishnamoorthy & Ventkatesh 2015).

In addition to inoculum size, inoculum age should also be evaluated due to the effect on product yield and variability, and because harvesting times determine the quantity of cells present in the inoculum (Baldrian & Gabriel 2002; Jeennor *et al*., 2008; Resende *et al*., 2014). Some biochemical properties related with the age of cultures are the accumulation of compatible solutes. As mentioned before, some compatible solutes decrease as cultures age. Regardless this situation, evaluation of the effect of inoculum age in specific product formation is necessary since there are no general responses when comparing young or aged inocula. For example in the production of fructooligosaccharides (FOS) from *Penicillium expansum*, the use of 1 or 3 week-old immobilized cells as inoculum was similar for FOS production, but *β*-fructofuranosidase, a hydrolase with transfructosylation activity, reached higher levels with 3 week-old inocula (Mussatto *et al*., 2012). In the case of *C. indica* the spawn age affects the yields of fruiting bodies, since spawns older than 30 days decrease yields of fruiting bodies (Pani, 2011).

The size and age of inoculum have a combined effect in the reduction of variability. In lipase production by *Geotrichum candidum*, experimental error of the process was successfully reduced from 30 to 20% by optimizing the size and age of inoculum, this improvement was further confirmed by the reproducibility in 4 trials (Resende *et al*., 2014). In addition, lipase production was also enhanced with optimized inoculum size and age.

5. STRONG INOCULA BY CO-CULTURE SYSTEMS

In contrast to most industrial bioprocesses, natural occurring biological systems rarely consist of a single population strain (Jones *et al*., 2011). In fact many populations work together not only to ensure their survival, but also to maintain the ecosystem. From this knowledge, new biological processes have been redesigned to employ two or more different populations, known as co-culture systems. In this sense, a strong inocula could also be obtained by co-culture, in which two or more strains cooperate during the fermentation process. In fact, co-culturing is not strictly carried out with eukaryotic-eukaryotic populations, some examples of the main advantages are listed below.

The advantages of co-culturing involve the stimulation of growth in *Agaricus blazei* with beneficial bacteria which results in up to 63% increase in fresh matter yield relative to non-bacterial inoculated control. In addition the time required for the mushroom harvest was reduced up to 27 days (Young *et al*., 2012). Similar results were obtained, in the stimulated growth of *A. bisporus* with *Pseudomonas* spp. (Zarenejad *et al*., 2012). According to these results bacterial strains capable of growth in 1-octen-3-ol (a fungal self-inhibitor) enhanced

mushroom yield up to 14%, probably by the consumption and degradation of 1-octen-3-ol. Furthermore, many bacterial strains are capable of solubilize inorganic phosphate by acid production or by chelating iron compounds from compost substrates by siderophore production.

Renewability of lignocellulosic wastes is an important topic in biotechnology since the potential of the fermentable sugars is limited, due to the high content of lignin and cellulose that difficult the availability of those sugars. Some efforts have been carried out using fungal monocultures, however a single strain is insufficient since one strain may not produce all the enzymes required for lignocellulose degradation. Co-culture of fungal strains has the potential to overcome this situation by producing high yields of reducing sugars (Cui *et al*., 2015). The co-culture of *Phanerochaete chrysosporium*, *Trichoderma koningii*, *Aspergillus niger* and *Aspergillus ficuum* (consortium-29) was evaluated and compared to the monoculture of *P. chrysosporium* in the degradation of un-pretreated vinegar residue. This consortium was able to double the yield of reducing sugars compared to the monoculture control. This improvement was related to a higher xylanase and cellulase activity in the consortium-29 relative to the monoculture of the strains. The use of monocultures may not provide sufficient amount or all the enzyme set to achieve an effective degradation. In this sense *A. niger* compensates the low β-glucosidase production of *Trichoderma reesei* in corn stover (Fang *et al*., 2010).

The last advantage that will be review is the induced production of secondary metabolites by co-culture. In many fungal genomes putative genes related to secondary metabolites have been identified, however expression of these genes remain silenced (Scherlach & Hertweck 2009). Several strategies have been carried out including co-culture, since monocultures are an uncommon growth environment and in a multiple population environment, foreign molecules may act as signals for secondary metabolites production that otherwise will be silenced (Köning *et al*., 2013). The co-culture of *A. nidulans* with the actinomycete *Streptomyces hygroscopicus* leads to the induction of orsellinic acid production, lecanoric acid and two human protease inhibitors. Interestingly the necessary signal that triggers this response is the contact between bacterial and fungal mycelia, and may be a defense response since lecanoric acid is an inhibitor of ATP synthesis (Schroeckh *et al*., 2009).

Although, not every co-culture interaction improves fermentation process. In the co-culture of *Pleurotus ostreatus* and *Pleurotus citrinopileatus*, the harvested fruiting bodies were significantly higher when both strains were cultured separated relative to co-cultured strategy (Carabajal *et al*., 2012). This disadvantageous effects in co-culture were attributed to a decrease in enzyme production by the co-culture system and a dominant growth of *P. citrinopileatus* over *P. ostreatus*. Other reports using different strains of white rot fungi indicate that stimulation of lignin-degradation enzymes are species-specific, since different responses are obtained depending of the fungi used in co-culture (Chi *et al*., 2007). Cui *et al*., (2015) also reported that not every interaction of strains used in co-culture for vinegar residue degradation were positive, the use of *Trichoderma viridae* and *Candida utilis* presented an inhibitory effect in vinegar degradation when used with other strains. And finally, in the induction of silent genes in *A. nidulans* by different soil-dwelling bacterial strains, only one out of 58 strains was capable to induce secondary metabolite production (Schroeckh *et al*., 2009).

6. CULTURES EXPOSED TO SUBLETHAL STRESS PRODUCE STRONG INOCULA BY CROSS-PROTECTION

Strong inocula and propagules share biochemical and physiological features as accumulation of compatible solutes, increased antioxidant activities, fast germination, high tolerance to temperature and osmotic stress, or high infectivity for conidia form entomopathogenic fungi. Conditions to achieve such features are interesting to determine because the practical implications for fermentation processes and biocontrol, even for the initial stages regarding strong inocula. Frequently, the exposure of a microorganism to a sublethal stress generates protection to that stress condition, but also to a different kind of stress. This condition is known as cross-protection, and involves interactions with stress response elements (STRE) in key genes (Rangel, 2011; Dhar *et al*., 2013). STRE are non-coding DNA sequences (*cis*-regulatory element), with a consensus sequence 5´-AGGGG-3´, located within a gene promoter of several genes involved in DNA damage repair (Kobayashi & McEntee 1993), catalases and SOD enzymes (Kandor *et al*., 2004; Hong *et al*., 2013), trehalose and polyols synthesis (Parrou *et al*., 1999; Kobayashi *et al*., 2013).

The practical implication of cross-protection is due to the recovery of strong inocula, resistant to many stress conditions, if harvested from one particularly kind of sublethal stress. Some of the stress conditions to which microorganisms can be submitted are: thermal and osmotic stress, nitrogen and carbon starvation, oxidative stress. Some of the conferred stress protection includes resistantance to UV, heat and oxidant states (Rangel, 2011). A variation of sublethal stress conditions to which microorganisms can be subjected and the cross-protection effects are discussed below.

6.1 Thermal stress

In some circumstances, fermentation processes require high temperatures from the early stages, because some advantages such as reduction of contamination risk and the avoidance of costs in cooling systems. Xylose is a sugar present in lignocellulosic biomass that can be fermented to obtain ethanol, with the advantage that lignocellulosic waste as raw material does not compete with food supplies. Sterilization of lignocellulosic wastes is a rare process in large-scale ethanol production by fermentation; therefore there is a need for yeast strains which withstand high-temperature. Wild type *Saccharomyces cerevisiae* is unable to use xylose as carbon sources, but some strains are able to growth at 38°C. Ismail *et al*. (2013) transformed 8 industrial strains of *S. cerevisiae* to utilize xylose as carbon sources and evaluated ethanol production. Transcriptomic analysis revealed that differential profiles between the 3 highest ethanol producer strains compared to the lowest ethanol producer strain (negative control). Many differential regulated genes were related to carbohydrate metabolism and cell wall maintenance, but genes involved in heath shock, tryptophan biosynthesis (tryptophan is thought to induce ethanol tolerance), glycerol synthesis, formic acid protection and phospholipids barriers against ethanol were also up-regulated compared to the negative control. For these reasons, heat was considered as an inducer of cross-protection against ethanol. In *S. cerevisiae*, trehalose accumulation occurs after treatment with mild-shock heat. The amount of accumulated trehalose is strain dependent, and hence

determines the capacity to obtain cross-protection against other stress conditions such as oxidative stress (Herdeiro *et al*., 2006). However, it is worth mentioning that not all microorganisms show cross-protection effects when exposed to sublethal stress, since *Candida albicans* lacks the general stress response observed in *S. cerevisiae* to thermal stress (Enjalbert *et al*., 2003). In addition, in *P. ostreatus* and *Pleurotus pulmonarius* the exposure to heath shock causes apoptosis cell death and ROS accumulation, however apoptotic symptoms were stronger observed in *P. pulmonarius* (Song *et al*., 2014). For these reasons it is important to determine to what extent the level, time and type of exposure to sublethal stress will show an advantage for a particular strain and fermentation process.

Conidia from *M. anisopliae* produced under thermal stress (45-50°C) showed increased resistance to UV-B and heat shock compared to non-stressed conidia. The time when the stress is applied determined if cross-protection is achieved, since only application at 3 days of culture showed enhanced resistance to UV-B and heat shock. This time was related to a physiological moment previous to conidiogenesis completion. The level of sublethal stress should also be determined, since temperatures above 50°C not only failed to achieve cross-protection but increased the susceptibility of conidia to UV-B and heat (Rangel *et al*., 2008). This was consistent with the basidiospore germination in *A. bisporus*, since spores germination increased after treated for 20 minutes at 45°C, however heating spores more than 2 hours leads to a 4-fold loss in germination (Feofilova *et al*., 2004).

Thermal stress also comprises low temperatures, and this is relevant for inocula in edible mushroom production, since spawns are frequently stored around 10°C. Prolonged spawn storage at lower temperatures (4°C) results in the damage or death of *Agaricus subrufescens* (Kerrigan, 2005). However spawn storage of 6 *Agaricus* spp. strains at 10 or 15°C increased cellulase and laccase activities compared to a fresh spawn in fresh mushroom compost. Furthermore, the chemical transformation of the substrate to long chain acids (related to phospholipid membrane) were higher in low temperature-stored spawns than fresh spawn. Hence, the colonization of substrate was enhanced by storage at 10 or 15°C (Farnet *et al*., 2014). These improvements were related to a further mycelial growth in stored spawns. But as first stated, temperatures of 4°C cause lethal stress, thus 10 to 15°C could be consider sublethal stress that may cause special cross protection with an improvement of general markers of the culture process.

6.2 Nutrient starvation

Nitrogen and carbon starvation refer to minimal medium cultures with very low nitrogen (e.g. 0.67% yeast nitrogen base (Shpilka *et al*., 2015)) or carbon sources (e.g. 0.2% glucose or inositol (Rangel *et al*., 2008)). Freeze-thaw stress causes a loss of viability in *S. cerevisiae*, however, increase of freeze-thaw tolerance was observed during the depletion of nutrient media, in other words, when nitrogen and carbon starvation occur. When yeast was cultured in nitrogen and carbon limiting conditions, cells increased freeze-thaw tolerance. Other inducers of freeze-thaw tolerance in yeast are H_2O_2, cycloheximide (bacterial-produced protein inhibitor in eukaryotic organisms), in addition to heat shock and osmotic stress caused by NaCl. Interestingly, tolerance to freeze-thaw was not induced by freeze-thaw stress itself or cold shock pretreatment (Park *et al*., 1997).

When harvested from carbon starvation cultures, *M. anisopliae* conidia showed two fold increase tolerance to UV-B radiation and thermal stress compared to conidia produced in potato-dextrose agar enriched with yeast extract (PDAY). The tolerance to UV-B radiation is particularly attractive to achieve in conidia from entomopathogenic fungi since field application leads to a loss of viability caused by UV exposure. Furthermore, the strong conidia obtained in carbon starvation accumulated more trehalose and mannitol than conidia obtained in PDAY medium (Rangel *et al*., 2008). One disadvantage of this methodology is the low production of conidia, hence production of strong inocula and conidia by nutrient starvation is an unsolved challenge when using nutritional limited media.

6.3 Oxidative stress

As mention previously, ROS production in aerobic cells is an inherent process with implications in molecular damage and cell differentiation. In order to evaluate whether oxidative agents induce cross-protection, *M. anisopliae* cultures were subjected to sublethal oxidative conditions with UV-A, H_2O_2 and menadione (intracellular O_2^- generator) (Rangel *et al*., 2008). From these three agents only menadione induced cross-protection against heat, however no cross-protection was obtained towards UV-B radiation. In contrast to nutritional stress, conidia production in cultures submitted to oxidative agents were similar to that obtained in non-oxidative conditions. Hence, 1) not every oxidative agent has the capacity to induce cross-protection, and 2) nutritional stress is considered the best inducer of cross-protection since the wide protective effect towards different stress conditions.

Diatomic oxygen from atmosphere diffuses in cell trough lipid membranes, during the electron transport chain in mitochondria, a small proportion of electrons reduce diatomic oxygen to O_2^- by escaping from the protein complexes (Hansberg, 2002). For these reasons diatomic oxygen in atmosphere has the potential to act as a cross-protection inducer. In two strains of *I. fumosorosea* (ARSEF3302 and CNRCB1) 26% oxygen enriched pulses increased osmotolerance, and thermotolerance in conidia compared to the control conditions, in which the strains were cultured under normal atmosphere (continuous 21% oxygen). In addition oxidant states increased conidial germination rate, catalase activity and infectivity towards *Galleria mellonella* larvae, relative to conidia harvested from normal oxygen conditions. However conidiation showed mixed results, the strain ARSEF 3302 increased conidia production, while CNRCB1 strain decreased (Miranda-Hernandez *et al*., 2014). The threshold of oxygen concentration able to induce a sublethal stress and therefore to obtain strong inoculum or conidia is unclear, since there are no general responses to same oxygen levels. For *M anisopliae*, 26% oxygen pulses increased conidia production compared to normal atmosphere, nevertheless germination, hydrophobicity and infectivity remained similar between both treatments (Tlecuitl-Beristain *et al*., 2010). In this regard, 30% oxygen pulses and 60 h were determined as the optimal oxygen level and time application to obtain cross-protection in conidia from *M. anisopliae*, since conidia obtained under this treatment enhanced thermotolerance, and conidia production also improved in comparison to normal oxygen conditions (Garcia-Ortiz *et al*., 2015). In *B. bassiana* two oxygen levels (16% and 26% oxygen pulses) were analyzed; enhanced conidiation was observed for 16% oxygen pulses compared to both normal atmosphere and 26% oxygen enriched pulses. However germination was negatively affected when atmospheres were modified, probably by oxidative stress, since lipids were higher oxidized in treatments with

modified atmosphere. Thus, modified atmospheres were confirmed as inducers of oxidative stress (Garza-López *et al*., 2012). These are examples of the manipulation of oxidant states to obtain better quality in conidia, either as bio pesticides or as inoculum with potential as seed cultures

7. CONCLUSION

The control and monitoring of bioprocesses should include initial stages, as quality of inoculum may determine and affect the time, yields and variability of products. It is also important to determine the biochemical and microbiological characterization of a strong inoculum, in order to design new methodologies for the recovery of improved inoculants. Isolation of new strains with biochemical and physiological features of strong inocula could improve the performance in biotechnological process. In addition, submitting the strains to sublethal stress conditions is a suitable option to enhance overall bioprocesses and effectivity of entomopathogenic fungi in field application. The quality of inoculum and propagules are an excellent opportunity in research and industry for the improvement of microbial cultures.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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