PYCNIDIA AND CONIDIA QUANTIFICATION OF *LASIODIPLODIA* USING A CULTURE MEDIUM ENRICHED WITH SUGARCANE BAGASSE

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ABSTRACT

The study of *Lasiodiplodia* species has been increasing lately due to the importance of this fungus as a pathogen of plants and animals, as well as its ability to produce chemicals of human importance. For morphological and taxonomic study of this fungus *in vitro*, the growth factor is not a problem as it easily develops in different culture media. However, some impediments to a sequential and complete morphological characterization of isolates may occur, such as fructification and sporulation. Nevertheless, molecular studies for this group of fungi are still inconsistent (Mohali, et al., 2005; Rosado et al., 2016). Taking into consideration that morphological characterization of an isolate must be widely and completely done, we propose in this paper a new culture medium composed of salt and sugarcane bagasse (SC medium) that allows production, quantification and extraction of pycnidia and conidia of *Lasiodiplodia*. Assays were carried out with isolates from different sources, three of them pathogenic to coconut, cocoa and mango fruits and one endophytic isolate from Restinga. Our results show that the proposed medium was more translucent and allowed detection and quantification of all pycnidia produced by the isolates. There was a rapid production of pycnidia that were strongly adhered to sugarcane bagasse fibers after seven days of culture, suggesting that *Lasiodiplodia* uses sucrose present in the fibers as a carbohydrate source. In addition, it was observed significant differences between the means of pycnidia production, allowing to distinguish each isolate. The SC medium proposed here is a practical and efficient way to quantify pycnidia, structures that had been greatly neglected in the characterization and differentiation of *Lasiodiplodia* isolates to date.

Keywords: fungi, morphological characterization, lignocellulosic fibers

1. INTRODUCTION

Lasiodiplodia fungal species present a worldwide distribution in tropical and subtropical regions, occurring on a very wide range of environments including plants. The isolation and cultivation in laboratory is relatively easy, however, many isolates may not sporulate or poorly sporulate in common media (Saha et al., 2008).

Currently, the description and differentiation of *Lasiodiplodia* isolates have been done, mostly by molecular analysis, which is incomplete under the classical taxonomic point of view (Abdollahzadeh et al., 2010). Morphological characterization is important and has been omitted for quantification of pycnidia produced by fungal isolates during *in vitro* cultivation (Maciel et al., 2015). In culture media normally used, one of the obstacles for obtaining a numeral that closely corresponds to pycnidia produced is the presence of an abundant, dense and dark mycelial mass. This characteristic prevents the accurate visualization and counting of the formed structures, both on the surface and inside the culture medium, even if observed in a reversed Petri dish (Lima et al., 2013). The formation of stroma containing grouped pycnidia, may also hinder or obstruct their quantification. Mainly, the conidia produced *in cirri* at medium surface are the only ones extracted by washing or vacuuming. However, they do not express the correlation to the total pycnidia produced since all the fungal structures internally formed in the culture medium are excluded (Latha et al., 2013; Locatelli, et al., 2015).

The objective of this study was to obtain a culture medium able to improve viewing, extraction and quantification of all conidia produced by the fungus, generating a more confident analysis allowing comparison of species and isolates of *Lasiodiplodia*.

2. MATERIAL AND METHODS

2.1. *Lasiodiplodia* isolates

Four *Lasiodiplodia* isolates were used to carry out the *in vitro* tests. Three isolates were obtained from coconut fruit (*Cocos nucifera* L.), mango fruit (*Mangifera indica* L.) and cocoa (*Theobroma cacao* L.) presenting the post-harvest rot typical symptoms. Fruits were collected in Rio de Janeiro and Bahia states, Southeast and Northeast regions of Brazil respectively. The fourth isolate was obtained from *Capparis flexuosa* (L.) L. leaves collected at Açu restinga (Freire et al., 2015). All the isolates are preserved in the LAQUIBIO mycology collection, in test tubes containing PDA (Potato Dextrose Agar) inclined medium, at 4°C.

2.2. SC Medium

The salt-cellulose (SC) medium presents the following composition (per liter): $NaNO_3$, 2.0 g; $KHPO_4$, 0.7 g; K_2HPO_4, 0.3 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; agar-agar, 20 g and fiber fragments obtained from milled sugarcane bagasse, 50 g.

The sugarcane bagasse was cut into pieces of 2.5 cm and minced in a blender until obtaining a fine powder containing cellulose fiber fragments that were passed through a 0.2 x 0.2 mm sieve. The medium was sterilized at 121 ^oC for 15 minutes. An aliquot of 10 mL of the sterilized medium was then poured in previously sterilized Petri dishes and allowed to solidify. Pathogens were transferred to the center of each plate by placing a 0.5 cm culture disc of the pathogen, previously growing for 7 days in PDA, and sealed with Parafilm® to prevent desiccation and incubated at 28 ^oC for 15 days. Subsequently, Parafilm® was removed from each plate to allow oxygenation and all Petri dishes were kept under the same conditions for additional 15 days.

2.3. Assessment of pycnidia production and sporulation

In order to evaluate pycnidia and conidia production a disk with 1.6 cm of diameter was removed from each plate to be the representative sample for each repetition. The disk surface was washed with 1 mL of 1% Tween 80 solution and the conidia produced were collected in a 1.5 mL Eppendorf® vial.

Then, all pycnidia from each disc were extracted under a stereomicroscope, quantified and transferred into another 1.5 mL vial containing 0.1 mL of the previous disc wash solution. After extraction, pycnidia were added to 0.1 mL of the disc wash solution and macerated with the aid of a pestle. Finally, the final volume of the conidial suspension obtained was adjusted to 1 mL. An aliquot of 10 uL from this suspension was removed and placed on a slide and observed in a light microscope with a 20x objective. The number of conidia counted was adjusted to 1 mL.

The number of pycnidia on Petri dishes was calculated by extrapolating the number obtained from each 1.6 cm sample disk to 8 cm diameter, which corresponded to the whole colonies growing on the plates.

The experimental design was completely randomized, carried out with three replicates per treatment for each fungal isolate. Means were subjected to analysis of variance and compared by Tukey test.

3. RESULTS AND DISCUSSION

The lignocellulosic biomass contains cellulose, hemicelluloses and lignin as main components (Leschine, 1995; Ogeda and Petri et al., 2010). Sugarcane bagasse is one of the biggest wastes produced by Brazilian agro-industry nowadays. This biomass is basically composed of lignocellulosic fibers with high carbohydrate percentage (Du Toi et al., 1984).

SC medium provided a rapid production of *Lasiodiplodia* pycnidia and allowed the visualization of these structures on the substrate (Figure 1). Pycnidia were formed and distributed through all the culture medium in the Petri dish: on the surface, internally and in the bottom of the plate. The latter was firmly adhered to cellulose fiber fragments (Figure 2), which formed a support to fungal growth and consequent pycnidia formation.

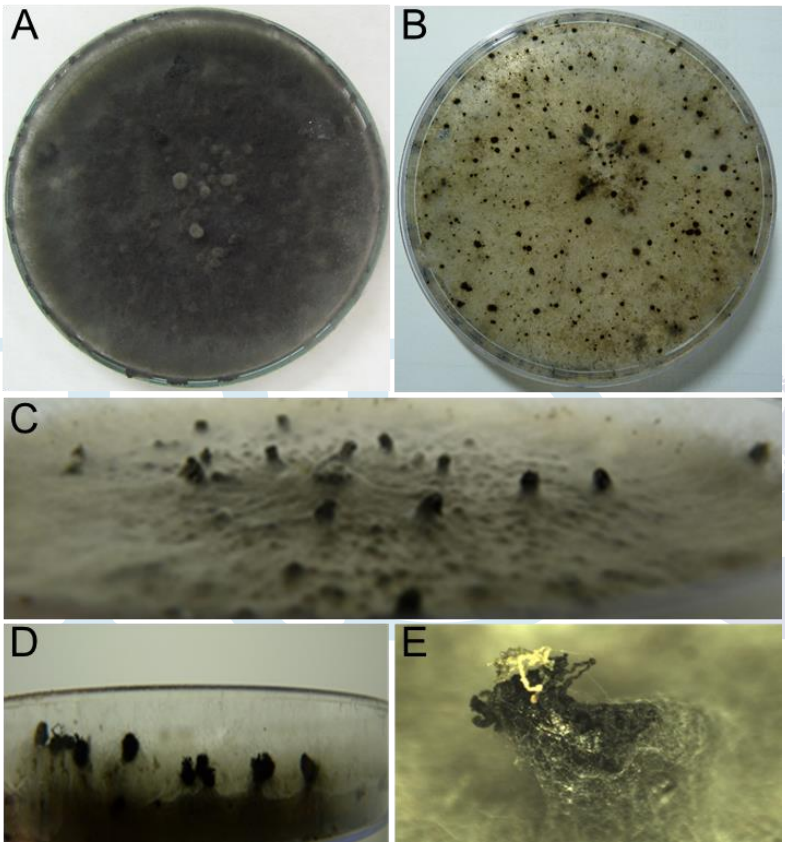

Figure 1. *Lasiodiplodia theobromae* cultivated *in vitro*. A) Colony growing on potato dextrose agar (PDA) presenting a dense and dark mycelia with non-uniformity production of pycnidia on a stroma; B) Colony growing on salt-cellulose (SC) translucent medium with low mycelia production and a uniform and individualized formation of pycnidia; C) Emergence of pycnidia on the surface of in SC medium with abundant sporulation; D) individual pycnidia on the edge of the plate containing SC medium; E) Sporulation in form of cirrus on pycnidia ostiole on the surface of SC medium.

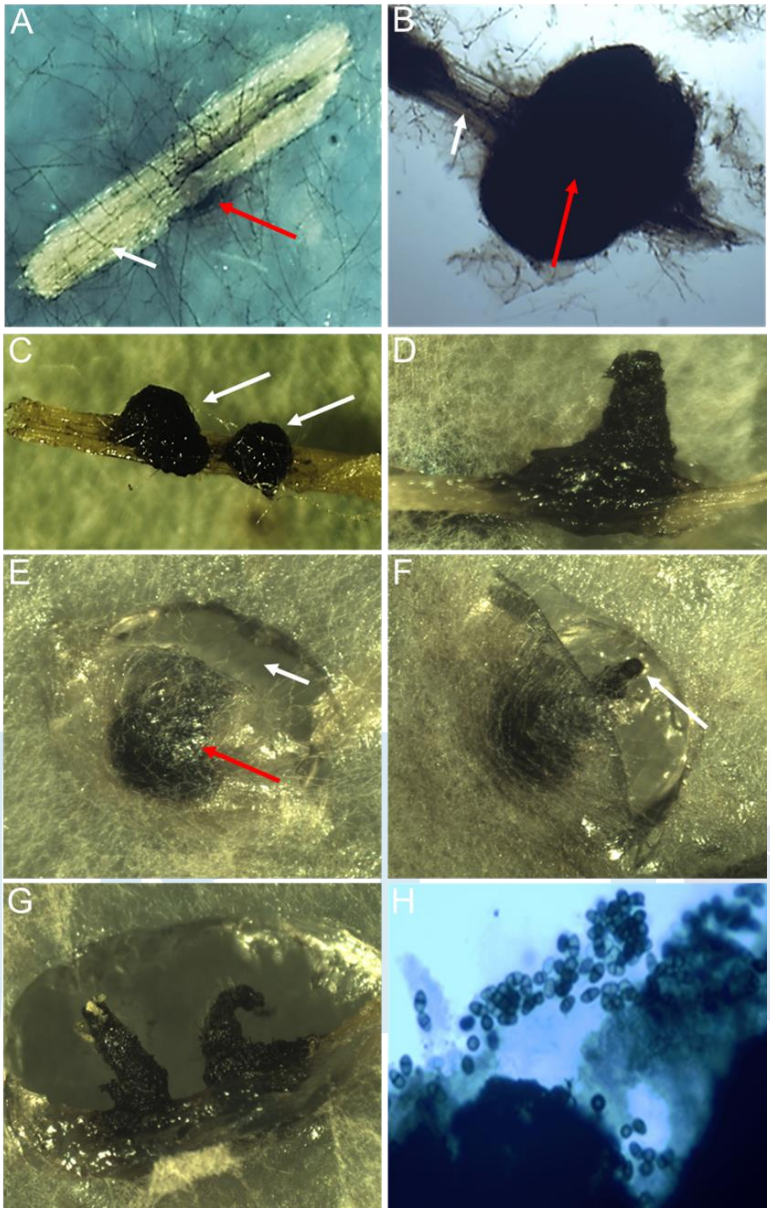

Figure 2. Colonization and sporulation of *Lasiodiplodia* on sugarcane fiber in salt-Cellulose culture medium (SC). A) hyphae penetration in sugarcane fiber (white arrow), clustered hyphae on the fiber and early formation of the fructifying body (red arrow); B) fungal colonization of sugarcane fiber (white arrow) and immature pycnidia involving fiber (red arrow); C) sequential stroma (arrows) formed on the sugarcane fiber; D) elongation of pycnidia on the sugarcane fiber; E) crack formed in the SC medium (white arrow) above the formation point of pycnidia (red arrow) after medium dehydration; F) pycnidia elongation and beginning of conidial liberation by the ostiole; G) completely formed pycnidia; H) conidia.

When compared to fungus growing on PDA medium (Figure 1A), it was observed a considerable reduction in *Lasiodiplodia* vegetative growth, generating more translucence to the medium and allowing the detection and quantification of all pycnidia formed (Figure 1B). The characteristic of SC medium of changing the growth form of the fungus can be attributed to the nutritional conditions of the medium that is poorer in carbohydrates and rich in plant substances, such as sugarcane bagasse, which induces or increases sporulation of phytopathogenic fungi (Cochrame, 1958).

Although different culture conditions such as light variations (Saha et al., 2008), temperature (Alam et al., 2001) or culture media containing pine needles (AP medium) have been indicated for fungus sporulation (Lima et al., 2013), there are no reports about the total quantification of pycnidia and conidia produced. It is believed that in AP medium these two morphological variables were restricted to acicula fragments making it difficult to count. Maciel et al., (2015) proposed a PDA medium containing pine needles where they observed pycnidia production and sporulation; however, the medium darkened in consequence of the dense mycelial growth, fact that prevented visualization and quantification of pycnidia. Even when mycelium surface was removed, the dark medium hindered the quantification of the reproductive structures. This is the reason why most studies involving *in vitro* culture of *Lasiodiplodia* suggest only the medium surface rinse to remove and quantify the conidia (Figure 1C; 1D and 1E), excluding pycnidia and conidia produced within the culture medium.

In order to morphologically describe and compare fungi isolates it should be considered both the vegetative growth and reproductive capacity, ie, the anamorphic and teleomorph phases. This can be confirmed in the researches of Maciel et al., (2015) who studied morphological characterization of *L. theobromae* transferring isolates growing on PDA to the same culture medium plus sterilized pine needles (*Pinus* sp.). Only 30 *Lasiodiplodia* sp conidia were evaluated in each isolate, considering just the length and width

measurements. Nevertheless, two important quantitative variables were neglected: total number of pycnidia and conidios generated by the different isolates.

In our study, we observed a rapid production of pycnidia that were strongly adhered to sugarcane bagasse fibers (Figure 2A and 2B) after seven days of culture on SC medium. This time interval was much smaller than that obtained by Khanzada et al, (2006) that noted the number of pycnidia after 15 days on Yeast Extract Manitol Agar (YEMA) medium. After two weeks most of the pycnidia produced on SC medium was in maturation process (Figure 2C and 2D) with some of them containing immature conidia.

In order to induce sporulation in our assays, it was necessary to improve oxygenation in the Petri dishes by removing the Parafilm® used to seal them. These conditions were maintained for more seven days, when it was observed a slight dehydration of the SC medium. This fact, apparently, exerted a pressure on the pycnidia produced internally and consequently caused some cracks in the culture medium at points where they were enwrapped (Figure 2E). Medium dehydration and cracks enabled the maturation and liberation of conidia through the pycnidia ostiole (Figure 2F and 2G). Although the detection of *Lasiodiplodia* conidia had been observed since the second week of cultivation, we decided to evaluate the plates on the thirtieth day of incubation as this was the maximum limit to prevent a complete medium dehydration (Figure 2H).

The fungus action mechanism suggested by our results proposes that the colonization process begins with the penetration of *Lasiodiplodia* hyphae in plant cells of sugarcane bagasse, followed by biodegradation of lignocellulosic materials (Figure 2A). A large variety of extracellular metabolites are generated altering the plant cell wall (Esposito Azevedo, 2010) and then fungus begins to use compounds produced in the substrate for its development (Katayama et al., 2014). In addition, it can be inferred that the fungus utilizes sucrose present in the fibers as a carbohydrate source (Sun and Cheng, 2002), since sucrose, carboxy methyl

cellulose (CMC) and glucose induced the highest *L. theobromae* mycelial growth and pycnidia production (Latha et al., 2013). Lignocellulosic fungi disrupt the plant cell wall, promoting the conversion of polysaccharides into easily assimilated sugars (Villas-Bôas et al., 2002).

Several studies show that enzymes are too large to penetrate the lignified cell wall (Yang and Wyman, 2008). Thus, some theories associate the biodegradation process to an initial step of disruption of lignified cell wall structure induced by the action of the low molar mass compounds, which will indeed penetrate cell wall, and act directly on macromolecular components. After this initial disruption step the pathways of oxidative and hydrolytic enzymes are initiated (Goodell et al., 1997). These extracellular metabolites may either act directly on the plant cell wall or operate as mediators for oxidative enzymes (Aguiar and Ferraz, 2011).

Studies on new microbial sources for the lignocellulolytic enzymes production are essential to reduce the cost of enzymatic complexes that act in the sugarcane bagasse degradation to obtain a second generation ethanol or to produce animal food (Santos et al., 2011). Although most commercial cellulases have been produced by *Trichoderma sp.* Pers. and *Aspergillus* P. Micheli, Faheina Junior (2012) evaluated production of cellulases by filamentous fungi with better cellulolytic potential, using a culture medium composed of salts, same one used in our study, plus microcrystalline cellulose instead of sugarcane bagasse. Their results showed that in submerged fermentation the best CMCase activity was obtained by *L. theobromae.* Katayama et al., (2014) also investigated this fungus aiming to propose a supplement to the *L. theobromae* enzymatic cocktail in order to make it more efficient to pretreated sugarcane bagasse hydrolysis.

These data reaffirm that bagasse fibers serve not only to support the growth of fungi studied in this work (Figure 2A, B, C, D and G), but also to provide energy to colonization

and development of their structures. Therefore, we strongly suggest the use of SC medium for *in vitro* cultivation of *Lasiodiplodia*.

The mean percentage of pycnidia and conidia produced by each Petri dish was evaluated by analysis of variance and significant means for treatments were compared by Tukey test at 5% probability. All isolates tested in this work produced pycnidia on SC medium, which were more extensively quantified, suggesting the effectiveness of the medium tested. There are no reports to date on the use of this variable in morphological or cultural comparison of *Lasiodiplodia*.

We observed significant differences between the means of pycinidia production allowing to differentiate them into three groups. The cocoa isolate produced the highest number of pycnidia followed by the mango isolate. It was not observed differences between the coconut isolate and the endophytic one which produced the lowest number of these structures (Figure 3).

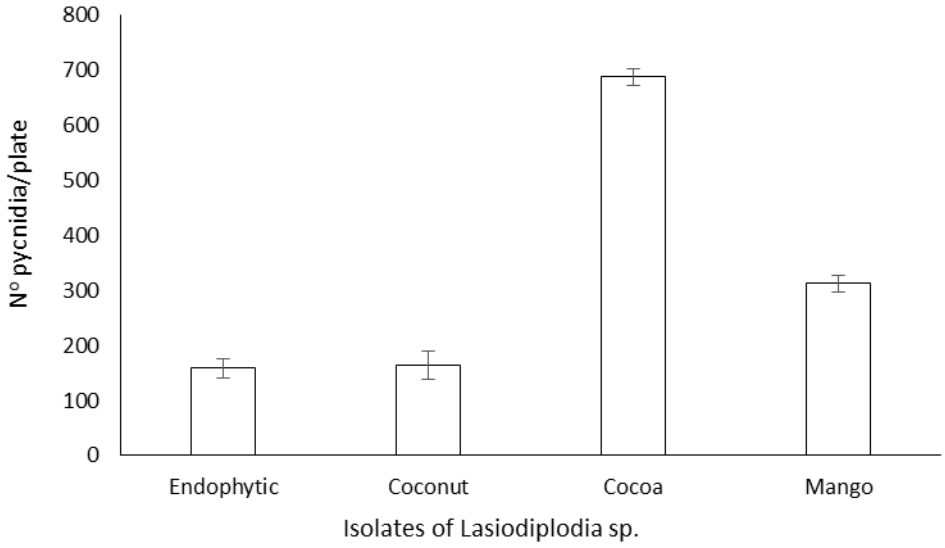

Figure 3. Pycnidia production by four *Lasiodiplodia sp.* isolates growing on salt-cellulose (SC) medium. Means were compared by the significance level based on Tukey's test ($p > 0.05$).

The evaluation of sporulation from all *Lasiodiplodia* isolates obtained on SC medium allowed us to separate them into two distinct groups, where mango and cocoa isolates produced a higher amount of conidia than the endophytic and coconut ones (Figure 4).

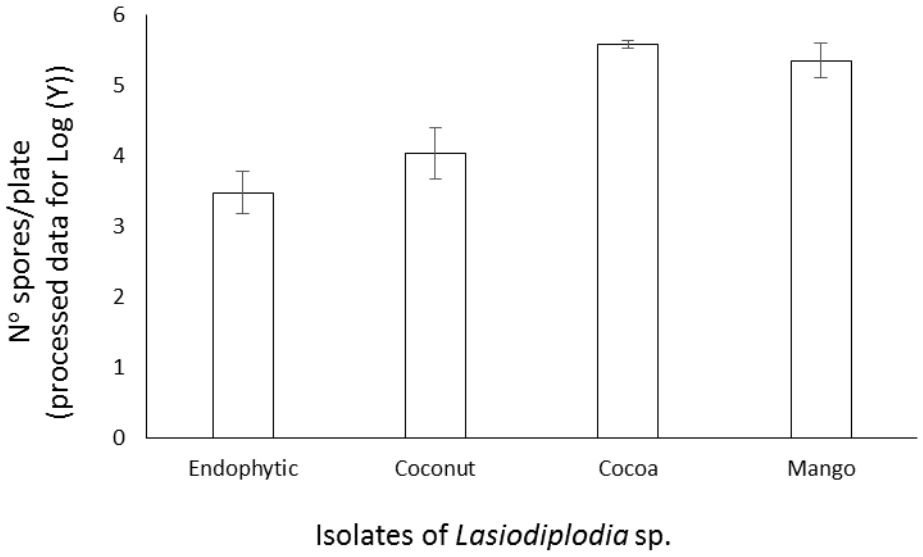

Figure 4. Conidia production by four *Lasiodiplodia sp.* isolates growing on salt-cellulose (SC) medium. The Y values were transformed to log (Y) and means were compared by the significance level based on the Tukey's test ($p > 0.05$).

Production of conidia and their viability may change according to various factors to which the colony of fungus is submitted (Freire et al., 2013). We determined variability in this study using SC medium, which allowed us to observe the highest positive correlation between pycnidia production and sporulation for cocoa and coconut isolates (data not shown). Correlation for mango isolate was also high however negative. It was not observed a significant correlation for the endophytic isolate.

It was observed differences in pycnidia formation for each isolate, both in shape as well in size. This characteristic may have contributed to the absence of correlation between

the two variables for the endophytic isolate and for the negative correlation for the mango isolate, since in small pycnidia conidial production can be reduced when compared to large ones.

Regardless of the correlation and variation in production of *Lasiodiplodia* reproductive structures on culture medium, the SC medium proposed here enabled a practical and efficient way to quantify pycnidia (Figure 5), structures that had been greatly neglected in the characterization and differentiation of *Lasiodiplodia* isolates.

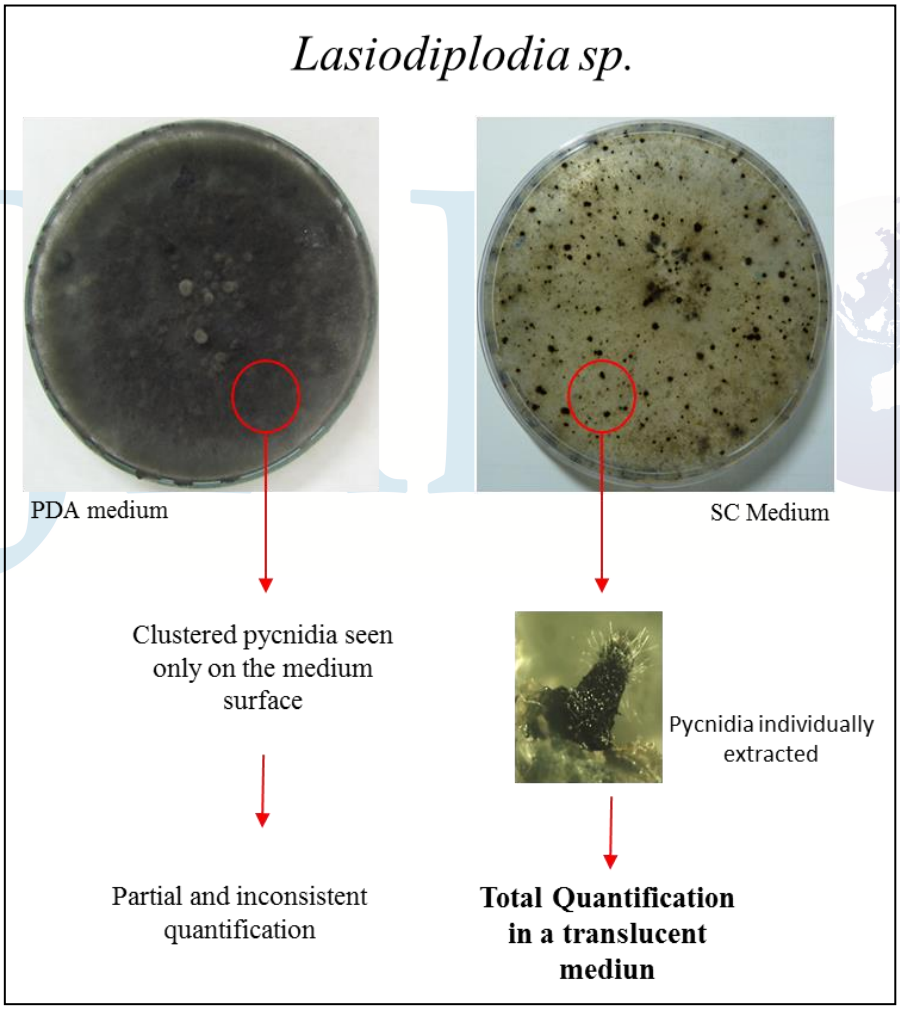

Figure 5. Efficiency of salt-cellulose medium (SC) in enabling the quantification of pycnidia when compared to potato-dextrose agar medium (PDA).

4. CONCLUSION

According to our results SC medium is indicated for *Lasiodiplodia* sporulation as it continues translucent after fungus growth, enabling visualization of all conidia formed, allowing their full quantification, characteristic that is not found in other media used for this fungus cultivation. In addition, the number of structures produced in SC medium allows the differentiation among isolates, indicating that conidial number is an important variable to be considered in the morphological characterization of *Lasiodiplodia*, an important feature that was ignored until now.

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