

Effect of Andiroba (*Carapa guianensis* Aubl.) Oil for Fungi Control in Maize (*Zea Mays* L.) Grains

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Abstract: The andiroba (*Carapa guianensis* Aubl.) oil (AO) antifungal effect was evaluated in strains of *Fusarium*, *Aspergillus* and *Penicillium* isolated from naturally contaminated maize (*Zea mays* L.). They were exposed to AO (10 to 40%), incubated and the antifungal effectiveness evaluated. AO had the best effectiveness at 40% for *Fusarium* and *Penicillium* (inhibition: 83.8 and 89.3%, respectively) genera, being *Aspergillus* not as efficient (6.2%) under the condition. Regarding new colonies formation, AO inhibited 100 and 75% of *Aspergillus* and *Penicillium* at its highest concentration. Further studies will be carried out in order to check sensory possible alterations.

Keywords: Amazon region, andiroba oil, food security, fungi, maize grains

Date of Submission: 06-09-2019

Date of Acceptance: 21-09-2019

I. Introduction

Maize (*Zea mays* L.) is a monocotyledonous plant, that belongs to the Family *Poaceae*, Subfamily *Panicoidae* (genus *Zea*, species *Zea mays* L.) and is produced in almost all continents (Magalhães, 2002). It is of extreme economic importance through its varied forms of application, ranging from feed for animals to high technological products for humans (Munirah et al., 2015). In addition to energy (carbohydrates) source, it is rich in lipids, vitamins (A and B complex) and minerals (iron, phosphorus and calcium) (Oliveira et al., 2004; Paes, 2006). According to the United States Department of Agriculture (USDA, 2017), Brazil is the third world largest maize producer, growing in different regions of the country, under different soil and climatic conditions, with an average of 74.0 million tons per year.

Fungi that invade grains are generally divided into two groups: field and storage (Magan & Aldred, 2006; Pitt & Hocking, 2009; Scussel et al., 2018). The main field fungi genera are *Fusarium*, *Helminthosporium*, *Alternaria* and *Cladosporium* that affect grains and other vegetables during ripening, where damage is observed before harvest (Scussel et al., 2018; Bullerman & Bianchini, 2009). However, storage fungi, such as *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor*, require less moisture and are found in silos, mills, equipment and places where grains are handled and or processed (Scussel et al., 2018; Bullerman & Bianchini, 2009).

Despite the conventional conditions to control fungi, alternative methods to control them are necessary (Savi et al., 2014). Several green methods have been reported, highlighting their decontamination (fungi / spores inactivation), due to the worldwide interest on using products that are less aggressive to the environment and healthier (Savi et al., 2014; Christ et al., 2016). Among these products, there is a great interest in the use of oils extracted from plants, mainly from the Amazon region, which are proved to be efficient in the control of microorganisms (Lima et al., 2009). Some have been highlighted such as the cashew nut (*Anacardium occidentale* L.) shell liquid, guaraná (*Paullinia cupana* Kunth) extract, copaiba (*Copaifera langsdorffii* Desf.) oil and the andiroba (*Carapa guianensis* Aubl.) oil – AO (Sousa et al., 2012; Martins, 2014).

Andiroba belongs to the *Meliaceae* Family and its oil is popularly known in the Amazon region (Lorenzi, 2002; Farias et al., 2016), as a healing product (anti-parasitic / malarial / inflammatory) (MacKinnon et al., 1997; Orellana et al., 2004; Roy & Saraf, 2006; Souza et al., 2006; Nayak et al., 2010; Silva, 2018). It is rich in essential fatty acids (linoleic and linolenic acids) and non-fatty components (triterpenes, tannins and alkaloids - carapine) (Souza et al., 2006; Silva, 2018). While the anti-parasitic and -malarial properties are attributed to the presence of gedunin, a meliacin component (MacKinnon et al., 1997), the anti-inflammatory and insect repellent properties are attributed to the andirobin, from the limonoids group (Orellana et al., 2004; Roy & Saraf, 2006; Nayak et al., 2010). AO has been studied as an alternative in the control of *Colletotrichum gloeosporioides* in pepper (*Capsicum baccatum*), as well as in *Botrytis*, *Cladosporium* and *Rhizopus*, and in

toxigenic fungi *Fusarium*, *Aspergillus* and *Penicillium* in bean (*Vigna unguiculata* L. Walp.). Despite that, there are still only a few studies of its use in food (Sousa et al., 2012; Farias et al., 2016).

Therefore, the present work aimed to evaluate the possible AO effect against fungi spores from different genera that contamination maize grains - and their different susceptibilities.

II. Material And Methods

Material

(a) Sample: maize grains (1 kg) naturally contaminated, kindly provided by the Integrated Company of Agricultural Development (CIDASC) from Santa Catarina Estate, Southern Brazil.

(b) Andiroba oil: commercial product (50 ml) purchased in the city of Belém, State of Pará (North Region of the country).

(c) Culture medium and others: potato dextrose agar - PDA, Neogen (Michigan, USA), malt extract - MEA and bacterial peptone, Himedia, (Mumbai, India), czapek – CYA and glycerol nitrate agar 25% - G25N, Vetec (Rio de Janeiro, Brazil); coconut agar - CAM, prepared with coconut milk, Serigy, FRUTEB S/A (Seará, Brazil). Others – cloranfenicol, Vetec (Rio de Janeiro, Brazil), Petry dishes (90x15 mm), Kasvi (Santa Catarina, Brasil).

(d) Equipment: laminar flow chamber, Veco (Campinas, SP, Brazil); autoclave, Phoenix (Araraquara, SP, Brazil); bacteriological oven, Fanem (São Paulo, SP, Brazil); analytical scale (range 0.01-210 g), Ohaus, (Parsippany, NJ, USA); light microscope, Olympus CX22, (Tokyo, Japan); stereoscopic, Opticam (São Paulo, Brazil); stomacher, Marconi (Piracicaba, SP); analog pachymeter (0.05 to 150 mm), Starrett (Massachusetts, USA).

Methods

(a) Sample collection: maize samples (total: 3 - G₁, G₂ and G₃), were collected by CIDASC, applying the Brazilian Ministry of Agriculture sampling official method (MAPA, 2007).

(b) Mycota from maize grains: the mycological tests were carried out from portions (25 g) of each maize sample (G₁, G₂ and G₃)* aseptically weighed as follows. **(b.1) total fungi count** - portions were transferred to polyethylene bags and added peptone water (0.1%) followed by homogenization (2 min at stomacher); then, a volume (100 µl) of each diluted sample (10⁻¹, 10⁻² and 10⁻³) was inoculated on PDA surface containing chloramphenicol (100 mg/L) in a flow laminar cabinet (n=3) and incubated at 25±1°C for 7 days (Silva et al., 2013; APHA, 2015). Their total count was read using the colony count and recorded as colonies forming units (CFU/g). Only the same dilution plates that had 15 to 150 colonies were counted. **(b.2) isolation and identification - (b.2.1) isolation** - by successive replications in 4 different culture medium (MEA, PDA, G25N and CAM) (n=3) followed by their **(b.2.2) identification** - it was performed by the microcutive technique (Weber & Pitt, 2000), briefly, a portion of each colony (presenting different morphology) was picked and inoculated onto Czapek agar medium (n=2) and incubated at 22-25°C for 5 days, followed by light microscopy identification (morphological characteristics, including the reproductive structures). From the macro and microscopic characteristics observation, the identification of the fungal genera was carried out according to the identification keys of Frisvad & Samson (2004). *Note: AO fungi contamination checking - the AO was also submitted to mycological test (total fungi load) by applying it on PDA for total fungi count (Section a.1) in order to certify its innocuity for further application in the decontamination study.

(c) Maize fungi genera isolated exposure to AO: (c.1) preparation of culture medium with AO - Petry dishes were added of PDA (20 ml) which were previously autoclaved and cooled (at 45-50°C), followed by AO addition at increasing volumes (AO-PDA: 2-20, 4-20, 6-20 and 8-20 ml), corresponding to 10, 20, 30 and 40%, respectively, then homogenized and solidified; **(c.2) maize fungi colonies inoculation** - the isolated colonies identified per genera at Sections b.2.1 & b.2.2, were transferred to AO-PDA, by taken a cylinder (disc colony + agar) of each one (7 mm diameter), placed in the agar center (n=3) and incubated at 22-25°C for 7 days (Sousa et al., 2012). **(c.3) evaluation of the AO antifungal effect** - during the incubation period, different parameters were investigated regarding each colony behavior against the AO-PDA medium (10 to 40%). **(c.3.1) development of the original colony** - measurement of the disc diameter (inoculum - 7 mm) of the AO treated and Control were carried out each they day by pachymeter and registered (n=3). **(c.3.2) aerial spores formation** - the number / intensity of new colonies (through aerial spores) formed away from the disc inoculation point were also registered and their intensity reported by a code (cross: +) and **(c.3.3) colonies development variation versus AO exposure** - it was carried out by comparing their development versus the AO percentages and incubation times (from Day Zero to the 3rd to 7th) to Controls.

III. Result And Discussion

From the results obtained using different AO concentrations as a fungal inactivation agent, variations were observed. They were both, on the colonies growth rate and fungi genera susceptibility. Figures 1-2 and Tables 1-3 show the characteristics of the isolated colonies from maize, antifungal AO effect as well the total load.

FUNGI LOAD AND ISOLATED GENERA FROM MAIZE GRAINS

(a) Fungi load

As expected, the total fungi spores load obtained from maize samples (G1, G2 and G3) was rather high and varied, ranging from 0.2 to 0.4×10^4 CFU/g. It was possible to be enumerated only at dilution 10^{-3} . Samples G1 and G2, mainly showed uncountable numbers of fungal colonies in the dilutions 10^{-1} and 10^{-2} (Table 1).

The presence of fungi in maize, especially whether they are from toxigenic strain, apart from deterioration are indication of the risk of mycotoxin contamination (Valmorbida et al., 2018). As other cereals, maize is often exposed to fungi contamination, with its proliferation starting from field (during plant development) and extending throughout its production chain (storage / transport / industry / consumer). The contamination can occur due to mycelial fragments and spores presence in the soil, plant and seeds remains or can be transported by wind, rain or insects (Scussel et al., 2018). During storage, the grains should be kept in adequate conditions to prevent damage and development of new fungal infections (Pezzini et al., 2005; Kumar & Kalita, 2017). The main, the colonies i.e., ones that grew in highest numbers and had consistent presence in the samples, were isolated - to be discussed in (b). Regarding the possible fungi contamination in the OA (to be utilized in the experiments), there was no significant development on the plates during the incubation period, thus able to be applied in the current study.

Table 1. Evaluation of fungi spores load from maize (*Zea mays* L.) grains and andiroba oil (*Carapa guianensis* Aubl.) to be utilized for the decontamination study

| Maize (code) | Total fungi load* (CFU / g) / dilution | | |
|-----------------|----------------------------------------|-------------------|-------------------|
| | 10^{-1} | 10^{-2} | 10^{-3} |
| G1 | CL ^a | CL | 0.4×10^4 |
| G2 | CL | 9.4×10^3 | 0.3×10^4 |
| G3 | 4.3×10^2 | 1.0×10^3 | 0.2×10^4 |
| AO ^b | 0.3×10^2 | NG ^c | NG |

^a countless

^b andiroba oil

^c no growth.

* average (n=3)

(b) Main fungi genera isolated from maize versus culture medium

The isolation and identification of the fungi colonies (obtained after the total load) inoculated in different culture medium (PDA, MEA, G25N and CAM), showed that all grain samples were contaminated, mainly by 4 genera, being 3 of them, of possible toxigenics (*Fusarium*, *Aspergillus* and *Penicillium*), and one (*Mucor*) atoxigenic (Figure 1). Through those medium application, it was possible to observe their growth characteristics as well as to corroborate their contamination presence in the maize samples. Several authors have reported that the genera *Fusarium* (field fungus) and *Aspergillus* & *Penicillium* (storage fungi) have been the most often isolated in maize and its products (Marques et al., 2009; Di Domenico et al., 2015). *Fusarium* - the most important fungus genera in maize coming from the field, is also of concern when its species are toxigenic (mycotoxins: fumonisins, deoxynivalenol, nivalenol, zearalenone). Their toxins can persist contaminating throughout the food chain as they are high temperature resistant (Gabriel et al., 2008 Scussel et al., 2018). The contamination of maize grains by *Fusarium* species can be explained by the fact that this fungus infects extensively certain portions of the grain, mainly the germen, apart from the external and internal grain tissues (Lazzari, 1997). On the other hand, *Aspergillus* - its species are considered storage initiators of seeds and grains deterioration, being able to grow with low moisture content (mc), followed by the *Penicillium* that needs somewhat higher mc (due to the metabolic activity of the first invaders). Both fungi genera are potentially toxigenic (toxins: aflatoxins, ochratoxin A, citrinine, penicillic / cyclopiazonic acids) and their presence in the maize samples is considered of concern too, given those toxins production possibility (Scussel et al., 2018). Regarding *Mucor* - it is usually present in the soil, grains, fruits and vegetables and are common contaminants of places where products are processed. That explains the presence of this genera in the samples (Scussel et al., 2018; Santos, 2018).

As far as the different culture media utilized versus fungi behavior are concerned (Table 2), it was observed that only the *Fusarium* and *Aspergillus* genera developed in all media. *Penicillium* and *Mucor* were not able to develop in the G25N and CAM (containing glycerol nitrate and coconut in their composition, respectively). Studies carried out by Mezzomo et al. (2018) also reported mycelial growth of *Fusarium* isolated in MEA medium. Nelson et al. (1983) registered the efficiency of G25N and PDA medium for that genus. It was

observed that the *Fusarium* developed on CAM culture medium (most commonly used and specific for aflatoxin-producing - storage fungi), inferring that this medium may also provide conditions for the development of that - field genera. Samson et al. (2014) and Pitt & Hocking (2000) reported *Aspergillus* grown in the four-medium utilized in the current work too. Pitt and Hocking (2000) observed that for the *Penicillium*, MEA and G25N provided adequate nutrients for their growth, corroborating our study.

Table 2. Behavior of field and storage fungal genera isolated from maize (*Zea mays* L.) samples in different culture medium

| Fungi genera | Culture medium maize fungi development* | | | |
|--------------------|-----------------------------------------|-------------------|------------------|------------------|
| | PDA ^a | G25N ^b | MEA ^c | CAM ^d |
| FIELD | | | | |
| <i>Fusarium</i> | G ^e | G | G | G |
| STORAGE | | | | |
| <i>Aspergillus</i> | G | G | G | G |
| <i>Penicillium</i> | G | G | G | NG ^f |
| <i>Mucor</i> | G | NG | G | G |

^a potato dextrose agar ^b czapek-dox 25% glycerol nitrate ^c malt extract agar ^d coconut agar ^e growth ^f no growth * n=3

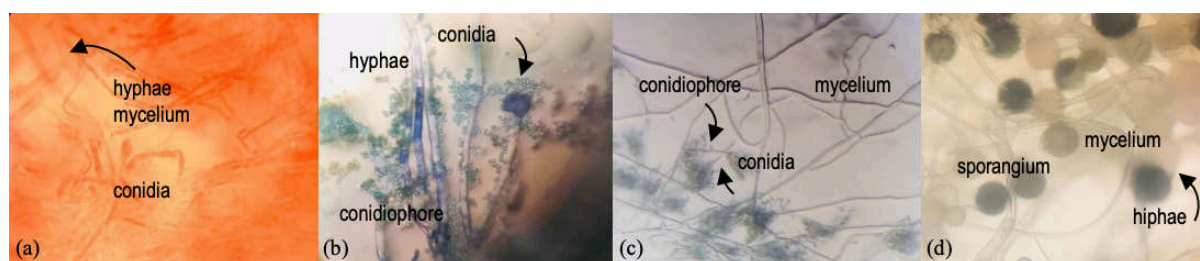


Figure 1. Major fungal genotypes isolated from maize (*Zea mays* L.) grains naturally contaminated and their characteristics: (a) *Fusarium*, (b) *Aspergillus*, (c) *Penicillium* and (d) *Mucor*.

ANTIFUNGAL EFFECT OF ANDIROBA OIL AGAINST ISOLATED GENERA FROM MAIZE

For the study of the AO antifungal effect against the main genera isolated from maize grains, there were selected only the toxigenic strains (*Fusarium*, *Aspergillus* and *Penicillium*) i.e., the potential mycotoxins producers. Their (a) diameters variation under AO presence measured during the colonies growth (from the application center point) period and the (b) new colonies formation (from the aerial spores) data (up to Days 3 and 7 of incubation) are shown in Table 3 and Figure 2.

From the data obtained it was possible to observe the AO antifungal effect on colonies diameters (inoculation point) and emergent colonies growth reduction in all the AO tested when compared to Control, its variation percentages wise as follows.

(a) AO control on fungi growth (diameter)

Regarding the AO inhibition on fungi colonies diameter (from 7 mm disc - the inoculation point), *Fusarium* and *Penicillium* showed reduction in all the tested concentrations (10, 20, 30 and 40%) when compared to Control (18 and 26.2 mm respectively). Therefore, registered the AO highest efficiency at 40% with 83.8 and 89.3% reduction (Table 3).

Fusarium (Control: 18 mm at Day 3) and *Penicillium* sp. (Control: 26.2 mm at Day 3) – their treated colonies diameter increased from original 7 to only 9.9 and 9.8 mm up to Day 3, keeping those dimensions still, until Day 7.

Aspergillus (Control: 3 mm at Day 7) – on the other hand, for *Aspergillus* no such an alteration was observed on its diameters in all PDA+AO medium (10-40%), throughout the whole experiment. It behaved similarly under AO exposure, growing only 3 mm by Day 3, reaching 10 mm diameter, then remained stable until Day 7 compared to Control. It is worth noting that the genus *Aspergillus* was the one that presented the highest new colonies growth since the 3rd days of incubation to be discussed in the next Section (Figure 2).

Table 3. *Andiroba* oil (*Carapa guianensis* Aubl.) antifungal effect on field and storage colonies development rate at different concentrations and incubation times*

| Fungus | | Incubation time (Day) | Colony development rate**versus AO concentration (%) | | | | | | | | | |
|--------------------|------------------|-----------------------------|------------------------------------------------------|-----------------|------------------|------|------------------|------|------------------|------|------------------|----|
| Origin | Genus | | Control | | 10 | | 20 | | 30 | | 40 | |
| | | | Diameter (mm) | AS ^a | Diameter (mm) | AS | Diameter (mm) | AS | Diameter (mm) | AS | Diameter (mm) | AS |
| FIELD | | | | | | | | | | | | |
| <i>Fusarium</i> | 0 ^b | 7.0 | NG ^c | 7.0 | NG | 7.0 | NG | 7.0 | NG | 7.0 | NG | |
| | 1 | 8.2 | NG | 10.1 | NG | 9.6 | NG | 9.0 | NG | 8.9 | NG | |
| | 2 | 12.1 | NG | 10.2 | NG | 10.0 | NG | 10.0 | NG | 8.9 | NG | |
| | 3 | 25.0 | NG | 16.5 | NG | 19.8 | NG | 22.9 | NG | 9.9 | NG | |
| | 7 | CL ^d | NG | CL | NG | CL | NG | CL | NG | 9.9 | NG | |
| | Total growth | 18.0 | NA ^e | 9.5 | NA | 12.8 | NA | 15.9 | NA | 2.9 | NA | |
| | AO reduction (%) | NA | NA | 47.2 | NA | 28.8 | NA | 11.6 | NA | 83.8 | NA | |
| STORAGE | | | | | | | | | | | | |
| <i>Aspergillus</i> | 0 | 7.0 | NG | 7.0 | NG | 7.0 | NG | 7.0 | NG | 7.0 | NG | |
| | 1 | 7.8 | + ^f | 9.5 | NG | 9.5 | NG | 9.1 | NG | 8.9 | NG | |
| | 2 | 9.2 | +++ | 9.5 | ++ | 9.5 | + | 9.2 | NG | 9.1 | NG | |
| | 3 | 10.2 | ++++ | 10.1 | +++ | 10.1 | ++ | 10.0 | + | 10.0 | NG | |
| | 7 | CL | +++++ | CL | ++++ | CL | +++ | CL | ++ | 10.0 | NG | |
| | Total growth | 3.2 | CL | 3.1 | 90.0 | 3.1 | 75.0 | 3.0 | 50.0 | 3.0 | NG | |
| | AO reduction (%) | NA | NE ^g | 3.1 | 10.0 | 3.1 | 25.0 | 6.2 | 50.0 | 6.2 | 100 | |
| <i>Penicillium</i> | 0 | 7.0 | NG | 7.0 | NG | 7.0 | NG | 7.0 | NG | 7.0 | NG | |
| | 1 | 11.2 | + | 9.3 | NG | 8.4 | NG | 8.2 | NG | 8.5 | NG | |
| | 2 | 31.3 | ++ | 9.3 | NG | 9.3 | NG | 8.2 | NG | 8.5 | NG | |
| | 3 | 33.2 | +++ | 10.1 | NG | 10.1 | NG | 10.0 | NG | 9.8 | NG | |
| | 7 | CL | +++++ | CL | ++++ | CL | +++ | CL | ++ | 9.8 | + | |
| | Total growth | 26.2 | CL | 3.1 | 90.0 | 3.1 | 75.0 | 3.0 | 50.0 | 2.8 | 25.0 | |
| | AO reduction (%) | NA | NE | 88.1 | 10.0 | 88.1 | 25.0 | 88.5 | 50.0 | 89.3 | 75.0 | |

^a aerial spores (emerging / new colonies formation) ^b Day zero (colony disc: inoculation and incubation) ^c no growth ^d countless ^e not applicable ^f formation of new colonies (number of +: corresponds to the intensity of colonies formed) ^g no AO effect * manly at Day 3 and 7 ** average

(b) AO effect on aerial spores (emerging colonies)

Only *Aspergillus* and *Penicillium* genera presented new colonies development (through AS) since Day 3 (*Aspergillus*) from 10 to 30% AO exposure (in decreasing other – Table 3) reaching total inhibition at Day 7. On the other hand, *Penicillium* AS started to spread and their colonies formed at Day 7. AO showed total efficiency (100% AS reduction / control) at 40% (no growth) for *Aspergillus* (Figure 2: a.1-a.4). No *Fusarium* genera presented similar behavior (AS).

The growth behavior of new colonies among *Aspergillus* / *Penicillium* genera and *Fusarium*, can be explained by their differences morphological characteristics. While FIELD FUNGI - *Fusarium* has its mycelium with cottony appearance and quite developed, its conidiophores are thin, simple and short, irregularly branched. STORAGE FUNGI - (a) *Aspergillus* has septate, branched mycelium, its conidiophores are simple erect and unicellular with spherical conidia. (b) *Penicillium* also has septate mycelium similar to *Aspergillus*, with aerial conidiophores, septates and may be branched or not, (with unicellular conidia) (Scussel et al., 2018). As the characteristic of the *Aspergillus* and *Penicillium* colonies are velvety and filamentous, they can spread more easily (forming new colonies away from the inoculation point), corroborating the achieved in the current result. On the contrary, *Fusarium* is cottony filamentous, its conidia doesn't spread across a broad area so easily.

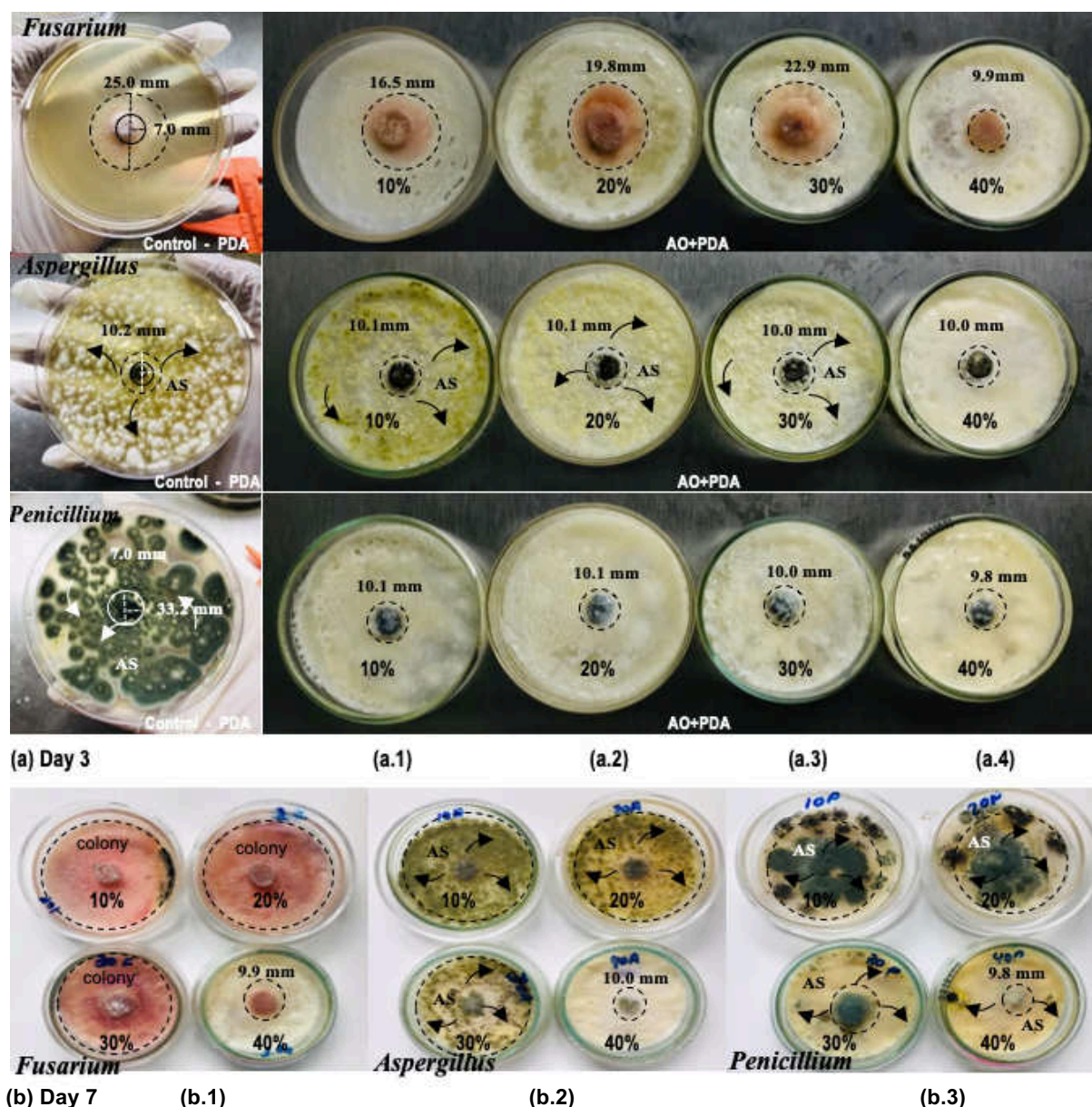


Figure 2. Andiroba oil - AO (*Carapa guianensis* Aubl.) antifungal effect on *Fusarium*, *Aspergillus* and *Penicillium* colonies development* and aerial spores - AS (new colonies formation) at: (a) Day 3 – diameter and AS (Control versus 10, 20, 30 and 40% AO) and (b) Day 7 – inhibition efficiency (Control versus 10, 20, 30 and 40% AO [*PDA, 25°C, original disc diameter: 7 mm / inoculum point])

IV. Conclusion

From the tested maize grains naturally fungi contaminated, *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor*, where the most frequent genera isolated. Regarding the different culture medium and the isolated fungi behavior, all of them grew on PDA, MEA, G25N and CAM except *Penicillium* and *Mucor*. It was observed a positive AO antifungal effect against the fungi isolated from maize as follows *Penicillium*>*Fusarium*>*Aspergillus* with 89.3, 83.8 and 6.2% growth reduction, mainly at 40% AO. Further studies will be carried out on AO mechanism of action on fungi, as well as sensorial evaluation, in order to check whether it interferes on food taste.

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