

RESEARCH ARTICLE

Effect of cold plasma on black beans (*Phaseolus vulgaris* L.), fungi inactivation and micro-structures stability

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ABSTRACT

The Cold Plasma (CP) treatment efficiency to inactivate naturally fungi contaminated beans (*Phaseolus vulgaris* L.), Class black, and the effects in micro-structures stability were investigated. Kernels were exposed to CP (8 kv intensity and 510W power) at different times (5, 10, 20 and 30 min) followed by incubation to verify the antifungal effect and their micro-structural resistance through microscopy (stereo –SM and scanning electron - SEM) observation. The treatment was effective to fungal inactivation when exposed to CP (10-30 min). It also inhibited bean germination during the longest treatment exposure (20-30 min). Regarding germination, only when CP was applied during the shortest period of time (5 min) had a positive effect (improved its speed: hypocotyl & radicle length increased), when compared to Control. Some morphological alterations were registered by SM, either *on testae* (wrinkling) and cotyledons (color changes). Indeed, through SEM, both *testae* and cotyledon structures showed the disrupting effects on their cell membranes during the treatment more power. The CP treatment, under the current conditions applied, showed effective for fungi inactivation, although it affected some kernel micro-structures and germination. Therefore, further studies will be carried out regarding adjustments on CP conditions, to improve treated beans quality.

Keywords: Non-thermal; Cold plasm; Bean; Fungus; Decontamination

INTRODUCTION

Beans (*Phaseolus vulgaris* L.) are one of the most consumed pulses worldwide (Yang et al., 2018). Brazil is the third largest producer, after India and Myanmar. It represents a significant aspect for the country economy, especially for small farmers (Blair et al., 2013).

However, when it is stored in inadequate conditions (high moisture and temperature), beans can suffer pests attack, causing qualitative and quantitative losses (Kumar and Kalita, 2017). Fungi infection is the main cause of stored grain damages worldwide after insects infestation (Verga and Teren, 2005). Fungi are indicators of grains quality (deterioration) and safety (mycotoxins producers), thus responsible for causing serious damages to human and animals health (Katsurayama and Taniwaki, 2017). Among

the toxins producers, the *Aspergillus* and *Penicillium* genera are the most important in the storage and need to be controlled (Scussel et al., 2018).

The traditional fungal control methods applied during storage (fungicides applications) and in the food industry (thermal treatments) can leave toxic compounds residues and/or induce texture alterations (Niemira, 2012; Misra et al., 2011; Freitas et al., 2017). Therefore, methods that are not aggressive for grains and effective on spores' inactivation are required (Savi et al., 2014). The CP is an example of a new technology. This non-thermal method is reported as a partially or totally ionized gas. It is composed of a subatomic and molecular elements mixture with electromagnetic radiation (UV photons and visible light) able to generate species such as the reactive oxygen (ROS), nitrogen (RNS) and halogen (RHS) (Bourke et al., 2017).

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Received: 04 July 2019; **Accepted:** 11 October 2019

The CP antifungal power occurs due to its effect on the cells membrane surface (wall) and in the inner components, causing dehydration through UV light and DNA chains breakdown, making difficult to replicate (Afshari and Hosseini, 2014; Devi et al., 2017). It's important to highlight that, among the gases that can be utilized in this process; the most common for microbiological decontaminations is the oxygen, it forms ozone, an important known oxidizer. One of the CP advantages in food, refer to minimizing effects, different from those caused by the *high* temperatures procedures. Besides, it decreases the use of chemical fungicides, thus meeting the consumer demand for safe foods (Niemira, 2012; Misra et al., 2011).

Regarding food CP application, several studies reported its effect against microorganisms (bacteria: *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* - and fungi: *Aspergillus flavus*, *A. parasiticus*, *Penicillium* spp.) (Selcuk et al., 2008; Han et al., 2016; Thomas-Popo et al., 2019; Hou et al., 2019). The investigations were carried out for different fruits, grains and pulses (Misra et al., 2011; Mitra et al., 2013; Dasan et al., 2016; Devi et al., 2017; Ukuku et al., 2019). It has also been studied for products such as, cereal bars and biodegradable films (starch-based/zein-with chitosan) (Chen et al., 2019; Nieves et al., 2019). However, for fungi decontamination in beans, CP detailed effects have still been unexplored.

Regarding changes in the food tissue caused fur CP and registered in microscopy, especially for scanning electron microscopy (SEM), some studies were performed in products such as apple and corn and peanuts (Dasan et al., 2016; Devi et al., 2017; Ukuku et al., 2019).

The objective of current study was to investigate the effectiveness of cold plasma on the inactivation of fungi present naturally in kernels beans, including its possible alterations on quality characteristics (inner and outer micro-structures) and germination capacity.

MATERIAL AND METHODS

Material

- Sample: beans, cultivar SCS204, Class: black (1 Kg), naturally contaminated, kindly provided by the Santa Catarina Integrated Company of Agricultural Development—CIDASC (moisture content—mc, water activity - a_w , and total fungi load – TFL of 17.5%, 0.6037 and 1.7×10^3 CFU/g, respectively).
- Culture medium and chemicals: potato dextrose agar (PDA), Kasvi (Pinhais, PR, Brazil), chloramphenicol, Vetec (Rio de Janeiro, RJ, Brazil) and methylene blue dye, Exôdo Científica (São Paulo, SP, Brazil).

- Equipment: CP system - reactor Tip Plane Type (glass chamber - 110.5 x 105.0 mm, with silicone cap and oxygen gas input) with dielectric barrier discharge (10 mm distance between the electrodes) and a VARIAC utilized as controller (input: 510 W) and a power supply (17 kv). Microbiological oven, Quimis (Diadema, SP, Brazil); microwave oven, Philco (São Paulo, SP, Brazil); autoclave, Phoenix (Araraquara, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil) and caliper, Santa Clara (São Paulo, SP, Brazil) Microscopes – (c.1) light –LM (100x), Olympus (São Paulo, SP, Brazil), (c.2) stereo- SM (180x), Opticam (Tokyo, Kt., Japan) and (c.3) scanning electron- SEM(5000x), model JSM6390LV, Jeol (Peabody, Mass., USA); gold coating machine, model EMScd500, Leica (Leider, Ill., USA).
- Other materials: mini-Petri dishes (60 mm \varnothing), Kasvi (Santa Catarina, Brazil).

Method

- Beans preparation prior CP treatment: beans kernels were arranged (n=3) on previously prepared Petri dishes with PDA - only one grain/dish. They were divided into two main groups: Control (CG - not CP treated) and Treated (TG - CP treated) for exposure times of 5, 10, 20 and 30 min. Total fungi load (TFL) was carried out prior the kernels treatment by applying the method prescribed by Samson, et al. (2006).
- CP treatment: the TG plates were placed into the reactor then applied 8 kV intensity (510W) and exposed during different length of times (5 to 30 min).
- Post-treatment CP effect analysis: the effect on fungal growth inhibition, germination capacity and micro-structures alterations were carried out through the following analyses. (c.1) *mycology tests* - the CP treatment on fungi spores was evaluated through plates incubation for 7 days, at 25°C (Berjak, 1984). The antifungal effect was then monitored through colony growth. The colonies that were able to grow, were identified through the microculture technique (Weber e Pitt, 2000; Samson et al., 2006). (c.2) *germination capacity* -each kernel (TG and CG) germination capacity was evaluated by monitoring their embryo tissues behavior through the development of the radicle (primary root) and hypocotyl (embryonic stem) during the incubation time (Day 1 to 7 at 25°C). Micrographs were taken from SM and SEM observation. (c.3) *micro-structures characteristics*- (c.3.1) *beans parts and sections preparation* – each bean kernel was prepared as follows, for SM- sectioned in two parts corresponding to the cotyledons and for SEM - different parts sectioned in cubes (*testae*/hilum/cotyledon/epicotyls/primary root) and bean cross sectioned. The stub preparation followed the specific protocol.

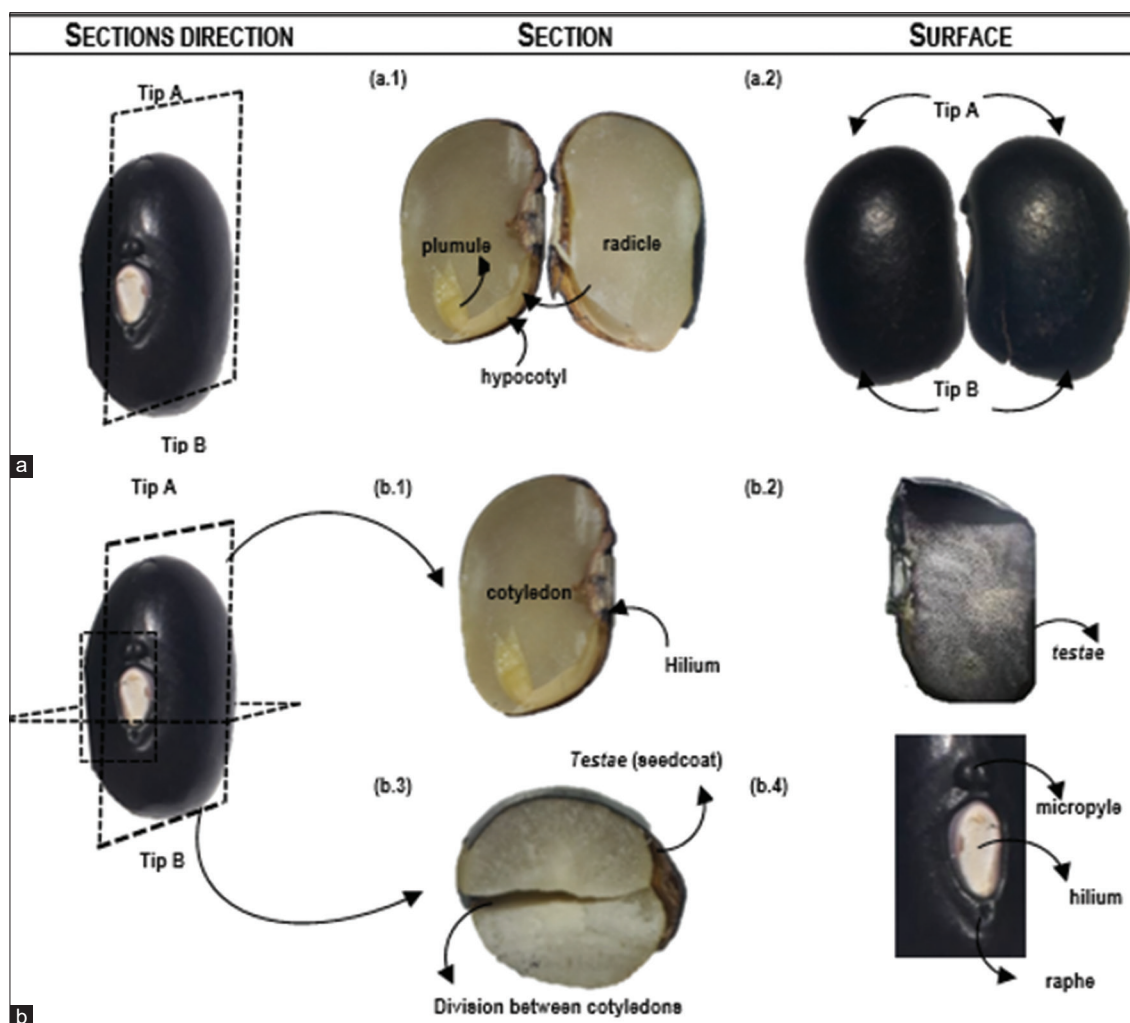


Fig 1. Bean (*Phaseolus Vulgaris* L.) kernels sections preparation for microscopy visualization by: (a) stereo (SM) (a.1) cotyledons, (a.2) testae and (b) scanning electron (SEM) (b.1) cotyledon (length wise - inner) and (b.2) testae (inner and outer surfaces); (b.3) whole kernel (cross sectioned) and (b.4) hilum surface.

Posteriorly, kernels were gold coated (40 nm layer) under vacuum in a planetary base and taken to the microscopes (Scussel et al., 2014). (c.3.2) *microscopy observations*- by SM- the structural characteristics of different bean parts and their morphology were registered through micrographs (taken at different amplifications:67-80x) with any tissue variations (TG) compared to CG. For a more precise analysis of the CP effects changes on the constituents of external and internal structures of the kernels, their tissues were evaluated by SEM - testae/hilum, cotyledons, starch and germination tissues modifications were observed, then registered through micrographs (taken at different amplifications:100-1000x) and their alterations compared to CG (Fig. 1).

RESULTS AND DISCUSSION

Data obtained on the beans Class black, CP treated (conditions: 8Kv/5-30 min, Tip Plane type) for antifungal

inactivation and micro-structures stability showed some variations regarding the conditions applied. Figs. 2-6 show the fungi genera contamination, SM and SEM microscopy characteristics, respectively.

Antifungal CP effects

Beans naturally contaminated – prior CP treatment

- Total fungi load- the bean samples utilized for the CP treatment presented a previous natural contamination of 1.1×10^3 CFU/g, as expected. Indeed, kernels had a rather high mc (17.5%) to allow fungi spores development. That mc is known as an adequate condition (together with temperature) for fungi development, especially the storage ones (Scussel et al., 2018).
- Fungi genera isolated- the fungi colonies isolated (Section 3.1.a) from the bean samples were identified as *Aspergillus* sp. and *Penicillium* sp., storage genera which need less mc/aw to proliferate (Fig. 2). Beans samples humidity were of 17.5% and 0.6037 for mc and aw, respectively. The *Aspergillus* was the mainly

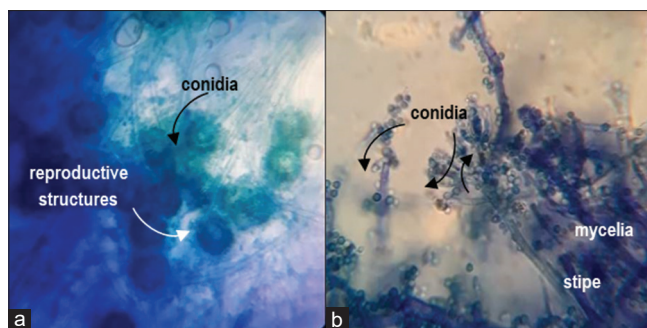


Fig 2. Fungi genera isolated from naturally contaminated bean (*Phaseolus vulgaris* L.) kernels, Class black: (a) *Aspergillus* and (b) *Penicillium* by light microscopy [40X].

genera isolated, with the highest number of colonies growth, compared the other genera. Attention should be paid, regarding these microorganisms, due to their possibility to produce toxins. *Aspergillus* species can produce mainly the aflatoxins (B1, B2, G1, G2) and ocratoxin A. On the other hand, *Penicillium* can produce citrinine, cyclopiazonic acid, among others (Scussel et al., 2018). Consequently, their presence can compromise the human and/or animal health, regarding their toxic characteristics, such as hepatotoxic, nephrotoxic, immunosuppressive, mutagenic and/or carcinogenic (Medeiros et al.; 2012; Prado, 2014; Zhu et al., 2018).

Beans naturally contaminated – post CP treatment

- (a) Anti-fungi CP effect and susceptibilities – treatment, under the CP Type (Tip Plane) and conditions (8Kv/5-30 min) applied showed to be effective on fungal inactivation (100% reduction - no growth) throughout all the exposure times (10-30 min), except for the shortest. That positive effect, was due to fungi cell walls being affected by the CP components (UV photons, charged particles, radicals, reactive species and ozone) (Afshari e Hosseini, 2014, Bourke et al., 2015). The reactive species of plasma are associated to oxidative effects in external surface of microbial cells. The lipids are the main components affected by the reactive species, as they are distributed (together with proteins) in the membrane surface and are easily hit for plasma. The protein are affected too, through denaturation and cell leak (Misra et al., 2011; Afshari et al., 2014). Regarding the shortest CP exposure (5 min), fungi spores took around three days to start hatch, they were still able to grow (develop into colonies) from Day 4th (both: *Aspergillus* & *Penicillium*), then kept on growing up to Day 7th (countless). That probably occurred due to spores resistance to the short CP oxygen ionized gas exposure (not enough to membrane disintegration). That finding was corroborated by Han et al. (2016) and Lauroussi (2009). Authors reported short contact time between the reactive gas species

and the fungi contaminant, promoting a less effective inactivation. Studies also related a better efficacy with longer exposure times (Suhem et al., 2013; Selcuk et al., 2008). Thus corroborating with the registered in the current study (100% inactivation – longer time -10 to 30 min). Selcuk et al. (2008) also reported satisfactory results on fungi decontamination of cereals and pulses naturally contaminated with *A. parasiticus* and *Penicillium* sp, regarding exposure time variation.

- (b) CP treatment variables versus effectiveness - several studies demonstrated a partial or total microorganism load reduction by CP. However, they were carried out by utilizing different conditions, either CP reactor geometries, the application parameters, level of fungi spores load and/or different species (Grzegorzewski et al., 2011; Suhem et al., 2013; Chaiwat et al., 2016). Other factor, responsible for fungal inactivation efficiency is the CP form of application onto a surface: directly, semi-directly or indirectly. The direct (used in this work) CP application in certain area provides a higher range of reactive species interaction with the microorganism than indirectly (Hertwig et al., 2015).
- (c) CP applications versus pulses: as far as fungi reduction by CP on pulses, some studies were carried out for beans, chickpeas, lentil, peanuts and soya. Despite that, they were reported CP application at different conditions, such as: Atmospheric Low Pressure, Gas Barrier Discharge, Bell-Jar-Type and Surface Micro-Discharge (Selcuk et al., 2008; Mitra et al., 2013; Devi et al., 2017). In the current work, the CP utilized was Tip Plane type, applied directly on the bean surface, keeping a gap (distance between plasma and substrate) of 10 mm, so the reactive species directly affected the sample, contributing to the fungi decontamination efficacy. Thomas-Popo (2019) by using CP with an argonium Gas Barrier Discharge, kept a 20 mm gap on wheat grains and was able to inactivate the natural microflora, including *Escherichia coli* and *Salmonella enterica*. Los et al. (2018) investigated the effect of Gas Barrier Discharge CP on wheat and barley to control potential of native and pathogenic microflora as well of fungi and reported reduction by direct treatment for 20 min. Those findings corroborate to the antifungal potential of the current study, although, by utilizing a different geometry (Tip Plane Type).

Bean kernels micro-structures CP stability - quality related

As far the susceptibility of the beans tissues micro-structures characteristics to CP application are concerned, by applying both SM & SEM microscopies, alterations were able to be registered. They were mainly on beans *testae* and cotyledons. Fig. 4 and 5/6 show the SM and SEM tissues alterations, respectively.

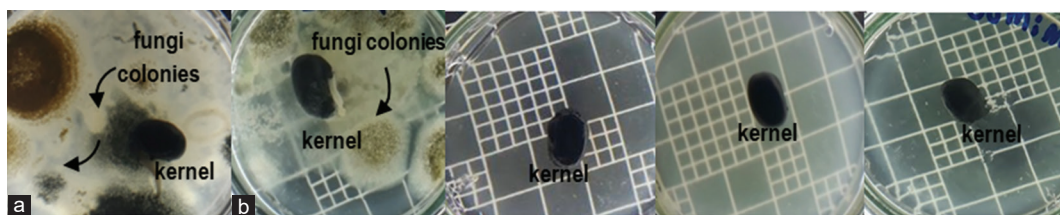


Fig 3. Fungi colonies on bean (*Phaseolus vulgaris* L.) kernels Class black:(a) Control (not CP treated) and (b) Treated (TG: at 5, 10, 20 and 30 min) - Day 7th.

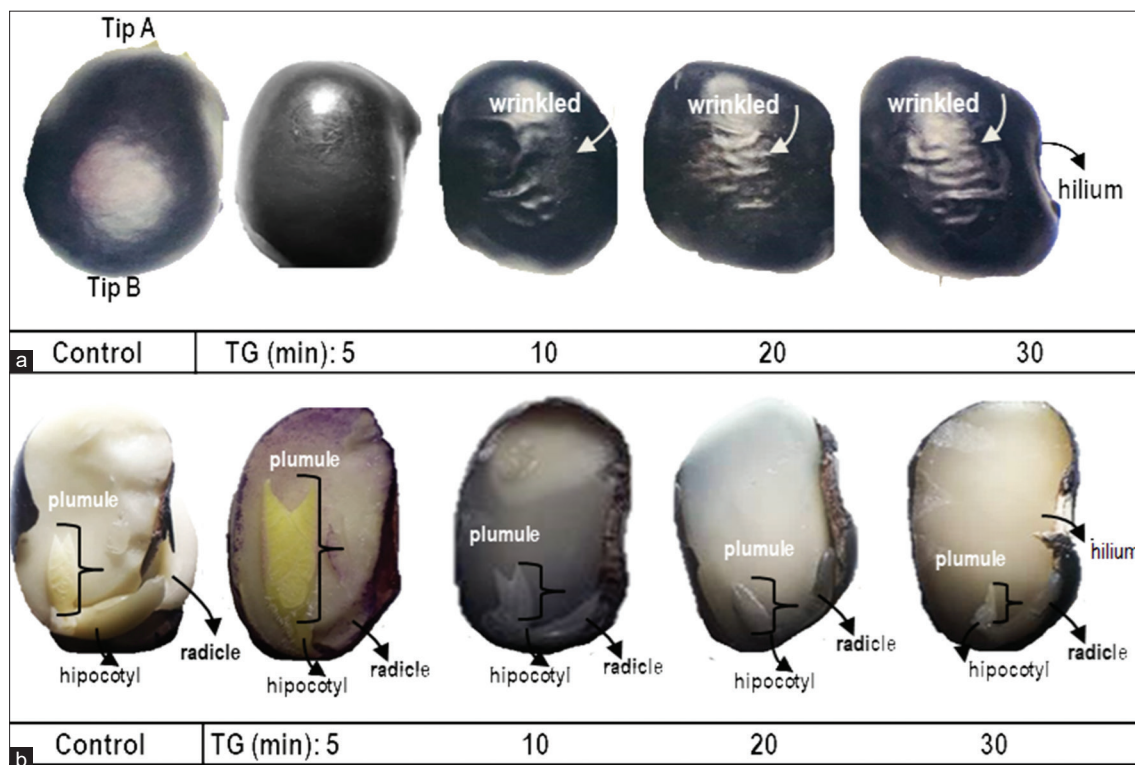


Fig 4. Bean (*Phaseolus vulgaris* L.) kernels Class black, – testae & cotyledons surfaces micro-structures characteristics: (a) testae (external) and (b) cotyledons (inner). Control (not treated) and Treated (Cold Plasma: 8 Kv, 5, 10, 20 and 30 min exposure time) [SM- 80X] TG: treated group.

SM of bean micro-structures CP treated– the morpho-histological characteristics alterations in beans kernel CP treated were registered on the *testae* and cotyledon tissues as follows.

- testae* (outer surface)- some changes on the bean external part could be visualized (Fig. 3). *Testae* developed a wrinkled texture in comparison to the CG (smooth). Dhayal et al. (2006) also observed considerable alterations on the *testae* caused by CP (low-pressure) in safflower seeds;
- cotyledons (inner surface)-the treatment affected somewhat the bean. It presented color alteration (Fig. 4). Similar changes were also reported by Mitra et al. in chickpea. Authors registered significant alterations related to roughness development when exposed to CP (micro-discharge surface type) during 5 min (treatment longer of the study) (Mitra et al., 2014). On the other hand, in blueberries (high moisture- fruits) it was noticed color and firmness

reduction when submitted to CP (tip: jet) (Lacombe et al., 2015). The food characteristics changes after CP exposure can be explained by the reactive species action on tissues causing expansion apart from the breaking down of cell membranes (Pasquali et al., 2016). The cotyledon color change, for example, can be explained by a possible beans water exudate when submitted to CP (Bormashenko et al., 2015). Changes in nutritional, sensory and other attributes linked to quality also were registered by Pasquali et al. (2016) for red chicory.

- germination: besides the structural characteristics of the bean, another aspect directly related with the application of CP is the germination. In this case, the kernels groups exposed only during 5 and 10 min germinated (both hypocotyl and radicle were formed). The other groups (exposed to 20 and 30 min) did not develop of the radicle (inhibited germination). During

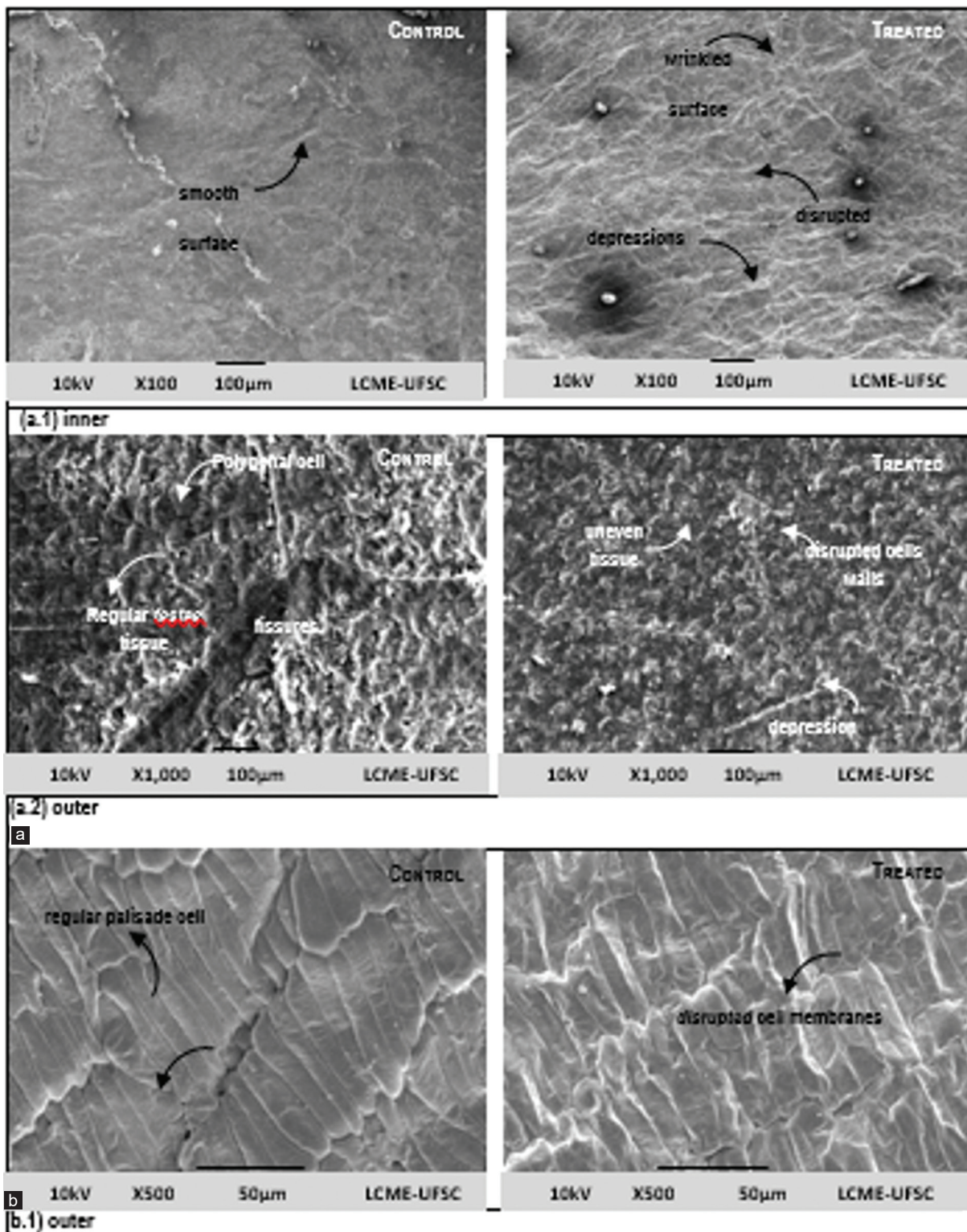


Fig 5. Bean (*Phaseolus vulgaris* L.) kernel, Class black Scanning electron microscopy of (a) TESTAE and (b) COTYLEDONS surfaces: Control and Treated (exposed to Cold Plasma - Tip Plane: 8 Kv, 30 min exposure time) [100-1000X].

the 5 min exposure (Fig. 4) the structures that give rise to stems and leaves showed size increase (to 16 mm or 33% length), than the Control kernel (12 mm). It demonstrated that CP has a positive effect under the

shorter time tested (5 min/8kv). On the other hand, the growth of the radicle under the 10 min exposure was visually inferior when compared to the CG (8 mm). Several authors verified that CP stimulated the

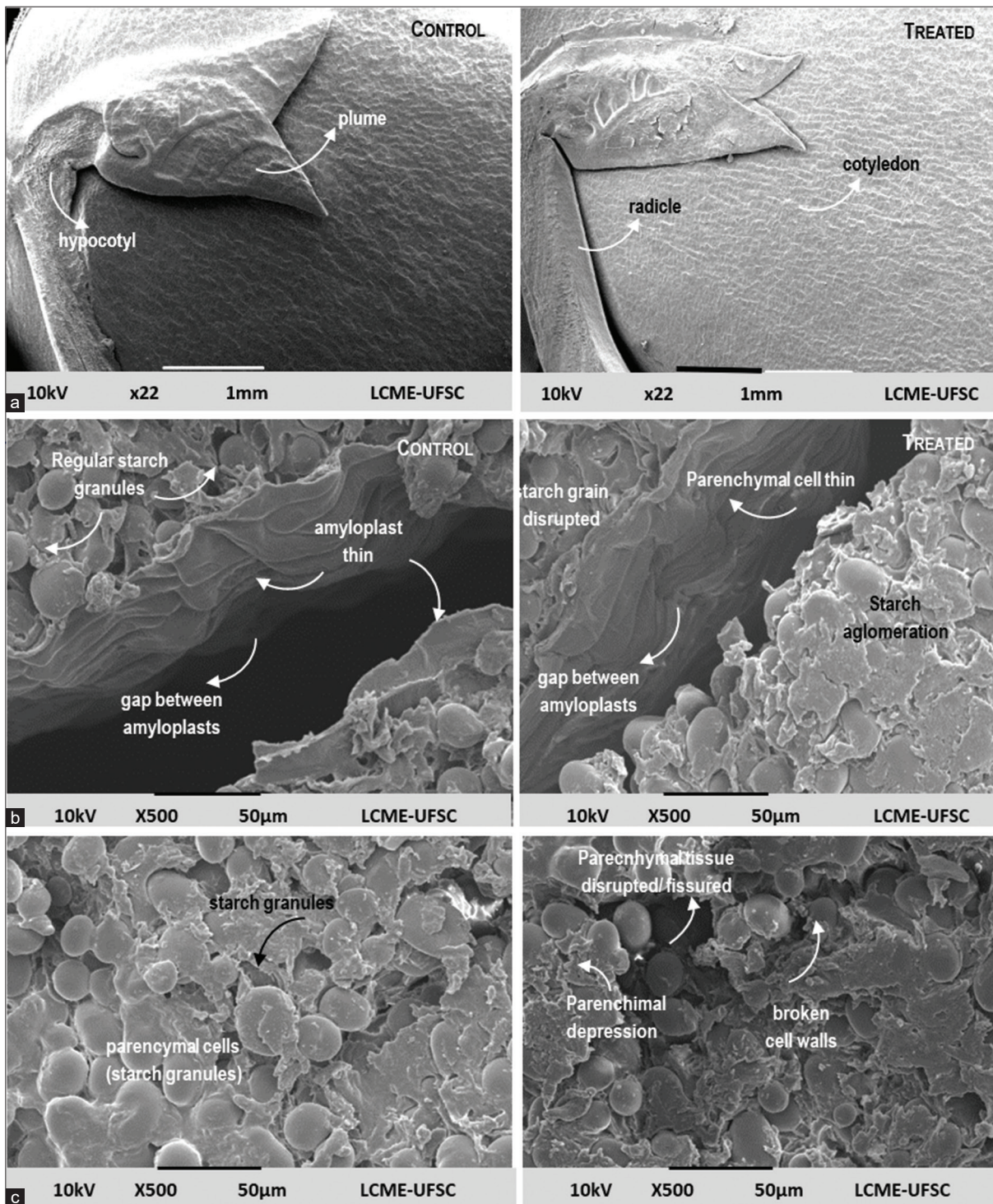


Fig 6. Bean (*Phaseolus vulgaris* L.) kernel, Class black scanning electron microscopy of cotyledon – starch/parenchymal cells in cross section: (a) germination structures, (b) membrane between amyloplasts and (c) starch morphology and alterations. Control and Treated (exposed to Cold Plasma -Tip Plane Cold Plasma: 8 Kv, 30 min exposure time) [500X].

germination process, as Ling et al. (2014) in soybean seeds (15 sec exposure to CP) and Mitra et al. (2014) in the chickpea. (0.5 to 5 min exposure to CP). In the current work bean kernels possibly did not germinate due to the damaged structures by long exposure time (germination responsible).

SEM of bean micro-structures CP treated - regarding beans treated micro-structures characteristics and observed by SEM, some subtle differences were noticed as follows (a) testae - control sample showed smoother surface in relation to treated kernel by CP (disrupted cells walls). This alteration can be observed in Fig. 5.

- (b) cotyledon- it also suffered some endosperm micro-structures alterations, when compared to Control (Fig. 5). Those kernels CP treated showed palisade broken (disrupted) cells which were mainly on their surfaces (membrane/wall), thus affecting the whole membrane integrity. Different to those of the CG group, that presented uniform and united regular palisade cells, with even membranes. Differences detected in the kernels before and after CP exposure analyzed by SEM was registered also by Grzegorzewski et al. (2011) on lettuce leaves using CP (Atmospheric Pressure). The author noticed erosions and ruptures in the epidermal tissue. In the research carried out by Thirundas et al. (2016) after exposure to CP (for 5 and 10 min, 30 and 40 W), SEM revealed cracks and depressions on the basmati rice surface. Ukuku et al. (2019) noticed changes in apples treated with a combination of antimicrobial and CP. Authors reported CP changed on the apple surface structures but not on bacterial cell structure.
- (c) germination: from the SEM micrographs, it was possible to observed that the structures responsible for the germination process, despite whether able to germinate or not, no CP tissues alteration were observed up to the SEM amplification applied [20 to 1000X] (Fig. 6). Therefore, the outer structures of plumule, radicle and hypocotyl were not CP affected (they remained intact). Probably to the fact that the Tip Plane CP did not reach strongly to the peripheric region of the kernels. However, as discussed at Section 3.2.c, the CP altered somewhat the germination capacity regarding the radicle length but no SEM micro-structures modifications were observed.
- (d) starch: no differences in the granular morphology of cotyledons starch texture were observed in the amyloplasts such as cracks and uneven surface when compared to control (Fig. 6). Although not observed differences on the morphology of the starch granule, some authors confer the effect of CP the formation of *cracks* and *cavities* in the starch (Thirumdas et al., 2016). Studies indicated that different CP reactors are able of altering the carbohydrate chemical composition (Chaiwat et al., 2016), the texture, cooking time and changes in the molecule structure (Thirumdas et al., 2016). This change on starches granules can be useful in the beans, for some characteristics when modified may have interesting consequences for the industry.

SEM is a technique that allows observing with details a material superficie, as foods. Besides structure alteration in beans kernels treated with CP, using this technique in this study, others aspects were registered, as absence of fungi

spores in samples. Hereby SEM was a tool very important in the current work.

CONCLUSION

The CP conditions applied (8 kv intensity, 510W power and time 5-30 min) showed potential to reduce the total fungal of the naturally contaminated beans exposed to the long time (20/30 min). Despite that, it affected some bean characteristics - cotyledons color and *testae* wrinkling (10-30 min), observed by SM and SEM. Regarding the CP application for beans during storage, germination inhibition in the time of 10-30 min can be an advantage, because it is not desirable (mainly on top of the silos). Other CP advantage is the to avoid of chemical fungicides application. The inactivation efficiency is dependent of several factors (the plasma generation source utilized, the parameters applied, samples type and microorganism physiological state) - therefore it is very important to take into account those factors when comparing CP application for different foods and/or literature studies. Further studies on CP effect on beans need to be carried out and also conditions adjusted to reduce sensory alterations.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support of Coordination of Improvement of Higher Level Personnel (CAPES) and National Council for Scientific and Technological Development (CNPQ) through the granting of research grants, the Federal University of Santa Catarina (UFSC) and the Laboratory of Mycotoxins and Contaminants for Food (LABMICO) for ceding their facilities for the development of this work and for Integrated Company of Agricultural Development of Santa Catarina (CIDASC) by the donation of the grains.

Authors' contributions

The authors Elisa Siegel Moecke and Vildes Maria Scussel correspondig the advisors this study (correction of work). The others one (Cristina Link Rüntzel, Juliana Regina da Silva, Bruna Aparecida da Silva) colobarrate doing the analyses, analyzing the data and writing the paper.

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