FUNGI DETECTION IN WEIGHT LOSS TEA

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RESUMO

Existem quatro diferentes tipos de chás provenientes da planta de origem asiática *Camellia sinensis*: o chá branco, o chá preto, o chá verde e o chá vermelho. A procura por fitoterápicos aumentou significativamente, já que estes chás podem ser recomendados para o processo de emagrecimento. O aumento nas vendas consequentemente promove aumento no volume de produção e de material armazenado. Quando manipulados e estocados de forma inadequada este produto pode ter um aumento no teor de umidade que, consequentemente, está associado a presença de fungos decompositores ou produtores de toxinas. Este trabalho tem como objetivo verificar a flora fúngica associada aos chás provenientes de *C. sinensis* comercializados em Campos dos Goytacazes, RJ. Isolamentos direto e indireto foram feitos a partir de fragmentos de quatro tipos de chás, encontrados no comércio local, em meio de cultura "Batata Dextrose Ágar – BDA". Também foi preparada câmara úmida das amostras. Foram isolados fungos dos quatro tipos de chás amostrados, sendo identificados os gêneros *Aspergillus*, *Curvularia*, *Fusarium*, *Monilia*, *Penicillium*, *Pestalotiopsis* e *Rhizopus*. No chá preto ocorreu maior incidência de fungos quando comparados aos outros três chás, embora tenha ocorrido colônias em todas as amostras. Foi possível verificar o afloramento de fungos quando se aumentou a umidade sobre os chás desidratados, indicando a importância na manutenção dos produtos em condições adequadas de armazenamento e nas suas respectivas embalagens para se evitar a deterioração com possível produção de micotoxinas por estes organismos comumente associados.

Palavras-chave: Plantas medicinais; micologia; endofíticos; deterioração de alimentos; micotoxinas.

ABSTRACT

There are four different types of teas from Asian origin plant *Camellia sinensis*: white tea, black tea, green tea and red tea. The demand for these herbal increased significantly since these teas are recommended for

those who are in weight loss process. With the increase in sales, as a result there was an increase in production and the volume of stored material. When handled and stored improperly this material, for the preparation of tea may have an increase in moisture content, which consequently is associated with the presence of bacteria or fungi decomposing and producing toxins. This study aims to determine the fungal flora associated with teas from *C. sinensis* marketed in Campos, RJ. Direct and indirect isolates were obtained from the four teas fragments in "PDA" culture medium for fungi. It was also prepared wet sample chamber. They were isolated fungi of the four types of teas sampled and noted the *Aspergillus, Curvularia, Fusarium, Monilia, Penicillium, Pestalotiopsis and Rhizopus*. In black tea showed a higher incidence of fungi when compared to the other three teas, although there was colonies in all samples. It was possible to verify the upwelling fungi when increased moisture on the dehydrated teas, indicating the importance of maintaining the products in their packaging in appropriate conditions to prevent deterioration with possible mycotoxin production by these organisms commonly associated.

Keywords: Medicinal plants; mycology; endophytic; food spoilage; mycotoxins.

1. INTRODUCTION

Medicinal plants can be used in different ways and for different purposes, even in natura, with whole parts or other forms for the preparation of tea and/or other homemade preparations. The teas has many biologically active compounds, such as flavonoids, catechins, polyphenols and alkaloids that can contribute to the prevention and treatment of various diseases and when added to the diet trigger metabolic or physiological processes in the body (SAIGG & SILVA, 2009).

Teas have long been used for weight loss, especially green, white and black teas. These teas are all derived from the same plant species, *Camellia sinensis* (L.) Kuntze, a large tree from Theaceae family, whose variation in the types of teas mentioned above, would be the way they are harvested and processed (PAGANINI-COSTA & CARVALHO DA-SILVA, 2011)**.**

As the demand for this type of material is large, the demand for production and marketing also increase. During normal production processes (drying, packaging and storage) and distribution, these products are not strictly subjected to an appropriate control in order to quality. In this context the microbiological control could be highlighted, whose purpose is to analyze the contamination by microorganisms, including filamentous fungi (RUSSOMANO & KRUPPA, 2009; MUSSI-DIAS, *et al.*, 2012).

Fungi can colonize leaves, branches and roots, without causing harm to host (PEIXOTO NETO *et al.,* 2003). These organisms belonging to Fungi Kingdom are eukaryotic and reveal remarkable adaptability and growth under humid conditions and extremely variable temperatures. As these microorganisms are not very demanding in relation to existing nutrients in the medium, can occur on almost any type of substrate or food (ALEXOPOULOS *et al.,* 1996).

Fungi can colonize leaf tissue and produce mycotoxins, which cover a group of secondary metabolites derived from certain species of fungi and whose intoxication is called mycotoxicosis. The contaminating microorganisms are usually from soil, water and air. Secondary contamination can still occur due to inadequate farming practices, and storage (SANTOMI *et al*., 2005). These metabolites are chemically different and may be contained within the spores in their mycelia, or be released in food colonized with these microorganisms (RUSSOMANO & KRUPPA, 2009). Thus, the aim of this work was to detect the fungal flora associated with samples of four teas for weight loss, available in supermarkets, and infer the health quality of these products.

2. MATERIALS AND METHODS

2.1 Collection and storage of samples of teas

For the research development we choose the tea plant from *Camellia sinensis* species, sold under four different ways, depending on the process used in its manufacture, as follows: green tea, black tea, white tea and red tea. Each type of tea, was separated and acquired in commercial establishments authorized for marketing these products in the municipality of Campos Goytacazes, RJ. After the samples acquisition, they were kept under recommended storage conditions, according to the indications for this type of product, in other words, in its original packaging, sealed, in a cool, dry and under cover out of direct sunlight.

2.2 Fungal Detection externally associated to teas

Each identified sample was analyzed for the detection of fungi associated externally to fragments of teas. For this purpose, about 10 g of the fragments was weighed, and mixed in becker, with 90 mL of sterile distilled water, constituting an inventory suspended 1:10 (p/v). The samples were shaken in a magnetic plate for a 20 minutes period. Thereafter, it proceeded to serial dilutions by adding 1 ml of this stock solution to 9 ml of sterile distilled water in a test tube, constituting a 1:10 dilution or 10^{-1}. From this dilution, 1 ml was removed and added to 9 mL of sterile distilled water in another test tube, constituting a dilution of 1: 100 or 10^{-2}, and so on until a dilution of 1: 10,000 or 10^{-4}. About 0.1 ml of each obtained dilution was seeded on the surface of petri plates containing PDA culture medium (potato, dextrose and agar) plus streptomycin sulfate and spread with the aid of Drigalsk handle, resulting a total of four plates for each dilution (repetitions). For statistical analysis it performed analysis of variance and comparison of averages of evaluations carried out for the amount of found fungal colonies. The treatment means (Teas) were compared by Tukey test at 5% probability. For statistical analysis we used the R program (R CORE TEAM, 2015), and the Agricolae package (MENDIBURU, 2014). The plates were kept at room temperature (approximately 26 ° C) and photoperiod of 12 hours light for five days.

2.3 Fungal Detection internally associated to teas

Fresh samples from the four commercial teas, taken to the stock suspension were used to detect fungi in plant tissues. With the use of forceps, the fragments have undergone a process of sequential surface disinfection, which were submerged in alcohol 70 % (1 minute), sodium hypochlorite solution 1 % (1 min, and sterile distilled water (2 times). Excess water was absorbed by sterile filter paper and each fragment was seeded in petri dishes containing PDA culture medium and streptomycin sulfate, which were maintained at the same temperature and light system described above, for 7 days. For each sample four repetitions were made, which received 5 of tea fragments, arranged equidistant from each other, totaling 20 pieces/sample.

2.4 Fungal Detection by moist chambe

Samples with about 50 g of each tea was packaged in a plastic box, suitably disinfected, moistened by spraying with sterile distilled water, in order to maintain saturated humidity (above 90 %). Similarly, four other samples were packaged, but without wetting, serving as assay control. The boxes were hermetically sealed and the samples kept for 48 to 72 hours in that condition until the time of assessment.

2.5 Subcultures , maintenance of the colonies and fungi identification

The isolated fungi from samples both of suspensions as the fragments derived from teas were individually subcultured to test tubes containing culture medium "PDA" inclined. After the growth and/or sporulation the tubes were kept at 7 ° C in refrigerator until the end of the experiments, if there were need to use new subcultures.

Petri dishes containing “PDA” isolates were used for storage of fungi. For this purpose, 0.6 cm diameter disks were obtained from the colonies with the aid of a cork borer. The discs were then submerged

in sterile distilled water (Castellani method) in “penicillin bottles”, sealed with rubber stopper (ALFENAS & MAFIA, 2007) and deposited in mycology collection from Laboratório de Química e Biomoléculas (LAQUIBIO) of the Institutos Superiores de Ensino do Censa (ISECENSA).

Preparations of each isolate structures were mounted on slides containing lactophenol with or without dyes, in order to verify the best contrast and visualization of structural morphology of each species (FIGUEIREDO et al., 2013). Semi-permanent slides were observed under a light microscope with objective 10 to 40x.

The identification was carried out at the gender level by comparisons of the structures formed, which were related to the phyla Ascomycota, Zigomycota, Basidiomycota and "Deuteromycota" (Mitosporic Fungi) from Fungi Kingdom. (HANLIN & MENEZES,1996; CANNON,1991; BARNETT & HUNTER, 1998; SUTTON,1980 and others).

3. RESULTS AND DISCUSSION

3.1 Fungal Detection externally associated to teas

All teas samples presented themselves visually fit for human consumption, within their original packaging cannot be detected with the naked eye or under a microscope stereoscopic the presence of contaminating organisms or decomposers. When placed in suspension was possible to detect the fungal flora associated with samples. All four teas had adhered to fungi fragments, whose quantification of the number of colonies was held in lower dilution (10^{-1}) for all of them (Figure 1).

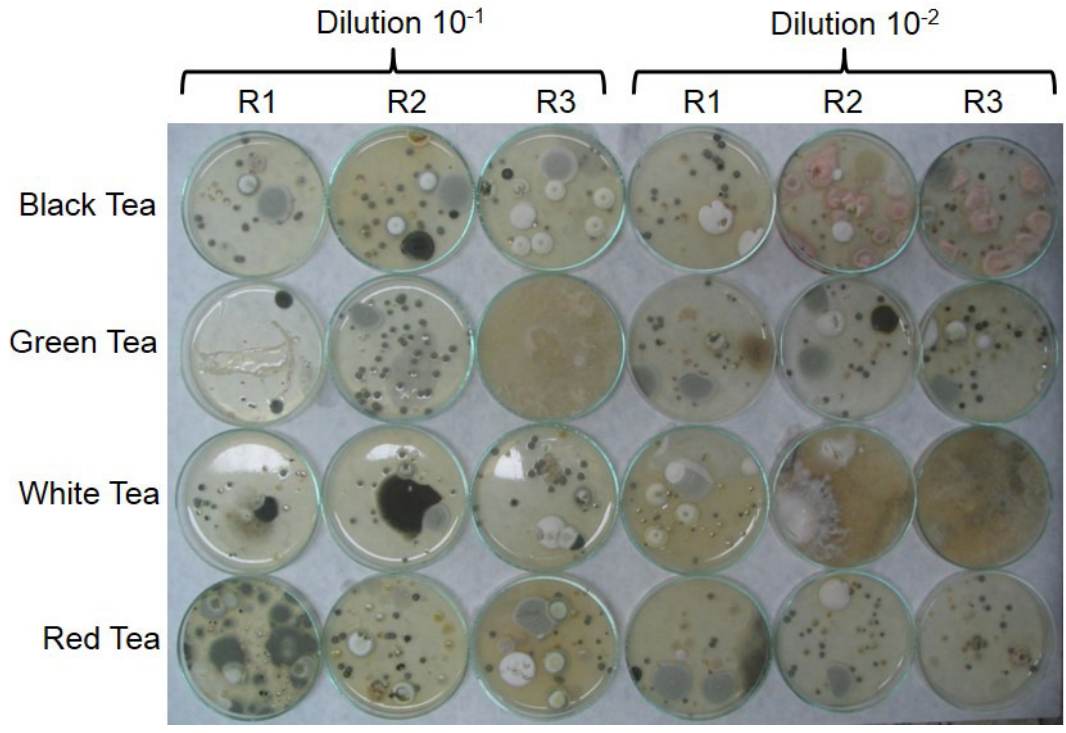

Figure 1. Fungal Colonies in “PDA” medium, from serial dilution of suspension containing teas fragments marketed in Campos, RJ-Brazil.

Although there was some variety of fungal genera recovered in both dilutions 10^{-1} provided less variation between 30 and 300 colonies, which is a threshold generally used for the calculation of colony forming units/g (CFU) of the used material.

According to the averages of test performed (Figure 2), the amount of fungi was significantly higher for black tea sample, red tea, white and green did not differ statistically from themselves. The results of CFU/g (Table 1) were compared with those obtained by Rocha *et al*. (2013), who used to limits set by the World Health Organization. Thus, it was possible to frame our results within those lower limits recommended for herbal medicines, equivalent to 104 CFU/g (WHO, 2007). Thus, it is suggested that the serial dilution method, the water used in the solution is heated to the temperature which is used for preparing infusions or decoctions, and the time elapsed to perform plating the samples in the culture medium is, at least the recommended time for the preparation of each type of tea. Probably, the number of formed colonies tend to be lower due to the deleterious effect of high temperatures to fungal structures in the material used to make the tea.

Table 1. Colony forming units (CFU) obtained from serial dilution (10^{-1}) aqueous suspension containing tea fragments marketed in Campos dos Goytacazes, RJ

Rep. \ Sample*	Black Tea	White Tea	Red Tea	Green Tea
1	$4{,}5 \times 10^3$	$3{,}0 \times 10^3$	$3{,}4 \times 10^3$	$3{,}2 \times 10^3$
2	$4{,}3 \times 10^3$	$3{,}5 \times 10^3$	$3{,}6 \times 10^3$	$3{,}3 \times 10^3$
3	$4{,}1 \times 10^3$	$3{,}4 \times 10^3$	$3{,}5 \times 10^3$	$3{,}1 \times 10^3$
Average	$4{,}3 \times 10^3$	$3{,}3 \times 10^3$	$3{,}5 \times 10^3$	$3{,}2 \times 10^3$

* Samples in stock suspension obtained from 10 g of tea in 90 ml of sterile distilled water.

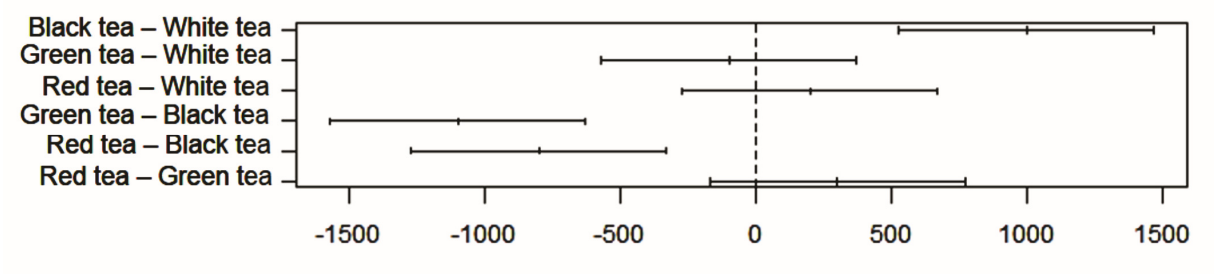

Figure 2. Confidence intervals that do not include zero indicate that couples have significant differences at 5 % probability by Tukey test.

Even black tea as red tea brewing process does not eliminate, in first time, the presence of fungi. Once fermentation is not a disinfection or sterilization process, the presence of fungi was expected, at least some genres more resistant to the process steps. Since there was no materials evaluation in the field or at the preparation and tea storage, it is unlikely that one can define precisely the origin of these fungi. In other words, if they occurred in association with *C. sinensis* in producing fields, infested, or infected plant material before, during or after the preparation of the product to be marketed.

3.2 Fungal Detection internally associated to teas

All teas samples showed fungi from the interior of the fragments, in other words, fungi that were probably colonizing the internal plant tissues (Figure 3). Although it has not quantified the obtained colonies were isolated all fungal genera from the fragments used in isolation.

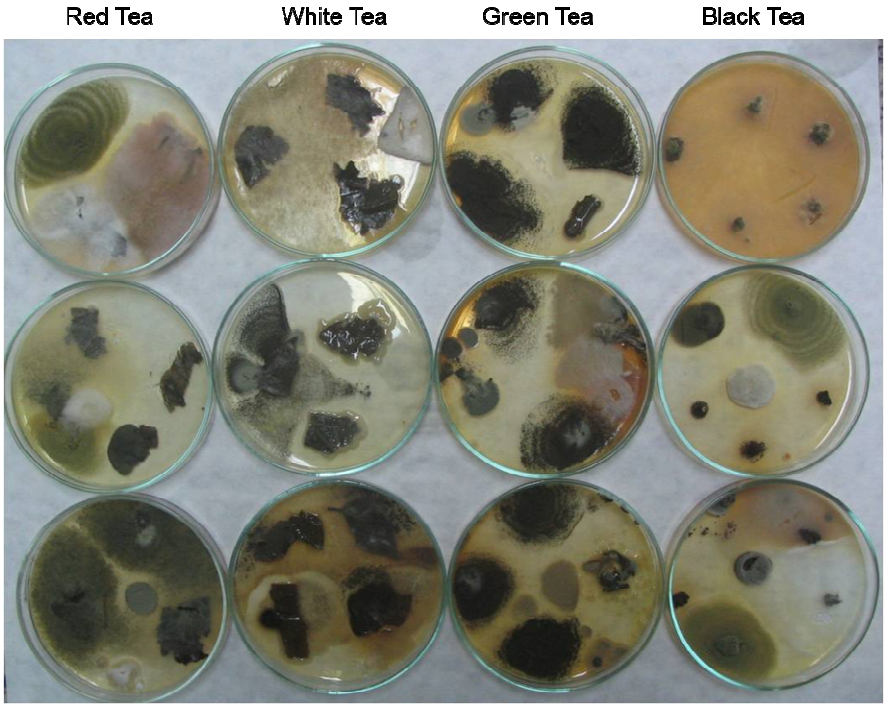

Figure 3. Fungal isolation in "PDA" culture medium, from teas fragments after surface disinfection

The presence of these organisms inside the fragmets of the marketed product indicates at first that the fungi may have infected/colonized the plants still in the field of culture or during harvesting, drying, storage and marketing. In all cases, the fungus requires a small percentage of humidity for the occurrence of spore germination and the infection process occurs. Even though these fungi are not pathogenic to the four teas plant species, they may have colonized the plants during the plant growth, without causing any obvious signs of its presence. In this case, we call these type of fungi as endophytes and simply occupy an ecological niche that is exactly inside the plant. Therefore, when we harvest a vegetable or part of it, we are also harvesting endophytic fungi associated to the material (MUSSI-DIAS & FREIRE, 2013). In processing plant for commercialization, the reduction in moisture also reduces the fungal metabolism, being this surviving in dehydrated or remain latently material until favorable conditions for its development to occur again, in other words, increase mainly moisture tea. Even if this material is in a dry and cool environment, a small moisture content other than that necessary to maintain the dry tea can trigger the onset of fungus growth. With this growth and a possible temperature increase within the package itself, provided by metabolic activity in that environment, it begins a tea degradation process with consequent loss of quality. In some cases may occur production of toxic compounds mycotoxins produced by several species of fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, *Myrothecium*, causing serious problems to human and animal health (MALLOZZI & CORRÊA, 1998).

The mycotoxins control through detoxification techniques has been done with relative success in some parts of the world. However, prevention through good agricultural practices is still the best way to get around

the problem. This prevention can be made even with the plant in the field, through proper crop driving, providing water conditions, fertilization and other cultural practices that enable plants a normal and healthy development; keeping aseptic during harvest; rapidly processing the collected material in order to prevent mold or saprophytic colonization by fungi; maintaining the curing warehouses and fresh air circulation between the greenhouse and plants or by using appropriate time to remove the moisture content required, thus preserving the quality of the product to be market supplied.

3.3 Fungal Detection by moist chambe

All four samples of teas kept in moist chamber presented fungi, mainly Aspergillus sp. For large volume of fungus surface growth, associated with a rapid samples colonization, it was not possible to distinguish most species present in teas. Regardless of the number of colonies or touched on species, the mere humidity presence above normal storage s provided the accelerated development of these contaminants. Accordingly, product handling at any stage of dehydration must be maintained; otherwise, it starts a process of decomposition with possible loss of quality and probably the mycotoxins production.

3.4 Subcultures, maintenance of the colonies and fungi identification

All fungal isolates obtained from both straightforward as indirect sampled from four types of teas were transferred and maintained in test tubes containing “PDA” culture medium tilted (Figures 4 and 5). A hydrophobic cotton swab, in the pipe end was efficient air filter of particles and other contaminants from the external environment, since when the absorbent cotton was used there was tubes contamination. Each tube was then sealed with parafilm, placed in plastic bags and stored at approximately 7°C in refrigerator. This storage technique was feasible in maintaining the successive cuttings of work during the test conduct. So it was avoided peaking isolated already definitively stored by Castelani method (in sterile distilled water).

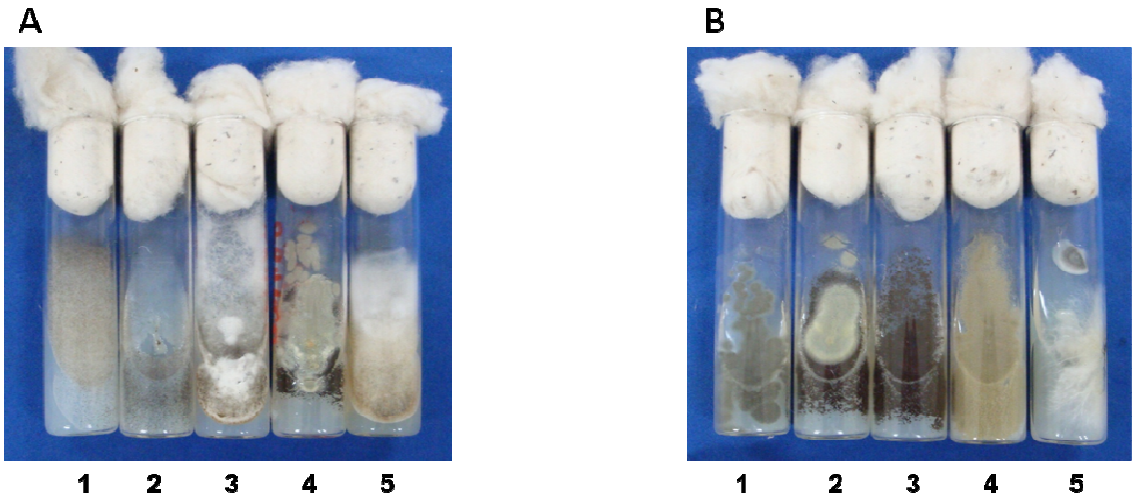

Figure 4. Isolated fungi from "in natura" fragments of Red Tea (A) and Black Tea (B) sold for consumption.

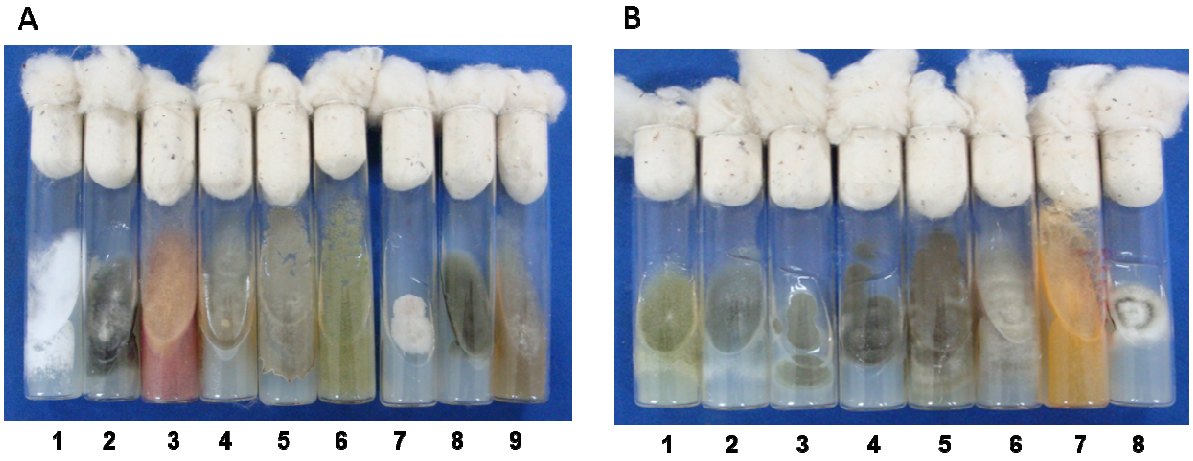

Figure 5. Isolated fungi from "in natura" fragments of White Tea (A) and Green Tea (B) sold for consumption.

The fungi identified to genus are somewhat fungi commonly seen on the environment and marketed food samples (Table 2). All four types of teas showed fungi belonging to group of toxins producers. Although there have been external and internal contamination of the fragments, it is difficult to determine at what stage there was a product predisposition to fungi. The recovered genres may be considered endophytic, being already present in the plant at harvest act, or have colonized upon contact at any time from the harvest, transport, processing, storage and finally during marketing.

The fungi presence in tea has been studied very little when one takes into consideration the amount of plant products marketed for this purpose. We can highlight the work done by Russomanno & Kruppa (2009) on the fungi occurrence in teas and herbs. These authors observed that among various recovered fungi, species as *Aspergillus* e *Penicillium* showed high percentages of occurrence in sampled material, furthermore concluded that the method of infusion, usually as preparing teas, not completely eliminated the fungi toxigenic, only decrease its occurrence.

Table 2. Fungi isolated from teas fragments marketed in Campos , RJ

Samples*	Fungi**
Black Tea	*Aspergillus* sp.1; *Aspergillus niger*; *Penicillium* sp.; *Curvularia* sp.; *Fusarium* sp.; *Monilia* sp.; *Pestalotiopsis* sp.; Nonsporulating hyphae
White Tea	*Aspergillus niger*; *Penicillium* sp.; *Fusarium* sp.; *Rhizopus* sp.
Red Tea	*Aspergillus niger*; *Aspergillus* sp.2; *Aspergillus* sp.3; *Penicillium* sp.; *Monilia* sp.; *Curvularia* sp.; Nonsporulating hyphae
Green Tea	*Aspergillus niger*; *Aspergillus* sp.4; *Aspergillus* sp.5; *Aspergillus* sp.6; *Penicillium* sp.; *Fusarium* sp.

* = disinfestation by indirect isolation method in 70% alcohol, 1% sodium hypochlorite and sterile distilled water; ** = growth in "PDA" culture medium

4. CONCLUSIONS

No matter the type of processing used to put in market the four types of teas studied, there is presence of fungi associated with marketed products, many of whom are potential producers of mycotoxins. Any increase in moisture content above the recommended standards, storage or packaging, can enable the development of fungi present in teas and as a result, change the sanitary quality of the product.

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