COMMUNITY OF CULTIVABLE ENDOPHYTIC FUNGI OF CASSAVA *Manihot esculenta* CRANTZ

Comunidad de hongos endofíticos cultivables de yuca Manihot esculenta Crantz

Revista Desafios

Artigo Original
Original Article
Artículo Original

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Artigo recebido em 05/08/2020 aprovado em 21/10/2021 publicado em 28/04/2022.

ABSTRACT

The objective of this work was to characterize the community of endophytic fungi of cassava *Manihot esculenta* cultivated in the state of Acre, Brazil. For that, *M. esculenta* root, stem and leaf were collected, washed and disinfected by immersion in 70 % ethanol (1 min), sodium hypochlorite 2 % (5 min), 70 % alcohol (30 sec) and washing in water sterile distilled (1 min) three times. The samples were fragmented and inoculated in BDA and Oat culture media and incubated at 28 ºC for 30 days. The isolated fungi were characterized by morphology and grouped into morphospecies. There was isolated a total of 39 endophytic fungi from *M. esculenta*, 19 (48.7 %) of stem 13 (33.3 %) of leaf and 7 (18 %) root. On culture medium, 23 (59 %) fungi were isolated in BDA medium and 16 (41 %) in Oat medium. *Penicillium* and *Phomopsis* were the most frequent genera, with 30.8% each, followed by *Fusarium* (10.2 %), *Aspergillus* (5.1 %), *Guignardia* (5.1 %), *Acremonium* (2.6 %), *Colletotrichum* (2.6 %), *Phoma* (2.63 %), and unidentified (10.2 %). This is the first study report of the endophytic fungi community of *Manihot esculenta.*
Key Words: Leaf, *Penicillium, Phomopsis*, Root, Stem.

RESUMEN

El objetivo de este trabajo fue caracterizar la comunidad de hongos endofíticos de yuca Manihot esculenta cultivados en el estado de Acre, Brasil. Para ello, la raíz, el tallo y la hoja de M. esculenta se recogieron, lavaron y desinfectaron por inmersión en etanol al 70 % (1 min), hipoclorito de sodio al 2 % (5 min), alcohol al 70 % (30 seg) y lavado en agua destilada estéril (1 min) tres veces. Las muestras se fragmentaron e inocularon en medios de cultivo BDA y avena y se incubaron a 28 ºC durante 30 días. Los hongos se caracterizaron por la morfología y se agruparon en morfoespecies. Se aisló un total de 39 hongos endofíticos M. esculenta, 19 (48, %) del tallo 13 (33,3%) de la hoja y 7 (18 %) de la raíz. En medio de cultivo, se aislaron 23 (59 %) hongos en medio BDA y 16 (41 %) en medio avena. Se identificaron ocho géneros de hongos. Penicillium y Phomopsis fueron los géneros más frecuentes, con un 30,8 % cada uno, seguidos de Fusarium (10,2 %), Aspergillus (5,1 %), Guignardia (5,1 %), Acremonium (2,6 %), Colletotrichum (2,6 %), Phoma (2,63 %) y no identificado (10,2 %). Este es el primer informe de un estudio de la comunidad de hongos endofíticos de Manihot esculenta.
Descriptores: Hoja, *Penicillium, Phomopsis, Raíz, Tallo.*

INTRODUÇÃO

Cassava is a plant of the Euphorbiaceae family of the genus *Manihot*. This genus has several species, with economic highlight *Manihot esculenta* Crantz (DA SILVA and DO AMARAL, 2020). *M. esculenta* is one of the most important crops in world agriculture, the basis for the manufacture of food and feed in the agribusiness. The most consumed part of the plant is the root, staple food, source of carbohydrate for more than 2 billion people in the world, mainly in underdeveloped countries (FERRARO et al., 2016). In addition, cassava has a social function such as reducing poverty and reducing rural exodus, it has simple cultivation and requires few resources. (LOBO et al., 2018). Due to its social importance, this crop stands out in the agricultural economy due to its multitude of industrial uses such as the commercialization of the starch produced (SILVA et al., 2018).

According to statistical data from the United Nations Food and Agriculture Organization, the cultivated area of cassava is responsible for occupying 24.5 million ha worldwide (FAO, 2018). Brazil alone is responsible for 1.2 million ha of cultivated area, equivalent to a total production of approximately 176 thousand tons per year of this root (FAO, 2018). These numbers represent the greatness of this culture to the world, especially Brazil, which is fundamental for food security in low-income families and a guarantee of livelihood for small farmers (LOBO et al., 2018).

M. esculenta is the focus of studies that allow the advancement of its cultivation technology, essential for solving agricultural problems that involve this culture. The agricultural limitations of cassava mainly include the physiological deterioration of its leaves and roots, caused by phytopathogenic microorganisms including, viruses, bacteria and fungi (MORAIS et al., 2013). The phytosanitary problem generates very low crop yields, some diseases generate losses of up to 95 %, as in the case of plants affected with Cassava Mosaic (ANDRADE and LARANJEIRA, 2019). Examples of diseases caused by bacteria include bacterial burning, spotting, rotting and wilting. Fungal diseases include anthracnose, leaf spot, over-stretching of cassava, rust and stem rot (MCCALLUM et al., 2017).

Thus, sustainable alternatives to control these diseases are required, for economic reasons and for the food security that cassava provides. In this context, the knowledge of the community of microorganisms that live in symbiotic association with *M. esculenta* becomes essential for the search for new technologies (DE SILVA et al., 2019). Potential microorganisms include endophytic fungi, where the study of this community in cassava can be a source of active molecules for the production of biofertilizers, toxic to the target organism and without producing environmental waste (SEGARAN and SATHIAVELU, 2019).

Endophytic fungi symbiotically inhabit the internal region of plant tissues, without causing apparent damage to the host, living part of its life cycle in association with the plant (AZEVEDO et al., 2000). Endophytes establish beneficial relationships to the plant, help in adapting to different environments, with different temperatures, and promote states of tolerance to stress, such as pH fluctuation, high levels of salinity and heavy metals, herbivory and mainly against pests and diseases through produced metabolites (BILAL et al., 2020; PETERS et al., 2020; GOPANE et al., 2021; SOPALUN et al., 2021). Thus, they can act not only in the control of pests and diseases, but also in technological areas of agriculture, the basis for sustainable development, producing *ex situ* technology for culture.

Among the endophytic fungi with proven biological activity for the control of pests and diseases, it is important to mention the genera *Colletotrichum*, *Phomopsis*, *Penicillium*, *Trichoderma*, *Fusarium*, and

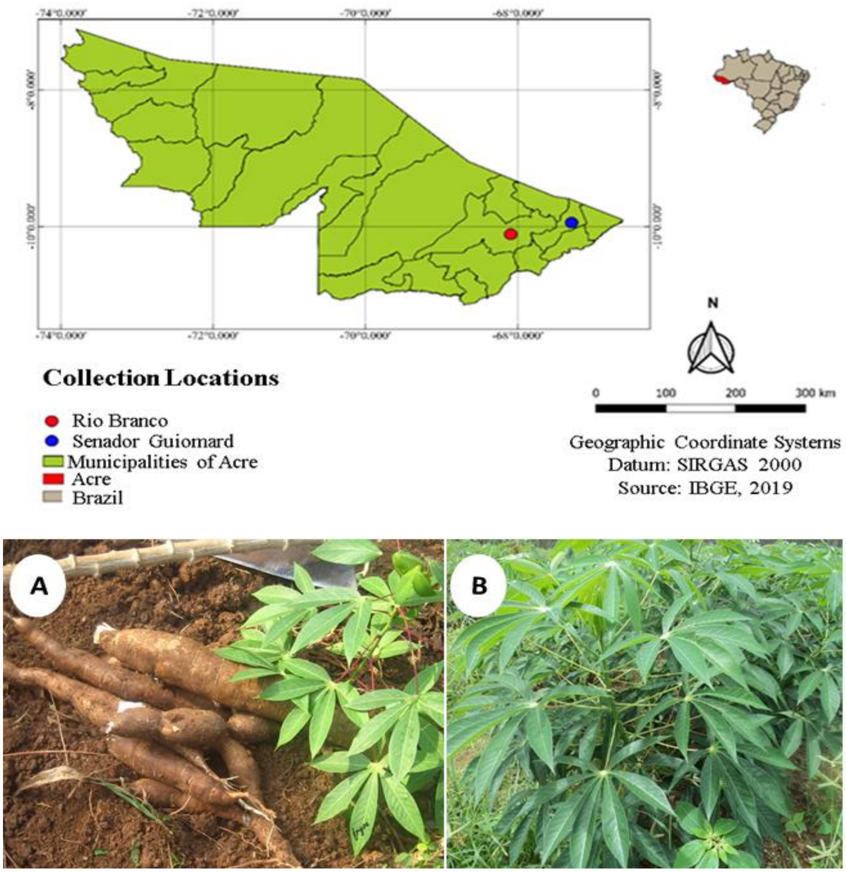
(LARRAN et al., 2016; TOGHUEO et al., 2016; PETERS et al., 2020). Despite the fungi strains reported in other cultures, there are no reports in the literature about the community of *M. esculenta* endophytic fungi, which represents an important source of microorganisms with potential to act in different technological areas of cassava cultivation, especially in the problems phytosanitary (SEGARAN and SATHIAVELU, 2019). Thus, the objective of this work was to characterize the community of endophytic fungi of cassava *Manihot esculenta* cultivated in the state of Acre, Brazil.

MATERIAL AND METHODS

Material Collection

Two specimens of de *Manihot esculenta* were collected in areas of sustainable production in the cities of Rio Branco and Senador Guiomard, state of Acre, Brazil (Figure 1).

Figure 1. Location map of *Manihot esculenta* collection sites. **A.** Root; **B.** Aerial part.

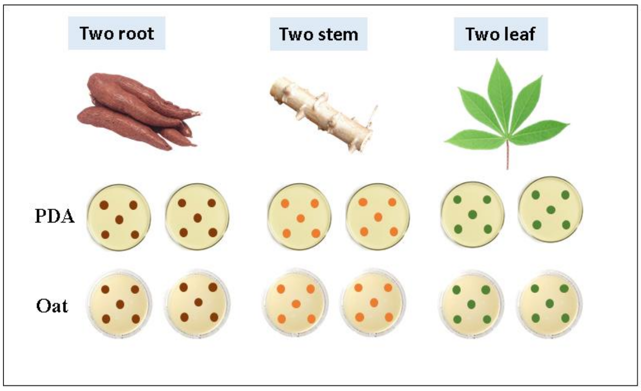


Isolation of Endophytic Fungi

Two branches of *M. esculenta* (1 branch per plant) containing healthy leaves and stems and two roots (one per plant) were collected. For each plant, 60 fragments were used, taken from samples of roots, stems and leaves (Figure 2). The plant material was washed with a sponge and detergent under running water to remove solid residues and epiphytic microorganisms. After washing, the material was subjected to surface disinfection by immersion in 70 % ethanol (1 min), 2 % sodium hypochlorite (5 min), 70 % alcohol (30 sec) and washing in sterile distilled water

(1 min) three times. Subsequently, 20 fragments were used for each plant tissue, 10 fragments inoculated in PDA culture medium (potato-dextrose-agar) and 10 in oat culture médium (Figure 2). Each disinfected fragment obtained a diameter of 5 mm and was inoculated into the culture medium with chloramphenicol antibiotic (100 mg. L-1), and the plates incubated at 28 ºC for 30 days (ARAÚJO et al., 2010).

Figure 2. Isolation of endophytic fungi from the root, stem and leaves of *M. esculenta* in PDA and Oat culture médium



Fungal colonies with distinct characteristics according to macroscopic observations (staining and growth) were purified using the streak depletion technique in Petri dishes with PDA culture medium and incubated for 48 h. The purified fungal colonies were inoculated in tubes with inclined PDA medium (ARAÚJO et al., 2010), and the fungi were preserved in distilled water (CASTELLANI, 1963) and mineral oil (BUELL and WESTON, 1947).

Morphological Characterization

The fungi were organized into morphospecies according to the characteristics of the colony, such as color, texture and pigment production. One representative of each morphospecies was used for micromorphological identification. To make the microculture, the fungi were inoculated in 1 cm^2 cubes of PDA and Oat medium and covered with coverslips, inside a Petri dish. The plates were incubated at room temperature for 7 days for mycelial growth and the coverslips stained with lactophenol blue for visualization of reproductive structures under an optical microscope (LACAZ et al., 1998; BARNETT and HUNTER, 1999).

Data analysis

To calculate the total frequency of isolates and genera by plant tissue and culture medium, the numbers obtained in each treatment were divided by the total number of isolates multiplied by one hundred, with the aid of the Excel program. GraphPad Prism 5.0 was used to make figures.

RESULTS

A total of 39 endophytic fungi were isolated from *M. esculenta*, 19 (48.7 %) stem, 13 (33.3 %) leaf and 7 (18 %) root were isolated. About the culture medium, 23 (59 %) were isolated in PDA medium and 16 (41 %) in Oat medium (Tabela 1)

Table 1. Relative frequency of endophytic fungi isolated from *Manihot esculenta* according to plant tissue and culture medium

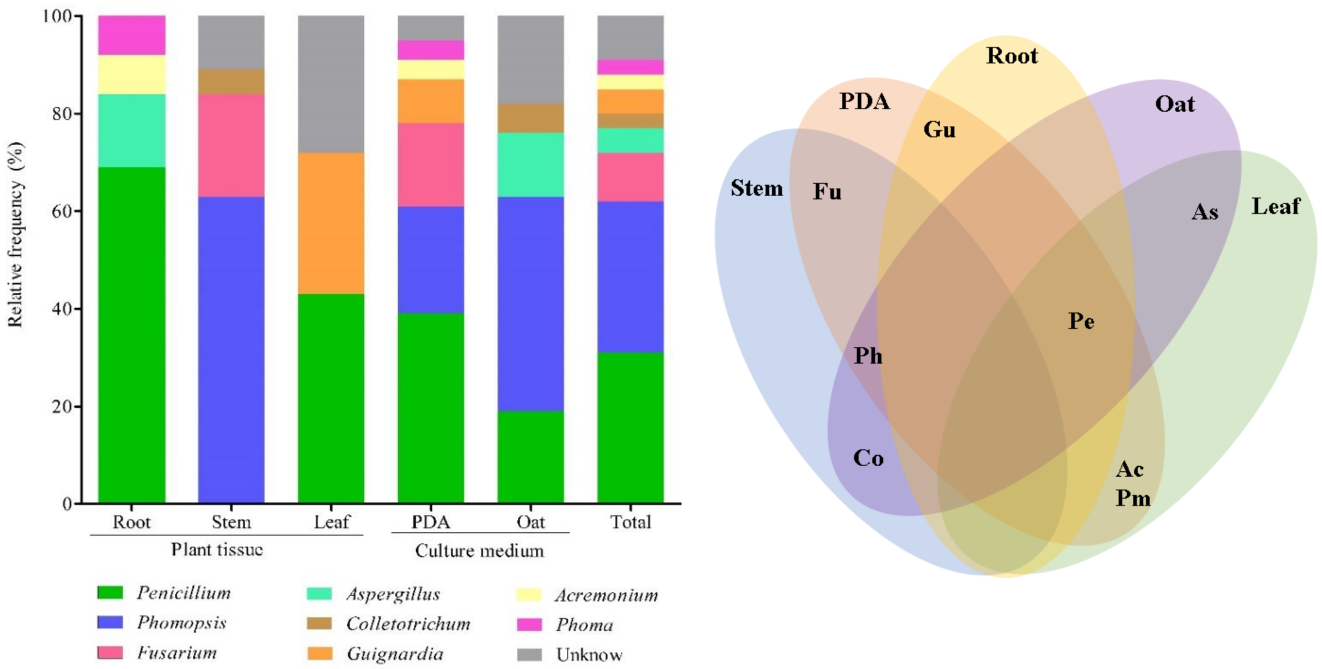
Table 1.	**Plant Tissue**			**Culture medium**		**Total**	**RF (%)**
Relative	Stem	Leaf	Root	PDA	Oat		
Phomopsis	12	-	-	5	7	12	30.8
Penicillium	-	9	3	9	3	12	30.8
Fusarium	4	-	-	4	-	4	10.2
Asperaillus	-	2	-	-	2	2	5.1
Guignardia	-	-	2	2	-	2	5.1
Colletotrichum	1	-	-	-	1	1	2.6
Acremonium	-	1	-	1	-	1	2.6
Phoma	-	1	-	1	-	1	2.6
Unknown	2	-	2	1	3	4	10.2
Total	**19**	**13**	**7**	**23**	**16**	**39**	**100**
RF (%)	**48.7**	**33.3**	**18.0**	**59.0**	**41.0**		

RF = Relative Frequency; PDA = Potato-Dextrose-Agar

35 fungi (89.7 %) were identified, distributed in eight genera. *Penicillium* and *Phomopsis* were the most frequent, with 30.8 % relative frequency each, followed by *Fusarium* (10.2 %), *Aspergillus* (5.1 %), *Guignardia* (5.1 %), *Acremonium* (2.6 %), *Colletotrichum* (2.6 %), *Phoma* (2.6 %), and not identified fungi (10.2 %) (Figure 3A). The presence of generalist and species-specific fungi was also observed (Figure 3B)

Figure 3. Frequency of endophytic fungi of *Manihot esculenta*. A) Frequency of endophytic according to plant tissue and culture médium. B) Venn diagram showing the intersection of the isolation conditions of *M. esculenta* endophytic fungi. Ph: *Phomopsis*; Pe: *Penicillium*; Fu: *Fusarium*; As: *Aspergillus*; Gu: *Guignardia*; Co: *Colletotrichum*; Ac: *Acremonium*; Pm: *Phoma*.

A **B**



DISCUSSION

Maniohot esculenta demonstrated in the present study to have a rich community of endophytic fungi. 39 fungi were isolated, and stem was the plant tissue with the highest frequency of isolates (48.7 %). Variations in the rate of colonization between plant tissues are commonly described in the literature, and can be directly affected by abiotic and biotic factors, plant age, seasonality, ecological conditions and geographic distribution (FAETH et al., 2002; OKI et al., 2009). In addition, the anatomy of the stem also favors colonization, as the stem contains specific substances, such as bisabolol, which has a protective function to the fungal community (OTERO et al., 2002).

Some studies corroborate our findings, such as the endophytic fungi of *Oenocarpus bacaba* and, *Mangifera indica* which also showed higher rates of stem colonization (DASHYAL et al., 2019; DINIZ et al., 2020). Despite the greater stem isolation in this work, any plant tissue can be used in the process of isolating endophytic fungi.

Another factor that plays a decisive role in the isolation of endophytic fungi is the culture media used. This work revealed that in the BDA medium there was a higher frequency of fungal isolation in relation to the Oat medium, totaling (59 %). This is the most used culture medium for isolating endophytic fungi and was used to isolate fungi from *Croton lechleri* (VARGAS et al., 2018), *Polygonum hydropiper* (YE et al., 2019), *Euterpe precatoria* (PETERS et al., 2020) and *Oenocarpus bacaba* (DINIZ et al., 2020). It is important to offer different nutritional conditions to simulate conditions similar to the niche where the fungi originated. Thus, the culture medium can be a determining factor in the variation of isolated morphospecies (CARNAÚBA et al, 2007)

Some fungal genera in the present study were generalists or specifists for the conditions in which they were isolated. Regarding the culture media, only *Phomopsis* and *Penicillium* were generalists, the others being specific to a type of media. As for plant tissue, only Penicillium colonized two tissues, leaf and root. Both fungi were the most frequent (*Penicillium* (30.8 %) and *Phomopsis* (30.8 %)), which colonization is reported in numerous species and plant tissues (VARGAS et al., 2018; WU et al., 2020).

Penicillium is a promising fungal genus for agriculture, has shown numerous benefits, promoting disease resistance and production of plant hormones in inoculated plants, such as *Penicillium citrinum* (WAQAS et al., 2015). Vegetative hormones, such as gibberellins and indole-3-acetic acid and solubilization of inorganic phosphate produced by endophytes of the genus *Penicillium*, biologically impact plants, even in stressful conditions, favoring assimilation of nutrientes (GÓMEZ-MUÑOZ et al., 2018; BILAL et al., 2018). On biological control of diseases, has promising results, such as inducing resistance in banana trees to *Fusarium* wilt, inhibiting the growth of pathogens that cause root rot *in vitro*, such as *Alternaria panax*, *Fusarium* sp. *Fusarium oxysporum*, *Fusarium solani*, *Phoma herbarum* and *Mycocentrospora acerine* (TING et al., 2012; ZHENG et al., 2017; ROJAS et al., 2020).

Phomopsis had the same colonization frequency as *Penicillium* in *M. esculenta*. This fungal genus is known to produce secondary metabolites of importance for agriculture, producing compounds such as dothiorelones A – C and H and cytosporones C and U with antiviral activity against the tobacco mosaic virus, in addition, the crude extract of a purified exopolysaccharide had strong inhibitory effects against infection with this virus (TAN et al., 2017). They also have polyketide-producing genes, a source of promising bioactive metabolites in the pharmaceutical industry (RAO and SATISH, 2015; SONG et al., 2017; QU et al., 2020).

Fusarium is one of the most studied fungi in the literature. It is known to cause great damage in agriculture, so the studies are focused on the control of the pathogen, which causes fusariosis (NEFZI et al., 2019; BÁEZ-VALLEJO et al., 2020). However, a study reported the species *Fusarium solani* producing the vegetable hormone ethylene, which acts to protect tomatoes against soil fungal pathogens. (GARANTONAKIS et al., 2018). Furthermore, it is a promising genus for the biological control of nematoids, to which the species Fusarium oxysporum appears nematicidal activity to *Radopholus similis*, one of the main banana pests in the world (NIERE et al., 2006; KUMAR; DARA, 2021).

The endophytes *Aspergillus* and *Guignardia* presented 5.1 % isolation frequency each. *Aspergillus* is a cosmopolitan fungus of industrial interest, especially for agriculture. Production of plant hormones such as gibberellins and production of isoflavonoids has been reported, in addition to promoting plant growth under saline stress (KHAN et al., 2011; LUBNA et al., 2018). It has also provided biological control, such as the defoliating caterpillar (*Spodoptera litura*) by the production of extracellular enzymes (ELANGO et al., 2020). *Guignardia* has few studies for biotechnological applications in agriculture, however they demonstrate the presence of secondary active metabolites such as meroterpenes, derived from dioxolanone, ergosterol and cyclo- (Phe-Pro) with proven anti-fungal activity (FENG-WU, 2012; LI et al., 2015; CHEN et al., 2019).

The *Acremonium* (2.6 %), *Colletotrichum* (2.6 %) and *Phoma* (2.6 %) genera had the lowest isolation rates. *Acremonium* is an important fungus that produces extracellular enzymes, such as cellulases, chitinases and xylanases and may have industrial and agricultural applications (MARQUES et al., 2018; CHUNG et al., 2019). It also has potential for biological control, such as *Meloidogyne incognita*, a nematode causal agent of root gall in tomatoes (TIAN et al., 2014).

Colletotrichum is known to be or cause anthracnose, causing great economic losses, such as *Fusarium* (RODRIGUES et al., 2020). This genus, when isolated as endophyte, provided great results in the production of active compost, as in the case of the metabolite piperine, produced by the *Piper nigrum* plant, which was also reported as non-endophyte *Colletotrichum gloeosporioides* (CHITHRA et al., 2014). This substance has antimicrobial and antitumor properties (MGBEAHURUIKE, et al., 2019; TURRINI et al., 2020; WANG et al., 2020). Or endophyte *Colletotrichum* sp. isolate of Uncaria tomentosa also confirmed that different non-extracted fungal compost, using the bioautography technique, has antibacterial activity for *Escherichia coli*, *Enterecoccus faecalis*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (GONÇALVES et al., 2019).

Phoma is an endophyte with interesting technological application for agriculture, it has biofertilizing potential for the production of vegetative hormones such as gibberellins, as well as promoting growth and induction of germination of milho seeds (*Zea mays*), when compared to non-inoculated plants (KEDAR et al., 2014; TUDZYNSKI et al., 2018). Furthermore, the secondary metabolites produced are of interest in the pharmaceutical industry because they have anti-inflammatory, anti-viral and anti-câncer activities (WU et al., 2018; KIM et al., 2019; LIU et al., 2019).

Thus, we can see that important endophytic fungi are capable of colonizing *M. esculenta*, with important industrial, pharmaceutical biotechnological applications and mainly in current agriculture. Knowing this, aspects such as promoting plant growth, biological control or enzyme production should be explored in these strains in future studies, in order to provide *ex situ* technology for cassava providing improvements to this crop.

Endophytic fungi are able to colonize all tissues of cassava (*Manihot esculenta*), being more frequent in stem tissue, and the genera *Penicillium* and *Phomopsis* observed more frequently. This is the first report of studies of the community of endophytic fungi of *Manihot esculenta*.

ACKNOWLEDGMENT

The authors would like to thank the Brazilian institution "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the support of the scholarship.

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