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ORIGINAL ARTICLE

# An efficient technique for in vitro preservation of *Agaricus subrufescens* (=*A. brasiliensis*)

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**Abstract** *Agaricus subrufescens* is one of the most important fungi produced biotechnologically in Brazil. After subculture, it shows a reduction in mycelial growth and difficulties with recovery. Hence, it is important to establish preservation and recovery techniques for its maintenance. Mycelium on a solid medium supplemented with activated charcoal (AC; technique A) was compared with culture under mineral oil (B) and mycelial disks in sterile water (C), all intended for storage of up to 12 months at 8°C. Mycelium viability, radial growth rate and genetic stability, assessed by random amplified polymorphic DNA (RAPD) analysis, were evaluated continuously. Technique A showed a 75% viability in the initial and final periods; technique B showed a 50% reduction in viability towards the end of the storage period; and technique C differed from the others insofar as viability was high until the 6th month, with a 50% drop after the 10th month, and a complete loss of viability after the 12th month. Overall, throughout the storage period, the rate of radial mycelium growth decreased. However, improved results were obtained when the media were also supplemented with AC. RAPD profiles demonstrated high genetic homogeneity of the mycelia maintained under techniques A, B and C (> 99%). In short, AC was shown to be an efficient ingredient for preservation within the 12-month storage period. Furthermore, it adds an important effect in that further recovery of *A. subrufescens* without significant morphological and genetic changes can be achieved.

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## Introduction

Fungal culture collections are maintained mainly for the preservation of germplasm. Preservation of such microorganisms is important for research and industrial applications, especially because of the active biological substances they may produce. By preserving, one can also maintain the genetic and ecological functions of these organisms (Cameotra 2007).

*Agaricus subrufescens* Peck (= *Agaricus brasiliensis* Wasser, Didukh, de Amazonas & Stamets[1])—a culinary-medicinal fungal species native to the Americas—is one of the more important basidiomycetes produced in Brazil. Consumption of this fungus (fruiting bodies) has increased in recent years mainly because of its nutritional and pharmaceutical properties (Ohno et al. 2001; Angeli et al. 2009), which are related to fungal cell wall β-glucans (Ohno et al. 2001; Camelini et al. 2005).

In order to meet the ongoing demand for fungal material of this and other medicinally important species, determining the best conditions for preserving mycelia is among the main interests for those cultivating *A. subrufescens*. For many fungal species, mineral oil and distilled water are often used for short-term preservation (Richter and Bruhn 1989; Croan et al. 1999). On the other hand, lyophilization and cryopreservation in liquid nitrogen, which arrest growth and may compromise some metabolic processes,

[1] Although the name *Agaricus blazei* Murrill *sensu* Heinemann is used widely as synonym for *A. subrufescens*, the name has been incorrectly assigned (Kerrigan 2005).

represent techniques for long-term preservation (Croan et al. 1999).

One of the main challenges in maintaining cultures of *A. subrufescens* is its low culture viability and frequent changes in growth habit, mainly after subculture (Neves et al. 2005). Although there are studies reporting the use of certain techniques for the preservation of fungal mycelium in vitro (Antheunisse 1973; Croan et al. 1999; Crespo et al. 2000), we have not found any related to either long- or short-term preservation of *A. subrufescens*. Furthermore, lyophilization, which, according to Chang and Elander (1986), can be quite efficient for fungal storage, especially when 10% trehalose and 10% skimmed milk are used, proved to be inefficient for *A. subrufescens* because the fungus did not survive under such treatment (C.M.C., unpublished data).

Another important aspect that needs to be taken into account is that many fungi are able to produce secondary metabolites, such as production of agaritine by species of *Agaricus* (Kondo et al. 2006). When such fungi are grown in culture conditions, these metabolites are introduced into the medium. Agaritine, in turn, has a mutagenic activity (Walton et al. 1997) and may be harmful to the species. Therefore, methods for the attenuation of the effects of such compounds are necessary. Activated charcoal (AC) has proven very helpful for removal of several toxic compounds in hemicellulosic hydrolyzates used for the cultivation of fungi (Mussatto and Roberto 2004; Chandel et al. 2007), and is used in tissue culture to improve cell growth of plants in vitro (Teng 1997). AC should also provide proper conditions in culture media for the preservation and evaluation of several characteristics of the microorganism in culture (Hays et al. 2005).

During a particular preservation period, it is important to investigate both viability and morphological characteristics of the mycelium on solid culture medium (Clark and Anderson 2004; Marín et al. 2008). It is crucial to observe the genetic stability of the preserved mycelium. The polymerase chain reaction (PCR) and other protocols for DNA analysis, such as random amplified polymorphic DNA (RAPD) markers, have become important tools in the evaluation of intraspecific variability in microorganisms (Atienzar et al. 2002; Neves et al. 2005) and detection of DNA damage and mutations (Lee et al. 2000).

The purpose of our study was to find an efficient technique for in vitro preservation of *A. subrufescens* under cold storage. To this end, we used mycelium on media supplemented with AC, mycelial culture under mineral oil, and mycelial disks in sterile water in search of a technique that would enable proper maintenance of such fungi for up to 12 months, under continuous evaluation of mycelium viability, radial growth rate and genetic stability by different evaluation methodologies.

## Materials and methods

### Microorganism

*Agaricus subrufescens* (isolate UFSC51) was isolated in 2004 from a standard cultivation farm in Biguaçu, Santa Catarina state, Southern Brazil (Camelini et al. 2005). Species were characterized according to Neves et al. (2005), and voucher specimens were deposited at the FLOR Herbarium (FLOR 11.797). After isolation, the fruiting bodies were dried and stored at −80°C until use. The mycelium was isolated on potato dextrose agar (PDA; Oxoid, UK) at pH 6 and cultivated at 26 ± 1°C.

### In vitro preservation techniques

Colonized agar disks (8 mm in diameter) of *A. subrufescens* were obtained from the margins of an actively growing culture using a sterile cork borer. In technique A, the disks were placed in PDA slant tubes, supplemented with 0.2% AC (Sigma, St. Louis, MO), and maintained until complete surface colonization was detected. For technique B, the disks were placed in PDA slant tubes and later covered with 5 mL mineral oil, which was added to the culture after full colonization. In technique C, four mycelium disks were placed in tubes containing 15 mL Milli-Q water (Millipore, Sao Paulo, Brazil). All tubes, prepared aseptically in accordance with the three different preservation techniques, were stored at 8°C for a period of 12 months.

### Mycelial viability

Four mycelial disks (8 mm) from each preservation tube were transferred to PDA media in Petri dishes at intervals of 2 months throughout an entire year. Viability was evaluated and considered positive upon observation of mycelial growth after 5 days. All experiments were carried out in a completely randomized design with three replications. Percent viability at each recovery period was evaluated.

### Mycelial growth recovery

To compare the in vitro radial mycelial growth rate of *A. subrufescens* on PDA culture media (pH 6) with the growth rate on PDA supplemented with AC (0.2%), viable mycelial disks preserved through the various techniques (A, B and C) were transferred onto 90 mm-diameter agar plates and incubated at 26 ± 1°C. Mycelial colony diameter (average of two perpendicular diameters) was assessed every 2 days for 16 days for both media, before preservation (BP), and after 4, 8 and 12 months of storage. No analysis was carried out for growth of disks before

transfer to each recovery treatment. Data on colony diameter (an average of three replications) were used to establish growth curves. Subsequently, regression analysis and best fitting lines were determined with nonlinear correlation coefficients and prediction intervals to establish statistically significance. The radial mycelial growth rate for the PDA supplemented with AC among all three techniques was also compared.

### RAPD analysis

Mycelia from aerial hyphae was collected aseptically after 4, 8 and 12 months of storage and compared with the BP material and fruiting body by RAPD analysis. Genomic DNA was isolated by phenol chloroform following the methodology described by Lee and Taylor (1990), with modifications. To improve DNA recovery, the buffer-suspended samples were frozen and thawed five times by passing the tubes between liquid nitrogen and a 37°C water bath for 60 s. Ten primers were used in this study as described by Lee et al. (2000) and synthesized by Invitrogen (Sao Paulo, Brazil). PCR was carried out according to Steindel et al. (1993). The PCR cycles were performed with Gene Mastercycler® Gradient (Eppendorf, San Diego, CA). Three microliters of amplified DNA were separated by electrophoresis on 6% polyacrylamide gel at 100 V and stained with silver nitrate. DNA fragment sizes were estimated with a standard 100 bp DNA ladder (Fermentas, Burlington, ON, Canada). The positive control was composed of *A. subrufescens* fruiting body DNA and the negative control was water. The PCR products were visualized in a Macro Vue UV-20 transilluminator (Hoefer, Holliston, MA). Samples on the gel were scored for the presence or absence of specific fragments with sizes between 100 and 1,000 bp by use of codes 1 and 0, respectively. Genetic similarity coefficients of the isolated strains in three replications of RAPD assays were calculated by the Unweighted Pair Group Method Analysis (UPGMA) and the FREETREE softwares. Dendrograms were generated using the TREEVIEW 1.6.6. software.

## Results

### Mycelial viability

Mycelial viability was determined by the percentage of mycelial growth observed in the disks after being treated under the different preservation techniques (Table 1). Preservation techniques A and B yielded improved and prolonged mycelial viability for up to 12 months. Samples tested under technique A maintained a 75% viability in the initial and final phases of the test, whereas viability for technique B was reduced from 87.5 to 50% during the same time span. Technique C differed from the others insofar as viability was high until the 6th month, but after the 10th month viability dropped by 50%, with a complete loss of viability after the 12th month of storage. Technique A was the most efficient for preserving *A. subrefescens* for storage up to 12 months.

**Table 1** Average viability of mycelial disks of *Agaricus subrufescens* after preservation in potato dextrose agar (PDA) slant tubes with activated charcoal (A), PDA slant tubes with mineral oil (B) and under sterile water (C), all under cold storage

| Preservation technique | Monthly % of mycelial recovery[a] | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 |
| A | 75.0 | 75.0 | 68.7 | 75.0 | 93.7 | 75.0 |
| B | 87.5 | 87.5 | 75.0 | 62.5 | 62.5 | 50.0 |
| C | 93.7 | 100.0 | 100.0 | 75.0 | 50.0 | 00.0 |

[a] Average of four viable disks for three replications

### Mycelial growth recovery

Radial mycelial growth rates of *A. subrufescens* before preservation (BP) on PDA culture medium (1) and PDA supplemented with AC (2) showed results of 4.7 and 5.5 mm/day, respectively. After the preserved mycelia were recovered, the radial growth rate decreased throughout the 12-month period (Fig. 1a). Best recovery results were obtained when the fungus was grown on PDA supplemented with AC, with growth rates of 2.6 and 2.2 mm/day for techniques A and B, respectively. On the other hand, mycelia recovery without charcoal reached approximately half of these growth measurements. Technique C showed similar growth rates as observed for the other preservation techniques, with the difference that it happened much earlier into the recovery cultivation period (in the 8th month). Techniques A and B on media supplemented with AC differed significantly from technique C. Furthermore, mycelium preserved by technique A showed a higher growth propensity observed by the accelerated growth rate when compared to the others (Fig. 1b).

All three preservation techniques presented deformed colonies after mycelium recovery on PDA, showing slow growth and growth sectoring. However, when the media were supplemented with AC, colonies showed typical morphological traits, such as equidistant mycelial growth, which became densely zonate with concentric bands. Cottony aerial mycelium was also observed (Fig. 2). The cold storage increased the lag phase, i.e., the time required for the colony to grow beyond the inoculation zone, where a decrease in growth rate was observed with time. However, normal growth was usually obtained after the

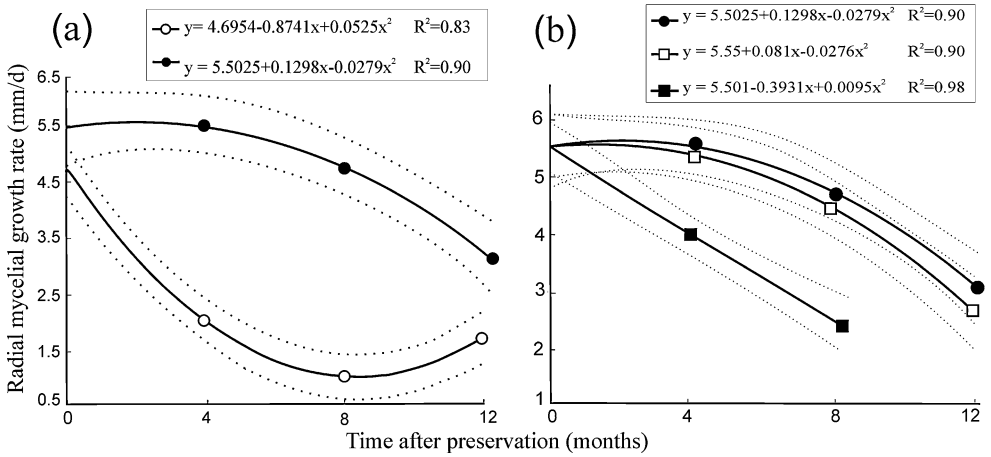


**Fig. 1** **a** Radial mycelial growth rate of *Agaricus subrufescens* after preservation in potato dextrose agar (PDA) slant tubes with activated charcoal (AC): ● recovered on PDA supplemented with AC; ○ recovered on PDA without AC. **b** Comparative results of preserved samples recovered on PDA supplemented with AC: ● mycelium in PDA slant tubes with AC; □ mycelium in PDA slant tubes under mineral oil; ■ mycelium disks under sterile water. Time zero (0) shows the growth rate of the mycelium before preservation. Growth rates were estimated by nonlinear regression analysis using correlation coefficients and 95% confidence intervals. Each graph shows equation and $R^2$ values of the curves, which were calculated by the average of three replications based on the colony diameter

second transfer onto PDA supplemented with AC. Higher radial mycelial growth rates were observed for techniques A and B with averages of 4.4 and 4.2 mm/day, respectively.

## Genetic stability determined by RAPD analysis

The genetic stability of *A. subrufescens* after different preservation techniques can be estimated successfully using the ten primers described by Lee and Taylor (1990). A typical result of a RAPD gel is shown for the primer OPF-1 in Fig. 3. Similar results were observed for the other tested primers, with the separation of two clusters: (1) fruiting body; and (2) mycelium recovered after the preservation by techniques A, B, C; all recovered by use of techniques 1 and 2, creating subgroups A1, A2, B1, B2, C1 and C2.

RAPD analysis showed low genetic variability in mycelia collected after 4, 8 and 12 months of preservation for all techniques when compared with the mycelium before preservation (BP), presenting genetic similarities above 95, 93 and 96%, respectively (Fig. 4). However, even lower similarity levels, close to 87.4% were detected when comparing the mycelium BP and the fruiting body with the preservation techniques for the mycelia recovered at the 8th month of storage. After 4 months of preservation (Fig. 4a), subgroups A1 and A2, B1 and B2, C1 and C2 showed the same RAPD profiles demonstrating high genetic homoge-

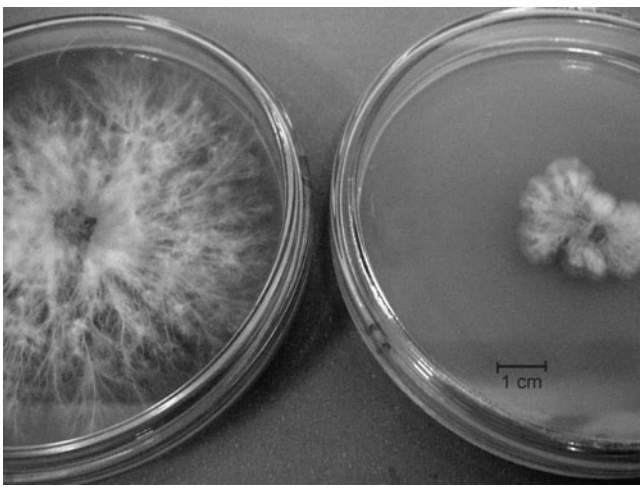


**Fig. 2** *Agaricus subrufescens* mycelial growth on PDA supplemented with 0.2% AC (*left side*) and on PDA without AC (*right side*)

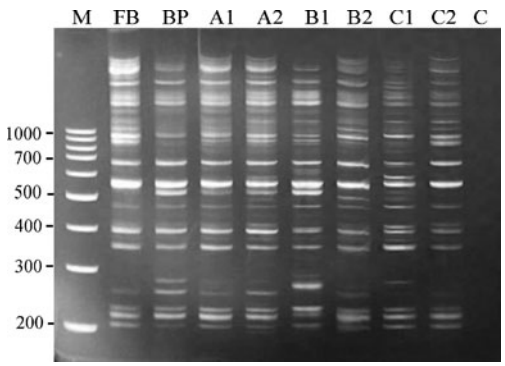


**Fig. 3** Random amplified polymorphic DNA (RAPD) pattern using the primer OPF-1 for DNA amplification of *A. subrufescens* before (BP) and after preservation under cold storage including mycelium in PDA slant tubes with AC (A1, A2), mycelium in PDA slant tubes under mineral oil (B1, B2) and mycelium disks under sterile water (C1, C2) recovered on PDA culture media (1) or PDA supplemented with AC (2) after a 8 month preservation period. *FB* Fruiting body, *C* water control, *M* 100 bp DNA ladder

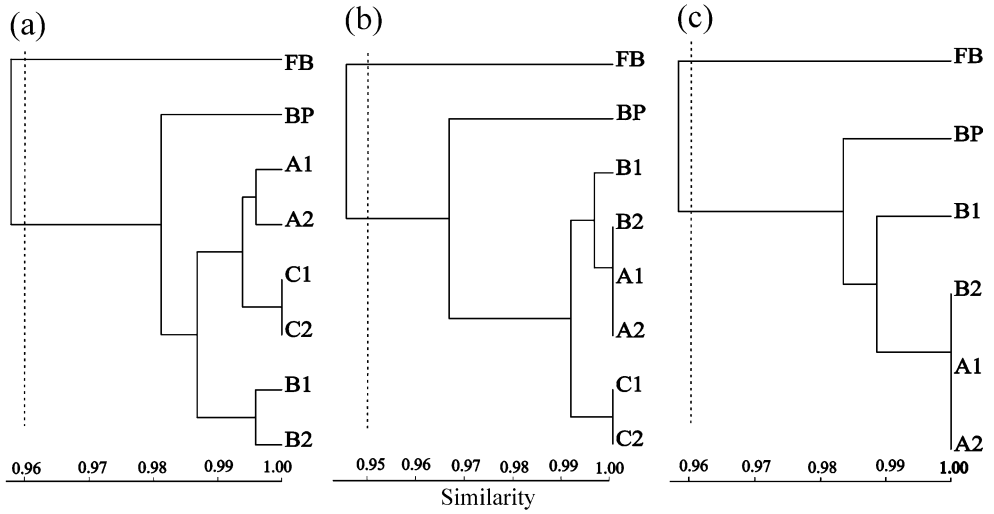


**Fig. 4** Dendrograms based on RAPD patterns (3 replications) of *A. subrufescens* after **a** 4, **b** 8, and **c** 12 months of mycelia preservation. Data show the comparison among the mycelium before preservation (BP) and after preservation maintained either under cold storage in PDA slant tubes with AC (A1, A2), PDA slant tubes in mineral oil (B1, B2), or mycelia disks maintained in sterile water (C1, C2). Numbers following each treatment indicate recovery on either PDA culture media (1) or PDA supplemented with AC (2). *FB* Fruiting body. Molecular genetic similarity was revealed by the unweighted pair group method analysis (UPGMA) with arithmetic averages. *Vertical dashed lines* represent the phenon lines

neity (> 99%), which was maintained in the 8- and 12-month storage (Fig. 4b,c).

## Discussion

The use of PDA medium in slant tubes supplemented with AC was the most efficient method for the preservation of *A. subrufescens* under cold storage and resulted in the longest period of successful storage (12 months). Mycelium in PDA slant tubes under mineral oil also showed similar recovery results, but the presence of oil prolonged the lag growth phase of the fungus.

Activated charcoal has been used previously to adsorb toxic compounds in fermentation media prepared from hemicellulosic hydrolysates used for the cultivation of fungal species (Mussatto and Roberto 2004; Chandel et al. 2007). It also proved an efficient additive in the in vitro preservation of fungi such as *A. subrufescens*. *Agaricus subrufescens* is the only species of *Agaricus* either harmed or killed by prolonged exposure to temperatures of 4°C or lower (Wasser et al. 2002; Kerrigan 2005). Therefore, employment of AC to detoxify secondary metabolites produced by fungi at lower temperatures has proven to be an important procedure for preservation up to 12 months, but mainly for the recovery of such fungi.

Mycelium disks of *A. subrufescens* preserved under sterile water lost viability completely within 12 months. However, Richter and Bruhn (1989) reported preservation at 5°C up to 48 months for several basidiomycete species and shorter periods for other species, with proven viability of up to 9 months. Smith et al. (1994) also reported 95% viability of 169 fungi tested after 20 months of preservation. Richter (2008) had 88% viability of 34 cultures after 20 years in sterile water. The overall survival rate of dermatophyte strains was 35 out of 39 (89.7%) after 12 years, with species-specific physiological characteristics maintained after this period (Qiangqiang et al. 1998).

An important condition observed for recovered mycelia of *A. subrufescens* maintained under all preservation techniques was deformity of the colonies. These deformities, however, were not observed when the recovery PDA media was supplemented with AC. These morphological characteristics found with species under PDA with AC have been described also by Wasser et al. (2002). Mantovani et al. (2007) evaluated the effect of different nitrogen sources on the radial mycelial growth rate of *A. subrufescens* in vitro, and observed no visible degeneration in the mycelium when compared to culture media with malt extract (positive control), which had a higher mycelium growth and density. Teng (1997) reported that the incorporation of AC in leaf cell suspension culture increased the number of regenerated sporophytes, even in media free of growth regulators, preventing abnormal morphogenesis and therefore normalizing cell development.

The preservation methods used in this study are classic techniques for in vitro maintenance of bacteria and fungi. Most fungi can be stored at temperatures between 4 and 12°C and subcultured at intervals of 6–8 months. This procedure can induce senescence in fungi, i.e., a progressive loss of mycelial growth potential, culminating in mutation and total cessation of growth, leading to fungal death (Maheshwari and Navaraj 2008).

DNA mutations in fungi can be induced by radiation (Lee et al. 2000), genotoxins (Delmanto et al. 2001), preservation (Clark and Anderson 2004) and senescence (Maheshwari and Navaraj 2008). A morphological mutation particularly detectable in the dikaryotic phase was found in *Lentinula edodes*. The mutant dikaryon was readily distinguishable from the normal dikaryon by its irregularly branched short hyphae, very slow hyphal growth, and sparse aerial hyphae (Shimomura and Hasebe 2006).

Without AC, *A. subrufescens* showed deformed sectional colonies and slow mycelial growth after all tested preservation techniques. However, the RAPD analysis showed high genetic similarity of mycelium collected at the 4th, 8th and 12th months for all techniques of preservation and recovery when compared to the mycelium BP. However, lower similarity levels were detected when comparing the mycelium BP and the fruiting body with the preservation techniques for the mycelia recovered at the 8-month storage. The greater genetic similarity of the preserved mycelium increased the homogeneity of the isolated culture. Therefore, low variability is not desirable for the genetic diversity, but it is necessary for the quality control during cultivation of *A. subrufescens* aimed at obtaining polysaccharides for biotechnological industrial applications.

On the other hand, the genetic distance between fruiting bodies and mycelium of *A. subrufescens* is possibly due to the differentiation of reproductive and vegetative phases in the life cycles of most basidiomycetes. The dikaryon is the predominant vegetative structure that, under appropriate conditions, produces the fruiting bodies within which meiosis occurs. In the dikaryon, the two haploid gametic types of nuclei are maintained indefinitely in paired association. A potential complication with the long-term culture of dikaryons in vegetative phase is the possibility of genetic exchange between the paired haploid nuclei, in which a dominant deleterious mutation in one nucleus is followed by a compensatory mutation in the other, resulting in an increasing linear growth rate of the mycelium (Clark and Anderson 2004).

Neves et al. (2005) examined six Brazilian productive isolates of *A. subrufescens* to determine genetic variability based on data from RAPD analysis, and demonstrated two genetically differing groups with a 11.5% variability. In our study, we found a 12.6% difference between the mycelium and fruiting body when characterizing isolate UFSC51. This higher difference may be due to the use of a different set of primers, which consequently gives a more complex genetic overview. Additionally, the use of silver nitrate to stain the electrophoresis polyacrylamide gel may also offer more accurate and sensitive results.

The RAPD technique has been used largely to detect the genetic variability of pathogenic and non-pathogenic microorganisms (Steindel et al. 1993; Oborník et al. 2000; Neves et al. 2005; Gasser 2006). This method is cheap, simple to perform and suitable for detecting DNA mutations—all features that are important for the evaluation of the genetic stability in microorganisms during in vitro storage (Mariano et al. 2007). The AC technique used for the preservation and recovery of *A. subrufescens* is an easy, quick, and inexpensive method that does not result in significant morphological and genetic changes within a 1-year storage period.

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