Alternative and safe dyes for staining Arbuscular Mycorrhizal fungi

Several methodologies are available for the study of Arbuscular Mycorrhizal fungi (AM). Most involving the colouring of typical symbiosis structures, with the use of dyes such as Trypan Blue, which can be harmful to users’ health (e.g., carcinogens) and the environment (e.g., pollutants). In order to use safe alternatives for AM fungi studies, a comparison and analysis of the use of industrial organic food dyes (Arcól®) in blue and red colours, organic dye based on Euterpe oleracea Mart. pulp (açaí) and the traditional Trypan Blue (control), all of them diluted in commercial vinegar, for observation and quantification of the typical structures of mycorrhizal infection in the roots of Vigna unguiculata L., Schizolobium amazonicum Huber ex Ducke, Zea mays L. and Brachiaria sp. The results indicated that the organic dyes visually colour the structures of the AM fungi. However, blue food colouring is as effective as Trypan Blue, in addition to being a good option in teaching activities evolving AM fungi, mainly because it presents less risk to users’ health. Thus, a good substitute for the Trypan Blue synthetic dye is presented, based on an effective, easily applicable, low cost and safe methodology for the study and teaching of AM fungi.

Keywords: Food dyes; Glomeromycota; Mycorrhizal staining; Symbiosis; Trypan blue.

Corantes alternativos e seguros para a coloração de fungos Micorrízicos Arbusculares

Diversas metodologias estão disponíveis para o estudo dos fungos Micorrízicos Arbusculares (MA). A maioria envolve a coloração de estruturas típicas de simbiose, com o uso de corantes como o Azul de Tripano, que pode ser prejudicial à saúde dos usuários (por exemplo, carcinógenos) e ao meio ambiente (por exemplo, poluentes). Com o objetivo de utilizar alternativas seguras para estudos dos fungos AM, foi feita uma comparação e análise do uso de corantes alimentares orgânicos industriais nas cores azul e vermelha (Arcól®), o corante orgânico à base de Euterpe oleracea Mart. polpa (açaí) e o tradicional Azul de Tripano (controle), todos diluídos em vinagre comercial, para observação e quantificação das estruturas típicas da infecção micorrízica nas raízes de Vigna unguiculata L., Schizolobium amazonicum Huber ex Ducke, Zea mays L. e Brachiaria sp. Os resultados indicaram que os corantes orgânicos colorem visualmente as estruturas dos fungos AM. Porém, o corante alimentar azul é tão eficaz quanto o Azul de Tripano, além de ser uma boa opção no ensino de atividades que envolvem fungos MA, principalmente por apresentar menor risco à saúde dos usuários. Assim, apresenta-se um bom substituto para o corante sintético Azul de Tripano, baseado em uma metodologia eficaz, de fácil aplicação, baixo custo e segura para o estudo e ensino dos fungos AM.

Palavras-chave: Corantes alimentares; Glomeromycota; Coloração micorrízica; Simbiose; Azul tripano.

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INTRODUCTION

Arbuscular Mycorrhizal Fungi (AM), subphylum Glomeromycotina (SPATAFORA et al., 2016), have existed for at least 400 million years (REDECKER et al., 2000). These fungi constitute a mutualistic relationship with the roots of the host plant, involving the intraradicular mycelium (including the symbiotic interface), the extraradicular mycelium (network of hyphae in the soil) and the spores (MERRYWEATHER et al., 1998) of the fungus.

AM fungi integrate all soil ecosystems and also some humid ecosystems (TURNER et al., 2000). However, their greatest relationship occurs with terrestrial plants, acting in symbiosis with 80% of known plants, in addition to some species of bryophytes and pteridophytes (ZHANG et al., 2007). The hyphae of the AM fungi grow exponentially and exploit large amounts of soil around the plants, and their effects on plant growth and on the resilience of ecosystems are well documented (AUGÉ et al., 2001, RAVEN et al., 2017; COMPART et al., 2019). This symbiosis makes plants ecologically more competitive based on intimate and reciprocal physiological and metabolic dependence and agronomically superior (TEDERSOO et al., 2020).

Mycorrhizal symbiosis study requires the use of methodologies that assist in visualising the typical structures of AM fungi in the roots of the host plant, such as spores, extraradical hyphae, intraradical hyphae, vesicles and arbuscules. Among the methodologies already known (GERDEMANN, 1955; NICOLSON, 1959; BRUNDRETT et al., 2004) the technique widely spread among the mycorrizologists proposed by Phillips et al. (1970) stands out, in which the roots are clarified in potassium hydroxide (KOH) (10%), acidified in hydrochloric acid (HCl) (1%) and the AM fungi stained with Trypan Blue (0.05%). This dye can be prepared by dissolving it in lactophenol (PHILLIPS et al., 1970), in an acidic glycerol solution (KOSKE et al., 1989) or in acetic acid (MAULER-MACHNIK et al., 1990).

However, the use of such chemicals should be reduced for the safety of users, as they can cause eyes, skin, nose, throat and lungs irritation (PROCTOR et al., 1978). Considering more severe cases, it causes mutagenicity or toxicity (ROBERTSON et al., 1982; CHUNG, 1983), in addition to the costs of obtaining reagents. Based on this concern, some scientists have modified the traditional mycorrhiza staining technique for less harmful and more accessible options. Following the example of Vierheilig et al. (1998) who suggested the use of vinegar to replace HCl, Ishil et al. (2004) who modified the technique by Koske et al. (1989), eliminating acidification with HCl, but maintaining the clarification step with KOH and staining with Trypan Blue and Silva et al. (2015) who replaced KOH with caustic soda and HCl with vinegar, both in their commercial form, thereby obtaining good quality in the decolourisation of the roots.

To replace the Trypan Blue, Kormanik et al. (1982) suggested the use of acid fuchsin, long recommended by Gerdemann (1955). Brundrett et al. (1984) proposed Chlorazole Black E (CBE) and Grace et al. (1991) and Vierheilig et al. (1998) indicated pen ink from different brands. However, these synthetic substitutes can be expensive, difficult to access and present health risks just like Trypan Blue (COMBES et al., 1982), in addition to the low efficiency in the colouration of specific structures of AM fungi (VIERHEILIG et al., 1998).
Silva et al. (2015) proposed the use of natural food dyes from Amazonian products, such as *Euterpe oleracea* Mart pulp. (açaí), *Bixa orellana* L. (annatto) and *Crocus sativus* L. (saffron), in contrasts for visualisation of AM fungi under microscopy. With satisfactory results, the authors indicated the use of these dyes as good substitutes for Trypan Blue, mainly açaí. However, the degree of purity of these products, as well as regionalism and seasonality, influences and impairs the use of the technique outside the Amazon domain. Thus, we seek to evaluate the potential use of two organic food dyes in the colouring of AM fungi, since they are produced industrially and widely disseminated, reducing risks to the health of users and the environment, facilitating studies on AM fungi.

**MATERIALS AND METHODS**

**Roots preparation**

Seeds surfaces of *Vigna unguiculata* L. (Fabaceae) (cowpea), *Schizolobium amazonicum* Huber ex Ducke (Fabaceae) (paricá-da-amazônia), *Zea mays* L. (Poaceae) (corn) and *Brachiaria* sp. (Poaceae) (grass) were sterilised by immersion in 0.75% sodium hypochlorite for five minutes, then washed under running water and germinated in a gully soil substrate containing AM fungi of the genus *Glomus* Tul. & C. Tul. from the collection of the Fisiologia Vegetal e Crescimento de Plantas Laboratory, from the Universidade Federal do Oeste do Pará, Santarém campus, Pará, Brazil. The plants were kept under artificial lighting with a 12-hour photoperiod at 27°C, receiving daily irrigation to maintain the field capacity according to the plant species. For each species, five pots were kept, with one plant in each pot, each plant being considered as a repetition. After 20 days of germination for *Z. mays* and *V. unguiculata*, 30 days for *Brachiaria* sp. and 60 days for *S. amazonicum*, the roots were collected and washed under running water.

**Roots clarification**

To clarify the roots, it was used the methodology suggested by Silva et al. (2015) in a modification of the Phillips et al. (1970) technique, where fragments of fresh root were submerged in a commercial caustic soda solution (10%) in a bain marie (± 90°C). After each period of discolouration, different for each plant species tested (Table 1), the roots were washed under running water, acidified in vinegar for one minute and then prepared for staining.

<table>
<thead>
<tr>
<th>Species</th>
<th>Time of discolouration in minutes</th>
</tr>
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<tbody>
<tr>
<td><em>Zea mays</em> L.</td>
<td>5</td>
</tr>
<tr>
<td><em>Vigna unguiculata</em> L.</td>
<td>5</td>
</tr>
<tr>
<td><em>Brachiaria</em> sp.</td>
<td>15</td>
</tr>
<tr>
<td><em>Schizolobium amazonicum</em> Huber ex Ducke</td>
<td>30</td>
</tr>
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</table>

**Roots Colouring**

To perform the colouring, two organic dyes were used individually, in liquid form, in the colours blue and red (containing: organic dye, neutral ethyl alcohol; available in bottles of 10 and 960 ml, Arcolor®, Brazil) at 5% (v/v). The dye solutions were prepared using vinegar as a solvent. The roots, together with 20 ml of
each dye solution, were placed in 50 ml test tubes and heated in a bain marie (± 90°C) for five minutes. As a control, the natural dye of açai pulp, obtained in the local market, was diluted in commercial vinegar at 5% (v/v), according to Silva et al. (2015), and the Trypan Blue dye (0.05%), formulated using the traditional technique. After staining, the roots were transferred to new tubes of equal capacity, containing 20 ml of glycerin solution (10%) and kept at room temperature until analysis.

Application of the method in the teaching of AM fungi

The dye solutions proposed in this study, in addition to the açai dye, were presented as a possibility of application in the teaching of AM fungi for undergraduate students in Biological Sciences (40), Agronomic Engineering (35) and Biotechnology (27) majors from the Universidade Federal do Pará, Santarém Campus, Pará, Brazil. In the teaching laboratory, students were divided into groups to prepare the dye solutions as previously described, using roots from Z. mays, with 15 days of cultivation and colonised with Glomus sp. The professor of the classes demonstrated the preparation of the colouring with Trypan Blue as a control. The time of each practical class consisted of two hours and the objective, in addition to comparing the dyes, was to present the mycorrhiza staining technique and to recognise the typical structures of AM fungi in the root system of plants. The participants signed the free and informed consent form and agreed with the dynamics and dissemination of the results of the class.

Statistical Analysis

To confirm the potential of alternative dyes, the typical structures of AM fungi in the roots of five plants of each species (V. unguiculata, S. amazonicum, Z. mays and Brachiaria sp.) were quantified. To facilitate visualisation, the roots were broken up into fragments of 1 and 2 cm, which were inserted between slide and cover slip with polyvinyl lactoglycerol (PVLG) in the direction perpendicular to the longitudinal axis. Five slides were observed for each plant, each slide containing 5 cm of root, totaling 25 cm of roots analysed per plant and 125 cm for each species in each dye. The slides were observed in an optical microscope (Carl Zeiss - Primo Star) with 400 times magnification. Each dye was considered a treatment, and the calculation of the average of the count of visible structures per centimeter of root was submitted to analysis of variance after logarithmisation \((x+0.5)^{0.5}\) and after the \(t a p<0.005\) test of reliability performed by SISVAR programme 5.6 (FERREIRA, 2011).

To assess the difficulty of the technique when applied to teaching, in addition to the students' preference for the dyes tested, a quantitative questionnaire was carried out containing two objective questions (CHART 1).

Chart 1: Evaluation of higher education students on alternative dyes and staining technique of Arbuscular Mycorrhizal fungi.

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<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blue</td>
<td>Red</td>
<td>Açai</td>
<td></td>
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</table>

1) How difficult is the technique?
2) Which alternative dye was most effective?
RESULTS

Both blue and red food colouring made it possible to distinguish between colonised and non-colonised roots, especially considering structures such as extraradical and intraradical hyphae, which were easily observed. In addition, the blue food colouring did not present a statistically significant difference in the spore, hyphae, vesicle and arbuscule counts in relation to Trypan Blue. The red dye and the one from the açai pulp visibly coloured some structures of the AM fungi, such as intra and extra root hyphae, but gave little clarity to the others, such as vesicles and arbuscules (Table 2).

Table 2: Average of typical structures of mycorrhizal association per centimeter of root in different plant species (Zm = Zea mays; Br = Brachiaria sp.; Vu = Vigna unguiculata; Sa = Schizolobium amazonicum) submitted to different dyes (AT = Trypan blue; FB = Food Blue; FR = Food Red; AÇ = Açai).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Structure of the AM fungus</th>
<th>Tested dyes</th>
<th>ANOVA</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>TB</td>
<td>FB</td>
</tr>
<tr>
<td>Zm</td>
<td>Spore</td>
<td>4.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Hyphae</td>
<td>10.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Vesicle</td>
<td>4.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arbuscule</td>
<td>2.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Br</td>
<td>Spore</td>
<td>8.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Hyphae</td>
<td>10.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Vesicle</td>
<td>4.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arbuscule</td>
<td>3.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vu</td>
<td>Spore</td>
<td>5.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Hyphae</td>
<td>9.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Vesicle</td>
<td>4.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arbuscule</td>
<td>2.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sa</td>
<td>Spore</td>
<td>5.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Hyphae</td>
<td>10.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Vesicle</td>
<td>5.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Arbuscule</td>
<td>5.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The averages followed by the same letter in the columns do not differ in the level of significance to the t test of confidence. F and p correspond to ANOVA.

Figure 1: Structures of Arbuscular Mycorrhizal Fungi (AM) in Zea mays L. root stained with different alternative dyes. A-C) Food blue; D-F) Food red; G-I) Açai pulp; J-M) Trypan blue. Structures of AM fungi: Hyphae (H), Spore (S), Vesicle (V), Arbuscule (A). Scale: A, B, D, E, J, L = 200 µm; C, F, G, H, I, M=150 µm.
However, for photographic and visualisation purposes, a better contrast was achieved with the blue dye, allowing a better distinction and quantification of structures typical of AM fungi (Figure 1).

The application of the AM fungi staining technique using food dyes was successful in the teaching activity, being found during the practical activity, the low degree of difficulty to be performed (Figure 2A). Based on the observations of the slides, the blue food colouring was the most effective according to the students (Figure 2B), as it provided the best contrast between fungal structures and plant tissue, although the other dyes also provided visualisation of the AM fungi structures.

The methodology proposed in this study, facilitated the activities developed in the laboratory, such as the training of new trainees, decreased the costs of basic research involving AM fungi and helped in the formation of higher education students.

**Figure 2:** Students' perception of the staining technique of Arbuscular Mycorrhizal fungi and the use of alternative dyes. A). Degree of difficulty in performing the technique; B) Effectiveness of the tested alternative dyes.

**DISCUSSION**

Even with the existence of molecular techniques to recognise mycorrhizal symbiosis (ALKAN et al., 2004; PITET et al., 2009), the colouring of the roots to quantify fungal structures through routine analysis under light microscopy, still remains the most used in the study of AM fungi. However, the efficiency of mycorrhizae staining involves the ability to visualise and quantify the typical structures of AM fungi in plant roots by means of specific staining. Although not a recent technique, the problem of the inappropriate use of the necessary chemicals in the colouring process is still an issue to be resolved.

This concern is directly related to the chemicals used, which cause risks to the health and safety of users (COMBES et al., 1982), in addition to environmental damage due to inappropriate waste disposal. Although it is possible to reduce the risks of the traditional technique (VIERHEILIG et al., 1998; ISHIL et al., 2004; SILVA et al., 2015), it is still not possible to fully replace the Trypan Blue dye, being one of the causes to avoid activities involving AM fungi by users with little experience.

Grace et al. (1991) and Vierheilig et al. (1998) proposed less harmful dyes formulated from pen inks of different colours such as purple (Waterman), green (Reynolds), red (Parker; Lamy; Pelikan), blue (Pelikan; Kreuzer; Shaeffer) and black (Pelikan; Carrefour; Shaeffer; Cross) for AM fungal staining. However, most of these products were not efficient in colouring the structures of these fungi. Silva et al. (2015) tested natural...
regional dyes from Amazon, such as the *E. oleracea* Mart. Pulp, popularly called in the Amazon as açaí, in addition to pen inks in the colours blue and red of popular brands, with encouraging results to replace the Trypan Blue. However, according to the authors, some pen inks coagulate during the staining process and do not penetrate the fungal tissue.

When tested in this study, the dye based on the açaí pulp showed promising results and, corroborating with Silva et al. (2015), represent a simple and low-cost alternative, which can contribute to research and teaching on AM fungi in regions with little technology and limited financial resources. However, the purity of the açaí pulp is directly related to the quality of the colour and its use may be compromised in a period of low demand, as this fruit presents seasonality, in addition to Amazonian regionalism, preventing the technique from being carried out in other regions of the world.

Organic food dyes, even if industrial, are substances that confer, intensify or restore colour when used in food, and thus may be good options due to the standardisation of the substance, low cost and possibility of obtaining them in a less expensive way than chemical substances such as Trypan blue. This source was explored by Thies et al. (2002), Thies et al. (2007), Al-Amura et al. (2012), Damasceno et al. (2016), Kobae et al. (2016) and Mohammed et al. (2016), who, like this study, aimed to replace dangerous dyes in their target groups.

Given the reports in the literature on the use of food dyes, it seems to be a relationship between the colour of the alternative dye and the colour of the dye to be replaced. Thies et al. (2002) replaced acid fuchsin and Phloxine B in the staining of nematodes by industrial food colouring of red color, with satisfactory results. These results were used by Al-Amura et al. (2012) and Mohammed et al. (2016) in the colouring of Helminths and Leishmania, respectively. Kobae et al. (2016) used an organic compound of Blue colour in the immunohistochemical staining of nucleic acids and proteins in substitution of 3,3'-Diaminobenzidine with significant effects in the staining of mycorrhiza. In this study, the blue food dye, presented colour intensity and contrast similar to Trypan Blue, being the most promising in the colouring of the AM fungi among the tested dyes.

**CONCLUSIONS**

We provide a simple, easily applicable and safe technique with accessible compounds that are low risk to the health of the user and the environment, which can be widely applicable and possibly expanded to other root fungi in scientific studies, especially in regions with little or low technology investment in scientific research. Although all of the proposed dyes have performed well, we recommend the blue food dye for its better contrast between plant and fungal tissue and its similarity to the synthetic dye traditionally used in the staining technique of Arbuscular Mycorrhizal fungi.

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