



# ALTERNATIVE STAINING TECHNIQUE TO DETERMINE MYCORRHIZAL COLONIZATION

## Alternativa de la técnica de tinción para determinar la colonización micorrízica

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**ABSTRACT.** The root staining technique is necessary in the work with arbuscular mycorrhizal fungi (AMF), which constitute the base of several Biofertilizers, as the EcoMic<sup>®</sup> product, each time more used on agricultural practices at world level. That's why the sample number evaluated at Mycorrhiza laboratories from diverse Research, Academic and even some productive Centers, increases every year. Those works require the application of such technique; nevertheless, it has the inconvenient to use highly toxic and carcinogenic reagents. The objective of the present work was to substitute those reagents by others non harmful to human health and environment. This is the case of Trypan blue that was substituted by the washable pen ink Parker QuinK. Moreover, Lactic acid and Glycerin were eliminated from the protocol. Results showed the pertinence of such modifications and revealed the usefulness and high quality of mentioned ink, which successfully stains all fungal structures allowing a clear visualization during long time.

*Key words:* arbuscular mycorrhiza, roots, colorants, health protection

**RESUMEN.** La técnica de tinción de raíces es necesaria en el trabajo con los hongos micorrizógenos arbusculares (HMA), que constituyen la base de algunos biofertilizantes, como EcoMic<sup>®</sup>, cada vez más utilizados en las prácticas agrícolas a nivel mundial. Cada año se incrementa el número de muestras a evaluar en los laboratorios de micorrizas de los distintos centros de investigaciones científicas, de enseñanza e incluso de algunos vinculados a la producción, cuya labor requiere de la aplicación de dicha técnica. Sin embargo, ésta tiene el inconveniente de utilizar reactivos cancerígenos y altamente tóxicos, por lo que fue objetivo de este trabajo sustituir los mismos por otros no nocivos a la salud humana y al medio ambiente. Tal es el caso del azul de tripano, que fue sustituido por la tinta de bolígrafo Parker QuinK lavable en agua. Se eliminó además del protocolo el ácido láctico y la glicerina. Los resultados demostraron la pertinencia de las modificaciones realizadas y revelaron la utilidad y alta calidad de la tinta mencionada, la cual tiñe exitosamente todas las estructuras fúngicas, permitiendo visualizarlas claramente durante un periodo de tiempo prolongado.

*Palabras clave:* micorrizas arbusculares, raíces, colorantes, protección de la salud

## INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) form a symbiotic association with more than 85 % of land plants, as a result, mutual benefits are attained for their development and growth (1). It is important to stand out satisfactory results in improving the nutritional status of plants and in increasing agricultural yields obtained with the inoculation of these microorganisms,

to different crops of economic interest in several Latin American countries including Cuba (2). So research in the establishment and operation of this association in natural ecosystems and agrosystems are essential. Therefore, it's necessary to have appropriate techniques to quantify the intraradical colonization of these fungi.

For this purpose a method for staining AMF structures in roots using Trypan blue was developed (3). This reagent is considered carcinogen according to the International Agency for Research on Cancer. Also, they are often used other techniques based on the use of suspected carcinogens as Black

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Chlorazol E (5) and the acid fuchsine. The use of those chemicals should be reduced for reasons of health and the personnel safety, they may cause skin irritation and vapours can irritate eyes, nose, throat and lungs (7). Likewise for environmental reasons and whenever possible it is preferable finding substitutes for harmful chemicals.

In an attempt to eliminate some of these dangerous compounds, a simple, reliable and cheap method was developed to dye the fungal structure in the root tissues interest and determine the mycorrhizal colonization (8). This technique replaces potential carcinogen chemicals, conventionally used, for non-washable ink pen in water that is harmless. However, the carcinogenic Trypan blue stain is commonly used even by most researchers including the INCA ones.

The aim of this work was to adapt the described technique for widespread use in various laboratories conditions (8), emphasizing on the replacement of reactants which are noxious for health and the environment, for non-toxic ones but equally effective.

## MATERIALS AND METHODS

### BIOLOGICAL MATERIAL

Fresh roots of different characteristics were used from different plant families (corn [*Zea mays* L], tomato [*Solanum lycopersicum* L] and cucumber [*Cucumis sativus* L]). The same is received in the INCA arbuscular mycorrhizal laboratory for processing. As inoculum of AMF two strains of the collection of the INCA : *Funneliformis mosseae* [(Nicol & Gerd ) WALKER & Schüßler] (9) and *Glomus cubense* (y Rodr & Dalpé) (10) INCAM-2 and INCAM-4 were used respectively; as well as a conglomeration of native AMF (11) belonging to *Glomus* genres (10 morphotypes), *Funneliformis* (2), *Rhizoglomus* (4), *Sclerocystis* (1), *Septoglomus* (1), *Claroideoglomus* (3), *Acaulospora* (5), *Gigaspora* (1) and *Scutellospora* (1), according to Glomeromycota classification. Inoculation in all cases took place at the moment of planting. Inoculated and non-inoculated roots were processed as control.

### PREPARATION OF STAINING SOLUTION BASED ON PEN INK

Several pen ink ,blue and black were used for preparing staining solutions: Pelikan blue 523 non-washable in water, China; Kores blue, ink tampon; Staedtler blue 745, Germany; blue correction fluid,

China; Parker QuinK blue ,washable in water, England; Parker black, England; Musso black ink to seal without oil. The same were prepared at 25 mL ink in 1000 mL of hydrochloric acid (1N) 2, 5 % (v/v).

### DETERMINATION OF INTRARADICAL MYCORRHIZAL COLONIZATION

Roots were washed carefully with plain water and were dried in an oven to 70 °C to constant weight for each vegetal species, several groups of 200 mg of secondary roots, were stained separately, according to different protocols, processing 20 replicates per treatment in each of them. The first was, the staining through the standard procedure using the Trypan blue (3) and the second was developed in this work that consisted in clarifying roots in potassium hydroxide solution at 10 % (m/v) incubating in an oven at 90 °C for 30 minutes to an hour and later the solution was eliminated washing roots with plain water several times Then the staining solution was added, it was to stand for 15 minutes at room temperature and later was placed in the oven for 10 to 15 minutes at 70 °C. The latter protocol is made repeatedly in correspondence with the number of staining solutions were selected to develop the technique.

The reading of examples was carried out in the stereoscope (Carl Zeiss, Stemi 2000/50x) based on the reading from the mycorrhizal colonization percentages and intensity, according to the methodology described in the Manual of procedures<sup>A</sup>. Fragments of stained small roots preparations were mounted and observations were made by optical microscope (Carl Zeiss, Axiostar Plus) to select the staining solution with better results in terms of sharpness to visualize fungal structures.

### STATISTICAL ANALYSIS

Percentage values and intensity of mycorrhizal colonization by evaluating for both protocols (standard method and ink with better results), were compared using Student T test for related samples and SPSS package, Version 21 for Windows.

<sup>A</sup> Trouvelot, A.; Kough, J.; Gianinazzi-Pearson, V. Mesure du Taux de Mycorrhization VA d'un Systeme Radiculaire. Recherche de Methodes d'Estimation ayant une Signification Fonctionnelle. Proceedings of the 1<sup>st</sup> European Symposium on Mycorrhizae: Physiological and Genetical Aspects of Mycorrhizae, Dijón, 1-5 July, 1985. Gianinazzi-Pearson, V.; Gianinazzi, S. (Eds.). Paris: INRA, 1986. pp. 217-222.

## RESULTS AND DISCUSSION

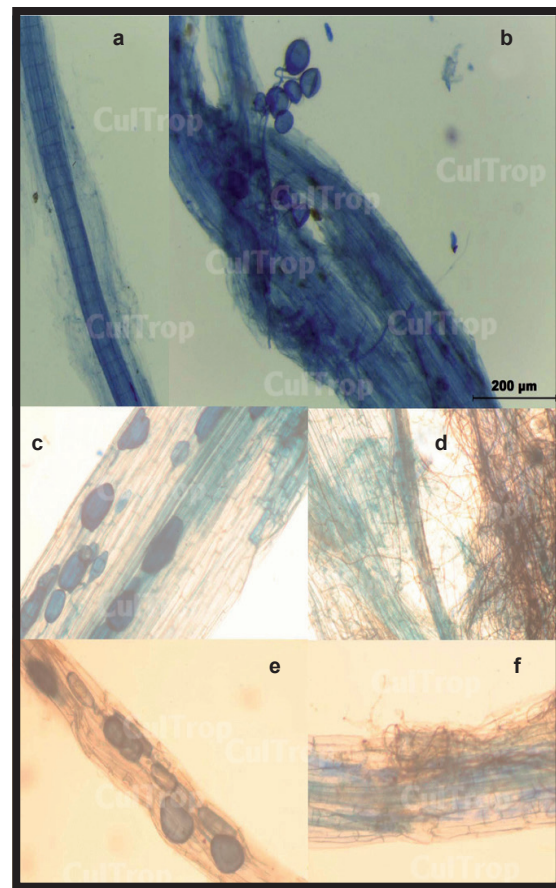
The staining solutions prepared using: Pelikan blue 523 non-washable in water and blue correction fluid both from China and Kores blue and Staedtler blue 745 from Germany, resulted in the formation of the precipitates and changed color when hydrochloric acid was added. So they were not selected in to be used in the technique trials because could hinder the visualization of the fungal structures in the subsequent microscopic observation considering them not useful for this purpose.

The two black inks tested, stained intraradical and extraradical structures of AMF and they made possible the stereoscopic observation, giving better results in terms of sharpness in the fungal structure visualization; specially Parker ink in comparison to the Musso one. The usefulness of black ink found in this work is consistent with previous results where black ink pen Schaeffer, not washable in water was reported as the best alternative for this purpose (8).

In contrast to the work mentioned, in the present study, a higher contrast was obtained when the blue ink Parker QuinK was used. In addition, if the sample is over stained, a few days should be waited before reading it and roots can be kept in the staining solution at room temperature around 6 months approximately. If the reading is made after this period and it has difficulties visualizing fungal structures, samples should be heated in an oven for 10 minutes at 70 °C.

It highlights Parker QuinK blue ink, washable in water, which dyed samples allowing a display with excellent clarity of fungal structures, inside and outside of the roots from different families of plants as shown in figure. In the developed technique to stain the AMF structures, this ink was the most appropriate one to use as colorant and to replace blue Trypan (3), so it is recommended for a higher quality of documentation photo in order to facilitate the evaluation itself microscopically. Thanks to this ink, colonized roots were distinguished easily from non colonized ones, (photos a, b), individual hyphae in root sections with partial or abundant mycorrhizal colonization was clearly observed (photos d, f). Also it permitted, clear visualization of vesicles, germinated spores and intra and extraradical hyphae with penetration structures (photos d, f).

In the figures can be appreciated that the staining solutions tested equally stained fungal structures of various inoculated fungi.



(A) Uncolonized cucumber root (B) Colonized tomato root by *Glomus cubense*. Spores and intraradical hyphae are observed. (C, D) Colonized tomato roots by native AMF belonging to the conglomerate. (C) Vesicles; (D) intra and extraradical hyphae, (E, F) abundant colonization. are observed, corn roots colonized by *Funneliformis mosseae*. Vesicles (e) intra and extraradical hyphae (f) and partial colonization are observed

### Root fragments colonized by AMF, stained with blue ink Parker solution –HCL

The same represent two orders (Glomerales and Diversisporales), four families (Glomeraceae, Claroideoglomeraceae, Gigasporaceae, and Acaulosporaceae) and nine represented genera mainly by species of *Glomus*, *Acaulospora*, *Rhizoglomus*, *Funneliformis*, and *Claroideoglosum*, among the others. The above demonstrates the magnitude of the proposed alternative technique with the use of Parker washable blue ink as a colorant. It worth mentioning that there were not significant differences of mycorrhizal colonization degree or intensity determined in plant roots with different characteristics assessed by both staining methods employing as dye, blue Trypan (standard method) or the pen washable ink that this work has proposed (Table).

## Results of the statistical analysis to compare percentages of mycorrhizal colonization and intensity determined in stained root samples with blue Trypan (Standard method) and blue washable ink Parker

Plant	Percentages of stained roots:				value t		Significance	
	Trypan blue		Washable blue ink		CM	I	CM	I
	CM	I	CM	I				
Corn	50±2,3	2,6±0,2	54±3,6	3,2±0,5	-0,75	-1,02	0,47	0,33
Tomato	72±0,03	4,3±0,9	71±3,07	3,9±0,6	0,53	0,92	0,64	0,40

(Standard ± error). Root samples average of twenty replicates for each plant species. T test for related samples ( $p < 0,05$ )  
MC-mycorrhizal colonization, I- intensity

## CONCLUSIONS

This study proposes modifications to the standard staining technique of AMF consisting of the replacement of Trypan blue dye for blue pen ink Parker Quink, washable in water, removal stage incubation of samples and elimination of the incubation stage of the samples in hydrochloric acid solution and the use of lactic acid and glycerine. While the alternative ink suitable for use in the previously described technique for this purpose. This represents a considerable saving of time water, financial resources. Equally important is contributing to the security of involved laboratory staff because its exposure is avoided to carcinogenic and toxic reagents and the environment conservation, because it avoids the emission of such reagents, either as gases or water which is washed the same.

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