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Amending storage vessel and media increase transfer interval of *Musa* spp. tissue culture plantlets[1,2]

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ABSTRACT

Musa **spp. are some of the most important fruit food crops in the world. The USDA-ARS TARS maintains a *Musa* spp. germplasm collection of 164 accessions in field plots and in medium-term storage in vitro. Accessions maintained in vitro require routine sub-culturing as nutrient medium is lost due to uptake by the plant. Culture transfer intervals occur every six months and the transfer process is a resource and time consuming effort. To lengthen the transfer interval, an experiment was conducted to evaluate storage medium modifications and storage vessels on four *Musa* spp. accessions. Treatments consisted of glass tubes, glass tubes with Parafilm®, and plastic culture bags with three medium alterations: Murashige and Skoog (MS) medium, ½ strength MS and MS with 4% D-mannitol. Treatment effects were estimated by measuring plantlet's overall appearance, shoot and leaf number, and rooting on a monthly basis. All medium formulations for all four accessions, in glass tubes with Parafilm® and in culture bags showed significantly increased sub-culture interval times. The ½ MS treatment initially retarded plantlet development and showed the shortest storage time for all accessions. Storage time could be extended to 12 months with tissue culture bags, and to over 16 months with sealed tubes. The simplicity of**

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using culture bags for distribution and the decrease in contamination during storage in bags were identified as additional advantages.

Key words: *Musa*, tissue culture, in vitro, germplasm

RESUMEN

Modificaciones al envase y al medio aumentan el intervalo de transferencia *in vitro* de plántulas de *Musa* spp.

Los bananos y plátanos (*Musa* spp.) son de los cultivos alimenticios más importantes del mundo. El USDA-ARS TARS mantiene una colección de alrededor de 164 accesiones de *Musa* spp. en el campo y almacenado in vitro. Las accesiones que se mantienen in vitro requieren transferencias rutinarias al absorberse los nutrientes. Los intervalos de transferencia se hacen, en promedio, cada seis meses en un proceso que requiere muchos recursos. Para extender el intervalo entre transferencias, se evaluaron tres modificaciones al medio nutritivo y tres envases de almacenamiento utilizando cuatro accesiones de *Musa* spp. Los envases de almacenamiento consistieron en tubos de cristal no sellados y sellados con Parafilm®, y bolsas de cultivo plásticas. Las tres modificaciones al medio nutritivo Murashige and Skoog (MS) fueron: concentración completa, ½ de concentración y concentración completa con 4% D-mannitol. Los efectos de los tratamientos se evaluaron mensualmente midiendo la apariencia general de la plántula, enraizamiento, y número de hojas y tallos. Para los tres diferentes medios y para las cuatro accesiones se vieron diferencias significativas en intervalos de transferencia para las plántulas en los tubos de cristal sellados con Parafilm® y en las bolsas de cultivo plásticas. El tratamiento de MS a la ½ de concentración retardó el crecimiento de las plántulas y mostró el tiempo más corto en almacenamiento para las cuatro accesiones independientemente del envase. El tiempo de almacenamiento se pudo prolongar a 12 meses con las bolsas de cultivo plásticas, y a 16 meses con los tubos sellados. Sin embargo, las bolsas de cultivo plásticas son más económicamente ventajosas debido a su simplicidad para propósitos de distribución y por su baja incidencia de contaminación.

Palabras clave: *Musa*, cultivo de tejido, *in vitro*, germoplasma

INTRODUCTION

Plant genetic resources are threatened by genetic erosion caused by habitat loss, natural disasters, insect and disease pests, and by poor management of field collections. To safeguard plant genetic resources, *ex situ* germplasm collections are established and serve as repositories for economically important agronomic and/or horticultural genetic traits from which plant breeders, along with producers, can benefit (Bretting and Bennet, 2007; Bretting, 2006; Clark et al., 1997; Rubenstein et al., 2006). For the majority of agronomic crops, genetic resources in germplasm collections are maintained in the form of seed. However, seed storage is not possible for crop species propagated vegetatively (i.e., cuttings, grafted, air-layered, or divided) (Postman et al., 2006; Reed and Chang, 1997; Scherwinksi-Pereira et al., 2010). This is especially true with many tropical crop species that must be vegetatively propagated due to their partheno-

carpic, apomictic, and/or seed-recalcitrant nature of reproduction (Ito et al., 1990; Ng and Ng, 1991; Dodds and Roberts, 1995). These crop species must be maintained by utilizing vegetative propagules for storage if any extended period of conservation is sought (Malaurie, 2001; Engelmann, 1998).

Bananas and plantains (*Musa* spp. Colla.), in the Musaceae family, are some of the most agriculturally important food crops worldwide. *Musa* spp. are native to tropical and subtropical regions of India and Southeast Asia (Uma et al., 2005; Volkaert et al., 2011), and cultivars are currently grown in more than 100 countries throughout the world. According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2012), total world production for bananas and plantains accounted for close to 139 million metric tons in 2010. The export of bananas and plantains accounts for a significant amount of the worldwide industry. However, *Musa* spp. are grown mostly as a staple crop by smallholder farmers where they provide important nutritional value (Davey et al., 2009).

Because of the lack of seed production in most cultivated germplasm and in order to maintain genetic integrity, *Musa* spp. genetic resources must be maintained vegetatively. Maintaining collections of *Musa* spp. in field settings has limitations when conserving genetic resources. The area needed for managing collections can be large, diseases and insect pests must be managed, and unpredictable environmental conditions (e.g., hurricanes) contribute to difficulties in managing and conserving field collections. In addition, germplasm from field collections are more prone to infestation by pest and diseases making distribution of propagative material difficult and more restricted (Diekmann and Putter, 1996). To avoid some of the above mentioned limitations, *Musa* spp. genetic resources may be propagated in vitro through tissue culture techniques and stored for intermediate periods of time (medium-term). Besides providing an aseptic, easy and effective way for distributing germplasm, in vitro collections may serve as a starting point for aseptic material for plant transformation (Tripathi et al., 2005) and for long-term backup cryopreservation (Panis et al., 1996) of plant material in field collections. Many plant tissue culture collections are stored under short photoperiod, low light intensity, and under relatively low temperatures (~15 to 20 °C) to reduce plant growth rate and allow for medium-term storage (Banerjee and Langhe, 1985; Van den Houwe and Jones, 1994; Oliveira et al., 2000). Even under these conditions, a resource-demanding sub-culturing routine is required (Bhat and Chandel, 1993; Van den Houwe et al., 1995).

The USDA-ARS Tropical Agriculture Research Station (TARS) in Mayagüez, Puerto Rico, is part of the National Plant Germplasm System and as such is charged with the maintenance of a number of tropical plant genetic resources including the *Musa* spp. collection. *Musa* spp. ac-

cessions are currently maintained in a field collection for characterization and evaluation purposes as well as in vitro for medium-term storage and for distribution of disease-free germplasm. The objectives of this study were to determine optimal medium-term storage medium and containers and to determine maximum sub-culturing intervals for micropropagated *Musa* spp. tissue culture plantlets maintained in the collection.

MATERIALS AND METHODS

Stock cultures. All stock plants were grown on Murashige and Skoog (1962) macro- and micro-nutrients and vitamins (Sigma-Aldrich, St. Louis, MO)[5] with 230 mg/L KH_2PO_4, 0.3 mg/L thiamine hydrochloride (Sigma-Aldrich, St. Louis, MO), 30.0 g/L sucrose, 8.0 g/L agar and 1.00 mmol BAP. The choice stock culture used was described in Vuylsteke (1989) with standard nutrients and low BAP to reduce cell proliferation and retard active growth.

Storage conditions. All cultures were stored at 23° C with a 12-hour light/dark cycle provided by Sylvania GRO-LUX 40 Watt T12 fluorescent bulbs.

Storage vessels. The storage vessels evaluated in the experiment included, standard Pyrex® culture tubes (25 mm × 150 mm) and Star*Pack® breathable 5-chambered (15.2 × 22.9 cm) tissue culture bags (Garner U.S. Enterprises, Inc., Willis, TX). Each of the four accessions were planted in the three storage vessel treatments: 1) culture tubes with a polypropylene 25 mm closure cap; 2) culture tubes with a cap and sealed in Parafilm®; and 3) tissue culture bags.

Growth medium. Three treatments were used with all containers: 1) full strength MS medium as listed above; 2) ½ strength MS salts with the vitamins and amendments as above; and 3) full strength MS (as above) with vitamins and amendments and with 4.0 mg/L D-Mannitol (Sigma-Aldrich, St. Louis, MO).

Plant materials. Four *Musa* spp. accessions/cultivars ('Valery', 'Dwarf Cavendish', 'Pelipita-Colombia' and 'Pelipita-Costa Rica') were included in the evaluation. These genotypes were selected because they were readily available at the onset of the experiment, plants multiplied quickly and they belonged to two separate genomic *Musa* spp. subgroups; the dessert banana or 'Cavendish' (AAA) sub-group ('Valery',

[5]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the U.S. Department of Agriculture or the Agricultural Experiment Station of the University of Puerto Rico.

and 'Dwarf Cavendish') and the starchy cooking-banana (ABB) subgroup ('Pelipita-Colombia' and 'Pelipita-Costa Rica').

Experimental design. The experiment consisted of a split-split plot design with three replications, using storage vessels as main plots, growth medium as subplots, and accessions as sub-subplots. Treatments consisted of 10 replicates for a total of 360 plants for the 36 treatments and each experiment was repeated three times.

Evaluation. The number of living, dead, and contaminated plants and a rating of the overall condition of the cultures were recorded 16 times at monthly intervals for the duration of the experiment. Rating of the roots, leaves and shoots was conducted for the first six months. The root rating was based on the following scale: 0 = no roots; 1 = one to five roots; 2 = five to 10 roots; 3 > 10 roots (Figure 1). Leaves were

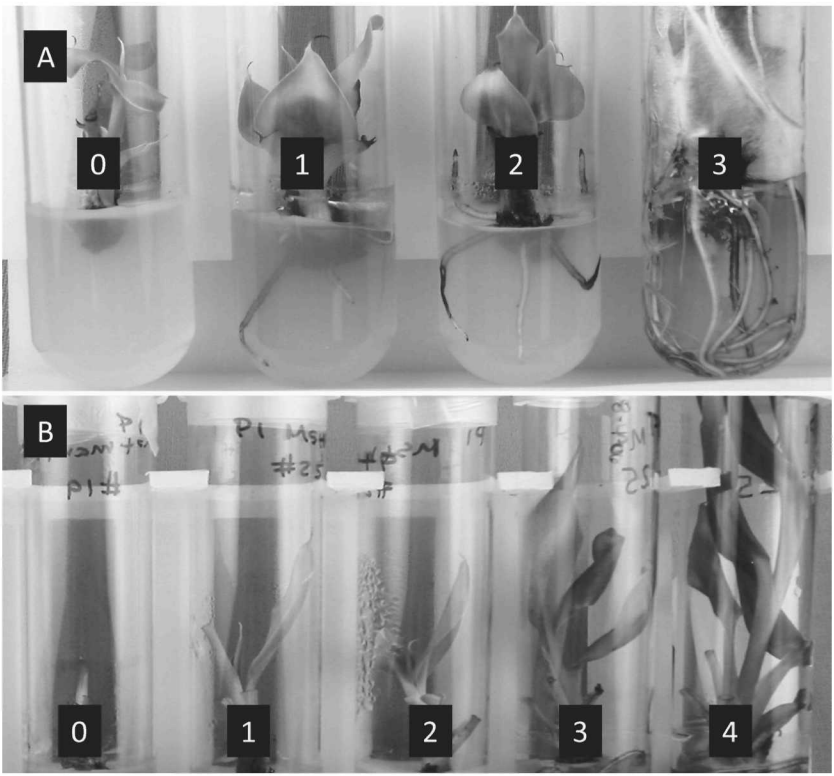

FIGURE 1. Images of representative *Musa* spp. tissue culture plantlets in culture tubes at different development stages used for scoring of A) roots and root categories (0 = no roots, 1 = one to five roots, 2 = five to 10 roots, 3 > 10 roots); and B) leaf number.

rated by counting the number of true leaves (completely unfolded) as they emerged (Figure 1). The number of shoots or adventitious 'suckers' arising from the initial mother rhizome was also counted. An overall appearance rating was used to evaluate plantlet condition for the length of the experiment. Rating scale for the overall appearance was: 5 = non-etiolated, dark green, older leaves drying up/senescing naturally; 4 = non-etiolated, medium-green color, two to three true leaves drying up/senescing; 3 = etiolated, retaining medium-green color, half true leaves drying up/senescing; 2 = etiolated, pale green color, 2/3 true leaves drying up/senescing; 1 = etiolated, pale tan, no green color, most true leaves drying up/senescing; 0 = dead, brown. Cultures rated as 2 or 1 were considered to be at the end of their storage life and would have been removed for re-propagation; however, to evaluate complete treatment effects, plants continued to be evaluated until they reached a rating of 0. All cultures were monitored for contaminants and removed if a contaminant was noticed.

Statistical analysis. Analysis of variance was carried out by using the GLM procedure of SAS ver. 9.1 (SAS Institute, Cary, NC). Significant treatments and/or interactions ($P < 0.05$) were compared by mean separation utilizing the Waller-Duncan test.

RESULTS AND DISCUSSION

Storage vessel

The storage vessel had a significant effect on three of the four variables evaluated. The overall mean rating over the 16 ratings for storage vessel, number of roots and leaves showed significant differences among the three treatments. No significant differences in shoot numbers were observed when evaluating the storage vessels. The culture tube plus Parafilm® vessel treatment performed the best, the culture bag was intermediate and unsealed culture tubes had the worst overall average ratings of the 16 evaluations (Table 1). No significant interactions were observed for storage vessel with any of the other factors evaluated. Although the overall number of cultures contaminated was relatively low for the experiments, there were clear differences associated with the vessels employed. The total number of contaminated plants in the glass tubes was the highest (22), followed by culture tube plus Parafilm® (17) and lowest for the culture bag (6) (data not shown).

Medium

Significant differences among mean ratings were observed for three of the four variables. Overall mean rating, number of roots and leaves

were significantly different among treatments, being highest for the standard MS medium and lowest for the ½ strength MS medium with MS plus mannitol intermediate (Table 1). When observations for mean root rating were compared with the medium treatments, significant differences were observed with the standard MS medium having the highest mean root rating (1.77), and the lowest was that of the ½ strength MS medium (1.43). Significant differences were also observed for the mean number of leaves with a range in ratings of 3.55 for the standard MS medium to 2.95 for the ½ strength MS medium. Differences were observed for mean number of shoots across medium treatments with the standard MS medium (0.32) being significantly distinct when compared to the MS plus mannitol (0.43) and the ½ strength MS media (0.48). No significant interactions were observed for medium with any of the other factors evaluated.

Accession

Significant differences among mean ratings were observed for all four variables. Significant differences in overall mean rating among accessions was observed and ranged from a 3.70 for the 'Dwarf Cavendish' accession to 3.37 for both the 'Valery' and 'Pelipita-Costa Rica' accessions (Table 1). Likewise, significant differences were observed

TABLE 1.—*Effect of vessel, medium and accession on overall plant health and number of roots, leaves and shoots on* Musa *spp. tissue culture plantlets.*

Treatments	Rating			
Storage vessel	Overall[1]	No. Roots[2]	No. Leaves	No. Shoots
Culture tube+Parafilm®	4.39 a[3]	1.83 a	3.68 a	0.40 a
Tissue culture bag	3.66 b	1.53 b	3.38 b	0.39 a
Culture tube	2.37 c	1.43 c	2.81 c	0.43 a
Medium[4]				
Standard MS	3.66 a	1.77 a	3.55 a	0.32 b
MS + Mannitol	3.51 b	1.58 b	3.36 b	0.43 a
½ X MS	3.28 c	1.43 c	2.95 c	0.48 a
Accession				
Dwarf Cavendish	3.70 a	2.21 a	3.93 a	0.45 b
Pelipita-Colombia	3.51 b	1.21 d	2.71 d	0.68 a
Valery	3.37 c	1.69 b	3.55 b	0.27 c
Pelipita-Costa Rica	3.37 c	1.27 c	2.99 c	0.23 c

[1]Mean overall score was obtained by averaging across all ratings for the 16 monthly evaluations.
[2]Mean for roots, leaves and shoots were obtained by averaging across all ratings for the first six monthly evaluations.
[3]Means followed by the same letter within a column are not significantly different (P = 0.05).
[4]Medium treatments were 1) standard Murashige and Skoog (MS) medium, ½ strength MS and the MS with 4% D-mannitol.

between mean number of roots and leaves among the four accessions. Unlike in the main plot and subplot, larger and significant differences in the mean number of shoots were observed within accessions (sub-subplot). The highest mean was for the 'Pelipita-Colombia' accession (0.68), whereas the lowest was for the 'Pelipita-Costa Rica' accession (0.23). No significant interactions were observed for accession with any of the other factors evaluated.

Time course experiment

The time course of plant quality ratings in the three storage container treatments is shown in Figure 2. The three containers provided good storage for the shoot cultures during the first eight months (ratings >2). Plants in sealed tubes remained in good condition with ratings >3 during 14 months and slowly declined for the next two months, but the rating remained above 2 at the end of the 16-month experiment. Plants in bags showed high ratings during 11 months and gradually declined to ratings <2 after 13 months. In contrast, plants in unsealed tubes began to decline rapidly after seven months, being rated <2 by about eight months and 1 at nine months. Only the Parafilm® sealed tubes were suitable for re-propagation by the end of the 16-month experiment. At the conclusion of the experiment and for the 16th evaluation, only the culture tube plus Parafilm® showed an average rating above a 2 (2.17), followed by the culture bag with a rating of 0.59 and

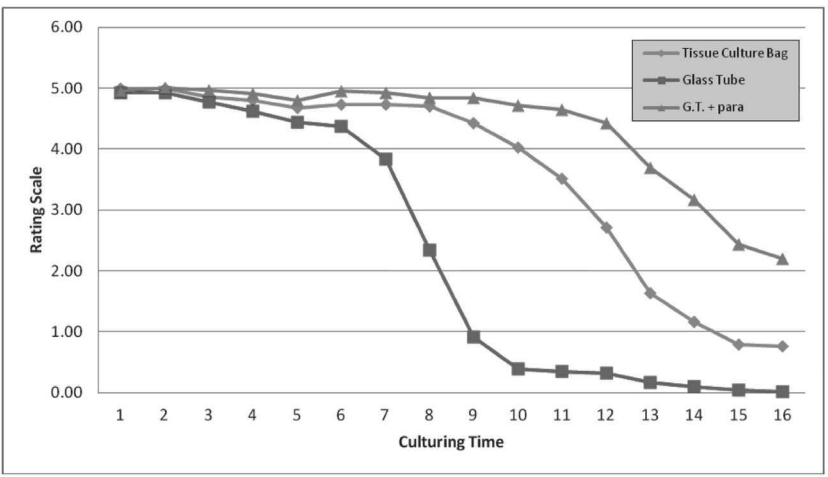

FIGURE 2. Relationship between culturing period (months) and overall rating of *Musa* spp. tissue culture plantlets grown in glass tubes, tissue culture bags, and glass tubes with Parafilm® (G.T. + para).

last the unsealed culture tubes with an average rating of 0.01 (Figure 2). Figure 3 provides an example of overall appearance and ratings for 'Pelipita-Colombia' at the 12-month rating date for the three storage vessels in the standard MS medium.

The in vitro *Musa* spp. collection held at the USDA-ARS TARS site currently requires transfer intervals every six months using unsealed culture tubes and full-strength MS medium. In efforts to lengthen standard transfer intervals for medium-term conservation of in vitro maintained tissue cultured plantlets, an experiment was conducted to determine the best storage vessel and storage medium utilizing four *Musa* spp. accessions. In this study, the sub-culturing transfer interval for all treatments was extended when compared to the control storage condition of full strength MS in unsealed glass culture tubes. The mean overall rating of unsealed glass tubes at the end of the experiment was 2.37 (Table 1). This finding contrasts with the best overall mean rating of the culture tube plus Parafilm® treatment, which had an overall rating of 4.39 at the end of the experiment. The low overall rating for the unsealed culture tubes meant that a large number of plants had a rating below 2, were dead, or in need of transfer to prevent loss. At the TARS site, the current transfer interval using unsealed glass tubes is carried out approximately every six months when plants begin to etiolate, lose their green color and when most of the true leaves begin to senesce/dry. This condition corresponds to a rating of 2 on the overall rating scale used in the current evaluation. With the standard methodology utilized, most plantlets are approximately six months into culture when this rapid decline is observed (Figure 2). Of the factors evaluated herein, storage vessel had the largest influence on overall plant health since nine out of the 10 highest overall mean ratings for all possible treatment combinations were for culture tubes wrapped

FIGURE 3. Example of overall appearance for *Musa* spp. 'Pelipita-Colombia' accession in vitro grown plantlets on the standard MS medium at the 12-month rating date for the three storage vessels: A) glass tube with Parafilm®; B) culture bag; and C) glass tube.

with Parafilm® (data not shown). For both treatments where plantlet was 'sealed', plant evapotranspiration and medium evaporation were minimized, maintaining plant health and extending the transfer interval. However, a disadvantage observed with the Parafilm® sealed glass tubes was the considerable amount of time involved in the removal of residual film from the glass tubes, prior to and during washing and re-use.

When the media treatments were compared, a general trend showed that shoots grown on MS had higher overall ratings, followed by those on the MS plus D-mannitol and the lowest ratings on the ½ MS medium. Regardless of storage vessel and accession, reduced initial growth was noticed for the ½ strength MS medium treatment when growth parameters were rated and this effect caused the lower values shown in Table 1 for mean number of roots and leaves. It was assumed that the half-strength MS medium treatment might outperform (i.e., have a higher overall mean rating and therefore have a longer transfer interval at the conclusion of the evaluation) the other treatments because of its slow growth and nutrient uptake. However, the plants never developed normally and were rated poorest among the treatments at the end of the evaluation period. Utilizing a three fourth-strength MS medium might be appropriate for extending the transfer interval while keeping plants healthy; efforts in this direction are being evaluated. Addition of mannitol, as a carbon source, to in vitro cultured plants reduces growth rate and extends transfer interval for *Musa* spp. (Bhat and Chandel, 1993) and other crops such as celery (Stoop and Pharr, 1993). Plants grown on MS amended with mannitol had mean overall ratings lower than the full strength MS medium and showed a reduced number of leaves, roots and shoots. The 4% mannitol used in the treatment was equal to the highest percentage rate at which no deleterious side effects were reported by Bhat and Chandel (1993). Unlike the findings of Bhat and Chandel (1993), the 4% mannitol in the current evaluation appeared to retard growth when compared to the full-strength MS medium. The standard MS medium treatment seemed to provide optimal nutrients for growth and thus, although plants grew faster initially, based on the ratings for number of roots and leaves, they also survived longer once nutrients became limited.

While significant differences in overall rating, number of roots, leaves and shoots were observed for the accessions evaluated, no clear pattern could be established between the accessions evaluated. The number of roots and leaves during the first six months of the experimental period was higher for 'Cavendish' AAA accessions when compared to the 'cooking banana' ABB accessions, but this was not the case for number of shoots where no clear pattern was evident. Oliveira et al.

(2000) found no effects on sub-culturing interval among distinct *Musa* spp. accessions within the (AA) diploid subgroup with intervals of six, 12 and 15 months, for the three temperatures evaluated (17, 22, and 26 oC). In contrast, Van den Houwe et al. (1995) reported finding significant differences in transfer interval when evaluating temperature effects for accessions from multiple *Musa* spp. subgroups (AA, AAB, ABB) with transfer intervals ranging from two months up to over a year and a half. In both of the previous described evaluations, standard glass test tubes and media were used with similar temperature ranges and the observed divergence in these two efforts might be that Oliveira et al. (2000) looked only at accessions within the diploid (AA) subgroup, while Van den Houwe et al. (1995) evaluated a larger number of accessions, including hybrids from several subgroups. In the present study significant differences were observed among two *Musa* spp. subgroups (AAA, ABB), although observed differences among accessions were minor in terms of storage time.

Data collected during the evaluation indicated marked and significant differences in storage transfer intervals among *Musa* spp. tissue culture plantlets. The development of roots, leaves and shoots would have indicated rapid or slow growth and might have an effect on the length of time maintained in storage prior to sub-culturing. Medium modifications to slow down growth and extend the interval between sub-culturing did not work. Half strength MS and the MS plus D-mannitol treatments reduced growth and translated to poor plant development, lower overall rating and less than optimal transfer interval times. In the current evaluation, all treatments improved storage interval and at least doubled the time between sub-cultures based on overall rating. Ideally, a plant would be in storage for a year with an overall rating of no less than a 2 prior to sub-culturing.

Although it was apparent that for storage vessels test tubes with the Parafilm® were the best treatment (4.39), when it came to the rating for mean overall appearance, the tissue culture bags rated well (3.66) and better than the current standard glass vessel (2.37). Culture bags showed very low contamination rates and have the additional advantage of 'off-the-shelf' shipment with no transfer from a glass container prior to shipment required. Therefore, we conclude that culture bags are the most economically-viable option for medium-term in vitro storage of *Musa* spp.

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