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Storage of *In Vitro* Banana Shoot Cultures at Low Temperature or under Mineral Oil Layer

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ABSTRACT

Banana micropropagated plantlets were either stored at 10ºC or covered under mineral oil for 10 weeks. Pronounced decline in growth rate was recorded in samples stored under mineral oil (8.15%) followed by 27.94% for those stored at 10ºC. Accumulation of chlorophyll and changes in protein profile were noticed during the storage period. Regrowth after recovery period proved that banana shoots could successfully restore activity after storage. Results obtained confirmed the possibility of storing banana micropropagation shoots *in vitro* by both treatments without losing viability.

Key Words: Storage; *in vitro*; Banana shoots; Low temperature; Mineral oil

INTRODUCTION

During the last thirty years, tissue culture techniques have been extensively developed and applied for many species. These techniques are of great interest for the collecting, selecting, multiplication and conservation of plant germplasm (Engelmann, 1991). However, the high multiplication rates which are usually achieved using *in vitro* culture techniques, lead to the regular production of large amounts of plant material. This creates problems for the management of large *in vitro* collections. In addition, risks of losing material through contamination or human error are present at each subculture. More importantly, the risks of losing the genetic integrity of the plant material through somaclonal variation increase with time in culture (Scowcroft, 1984). Thus, *in vitro* conservation of cultures is urgently needed to minimize the previously mentioned risks in particular, preserve the genetic integrity of the plant material. *In vitro* conservation of cultures has been applied with varying degrees of success to wide range of species and culture systems, and successful slow growth systems were developed for different species (Withers *et al*., 1990; Engelmann, 1991; Janiero *et al.*, 1995; Negri *et al.*, 2000).

Different *in vitro* conservation methods were employed depending on the storage duration required. For long-term storage, cryopreservation (Lecouteux *et al*., 1991; Kendall *et al*., 1993; Fretz & Lorz, 1995; Mycock *et al*., 1995; Thierry *et al*., 1999) and encapsulation (Maruyama *et al*., 1997; Castillo *et al*., 1998; Vandenbussche & De Proft, 1998; Pattnaik & Chand, 2000; Soneji *et al*., 2002) were used. For short- or medium-term storage, the aim is to reduce growth and to increase the intervals between subcultures. Slow growth *in vitro* may be obtained by low temperature (Moriguch *et al*., 1988; Bertrand-Debrunais *et al*., 1992; Hausman *et al*., 1994; Janeiro *et al*., 1995; Romano & Martin-Loucao, 1999; Negri *et al*., 2000), osmotic stress (Grout, 1991; Withers, 1991; Dodds & Roberts, 1995), a low concentration of nutrients (Mannonen *et al*., 1990; Mathur *et al*., 1991; Berjak *et al*., 1996; Negri *et al.*, 2000) or under mineral oil (Augereau *et al*., 1986; Gillis & Debergh, 1989; Lee & Lam-Chan, 1995; Zamora *et al*., 1999).

The aim of the present work was to study the effect of storage under mineral oil or low temperature on the growth of banana micropropagated shoots.

MATERIALS AND METHODS

In vitro cultures of banana shoots (*Musa* Cv. Grande) were initiated from terminal buds and were proliferated and maintained by monthly subcultures on the medium described by Drew *et al.* (1989). To study the effect of short-term storage, some micropropagated banana shoots were incubated at 10ºC or covered with autoclaved mineral oil (Light White Oil, ICN Biochemical Inc., Ohio, USA) and incubated at 27ºC. Ten weeks latter, the *in vitro* cultures were transferred to standard culture conditions on fresh medium and were allowed to recover for four weeks. The tissues growing under mineral oil (Fig. 1) were first washed with hexane and then rinsed 3-4 times in sterile distilled water.

Fresh weight and chlorophyll content. The fresh weight (expressed as % of fresh weight increase) as well as chlorophyll content (measured according to Wintermans & De Mots, 1965) were determined at the end of storage experiment (10 weeks) and after recovery (4 weeks).

Protein analysis (determined according to Laemmli, 1970)**.** Samples, taken at the end of storage experiment and after recovery, were frozen in liquid nitrogen and ground in a mortar in the presence of Tris-HCl buffer (pH 6-8) containing 8% (W/V) insoluble polyvinyl pyrolidone. Then, complete destruction of cell walls was

achieved using "Hielscher Ultrasonic Processor UP50H". The homogenate was then centrifugated for 12 minutes at 12000 rpm. The supernatant of the samples was concentrated by freeze-drying using "Automatic Environmental Speed Vac.". Protein content was assayed using the method originally described by Bradford (1976). Samples (30 µg protein) were loaded on 12.2% SDS-polyacrylamide gel and allowed to run at constant electric current (25 mA) for five hours. The molecular weight markers were run alongside the protein samples. The gel was then stained for 6 hours with 0.125% coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and destained in methanol: acetic acid: water (5: 1: 4, V: V: V) for several hours.

Students't Test was used for the comparison of different means.

RESULTS AND DISCCUSION

A pronounced decline in growth rate (expressed as % of fresh weight increase) of banana micropropagated shoots was observed following storage for 10 weeks at 10ºC (27.94%, Table I). This reduction in growth rate was significantly more in shoots stored under mineral oil overlay (8.15%). However, these shoots restored their ability to grow after transferred to standard cultured conditions. It is interesting to mention that the difference in recovery of growth of shoots stored at 10ºC (49.07%) and those stored under mineral oil (41.56%) was non-significant. Both techniques of storage have been reported earlier to be suitable for a variety of *in vitro* cultures. When Augereau *et al*. (1986) stored callus cultures of three strains; *Catharanthus roseus*, *Voacanga thouarsii* and *Coffea arabica* at 5, 10 or 15ºC or under a layer of mineral oil, they found that growth characteristics and secondary metabolite production were preserved for 4-6 months. Gillis and Debergh (1989) also stored tissue-cultured materials of a number of species under autoclaved paraffin oil for 4-6 months. Lavieville *et al*. (1991) stored callus culture of *Datura innoxia* at low temperature of 10 or 15ºC for 19 weeks or under anoxic conditions under liquid paraffin for 11 weeks. They reported that the survival rate was more than 90% but the capacity for alkaloid synthesis declined after recovery. Survival of callus was reported by Lee and Lam-Chan (1994) when they stored callus tissues of Khaw Phaik Suan under mineral oil. Zamora *et al.* (1999) stored bamboo node cultures under mineral oil for 12 weeks then transferred them to proliferation medium to assess viability. They reported that mineral oil proved to be an effective storage technique (up to 3 months) preventing loss of moisture and slowing growth. On contrast to the previous data, Mathur *et al.* (1991) reported that rate of growth and morphogenesis in cultures covered with mineral oil was greatly reduced compared to the control. Mannonen *et al.* (1990) also found that preservation of *Paxa ginseng* and

Fig. 1. Micropropagated banana shoot covered with mineral oil

Fig. 2. SDS-PAGE of *Musa* micropropagated shoots: lane (1) the molecular weight markers; lane (2) control; lanes (3 & 4) banana shoots stored at 10ºC or under mineral oil for ten weeks, respectively; lanes (5 & 6) represent shoots recovered for 4 weeks after low temperature or mineral oil storage, respectively

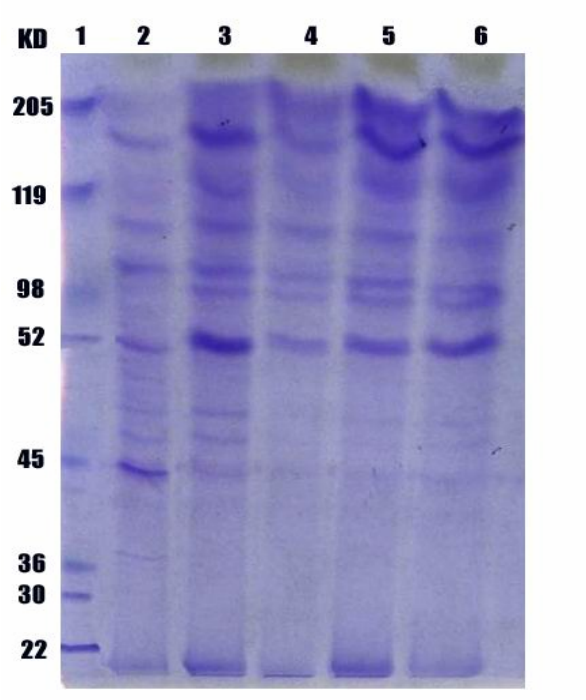

Table I. Effect of storage at 10ºC or under mineral oil overlay on the % of fresh weight increase and total chlorophyll of *in vitro* banana shoot cultures. Each value is the mean ± SE of 10 replications

Treatment	% of fresh weight increase**		Total chlorophyll (mg/g dry weight)	
	After storage	After Recovery	After Storage	After Recovery
Control	80.12±2.89*		3.28±0.043	
At 10ºC	27.94±1.86	49.07±5.98	6.19±0.33	4.84±0.42
Mineral oil	8.15±1.84	41.56±2.41	6.69±0.46	4.94±0.5

* Control was subcultured regularly every 4 weeks;** = $\frac{\text{Final fresh weight – Starting fresh weight}}{\text{Starting fresh weight}} \times 100$

Catharanthus roseus under mineral oil does not preserve the production of cell cultures.

In addition, several reports have been published on *in vitro* storage of tissue cultured materials at low temperatures. Fortes and Pereira (2001) reported that the growth rate of micropropagated asparagus shoots maintained at 6ºC for 4 months was 15.6% and the survival rate after 9 months, regardless of treatments, was 25%. Yu *et al*. (2003) stored 22 varieties of strawberry at 4ºC and reported that only 8 varieties could be preserved for 4 months with a survival percentage of over 50%. Benelli *et al*. (2003) reported that grape shoots kept at 10ºC had higher survival rate (80%) than those conserved at 4ºC. On the other hand, Wilson *et al*. (1998) indicated that, regardless of media composition or illumination, *in vitro* storage of broccoli seedlings at low temperatures for more than 8 weeks resulted in dramatic losses and recovery as well as photosynthetic ability.

Total chlorophyll of micropropagated banana shoots showed high accumulation in samples stored at 10ºC and under mineral oil (Table I). This result was in agreement with Kubota and Kozai (1994) who reported that storing broccoli micropropagated shoots at low temperature was accompanied by an increase in chlorophyll content. Similar results were also reported by Wilson *et al*. (1998) working on low temperature *in vitro* storage of broccoli seedlings.

Fig. 2 shows the SDS-PAGE of *Musa* micropropagated shoots stored at 10ºC or under mineral oil for 10 weeks (lanes 3 & 4, respectively) or recovered after 4 weeks at standard culture conditions (lanes 5 & 6). Lane 2 (control) shows the presence of 4 major bands of 200, 100, 54 and 43 KD and 5 minor bands of 119, 98, 50, 48 and 38 KD. Preserving *Musa* plantlets at 10ºC stimulated the over-production of the polypeptides of 200, 98 and 54 KD, which shows high intensity in lane 3. All the other polypeptides present in the control were still synthesized under 10ºC except that of 38 KD. Preserving *in vitro* banana shoots under mineral oil (lane 4) showed less accumulation of polypeptides between 200 and 50 KD, while low molecular weight ones disappeared. The same results were observed in plantlets recovered from 10ºC or mineral oil (lanes 5 & 6, respectively) where high molecular weight fractions showed high intensity while low molecular weight polypeptides (between 200 & 54 KD) almost disappeared. Robertson *et al.* (1988) reported that low temperature treatment inhibited the synthesis of a 22 KD protein. Changes in protein pattern of *Bromus inermis* cell suspension culture pretreated with ABA to induce cold tolerance was indicated by Robertson *et al*. (1995). The same result was also showed for acquisition of tolerance to cryopreservation of carrot somatic embryos (Thierry *et al*., 1999). Recently, Hassan (2004) reported that exposing micropropagated shoots to low temperature stimulated the synthesis of a new polypeptide of 47 KD.

CONCLUSION

The results obtained in the present investigation confirmed that storage of micropropagated banana shoots at 10ºC or under mineral oil preserved their viability for 10 weeks without the need of subculturing. Frequent subcultures make cultured materials vulnerable to be lost. This may be due to: chromosomal aberrations, mutations and changes in ploidy level besides the risk of contamination. In agreement with Augereau *et al*. (1986), the study suggests that storage under mineral oil is a very attractive method for the conservation of numerous strains cultivated on solid medium. As this method does not need any specific investment, it is available to any laboratory using normal culture conditions for plant tissues. Mineral oil is known to dissolve 4 times the amount of oxygen solubilized in water and supplies the minimum quantity of oxygen required for the shoots to survive (Moriguchi *et al.*, 1988). On the other hand, cryopreservation, applied to a number of strains, takes a long time to be perfectly adjusted and often requires sophisticated and high-cost cooling apparatus which is not accessible to numerous laboratories.

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