DEVELOPMENT OF *IN VITRO* TECHNIQUES FOR THE ELIMINATION OF CUCUMBER MOSAIC VIRUS FROM BANANA (*MUSA* SPP.)

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Abstract

Bananas and plantains (*Musa* spp.) are threatened by various pests and diseases, including a number of important viral diseases which represent a constraint on banana production and on germplasm movement from country to country. The latter is especially important for farmers who are waiting to benefit from pest- and disease-resistant varieties, either naturally occurring germplasm or improved varieties produced by breeding programmes or by mutation techniques, as produced in the framework of the IAEA Co-ordinated Research Programme. INIBAP has therefore been very active in establishing a system for the safe international movement of *Musa* germplasm using different virus-indexing centres (South Africa, Australia and France). At present, about 25% of the International *Musa* Germplasm Collection maintained at K.U.Leuven (Belgium) by INIBAP, and in particular a significant number of potentially important and improved varieties from breeding programmes, are infected with viruses. Most of this germplasm is infected with BSV but also with CMV, BBTV, BanMMV and BBrMV.

To make these accessions available, a programme of virus elimination is currently carried on in the Plant Pathology Unit (FUSAGx, Belgium) in collaboration with the Laboratory of Tropical Crop Improvement (K.U.Leuven, Belgium). Different *in vitro* techniques, such thermotherapy, chemotherapy, electrotherapy or meristem culture, as well as more innovative ones, such as cryotherapy, were tested for their virus elimination capacity. For this, Williams BSJ banana plants (AAA) mechanically infected with CMV were used. Initially, the health status of regenerated material was checked on *in vitro* plants through ELISA. The putative virus-free material was then tested a second time after greenhouse acclimatisation.

1. INTRODUCTION

Bananas (genus *Musa*) are cultivated on five continents in about 120 countries, and provide a staple food for millions of people [1]. Banana production is threatened by different biotic agents such as bacteria, fungi or viruses, such as the Cucumber Mosaic Virus (CMV) [2]. CMV, which consists of a spherical particle of 28–30 nm in diameter containing ssRNA, is naturally transmitted by aphid vectors or by seed [2]. Hu et al. [3] have identified banana isolates that belong to the subgroup I, DTL serotype. Symptoms depend on the strain of the virus pathogen and growth temperature. Thus, mild or severe chlorosis and necrosis on leaves and pseudostem can be observed, causing significant yield losses.

Since Morel [4] proposed the hypothesis of meristem 'immunity' towards viruses, meristem culture has been developed to eradicate viruses of various vegetative propagating plants, as reported by Bhojwani and Razdan [5] for CMV. Later, it was found that CMV particles could invade meristems

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and that a gradient of increasing virus concentration from the dome to the successive primordia exists [6]. This means that the possibility of obtaining virus-free plants is inversely related to the size of the meristem excised. Using this principle, Mori and Hosokawa [7] and Logan and Zettler [8] reported on the production of 100% CMV-free lily and gladiolus respectively.

To improve pathogen elimination, thermo- and chemotherapy coupled with meristem culture can be used when meristem culture alone fails [9]. Thermotherapy can be carried out on mother plants before meristem excision, or directly on *in vitro* meristem cultures. Since prolonged exposure to heat may damage host tissue, the use of alternated temperature could be beneficial for regeneration rate and eradication. Walkey [10] observed a complete CMV eradication from *Nicotiana rustica* when cultures were kept continuously at 32°C for 25 days. Virus elimination was greatly accelerated at 40°C, but with a subsequent deterioration of the cultured tissue. Inactivation of CMV in cultured *Nicotiana rustica* by alternating diurnal periods (40°C for 16 h and 22°C for 8 h over a period of 12 days) was later proposed by Walkey and Freeman [11] to increase culture survival.

Chemotherapy has also been applied to meristem cultures, but this has a negative effect on meristem growth. Virazole® (Ribavirin, 1-β-D-ribofuranosyl-1-2-4-triazole-3 carboxamide) [12], a synthetic analogue of guanosine, is a widely used antiviral substance [13]. When incorporated into the medium at 100 mg/l for up to 127 days, Virazole was quite effective in eradicating CMV from infected cultures of *Nicotiana rustica*, although the growth of tissue is reduced [14].

The application of electrical pulses to eliminate viruses from plant tissue has recently received much attention. Quacquerelli et al. [15] obtained symptomless almond plants and Lozoya-Saldana et al. [16] reported on the elimination of PVX from different clones of potato. Using an electrotherapy apparatus developed in Cuba (Patent Cuba 37/95 AO 1C/08 1524/97), Hernandez et al. [17] treated garlic (*Allium sativum* L), sugar cane (*Saccharum* sp. *hibrido* L.), potatoes (*Solanum tuberosum* L.) and araceas (*Xanthosomas* and *Colocasia*) for Potyvirus, Luteovirus and Carlavirus elimination respectively. For banana (cv. W. Bungulan (AAA)), Hernandez et al. [18] reported BSV elimination in approximately 40–80% of regenerated plants.

For many crops, cryopreservation is currently only being applied to overcome the serious limitations encountered by traditional germplasm conservation strategies in field-, seed- and *in vitro* culture collections. The conservation at ultra-low temperatures, usually -196°C (which is the temperature of liquid nitrogen), allows long-term, contamination-free storage of plant genetic resources. In 1997, Brison and co-workers [19] demonstrated for the first time that cryo-treatment can not only be used for germplasm conservation but also for virus eradication. Cryopreservation resulted in 50% virus-free *in vitro* plants from plum shoots infected with plum pox virus.

Thus far, reports on CMV elimination from infected banana plants are very scarce. Berg and Bustamante [20] observed that heat treatment (35–43°C for 100 days) performed on rhizomes (2.5 cm square and 5–7.5 cm long) in conjunction with meristem culture was inefficient for cleaning commercial banana cultivars. However, 56 plantlets out of 73 regenerated by culturing meristems of heat-treated rhizomes did not produce symptoms when extracts were used to inoculate indicator plants. CMV eradication was also achieved by Gupta [21] in approximately 100% of the regenerated plants when using meristem culture in combination with a two-week heat therapy (38–40°C). Again, heat therapy and meristem culture alone were not effective. In contradiction to the previous observations, Allam *et al.* [22] reported on CMV eradication from three different cultivars (Grand Naine, Dwarf and Williams) after 1 mm meristem culture alone as well as combined with thermotherapy, while the application of chemotherapy using 6-benzylaminopurine (BAP) to supplement the nutritive medium was unfruitful. Recently, we reported [23,24] on successful CMV eradication by cryopreservation of highly proliferating meristems of banana (cv. Williams BSJ, ITC.0570, AAA). Thirty percent of the regenerated plants were found to be healthy by DAS-ELISA, and this eradication rate was confirmed after a 6-month acclimatisation of plants in the greenhouse. The present study aims to evaluate the efficiency of the different *in vitro* techniques for CMV elimination in *Musa*.

2. MATERIAL AND METHODS

2.1. Plant material

The dessert banana cultivar Williams BSJ (ITC. 0570, AAA Cavendish subgroup) [25] was provided by the INIBAP Transit Center (ITC, Leuven, Belgium).

2.2. CMV transmission

The CMV isolate (sub-group DTL), originating from Colombia, was mechanically transmitted to banana plants. Leaves of CMV-infected tobacco were ground in a phosphate buffer (0.05 M KH_2PO_4, 0.01 M DIECA, pH 7.2) using carborundum (75 mg/ml) as an abrasive. The sap was applied for 10 min on leaves which were kept under low light conditions for 12 h. Leaves were then rinsed with water and plants were kept under low light conditions for 4–5 h before they were grown under normal conditions (23°C–16 h light/18°C–8 h dark regime with a light intensity of 39 µMole/m^2/s).

2.3. Production of proliferating meristem clumps

In vitro shoots were placed on p4 medium: Murashige and Skoog semi-solid medium [26] supplemented with 100 µM BA, 1 µM IAA and 3% sucrose. Every 1–2 months, the material was subcultured and only small white clumps of highly proliferating meristems were selected, and transferred to fresh medium.

2.4. Meristem tip culture

Apical meristem tips (domes with 1–2 leaf primordia) were excised in sterile conditions either from *in vivo* or *in vitro* plants or highly proliferating meristems, and transferred to glass tubes on 10 ml of solid MS medium. Tubes were maintained in a growth cabinet (culture room) at 24 ± 1°C in dark conditions for 3 days, and then under standard illuminated conditions.

2.5. Thermotherapy

CMV-infected *in vitro* or *in vivo* plants of banana cv. Williams BSJ (ITC.0570) were kept for one day in a growth cabinet under artificial light with diurnal alternating periods (16 h light/8 h dark). Day temperature in the growth cabinet was initially 28 ± 1°C and was increased at 2 ± 1°C per day until 40 ± 1°C was obtained. Night temperature was kept at 25 ± 1°C. When 40°C was reached, plant material was kept at this temperature for 4 weeks. Meristems (domes with four-leaf primordia) were then excised from *in vitro* or *in vivo* plants and transferred to MS medium.

2.6. Chemotherapy

2.6.1. Antiviral substances

The acyclic adenosine analogue (RS)-9-(2, 3-dihydroxypropyl) adenine [(RS)-DHPA] was kindly provided by J. Neyts (Rega Institute, K.U.Leuven, Belgium) while ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole carboxamide; Virazole®) was provided by ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA).

2.6.2. Phytotoxicity test

To monitor the phytotoxic effects of Virazole and (RS)-DHPA, meristem culture was performed on MS medium supplemented with 10, 50, 100 or 200 mg/l of Virazole or (RS)-DHPA. After a 3-month treatment, regenerated shoots were transferred to an MS medium. Regular MS medium is used as control. The phytotoxic effect was evaluated based on plant growth and mortality. Experiments were repeated twice.

2.6.3. Chemotherapy

Two types of experiments were carried out. In the first set, meristems excised from CMV-infected banana were grown in a culture room (standard conditions) for 3 months on MS medium to which 50 mg/l of Virazole or (RS)-DHPA was added. In the second set of experiments, highly proliferating meristems were chopped, mixed and transferred for 3 months to MS medium in glass tubes to which 50 mg/l of Virazole or (RS)-DHPA was added. After the treatment, material was transferred to fresh MS medium. Concentrations of chemical compounds used for the chemotherapy assays were determined after the phytotoxicity tests.

2.7. Electrotherapy

Electrotherapy assays were carried out either on infected *in vivo* or *in vitro* plants. For this, 2–3 cm long explants containing the apical meristem were excised from the plant material. Explants were washed for 5 min in a detergent bath, and then fixed in the electrotherapy chamber containing a NaCl solution (1 M). Pulses of 15 V were applied for 5 min using the electrotherapy kit developed in Cuba [18]. After the treatment, explants were surface-sterilised in 70% ethanol and then in a 3% commercial bleach solution. Meristems were then excised and placed on an MS culture medium. Temperature variations in the explants during the electro-treatment were recorded with a thermo-probe developed in the Physics Department of the University of Agricultural Sciences (FUSAGx, Gembloux, Belgium).

2.8. Cryopreservation

2.8.1. Preculture

White meristematic clumps (4 mm in diameter) containing at least four apical domes were transferred onto the preculture medium. This medium is identical to p4 medium except that the BA concentration is lowered to 10 µM and the sucrose concentration increased to 0.4 M. These cultures are kept for 2 weeks.

2.8.2. Loading

Sucrose-precultured meristematic clumps (1.5–3 mm in diameter) were isolated and kept for 20 min in a filter-sterilised loading solution (2 M glycerol and 0.4 M sucrose dissolved in MS medium, pH 5.8) at room temperature.

2.8.3. Treatment with vitrification solution (dehydration) and freezing

The loading solution was replaced by ice-cooled and filter-sterilised PVS-2 solution [27] containing 30% (3.26 M) glycerol, 15% (2.42 M) ethylene glycol (EG), 15% (1.9 M) DMSO and 0.4 M sucrose dissolved in MS medium (pH 5.8). Meristematic clumps were immersed in the PVS-2 solution for 120 min at 0°C, transferred to 2 ml cryotubes and immersed in liquid nitrogen for a minimum of 1 h.

2.8.4. Thawing and deloading

Tubes containing meristematic clumps were rapidly thawed in a warm water bath (40°C) for 80 s. The PVS-2 solution was replaced by the filter-sterilised deloading solution (1.2 M sucrose dissolved in MS medium, pH 5.8) for 15 min at room temperature.

2.8.5. Recovery

Control meristematic clumps (*C*) that were loaded, dehydrated and deloaded but not frozen, and frozen meristems (*F*) were taken from the deloading solution and placed onto two sterile filter papers on top of semi-solid hormone-free MS medium containing 0.3 M sucrose in 9 cm plastic Petri dishes. After

2 days, the meristem clumps were transferred onto regeneration medium. The first week of culture always takes place in the dark.

2.9. Control treatment

Control treatments were included to allow comparison of virus eradication following therapy with the spontaneous eradication obtained after direct regeneration after several subculturings of either *in vitro* plants or highly proliferating clumps. For this, proliferating clumps containing several meristem tips were regenerated on a Murashige and Skoog [26] semi-solid medium supplemented with 1 µM BA and 1 µM IAA (regeneration medium).

2.10. Plant regeneration

Shoots produced at the end of each assay were rooted on Murashige and Skoog medium supplemented with 0.5 g/l active charcoal. The first week of culture always takes place in the dark.

2.11. Virus screening

Initially, the health status of regenerated material was checked on *in vitro* plants through DAS-ELISA with the Loewe kit (Biochemica GmbH) for CMV according to the manufacturer's instructions. The cut off value for the threshold is 0.2 absorbance units. The putative virus-free material was tested a second time after a greenhouse acclimatisation phase of 6 months.

The eradication rate (ER) observed *in fine* for each treatment was calculated as follows:

- First ELISA test performed on *in vitro* plants:

$$ER = \left(\frac{\text{negative } in\ vitro \text{ plants}}{\text{tested } in\ vitro \text{ plants}} \right) \times 100$$

- Second ELISA test performed on *in vivo* plants:

$$ER = \left(\frac{\text{negative } in\ vitro \text{ plants}}{\text{tested } in\ vitro \text{ plants}} \right) \times \left(\frac{\text{negative } in\ vivo \text{ plants}}{\text{tested } in\ vivo \text{ plants}} \right) \times 100$$

3. RESULTS AND DISCUSSION

According to the literature, meristem culture is considered to be the reference tool for virus disease eradication [28]. The application of meristem-tip culture to eradicate viral particles is based on the meristem 'immunity' towards viruses [4]. For banana, however, we observed that the virus-free state of plants regenerated after meristem culture alone is very low and reaches only 1% (one plantlet out of 70 tested) when meristems were excised from *in vivo* plants (Figure 1a) whereas 7% (11 plantlets out of 165 tested) of plants were virus-free when they were taken from *in vitro* plants (Figure 1b). This low CMV eradication rate confirms previous results obtained by Berg and Bustamante [20] who still observed mosaic disease symptoms on 11 indicator plants when inoculated with an extract of Cavendish banana plants regenerated after meristem culture.

In order to eradicate CMV, a more severe treatment is thus needed. The percentage of healthy plants regenerated after a combination of meristem culture and thermotherapy reached 38% (33 plantlets out of 87 tested) and 70% (118 plantlets out of 167 tested) when meristems were excised from *in vivo* (Figure 1a) or *in vitro* (Figure 1b) plants respectively. The latter eradication rate almost reached the 75% of virus-free plantlets obtained earlier by Berg and Bustamente [20], but is lower than the 100% obtained by Gupta [21] by using apical meristem culturing in combination with heat treatment of CMV-infected rhizomes or *in vivo* plants. Despite the diurnal alternating heat treatment, 60%

mortality was still observed when treatment was performed on *in vitro* plants, while *in vivo* plant material was less sensitive (4% mortality).

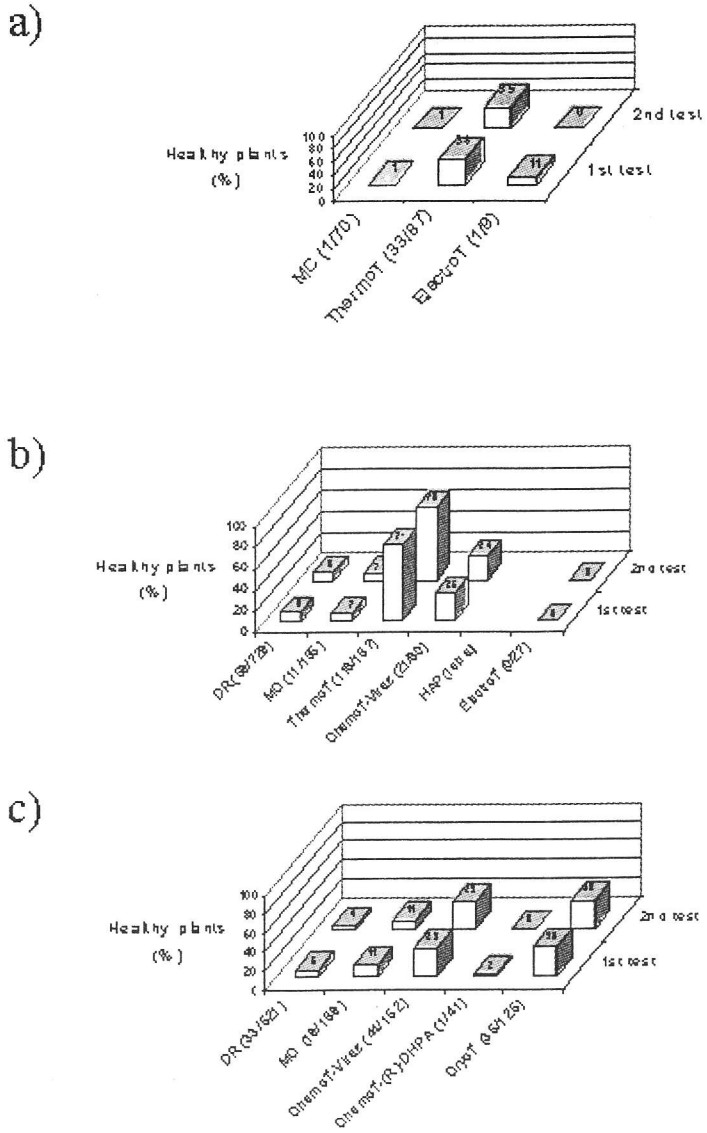

Figure 1 Eradication rates obtained for plants regenerated from CMV-infected *in vivo* plants (a); *in vitro* plants (b); *in vitro* clumps (c) of banana (cv. Williams BSJ, ITC.0570, AAA), as a result of different treatments. Viral status of plants was assessed by ELISA on *in vitro* plants (first test) or on *in vivo* plants (second test) performed following a 6-month acclimatisation in the greenhouse. The number in brackets is the number of CMV-free plantlets per total number of plantlets after the second test. DR, direct regeneration on MS semi-solid medium supplemented with 1 µM BA and 1 µM IAA; MC, meristem culture on solid MS medium (domes with 1–2 leaf primordia); Thermotherapy, meristems (domes with four leaf primordia) excision and culture on MS medium, 4 weeks at 40°C (day temperature)/25 ± 1°C (night temperature); Chemotherapy, MS medium supplemented with 50 mg/l Virazole or (RS)-DHPA for 3 months; Electrotherapy, 5 min in a detergent bath, pulses of 15 V for 5 min, surface sterilisation in 70% ethanol and then in 3% commercial bleach solution, meristem excision and culture on MS medium; Cryopreservation: *Preculture*, 2 weeks on p4 medium with 0.4 M sucrose; *Loading*, 20 min at room temperature in MS medium containing 2 M glycerol and 0.4 M sucrose, pH 5.8; *Dehydration*, 120 min at 0°C in PVS-2 (3.26 M glycerol, 2.42 M ethylene glycol (EG), 1.9 M DMSO, 0.4 M sucrose in MS medium, pH 5.8); *Freezing*, 1 h in liquid nitrogen in 2 ml cryotubes; *Thawing*, water bath, 40°C, 80s; *Unloading*, 15 min at room temperature in MS medium containing 1.2 M sucrose, pH 5.8: *Recovery*, semi-solid hormone-free MS medium containing 0.3 M sucrose, 2 days, followed by regeneration medium.

Chemotherapy is an alternative *in vitro* technique traditionally used for virus eradication [9]. According to the phytotoxicity assay (data not shown), we decided to use either 50 mg/l of Virazole or (RS)-DHPA. Eradication rates obtained for the material regenerated after meristem culture (Figure 1b) on MS medium supplemented with 50 mg/l of Virazole reached 26% (21 plantlets out of 80 tested). Previously, Simpkins et al. [14] obtained virus-free *N. rustica* cultures from CMV-infected meristem tips irrespective of the presence (50 or 100 mg/l) or absence of Virazole. No plantlet survived the 50 mg/l (RS)-DHPA-treatment (Figure 1b). De Fazio et al. [29] reported on the selective inhibitory effect of (RS)-DHPA on ss (+) RNA plant viruses. The toxic effects of these two compounds were confirmed when developmental competence parameters such as the regrowth rate and the multiplication rate per meristem were considered (data not shown). Faccioli and Colombarini [30] also reported the negative effect of Virazole on potato meristems and Stevenson and Monette [31] observed a high phytotoxicity of (RS)-DHPA at a concentration as low as 10 mg/l when applied to *Vitis vinifera* tissue cultures.

Simpkins et al. [14] emphasized that the efficiency of Virazole in suppressing virus diseases was dependent on its concentration, the host species, and the infected tissue treated. They observed that Virazole at 100 mg/l could suppress virus infection completely when it was applied to proliferating meristematic tissue of *Nicotiana rustica*. Similar experiments performed on highly proliferating banana meristems treated for 3 months either with Virazole or with (RS)-DHPA at 50 mg/l, however, did not improve the eradication rate (Figure 1c) previously obtained with treated-meristem culture (Figure 1b). The percentage of healthy plantlets regenerated from Virazole-treated highly proliferating meristems reached 29% (44 plantlets out of 152 tested) while only 2% (1 plantlet out of 41 tested) of plantlets were found virus-free when regenerated from (RS)-DHPA-treated highly proliferating meristems. Mortality rates of highly proliferating meristem cultures after Virazole and (RS)-DHPA treatment were comparable with those of *in vitro* plants (data not shown).

The efficiency of electrotherapy has previously been investigated by Hernandez *et al.* [18] on BSV-infected banana plants (cv. W. Bungulan); 40–80% of virus-free plants were regenerated. Thus, using the same parameters of treatment (15 V for 5 min), the preliminary percentage of healthy plantlets regenerated from electrically treated explants obtained from *in vivo* plants (Figure 1a) reached 11% (one plantlet out of nine tested) while no plantlet out of 27 tested was found to be virus-free when explants were obtained from *in vitro* plants (Figure 1b). A higher mortality rate was obtained for meristems excised from electrically treated explants, but no significant temperature increase was registered during the treatment compared with untreated meristems (data not shown).

To evaluate the efficiency of cryotherapy for virus eradication in *Musa*, highly proliferating meristems of banana were used instead of excised meristems, since very low post-thawing regeneration rates were reported for the latter [32]. The absence of leaf primordia on highly proliferating meristem cultures may facilitate the penetration of the cryoprotective PVS-2 solution and its dehydration effect. Moreover, the air bubble that is enclosed between the dome and the leaf primordia could act as thermal isolation, slowing down the rate of freezing. Cryopreservation of proliferating meristems resulted in regrowth of about 50%, but growth was delayed compared with the control. Moreover, plants regenerated after cryopreservation showed no morphological differences from control plantlets. After cryopreservation, 30% (38 plantlets out of the 125 tested) of regenerated plants were virus negative after the acclimatisation in the greenhouse (Figure 1c).

The control treatments, i.e. plants directly regenerated after *in vitro* multiplication of plantlets (Figure 1b), or highly proliferating meristem cultures (Figure 1c) had a spontaneous virus eradication rate of 8% (59 plantlets out of 729 tested) and 4% (33 plantlets out of 521 tested) respectively.

In conclusion, meristem culture, chemotherapy and electrotherapy are not efficient techniques for CMV eradication from *Musa*, whereas thermotherapy seems to be more attractive in the context of germplasm preservation and international diffusion. However, heat treatment is time-consuming, and mortality of treated material is high. Therefore, cryopreservation seems to be an attractive alternative.

It avoids the time-consuming meristem excision and the consequent problem of blackening due to polyphenol oxidation. Results of this report might lead to wider application for the eradication of other viruses from other banana accessions. However, in the case of banana varieties with BSV integrated into their genome, eradication of viral particles obtained by heat or cryo-treatment could be counterbalanced by the activation of these integrated sequences induced by the cryoprocedure and/or by the *in vitro* procedure.

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REFERENCES

[1] FRISON, E.A., SHARROCK, S., "The economic, social and nutritional importance of banana in the world", Banana and Food Security, (PICQ C., et al., Eds), INIBAP, Montpellier, France, (1999), 21–35.

[2] LOCKHART, B.E.L., JONES, D.R., "Banana Mosaic and Banana Streak, diseases caused by viruses", Diseases of Banana, Abaca and Enset, CABI Publishing, Wallingford, UK (2000) 241–293.

[3] HU, J.S., et al., Comparison of dot blot, ELISA and RT-PCR assays for detection of two cucumber mosaic virus isolates infecting banana in Hawaï, Plant Disease **79** (1995) 902–906.

[4] MOREL, G., Recherches sur la culture associée de parasites obligatoires et de tissus végétaux, Ann. Epiphyt **1** (1948) 123–234.

[5] BHOJWANI, S.S., RAZDAN, M.K., "Production of pathogen-free plants", Plant Tissue Culture: Theory and Practice, Elsevier, Amsterdam, The Netherlands, (1983), 287–312.

[6] WALKEY, D.G.A., WEBB, M.J.W., Virus in plant apical meristems, J. Gen. Virol. **3** (1968) 311–313.

[7] MORI, K., HOSOKAWA, D., Localization of viruses in apical meristem and production of virus-free plants by means of meristem and tissue culture, Acta Hort. **78** (1977) 389–396.

[8] LOGAN, A.E., ZETTLER, F.W., Rapid *in vitro* propagation of virus-indexed gladioli, Acta Hort. **164** (1985) 169–180.

[9] KARTHA, K.K., "Production and indexing of disease-free plants", Plant Tissue Culture and its Agricultural Application, (WITHERS, L.A., ALDERSON, P.G., Eds), Butterworths, London (1986), 219–238.

[10] WALKEY, D.G.A., High temperature inactivation of cucumber and alfalfa mosaic viruses in *Nicotiana rustica* cultures, Ann. Appl. Biol. **84** (1976) 183–192.

[11] WALKEY, D.G.A., FREEMAN, G.H., Inactivation of cucumber mosaic virus in cultured tissues of *Nicotiana rustica* by diurnal alternating periods of high and low temperature, Ann. Appl. Biol. **87** (1977) 375–382.

[12] WITKOWSKI, J.T., et al., The design, synthesis and broad spectrum antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides, J. Med. Chem. **15** (1972) 1150–1154.

[13] KUMMERT, J., TOUSSAINT, A., Utilisation du Virazole pour l'assainissement des plantes par cultures *in vitro*, Parasitica **40** (1984) 121–137.

[14] SIMPKINS, I., et al., Chemical suppression of virus in cultured plant tissues, Ann. Appl. Biol. **99** (1981) 161–169.

[15] QUACQUERELLI, A., et al., The use of electric current (RACE) for obtaining mosaic free almonds, Acta Phitopathol. Acad. Sci. Hungaricae **15** (1980) 251–255.

[16] LOZOYA-SALDANA, H., et al., Electrotherapy and shoot tip culture eliminate Potato Virus X in potatoes, Am. Potato J. **73** (1996) 149–154.
[17] HERNÁNDEZ, P. R; et al., Electroterapia nueva técnica para el saneamiento a virus en ajo (*Allium sativum* L.) Res. Patent. 37/95 A0 1C/08 Res. 1524/97, OPIC, Cuba (1995).
[18] HERNÁNDEZ, P.R; et al., Nuevo método para el saneamiento a bacterias y virus en caña de azúcar (*Saccharum* sp. *híbrido)*, Rev. Cuaderno Fitopatología, Levante, España **3** (1997) 153–157 (in Spanish).
[19] BRISON M., et al., Effect of cryopreservation on the sanitary state of a cv. *Prunus* rootstock experimentally contaminated with Plum Pox Potyvirus, Plant Sci. **123** (1997) 189–196.
[20] BERG L.A., BUSTAMANTE M., Heat treatment and meristem culture for the production of virus-free bananas, Phytopathology **64** (1974) 320–322.
[21] GUPTA P.P., Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture, Plant Cell Tissue Organ Culture **6** (1986) 33–39.
[22] ALLAM E.K., et al., Eradication of Banana Bunchy Top Virus (BBTV) and Banana Mosaic Virus (BMV) from diseased banana plants, Ann. Agric. Sci. **45** (2000) 33–48.
[23] HELLIOT B., et al., Cryopreservation for the elimination of cucumber mosaic or banana streak viruses from banana (*Musa* spp.), Plant Cell Rep. (2002) (in press).
[24] HELLIOT, B., et al., Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristem, Plant Cell Rep. (2002) (submitted).
[25] DANIELLS, J., et al., "Williams, Cultivated varieties AAA", *Musa*logue: A catalogue of *Musa* germplasm – Diversity in the genus *Musa,* (ARNAUD, E., SHARROCK, S., Eds), INIBAP, Montpellier, France (2001), 67.
[26] MURASHIGE, T., SKOOG, A., A revised medium for rapid growth and bioassays with tobacco tissues cultures, Physiol. Plant. **15** (1962) 473–497.
[27] SAKAÏ, A., et al., Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Obs. var. *Brasiliensis tanaka*) by vitrification, Plant Cell Rep. **9** (1990) 30–33.
[28] FACCIOLI, G., MARANI, F., "Virus elimination by meristem tip culture and tip micrografting", Plant virus diseases control, (HADIDI, A., et al., Eds), APS Press, The American Phytopathological Society, St Paul, MN, USA, (1998) 346–380.
[29] DE FAZIO, G., et al., Antiviral effects of dihydroxypropyladenine [(RS)-DHAP] and bromovinyldeoxyuridine (BVDU) on plant viruses, Antiviral Res. **8** (1987) 163–169.
[30] FACCIOLI, G., COLOMBARINI, A., Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets produced *in vitro*, Potato Res. **39** (1996) 129–140.
[31] STEVENSON, J.H., MONETTE, P.L., Delay of onset of leafroll symptom expression in *Vitis vinifera* "Liemberger" from ribavirin-treated *in vitro* cultures, Can. J. Plant Sci. **63** (1983) 557–560.
[32] PANIS, B., et al., "Cryopreservation of proliferating meristem cultures of banana", Cryopreservation of Tropical Plant Germplasm, Current Research Progress and Applications, (ENGELMANN, F., TAKAGI, H., Eds.), Proceedings from JIRCAS/IPGRI Joint International Workshop, Tsukuba, Japan, 20–23 October 1998 (1998) 238–243.