In vitro: Osmotic potential for virus elimination and preservation of infected banana shoot tip

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Abstract

Osmotherapy of virus infected plant materials is a new method for virus eliminationbased on osmopreservation techniques. Osmotherapy wasapplied for eliminationbanana bunchy top virus(BBTV) through preservation of shoot tips on MS media containing high sucrose concentration at low temperature. Shoot tips and subculture 1st of banana plantlets were preservedsuccessfully for 12 months on storage MS medium supplemented with 40, 50, 60 and70 gL^{-1} at 10, 15 and 20^{O}C. The highest survival percentage 100% with recorded with 40 and 50gm sucrose concentrations at 10, 15 and 20°C while 70 gL^{-1}at 20°C recorded the lowest survival percentage, 70.5% sucrose at 12 months. The lowest shoot length and roots number were recorded with 40 and 50gm sucrose conc.especially at 15°C for 12 months will higher shoot length were recorded with 60 and 70% sucrose at 15°C. All survival shoot tips at 1st subculture recultured on fresh modified MS media revealed viability and resumed growth within three weeks. These plantlets were tested against BBTV by DAS-ELISA. The highest plantlets virus free percentage 95 and 100% were recorded with 60 and 50 gsucrose concentration at 15°C, respectively. The results of shoot tips virus tested were confirmed by DAS ELISA.

Keywords: Osmotherapy, Osmopreservation, Tissue culture, Viruses, Plants

Introduction

The availability of pathogen-free plant materials is crucial for high yields and quality of all crops. Plant diseases threaten the productivity and sustainability of agricultural production. Crop species such as potato sweet potato, cooking bananas and cassava that ensure food security in many parts of the world are vegetatively propagated and therefore particularly prone to losses caused by viruses that are transmitted from generation to generation in the planting materials **(Loebenstein and Thottapilly, 2003)**. Similar problems also occur in many

economically important horticultural crops such as citrus, pome and stone fruit trees, berry crops and in ornamental plants **(Hadidi*et al.*, 1998)**.Shoot tips used in osmopreservation or osmotherapy are anatomically defined as structures that consist of the apical or lateral shoot meristem (1-1.5 mm in size) with three to few leafprimorda**(Benson, 2007)**. Sizes of cells and vacuoles increase and nucleocytoplamic ratio decreases with increasing distance from the apical dome **(Wang *et al.*, 2008)**.Meristematic cell division and differentiation are two basic physiological processes required for shoot regeneration from meristems. Therefore this study conduct to employ osmatic potential for production shoot tips virus free and preservation using osmotherapy and tissue culture techniques.

Materials and Methods

Plant materials:

The suckers of banana mother plants cv. Grand Nain showed virus like symptoms were carefully cut with about 50 to 75 cm. All suckers about 100 samples were tested for Banana bunchy top nanavirus (BBTV) by DAS-ELISA kit as described by **Clark and Adams (1977)**.

BBTV infected suckers were excised with rhizomatous base and washed under running tap water. The explants were surface sterilized by soaking in Clorox (15%) for 30 min and then rinsed with sterilized water containing 0.1 gL^{-1} of each citric and ascorbic acids. The explants were excised with 1 cm length x 1 cm diameter and soaked in ethanol 70% for 5 sec.

*In vitro*micropropagation:

The MS medium for tissue culture was prepared according to **Murashige and Skoog (1962)** supplemented with benzyl adenine (5 mgL^{-1}), Myo-inositol (0.1 gL^{-1}), sucrose (30 gL^{-1}) and agar (7 gL^{-1}). The explants (meristems) were cultures on MS medium individually in pyrex glass jars. The meristems cultures were incubated at 26 ± 2°C under photo period cycle of 16/8 h as light/dark for 4 weeks.

Osmotherapy and preservation of shoot tips:

The meristems were transferred to storage medium for 12 months. MS medium supplemented with 0.4 mgL^{-1} thiamin HCL, 100 mgL^{-1} L-arginine, 100 mgL^{-1}Myo-inositol, 2 mgL^{-1}indol 3-acetic acid (IAA), 5 mgL^{-1}benzyladenine (BA), 160 mgL^{-1} adenine sulphate, 0.2 gL^{-1} charcoal and 7 gL^{-1} agar as recommended by **Ko*et al.* (1991)**. four sucrose concentrations 40,50, 60 and 70 gL^{-1}were added to storage medium. The cultures were incubated at different temperatures 10, 15 and 20°C for each sucrose concentrations.

The data were recorded after 6 months and 12 months on survival percentage, average shoot number and length, and average root number/explant.

After storage period 12 months, the meristems of all sucrose concentrations were transferred and sub-cultured to fresh propagated medium. Percentages of virus free shoots were determined by DAS-ELISA test as well as survival shoots.

Results

Germplasm storage of BBTV-free banana cv. Grand Nain meristems and planlets depends on micropropagation*in vitro* under standard condition of tissue culture technique. The obtained results were explained the role of osmotic potential for virus elimination and is considered as an ideal for long-term storage of shoot tips germplasm.

One hundred banana suckers were collected from open field. These suckers divided into 2 groups according external viral symptoms and tested with DAS-ELISA assay. First group included 75 healthy suckers BBTV-free; which gave negative results with DAS-ELIS against BBTV polyclonal antibodies were used in storage. Second group 25 infected suckers with distinct viral symptoms of BBTV; which gave positive results with DAS-ELISA against BBTV polyclonal antibodies were used in production of shoot tips free BBTV and storage.

The healthy and BBTV infected explants (meristems) were cultured on MS starting medium and incubated at 26°C for 21 days. The proliferating meristems were cultured on storage MS medium plus sucrose 40, 50, 60, and 70 $gL^{-1}$and incubated at different 10, 15 and 20°C through 12 months. The obtained results investigated the effect of osmotic potential on BBTV elimination from infected shoot tips and preservation.

Shoot tip explant growth *in vitro*:

The effect of sucrose concentrations and incubation temperature on survival of shoot tip explants during storage for 6 and 12 months recorded in table (1). The survival percentage of shoot tips was recorded 25 out of25 for 40, 50 and 60 gL^{-1} at 10, 15 and 20°C but under 70 gL^{-1} was decreased 88, 84 and 76% at 10, 15 and 20°C, respectively at 6 months. On the other hand, at 12 months, the percentage of shoot tips survival was decreased non-significantly at 50 and 60% sucrose concentration while was significantly at 70% sucrose concentration 17, 15, 10 out of 25 shoot tips at 10, 15 and 20°C, respectively for 12 months (Table, 1 and fig.1).

The number of shoots per explant during in vitro storage for 6 and 12 months under sugar concentrations and temperature periods showed that, the lowest

shoot number was recorded with 60 gL^{-1} and 70 gL^{-1} sucrose at 10, 15 and 20°C for 6 and 12 months. On the other hand, the shoot number were increased with the increase storage period 12 months than 6 months (Table, 1 and fig. 1).

After 6 months storage period, the effect of sucrose concentrations data revealed that, one shoot number produced at all sucrose concentrations. As for the effect of storage temperatures, the lowest number of shoot/explants was obtained at 15 and 17°C. Regarding the interaction between the two studies factors recorded that the lowest shoot number produced with all sucrose concentrations under all storage temperatures. Meanwhile, after 12 months storage period concerning the effect of sucrose concentrations showed that, the lowest values (1.00) were recorded by explants stored on storage medium supplemented with 40, 50, 60 and 70 gL^{-1} sucrose concentrations. The effect of storage temperatures gave variation with among storage temperatures. Regarding the interaction between the two factors showed that, the lowest shoot number per explants was obtained on medium containing 40, 50, 60 and 70 gL^{-1} sucrose concentrations and 15, 17 and 20°C storage temperatures.

Data in table (1) showed the effect of different sucrose concentrations and temperatures on average shoot length of shoot tip explants *in vitro* storage for 12 months. After 6 months as for the effect of sucrose concentrations data showed that the highest shoot length were used 75 gL^{-1} (4.5 cm) but recorded shortest shoots values at 40 gL^{-1}(3.0 cm). The effect of storage temperatures data showed that the highest shoot length were recorded at 20°C and shortest at 10°C. The interaction between two sucrose and temperature under study showed that, highest shoot length were recorded by explants stored on 70 gL^{-1} sucrose at 20°C (4.0 cm) and lowest was recorded with 40 gL^{-1} at 10°C (0.5 cm). Storage period, 12 months, concerning the effect of sucrose concentrations, the highest shoot length was recorded at 70 gL^{-1} and 20°C (4.5 cm) and the lowest recorded by 40 gL^{-1} (1.5 cm) at 10°C.

The average root number per shoot tip grown on MS medium supplemented with 40, 50, 60 and 70 gL^{-1} sucrose and storage at 10, 15 and 20°C, revealed that no formation rooting on 40, 50 and 60 gL^{-1} sucrose at 10, 15 and 20°C except 70 gL^{-1} sucrose at 10, 15 and 20°C formed 2.0, 1.5 and 1.3 roots, respectively after 6 months storage period. On the other hand, after 12 months of storage on 40, 50, 60 and 70 gL^{-1}sucrose at 10, 15 and 20°C; it was found the highest roots number were formed on 40 gL^{-1} sucrose and lowest were formed on 70 gL^{-1} sucrose (Table ,1 and fig.1).

The interaction between the sucrose concentrations (40,

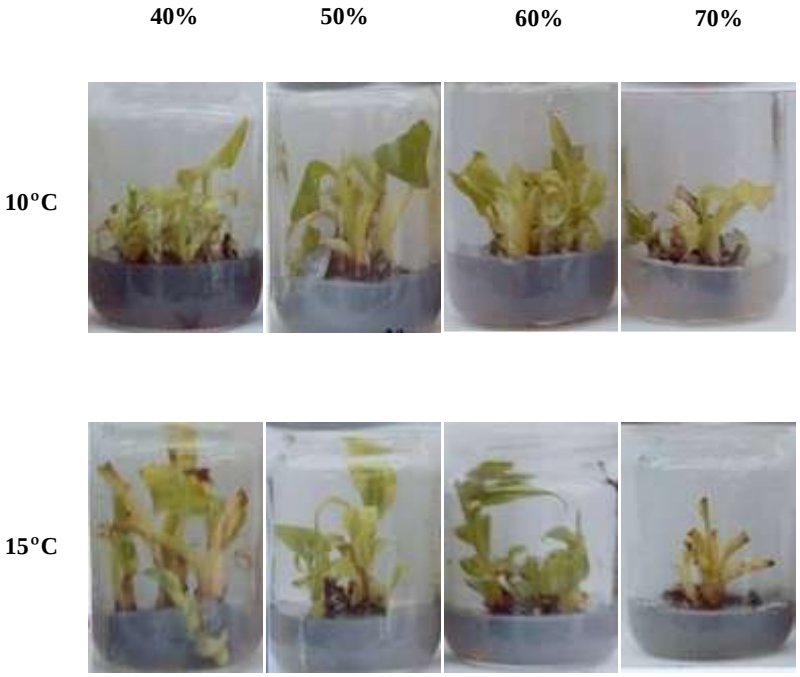
50, 60 and 70 gL^{-1}) and temperature incubation (10, 15 and 20°C) with shoot explant growth at the 6 and 12 months storage periods revealed that, the increase of sucrose concentration due to increase the growth parameter of shoot explants. Also, the increase of temperature due to the increase of shoot length (length and number of roots) (Table 1).In addition, the increase of storage period due to the increase of shoot growth (length and number of roots) (Table 1).

BBTV elimination:

The highest sucrose concentration 70 gL^{-1} gave the low shoot tip survival 20/25, 18/25 and 15/25 at 10, 15 and 20°C, respectively at 6 months storage period. It was decreased at 12 months storage period 17/25, 15/25 and 10/25 at 10, 15 and 20°C, respectively. On the other hand, 70 gL^{-1} sucrose in MS medium due to increase of shoot tip BBTV free 18/20, 18/18 and 15/15 at 6 months storage period and 17/18, 15/15 and 10/10 at 10, 15 and 20°C temperature incubation for 12 months storage period respectively. The lowest sucrose concentration 40 gL^{-1} gave the highest shoot tip survival 25/25 at 10, 15 and 20°C for 6 and 12 months storage period.

Table 1. The effect of sucrose concentrations on BBTV elimination from infected banana shoot tips and preservation.

Sucrose Conc.	Incubation (Tm)	6 months					12 months				
		Survival (No.)	Virus free (No.)	Shoot (No.)	Length (cm)	Root (No.)	Survival (No.)	Virus free (No.)	Shoot (No.)	Length (cm)	Root (No.)
40%	10°C	25	9	3	0.5	0	25	15	3	1.5	2.2
	15°C	25	11	3	2.0	0	25	18	3	2.7	2.0
	20°C	25	12	3	3.0	0	25	18	3	3.5	1.2
50%	10°C	25	12	2	1.5	0	23	19	3	3.3	26
	15°C	25	13	2	2.0	0	22	19	3	3.0	2.8
	20°C	25	15	2	3.0	0	22	19	3	4.0	2.5
60%	10°C	25	20	1	1.6	0	20	20	2	3.0	2.5
	15°C	25	20	1	2,05	0	20	20	2	3.4	2.0
	20°C	25	20	1	4.0	0	20	19	2	4.5	1.0
70%	10°C	20	18	1	2.0	2.0	18	17	2	3.0	2.5
	15°C	18	18	1	2.5	1.5	15	15	2	3.5	2.0
	20°C	15	15	1	4.0	1.3	10	10	2	4.5	1.5



20°C

Fig.(1) : Banana plantlets preserving in MS mediumsupplement with 40, 50, 60 and 70 % sucrose at 10, 15 and 20 ^{o}C

Discussion

The main target of this study was more conductive to achieve the BBTV illumination and the highest survival percentages of shoot tip(mrristem) or the plantlets(1st subculture) by using osmotic potential MS medium (osmotherapy) and without subculturing for longer period storage (osmopreservation). Generally, it could be concluded that shoot tip or 1st subculture plantlets banana cv. Grand Nain were successfully eliminated BBTV and preserved for 12 months on MS medium supplemented with 60 or 70 gL^{-1} sucrose at 15 or 20°C incubation. The stored explants resumed growth and started the regeneration during 20 days after transferring on proliferation MS medium at 26±2°C. These results suggest that, tropical plants may survive in osmopreservation at intermediate temperatures (10-20°C). Alternatively growth media may be altered to slow growth. Rooted epically dominate plantlets of banana were maintained for 12 months without subculture when 60 gL^{-1} sucrose in the growth medium (**EmanYounis, 2006**). In general the carbohydrates play a prominent part in the nutrition and structure of a plant. The carbohydrates could have been caused by effects on water potential or metabolism uptake differences (**El-Habashy, 2000**). Temperatures range 15 and 20°C in preservation chambers are frequently utilized to reduce the growth rate (**Van Den Houw*et al.*, 1995**).

Concerning to extend the preservation duration to 12 months under 15°C or 20°C, it could be observed that increasing or decreasing the sucrose on storage media more or less than 0.5 M decreased significantly the survival percentage. Sucrose concentration at 0.3, 0.5 and 0.7 M induced proline accumulation and correlated linearly with the concentration sucrose. The correlation between sucrose and

proline accumulation could be exploited to improve further studies of minimal growth storage and cryopreservation of oil palm embryogenic cultures (**Tarmizi*et al.*, 1993**).

The increase of the sucrose levels from 60 to 70 gL^{-1} on MS medium results in a lowered survival rate of banana shoot tips cultures 20°C and increased continuous virus illumination. These results suggested that, high sucrose levels cause hyper-methylation of DNA, possibly as on adaptive response to conserve cellular resources during osmotic stress. The growth rate of explants decreased when the sucrose concentration revealed 60%. The moisture content was reduced in all sucrose concentration (**Sokolova, *et al* 1974 and Uargami 1991**). The dry materials were accumulated of banana plants during *in vitro* growth appears to be linked to the quantity of sucrose concentration to be used during this stage between 70 to 80 mg/L (**Murchal and Folliot, 1992**). The proposed modes of sucroses action pr-eculture in enhancing freeze resistance are numerous. The results in a slow reduction in moisture content. Histological studies on pre-cultured banana meristem revealed the synthesis or accumulation of sugar-like compound inside the cytoplasm after pre-culture. This is confirmed by the sugar analysis. At 0.6 M sucrose, only 17% of the inoculated bud display growth. At 0.75 M, no buds growth and all become brown. It is assumed that, the osmotic check reaction of living tissues to stress as well as reduce growth by osmotic stress. These results suggested that, high sucrose levels can there for be used to maintain cultures in a dormant conditions for long period, this appears to be an osmotic effect (**Tarmizi e*t al.*, 1993; Harding, 1994; Panis*et al.*, 1996; Hassan, 2002; EmanYounis, 2006**).

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