academicJournals

Vol. 15(13), pp. 490-496, 30 March, 2016
DOI: 10.5897/AJB2015.14446
Article Number: 7DF330957762
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of osmotic stress on *in vitro* propagation of *Musa* sp. (Malbhog variety)

Swapan Kumar Sinha* and Alak Chandra Deka

TERI, Northeastern Regional Centre, Express Highway, Barbari, Guwahati-781036, Assam, India.

Received 22 January, 2015; Accepted 21 December, 2015

In vitro **propagation of banana preferably use sword sucker as explant source where microbial contamination poses a great problem in establishment of aseptic cultures. This study demonstrates up to 36% reduced microbial contamination in aseptic culture establishment and subsequent micropropagation due to osmotic stress induction in the banana suckers. Osmotic stress was induced by keeping the freshly collected suckers in shade and measuring fresh weight at 0, 7, 14, 21, and 28 days interval to ascertain loss of moisture. Stress induced for 21 days showed 58.85% moisture loss showing lowest contamination upto 40% against 76% for fresh suckers. Micropropagation of *Musa* sp. (Malbhog variety) through shoot tip culture of stressed suckers was carried out in Murashige and Skoog (MS) medium supplemented with 1 to 2 mg/l 6-benzyl-aminopurine (BAP), 2 to 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l α-naphthalene acetic acid (NAA). Multiplication of plantlets was observed till 6th passage. From 19 aseptically established explants of 21 days stressed sucker sub cultured in MS medium fortified with 1.0 mg/l BAP and 0.5 mg/l NAA, for 6th passage produced 5122 plantlets. Regenerated micro shoots were rooted in MS medium fortified with 0.5 mg/l IAA. The plantlets were hardened in polybag containing soil and seasoned cow dung.**

Key words: Osmotic stress, shoot tip culture, *in vitro*, *Musa,* Malbhog.

INTRODUCTION

Banana is an important group of plants that provides millions of livelihood. India has a rich genetic diversity of banana with more than 90 distinct clones. Banana is grown under diverse conditions and production systems and hence selection of varieties is based on needs and situation. Around 20 cultivars viz. Dwarf Cavendish, Robusta, Monthan, Poovan, Nendran, Red banana, `Safed Velchi, Basrai, Ardhapuri, Rasthali, Karpurvalli, Karthali, Grande Naine, Malbhog etc. are commercially cultivated. Malbhog is a Musa cultivar of AAB genomic group under subgroup of silk type having reference accession number TRY0077 (Daniells et al., 2001) commonly found in Assam, India. Malbhog banana is very popular and has high commercial value in Assam due to its sweet aroma, taste and high post-harvest life. However, shortage of quality planting materials remains the bottleneck for the banana farmers of the state. Tissue culture techniques have been employed for large scale banana planting material production. Many regeneration protocols dealing with different *Musa*

*Corresponding author. E-mail: sksinha@teri.res.in. Tel: +913612334790.

species has been reported for *in vitro* propagation of banana and plantains (El-Saghir,1997; Kodym and Zapata-Arias, 2001; Vidhya and Nair, 2002; Madhulatha et al., 2004; Sebastian and Mathew, 2004; Anilkumar and Sajeevan, 2005; El-Dougdoug et al., 2006; Kulkarni et al., 2006; Darvari et al., 2010; North et al., 2012; Ngomuo et al., 2014; Qamar et al., 2015). However establishment of aseptic culture remain a great challenge for banana micropropagation. Endo-bacterial contamination is one of the major problems for aseptic establishment of banana where the contaminants may survive in the plant material for several subculture cycles and it may over extend the period of time without expressing symptoms in the tissue or visible signs in the medium (Van Den Houwe and Swennen, 2000). The use of antibiotic, commercial fungicide, thermotherapy, warm water treatment and ultrasonic treatment are some of the commonly used methods to control *in vitro* contamination (Cole, 1996). In this report, osmotic stress induction of banana suckers as a technique to reduce microbial contamination and its subsequent influence on multiplication is emphasized.

MATERIALS AND METHODS

Stress induction

Two months old sword suckers of banana variety Malbhog were collected from farmer's field of Assam, India. Sucker were washed thoroughly under running tap water for half an hour and stress was induced by keeping the cleaned suckers in shade and fresh weight were recorded at 0, 7, 14, 21, 28 days interval to ascertain loss of moisture. Loss of moisture was calculated as follows:

Moisture loss (%): a-b/a x 100

where, a = initial fresh weight of suckers; b = fresh weight of suckers after stress for d.

Establishment of explants in aseptic media

After each interval of stress induction, 25 suckers were washed under running tap water for 30 min and trimmed into square block ranging from 5 to 8 cm in sizes and dipped in fungicide solution (2 gm/l Bavistin and 100 mg/l ascorbic acid) for 30 min followed by washing with liquid detergent (Extran, Merck, 0.05 ml/l) for 20 min and 4 to 5 times rinsed with clean water. Thereafter, sucker cubes were treated with Savlon (Johnsons and Johnsons) for 30 min and brought under laminar hood. Surface sterilization was carried out with 0.1 mg/l $HgCl_2$ fortified with 2 to 3 drops of Tween 20 (Merck) for 10 to 12 min and the disinfectant was removed by 4 to 6 rinse with sterile distilled water. After surface sterilization the cut ends of explants were trimmed with a sterile surgical blade and the shoot tips of 8 to 10 mm from decapitated shoot apexes of suckers are carefully inoculated in pre sterilized MS medium (Murashige and Skoog, 1962) supplemented with 1 to 2 mg/l 6-Benzyl-aminopurine (BAP), 2-4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l α-naphthalene acetic acid (NAA), 30 g /l sucrose, 2.2 g /l Gelrite. The pH of the medium was adjusted to 5.8 using 0.1 M NaOH before autoclaving for 20 min at 121°C at 15 lbs psi pressure. The inoculated media with explants were incubated in the growth room at 25±2°C with 16 h illuminations. The influence of stress on contamination reduction was calculated as follows:

Contamination % = c-d/c*100

where c = contamination of explant of fresh suckers d = contamination of sucker explants stressed for d.

Plant multiplication

Aseptically established explants were subcultured into basal medium (MS) with varying combination of 0.5 to 1 mg/l of BAP, 0.5 mg/l NAA and 2 to 4 mg/l 2, 4-D. Number of plantlets from each explant was recorded for subsequent subculture till 6th passage. Subculture was carried out at every 3 weeks interval.

Root induction and hardening

Microshoots of 4 to 5 cm length were separated from the cluster and inoculated into MS medium supplemented with 0.5 mg/l IAA. Rooted plantlets of 6 to 8 cm length were taken out from culture bottle and washed under running tap water for 10 min to remove media attached to root system. Thereafter plantlets were treated with 1 g/l Bavistin for 5 min and transferred to polybag (size 12 × 18 cm) containing river sand and kept inside the polyhouse for 21 days. Subsequently, these plantlets were transferred to larger polybag (size 15 × 21 cm) containing soil, seasoned cow dung at 1:1 and kept under agro shade net house with provision of 50% penetration of natural light. Irrigation was ensured depending on the requirements.

RESULTS

Stress induction and impact on contamination reduction

Fresh weight of banana suckers decreased with increasing storage duration due to loss of moisture and is expressed as induced osmotic stress. The moisture loss ranged between 15 to 70% during 7 to 28 days of shade storage (Figure 1a, b). Bacterial contamination was visible within 3 days of the inoculation. The lowest contamination (36%) was recorded in 21 days of shade storage where the sucker has lost 58% of moisture (Figure 1c, d). Increasing moisture loss has negative relation with microbial contamination till 21 days (Table 1). This could be attributed to the microbial load present at the periphery of suckers reduced due to drying of suckers.

Influence of pre induced stress on regeneration potential

Initially, the aseptic shoot tip cultures swell at base and developed green colour irrespective of growth hormone supplementation after 3 weeks of inoculation. In the subsequent week, it formed shoot with swollen corm. Corms after dissection into halves and subcultured in MS medium produced microshoots from the base. Regeneration of microshoots in MS basal medium increased from 69 microshoots for fresh suckers to 586 for 21 days stored

Figure 1a. Fresh collected Malbhog sucker.

Figure 1b. Malbhog suckers stored for 21d in shade.

sucker after six passages. Effect of different combination of BAP, NAA and 2,4-D were studied for *in vitro* multiplication of the stress induced explants. Among the various combinations, the effective results were obtained on the MS medium supplemented with BAP and NAA. The shoot proliferation was found to be best in MS basal medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l). Subculturing of the *in vitro* raised microshoots for multiplication on the same medium induced multiple shoots. Shoot multiplication and clump formation at the base was observed after two to three subcultures. After attaining vigorous growth and proliferation, the clumps were divided into smaller clumps and transferred to the same medium for further multiplication. In this combination, the micro shoot regeneration increased to 5221 after 6th passage (Table 2 and Figure 1e, f). The fully grown healthy plantlets were aseptically transferred to the MS basal medium supplemented with 0.5 mg/l IAA for in

Figure 1c. Malbhog explant from stressed sucker ready for surface sterilization.

Figure 1d. Aseptically established Malbhog explant.

Table 1. Effect of different storage duration on moisture loss of Banana Malbhog suckers and its impact on contamination reduction during *in vitro* culture.

Parameters	Stress duration (days)				
	0	**7**	**14**	**21**	**28**
Average weight loss (%) ± SE	0	15.65 ± 0.10	37.79 ± 0.19	59.36 ± 0.21	72.98 ± 0.22
Contamination (%)	76	72	48	40	56

Contamination recorded 7 days after inoculation. 25 explants were used for each treatment.

Table 2. Effect of different storage duration of Banana Malbhog suckers on multiplication efficiency.

MS + PGR (mg./l)			Stress duration (days)	Number of explants aseptically established	Number of microshoots after each passage					
BA	NAA	2,4-D			1	2	3	4	5	6
-	-	-		6	6	10	16	25	57	69
2				6	10	17	38	55	78	95
1	-	2	0	4	7	16	48	71	156	210
1	-	4		8	15	36	66	82	187	402
1	0.5	-		9	16	47	70	133	228	562
-	-	-		8	12	26	40	58	73	108
2				9	16	52	106	218	345	569
1	-	2	7	8	16	43	94	238	457	734
1	-	4		11	24	50	114	190	558	1132
1	0.5	-		13	37	63	124	218	772	1296
-	-	-		13	20	45	68	109	203	269
2				13	24	50	106	243	746	1154
1	-	2	14	12	23	65	124	462	773	1344
1	-	4		14	40	64	213	616	1044	2256
1	0.5	-		16	43	70	228	697	1349	2416
-	-	-		15	36	69	113	302	445	586
2				15	40	74	173	398	774	1119
1	-	2	21	16	42	80	190	452	827	1240
1	-	4		18	56	117	360	1159	3012	5170
1	0.5	-		19	62	150	457	1327	3610	5221
-	-	-		11	20	39	62	120	196	287
2				13	28	46	111	236	686	774
1	-	2	28	10	26	47	92	467	570	1093
1	-	4		15	30	55	109	480	910	1215
1	0.5	-		16	41	60	94	551	1012	2312

Explants inoculated for each treatment are 25.

Figure 1e. Micro shoot cluster of 21d stress induced sucker in MS supplemented with 1mgl BAP and0.5mgl NAA.

Figure 1f. Shoots ready for root induction.

vitro root induction and recorded 9 numbers of healthy roots per shoot within 9 days of culture. The *in vitro* rooted plantlets were washed thoroughly in running tape water and planted in sterile potting mixture and hardened in a agro shade house under 50% penetration of natural light and 70% relative humidity (RH) for two weeks. After four weeks of hardening, the established plants were transferred to the field. This clearly indicates that pre inoculation stress induction to suckers was beneficial for reduction of microbial contamination and subsequent microshoot production.

DISCUSSION

Banana is cultivated in humus and organic matter rich soil where microbial load in rhizosphere region is very high and poses challenge to establish aseptic culture. Various techniques to contain contamination begin with pretreatment of donor plants to reduce contamination (Holdgate and Zandvoort, 1997) with antibiotics and fungicides (Kritzinger et al., 1997) as well as anti-microbial formulations, such as plant protection mixture (PPM) (Guri and Patel, 1998). The explants are also culture indexed for contamination by standard microbiological techniques, which are occasionally supplemented with tests based on molecular biology or other techniques (George, 1993; Leifert and Woodward, 1998; Leifert and Cassells, 2001). Since plants do not have an immune system to antibiotics and as such many of the antibiotics, that are effective against bacteria, fungi, *and* phytoplasmas, are toxic to plants as well. Use of antibiotics is not full proof or the desired method to get rid of microbial contamination (Pierik, 1989). Islam and Zobayed (2000) reported microbial contamination in banana using sugar free medium. We report inexpensive and effective mode of microbial contamination reduction due to stress induction in suckers. Besides reducing contamination, this technique also has the advantage of stretching inoculation period after material collection.

Plants cell experience severe strain and stresses during multiplication of plantlets under artificial medium depending on the hormonal combination. Thus, synergistic effect of BAP and auxin on shoot proliferation has already been reported in banana tissue culture (El-Saghir, 1997; Nauyen and Kozai, 2001; Kagera et al., 2004; Kalimuthu et al., 2007; Sheidai et al., 2008; Qamar et al., 2015). In the present experiment, plants tissue were exposed to osmotic stress during pre-inoculation stage and its subsequent exposure to hormonal combination in the artificial medium responded exceptionally well for microbial contamination reduction and multiplication of microshoots. This is the first report to study the influence of stress on contamination free *in vitro* establishment and multiplication of microshoots (Tables 1 and 2). Root induction is one of the important steps in plant tissue culture. In case of banana, rooting occurs simultaneously for 4 weeks old cultures. However, micro shoots when subcultured in MS medium fortified with 0.5 mg/l IAA induced 9 numbers of healthy roots per shoot within 9 days of culture. Rooting of microshoot in IBA supplemented media has been reported (Haq and Dahot, 2007).

Hardening of plantlets in two stages was found beneficial. Initially, plantlets were transplanted into river sand with intermittent watering for 21 days in polyhouse followed by subsequent transplanting into larger polybag with river sand and seasoned cow dung in agroshade resulted to satisfactory survival and growth.

Conclusion

Microbial contamination in tissue culture could be curtailed for sucker based banana explant by inducing stress for 21 days storage in shade. This technique also increases the multiplication efficiency of microshoots in the subsequent subculture. The present protocol with 21 days osmotic stress induction of banana suckers with MS medium fortified with BAP (1.0 mg/l) and NAA (0.5 mg/l) for *in vitro* establishment, shoot initiation, mass scale multiplication, rooting and two step hardening could be used for mass scale production of banana planting material.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. R. K. Pachauri, Director General, The Energy and Resources Institute for his encouragement and support. Authors are also thankful to Department of Science and Technology, Government of India, for the research grant vide DST No: SR/SO/PS-47/06 dated 10-9-2007.

REFERENCES

Anilkumar M, Sajeevan RS (2005). Micro-propagation of *Musa accuminata* Colla. Plant Cell Biotechnol. Mol. Biol. 6:159-162.

Cole M (1996). Microbial contaminations and aseptic technique in plant tissue culture. In: Taji A and Williams R. (eds.): Tissue culture of Australian plants. University of New England, Armidale, Australia. pp. 204-228.

Daniells J, Jenny C, Karamura D, Tomekpe K (2001). Musalogue: a catalogue of Musa germplasm. Diversity in the genus Musa (E. Arnaud and S. Sharrock, compil.). International Network for the Improvement of Banana and Plantain, Montpellier, France.

Darvari FM, Sariah M, Puad MP, Maziah M (2010). Micropropagation of some Malaysian banana and plantain (*Musa* sp.) cultivars using male flowers. Afr. J. Biotechnol. 9(16):2360-2366.

El-Dougdoug KA, Hazaa MM, Hanaa Gomaa HA, El-Maaty SA (2006). Eradication of banana viruses from naturally infected banana plants. 1 - Biological and molecular detection of cucumber mosaic virus and bunchy banana top virus in naturally infected banana plants. J. Appl. Sci. Res. 2(12):1156-1163.

El-Saghir MM (1997). Production of virus-free banana plants. Ph.D. Thesis, Faculty of Agriculture, ElMenia University, Egypt. p. 179

George EF (1993). Plant Propagation by Tissue Culture. Part 1. The Technology. Exegetics Ltd., Edington.

Guri AZ, Patel KN (1998). Compositions and methods to prevent microbial contamination of plant tissue culture media. U. S. Patent No. 5,750,402.

Haq I, Dahot MU (2007). Morpho-physiological aspects of micro-propagating banana under different hormonal conditions. Asian J. Plant Sci. 6:496-501.

Holdgate DP, Zandvoort EA (1997). Strategic considerations for the establishment of microorganism free tissue cultures for commercial ornamental micropropagation. In: Cassells, A.C. (ed.): Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Acad. Pub. Dordrecht. pp.15-22.

Islam N, Zobayed SMA (2000). Microbial contamination under photoautotrophic culture system. In: C. Kubota and C Chun (eds.). Transplant production in the 21st century. Kluwer Academic Publishers. Netherland. pp. 284-288.

Kagera AG, Kagera GR, Kegera CBM, Van den Houwe I, Swennen R (2004). Rapid mass propagation and diffusion of new banana verities among small. Scale farmers in north western Tazania. Afr. Crop Sci. J. 12(1):7-17.

Kalimuthu K, Saravanakumar M, Senthilkumar R (2007). *In vitro* micropropagation of *Musa sapientum* L. (Cavendish Dwarf). Afr. J. Biotechnol. 6(9):1106-1109.

Kritzinger EM, Jansen Van Vuuren R, Woodward B, Rong IH, Spreth MH, Slabbert MM (1997). Elimination of external and internal contaminants in rhizomes of *Zantedeschia aethiopica* with commercial fungicides and antibiotics. In: Cassells, A.C. (ed.): Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Acad. Pub. Dordrecht. pp.61-167.

Kodym A, Zapata-Arias FJ (2001). Low-cost alternatives for the micropropagation of banana. Plant Cell Tissue Organ Cult. 66:67-71.

Kulkarni VM, Suprasanna P, Bapat VA (2006). Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli. Curr. Sci. 90(6):842-846.

Leifert C, Cassells AC (2001). Microbial hazards in plant tissue and cell cultures. *In vitro* cellular and developmental biology. Planta 37:133-138.

Leifert C, Woodward S (1998). Laboratory contamination management: the requirement for microbiological quality assurance. Plant Cell Tissue Organ Cult. 52:83-88.

Madhulatha P, Anbalagan M, Jayachandran S, Sakthivel N (2004). Influence of liquid pulse treatment with growth regulators on in vitro propagation of banana (*Musa* sp. AAA). Plant Cell Organ Cult. 76: 189-191.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Nauyen QT, Kozai T (2001). Growth of *in vitro* banana (*Musa* sp.). shoots under photomixotrophic and photoautotrophic conditions *in vitro* Cell. Dev. Biol. Plant 37:824-829.

Ngomuo M, Mneney E, Ndakidemi PA (2014). The *in vitro* propagation techniques for producing banana using shoot tip cultures. Am. J. Plant Sci. 5:1614-1622.

North JJ, Ndakidemi PA, Laubscher CP (2012). Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of strelitzia reginae. Int. J. Phys. Sci.7(4):638-646.

Pierik RLM (1989). *In vitro* Culture of Higher Plants. Martinus Nijhoff Pub. Dordrecht.

Qamar M, Qureshi ST, Khan IA, Raza S (2015). Optimization of *in vitro* multiplication for exotic banana (*Musa* spp.) in Pakistan. Afr. J. Biotechnol. 14(24):1989-1995.

Sebastian L, Mathew MM (2004). Micro-propagation of Nendran and Poovan varieties of Musa through inflorescence tip culture. Plant Cell Biotechnol. Mol. Biol. 5:141-148.

Sheidai M, Yahyazadeh F, Farahanei F, Noormohammadi Z (2008). Genetic and morphological variations induced by tissue culture in tetraploid cotton (*Gossypium hirsutum* L.) Acta Biol. Szeged. 52:33-38.

Van den houwe I, Swennen R (2000). Characterization and control of bacterial contaminants in *in vitro* cultures of banana (*Musa* spp.). Meeting: International Symposium on Methods and Markers for Quality Assurance in Micropropagation. Acta Hortic. 530:69-79.

Vidhya R, Nair AS (2002). Molecular analysis of somaclonal variation in *Musa acuminata* (AAA) cv. Red. Phytomorphology 52:293-300.