RESEARCH PAPER International Journal of Agricultural Sciences, Vol. 5 Issue 1, January to May, 2009 : 254-257

Performance of liquid culture on *in vitro* mass multiplication of woolly aphid (*Ceratovacuna anigera* Zehntner) resistant sugarcane cultivar SNK-44

MALLIKARJUN KENGANAL*, R.R. HANCHINAL AND H.L. NADAF
Sugarcane Tissue Culture Laboratory, University of Agricultural Sciences, DHARWAD (KARNATAKA) INDIA

ABSTRACT

Meristemculture culture on solid medium was the routine practice in micropropagation of most of the vegetatively propagated crops *in vitro*. Mass multiplication of sugarcane woolly aphid resistant cultivar SNK-44 with the help of meristem culture using liquid culture was the aim of present study. In the current experiment liquid medium was employed in all the stages of micropropagation in combination with different growth regulators for different stages. The meristem from 8 month old mother pant were inoculated on MS medium supplemented with 0.2 mg/l BAP + 0.1mg/l GA3 and 0.1mg/l NAA and 20 g/l sucrose for initiation. Shoots emerged from meristems were subcultured to multiplication medium consisting of MS + 0.2 mg/l BAP + 0.1mg/l NAA and 20 g/l sucrose. The culture bottles were kept with continuous shaking at five differ speeds *viz*., 25, 50, 75, 100 and 125 RPM on orbital shaker to determine the optimum speed for multiplication of large number of shoots in short period. The highest multiplication with an average of 6.45 seedlings per incoula on each cycle of multiplication was found at 100 RPM and lowest was at 25 RPM with 4.06 seedlings. The highest speed of 125 RPM did not increase the rate of multiplication rather it declined with an average of 4.83 seedlings per incoula per cycle. The meristems inoculated on static position responded poorly with an average of 2.29 seedlings per cycle of multiplication. The multiplied shoots were rooted in rooting medium (MS + 0.1 mg/l IBA and 20 g/l sucrose) and seedlings were hardened before discharge for field plantation. The rate of shoot multiplication was found increasing form 25 to 100 RPM but declined at 125 RPM revealing 100 RPM is the most suitable for large scale multiplication of sugarcane cultivars using liquid culture medium on orbital shaker. The cost of production for each seedling was also found less compared to gelled medium. The method was found most successful up to six to seven cycles. It is a novel way for popularizing the newly developed sugarcane cultivars in short time for larger areas.

Key words : Woolly aphid resistant, Sugarcane, Meristems, Multiplication, Seedlings

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is an economical cash crop extensively grown all over the world. It is polysomic, highly heterozygous, clonally propagated crop that accounts for more than 60 per cent of the global sugar production (Guimarces and Sobral, 1998) with an employment opportunity to over more than 100 million people across the world, both in rural and urban industrial sector. The importance of this crop has increased many folds in recent years, since the cane has become an important raw material for sugar industries and allied industries engaged in production of potable alcohol, acetic acid, butanol, paper, plywood, industrial enzymes, animal feed (Arencibia, 1998). Ethanol is commercial product of sugarcane. Blending of petrol, diesel and gasoline with ethanol has added an additional demand for sugarcane production world over. Many of the countries have adopted blending of automobile fuels up to 10% with ethanol to reduce dependence on declining resources of fossil fuels, which also helps in reducing the environmental pollution.

Commercial sugarcane varieties play key role in meeting required demand in the sugar industry. They are obtained through crossing, prolonged breeding and multistage evaluation, which involves selection over a period of years. The cost of complete development of such elite varieties reaches millions of dollars (Birch, 1996) and their multiplication for large area coverage is difficult by conventional method of set multiplication. Tissue culture technology is the best suited procedure for large scale multiplication of vegetative propagated crops such as sugarcane and banana. The technology enables to reduce the time between development and release of a new varieties (Feldmann *et al*., 1994; Taylor and Dukic, 1993). The technology offers best methodology to obtain quality seed material in shorter period of time. Numbers of attempts have been made to standardize large scale micro propagation protocols. Achieving high rate of seedling multiplication is a challenge without affecting the genetic makeup and physical characters of planting material (Williams and Taji, 1991). Lowering the cost of production is another concern in case of commercial production under large scale, which enables to purchase by maximum growers. But, the current situation is still not so satisfactory in production of required quantity of planting material within time at affordable prices. Efforts by many of the workers are still undergoing.

The presently available protocols of *in vitro* propagation of sugarcane allow use of medium semi solidified with agar agar (0.5 to 0.8 %) in all the stages, since from initiation to rooting of the seedlings. Use of liquid medium is often considered as superior for shoot multiplication, it also enables the earlier detection of any

* Author for correspondence

microbial contamination. The purpose of present study was to develop a low cost protocol for high rate of multiplication of woolly aphid resistant sugarcane cultivar SNK-44 *in vitro* using liquid culture medium in all the stages, without addition of any culture supportive material to reduce the cost of production per unit seedling.

MATERIALS AND METHODS

***Explant preparation*:**

Sugarcane shoots from 8 to 10 months old mother plant resistant to woolly aphid were collected and meristem buds of 1cm diameter and 4 cm length were taken out from these shoots. The buds were thoroughly washed with detergent Tween 20 (2 drops per liter) to remove soil and litter adhered on them. The buds were surface sterilized with 0.1% Bavistin for 8 minutes followed by three times washing with clean water. Second surface sterilization was carried out aseptically under laminar air flow cabinet with 70 % alcohol for 30 seconds followed by three times washing with double distilled sterile water. Final sterilization was done with 0.1 % $HgCl_2$ for 5 minutes followed by three washings with sterile double distilled water.

***Inoculation of explant*:**

The outer three to four leaf sheath layers of meristem bud were removed carefully with the help of sterile forceps and scalpel, retaining innermost two to three leaf whorls intact with the base measuring 3-4mm diameter and 20mm height. These meristems were inoculated for initiation on Murashige and Skoog (1962) (MS) medium supplemented with 20 g/l sucrose, 0.2 mg/l BAP, 0.1mg/l GA3 and 0.1mg/l NAA, adjusted to 5.8 pH, autoclaved at 15lbs for 15 minutes at 121°C under steam sterilizer (Nat Steel, Mumbai) and dispensed in test tubes 15ml each. Since the medium was liquid, to avoid submergence of inoculated meristem a wedge shaped paper bridge was provided inside the tube and meristem was placed on the bridge vertically. The cultures were maintained under diurnal day and night of 16 hr light and 8 hr dark conditions at 23±2°C temperature and 3000 Lux light intensity.

***Initiation and multiplication*:**

Meristms inoculated on initiation medium were subcultured on same medium on every 10 days interval for three times. The position of the meristem was changed in few cases on the paper bridge initially, to avoid the adverse effect of phenolics exuded by the cut end surface of the base. Since, phenolics were known to obstruct the growth and uptake of nutrients. Proliferated meristems after 30 days were transferred to multiplication medium consisting of MS medium, 20 g/l sucrose, 0.2 mg/l BAP and 0.1mg/l NAA dispensed in transparent culture bottles of 300ml capacity at 30 ml in each bottle having autoclavable poly propylene caps. These bottles were incubated on continuous rotating illuminated orbital rotary shaker (Steel Met, Puna) with five different level of speed (25, 50, 75, 100 and 125 RPM). In each bottle two clumps were maintained and same number was maintained in static position. The fully developed large shoot clumps after 20 days of multiplication were separated in to single shoot clumps, transferred to fresh medium and re incubated on shaker for next cycle of multiplication and cycles were continued up to three. The best performed speed was continued for another two cycles to check the efficiency.

***Rooting and hardening of the seedlings*:**

After multiplication cycle's elongated shoots were rooted in liquid medium consisting of half strength MS medium consisting of 0.1 mg/l IBA along with 20g/l sucrose. The rooted seedlings after 25 days of incubation were planted in poly bags consisting of soil, sand and FYM (1:1:1) in equal proportion. Pre hardening was done in green house and secondary hardening under shad net before supplying to main field for planting. The rate of shoot multiplication in each speed was recorded. Increase in number of shoots multiplied was compared with shoots multiplied on static positions.

RESULTS AND DISCUSSION

The meristems inoculated on initiation medium produced heavy phenolics as a sign of growth and development (Wolf *et al*., 1976). However, shifting of meristem positions on paper bridge avoided growth hindrance due to accumulation of phenolics. In alfalfa, leaves used for callus formation developed phenolics, which decreased the proliferation of callus simultaneously with increase in content of phenolics produced (Cvikrová *et al*., 1996). The meristems started proliferating on 16th to18th days after inoculation. Meristems which, submerged in the medium accidentally delayed shoot induction as found in *Rheum emodi* (Nand Lal and Ahuja, 1993) and failed to establish in some cases where, they turned to brown discoloration leading to death due to anaerobic condition.

The meristms on multiplication medium performed depending on speed of the shaker. The five level of speeds maintained on orbital shaker gave varied (Table 1) response. The highest multiple shoots were produced on 100 RPM followed by 75 RPM. The multiplication rate

Table 1 : Response of sugarcane meristems for multiplication on different speed of orbital shaker

Sr. No.	RPM	Inoculants used	I cycle		II cycle		III cycle		Average rate of multiplication
			Rate of multiplication	Shoots produced	Rate of multiplication	Shoots produced	Rate of multiplication	Shoots produced	
1.	25	50	3.56	178	4.00	712	4.64	3310	4.06
2.	50	50	5.08	254	4.78	1214	5.64	6848	5.16
3.	75	50	5.12	256	5.30	1357	5.80	7876	5.40
4.	100	50	5.26	263	6.23	1638	7.87	12895	6.45
5.	125	50	4.14	207	5.14	1064	5.22	5554	4.83
6.	0	50	1.36	68	2.70	184	2.83	521	3.02

at 25 RPM produced an average of 4.06 seedlings per cycle. The average was 5.16 per cycle at 50 RPM followed by 5.40 at 75 RPM. The highest rate of multiplication 6.45 per cycle was at 100 RPM. This speed might be the appropriate in providing nutrients and phytohormones. Since, the close contact of the tissue with the medium may stimulate and facilitate the uptake of nutrients and phytohormones leading to better shoot growth (Ziv, 1989; Smith and Spomer, 1994). The poor expression or disappearance of apical dominance due to continuous shaking condition of the tissues in the medium is another important feature of liquid cultures, which generally leads to induction and proliferation of numerous axillary buds. This leads to the development of bud clusters. The formation of condensed organized structures in which the shoots are reduced to buds / meristematic tissue in liquid media has been reported for several plant species. These clusters are made up of densely packed meristematic cells, actively dividing and forming new meristemoids on outer surface (Ziv *et al*., 1998; Young *et al*., 2000). Promotion of larger number of axillary bud development favors the production of large number of plants which are more or less true-to-type.

The highest speed maintained in the experiment 125 RPM produced an average of 4.83 seedlings per cycle, which was not ideal in producing large number of seedlings compared to 100 RPM. The higher rate of speed might have caused uncomfortable for uptake of nutrients due to rotational force of liquid medium in the culture bottle on the shoots. The shoot cultures maintained at static position did not perform well, here the average rate of multiplication was lowest compared to any other kept on orbital shakers. In shaking culture conditions the growth and multiplication rate of the shoots was enhanced by forced aeration, since continuous shaking of the medium provides sufficient oxygen supply to the tissue, which ultimately leads to their faster growth. In addition to these advantages, the preparation of liquid medium and handling of shake cultures is easier as compared to the semi-solid one. On the other hand, vitrification in the tissues is the common disadvantage in plant tissues during their *in vitro* propagation using liquid medium on still position (Kevers *et al*., 1984; Gasper *et al*., 1987; Debergh *et al*., 1992). The liquid culture medium added an additional advantage of reducing the cost of production of each seedling. In gelled medium sugarcane meristems of hybrid 'Co 86032' started proliferating after 57 days of inoculation and multiple shoots after another 45 days (Jadhav *et al*., 2001). But, in the present experiment the time for multiple shoot induction was reduced by half the time taken in gelled medium.

The shoots at 100 RPM were subjected for an additional two more cycles of multiplication and found to maintain same rate of multiplication in each cycle with an overall excellent response. The higher number of multiplication cycles by callus culture procedure may expose somatic variation among the seedlings produced and the threat can be minimized using this procedure. As meristem used as source of explant does not exhibit such variation in its offspring's produced. After multiplication seedlings were subjected to pre and post hardening in green house and shade net, respectively and found less than 2 per cent mortality during the hardening period. Hence, this methodology found to be very potential for mass multiplication of newly developed elite sugarcane cultivars in short time for larger area distribution among the growers. The procedure can be executed for large scale on commercial level for other cultivars.

Abbreviations:

MS	Murashige and Skoog
BAP	6-Benzyl aminopurine
NAA	Naphthaene acetic acid
GA3	Gibberellic acid
IBA	Indole-3-butyric acid
RPM	Rotations per minute

REFERENCES

Arencibia, A. (1998). Gene transfer in sugarcane. In: Hohn T, Leisinger KM (eds) *Biotechnology of Food Crops in Developing Countries*, Springer-Verag, New York, pp. 79-104.

Birch, R. G. (1996). New gene technologies and their potential value for sugarcane. *Outlook on Agriculture*, **25**: 219-226.

Cvikrova, M., Mala, J., Eder, J., Hrubcova, M. and Vagner, M. (1998). Abscisic acid polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. *Plant Physiol. Biochem*., **36**: 247–255.

Debergh, P. C., Aitken-Christic, J., Cohen, B., Von Arnold, S., Zimmerman, R. and Ziv, M. (1992). Reconsideration of the term "vitrification" as used in micropropagation. *Plant Cell Tiss.Org. Cult*., **30**: 135-140.

Feldmann, P., Sapotille, J., Gredoire, P.and Rott, P. (1994). Micropropagation of sugarcane. In: Teisson, C., ed. *In vitro culture of tropical plants*. France: CIARD: 15-17

Gasper, T. H, Kevers, C., Debergh, P., Maene, L., Paques, M. and Boxus, P. H. (1987). Vitrification: morphological, physiological and ecological aspects. In: Bonga JM, Duran DJ (eds.) *Cell and tissue culture in forestry*. Vol. 1. General principles and Biotechnology, Martinus Nijhoff, Dordrecht., pp. 152-166.

Guimarces, C. T. and Sobral, W. S. (1998). The Saccharum complex: relation to other andropogoneae. *Plant Breed. Rev*., **16** : 269-288.

Jadhav, A. B., Vaidya, E. R., Aher, V. B. and Pawar, A. M. (2001). *In Vitro* multiplication of 'Co-86032' sugarcane (*Saccharum officinarum*) hybrid. *Ind. J. agril. Sci*., **71**:113-115.

Kevers, C., Comans, M., Coumans-Gilles, M. E. and Gasper, T. H. (1984). Physiological and biochemical events leading to vitrification in plant cultured *in vitro*. *Physiol. Plantarum*., **61**: 69-74.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum.*, **15** : 473—497.

Nand Lal and Ahuja, Paramvir Singh (1993). Assessment of liquid culture procedures for in vitro propagation *of Rheum emodi*. *Plant Cell Tiss. Org. Cult*., **34** : 223-226.

Smith, M. A. L. and Spomer, L. A. (1994). Vessels, gels, liquid media and support systems. In: Aiktken-Christie J, Kosai T, Smith MAL (eds.). (1994). *Automation and Environmental control in plant tissue culture*. Kluwer Academic Publishers, Dordrecht., 371-404.

Taylor, P. W. J. and Dukic, S. (1993). Development of an *in vitro* culture technique for conservation of *Saccharum* spp. Hybrid germplasm. *Plant Cell Tiss. Org. Culture*, **34**:217-222.

Williams, R. R. and Taji, A. M. (1991). Effect of temperature, gel concentration and cytokinins on vitrification of *Olearia microdisca* (J.M. Black) *in vitro* shoot cultures. *Plant Cell Tiss. Org. Cult.*, **26**: 1–6.

Wolf, F.T., Tilford, R.H. and Martinez, M. L. (1976). Effect of phenolic acid and their derivatives upon the growth of *Avena* coleoptiles. *Z. Pflanzenphisiol*., **80**: 243–250.

Young, P. S., Murthy, H. N. and Paek, K. Y. (2000). Mass multiplication of protocorm like bodies using bioreactor system and subsequent plant regeneration in Phalanopsis. *Plant Cell Tiss.Org. Cult*., **63**: 67- 72.

Ziv, M. (1989). Enhanced shoot and cornlet proliferation in liquid cultured gladiolus buds by growth retardants. *Plant Cell Tissue Organ Cult.*, **17**: 101-110.

Ziv, M., Ronen, G. and Raviv, M. (1998). Proliferation of meristematic clusters in disposable presterlized plastic bioreactors for large scale micropropagation of plants. *In vitro Cell Dev. Biol. Plant*., **34**: 152- 158.

Received : July, 2008; Accepted : December, 2008