



THE STANDARDIZATION OF PROTOCOL FOR LARGE SCALE PRODUCTION OF SUGARCANE (CO-86032) THROUGH MICRO PROPAGATION

Jai Godheja^{a*}, Sudhir K. Shekhar^b, D.R. Modi^c

^{a,b}Research Scholar, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow

^cAssociate Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow

*Corresponding author email: jaigodheja@rediffmail.com

ABSTRACT: Sugarcane belongs to the grass family (Poaceae), an economically important seed plant family that includes maize, wheat, rice, and sorghum and many forage crops. The main product of sugarcane is sucrose, which accumulates in the stalk internodes. India is the second largest producer of sugar in the world. Sugarcane is an important agro-industrial crop in India, occupying 4.0 million hectare area. The average cane yield in India is about 70.0 tonnes per hectare while the sugar recovery is around 10.0 percent. The present study was carried out to standardize micro-propagation of sugarcane CO-86032 for large scale production of sugarcane planting material. Standardization and optimization of shoot induction and root induction medium were the major objectives of the study. Initiation of explant was found maximum in BAP (1 mg/lit) in MS medium. For shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Though number of multiple shoots were more in SM – III media, but was not appropriate for sub-culturing. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA. Maximum survival percentage (97.5 %) was found in SM – II media. From the present study it can be concluded that MS media supplemented with 0.3 mg/lit is best for shoot multiplication and for root induction ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA is best. For commercial production of sugarcane ½ strength MS is found to be more economically viable.

Key words: Sugarcane, micro propagation, MS, BAP, NAA, IBA.

INTRODUCTION

Sugarcane belongs to the grass family (Poaceae), an economically important seed plant family that includes maize, wheat, rice, and sorghum and many forage crops. The main product of sugarcane is sucrose, which accumulates in the stalk internodes. Sucrose, extracted and purified in specialized mill factories, is used as raw material in human food industries or is fermented to produce ethanol. Ethanol is produced on a large scale by the Brazilian sugarcane industry. It is native to the warm temperate to tropical regions of South Asia, and used for sugar production. In 2012, FAO estimates it was cultivated on about 26.0 million hectares, in more than 90 countries, with a worldwide harvest of 1.83 billion tons. Brazil was the largest producer of sugar cane in the world. The next five major producers, in decreasing amounts of production, were India, China, Thailand, Pakistan and Mexico. The world demand for sugar is the primary driver of sugarcane agriculture. Cane accounts for 80% of sugar produced; most of the rest is made from sugar beets. Sugarcane predominantly grows in the tropical and subtropical regions, and sugar beet predominantly grows in colder temperate regions of the world. Other than sugar, products derived from sugarcane include falernum, molasses, rum, *cachaça* (a traditional spirit from Brazil), bagasse and ethanol.

Development of tissue culture method for sugarcane production was initiated almost three decades before in late 1970s using meristem culture [1]. Till date several somaclones has been developed through tissue culture with improved productivity and eliminating certain defects like spines, leaf drying, disease susceptibility etc. Somaclones with smut resistance were developed from susceptible clones. Improvement in agronomic characters also have been reported in somaclones and some of the recent 'Co' releases like Co 86032, Co 92007, Co 92029, Co 93005, Co 94003, Co 94012, Co 94003, Co 95016, Co 99011 and Co 99012.

The sugarcane variety Co 94012 is a somaclonal variant that was released for cultivation in Maharashtra and is found to give high sugarcane recovery. However there is still a large gap between demand and supply of tissue culture raised sugarcane plants developed from above mentioned somaclones. Difficulty in standardizing tissue culture protocols for large scale production of sugarcane and lack of commercial interest in the same due to poor survival of the in-vitro regenerated plantlets of sugarcane during primary and secondary hardening procedures are the major reason behind such a large difference. The project entitled “Standardization of Protocol for Large Scale Production of Sugarcane (CO-86032) through Micro-propagation” was thus undertaken to study and understand important growth parameters of in-vitro regenerated sugarcane plants in order to standardize tissue culture protocol for large scale production of sugarcane.

Distribution of sugarcane world wide

Sugarcane area and productivity differ widely from country to country. Brazil has the highest area, while Australia has the highest productivity. Out of 90 sugarcane producing countries, 15 countries (Brazil, India, China, Thailand, Pakistan, Mexico, Cuba, Columbia, Australia, USA, Philippines, South Africa, Argentina, Myanmar, Bangladesh) 86% of area and 87.1% of production.

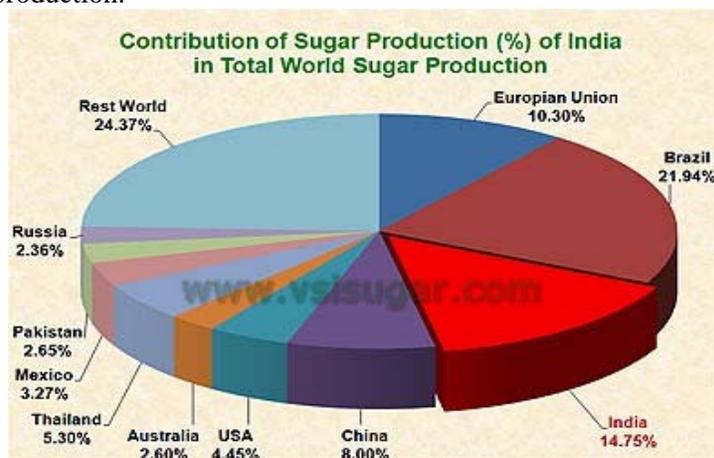


Fig. 1: Average production of Sugarcane during 2002-2012. (www.google.com)

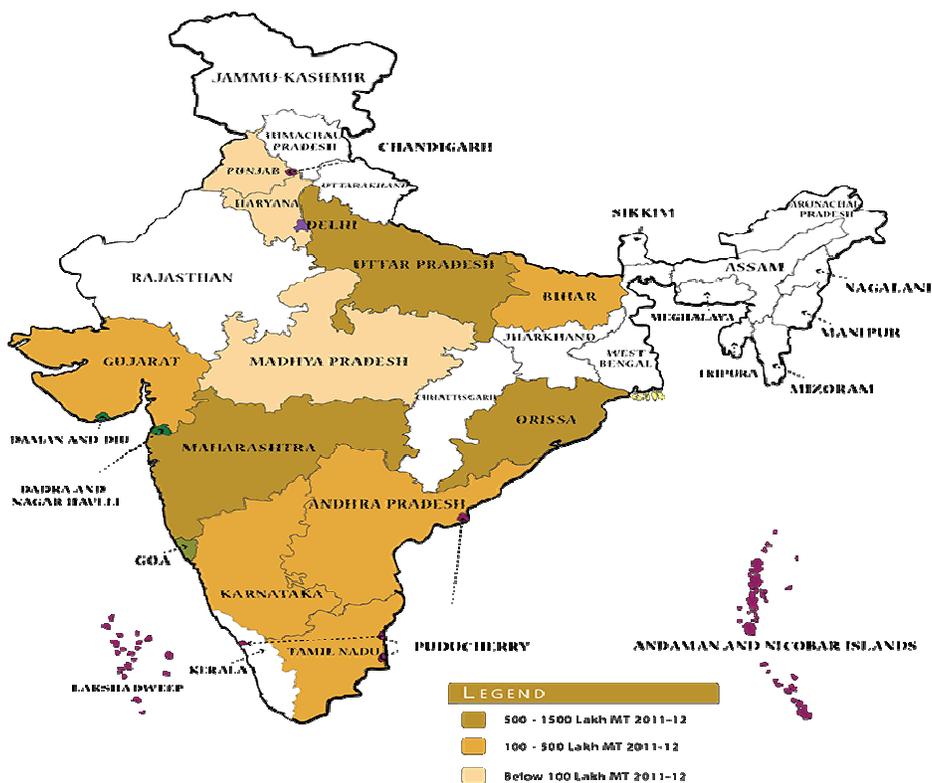
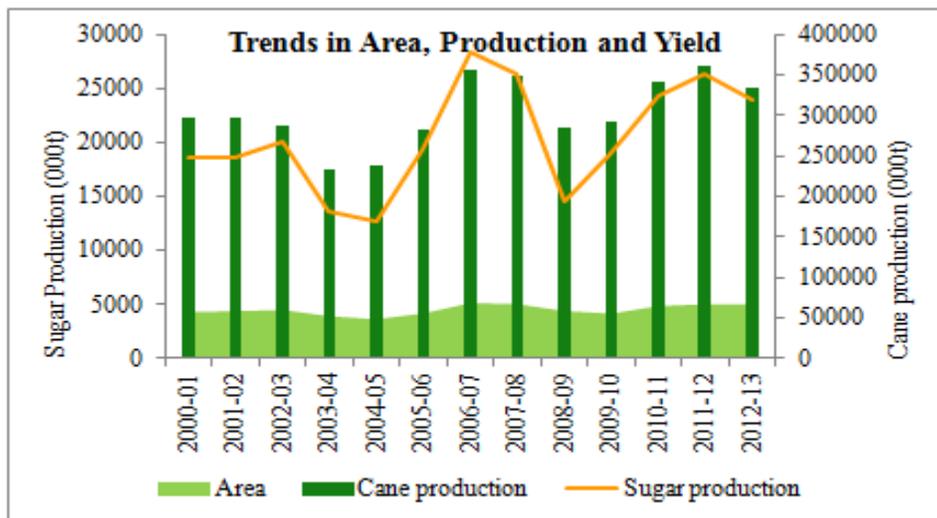


Fig. 2: Production of Sugarcane in different states of India (www.sugarcane.res.in)

Sugar industry in India

India is the second largest producer of sugar in the world. Sugarcane is an important agro-industrial crop in India, occupying 4.0 million hectare area. The average cane yield in India is about 70.0 tonnes per hectare while the sugar recovery is around 10.0 percent. However, there is potential of increasing the average cane yield to 100 tonnes per hectare and sugar recovery to 11.0 percent, if new technologies are transferred to the farmers fields.



Source: Ministry of Agriculture, Govt. Of India

Fig. 3: Trends in area, Production and Yield (Govt. Of India)

The various sugar canes are native to tropical locations around the world. Africa, South America, parts of Asia, and India are examples of some of the areas where one or more types of canes are cultivated, grown, and harvested. Just under two hundred different countries around the world are involved in the production of the canes and various Cane sugar products of some type. Sugar is a broad term applied to a large number of carbohydrates present in many plants and characterized by a more or less sweet taste. The primary sugar, glucose, is a product of photosynthesis and occurs in all green plants. In most plants, the sugars occur as a mixture that cannot readily be separated into the components.

In vitro Regeneration of planting material

The family poeaceae includes grasses consisting of about 620 genera and 10,000 species, occupying maximum land and water bodies across the globe. Constitute a natural and homogeneous family [2] and are among the most successful of all angiosperms in their spread. They provide the most important source of food for human and animal. Over 70% of the calories consumed by humans come from grasses, either directly in the form of flour, cereals, sugar, and alcohol, or indirectly, in the form of meat from animals that consume grass [3].

Tissue culture is based on the principle of totipotency, first described by German plant physiologist Haberlandt in 1902. He predicted the indefinite culture of tissues, cells, and organs. However, it was in 1939 Nobecourt and Gautheret in France and White in the United States independently reported the indefinite culture of plant callus tissue on a synthetic medium supplemented by auxin. Thus the first requirement for micropropagation was provided, that is the ability to grow plants in artificial culture. A second requirement for successful micropropagation was the regulations of shoot and root regeneration. This was elegantly presented in the classic work by Skoog and coworkers utilizing tobacco pith callus [4]. In recent years, tissue and cell culture techniques have been recognized as potentially valuable tool in crop improvement program. It is now possible to regenerate plants from single cells and protoplasts of numerous species [5], foreign genes can be introduced into cultured cells where they are expressed [6, 7].

Hendre *et al*, 1983, [8] reported that about 2 lac plants can be produced in six months from a single shoot tip. *In vitro* micropropagation provides for the rapid multiplication of sugarcane. By this technology nearly 10,00,000 healthier, virus-free, type sugarcane plantlets can be produced from single plant in one year. It was also studied micro propagation of sugarcane using two procedures [1] shoot tip culture, [2] indirect somatic embryogenesis from callus and reported that Shoot tip culture produced plants phenotypically similar to the mother plant and gave a much more rapid multiplication rate compared to the other procedure [9].

Chen *et al*, 1988, [10] reported sugarcane spindles of 6-12 months old plant were successful for shoot regeneration. Lal and Singh, 1994, [11] reported use of different gelling agents like (agar and agarose), and support materials (filter paper bridge, cotton cloth bridge and adsorbent cotton) as well as shaken and static liquid (control) cultures to improve in vitro shoot multiplication and vigor in sugarcane. Dhumale *et al*, 1994, [12] demonstrated that 2-3 mm shoot tips of sugarcane plant give significant shoot regeneration. Burner *et al*, 1995 [13] propagated sugarcane in vitro from shoot tip. Mullegadoo and Dookun, 1999, [14] studied the explant source of sugarcane and its effect on multiplication. Explants from three sources, axillary bud, apical bud and shoot apex, were cultured. Severe bacterial contamination occurred in axillary buds resulting in necrosis and death of the explants. Growth responses were better with apical buds than with axillary buds in two of the three varieties cultured. By micropropagation over 1.5 million plants can be produced from a single shoot tip in six months [15]. Ali and Afghan, 2001, [16] reported micro propagation of sugarcane using meristem culture method and found that micropropagated plants were phenotypically similar to the mother plants. Noguera *et al*, 2002, [17] described the micropropagation of sugarcane from shoot apical. Hoy *et al*, 2003, [18] studied the effect of tissue culture explant source on sugarcane yield components. Chen *et al*, 1998, [19] has described surface sterilization of leaf explants with 95% ethanol for 5 minutes. Gallo-Meagher, 2000, [20] reported surface sterilization of leaf explant by 0.5% sodium hypochlorite for 20 minutes. Mamun *et al*, 2004, [21] reported that sterilization of leaf explant with 0.1% mercuric chloride for 8 to 10 min gave satisfactory results with, 85-90% of the explants being contamination free. Chen *et al*, 1987, [22] reported that at lower concentration of BAP (0.5 mg/l) and Kinetin (0.5 mg/l), there was loss of proliferation and vigour. Naritoom *et al*, 1993 [23] reported plant regeneration from shoot tip culture of sugarcane using MS medium supplemented with cytokinin. Ali and Afghan, 2001, [16] reported that basal medium (MS) supplemented with benzyl amine purine (BAP) and kinetin (Kn) gave rapid shoot multiplication. It was observed that at lower concentration of BAP, shoot proliferation was significant. Patel *et al*, 2001 [24] also recorded highest multiplication on medium containing 1.5 mg/l Kinetin. The treatment 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivar. Pawar *et al*, 2002 [25] reported effect of growth regulators on in vitro multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014) and studied the effect of different levels of kinetin, BAP and coconut water (CW) on shoot multiplication, length of main shoot and number of leaves on main shoot using MS medium supplemented with three levels of kinetin (0.5, 1.0 and 1.5 mg/l), four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and two levels of CW (10 and 20%). The treatment MS + 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivars. Baksha *et al*, 2002, [26] reported multiple shoots from shoot tip explant of sugar-cane (*Saccharum officinarum*) by culturing on MS medium supplemented with BAP (0.5-2.0 mg/l), Kn (0.1-0.5 mg/l). Mamun *et al*, 2004 [21] reported that *in vitro* micro propagation for sugarcane variety viz., Isd-28 and Isd-29 showed best shooting when media were supplemented with BA 1.5 mg/l + 0.5 mg/l NAA. Shenk and Hildebrandt, 1972, [27] have reported requirement of high concentration of auxin for rooting in sugarcane. Nadar and Heinz, 1977, [28] reported that preferred auxin for root initiation was NAA. Rooting was induced in clumps. Chen *et al*, 1988, [10] reported that rooting in sugarcane takes place in the clumps. Auxins are reported to be essential for root induction in sugarcane, 70 gm/l sugar in MS media supplemented with 500 mg/l casein hydrolysate and 5 mg/l of NAA induced rooting. Naritoom *et al*, 1993, [23] initiated rooting on MS medium with 1 mg IBA/l. Dhumale *et al*, 1994, induced roots on half strength MS medium containing 2 mg/l IBA + 1 mg/l IAA. Cheema *et al*, 1995, [29] demonstrated rooting on MS medium supplemented with 7% sugar and 5 mg/l NAA. Singh *et al*, 2001, (30) transferred clumps with 5-10 well grown shoots on 1/2 strength MS liquid medium supplemented with NAA (5.0 mg/l) and elevated sucrose level (60 g/l). All cultures were incubated at 25 ± 20C under 12 h illuminations at a photon flux density of 50-70 micron. The development of fine roots, which began after 7-15 days, ranged from 75% (CoJ 85) to 95% (CoJ 86). Profuse rooting was achieved within 30-40 days. Plants transplanted in the field 45-60 days after hardening in the greenhouse showed uniform growth and asynchronous tillering within 60 days after transplanting. Pawar *et al*, 2002, [25] induced rooting (85-92%) by transferring shoot clumps on 1/2 MS medium containing 2 mg/l NAA and 1.0 mg/l IBA. Mamun *et al*, 2004, [21] reported that. Best results of rooting were observed on modified MS with auxins. Eighty to ninety percent regenerated plantlets were viable at normal temperature with 85% humidity while transferred sterilized.

MATERIALS AND METHODS

Explant preparation

Healthy explants of CO-86032 were collected from the mother plant nursery of sugarcane from Aditya Biotech Lab's field. The mother plants of Sugarcane variety CO-86032 were obtained from National Sugarcane Research Institute, Coimbatore. Selection of explants was done from mother plant nursery protected with insect proof nets.

Healthy mother plants with maximum numbers of tillers, good girth, appropriate color were selected and tagged with respective accession numbers. Soil was removed from the explants and washed with running tap water. The explants were then dipped in bavistin solution (1 gm/liter) 5 minutes followed by washing in running tap water to remove all traces of detergent. Then the explants were treated with 10 % sodium hypo chloride solution for 15 minutes. The explants were taken to the laminar airflow bench. Sodium hypo chloride solution was discarded and the explants were washed 3 times with sterilized distill water.

Media preparation and Initiation of Culture

The Murashige and Skoog's (MS) (1962) basal medium was used for the study. Stock solutions were prepared by using required quantities of macronutrient and micronutrient for convenience. Medium pH was adjusted to 5.8 and solidified with 0.7 % agar prior to autoclaving at 121°C and 15 psi pressure for 20 minutes. Media was stored at media storage room for at least 3 days prior to inoculation. Initiation of *in-vitro* culture of *Sachharun officinarum* is an important step. For culture initiation Murashige and Skoog MS) medium + Benzyl Amino Purine (BAP) (1 mg/L) was used. For multiplication of shoots MS media was prepared and was supplemented with 3 different concentrations BAP and designated SM – I, SM – II and SM – III. For root induction ½ strength MS media was prepared. After 3 - 4 cycle of sub-culturing, well developed shoots were induced for root multiplication in ½ strength MS medium with different concentrations and combinations of Indole Butyric Acid (IBA) and Napthalene acetic acid (NAA). These cultures were designated as RM1, RM2 and RM3.

RESULT AND DISCUSSION

Initiation of *in-vitro* regeneration

Shoot tip containing auxiliary meristem of cultivar Co-86032 were used as explants for *in-vitro* regeneration. The shoot tips containing axillary meristem were inoculated in MS medium with a fixed concentration of BAP (1mg/lit) for shoot tip initiation and establishment. Small shoots started appearing with in 7-10 days in all cultures bottles. Similar results have been reported by Dhumale *et al* (1994) for initiation where BAP @ 3 mg/lit. and NAA 1mg/lit. were used. Biradar *et al* (2008) also reported that axillary bud is the most suitable explant for initiation with MS medium containing BAP at 2 mg/lit concentrations.



Fig. 4: Sugarcane Node used as Explant



Fig. 5: Culture Initiation in MS

Shoot Elongation

Ten random bottles were selected for recording average shoot length of the shooting cultures during three cycles of multiplication. It was observed that maximum shoot lengths were obtained in the medium coded as SM-II i.e. MS supplemented with 0.3mg/lit BAP (Table 1). After 30 days of incubation the average shoot length recorded in SM-II medium was found to be 2.88cm, whereas minimum shoot length of 1.44cm was observed in SM-I i.e. MS supplemented with 0.1mg/lit BAP. It was also recorded that shoot growth in SM-3 were as elongated as SM-II but the shoot quality was relatively poor being succulent and weak. The shoots obtained in SM-III were not appropriate for further sub culturing due to curled morphology and fragile nature.



Fig. 6: Shoot Elongation.



Fig.7: Initiation of multiple shooting



Fig. 8: First sub culturing for shoot multiplication



Fig. 9: Second sub culturing for shoot multiplication in sugarcane

Multiplication of Shoots

For recording average no of multiple shoot similar procedure was followed as in shoot elongation. After 4-5 week of shoot growth, actively growing shoots were transferred to fresh medium in jars for further growth and proliferation. Proliferation of shoot started & during secondary proliferation stage, lateral shoots developed from base of newly initiated shoot. As a result a dense mass of shoot (25-30) was developed in each culture. After 30 days of incubation at about 25-28°C temperature and 60% relative humidity the dense shoot growth were separated and sub-divided in bunches containing 4-5 shoots each. Each of bunches was then cleaned and transferred in to fresh shooting medium in jars. In these way shoot multiplication was maintained for several passages by regular transfer to fresh medium. The best shoot multiplication response for sugarcane variety Co-86032 was obtained in SM-2 medium i.e MS Medium supplemented with 0.3mg/lit. BAP (Table-1). The medium showed about 13.18 shoots in an average after 1 month of incubation in 3rd cycle. Though SM-3 medium i.e MS medium supplemented with .5mg/lit. BAP also showed high numbers of multiple shoot the shoots were succulent and curly in nature that made them highly unsuitable for rooting.



Fig.10: Profused multiple shooting in Sugarcane



Fig. 11: Initiation of rooting

Survival percentage

The percent of survival varied between different shooting media carrying different levels of BAP. Maximum survival rated was found in medium containing BAP @ 0.3mg/lit (97.5%), on which shoots were active and healthy compared to other treatments. It was observed that survival percentage increased with increase in no of sub culturing.

Rooting of regenerated shoots

The regenerated shoots were used for root induction in root forming media. Half strength MS medium supplemented with 3 different combinations of NAA & IBA was used. The frequency of root formation was different in all media. Best root formation response was obtained in RM-III (table-2). MS medium having 3 mg/lit NAA & 3 mg/lit IBA. Average number of plants showing roots were 9.2 in RM-III, where as In RM-I & RM-II was 5.6 & 5.3 respective. Frequency of root formation was always recorded best in RM-III. Heavy bunch, healthy roots more than 20 roots per plant & whitish color.

Table 1 Effect of different concentration of BAP on multiple shoot induction of CO-86032

Media Type	Media Composition (mg/lit.)	Days to Shoot Formation	General Shoot Health	Cycle		
				First	Second	Third
SM - I	MS + 0.1 BAP	10.8	Less multiple shoots, shorter in length.	1.45	1.47	1.54
SM - II	MS + 0.3 BAP	10.3	High multiplication, longer in length.	2.04	2.24	2.88
SM - III	MS + 0.5 BAP	9.8	Very high multiplication, longer in length, succulent & curled leaves	2.11	2.4	3.01

Table 2 Effect of different concentration of BAP on number of multiple shoots in CO-86032

Media Type	Media Composition (mg/lit.)	Days to Shoot Formation	General Shoot Health	Cycle		
				First	Second	Third
SM - I	MS + 0.1 BAP	10.8	Less multiple shoots, shorter in length.	9.41	10.52	11.6
SM - II	MS + 0.3 BAP	10.3	High multiplication, longer in length.	11.46	11.99	13.18
SM - III	MS + 0.5 BAP	9.8	Very high multiplication, longer in length, succulent & curled leaves.	11.66	12.66	13.04

Table 3 Effect of different concentration of NAA & IBA combination on Root Formation

Media Type	Media Composition (mg / Liter)	Days to Root induction (Mean Value)	No. of Plants Showing Roots (Mean Value)	Average Frequency of Roots
RM - I	½ MS + 1 NAA + 1 IBA	16.7	5.6	Low (2-5 Roots, poor health, Blackish color)
RM - II	½ MS + 3NAA + 1 IBA	15.2	8.3	Medium(5-10 Roots, Normal Health, Whitish Color)
RM - III	½ MS + 3NAA + 3 IBA	13.56	9.5	High (<20 Roots, Heavy Bunch, Healthy Roots, Whitish Color)

Hence from the above result & discussion we can summarize the best result obtained in terms of media used for different stages of sugarcane cultures. So, for initiation it was found that BAP 1 mg/lit in MS medium was optimal for regeneration & multiplication. Similarly for shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA.

CONCLUSION

The present study was carried out to standardize micro-propagation of sugarcane CO-86032 for large scale production of sugarcane planting material. Standardization and optimization of shoot induction and root induction medium were the major objectives of the study. It was observed in the present study that an efficient protocol is needed for any new variety or clone to get rapid shoot initiation, shoot multiplication, root induction & elongation. Initiation of explant was found maximum in BAP (1 mg/lit) in MS medium. For shoot proliferation & multiplication SM - II media i.e. MS salts supplemented with 0.3 mg/lit BAP, was found to be the best compared to other media prepared. Though number of multiple shoots were more in SM – III media, but was not appropriate for sub-culturing. Best rooting result obtained in RM – III media i.e. ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA. Maximum survival percentage (97.5 %) was found in SM – II media. From the present study it can be concluded that MS media supplemented with 0.3 mg/lit is best for shoot multiplication and for root induction ½ strength MS media supplemented with 3 mg/lit NAA & 3 mg/lit IBA is best. For commercial production of sugarcane ½ strength MS is found to be more economically viable.

REFERENCES

- [1] Ali A, Naz S, Siddiqui S. A. and Iqbal J, 2008. An efficient protocol for large scale production of sugarcane through micropropagation. Pak. J. Bot, 40; 139-149
- [2] Purseglove JW 1972. Tropical Crops, Monocotyledons. Longman, London, UK.
- [3] Alderson, J., and W.C. Sharp. 1995. "Grass varieties in the United States." USDA. CRC Press, Boca Raton, Fla.
- [4] Skoog, F. and C.O. Miller 1957. Chemical regulation of growth and organ formations in plant tissue cultures *in vitro*. Symp.Soc. Exp. Biol., 11: 118-131.
- [5] Narayanaswamy S. 1977. Regeneration of Plants from tissue culture. In: Plant Cell Tissue and Organ Culture (Ed: J. Reinert and Y.P.S. Bajaj) Springer Berlin, pp. 179-206
- [6] Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R. Gallupi, S.B. Goldberg, N.L. Hoffman and S.C. Woo, 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA, 80: 4803–4807
- [7] Murai NM, Sutton DW, Murray MG, et al. 1983. Phaseolin from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222, 476–482.
- [8] Hendre, R.R, Fyer, R.S. Kotwal, M. Khuspe, S.S. and Mascarenhas, A.F. 1983. Rapid multiplication of sugarcane. Sugarcane 1: 5-8.
- [9] Lee 1987. Micropropagation of sugarcane (*Saccharum* spp.) Plant Cell, Tissue and Organ Culture 10(1): 47-55.
- [10] Chen, W. H.; Davey, M. R; Power, J. B. and E. C. Cocking 1988. Control and maintenance of plant regeneration in sugarcane callus cultures. Journal of Experimental Botany, 39: 251-261.
- [11] Lal, N and Singh, H.N. 1994. Rapid clonal multiplication of sugarcane through tissue culture. Plant Tissue Culture, 4: 1-7.
- [12] Dhumale, D.B; Ingole, G. L. Durge, D.V. 1994. In vitro regeneration of sugarcane by tissue culture. Annals of Plant Physiology, 8(2): 192-194, 5.
- [13] Burner, D.M. and Grisham, M. P. 1995. Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. Crop Science, 35(3): 75-880, 40.
- [14] Mulleegadoo, K. D.; Dookun, A. 1999. Effect of explant source and genotype on growth of sugar cane In vitro Revue Agricole et Sucrière de l'Île Maurice, 78(2):35- 39; 18 ref.
- [15] Anita, P.; Jain, R.K.; Sehrawat, A.R and. Punia, A. 2000. Efficient and cost- effective micropropagation of two early maturing varieties of sugarcane (*Saccharum* spp.). Indian Sugar, 50: 611-618.

- [16] Ali, Kazim and Shahid Afghan 2001. Rapid multiplication of sugarcane through micropropagation technique. Pakistan Sugar Journal, 16(6): 11- 14, 14 ref.
- [17] Noguera, A., E. Diaz, N. Paz and J. Ramallo, 2002. Sugarcane: micropropagation of recalcitrant varieties. Advance Agroindustrial, 23: 25-27.
- [18] Hoy, J.W.; Bischoff, K. P.; Milligan, S. B.; Gravois, K. A. 2003. Effect of tissue culture explant source on sugarcane yield components. Euphytica, 129(2): 237-240.
- [19] Chen, W. H.; Davey, M. R; Power, J. B. and E. C. Cocking 1998. Sugarcane protoplasts: factors affecting division and plant regeneration. Plant Cell Reports, 7(5): 344 – 347.
- [20] Gallo-Meagher, M., English, R. G. and Abouzid, A. 2000. Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cell Dev. Biol. Plant, 36: 37–40.
- [21] Mamun, M.A; Sikdar, M.B.H.; Paul, Dipak Kumar, Rahman, M.; Mizanur 2004. In vitro Micropropagation of some Important Sugarcane Varieties of Bangladesh. Asian Journal of Plant Sciences. 3 (6): 666-669.
- [22] Chen, W. H.; Davey, M. R; Power, J. B.; Cocking E. C.; Davey, M. R.; Sotak, R.; Gartland, J. S.; Mulligan, B. J 1987. Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. Plant Cell Reports, 6(4): 297-301.
- [23] Naritoom, K.; Sooksathan, K.; Korpradittskul, V.; Suksathan, I.; Visessuwan, R.; Burikam, S.; Klingkong, S.; Suriyachaiyakorn, M. 1993. Plant regeneration from shoot tip culture of sugarcane. Kasetsart Journal of Natural Sciences, 27: 286-291.
- [24] Patel, A.A.; Patel, S.R; Patel, C.L. and Prajapati, B.S. 2001. Effect of media composition on *in vitro* multiplication of sugarcane varieties. Ind. J. Gen. Pl. Breed., 61: 82-83.
- [25] Pawar, S. V.; Patil, S. C.; Jambhale, V. M.; Naik, R. M.; Mehetre, S. S 2002. Rapid multiplication of commercial sugarcane varieties through tissue culture Indian Sugar, 52(3):183-186; 9 ref.
- [26] Baksha, R., Alam R; Karim, M.Z; Mannan, Sk; Podder, B.P and Rahman, A.B.M.M. 2003. Effect of Auxin, Sucrose and pH Level on *in vitro* Rooting of Callus Induced micro Shoots of Sugarcane (*Saccharum officinarum*). Journal of Biological Sciences, 3 (10): 915-920.
- [27] Shenk, R.U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., 50:199-204.
- [28] Nadar, H.M. and Heinz, D.J. 1977. Root and shoot development from sugarcane callus tissue. Crop Science, 17: 814-816.
- [29] Cheema, Ravinder Kaur; Gupta, J. N 1995. Plants regenerated from protoplasts of sugarcane (*Saccharum officinarum*). Current Science, 68(6): 650-653, 23 ref.
- [30] Singh, B.; Yadav, G. C.; Lal, M. 2001. An efficient protocol for micropropagation of sugarcane using shoot tip explants. Sugar Tech, 3(3):113-116; 10 ref.