Improvement in Micropropagation Protocol for Rapid Seed Production and Fidelity Analysis of Plants using Molecular Markers in Sugarcane

Summary

Submitted to

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
LUCKNOW

FOR THE DEGREE OF

Doctor of Philosophy

IN

Biotechnology

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Enrolment No: 235/13

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(2018)

Title : Improvement in Micropropagation Protocol for Rapid Seed Production and Fidelity Analysis of Plants using Molecular Markers in Sugarcane

Sugarcane is a cash crop which is cultivated in large agricultural land of India. India is the second largest producer of sugarcane. Sugarcane breeders develop new elite varieties of sugarcane which are available in a very limited quantity at the time of its release and it takes about 8-10 years to produce sufficient seed for occupying the vast area of the state for commercial planting if multiplied through conventional sett planting method of seed multiplication. By the time the variety begins deteriorating in yield and quality traits. Thus, the increasing requirements for seed cane of newly released varieties could not be met timely through conventional methods of multiplication. Micropropagation is one of the most important and the most utilized technique of plant tissue culture emerging as an important tool of biotechnology for rapid seed multiplication in various crops.

The present investigation was undertaken with a view to improve the existing protocol for micropropagation of sugarcane for rapid seed production. Different growth regulators (cytokinins and auxins) including Thidiazuron, a compound possessing cytokinin like activity were evaluated for their responses on *in vitro* morphogenetic events using shoot tip explants of a popular midlate maturing variety of sugarcane *viz.* Co 05011. Efforts were also made to investigate the best source of explants and season of explanting to achieve the higher rate of shoot multiplication. Effect of various environmental conditions on shoot multiplication and growth was also studied. Keeping the above points in view, the present work was proposed to be carried out with the following objectives:

- To develop an improved protocol for rapid multiplication of sugarcane through *in vitro* micropropagation technique for seed production.

- Studies on acclimatization and survival of micropropagated plants under greenhouse conditions.
- Evaluation of micropropagated plantlets for clonal fidelity through molecular techniques

This work was aimed with above objectives for developing an improved and cost-effective protocol for *in vitro* micropropagation of aforesaid variety. The breeder seed nurseries were raised using the micropropagated plantlets and evaluated in the field for ago-morphological performance by comparing with the equivalent population of donor plants grown through conventional method of planting using sugarcane setts. The salient results of the present study have been briefly summarized in the following pages:

Sterilization of the explant is very important for successful establishment of shoot cultures. An experiment was conducted to find out appropriate concentration of mercuric chloride for effective sterilization of different explants of sugarcane. Results revealed that maximum 70% shoot tip explants sterilized with 0.2 % mercuric chloride solution and 50% meristem explants sterilized with 0.1% solution of mercuric chloride were able to develop sterile and viable cultures which subsequently established into the shoot cultures. The explants treated with higher concentrations (0.4 and 0.5%) of the chemical gave minimum 10% sterile cultures that were able to survive. The results also showed that sterilization of explants with mercuric chloride at the concentrations above 0.2% in case of shoot tips and above 0.1% in case of meristems were found to be detrimental to the explants due to high toxicity level of the chemical. On comparison, it was observed that shoot tip explants responded better than the meristem explants regarding the frequency of establishment of shoot cultures indicating that the shoot tip explants could be more efficiently utilized than meristems in seed multiplication programme through *in vitro* techniques.

Three different types of sugarcane crop *viz*., spring planted, autumn planted and ratoon of spring planted crops were used as the explant source to study the morphogenetic responses of shoot tip explants. Results demonstrated that the percentage of shoot initiation, establishment of shoot cultures and number of shoot/culture were highest in explants collected from spring planted crop followed by those collected from autumn planted crop and ratoon of spring planted crop. Consequently, the highest multiplication ratio (1:6) was recorded in cultures raised from spring planted crop followed by autumn planted crop (1:4) and ratoon of spring planted crop (1:3). On transferring the shoot cultures raised from explants collected from spring planted crop on rooting medium, gave the highest frequency of rooting in cultures raised from spring planted crop followed by those from autumn planted and ratoon of spring planted crop. Conclusively, the spring planted crop of sugarcane emerged as the best source of explants for *in vitro* micropropagation followed by autumn planted crop and ratoon crop. These results suggested that the source of explant is an important factor which should be taken into consideration during micropropagation of a sugarcane variety.

With a view to find out the best explanting season for achieving optimum morphogenetic responses, an experiment was conducted using shoot tip explants collected at monthly interval on 20th of each month from the field grown crops of sugarcane variety Co 05011. The findings of the experiment represent that the uppermost percentage of establishment of shoot cultures took place in explants inoculated in the month of March followed by October, February, September, April and November. The frequency of culture establishment was below 30 percent during extremely hot and cool months. Minimum explants could be established in the month of January when the average temperature was extremely low. Explants inoculated in the months of March to November required a minimum period of about 40 to 45 days for establishment of shoot cultures whereas those inoculated in the months of December and January required about

60 to 70 days in completing the establishment. The results indicated that the season could be one of the limiting factors under *in vitro* condition also as in the case of natural condition in the fields.

The results of an experiment carried out to study the effect of semisolid and liquid medium on establishment and multiplication of shoot cultures, showed that the frequencies of shoot initiation and culture establishment were significantly higher in shoot tip explants inoculated on agar-gelled semisolid medium than on liquid medium over filter paper supports. It was also observed that the cultures established on liquid medium gave an average of 18.3 shoots per culture which was significantly higher than those established on semisolid medium. The process of shoot initiation and culture establishment completed 3-4 days earlier on semisolid medium than on liquid medium. The shoot cultures established on semisolid and liquid media were multiplied for two more cycles by subculturing on the respective media to enumerate the multiplication ratio. The results revealed higher multiplication ratio of 1:6 in cultures multiplied on liquid medium as compared to those on semisolid medium (1:4). Based on results it may be concluded that semisolid medium proved to be more suitable for establishment of shoot cultures whereas shoots can be multiplied more rapidly in liquid medium.

In the experiment on the role of light intensity on multiplication and growth of shoot cultures it was observed that cultures incubated under 4000 lux light intensity produced maximum average number of shoots followed by those incubated under 5000 and 3000 lux light intensity. The shoots produced under 4000 lux of light intensity were most vigorous in growth, longer and healthier with dark green leaves as compared to those maintained under 3000 lux and lower intensities. Most of the shoots were poor to moderate in vigour, thin, stunted and smaller in size with yellowish green leaves under low light intensities. Regeneration of new shoots could not be observed in cultures maintained under complete dark. These results indicated that the intensity of light not only

influenced the multiplication ratio in the form of shoots /culture but also affected the growth and vigour of shoots. The results indicated that a high light intensity was essential for maximum establishment of shoot cultures, however, there was a threshold level of light (e.g. 4000 lux in the present case) beyond which the responses were markedly reduced.

An experiment was conducted to study the effect of photoperiod on multiplication and growth of shoot cultures. Results demonstrated that the rate of shoot multiplication, on the basis of average number of shoot per culture, enhanced significantly with the increasing photo-period from 8 h to 16 h. The results indicated that a photoperiod of 16 h was optimum for proper growth and multiplication of shoots whereas photoperiods beyond 16 h (i.e. 20 h or continuous light) did not prove beneficial for *in vitro* micropropagation. Further, the shoots produced under 16 h and 12 h photoperiods were mostly vigorous in growth, longer in size, bore dark green leaves and showed healthy appearance as compared to those maintained under other photoperiods whereas those produced under 8 h, 20 h and continuous light were poor to moderate in growth and vigour, thin in appearance and bore yellowish green leaves. Conclusively, photoperiod exerted remarkable effects of on growth and multiplication of shoot cultures and a photoperiod of 16 h proved to be most suitable for micropropagation of variety Co 05011.

Among various environmental factors, the temperature of growth room exerted remarkable effect on rate of shoot multiplication and shoot growth as evident by the result obtained in an experiment. The number of shoot per culture increased with the increasing temperature of growth room up to 25ºC and thereafter it decreased gradually. Maximum number of shoots were obtained in cultures grown at 25ºC followed by those incubated at 30ºC. The temperature of growth room also influenced the growth and vigour of regenerated shoots. Shoots grown at 25ºC were more vigorous in growth and appeared healthier with dark

green leaves than those grown at other temperatures. Conclusively, the maximum rate of shoot production in the form of shoots /culture was obtained at 25ºC and the shoots were vigorous in growth, attained better heights and bore dark green leaves.

An experiment was performed to study the effect of pH of nutrient medium on multiplication and growth of cultures and to optimize the multiplication ratio in sugarcane variety Co 05011. The maximum rate of shoot production on the basis of no. of shoot per culture was observed in cultures grown on medium having a pH value of 6.0 which produced 26.5 shoots per culture. There was a gradual increase in average number of shoots with the increasing pH of the medium from 5.6 to 6.0, thereafter it gradually declined. Shoots grown at pH 6.0 and 6.2 were more vigorous in growth and bore dark green leaves as compared to those produced at other pH values tried. Minimum shoots were obtained in cultures grown on a medium with a low pH value (5.6) which showed poor growth and yellowing of leaves while those growing at pH 6.4 turned brownish-black at their basal portion. These results indicated that the pH of medium exerted considerable effects on growth and morphogenesis of shoot cultures *in vitro*.

Zoom-stereomicroscopic observations made on proliferating shoot tip explants revealed that the shoot initiation took place by the germination of pre-existing young buds or meristems indicating their origin via axillary shoot development pathway. The initiated shoots grew further and gradually formed a crown of proliferating tissues around its base. Several new shoots primordia developed along the margin of crown through axillary shoot proliferation which grew further and subsequently formed a clump of shoots. Careful examination of proliferating cultures under Zoom-stereomicroscope revealed the formation of little callus at places as a thin layer of cells at the base of shoot cultures.

During micropropagation, an examination of shoot cultures subcultured for further multiplication revealed that mostly new shoots proliferated through axillary shoot development pathway. However, regeneration of a few shoots from the callus developed at the base of shoot cultures was also observed indicating their development through adventitious pathway but the frequency of such adventitious shoots was limited to less than 1.0 percent. This observation indicated the occurrence of a ‘mixed multiplication pathway during micropropagation of shoot cultures.

To overcome the problem of occasional microbial contamination during micropropagation, an experiment was conducted to find out a safe and effective dose of an antibiotic compound that could control the microbial growth without adversely affecting the growth and multiplication of shoots. The shoot cultures contaminated due to development of microbes during *in vitro* micropropagation were selected and subcultured on MS shoot multiplication medium supplemented with three different antibiotics *viz*., Streptomycin, Tetracycline or Cefotaxime at various concentrations (i.e. 250, 500 and 750 mg/l each). It is concluded from the result that Cefotaxime was the most effective antibiotic followed by Streptomycin and Tetracycline in controlling the bacterial growth. The frequency of contaminated cultures was up to minimum (2.7 percent) when the medium was supplemented with 500 mg/l of Cefotaxime. The rate of shoot production in the form of shoots /culture was also affected by the type and concentration of different antibiotics. The highest number of shoots could be recorded in presence of 500 mg/l Cefotaxime indicating the highest multiplication rate. The shoots developed in 500 mg/l of Cefotaxime present in the medium were vigorous in growth and healthy in appearance indicating that Cefotaxime exerted no adverse effects on shoot multiplication and growth. Based on results it may be suggested that 500 mg/l of Cefotaxime can be used for controlling microbial contamination,

especially bacteria, without any adverse effect on multiplication and growth of shoot cultures during *in vitro* micropropagation of sugarcane.

Three antioxidant compounds *viz*., Polyvinylpyrrolidone, Citric acid and Ascorbic acid were tested to investigate their role in reducing the adverse effects of phenolics during micropropagation. The results revealed that the compounds were able to reduce the secretion of phenolic substances to various degrees. The addition of PVP in the medium @ 0.20% was the most effective treatment in reducing the secretion of phenolics along with minimum adverse effects on growing cultures. At this concentration of PVP, the frequency of shoot initiation and culture establishment were markedly enhanced whereas the browning of medium was reduced to minimum. Conclusively, among the three antioxidant compounds used in the medium, Polyvinylpyrrolidone (PVP) at a concentration of 0.20 % was found most effective in reducing the secretion of phenolic substances from the explants during shoot initiation and subsequent establishment of shoot cultures from shoot tip explants of sugarcane variety Co 05011.

The cytokinin (BAP or Kinetin, alone) in the medium significantly increased the frequency of establishment of shoot cultures from shoot tip explants as compared to the medium containing no cytokinin. The numeral of shoot per culture was also affected due to the cytokinins. There was a significant increase in frequency of culture establishment and number of shoot per culture when BAP was used along with Kinetin, the highest responses in medium present with BAP and Kinetin (0.5 mg/l each). Raising the amount of these cytokinins to 1.0 mg/l, however, reduced the frequency of culture establishment and number of shoots formed per culture indicating that the cytokinins have a threshold level for expressing and optimum response. Conclusively, BAP along with Kinetin (each at 0.5 mg/l) appeared as the most excellent combination for obtaining maximum establishment of shoot cultures bearing maximum number of shoots. Most of the cultures established on this medium were healthy and vigorous in growth and

entered into the multiplication process successfully upon transfer to the shoot multiplication medium. The result indicated that BAP or kinetin alone was not as effective as if they were used in combination.

Pre-treatment of the explant with different quantity of thidiazuron (5, 10, 15 and 20 mg/l) produced encouraging results in comparison to the untreated explants. The maximum percentage of shoot initiation was obtained in explants pretreated with 10 and 15 mg/l of TDZ and maximum culture establishment was observed in explant pretreated with 10 mg/l of TDZ. Pretreatment of explants with TDZ also showed remarkable effect of number of shoot in the established shoot cultures which were significantly higher than control explants. Since the pre treatment of explants with 10 mg/l of TDZ gave the best results regarding establishment of shoot cultures in the aforesaid experiment, another experiment was carried out to optimize the pretreatment duration using TDZ (10 mg/l). It is shown from the result that the shoot tip explants pretreated for 24 h gave the best morphogenetic responses regarding culture establishment, shoot formation and shoot growth. Pretreatment of explants with 10mg/l of TDZ needed least number of days i.e. 40 days for culture establishment as compared to those receiving other treatments which required 45 to 60 days. Scanning electron microscopy (SEM) of pretreated and control explants showed that the mother shoots not only developed earlier but also induced early appearance of side shoots (5^{th} day after inoculation) in pretreated explant in comparison to the control representing that TDZ enhanced precocious growth of shoot primordia and lateral shoot formation.

The role of TDZ in increasing the rate of shoot multiplication and sustaining it during further cycles was evaluated. The continuously growing shoot cultures were transferred to the MS shoot multiplication medium augmented with various quantity (i.e. 0.001, 0.002, 0.004 and 0.006 mg/l) of TDZ along with BAP and Kinetin (0.5 mg/l each). MS medium devoid of TDZ served as control. The results showed that addition of TDZ in the medium increased the rate of

multiplication in the form, number of shoot/culture with the increasing concentration up to 0.002 mg/l. Maximum shoots were obtained in 0.002 mg/l of TDZ present in medium, which was significantly higher as compared to all other treatments tried. However, raising the concentration of TDZ to 0.004 mg/l and 0.006 mg/l significantly reduced the formation of shoots indicating that micro propagation with higher multiplication ratio can be achieved at an optimum concentration of TDZ. The shoots produced in presence of 0.002 mg/l of TDZ showed active growth and were most vigorous in appearance as compared to those produced in presence of other concentrations of TDZ and control. Further, the cultures grown on medium containing 0.002 mg/l of TDZ were subcultured and micropropagated successfully up to 12 cycles with improved rate of multiplication. Conclusively, incorporation of TDZ in the multiplication medium proved beneficial for shoot multiplication at an improved rate for several cycles of multiplication. Carbohydrate, being an important constituent of nutrient media, contributes significantly to the cost of plantlets in a micropropagation programme. Sucrose is a widely used (2-3%) carbon source in tissue culture studies of most plant species including sugarcane. With a view to reducing the production cost of tissue cultured plantlets, an experiment was conducted using cheaper sources of carbohydrate like Mill sugar, Sugar Cubes and Glucose. The results showed that the shoot cultures on transfer to MS liquid multiplication medium containing sucrose (30 g/l) formed maximum shoots followed by that containing sugar cubes, the results being statistically at par. Morphologically, the shoots produced in presence of sucrose or sugar cubes were also similar in growth and vigour. The average number of shoos produced on media containing mill sugar or glucose was significantly lower than those recorded on media containing sucrose or sugar cubes. The shoots produced on medium containing mill sugar were healthy in appearance and bore green leaves while those produced in presence of glucose had a moderate growth and bore slender, often yellowish green and curved leaves. The results suggested that sugar cubes can effectively be used as a cheaper alternative

of sucrose for micropropagation of sugarcane varieties on commercial scale. This practice may be helpful in reducing the cost of plant production considerably. These results suggested that after establishing shoot cultures on sucrose containing medium, further multiplication of shoots can successfully be carried out on medium containing sugar cubes which is comparatively much cheaper than sucrose. This may be helpful in reducing the cost of production to a considerable extent.

Among the three auxins tested, NAA was found to be most effective in inducing the rooting of shoots followed by IBA and IAA. The highest frequency of rooting was observed in shoots transferred on medium augmented with 5.0 mg/l of NAA. An increase in quantity of NAA from 5.0 mg/l to 10 mg/l significantly reduced the frequency of root formation. Maximum average number of root was also obtained in the medium having 5.0 mg/l of NAA and the roots were comparatively more thick, healthier and vigorous in growth than in other treatments tried.

The role of TDZ in root induction was revealed by an experiment which was carried out to find out an appropriate concentration of TDZ for optimum rooting in regenerated shoots. The results showed a sharp increase in the frequency of root formation in the medium having of TDZ (0.002 mg/l) in combination with 0.5 mg/l of NAA whereas low frequency rooting was recorded in presence of 0.002 mg/l of TDZ alone. The results indicated that a combination of both the growth regulators was essential for efficient rooting of shoots. Accordance with the previous and above mentioned results it may be argued that Thidiazuron possibly stimulated the activity of an added or endogenous auxin resulting into an enhanced proliferation of roots. The results also suggested that an optimum concentration of TDZ was necessary for activation of endogenous auxins.

Sucrose role in rooting of tissue culture derived shoots was found and an appropriate concentration of sucrose for enhanced rooting was also recorded. In this experiment, sucrose at various concentrations (20, 30, 40, 50, 60 and 70 g/l) was used in MS medium containing NAA (0.5 mg/l) in group with TDZ (0.002 mg/l) as optimized in the previous experiments. The results showed that an increase in the concentration of sucrose from 20 to 30 g/l significantly enhanced the frequency of rooting, however, further increase in sucrose level gradually reduced the frequency of rooting, number of root per shoot and quality of roots.

To achieve high rate of plant survival during hardening in the green houses, an experiment was laid out using soil alone or mixed with different combinations of sand and cattle-dung based compost for hardening of plants. It is shown from the result that the incidence of plantlet survival was highest (92.4%) in soil mixture containing soil, sand and compost in the ratio of 1:1:2, followed by 81.2 % survival in mixture comprising soil and compost in proportion of 2:1. These results being statistically similar were significantly higher than all other treatments tried. Minimum survival was observed in the mixture containing soil and sand (1:1). Observations made after 30 days of hardening revealed that the plantlets hardened in soil mixture including soil, sand and compost (1:1:2) attained maximum average height which was statistically at par in case of mixture containing soil and compost (2:1) but significantly higher than the plants grown in other soil mixtures or soil alone. The results indicated that sand was an important component of the soil mixture as it not only prevented the soil from being compact but also provided sufficient aeration to the roots. Compost, on the other hand, was helpful in supplying the required nutrients/micronutrients to the plants and in increasing the water holding capacity of the soil/mixture.

After proper hardening, the micropropagated plantlets were transplanted in well prepared field at spacing of 45 cm between the plants and 90 cm between the rows. Before transplantation, the leaves of plantlets were partially trimmed

towards the tip region and the polythene bags were carefully removed. For transplantation, the root zone along with the ball of soil was put in furrows at the spacing mentioned above, covered with the nearby field soil and gently pressed using the hands. A light irrigation was given to the plants immediately after transplantation. Further irrigations were given as per requirement and cultural operations were performed timely as per recommendations. Single bud setts obtained from the seed plot of the donor variety were planted by conventional method at the similar spacing for comparison. Various agro-morphological traits of micropropagated plantlets were evaluated against conventionally raised plants of the donor variety.

The agronomical data showed that the number of tillers/clump, number of millable cane/clump and cane height were significantly higher in tissue culture raised plants than in donor plants. The average number of internodes and cane height were almost similar in both the crops whereas the cane diameter was significantly lower in micropropagated plants than in donor plants. The average cane weight was comparatively lower in tissue cultured plants than in the donor plants but the differences were non-significant. The data recorded on cane yield during the harvest showed that the cane yield was marginally lesser in crop raised through tissue culture as compared to the conventionally grown crop. The results suggested that the improvement in traits like tillers count, millable canes count and cane height was possibly due to carryover effects of growth regulators used in the medium during micropropagation process.

Morphologically, tissue culture raised plants appeared to be more vigorous in growth with more green foliage than the donor plants. Other traits like length and number of internodes, length and width of leaves and leaf/bud characteristics (leaf colour, leaf angle, leaf ligules, shape and size of buds, bud groove etc) were almost similar in both tissue cultured and donor plants indicating that the tissue cultures plants were genetically stable and did not show any somaclonal variation.

Tissue culture generated plants were generally free from disease and insect/pest infestations, while 3-5 % plants raised through conventional method were found infested with wilt, GSD and borers.

Analysis of juice samples collected from randomly selected 10 plants raised through tissue culture and conventional methods was done at 60 days interval in the months of November, January and March. Observations were made on brix percent, sucrose percent in juice and purity percent. Results showed that the tissue culture raised crop had comparatively higher brix, sucrose percent in juice and purity percent than the donor plants. The sucrose percent in juice was found to increase gradually from November to March in both the crops. The aforesaid traits were numerically higher in tissue cultured plants than in donor ones in the respective months, however, the differences were non-significant for each trait. The results indicated that the micropropagated plants were almost similar to the donor plants regarding various quality traits. Marginal improvement in quality parameters in tissue cultured plants possibly occurred due to rejuvenation of plants rendered by *in vitro* technique.

Fidelity analysis of micropropagated plants through SPAR method revealed that the micropropagation protocol optimized in the present study is good as 98% of the plants were found to be true copy of the parental genotype indicating a high clonal stability. RAPD 10 markers produced 47 distinct bands with the average of 4.7 bands per primer while four DAMD primers produced 31 bands with average of 7.75 bands per primer. One polymorphic band was detected among RAPD band while six polymorphic bands were found among DAMD bands. DAMD primer 33.6 produced 5 polymorphic bands among total of 110 bands produced by 10 regenerates. RAPD primer OPA08 produced one polymorphic band.

Conclusions

- Sterilization of shoot tip explants with 0.2% and those of meristem explants with 0.1% aqueous solution of mercuric chloride proved beneficial for raising sterile cultures of sugarcane.

- Spring planted crop of sugarcane emerged as the best source of explants for *in vitro* micropropagation followed by autumn planted crop and ratoon crop.

- The highest frequency of establishment of shoot cultures took place in explants inoculated in the month of March followed by October, February, September, April and November. Explants inoculated during aforesaid months required a minimum period of about 40 to 45 days for establishment of shoot cultures.

- Use of semisolid medium proved to be more suitable for establishment of shoot cultures whereas shoots can be multiplied more rapidly in liquid medium.

- Shoot cultures incubated under 4000 lux light intensity produced maximum average number of shoots during micropropagation. The intensity of light not only influenced the multiplication ratio in terms of number of shoots per culture but also affected the growth and vigour of shoots.

- Photoperiod exerted remarkable effects of on growth and multiplication of shoot cultures and a photoperiod of 16 h proved to be most suitable for micropropagation of sugarcane.

- The pH of medium exerted considerable effect on multiplication and growth of shoot cultures *in vitro*. The highest rate of shoot multiplication in terms of number of shoot per culture was obtained in cultures incubated at

25ºC. The shoots were vigorous in growth, attained better heights and bore dark green leaves.

- Cefotaxime at a concentration of 500 mg/l can be used for controlling microbial contamination, specially bacteria, without any adverse effect on multiplication and growth of shoot cultures during *in vitro* micropropagation of sugarcane.

- Polyvinylpyrrolidone (PVP) at a concentration of 0.20 % was found most effective in reducing the secretion of phenolic substances from the explants during shoot initiation and subsequent establishment of shoot cultures from shoot tip explants of sugarcane.

- BAP along with Kinetin (each at 0.5 mg/l) emerged as the best combination for obtaining maximum establishment of shoot cultures bearing maximum number of shoots.

- Shoot tip explants pre-treated with 10 mg/l of Thidiazuron (TDZ) for 24 h, before inoculation on nutrient medium, resulted in high frequency establishment of shoot cultures with more number of vigorously growing shoots and required minimum period for establishment of shoot cultures.

- Incorporation of TDZ (0.002 mg/l) in the multiplication medium improved the rate of shoot multiplication and the cultures could be successfully multiplied for several cycles of multiplication.

- Sugar cubes can effectively be used as a cheaper alternative of sucrose for micropropagation of sugarcane varieties on commercial scale.

- Among the three auxins tested, α-Naphthaleneacetic acid (NAA) was found to be most effective for inducing root formation with maximum number of

roots followed by Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA).

- Incorporation of TDZ (0.002 mg/l) in combination with 0.5 mg/l of NAA enhanced the frequency of root formation in regenerated shoots of sugarcane.

- Zoom stereoscopic observations revealed the development of some adventitious shoots (<1.0 %) along with the axillary shoots during rapid micropropagation of shoot cultures which suggested strict monitoring of micropropagated material for clonal stability before using as seed for planting.

- The frequency of plantlet survival was highest (92.4%) in soil mixture containing soil, sand and compost in the ratio of 1:1:2 and the plantlets attained maximum average height as compared to plants grown in other soil mixtures or soil alone.

- During field evaluation, the number of tillers/clump, number of millable cane/clump and cane height were found significantly higher in tissue culture raised plants than in donor plants whereas the cane diameter was significantly lower in micropropagated plants than in donor plants.

- Morphological traits like length and number of internodes, length and width of leaves and leaf/bud characteristics (e.g. leaf colour, leaf angle, leaf ligules, shape and size of buds, bud groove etc) were almost similar in both tissue cultured and donor plants indicating that the tissue cultures plants were genetically stable and did not show any somaclonal variation.

- The cane yield was significantly lower in tissue cultured breeder seed plants as compared to the donor plants.

- The micropropagated breeder seed nursery was free from disease and insect-pest infestations whereas 2-3% plants raised through conventional method were found infested with smut, GSD and borers.

- Qualitative traits *i.e.* H.R. brix percent, sucrose percent in juice and purity percent recorded in tissue culture raised breeder seed nursery were almost similar to those recorded in conventionally raised donor crop.

- Based on molecular analysis of plants using RAPD and DAMD molecular markers, 98% micropropagated plants were found similar to the donor plants indicating that the developed protocol maintains the genetic integrity among the micropropagated population.

On the basis of above observations, it may be suggested that the aforesaid improved micropropagation protocol can be beneficially utilized for efficient production of quality seed material of sugarcane varieties.

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