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AN EFFICIENT PROTOCOL FOR LARGE SCALE PRODUCTION OF SUGARCANE THROUGH MICROPROPAGATION

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Abstract

A rapid propagation and acclimatization response of two different varieties of sugarcane (CP 77,400 and BL-4) was obtained in this study. The shoot apical meristem of different sizes was cultured on MS medium supplemented with different concentrations and combinations of BAP and kinetin either alone or in combination with each other or GA_3. Best shoot formation response in CP 77,400 was obtained on MS medium containing 1.5mg/l BAP while in BL-4 the combination of 0.5 mg/l BAP with 0.25 mg/l Kinetin showed best shoot formation response from apical meristem. Meristem of 3.0 mm size proved to be the best size for micropropagation of sugarcane. Excellent multiplication response of *In vitro* formed shoots was obtained when the concentration of BAP was decreased to 1.0 mg/l in CP 77, 400 and 0.25 mg/l BAP & Kin in BL-4 (i.e. 0.25 mg/lBAP + 0.25 mg/l Kinetin. MS medium containing 1.0 mg/l NAA and 2.0 mg/l IBA showed 100% rooting response of *In vitro* regenerated shoots of both the varieties of sugarcane within eight days of inoculation. Best hardening response was obtained in Sand+ Soil + Peat (1:1:1) after three week of transplantation in glass house.

Introduction

Sugarcane is an important cash crop of Pakistan. It constitutes a major source of edible sugars. Sugar is a highly placed commodity in consumer products. Day by day increasing use of sugar and its relevant products have created a challenging situation for sugarcane researcher and growers. Although Pakistan is the fifth largest sugarcane growing country of the world but our yield per hectare yield of this crop is lowest all over the world. The average yield of sugarcane in Pakistan is about 47.33 tons per hectares which is far below the existing potential being exploited at research stations and progressive growers farms. There are many reasons for this. The most important of which is the non availability of disease free elite stock for seeding and lack of implementation of advance technologies in sugarcane propagation. Malik (1990) reported that yield potential of sugarcane varieties is deteriorating day by day due to segregation, susceptibility to diseases, insects, admixture, changes adaphic and climatic environment. Moreover, the lack of rapid multiplication procedures has long been a serious problem in sugarcane breeding programs as it takes 10-15 years of work to complete a selection cycle.

Recently, plant biotechnology and molecular biology have created unprecedented opportunities and promises in the field of agriculture. The spectular findings in plant tissue culture have generated outstanding interest, enthusiasm and optimism over the

world. Methods have been developed for the propagation of genotype, more and efficient regeneration through micropropagation.

Micropropagation (propagation through apical meristem) is established, not only a popular mean of clonal propagation but also most viable and successful method for production of pathogen free stock material. The main advantage of micropropagation is the rapid multiplication of new varieties, improved plant health and its usefulness in germplasm storage. It is the best method for propagation as it produces plants phenotypically similar to the mother plant and gives much more rapid multiplication rate. Shaw (1990) also reported that micropropagation is being used in some sugarcane industries, for the development of disease free clones, mostly to facilitate their safe and speedy movement through quarantine. Lal *et al*., (1996) demonstrated that micropropagated system exhibited a potential *In vitro* production of 75600 shoots from a single shoot apex explant in a period of about 5.5 months. Nand & Ram (1997) have also reported that the mericlones derived seed cane was superior in sprouting, cane yield, sugar concentration and sugar yield. Gosal *et al*., (1998) have reported that micropropagated plants of cv. Co 83 grown in the field had up to 44.96% more canes/plots and up to 22.9% greater cane yield/plot than plants conventionally propagated from three-budded setts.

In order to ensure highest possible yield and quality of field crops the disease free stock can be extended to the growers by using this technique. The present research work was under taken by keeping in view the importance of tissue culture technology in sugarcane improvement and establishment of efficient protocols for mass scale propagation of healthy, disease free and premium quality planting material through micropropagation to enhance the yield and recovery per hectares of sugarcane.

Material and Methods

Apical portion from the shoot of sugarcane were excised from field growing plants. As sugarcane apical meristem has covering of rolled leaves, therefore, apical meristem is wrapped deep in leaf sheaths and is naturally sterilized. Therefore, it is not necessary to disinfect them. However, to prevent any contamination from outer covering of leaves, surface sterilization was carried out as follows.

For sterilization, explant was first washed with running tap water. Then treated with house hold detergent for five minutes. This was followed by second washing with tap water to remove all the traces of detergent. The explant was then treated with 10% Sodium hypochlorite solution for 15 minutes. After discarding Sodium hypochlorite, the explants were washed three times with sterilized distilled water to remove all the traces of Sodium hypochlorite. The sterilized explants were then inoculated by proper dissecting and sizing the meristem (0.5-1.0 cm) on MS (Murashige & Skoog, 1962) medium supplemented with different concentrations of BAP either alone or in combination with Kinetin or GA_3. For multiplication of induced shoots hormonal concentration was decreased and shoot multiplication was observed after 24 days of shoot induction. For *In vitro* rooting MS medium containing different concentrations of NAA and IBA was used either alone or in combination with each other. Sucrose 3% was used in all the media. The pH of the medium was adjusted to 5.74 with 0.1 N solution of NaOH or HCl. MS medium was used both in solid and liquid farms. For solidification 0.7% agar was used. In case of liquid medium autoclaved cotton was used to support the plant tissues. The medium was autoclaved at 121ºC and 15 Ibs/inch^2 pressure for 15 minutes. Cultures were

maintained under fluorescent light having 2500 lux light intensity. The incubation temperature was 26◦C ± 1◦C with 16 hour light and 8 hour dark period in every 24 hour cycle. First sub-culturing was done after five weeks and rest sub-culturing after two weeks. During each sub-culturing all dead or dis-coloured or vitrified shoots were removed. Hardening was carried out in glass house under natural light conditions.

Results

Shoot formation from apical meristem: The criterion of good growth for newly formed shoots from apical meristem was based on the production of broad and dark green colored leaves, healthy stems and number of small germinating buds at the base of stem.

From Table 1a it is evident that in CP 77400 best results for shoot formation were obtained in AM_2 medium (MS medium containing 1.5 mg/l of BAP). In this medium all explants showed shoot proliferation response within 10 days with maximum number of 1.8 shoots per explant (Fig. 2 a). By increasing the concentration of BAP, frequency of shoot proliferation was decreased and time taken for shoot formation was also delayed, In case of BL-4, shoot formation response varied from 9 to 13.5 days. Best response was obtained in AM_6 medium (BAP 0.5 mg/l + Kinetin 0.25 mg/l). All explants showed 100% shoot formation within 10 days with 1.7 shoots per explant (Table 1b, Fig. 2b). AM_1 and AM_8 media also showed hundred percent shoot formation but time taken was more and the number of shoots per explants were less. All other combination did not prove good for shoot formation in BL-4 (Table 1b).

In the present investigation shoot apical meristem of different sizes ranging from 0.5-5 mm was used. As shown in Table 2, time for shoot formation was increased by decreasing the size of meristem. Maximum rate of survival was achieved when meristem of 3 mm size was used. This size exhibited 100% survival with 90% regeneration potential within 12 days of inoculation. For shoot formation both solid and liquid media were used. Best results were obtained on media solidified with Phytagel at 3.0 mg/l.

Multiple shoot formation: After 4–5 weeks of shoot growth, actively growing shoots were transferred to fresh medium in jars for further growth and proliferation. Both solid and liquid media were tested. Best results for shoot multiplication were obtained in liquid medium. Proliferation of shoot started and during secondary proliferation stage, lateral shoots developed from the base of newly initiated shoot. As a result a dense mass of shoots (25-30) was developed in each culture jar (Fig. 3a and b). After 20 days these bunches were further sub-divided in bunches containing 4-5 shoots and were transferred into fresh medium in jars. In this way shoot multiplication was maintained for several passages by regular transfer to fresh medium.

The best shoot multiplication response in CP 77400 was obtained in SM_1 medium i.e. MS medium containing 1.0 mg/l BAP (Fig. 4a). In this medium 29 shoots were obtained after four weeks of sub-culturing. Addition of kinetin and GA_3 did not show any support to shoot multiplication in CP 77400. In case of BL-4, best shoot multiplication was achieved in SM_5 medium i.e., MS media containing 0.25 mg/l BAP and Kin each (Fig. 4b). From Table 3 it is evident that in both the varieties rate of shoot multiplication increased by decreasing the concentration of BAP. It was also observed that shoot multiplication response was enhanced in liquid medium while solid medium delayed shoot multiplication response.

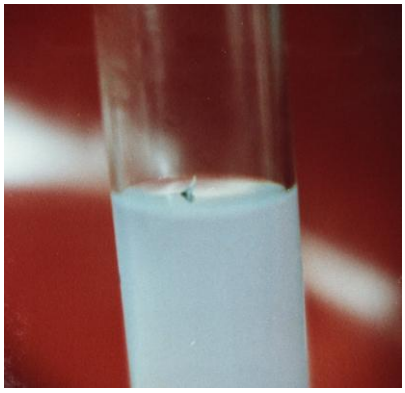

Fig. 1a. Sugarcane shoot apical meristem inoculated on MS medium medium containing 1.5 mg/l BAP Var. CP 77,400 (2.0X).

Fig. 1b. Sugarcane shoot apical meristem inoculated on MS medium containing 0.5 mg/l BAP+ 0.25 mg/l Kin Var. BL-4 (3.5X).

Fig. 2a. Shoot formation from apical meristem after 10 days of inoculation on MS medium containing 1.5 mg/l BAP Var. CP 77,400 (4.0 X).

Fig. 2b. Shoot formation from apical meristem after 10 days of inoculation on MS medium containing 0.5 mg/l BAP + 0.25 mg/l Kin. Var. BL-4 (4.0X).

Fig. 3a. Induction of shoot multiplication after four week of inoculation on MS medium containing 1.0 mg/l BAP(Var. CP 77,400) (4.0 X).

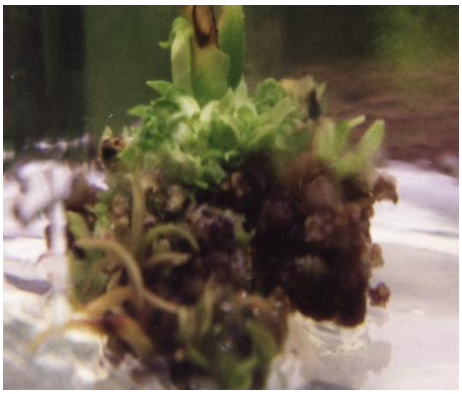

Fig. 3b. Induction of shoot multiplication after four week of inoculation on MS medium containing 0.25 mg/l BAP+ 0.25mg/l Kin. Var. BL-4 (4.0 X).

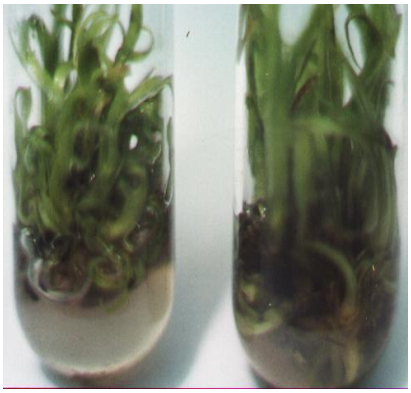

Fig. 4a. *In vitro* shoot multiplication after six week of noculation MS medium containing 1.0 mg/l BAP. Var. CP 77, 400 (1.5X).

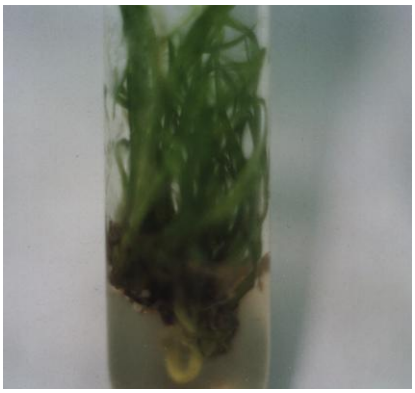

Fig. 4b. *In Vitro* shoot multiplication after six week of inoculation on MS medium containing 0.25 mg/l BAP + 0.25 mg/l Kin. Var. BL-4 (1.5X).

Fig. 5a. Well developed multiple shoots after seven week of inoculation on MS medium containing 1.0 mg/k BAP. (Var. CP 77, 400) (1.5X).

Fig. 5b. Well developed multiple shoots after seven week inoculation on MS medium containing 0.25 mg/l BAP + 0.25 mg/l Kin. (Var. BL-4) (1.5X).

Fig. 6a *In vitro* root formation after eight days of inoculation on MS medium containing 1.0 mg/l NAA+2.0 mg/l IBA. (Var. CP 77,400) (2.0 X).

Fig. 6b *In vitro* root formation after eight days of inoculation on MS medium containing 1.0 mg/l NAA+2.0 mg/l IBA(Var. BL-4) (1.0 X).

Rooting of regenerated shoots: The regenerated shoots were used for root induction in root forming media. Full and half strength MS medium supplemented with 12 different auxin concentrations was used. Frequency of root formation was different in all the media. Best root formation response was obtained in MR_{10} medium i.e., MS medium containing 1.0 mg/l NAA with 2.0 mg/l IBA. At this concentration 100% shoots formed roots within 8 days of inoculation with 2.8 roots per shoot in CP 77,400 and 3.1 roots per shoot in BL-4 (Fig. 6a and b, Table 4 and b). Half strength MS medium supplemented with the same auxins as used for full strength MS medium were also tested. No significant effect of half strength MS medium was found for root initiation and development (Table 4a and b).

Hardening of *In vitro* raised plants: For hardening *In vitro* raised plants were shifted in the glass house in three different medium compositions. Best hardening response was obtained in a mixture of sand + soil + Peat at 1:1:1 after three week of transplantation in glass house.

Discussion

The method of producing large number of identical clones by *In vitro* culture is being routinely used for wide range of plant species (Biondi, 1986). The results of present study demonstrates the regeneration potential of shoot apex of different sizes into plants.

Kartha (1986) and Siddiqui (1993) also reported the role of size of meristem shoot formation and proliferation. In the present investigation, best results for shoot formation and proliferation were obtained when meristem of size 3.0 mm was used. The present study also demonstrates the effect of phytohormones for shoot formation and multiplication. Among various phytohormones mainly two cytokinins i.e., BAP and Kinetin were used in MS medium either alone or in combination with each other or with GA3. The effect of cytokinin was different in both the varieties. In case of CP 77,400, BAP alone provided good results for shoot formation. Among different concentrations of BAP used, 1.5 mg/l provided best shoot formation response (Table 1a, medium AM_2). In case of BL-4, 0.5 mg/l of BAP with 0.25 mg/l Kinetin provided good shoot formation response (Table 1b, medium AM_6 and Fig. 2b). Many workers have reported the use of kinetin with BAP for shoot formation in sugarcane (Shukla, 1994; Geeta, 2001; Dhillon, 2002).

The regenerated shoots obtained were further multiplied by sub-culturing on fresh medium and maintained for one year (Fig. 4a & b and 5a & b). Maintenance and multiplication of germplasm stock in reduced space for several months was also reported by Engelman (1995).

The present study also highlights the role of cytokinin particularly BAP for shoot formation. The primary mode of action of plant growth regulators involves binding of active substances to a specific receptor molecule which bind either on cell surface or within the cytoplasm. The concentration of the receptors to target tissue determines the response potential (Flores & Tobin, 1988). Bud formation begins with an asymmetric division of target cell, several cells back from the tip. This step is initiated by cytokinin binding to an unidentified receptor within the target cell and its further development requires the continuous presence of cytokinins (Saunders & Hepler, 1982).

The initial response of cytokinin may be mediated by an increase in the cytosolic calcium concentration by promoting calcium uptake from the medium. Calcium affects

the cytoskeleton, which can regulate exocytosis (Hager *et al.,* 1991). Calcium ions have been shown to act through the regulating protein calmodulin. Each calmodulin have four high affinity calcium binding sites. Calmodulin alone is inactive as a regulator but the calmodulin-calcium complex can bind to and activate a number of enzymes including protein-kinase enzymes that add phosphorous to the serine or tyrosine hydroxyl group of proteins. The phosphorylation of enzyme can change their activity. So the calcium-calmodulin complex acts as a master switch, regulating alternative metabolic pathways within the cell. Therefore, the calcium ions may act as secondary messenger, transforming the hormonal signal into a biochemical switch regulating the initial stages of bud formation (Overvoodre & grimes, 1994).

In the present study best rooting response was obtained in full strength MS medium supplemented with 1.0 mg/l NAA with 2.0 mg/l IBA (Table 4a and b Fig. 6a and b). Anbalagan *et al.,* (2000) and Nadgauda (2002) reported high concentration i.e., 5.0 mg/l of NAA or combination of two auxins NAA and IBA for rooting in sugarcane. Yi *et al.,* (2004) also favoured the combination of NAA and IBA for rooting in *Phragmites communis.* Pruski *et al.,* (2005) found the combination of IBA and NAA best for rooting. However, Lal & Singh (1999) reported that 1.0 mg/l NAA for best rooting response in sugarcane. Cooke (2002) also preferred low auxin concentration as most suitable one for rooting in sugarcane. This inhibition of root initiation and elongation on higher concentration of auxins may be due to deposition of ethylene. As auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene retard root elongation (Weiler, 1984; Pau & Chi, 1993). The study indicates that micropropagation is not only feasible but it can be used as the helpful tool for rapid multiplication of disease free, high yielding and premium quality planting material of highly adapted, genetically stable and newly released varieties of sugarcane.

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