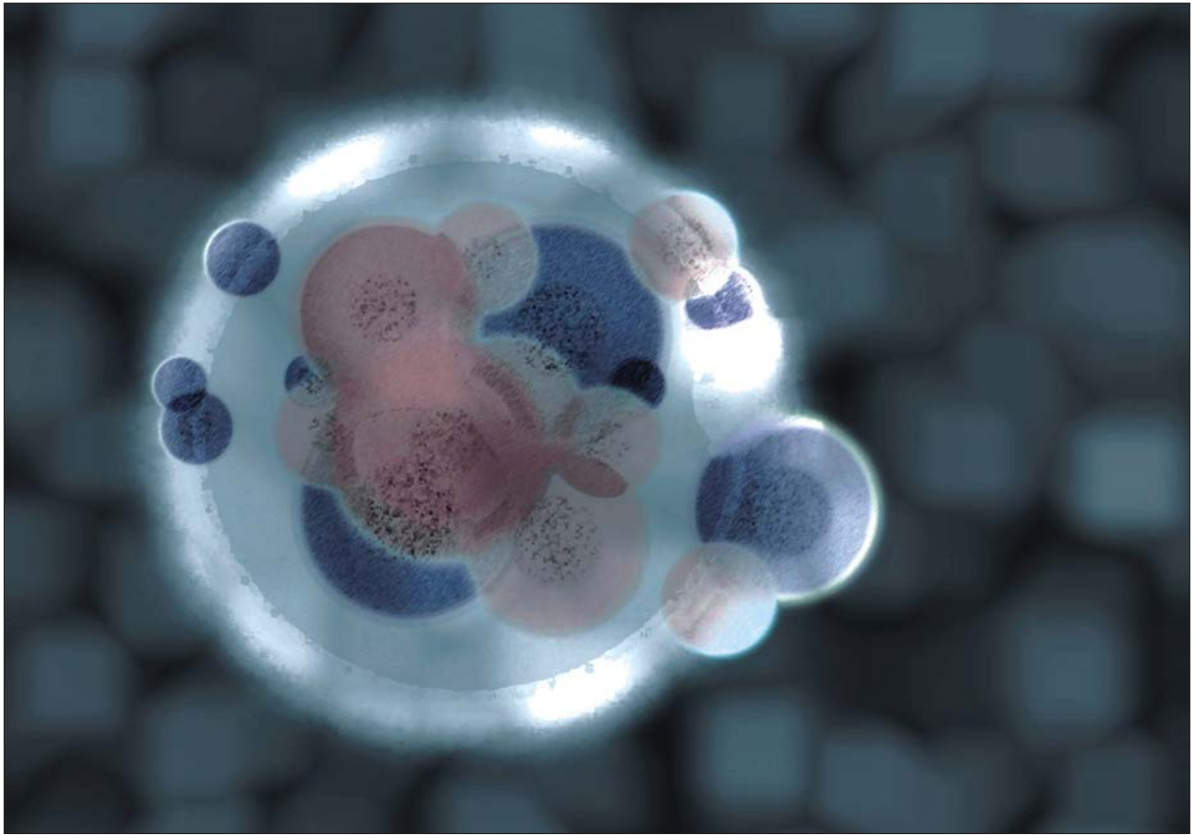




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Hormone Dependent Growth Promotion and Growth Retardation of Sugarcane Tissue Cultures for Germplasm Conservation

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Abstract: Two sugarcane (*Saccharum Officinarum* L.) varieties namely, Katha and BL₄ were used in this study in order to compare their response for culture establishment, shoot proliferation, root induction and growth retardation. Shoot number, mass and length were the growth parameters measured. Genotype dependent response was found against different growth conditions such as growth media composition and growth regulators. Liquid Murashige-Skoog (MS) media was found to be more suitable for culture proliferation as compared to the solid media of the same composition, suggesting a positive and favorable effect of aeration and homogenization on culture performance. A better organogenesis response i.e. maximum shoot length, shoot mass and shoot number was observed when explants were cultured on the media containing 4.4 µM BAP for both the varieties but BL₄ appeared to be more responsive genotype to shoot proliferation as compared to Katha at any given level of growth regulator. Growth retardation was best achieved on the media containing 4 g L⁻¹ mannitol among the concentrations used and degree of growth retardation was also found to be genotype dependent. There was a linear relationship between degree of growth retardation and concentration of the osmotica used. Root induction response (root number and root length) was the highest in Katha cultures grown on half strength MS media containing 1.9 µM Indole butyric acid among the auxins, Indole butyric acid, indole acetic acid and naphthalene acetic acid, that were used in this study.

Key words: Sugarcane, shoot proliferation, root induction, growth retardation

INTRODUCTION

Sugarcane is grown in tropical and subtropical regions of the world in a range of climates from hot dry environment near sea level to cool and moist environment at higher elevation. It is an important industrial and cash crop in Pakistan and in many countries of the world. Sugar production in the country mostly depends on this crop though a small quantity of sugar is also produced from sugar beet. Its shares in value added agriculture and GDP are 6.2 and 1.5%, respectively. Sugarcane was cultivated on an area of 1086 thousand hectares during the current year. The average production of sugarcane at national level is 52049 thousand tones. The average cane yield in Sindh, Punjab and NWFP is 53, 45 and 40 t ha⁻¹, respectively.

Pakistan occupies an important position among the cane producing countries of the world. It ranks fifth in cane acreage and production and 13th in sugar production in the world.

Sugarcane research in Pakistan is handicapped due to inappropriate breeding facilities (cane fuzz seed production) and shortage of funds to run research programs effectively to meet the existing as well as future challenges of cane breeding, variety development, cane agronomy and industrial research. Sugarcane flowers only in lower Sindh, Jabban valley in Malakand agency and at Murree hills. However, viable seed setting is still a problem. Hence the variety development programs depend on the import of exotic varieties and fuzz for which heavy investment in foreign exchange is required.

New technologies such as biotechnologies may be complemented to remove the bottlenecks for further improvements in productivity. Plant tissue culture has often been acknowledged as one of the most promising avenues to plant improvement. It can facilitate inter specific hybridization and genetic manipulations through molecular breeding approaches. Plant tissue culture serves as a means of vegetative propagation of economically important species. Tissue culture is also

used as an adjunct to more traditional means in plant modification. One of the important benefits of tissue culture is that it increases the mutation rate of plant cells. Such mutation is referred to as Somaclonal variation, which significantly affects plant improvement. Plant tissue culture technology has been most widely utilized in research and has found applications in industry as well.

About 20% of tissue culture regenerated plants have new mutations^[1], compared to less in frequency in field situations. The increased mutation rate is extremely useful for selecting plants with desired agronomic traits^[2]. Tissue culture regenerated soma clones exhibited more than 90% survival in the field. However, tissue culture serves to maintain the clonal fidelity of the germplasm when used as a tool for germplasm conservation. In this study many plant growth hormones and osmotic were used in tissue cultures for both shoot multiplication and retardation and root induction in two sugarcane varieties namely Katha and BL4. Moreover shake cultures and stationary cultures were compared for efficiency of growth of *in vitro* cultures. Discerning how the different hormones interact and how their quantities are effected by environmental factors will be important and necessary to improve traits of agricultural and therefore, economic importance in sugarcane and also for germplasm conservation which is vital for genetic improvement of crops.

MATERIALS AND METHODS

Plant material and explant source: Explants of sugarcane varieties namely BL4 and Katha were obtained from the existing collections of the *in vitro* Preservation Laboratory of the Institute of Agricultural Biotechnology and Genetic Resources, National Agricultural Research Center, Park Road, Islamabad. These cultures were established from the buds taken from the field grown plants planted at the research farm of the Sugarcane Research Program at NARC, as apart of the activities for *in vitro* conservation of germplasm of vegetatively propagated plant species.

Plant culture media: Full strength Murashige-Skoog^[3] media was used for all the experiments except the experiment on root induction wherein half strength media of the same kind was used. The scheme for preparation of MS media is described below.

The pH of all media was adjusted to 5.8 before they were boiled after addition of agar (8 g L⁻¹). The media were then dispensed into glass jars or test tubes. Culture vessels were sterilized by autoclaving them at 121°C for 15 min.

Plant culture establishment: The single isolated shoots were excised aseptically from the clusters of *in vitro*

grown cultures, obtained from the above-mentioned source. These shoots were established on Murashige and Skoog^[3] media containing BAP (4.6 µM) for the culture maintenance and proliferation to generate adequate plant cultures for further experiments on shoot proliferation, root induction, growth retardation, comparison of solid and liquid media for *in vitro* cultures of sugarcane.

Culture incubation: Cultures were incubated at 25°C at ±1°C under 16 h photoperiod and light intensity of 2000 lux was illuminated on to the culture vessels. Auto-clavable, translucent polypropylene lids were used to seal the glass jars to allow light to illuminate the cultures.

Shoot proliferation: Basal MS media containing salts, vitamins, myo-inositol (100 mg L⁻¹), sucrose (30 g L⁻¹) were supplemented either with BAP (0, 2.3, 4.6 and 9.3 µM), adenine sulfate (0, 0.43 and 0.86 mM) or kinetin (0, 2.3, 4.6 and 9.3 µM). When kinetin was used in the media, BAP was either omitted or included. BAP was also used either with or without IAA in the media described as above. A comparison was also made between continuously shaken liquid cultures and stationary cultures grown on semi solid media containing BAP at 4.4 µM concentration.

Root induction: The media for root induction contained half strength MS salts and all the rest of the ingredients i.e. sucrose, agar and vitamins were the same as in full strength media. Root induction media was enriched either with four different levels of IAA (0, 0.57, 1.7 and 2.8 µM), four different levels of NAA (0, 0.54, 1.6 and 2.7 µM) or 5 different levels of IBA (0, 0.5, 1.0, 1.4 and 1.9 µM) for two different sugarcane varieties tested in this study.

Growth retardation: The effects of mannitol and sorbitol were tested by culturing the plants on media containing five different levels of sorbitol (0, 1, 2, 3 and 4 g L⁻¹) and five levels of mannitol (0, 1, 2, 3 and 4 g L⁻¹) in separate experiments. These levels of growth retardants were incorporated into media containing basal MS salts, myo-inositol (100 mg L⁻¹), vitamin stock (1 ml L⁻¹), 3% sucrose and 0.8% agar. Physical incubation conditions were kept the same as in other experiments.

RESULTS AND DISCUSSION

Shoot proliferation: A series of experiments were conducted to study the effects of different levels of plant growth regulators, culture conditions and the genotypes on the growth of shoots and roots as manifested by shoot and root mass, length and number. The explants used in these experiments were taken from the sugarcane clusters

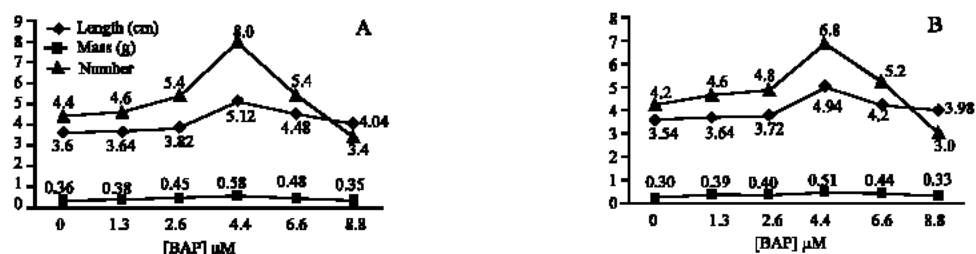


Fig. 1: Effect of varying concentrations of BAP (0, 1.3, 2.6, 4.4, 6.6 and 8.8 µM) on the growth of *in vitro* cultures measured as length, mass and number of shoots of sugarcane varieties namely BL4 (A) and Katha (B)



Fig. 2: A rapidly proliferating *in vitro* culture of sugarcane grown in liquid MS media containing 4.4 µM BAP

of shoots obtained from the *in vitro* cultures being maintained in the *in vitro* laboratory of the Institute of Agricultural Biotechnology and Genetic Resources, National Agricultural Research Center, Islamabad. The individual shoots were isolated from the clusters of shoots and aseptically transferred on the media combinations, which were prepared separately for each set of experiments.

In one of the experiments, effect of varying concentrations of benzyl amino-purine (BAP) ranging from 0, 1.3, 2.6, 4.4, 6.6 and 8.8 µM were tested on the growth of cultures of two varieties namely, BL4 and Katha. It was found that increasing levels of the growth regulator in the media had a positive effect on the shoot length, mass and number of shoots up to a certain extent beyond which there was found a gradual decline in the growth measured on these parameters. There was a difference in the behavior of the two genotypes tested. Figure 1 shows the pattern of response of the two genotypes against the hormonal regimes tested. The maximum number of shoot, the maximum shoot length and mass were obtained on MS media containing 4.4 µM BAP (Fig. 2). Patel *et al.*^[4] have shown that 4.4 µM BAP in the

MS media yielded maximum shoot length and number of *in vitro* grown sugarcane cultures. However, BL4 produced more biomass on this concentration of BAP than Katha over a period of 4 weeks in culture. The maximum numbers of shoots were 8 and 6.8, respectively for BL4 and Katha, at the level of BAP prescribed. Beyond this level, BAP has shown to impart a negative effect on the growth of sugarcane cultures.

In an other experiment, it was observed that the decline in effect of BAP could be further shifted to higher BAP levels if IAA was also incorporated in the media containing BAP. This phenomenon of shift in decline of response is demonstrated in Fig. 3, showing the effects of increasing levels of BAP at the same levels as tested before along with the enrichment of the media with IAA (1.7 µM). It was also observed that addition of IAA in the media keeps the plants greener than those plantlets grown on media devoid of IAA. Moreover, rooting of shoots was also visible among the cultures grown on the control media i.e. media that did not contain any growth hormones. Effects of varying levels of adenine sulfate on the shoot growth were tested. Adenine sulfate was added into MS media at 0, 0.43 and 0.86 mM levels and individual shoots were cultured on the media for a period of 4 weeks. A linear relationship between growth parameters and the concentration of adenine sulfate was found. The shoot mass, number and length of BL4 were higher than those of Katha at each level of adenine sulfate tested in this experiment (Fig. 4).

In another experiment, eight different combinations of growth hormones namely kinetin and BAP were tested for response of the two sugarcane varieties. In one such study, Kinetin level was increased either without any BAP added or with 4.4 µM BAP in the MS media. Four different levels of Kinetin were tested (0, 2.3, 4.6, 9.3 µM). Kinetin affected the growth of cultures depending upon whether or not BAP was present. In the presence of BAP, increasing the kinetin levels in the media resulted in the decline of shoot mass, length and number whereas in the

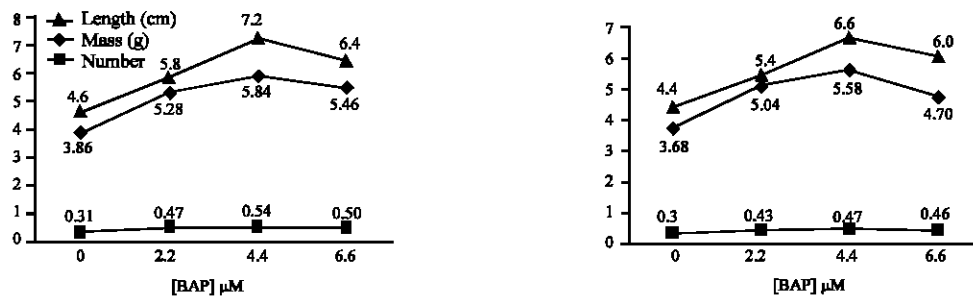


Fig. 3: Effect of varying concentrations of BAP (0, 2.2, 4.4 and 6.6 µM) on the growth of *in vitro* cultures measured as length, mass and number of shoots of sugarcane varieties namely BL4 (A) and Katha (B) cultured on MS media containing same level of IAA (1.7 µM)

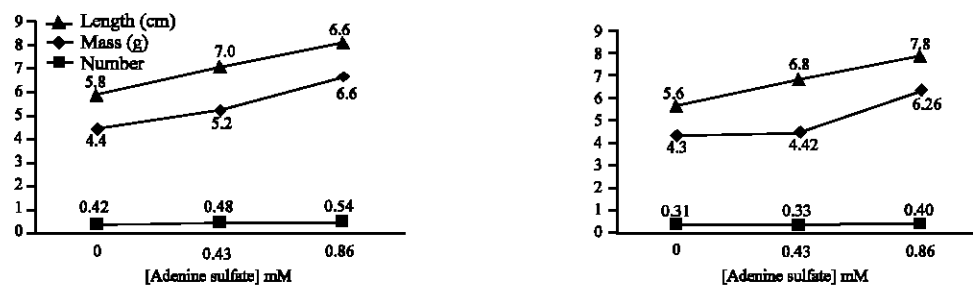


Fig. 4: Effect of different concentrations of adenine sulfate (0, 0.43 and 0.86 mM) on the, growth of *in vitro* growth of *in vitro* cultures measured as length, mass and number of shoots of sugarcane varieties namely BL-4 (A) and Katha (B)

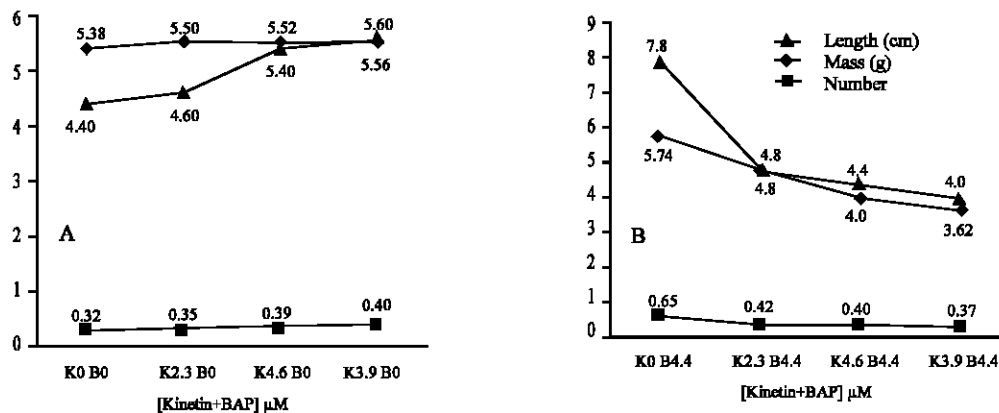


Fig. 5: Effect of varying concentrations of growth regulators namely, A: Kinetin ranging from 0 to 9.3 µM without BAP in the media on the growth of *in vitro* cultures of BL-4 variety. B: Kinetin ranging from 0 to 9.3 µM with constant level of BAP 4.4 µM on the growth of *in vitro* cultures of BL-4 variety

absence of BAP, the increasing levels of Kinetin proved to be stimulatory for growth of cultures in all respects. The same pattern was observed for both the varieties as depicted in Fig. 5.

Shake cultures vs. stationary cultures: The performance of cultures was compared between semi solidified and liquid media conditions. The same growth media as described above was either solidified by adding 0.8% agar

or was devoid of agar and enriched with 4.4 µM BAP and inoculated with isolated shoots of both the varieties. There was a profound difference in the growth of cultures of the two varieties in liquid and solid media. BL4 showed a better performance in the liquid media than the solid one. More than 16 shootlets were obtained in a cluster of sugarcane cultures when grown on liquid media as compared to merely 7 shootlets produced on the semi solid media of the same composition. Shukla *et al.*^[5]

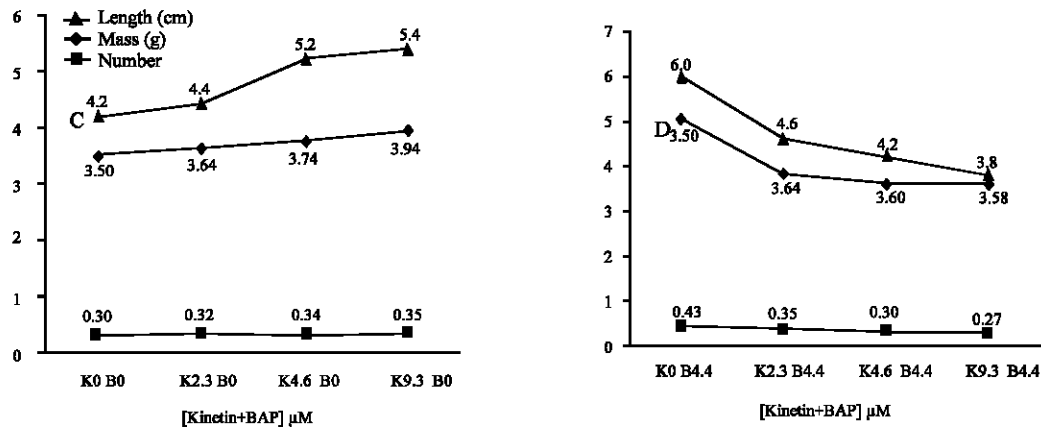


Fig. 5: C: Kinetin ranging from 0 to 9.3 (μM) without BAP in the media on, the growth of *in vitro* cultures of Katha variety. D: kinetin ranging from 0 to 9.3 (μM) with constant level of BAP 4.4 (μM), on the growth of *in vitro* cultures of Katha variety

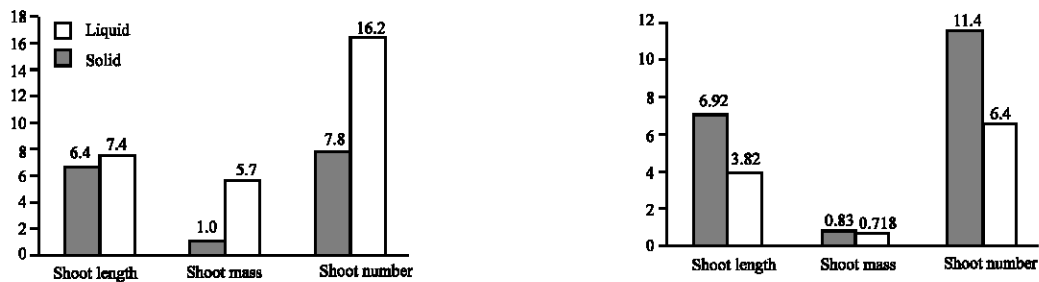


Fig. 6: Comparison between growth of *in vitro* cultures measured as length (cm), mass (g) and number of shoots of sugarcane varieties BL-4(A) and Katha (B) grown on solid and liquid MS medium of supplemented with 4.4 μM BAP

cultured the sugarcane variety Co84211 on liquid and solid media and showed that the liquid media performs better than the solid media for shoot proliferation. On the other hand, Katha gathered fewer mass, length and shoot number when cultured on the liquid media as compared to the solid media. Thus, liquid media are not recommended universally for better performance of all genotypes (Fig. 6).

Root induction: The best rooting response shown by the sugarcane varieties in our studies was obtained on half strength MS media containing 1.9 μM IBA. These results agree with the reports of Mannan and Amin^[6] who have used a combination of IBA and NAA each at 0.5 mg L^{-1} for two different varieties and obtained 96% rooting. In our experiment, the root number and root length in case of both the varieties increased with the increase in the level of NAA in the media. Singh *et al.*^[7] have reported similar results although they have used a much higher concentration of NAA (5 mg L^{-1}) in half strength MS

media. Ability of a defined media for successful root induction makes it possible to reduce the mortality of plants upon transferring them into pot or field conditions. Thus, the entire process of micro propagation and conservation culminates in a productive end due to successful rooting of the cultures. The results of these experiments are shown in Fig. 7-10.

When different concentrations of IAA (0, 0.57, 1.7 and 2.8 μM) were compared for their root induction ability, it was observed that there was differential response of the two genotypes used. Katha was more responsive to lower concentration of IAA as compared to BL4. In fact, higher concentration of IAA had an inhibitory effect on rooting of the former variety whereas the latter rooted better as the concentration of IAA was elevated. This pattern was consistent for both root number and root length parameters. The best rooting of Katha was obtained at 0.57 μM IAA and that of BL4 occurred at 2.8 μM IAA among the levels of this hormone tested in this experiment.

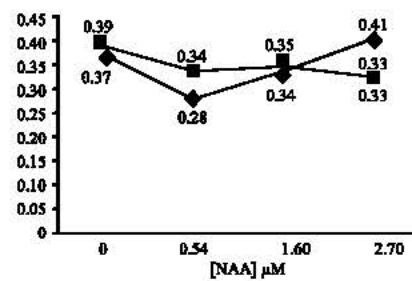
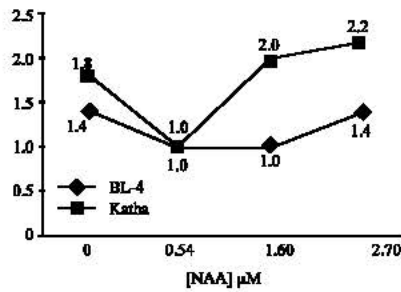


Fig. 7: Effect of varying concentrations of NAA (0, .5, 1.6 and 2.7 μM) in $\frac{1}{2}$ strength, MS media of *in vitro* cultures of rooting measured as number A and length B of roots, of two different varieties of sugarcane namely BL4 and Katha

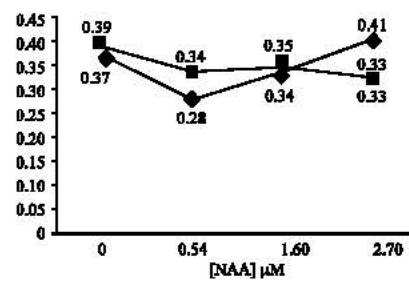
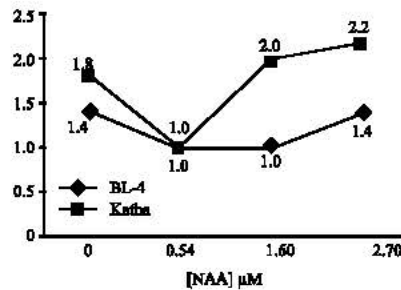


Fig. 8: Effect of varying concentrations of IBA (0, 0.5, 1, 1.4 and 1.9 μM) in $\frac{1}{2}$ strength, MS media of *in vitro* cultures of rooting measured as number A and length B of roots, of two different varieties of sugarcane namely BL4 and Katha

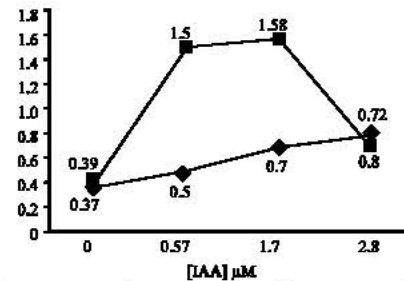
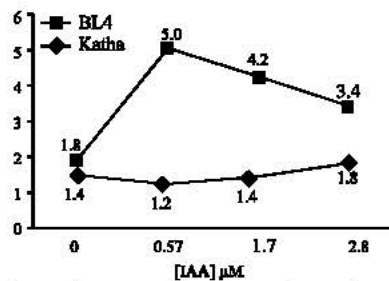


Fig. 9: Effect of varying concentrations of IAA (0, .57, 1.7 and 2.8 μM) in $\frac{1}{2}$ strength, MS media of *in vitro* cultures of rooting measured as number A and length B of roots, of two different varieties of sugarcane namely BL4 and Katha



Fig. 10: Roots induced in sugarcane plantlets grown *in vitro* on MS media containing 1.9 μM IBA

In a separate study, a range of concentrations of NAA (0, 0.54, 1.6 and 2.7 μM) were tested, rooting was best obtained in both the varieties at the highest concentration used. Katha rooted better than BL4 with NAA as well, like it did with IAA. There was a more linear relationship between rooting response and IBA levels used. As the concentration of IBA was increased, the rooting response improved correspondingly. The highest rooting response was obtained at the highest level of IBA used in the present study for both the varieties. But again, genotypic differential was visible and Katha was found to be more responsive to rooting than BL4 at any level. The rooted plantlets of both the varieties were transplanted into the pots containing a mixture of peat and coconut coir and sand in equal proportions. Humidity was maintained by placing the pots in a bell jars covered with

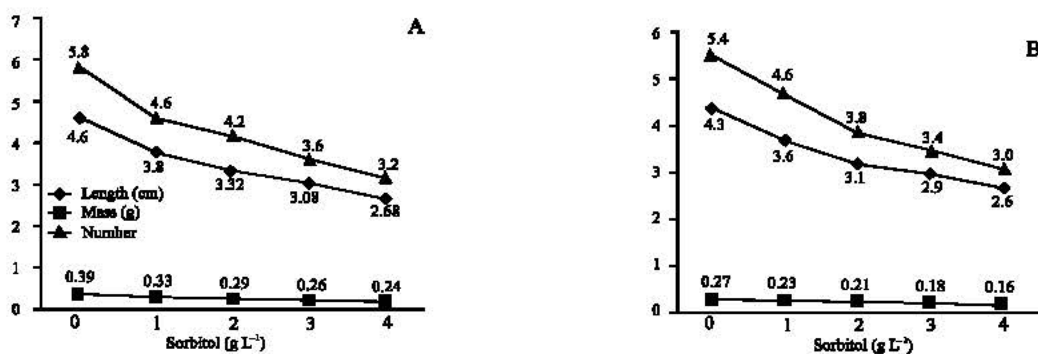


Fig. 11: Effect of varying levels of sorbitol 0, 1, 2, 3 and 4.0 g L⁻¹ on the growth of *in vitro* cultures, measured as length, mass and number of shoots of sugarcane varieties BL-4 (A) and Katha (B)

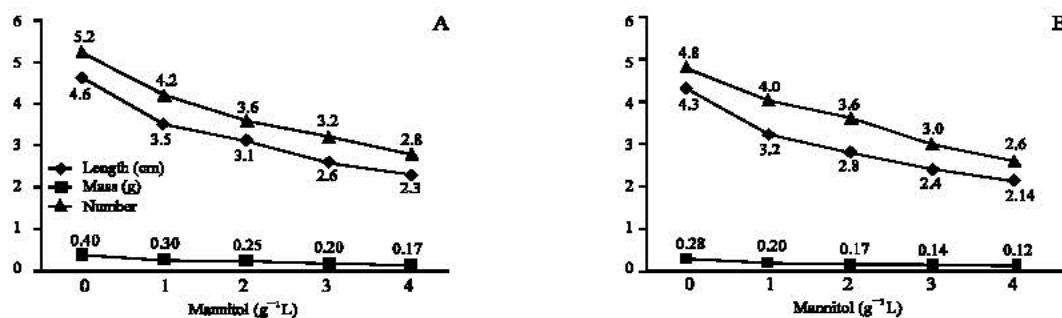


Fig. 12: Effect of varying levels of Mannitol (0, 1, 2, 3 and 4.0 g L⁻¹) on the growth of *in vitro* cultures, measured as length, mass and number of shoots of sugarcane varieties BL-4 (A) and Katha (B)



Fig. 13: The rooted plantlets of sugarcane surviving successfully after transplantation in to pots

transparent lid. The plants were irrigated on alternate days with half strength MS solution devoid of hormones and sucrose. The plants showed signs of new growth at the time of writing these lines (Fig. 11).

Growth retardation: Growth retardation is important to prolong the life of cultures between successive subculturing in order to economize the labor, space and resources for efficient conservation of germplasm. Growth

retardation of *in vitro* grown sugarcane cultures was experimented by employing media containing two kinds of osmotica, namely, sorbitol and mannitol. Each of the two was separately supplemented in to the full strength MS media at four different concentrations i.e. 0, 1, 2, 3 and 4 g L⁻¹. The cultures of the two varieties were grown on such media under standard light and temperature conditions as prescribed elsewhere. The growth parameters measured after a period of four weeks, are presented in Fig. 12 and 13. There was a linear decline in the growth of cultures along with the increase in the concentration of either sorbitol or mannitol. The retardation effect was found to be the maximum at the highest concentration (4 g L⁻¹) of either sorbitol or mannitol. The latter proved slightly better than the former in imparting growth retardation to both the varieties tested. The growth retardation achieved with the highest level of the osmotica was around 50% in either of the osmotica used in this study. Thus, it made it possible to prolong the life of the cultures without the effort of sub-culturing them repeatedly, thereby saving the labor and material resources for germplasm conservation. Present findings are in conformity with the results of Lemos *et al.*^[8] who used mannitol and sorbitol as osmotic

regulators along with other reagents such as sucrose, abscisic acid and found that they have a positive effect on conservation of sugarcane germplasm as they could grow these plants for 52 weeks without any sub-culturing.

Osmotic regulators resulted in growth retardation of both the sugarcane varieties tested in this study and the degree of retardation was directly proportional to the concentration of the osmotic regulator. Similar results have recently been reported by Ishtiaq *et al.*^[9] in case of potato cultures of a number of accessions obtained from the International Potato Center, Lima, Peru. These results suggest that growth retardation of sugarcane culture can prove to be helpful in maintaining the cultures for a longer time and can, therefore, facilitate germplasm conservation strategies. These treatments may improve even further the effectiveness of conservation protocols if they are used in conjunction with other treatments such as growing the cultures at low temperatures. The germplasm conservation at ultra low temperatures i.e cryopreservation may be costly for countries of hotter climates such as Pakistan but the osmotica-mediated conservation may be more economical.

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