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BIOREACTOR MEDIATED GROWTH, CULTURE VENTILATION, STATIONARY AND SHAKE CULTURE EFFECTS ON *IN VITRO* GROWTH OF SUGARCANE

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Abstract

Physical and chemical culture conditions are crucial in large scale micropropagation of clonally propagated plant species. In this study, two lines of sugarcane were used in a series of experiments to assess their *In vitro* growth performance under specified treatments of physical culture conditions. In one experiment, a Sibata glass double chamber bioreactor was used to evaluate the growth of cultures in two compartments of the bioreactor and it was found that the lower compartment proved better in supporting the culture growth as compared to the upper compartment. Lower compartment generated almost two times greater shoot mass and 1.5 times higher shoot numbers as compared to the upper compartment of the bioreactor. In a separate experiment, *In vitro* growth of sugarcane cultures was compared in vented vessels with the non-vented vessels. Ventilation was substantiated with microfilters (0.4 um membrane filters). It was observed that growth is initially better in vented vessels but soon extreme desiccation ensues due to loss of moisture from the media and plants and it proved detrimental to culture health. In yet another experiment, liquid and semi solid media were compared for their effect on *In vitro* culture of two sugarcane varieties. Liquid media exhibited better culture growth in case of BL-4 but Katha behaved opposite indicating thereby that there is a differential genotype dependent response to such culture conditions. Culture conditions described herein have proved very useful in maintaining the *In vitro* gene bank of Plant Genetic Resources Institute (PGRI) which undertakes collection, maintenance, evaluation, and distribution of germplasm of all plant genetic resources including the clonally propagated species.

Introduction

Sugarcane is an important food crop of the tropics and sub tropics that is cultivated in about 74 countries between 40°N and 32°S, encompassing approximately half the globe (Anon., 1998). It is a high value industrial and cash crop of Pakistan, a major raw material for sugar industry and plays a very important role in the economy. It is a multipurpose crop, creates employment at various stages and earns great amount of national exchange. In Pakistan, sugarcane is grown on about 1.099 million hectares with average cane yield of 47.32 tones per hectare and sugar recovery of 8.7 % as compared to world average of 63.7 tones per hectare and sugar recovery of 10.6% (Anon., 2005). Pakistan occupies an important position in cane producing countries of the world as it ranks fifth in cane acreage and production and almost fifteenth in sugar production. Although Pakistan has crossed the level of self-sufficiency in sugar production i.e., 3.65 million tones while the requirement is 3.55 million tones per annum (Anon., 2005), our national average cane yield is far below the true potential (Anon., 1998). Introduction and multiplication of new promising standard varieties would surely increase the yield

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potential and promote socio-economic development of both public and industrial sector as a whole. It has a potential of becoming an earner of foreign exchange as Pakistan has achieved self sufficiency in sugar production, albeit temporary sugar crisis in the recent months. Its export potential can be materialized with the adoption of new genetic material and technologies to fetch new world markets for sugar. New sugarcane varieties have been evolved and introduced in the agricultural farming system of Pakistan for enhanced yields and sucrose recovery.

Sugarcane research in Pakistan is limited by inappropriate breeding facilities (cane fuzz seed production) and financial constraints to effectively run research programs to meet the existing and 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 variety development programs rely on the import of exotic varieties and fuzz. New technologies such as biotechnologies may serve as complementary tool to address some problems in further improvements in productivity. *In vitro* technology has often been used as a promising avenue to plant improvement for research and practical applications. It can facilitate inter-specific hybridization and genetic manipulations through molecular breeding approaches. It serves as a means of clonal propagation of economically important species and as an adjunct to traditional means of plant modification as it increases the mutation rate of plant cells, resulting in somaclonal variation, which significantly affects plant improvement. About 20% of tissue culture regenerated plants have new mutations (Evans *et al*., 1984) which occur less frequently in field situations. Increased mutation rate is extremely useful for selecting plants with desired agronomic traits (Maddok *et al*., 1983). Tissue culture regenerated somaclones exhibit more than 90% survival in the field. Moreover, tissue culture serves to maintain the clonal fidelity of the germplasm when used as a tool for germplasm conservation. Clonally true to type propagation of plants by a variety of tissue and cell culture methods better known as microprpagation, is now the most commercially efficient and practically oriented plant biotechnology.

Organogenessis from explants result in *de novo* formation of both shoot and roots. These two events take place simultaneously during culture but frequently shoots are formed first. Regeneration of the complementary organ occurs only latter, either in same medium or after subculture to another medium and under different physical and chemical environmental conditions. Organogenesis starts with the distinct organization of shoot or a root meristem within the explant. This is controlled by the type of explant, composition of the culture medium and physical, chemical and environmental conditions during culture. Proper organization and differentiation, including the formation of functional vascular connections between the developing shoots and roots, finally gives rise to plantlets *In vitro*. Earlier, we reported the chemical conditions for growth promotion and retardation of sugarcane cultures (Saira *et al.*, 2005), wherein hormonal regimes, sugar content and osmotica compounds were used as variable treatments for growth promotion or retardation effects. This paper focuses on assessment of some physical conditions simulated in bioreactor, stationary and shake cultures and also ventilated and non ventilated culture vessels for their effects on *In vitro* growth performance of two sugarcane germplasm accessions. These findings will further substantiate the use of *In vitro* techniques in sugarcane research and practical applications.

Materials and Methods

Plant material and explant source: *In vitro* cultures of plants of sugarcane germplasm were established in the *In vitro* gene bank of Plant Genetic Resources Program of the Institute of Agricultural Biotechnology and Genetic Resources (IABGR), National Agricultural Research Center (NARC), Park Road, Islamabad. These cultures were derived from the buds taken from the field grown plants at the research farm of the Sugarcane Research Program at NARC. Explants from cultured sugarcane varieties viz., BL-4 and Katha were subjected to diverse treatments viz., ventilation, absence of ventilation, bioreactor mediated growth and growth on solid and liquid phases of media.

Media and culture incubation conditions: Full strength Murashige-Skoog (1962) media containing MS salts, sucrose (3%), myo-inositol (100mg/l), vitamins and BAP (4.4uM) was prepared with (8gm/l) or without agar. pH was adjusted to 5.8 before they were dispensed into glass jars. Culture vessels were sterilized by autoclaving. Chemicals used were of Sigma Chemical Company. Aseptic conditions were maintained during culture transfer and culture manipulation. After transferring the cultures on specified media, they were incubated at a temperature of 25ºC ± 1ºC under 16 hours photoperiod and light intensity of 2000 lux was illuminated on to the culture vessels. Cultures grown on liquid media were shaken at a speed of 80 rpm on a rotary shaker while cultures grown on solid media were incubated without shaking under otherwise similar conditions. For Bioreactor Mediated growth performance of cultures, a bioreactor (Sibata, Japan) having two compartments, lower and upper, was used. Air was filtered through a sterile filter (0.4 uM pore size, Millipore, Japan) before bubbling it into the bioreactor serially through the lower into the upper compartment. Entire media was replaced four times during growth cycle of cultures after which they were harvested from both the compartments for data measurements. pH of the spent media was monitored to observe changes in pH before discarding it. To evaluate the effect of air exchange on *In vitro* growth performance of sugarcane cultures, polypropylene lids of culture vessels were either or not equipped with autoclavable micro filters (Milli seal filter vents, Millipore, Japan). *In vitro* growth parameters namely shoot length, shoot mass and shoot number were recorded after a period of 4 weeks of incubation under aforementioned conditions.

Results and Discussion

Semi-solid and liquid media: In one of the experiments, *In vitro* growth performance of sugarcane cultures was compared between semi-solid and liquid media. Growth media was either solidified by adding 0.8% agar or was devoid of agar and enriched with 4.4uM BAP and inoculated with isolated shoots of both the varieties. There was a profound difference in the growth of cultures of the two varieties on liquid and semi-solid media. BL-4 showed a better performance in liquid media than on the semi-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 after the same duration. Shukla *et al*., (1994) cultured a sugarcane variety Co84211, on liquid and solid media and showed that its shoots proliferate better on liquid media than on the solid media. In our studies, BL-4 gathered significantly greater shoot mass (5.77gm) on liquid media than on the solid media (1.008 gm). Shoot length of this variety also was greater on liquid media as compared to the solid media. On the other

hand, Katha gathered less shoot mass, shoot length and shoot number when cultured on liquid media as compared to the solid media (Fig. 1). Similar genotype dependent growth responses were recorded in case of grape germplasm when cultured on media containing diverse hormonal regimes (Sajid *et al*., 2006). Liquid media have been used in many ways for different crops and have been found to promote better plant growth due to enhanced oxygenation and homogenization of the media in addition to continuous movement of plant cultures. Firozabady & Gutterson (2003) designed a temporary immersion bioreactor in which cultures were periodically immersed in liquid media for a few minutes and then the media was drained off for a few minutes. They found it more cost effective for commercial scale micropropagation of pineapple. Liquid cultures provide far better homogenization, aeration and oxygen transport both in the aerial and root zones as compared to solid media. CO_2 levels may not be crucial in the well sealed *In vitro* culture vessels as photosynthesis in these cultures is far less than normal due to low irradiance as compared to *In vivo* conditions and due to the presence of sucrose. However, microclimate conditions within the culture vessels need to be investigated in order to refine the culture conditions.

Bioreactor mediated culture: In yet another experiment, a bioreactor (Sibata, Japan) consisting of two chambers placed one above the other with an air permeable spacer in between and equipped with an air pump to flush filtered air through the lower chamber to the upper chamber, was used to culture the plants aseptically into full strength liquid MS media containing 4.4 uM BAP. Sugarcane variety, BL-4 was used in both the chambers and media was replaced periodically four times during the duration of experiment and each time, pH of the spent liquid media was measured and recorded for any change during culture growth. It was observed that pH of the media dropped during culture growth, necessitating the weekly replacement of liquid media with freshly prepared one (Table 1). Moreover, it was observed that the drop in pH during culture growth became less severe in every progressive week of culture growth. A similar pattern of pH modification was observed in case of grape cultures grown in bioreactor (Ajaz *et al*., 2002). Stability of pH after successive media replacements may be due to buffering ability of cultures themselves, to culture habituation phenomenon after successive transfers to fresh media and to reduced reaction after every successive transfer. Buffers are sometimes used in plant cultures to stabilize pH of the media but they may prove toxic especially if they are assimilated by plants.

Culture growth was measured upon harvesting the plants from both the chambers of bioreactor after 15 days incubation under a 16 h photoperiod and at a temperature of 25ºC. Shoot number, mass and length were recorded and data is presented in Fig. 2. Culture growth was found to be better in lower compartment of the bioreactor than the upper one**.** Shoot length, shoot mass and shoot number were 16, 67 and 102% respectively higher in the lower compartment as compared to the upper one**.** Better growth response of cultures placed in the lower compartment may be due to the fact that it received direct aeration from the air pump, thus bathing the cultures with well aerated media whereas the upper compartment receives air after it has passed through the lower compartment and therefore, media in this compartment became deprived of oxygen. Bioreactor has been shown to be very successful in achieving high volume micropropagation of grape plantlets (Ajaz & Sajid, 2002). Bioreactors theoretically offer many advantages over the conventional micropropagation procedures due to automation options to save labor, enhancing the speed of plant production and reducing the cost of

Table 1. Change in pH values of the liquid media recovered periodically during the *In vitro* culture of sugarcane variety BL- 4 in SIBATA Bioreactor.

Time of recovery	pH of fresh media	pH of recovered media	Drop in pH
1st week	5.8	4.76	1.04
2nd week	5.8	4.89	0.91
3rd week	5.8	4.96	0.84
4th week	5.8	5.03	0.77

planting material production. Since gaseous conditions, in particular those of CO_2 and O_2 affect culture growth, shake flask is practically closed system in which gas exchange is slow and as the culture grows, O_2 diminishes and CO_2 builds up. In contrast, bioreactor is supplied with a constant flow of air, which can keep the dissolved oxygen level high and thus, growth of plant cultures in bioreactor increases in biomass to increase productivity. Despite the advantages, bioreactor exploitation is limited due to high incidence of somaclonal variations in the progenies produced in large volumes. Thus, there is a need to optimize the culture conditions in order to keep the off types at the minimum to maintain the clonal fidelity.

Ventilation of culture vessels: In test tubes and other culture vessels, well sealed caps and closures are generally used to prevent desiccation and entrance of bacteria and other infectious agents. But such closures also prevent exchange of gases between the interior and exterior of culture vessels, resulting in O_2 depletion, enrichment of CO_2 and ethylene accumulation in culture vessels, which may prove detrimental to culture growth and even viability. In this study, it was observed that vented test tubes upon prolonged incubation, showed an excessive dehydration of the media as shown by the cracks in the media and a significant drop in the depth of media. Moreover, vented tubes showed no condensation of water vapors, which were clearly visible in the non-vented test tubes. It was observed that there was a 24% greater shoot length, 19% greater shoot mass and 31.5% higher shoot number in vented test tubes as compared to their non-vented counterparts in case of BL-4 variety (Fig. 3). Similarly, the other variety, Katha exhibited a 34% greater shoot length, a 10% larger shoot mass and 27% higher shoot number when cultured in vented test tubes than when cultured in non-vented test tubes. Better performance of vented cultures may be attributed to better air exchange that leads to prevention of accumulation of ethylene in the vessels and warrants the importance of designing new culture vessels or vessel closures. Condensation of water droplets inside the walls of non-vented vessels and cracks in the media in vented vessels respectively indicated high humidity and excessive desiccation due to water loss from the media. Marino *et al*., (2004) have reported that PVC films perform better than Para film used as sealing material for Petri plates that contained Quince cultures for regeneration and shoot development. They found that more quantities of CO_2 and ethylene accumulate in Para film sealed Petri plates than PVC sealed ones. Mills *et al*., (2004), however, found over-ventilation to pose worse effects on shoot proliferation of Jojoba plants than the moderate ventilation. They also reported reduced hyperhydricity and better resistance to ambient water loss in cultures provided with moderate ventilation, thereby helping better acclimatization upon transfer to greenhouse conditions after micropropagation stage. Sallanon & Maziere (1992) also reported that excess ventilation in culture vessels could impart unwanted

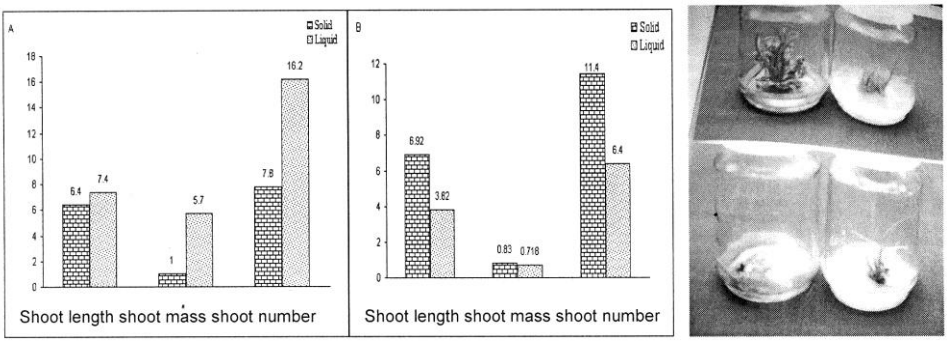

Fig. 1. Comparison between growth of *In vitro* cultures of sugarcane varieties BL-4 (A) and Katha (B) grown on solid and liquid MS medium supplemented with 4.4µM BAP. Right: Sugarcane cultures of BL-4 (upper)) and Katha (lower) grown on liquid (left) and solid media (right).

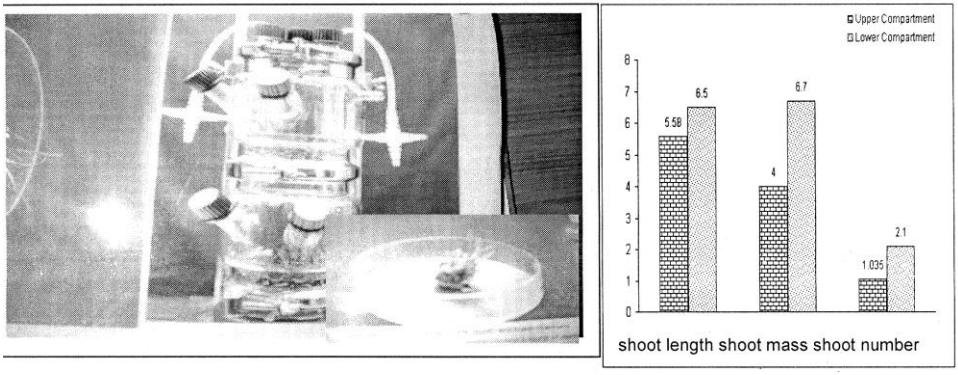

Fig. 2. Comparison between upper and lower compartments of the bioreactor (SIBATA CW-301) for growth of *in vitro* cultures of sugarcane variety BL-4 cultured on MS media supplemented with 4.4µM BAP. The insert shows a cluster of cultures harvested form the bioreactor.

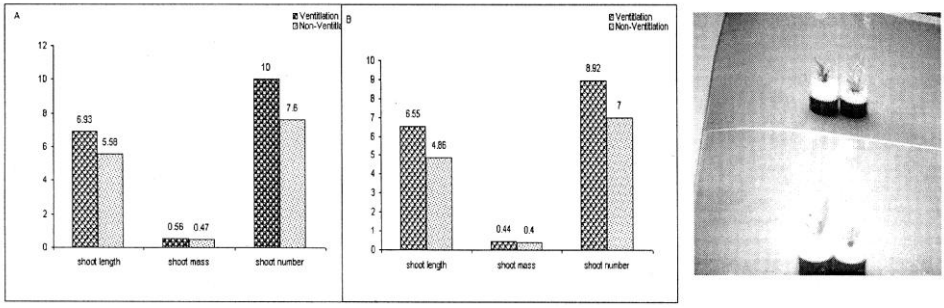

Fig. 3. Comparison between growth of *in vitro* cultures of Sugarcane varieties BL-4 (A) and Katha (B) grown with or without air exchange on MS media enriched with 4.4µM BAP. Right: Cultures of BL-4 (upper) and Katha (lower) grown with (left) or without (right) air exchange.

effects of producing fast desiccation of culture medium and reduction of tissue growth. Fal *et al*., (2002) have reported that physical environment in non-ventilated culture vessel affects micropropagation of Dianthus species. Kumar *et al*., (1998) have obtained better morphogenic response in tissue cultures by reducing ethylene production in the headspace or its inhibition of action. In our experience, however, ventilation despite promoting better culture health in short term cultures and thus recommendable for large scale micropropagation projects, also results in over-desiccation after prolonged culture and is, therefore, not suitable for long term germplasm conservation. Over-desiccation may be prevented by using hydrophobic type of microfilters or humidifying the entire culture room in order to save the desiccation sensitive cultivars. Studies may be initiated to reduce desiccation among ventilated cultures. Findings reported here may pave the way for designing new culture vessels and caps for obtaining better *In vitro* growth performance of sugarcane cultures and other species especially those which are required in large numbers for large scale industrial crop plantations.

Sugarcane improvement strategies for higher yields, disease resistance and quality enhancement for higher sugar content rely on a number of conventional and modern techniques. Among them, cell and tissue culture techniques occupy a central position, as sugarcane is a vegetatively propagated species. Conventional techniques for multiplication of planting material are slow and cannot keep pace with the demand for production of planting material especially of newly evolved varieties of sugarcane. It may take several years to produce enough planting material to plant thousands of hectares with a new variety, which may take equally long time to be developed. Thus, tissue culture techniques are extremely important in multiplication, germplasm conservation and even development of new varieties of sugarcane through callus mediated variability, screening of variants, protoplast hybridization and transformation. This paper in combination with our previous paper (Saira *et al*., 2005) sets out analysis of physical and chemical conditions which are best suited for successful *In vitro* culture establishment, shoot proliferation, germplasm conservation, root induction and transplantation of *In vitro* grown plants in field conditions.

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