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Phytohormones Influence on the In-Vitro Regeneration of *Saccharum Officinarum* L. from Apical Meristem Culture

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Abstract

Growth regulators especially auxins and cytokinins are critical for plant *in-vitro* regeneration. The effect of these plant growth regulators on *in-vitro* propagation of *Saccharum officinarum* L (Sugarcane) was investigated. *In vitro* response of two different varieties of sugarcane (NCS 005 and NCS 008) to Plant Growth Regulators was obtained in this study. Formation of buds was obtained on shoot apical meristem when cultured on MS (Murashige and Skoog) medium supplemented with 0.1mg/l BAP (6-Benzylaminopurine). After two weeks of initiation, regenerated meristem was inoculated into MS (Murashige and Skoog) fortified with different concentrations and combination of cytokinins. Shoot multiplication was optimal on 0.5mg/l BAP + 0.25 mg/l Kin(Kinetin) for NCS 005 variety while for NCS 008 variety, no significant ($P \geq 0.05$) difference was observed between 1.5mg/l BAP and 1.5mg/l BAP +0.5mg/l Kin. The best root induction for *in vitro* derived shoots was obtained on 1.0 mg/l NAA (Naphthalene acetic acid) and 2.0 mg/l IBA(Indole butyric acid) for both varieties of sugarcane within ten days of culture transfer. Successfully established plantlets showed excellent growth response when weaned under regulated green house conditions.

Keywords: Plant growth regulators, Murashige and Skoog, 6-Benzylaminopurine, Naphthalene acetic acid, Indole butyric acid, Kinetin

1. Introduction

Saccharum officinarum L (Sugarcane) a perennial herb belonging to the Poaceae family.It is one of the most important sugar crops in the world which accounts for about 70% of the world's total sugar production (Anonymous, 2005). This distinguishing quality place a high value on the species as a commercial crop especially in Nigeria where it has been highly cultivated. *Saccharum officinarum* is a polyploid plant that is grown in different parts of the world. Sugarcane importance has increased over time and in recent years becoming an important industrial raw material for sugar industries and other industries producing alcohol, and animal feed. *Saccharin officinarum* is mainly propagated vegetative by two to three budded sets. This traditional practice results in high production cost and also a large amount of sugarcane involvement in the cultivation process. Considering the importance in the agricultural industry, concerted efforts are being made for its improvement (Desai et al 2004). Tissue culture technique is an alternative method to overcoming these challenges and to produce uniform propagules for the cultivation, (Nickell, 1964, Heinz and Mee 1969). Micro propagation is important for rapid multiplication of elite genotypes/clones and for the quick spread of new varieties (Nickell and Heinz, 1973). Micro propagation is established, not only as means of clonal and rapid propagation it is also more efficient in a viable and successful method for the production of pathogen free planting materials. In recent decades, *in vitro* shoot tip culture for mass propagation of sugarcane has been reported by (Hendre et al, 1983; Lee, 1987; Nand and Singh,1994.). *In vitro* regeneration through leaf sheath culture and plant regeneration through callus culture (Nickell, 1977) has also been reported. The *in vitro* nutrition requirements for *Saccharum officinarum* vary according to genotypes as well as explants used. *In vitro* cultivation method has proven to be an efficient regeneration method for mass propagation of this crop and production of uniform and disease free propagules for

potential and quality production of sugarcane. In order to ensure highest possible yield and quality of field crops, the disease free stock can be extended to the growers and out growers by using this technique (Aamir et al 2008). Present research focuses on the importance of tissue culture technology in *Saccharum officinarum* improvement and establishment of efficient micro propagation protocols for healthy, disease free and premium quality planting materials in Nigeria.

2. Material and Methods

Two varieties, NCS005 and NCS008 were obtained from National Cereal Research Institute (NCRI), Badeggi, Nigeria. The varieties were established on the field gene bank of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan. Apical sections, containing 6-8cm long spindle segment with growth tips and young leaf shealths were collected from the two varieties within 2-4 months old. These collected plants materials were taken into the NACGRAB Biotech lab, where they were washed under running tap water with addition of few drops of household liquid detergents for 20-30 minutes. The apical portions were immersed in 70% Ethanol for 5 min followed by disinfection with 50% v/v (volume per volume) commercial bleach for 20mins. These were further rinsed several times with sterile distilled water. Meristems were kept in 0.001%w/v (weight per volume) citric acid solution for 30 minutes before excision. All surface sterilization was done under sterile conditions. Using the left and right leaf shealths remover technique, the surface sterilized spindle segments were aseptically dissected out into 2 cm long meristems with the help of sterile surgical blades and forceps. The apical meristems were immediately inoculated in MS medium (Murashige and Skoog, 1962) containing 3%(w/v) sucrose, B5 vitamins, 0.01g/L ascorbic acid and 0.7%(w/v) agar. MS basal media was supplemented with different concentrations and combinations of Plant Growth Regulators. The pH of the medium was adjusted to 5.8 and autoclaved at 121^0C and 15 lb for 15 min (Desai et al 2004). Cultures were maintained in the growth room at an incubation temperature of $23^{\circ}C \pm 1^{\circ}C$ with 8 to 16 hour photoperiod.

For axillary shoot proliferation, budding meristems were transferred to MS containing increased cytokinin concentrations. Shoot formation and proliferation was observed after 7 - 20 days of shoot initiation. First sub-culturing was done after three weeks and subsequent sub-culturing after every fourth night. All dead shoots were removed at each sub-culture.

For in-*vitro* rooting, MS medium containing different concentrations and combination of NAA and IBA was used. Successfully rooted *in-vitro* sugarcane plantlets were acclimatized in a humid environment, prior to transfer to outside conditions. During acclimatization, plantlets were protected from high temperature and irradiance.

All data were subjected to analysis of variance (ANOVA) using SAS software package and the means were separated using Duncan Multiple Range Test at 5% level of significance (Gbadamosi et al 2013).

3. Results

3.1 Shoot Formation from Apical Meristem

Enlargement, swelling and browning of the sugarcane explants were observed after 8 day of culture in all the media combinations. After 10 – 15 days of culture auxiliary shoot proliferation occurred from the base of explants, these differ from each media due to the varieties.

3.1.1 Multiplication of Shoot

Generally, best results for shoot multiplication were obtained in semi solid medium. The effect of plant growth regulators (PGRs) revealed differences in growth responses for both varieties (see Tables 1 and 2). During secondary proliferation stage, lateral shoots were formed at the base of newly initiated shoots in both varieties. As a result, a dense mass of shoots (15-20) was developed in each culture bottle. Shoot multiplication was maintained by regular transfer to new medium. This was achieved by sub-dividing shoots in clusters containing 4-5 shoots and transferring them into fresh MS medium augmented with different concentrations and combinations of cytokinins. For NCS 005, BAP in combination with KIN provided better results for shoot formation as compared to the use of BAP alone. Among different concentrations used, 0.5 mg/l of BAP with 0.25 mg/l Kinetin conferred the best shoot formation response (Table 1). For NCS 008, the best results for shoot formation (4.0 ±0.0) was observed on BAP at concentration 1.5mg/L, with this being in proximity with shoot formation (3.6 ±0.33)observed on 2.0mg/L BAP. However, for NCS 008, there was no significant difference ($P \geq 0.05$) observed between results obtained on MS +1.5mg/L and MS +1.5mg/L BAP +0.5mg/L Kin (Table 1).

3.1.2 Rooting of Regenerated Shoots

Root initiation in the successfully established shoots was achieved on MS medium supplemented with different auxin concentrations. The effect of different plant growth regulators on root length in *Saccharum officinarum* was significant ($P \leq 0.05$) (Table2). The best root induction for *in vitro* derived shoots was obtained on 1.0 mg/l NAA and 2.0 mg/l IBA for both varieties of sugarcane within ten days of culture transfer. An average shoot number of 6.0 ±0.57 and 10.0 ±1.67 were produced for NCS 005 and NCS008 respectively (see Table 2). The lowest mean value of 2.00 ± 0.00 cm was obtained in MS media that had 1.00 mg/l NAA and 0.50 mg/l IBA in case of NCS 005 while for NCS 008 MS media that had 0.50 mg/l NAA (Table 2).

Table1. Effect of different plant growth regulators and their concentration on establishment of shoot Variety, NCS 005

Media	Concentration mg/l	Number of explants	Shoot number/explants	Shoot length(cm)
MS+BAP	0.5	20	1.00 ± 0.00^{e}	1.32 ± 0.01^{d}
MS+BAP	1.0	20	1.33 ± 0.33^{de}	1.27 ± 0.10^{d}
MS+BAP	1.5	20	1.67 ± 0.33^{de}	1.72 ± 0.05^{d}
MS+BAP	2.0	20	2.00 ± 0.00^{cd}	1.81 ± 0.10^{d}
MS+BAP	2.5	20	2.00 ± 0.00^{cd}	1.56 ± 0.23^{d}
MS+BAP+KIN	0.5+0.25	20	5.00 ± 0.00^{a}	4.56 ± 0.37^{c}

MS+BAP+KIN	1.0+0.25	20	3.00±0.00^{b}	5.20±0.15^{c}
MS+BAP+KIN	1.5+0.50	20	2.66±0.33^{b}	6.09±0.21^{b}
MS+BAP+KIN	2.0+0.50	20	2.66±0.66^{bc}	6.66±0.33^{b}
MS+BAP+KIN	2.5+0.50	20	2.43±0.21^{bc}	7.70±0.60^{a}

Variety, NCS 008.

Media	Concentration mg/l	Number of explants	Shoot no/explants	Shoot length (cm)
MS+BAP	0.5	20	2.00±0.00^{c}	3.25±0.00^{ab}
MS+BAP	1.0	20	2.33±0.33^{c}	3.90±0.00^{ab}
MS+BAP	1.5	20	4.00±0.00^{a}	4.60±0.06^{a}
MS+BAP	2.0	20	3.60±0.33^{a}	2.25±0.00^{b}
MS+BAP	2.5	20	2.66±0.33^{bc}	4.50±1.25^{a}
MS+BAP+KIN	0.5+0.25	20	2.00±0.00^{c}	3.60±0.35^{ab}
MS+BAP+KIN	1.0+0.25	20	2.33±0.33^{c}	4.00±0.45^{ab}
MS+BAP+KIN	1.5+0.50	20	4.00±0.00^{a}	4.70±0.05^{a}
MS+BAP+KIN	2.0+0.50	20	3.33±0.33^{ab}	3.80±0.11^{ab}
MS+BAP+KIN	2.5+0.50	20	2.30±0.33^{c}	4.50±1.23^{a}

Values (mean ± standard deviation) in a column followed by same letter are not significantly different ($P \geq 0.05$)

Table2. Effect of different auxin and their concentrations on root initiation variety NCS 005.

Media	Concentration mg/l	Number of explants	Root number/explants	Root length (cm)
MS+NAA	0.5	20	2.33±0.33^{de}	1.10±0.01^{def}
MS+NAA	1.0	20	2.00±000^{e}	1.76±0.56^{cd}
MS+NAA	1.5	20	3.00±0.00^{d}	0.70±0.00^{f}
MS+NAA	2.0	20	2.66±0.33^{de}	1.23±0.07^{def}
MS+IBA	0.5	20	2.00±0.00^{e}	0.80±0.00^{f}
MS+IBA	1.0	20	2.00±0.00^{e}	0.90±0.20^{ef}
MS+IBA	1.5	20	3.00±0.00^{d}	1.10±0.00^{def}
MS+IBA	2.0	20	2.33±0.33^{de}	1.60±0.40^{cde}
MS+NAA+IBA	1.0+1.0	20	4.00±0.00^{c}	2.10±0.00^{bc}
MS+NAA+IBA	1.0+2.0	20	6.00±0.57^{a}	3.50±0.23^{a}
MS+NAA+IBA	2.0+1.0	20	5.00±0.00^{b}	3.90±0.00^{a}
MS+NAA+IBA	2.0+2.0	20	5.00±0.00^{b}	2.60±0.10^{b}

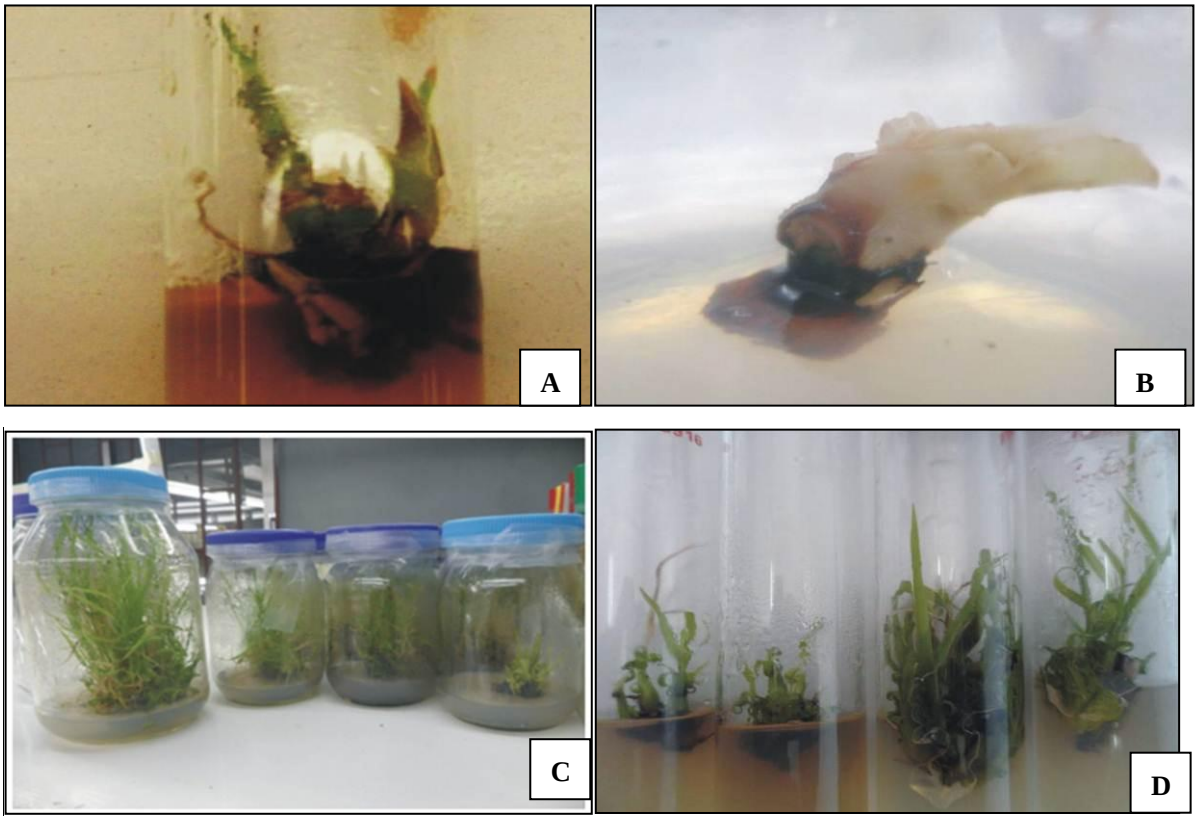
Variety NCS 008

Media	Concentration mg/l	Number of explants	Root number/explants	Root length (cm)
MS+NAA	0.5	20	2.33±0.33^{d}	0.70±0.00^{f}
MS+NAA	1.0	20	2.33±0.33^{d}	2.30±0.23^{cd}

MS+NAA	1.5	20	3.00 ± 0.00^{cd}	2.30 ± 0.00^{cd}
MS+NAA	2.0	20	4.30 ± 0.66^{bcd}	0.80 ± 0.00^{f}
MS+IBA	0.5	20	4.00 ± 0.00^{bcd}	0.80 ± 0.00^{f}
MS+IBA	1.0	20	5.00 ± 0.00^{bc}	2.00 ± 0.00^{d}
MS+IBA	1.5	20	5.00 ± 0.00^{bc}	2.40 ± 0.00^{cd}
MS+IBA	2.0	20	4.00 ± 1.00^{bcd}	2.26 ± 0.23^{d}
MS+NAA+IBA	1.0+1.0	20	6.00 ± 0.00^{b}	2.90 ± 0.00^{b}
MS+NAA+IBA	1.0+2.0	20	10.00 ± 1.67^{a}	3.50 ± 0.30^{a}
MS+NAA+IBA	2.0+1.0	20	$5.00 \pm 0.00b^{c}$	1.50 ± 0.00^{e}
MS+NAA+IBA	2.0+2.0	20	5.66 ± 0.66^{b}	2.73 ± 0.17^{bc}

Values (mean ± standard deviation) in a column followed by same letter are not significantly different ($P \geq 0.05$)

Figures 1. Photograph showing the tissue cultured explants at different steps of the micropropagation



Key of Photograph showing the tissue cultured explants at different steps of the micropropagation

A. Apical meristem cultured in regeneration medium

B. Shoot induction of variety NCS 008 with auxiliary shoot after 10 days

C. Sub cultured sugarcane variety NCS 005 shoots after 2 weeks

D. Sugarcane in culture bottles

E. Sugarcane in the screen house.

3.1.3 Hardening and Acclimatization of Plants of in Vitro Raised Plants

The in-vitro plants were hardened and acclimatized in medium containing a mixture of in River Sand, Topsoil, coconut fibers (1:2:2) after three weeks of transplanting in screen house. After 21 days the potted plant were nurtured in poly pots placed under sunlight for further hardening of the plantlets. These seedlings were transfer to the field after another two weeks and a high rate of seedlings were transferred successfully. Hardening was carried out in screen house under natural light conditions.

4. Discussion and Conclusion

In graminaceous species, plant regeneration has been facilitated by the identification of appropriate explant and *in vitro* culture conditions (Vasil, 1987, Gbadamosi et al 2013). 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, Aamir Ali et al 2008). In this study, different concentration and combination of auxin and cytokinins in MS Medium was successfully utilized for shoot organogenesis and multiple shoot regeneration from apical meristems of sugarcane. This concurs with report by Yutaka *et al* 1998, that combination of phytohormones often determines the course of morphogenesis (shoot organogenesis or embryogenesis). This study also reconfirms the new phase for plant multiplication for the production of efficient, genetically stable clonal germplasm and pathogen free stock (Hussey, (1983); and Kartha (1986). The best shoot formation and proliferation for both varieties was observed on BAP in combination with KIN. Many authors including Shukla, 1994; Geeta,

2001; Dhillon, et al 2002 and Aamil Ali et al 2008 have reported the use of kinetin in combination with BAP for shoot formation in sugarcane

When auxins were used singly, an average of two to three roots was observed in NCS005. Conversely, NCS008 showed a more roubust root formation on MS fortified with IBA only, as compared to MS fortified with NAA. This indicates that IBA is a more preferred auxin regarding effeiciency and yield for invitro root initiation in the NCS008 variety.

A combination of NAA and IBA was however observed to yield the best results for root formation in both varieties. (Anbalagan *et al.,* 2000 and Nadgauda 2002) had earlier reported that a combination of two auxins (NAA and IBA) promotes rooting in sugarcane, also favoured the combination of NAA and IBA promote for rooting. Also the combination of IBA and NAA best for rooting, and this favoured both when he combined NAA and IBA.

Several authors including Yi et al,(2004), Pruski et al.,(2005) and Aamil Ali et al.,(2008) has also reported optimum results in the combination of NAA and IBA for rooting in *Phragmites communis* and two different varieties of sugarcane.

Following Sreenivasan and Sreenivasan (1992) which observed 90-95% survival of plantlets in poly screen house under shade, the simple method of hardening, adopted in this study facilitated the successful transfer of $90^0/_0$ of *Saccharum officinarum* plants from *in vitro* to *ex vitro* conditions.

This study indicates and strongly support the statement by (Aamil Ali et al 2008) that micro propagation 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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