RESEARCH PAPER

In vitro response of sugarcane buds by the application of various sterilants

Ahmad Ali[1]*, Aysha Saddiqa[1], Sayed Tariq Shah[1] and Huma Fatima[2]

[1]Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan
[2]Department of Biology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

***Corresponding author's email: ahmadalirp@gmail.com

Received: 5 September 2021
Accepted: 22 December 2021

Key Message: In this research study, application of $HgCl_2$ at 0.1% concentration resulted in the lowest contamination frequency and highest survival rate of explants. Furthermore, the use of MS basal media supplemented with 1.0 mg/l BAP proved to be effective for *in vitro* shoot regeneration in Cp77flash400 variety of sugarcane.

Abstract

The high production costs in Pakistan make the country less competitive in sugar markets, both locally and globally, due to factors such as poor performance and lower sugar recovery. Numerous factors contribute to the low yield, with one notable issue being the absence of a rapid method for multiplying seeds. Furthermore, the identification of a desired clone typically requires six to seven years to produce enough better seed. This extended duration creates a bottleneck in crucial breeding programs. Now-a-days, the method of plant tissue culture has grown to be an effective tool for research, resolving both fundamental and applied issues in plant biotechnology. Therefore, the current study was carried out at Nuclear Institute for Food & Agriculture (NIFA), Tarnab, Peshawar. The apical buds from 6-8-months old, in good health, disease free Cp77flash400 variety seed canes were used as explants sources. The results revealed that minimum contamination frequency (60%) and the highest survival rate of (40%) of explants was recorded by the application of $HgCl_2$, 0.1% for minutes. During this study various concentration of BAP supplemented with MS basal media were also assessed on *in vitro* shoot regeneration frequency, days taken to initiate the shoot induction responses and the total number of initial shoots each explants. The highest regeneration frequency (86.45%), minimum days (12.8) spent to initiate shoot. On MS basal media supplemented with 1.0 mg/l BAP, the greatest number of primordial shoots per explants (9.5) was observed. The current study has led to the conclusion that every new variety that wants to achieve quick shoot initiation, shoot multiplication, and shoot elongation needs an effective protocol. MS basal media enriched with 1.0 mg/l was proved to be ideal for *in vitro* shoot regenerating in sugar cane variety Cp77flash400.

Keywords: BAP, Explant, *In vitro*, MS basal media, Sugarcane, Tissue culture

Citation: Ali, A., Saddiqa, A., Shah, S. T., & Fatima, H. (2021). *In vitro* response of sugarcane buds by the application of various sterilants. *Advances in Agriculture and Biology*, *4*(1), 34-40.

Introduction

Agriculture is a key sector in Pakistan's economy, directly sustaining the nation's population and contributing significantly to its GDP. The rising population exerts pressure on the agricultural sector to enhance production, aligning with the increased demand. In the context of cash crops, sugarcane holds particular importance in Pakistan. As a C4 crop primarily flourishing in tropical and sub-tropical regions, sugarcane assumes a crucial role as a global source of bioenergy and sugar. The perennial cultivation of sugarcane spans 20 million hectares, yielding approximately 1,325 million tons annually. This substantial output serves diverse purposes, including sugar, energy and chemicals (Saunders et al., 2009). Sugarcane, a key contributor to world food production, furnishes around 75% of the global sugar supply for human consumption (Uzma et al., 2012; Abbas et al., 2013a; Abbas et al., 2013b; Dehlavi et al., 2015). In the specific context of Pakistan's agricultural landscape, the cultivation of sugarcane during the 2015–2016 period encompassed 1,132 thousand hectares, slightly reduced from the previous year's 1,141 thousand hectares. This cultivation yielded 65,475 thousand tonnes (Muslehuddin & Faisal, 2006).

Various nations are recognized as prominent sugarcane producers, with Colombia leading at 123.0 tons ha^{-1}, afterward 99.3, 87.3, and 74.6 tons ha^{-1} produced in Australia, Egypt, and the USA, in that order (Devaraja, 2008). Pakistan contributes approximately 43 tons ha^{-1}, a comparatively smaller output in contrast with other important sugarcane-producing nations. The areas of Sindh, Punjab, and Khyber Pakhtunkhwa (KPK) in Pakistan are the main centers for sugarcane cultivation, with Punjab holding the top position in sugarcane production (Rehman et al., 2012). Sugarcane serves not only as a revenue source for sugar manufacturers but also for individuals in Pakistan engaged in reselling sugars and sugar products. Approximately 64% of Sindh's total agricultural output is derived from sugarcane (Haq et al., 2013). There are 91 sugar mills in Punjab, Sindh has 48, and KPK has nine, collectively producing around 3.2 million tons of sugar to fulfill Pakistan's consumer demand. However, a clear disparity exists among the genuine sugarcane production and the national demand. Factors contributing to this disparity encompass suboptimal farming practices, environmental conditions, and various challenges, including the absence of government and other stakeholder support (Rehman et al., 2015).

Sugarcane shoots originating from sugarcane seedlings exhibit a meager germination rate of approximately 30-35%, consequently resulting in reduced sugarcane production and contributing almost 25% of operating expenses (Patel & Patel, 2014). To address this challenge, concerted efforts are essential to devise techniques aimed at enhancing both the sugarcane yield as a whole and the growing proportion. One essential factor influencing the developments of sugarcane cutting and the early seedlings to grow is the presence of growth regulators and stored food (Yulianingtyas et al., 2015). Plant growth regulators, commonly referred to as growth hormones, play a significant role in improving relationships between source and sink. They promote translocation of adaptation, facilitating the development of seedlings and finally leading to enhanced plant growth (Hayat et al., 2012).

Sucrosin, a biostimulant utilized in planting material, is a product of the research conducted by the Bogor institute for Biotechnology and Bioindustry Research. This inducer includes macro- and micronutrients, activators, organic acids, vitamins, and antioxidants, as well as growth hormones like auxins, cytokinins, and gibberellins. When applied to sugarcane, sucrosin has been shown to enhance plant metabolic processes, leading to increased biomass and sugar content in the cane (Calvo et al., 2014). In commercial sugarcane cultivation, which relies on vegetative propagation, a substantial quantity of seeds is required. The quality of planting material significantly influences yields, with healthy seed cane being crucial for enhancing sugarcane crop productivity (Tiwari et al., 2012). However, the absence of adherence to the scientific seed production system consisting of first stage is cultivator seed; second stage is foundation seed; and third stage is approved seed production poses a challenge for sugarcane farmers, resulting in a shortage of high-quality seed material for planting (Ali et al., 2008; Rithesh, 2013). Since sugarcane is vegetatively propagated, it is susceptible to the accumulation of pathogens. Consequently, pathogens that cause disease are added to the fresh region along with seed canes. The gradual buildup of different pathogens over time can transform minor diseases into major epidemics (Ruchika & Sushma, 2015). Historical instances of epidemics caused by wilt, red rot, ratoon stunting, smut, yellow leaf, grassy shoot and leaf scald highlight the significant role played by disease-infected seed canes in their emergence and subsequent spread (Kumar & Saxena, 2008).

In contemporary times, tissue culture of plants has emerged as a potent method for investigating and addressing fundamental and practical concerns in plant biotechnology (Yadav et al., 2012). Over the past three decades, micropropagation and *in vitro* techniques have gained increased prominence in market farming and gardening. *In vitro* multiplication of sugarcane has been a focal point of research due to its commercial value as an income generator crop (Khan et al., 2004). The regeneration of plants via tissue culture method presents a promising approach for raising the quality and yield of sugarcane. While numerous reports on the tissue culture of sugarcane have been published globally (Dibax et al., 2011; Nawaz et al., 2013; Takahshi & Takamizo, 2013). Naz (2003) carried out research to regenerate plants using *in vitro* methods. Several authors have contributed to standardizing procedures for sugarcane *in vitro* multiplication using callus, axillary bud, and shoot tip cultures (Beard et al., 1978; Nadar et al., 1978; Bhansali & Singh, 1984; Nagai, 1998; Anita et al., 2000). The conventional approach yields a multiplication rate of 1-10 throughout a year (Gosal et al., 1998). Gosal et al. (1998) observed fast growth in liquid medium containing kinetin (0.5 mg/L) and BAP (0.5 mg/L), along with sucrose (7.0%) and NAA rooting (5 mg/L). A protocol for sugarcane micropropagation on MS medium (Murashige & Skoog, 1962), enriched with NAA, IBA and BAP, kept at

23 °C with constant lighting was developed by Jadhav et al. (2001). Their technique showed potential for a high-frequency, efficient method that would enable easy microplant division through *in vitro* shoot tip culture. This research study was conducted to evaluate the sugarcane variety Cp77flash400's *in vitro* regeneration ability because of the significance of sugarcane multiplication for the world's expanding population. The evaluation focused on the impact of various sterilants on contamination and the survival rate of buds.

Materials and Methods

Collection of germplasm

Plant material was collected from Nuclear Institute for Food & Agriculture, Tarnab, Tarnab farm, Peshawar. The samples were obtained from disease-free seed canes of the Cp77flash400 variety, which were 6-8 months old and in a healthy condition. Apical buds with 0.5 to 1 cm size were taken as explants.

Media preparation

The basic medium MS medium (Murashige & Skoog, 1962) was used for sugarcane culture. Before adding distilled water to reach the final volume, auxins were dissolved in 1N KOH and cytokinins in 1N HCl. A bottle with an amber shade was used to store an iron EDTA stock solution. The medium was made by mixing suitable amounts of stock solutions and the pH was adjusted to 5.6-5.8 using $NaOCl_2$, $CaOCl_2$, and $HgCl_2$. For culture initiation/establishment, agar at 6-7 g/l and sucrose at 30 g/l were used for solidifying medium. The media were filled into test tubes (up to 20 ml) or culture bottles (15 - 20 ml for liquid culture) that had been cleaned, dried, and appropriately labelled before being autoclaved at 121°C for 15 minutes at 15 PSI pressure. After that, they were moved to the inoculation room where they were kept aseptic as long as needed. In this study, various sterilizing agents were employed as disinfectants to reduce contamination frequency and increase the survival rate of explants in the sugarcane variety Cp77flash400. Following this experiment, different concentrations of the plant growth regulator (BAP) supplemented with MS basal media were examined for *in vitro* regeneration of shoots. Parameters like the number of primordial shoots per explant and the duration of the *in vitro* regeneration of shoots stimulation were investigated in the sugarcane variety Cp77flash400.

Results

Assessment of various sterilants on contamination frequency and survival rate of buds

During this study, the various sterilant namely mercuric chloride, sodium hypochlorite and calcium hypochlorite were assessed on contamination frequency and survival rate of explants in sugarcane variety Cp77flash400. Different concentrations of these sterilants were applied for different time periods (Table 1). Data showed that the minimum contamination frequency (60%) was recorded by the application of $HgCl_2$ 0.1% for 2 minutes. It was followed by $CaOCl_2$ 4% by for 2 minutes which gave 74% contamination frequency in Cp77flash400. On the other hand, the highest contamination frequency (80%) was recorded when the explants were sterilized by $NaOCl_2$, 2% for 5 minutes. Likewise, the rate of survival of the explants was also noted after the sterilization of these explants with various sterilizing agents (Table 1). The highest survival rate (40%) of explants was recorded by the application of $HgCl_2$, 0.1% for 2 minutes. It was followed by $CaOCl_2$, 4% for 2 minutes which yielded 26% survival rate of explants (Table 1).

Assessment of cytokinin (BAP) supplemented with MS basal media on *in vitro* shoot regeneration frequency

All the concentration of BAP was measured in mg/l and supplemented with MS basal media (Murashige & Skoog, 1962). Data was taken *in vitro* shoot regeneration frequency in sugar cane variety Cp77flash400. Data indicated the average of three replicates. Bold figure indicated the best results. The current study indicated that cleanly inoculated sugarcane buds in BAP showed concurrent shoot induction and shoot multiplication at the equivalent concentration. Specifically, at 1.0 mg/l of BAP, the multiplication rate reached as high as 86.45% (Table 2). It was followed by the *in vitro* shoot regeneration frequency (80.93%) at 1.5 mg/l concentration of BAP in Cp77flash400 variety, the *in vitro* shoot regeneration frequency was lowered when the BAP concentration was gradually increased up to 4.0 mg/l. Hence it was found in this study that BAP at1.0 mg/l was the paramount level for the maximum *in vitro* shoot regeneration frequency in sugarcane variety Cp77flash400 (Table 2).

Assessment of cytokinin (BAP) on number of days for *in vitro* shoot regeneration

All the concentration of BAP was measured in mg/l and supplemented with MS basal media (Murashige & Skoog, 1962). Data was taken about the number of days for shoot regeneration induction in sugarcane variety Cp77flash400. Data indicated the average of three replicates. Bold figure indicated the best results. The results regarding the days needed for shoot induction indicated that different concentrations of BAP had an impact on the shoot formation of the sugarcane variety (Table 3). The regeneration study demonstrated that the type and concentration of growth regulator used had a major impact on shoot growth. Within different concentrations, the most ideal efficiency for the days necessary for shoot initiation was observed at 1.0 mg/l BAP in the sugarcane variety Cp77flash400, with an average of 12.8 days taken for shoot induction. In term of concentration, MS medium enriched with 1.0 mg/l of BAP was discovered to be most effective for the sugarcane variety Cp77flash400 at higher level of cytokinin (BAP) the days taken to initiate *in vitro* shoot regeneration response of this variety was gradually increased, hence it was confirmed from this study that BAP (1.0 mg/l) was optimum level for quick response of *in vitro* shoot regeneration in sugarcane variety Cp77flash400.

Assessment of cytokinin (BAP) on number of primordial shoots per explants

All the concentration of BAP was measured in mg/l and supplemented with MS basal media (Murashige & Skoog, 1962). Data was taken in about number of primordial shoots per explants in sugar cane variety Cp77flash400. Data indicated the average of three replicates. Bold figure indicated the best results. The impact of different concentration of BAP on development of primordial shoots per explants, demonstrated that on an average, the highest number of shoot primordial per explants (9.5) was indicated I variety Cp77flash400 (Table 4) at 1.0 mg/l BAP, while the minimum number of primordial shoots per explant (2.13) was noticed in Cp77flash400 at 4.0 mg/l BAP. Among the media concentration the highest shoot regeneration ability was found at 1.00 mg/l BAP for Cp77flash400.The number of primordial shoots per explants was gradually decrease on gradual increase of BAP. Therefore, the efficient number of primordial shoots per explants was recorded at BAP (1.0 mg/l) fortified with MS basal media. However, when grown on MS medium supplemented with 1.0 mg/l BAP, the best development of shoots was obtained and on gradual increase of cytokinin concentration, shoot induction response was decreased.

Table 1 Assessment of various sterilant on contamination frequency and survival rate of buds as explants sources in sugarcane

Sterilant with strength sterilizing time	Number of explants cultured	Number of explants contaminated	Number of explants survived	Contamination frequency (%)	Survival rate (%)
$HgCl_2$, 0.1 % for 2 minutes	50	30	20	60	40
$HgCl_2$, 0.1 % for 5 minutes	50	42	08	84	16
$NaOCl_2$, 2% for 2 minutes	50	40	10	80	20
$NaOCl_2$, 2 % for 5 minutes	50	44	06	88	12
$CaOCl_2$, 4 % for 2 minutes	50	37	13	74	26
$CaOCl_2$, 0.1 % for 5 minutes	50	41	09	82	18

$HgCl_2$: Mercuric chloride; $NaOCl_2$: Sodium hypochlorite, $CaOCl_2$: Calcium hypochlorite

Table 2 Assessment of various concentrations of BAP on *in vitro* shoot regeneration frequency in sugarcane variety Cp77flash400

S. No.	Concentration of BAP (mg/l) frequency (%)	*In vitro* shoot regeneration
1	**1.0**	**86.45**
2	1.5	80.93
3	2.0	74.55
4	2.5	72.10
5	3.0	66.69
6	3.5	65.25
7	4.0	42.13

Table 3 Assessment of various concentrations of BAP on the number of days taken for *in vitro* shoot regeneration in sugarcane variety Cp77flash400

S. No.	Concentration of BAP (mg/l) regeneration	Number of days taken for *in vitro* shoot
1	**1.0**	**12.80**
2	1.5	13.90
3	2.0	14.50
4	2.5	16.10
5	3.0	17.60
6	3.5	18.25
7	4.0	20.13

Table 4 Assessment of various concentrations of BAP on on number of primordial shoots per explants in sugarcane variety Cp77flash400

S. No.	Concentration of BAP (mg/l)	Number of primordial shoots
1	**1.0**	**09.50**
2	1.5	07.30
3	2.0	05.50
4	2.5	05.10
5	3.0	04.50
6	3.5	03.25
7	4.0	02.13

Discussion

Plant tissue culture is a comprehensive term encompassing plant protoplasts, cells of plant, plant tissues, plant organs, and plant cultures. These diverse culture types share a common feature namely the cultivation of plant materials free of microbes in a sterile condition, typically using nutrient media that has been sterilized and placed in test tubes. The scope of plant tissue culture research is broad, with direct applications in commerce and relevance to fundamental investigations in cell biology, biochemistry, and genetics. Techniques employed in this field include the culture of anthers, cells, embryos, and ovules on a spectrum ranging from experimental to industrial scales. Additionally, methods such as cell selection, protoplast isolation and fusion, and bud and meristem culture are integral components.

Contrary to its initial role as a tool for fast proliferation, tissue culture has evolved into an alternative method for breeding. This involves the selection and testing of advantageous variants (Cassells & Doyle, 2005). The findings of the current study reported that the MS medium enriched with 1.0 mg/l BAP demonstrated notably sufficient (86.45%) regeneration ability in the sugarcane variety Cp77flsh400. Furthermore, it was observed that the MS medium supplemented with 1.0 mg/l BAP yielded the most favorable outcomes for the number of primordial shoots per explant in the Cp77flsah400 variety. With a rise in BAP concentration, there was a decrease in the rate of shoot proliferation and a longer time required for shoot formation. The Cpflash400 variety of sugarcane exhibited the highest number of shootlets (9.5 per explant). The findings of our investigation revealed that the concentration of auxins and cytokinins influenced shoot regeneration, consistent with the findings reported by Akin-Idowu et al. (2009). The duration required for shoot induction was also influenced by varying concentrations of BAP in the sugarcane variety under scrutiny. Throughout the study, the process of shoot development was significantly impacted by the concentration growth hormone employed in the experiment. Among the diverse concentrations tested, the most favorable achievements, with duration of 12.8 days required for shoot induction, was observed.

Concerning concentration, the optimal medium for sugarcane Cp77flah400 was determined to be BAP at a dose of 1.0 mg/l added to MS medium, resulting in the shortest time for shoot induction. Similarly, the most favorable adaptation in terms of formation of multiple shoots occurred in MS medium supplemented with 1.0 mg/l BAP, which proved to be effective in bud production. Comparable results were reported by Hesami et al. (2018) and Garcia-Gonzales et al. (2010). The study found that variety Cp77flash400 showed the highest average number of shootlets per explant (9.5 shootlets). Within the range of media concentrations, the most significant ability for shoot regeneration (9.5) was observed at 1.0 mg/l BAP for Cp77flash400. Conversely, the highest number of shootlets in each explant (2.13) was observed at 4.0 mg/l BAP in Cp77flash400, which proved to be less effective in generating shootlets.

Our findings align with those of Ali et al. (2008) who conducted *in vitro* shoot regeneration in sugarcane. They concluded that MS medium enriched with 1.0 mg/l BAP improved *in vitro* shoot proliferation in sugarcane. Additionally, our research group concurred with Ali et al. (2008) in noting that BAP concentrations exceeding 1.0 mg/l resulted in a decrease in shootlet induction in sugarcane. Our findings indicate that the Cp77flash400 sugarcane variety is preferable, exhibiting the highest frequency of *in vitro* shoot regeneration, the shortest duration for initiating shoot induction, and the maximum number of primordial shoots per explant. Across all concentrations of cytokinin, rapid growth was induced with the 1.0 mg/l BAP concentration yielding the most efficient response in *in vitro* shoot regeneration. This leads to the conclusion that phytohormones fundamentally impact cell division, differentiation, and elongation thus influencing the total growth of shoot formation. Corresponding results were documented by Iqbal et al. (2017); Hesami et al. (2018). George (1993) highlighted that young tissue contains higher levels of cytokinin compared to older tissue. The variable quantity of cytokinin likely plays a crucial role in adjusting cytokinin levels in the media to facilitate shoot development. Another factor affecting shoot formation is the concentration of phenolic compounds released by explants, which can limit the availability of nutrient. This aligns with the challenges mentioned by George (1993) in the context of shoot development.

Conclusion

Based on the current research, it is determined that the establishment of an effective protocol is essential for each novel variety or clone to achieve rapid multiplication elongation and initiation of shoots. The most favorable conditions for *in vitro* shoot regeneration and elongation in sugarcane were identified to be the use of MS basal media supplemented with 1.0 mg/l of BAP.

References

Abbas, S. R., Gardazi, S. D. A., Iqbal, M. Z., Khan, M. Y., Batool, A., Abbas, M. R., Shazad, S., Khan, A. M., & Shah, S. H. (2013b). Characterization of twenty-six genotypes of sugarcane using SSR markers. *International Journal of Scientific & Engineering Research*, *4*(7), 1002-1007.

Abbas, S. R., Gardazi, S. D. A., Sabir, S. M., Aziz, W., Batool, A., Abbas, M. R., & Shah, S. H. (2013a). Screening of drought-tolerant genotypes of sugarcane through biochemical markers against polyethylene glycol. *International Journal of Scientific and Engineering Research*, *4*(7), 980-988.

Akin-Idowu, P. E., Ibitoye, D. O., & Ademoyegun, O. T. (2009). Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology*, *8*, 3782-3788.

Ali, A., Naz, S., Siddiqui, F. A., & Iqbal, J. (2008). An efficient protocol for large scale production of sugarcane through micropropagation. *Pakistan Journal of Botany*, *40*(1), 139.

Anita, P., Jan, R. K., Schrawat, A. R., & Punia, A. (2000). Efficient and cost-effective micropropagation of two early maturing varieties of sugarcane (Saccharum spp.). *Indian Sugar*, *50*, 611-618.

Beard, J. B., Rieke, P. E., Turgeon, A. J., & Vargas, J. M. (1978). Annual bluegrass (*Poa annua* L.) description, adaptation, culture, and control. Research Report 352. Michigan State University, East Lansing, MI.

Bhansali, R. R., & Singh, K. (1984). Callus and shoot formation from leaf of sugarcane in tissue culture. Phytomorphology, pp: 167-170.

Calvo, P., Nelson, L., & Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant and Soil*, *383*(1), 3-41.

Cassells, A. C., & Doyle, B. M. (2005). Plant Tissue Culture: Current Status and Opportunities. In: Loyola-Vargas VM, Vázquez-Flota F, editors. Plant Cell Culture Protocols. Humana Press. New York, USA: 3550.

Dehlavi, A., Groom, B., & Gorst, A. (2015). Climate change adaptation in the Indus ecoregion: A microeconometric study of the determinants, impacts, and cost effectiveness of adaptation strategies. Islamabad: World Wide Fund for Nature (WWF) Pakistan.

Devaraja, T. S. (2008). Cost of production of sugar from sugarcane in Karnataka-a comparative analysis approach. *Cooperative Sugar*, *39*(6), 15.

Dibax, R., de Alcântara, G. B., Filho, J. C. B., Machado, M. P., de Oliveira, Y., & da Silva, A. L. L. (2011). Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. *Journal of Biotechnology and Biodiversity*, *2*(3), 32-37.

Garcia-Gonzales, R., Quiroz, K., Carrasco, B., & Caligari, P. (2010). Plant tissue culture: Current status and opportunities. *Ciencia e Investigación Agraria*, *37*(3), 353-360. doi: 10.7764/rcia.v37i3.530

George, E. F. (1993). Plant Propagation by Tissue Culture (2nd ed.). Exegetics Ltd., Edington, England.

Gosal, S. S., Thind, K. S., & Dhaliwal, H. S. (1998). Micropropagation of sugarcane—an efficient protocol for commercial plant production. *Crop Improvement*, *25*, 1-2.

Haq, A., Aslam, A., Chaudhry, A. A., Naseer, A., Muhammad, K., Mushtaq, K., & Farooqi, M. S. (2013). Who is the 'arthi': Understanding the commission agent's role in the agriculture supply chain. International Growth Centre (IGC) Working Paper.

Hayat, R., Ahmed, I., & Sheirdil, R. A. (2012). An Overview of Plant Growth Promoting Rhizobacteria (PGPR) for Sustainable Agriculture. In M. Ashraf, M. Öztürk, M. S. A. Ahmad, & A. Aksoy (Eds.), Crop Production for Agricultural Improvement (pp. 557-579). Springer Netherlands. https://doi.org/10.1007/978-94-007-4116-4_22

Hesami, M., Daneshvar, M. H., Yoosefzadeh-Najafabadi, M., & Alizadeh, M. (2018). Effect of plant growth regulators on indirect shoot organogenesis of *Ficus religiosa* through seedling-derived petiole segments. *Journal of Genetic Engineering and Biotechnology*, *16*(1), 175–180.

Iqbal, N., Khan, N. A., Ferrante, A., Trivellini, A., Francini, A., & Khan, M. I. R. (2017). Ethylene role in plant growth, development and senescence: Interaction with other phytohormones. *Frontiers in Plant Science*, *8*, 475. doi: 10.3389/fpls.2017.00475

Jadhav, A. B., Vaidya, E., Aher, V. B., & Pawar, A. M. (2001). *In vitro* multiplication of 'Co–86032' sugarcane (*Saccharum officinarum*) hybrid. *The Indian Journal of Agricultural Science*, *71*, 113–115.

Khan, I. A., Khatri, A., Raza, S., Nizamani, G. S., Siddiqui, M. A., Seema, N., Dahot, M. U., & Naqvi, M. H. (2004). Effect of different phytohormones on sugarcane (Saccharum Spp.) regeneration. *Pakistan Journal of Biotechnology*, *1*(2), 17–22.

Kumar, J., & Saxena, S. C. (2008). Seed health management for better productivity. Proceedings of the 20th Training, Centre for advanced training in plant pathology, ICAR, New Delhi.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth. *In Vitro Cellular & Developmental Biology - Plant*, *18*(5), 249-271.

Muslehuddin, M., & Faisal, N. (2006). Long range forecast of Sindh monsoon. *Pakistan Journal of Meteorology*, *3*(5), 35-44.

Nadar, H. M., Soepraptop, S., Heinz, D. J., & Ldd, S. L. (1978). Fine structure of sugar cane (Saccharum Spp.) callus and the role of auxin in embryogenesis. *Crop Science*, *18*, 210-216.

Nagai, C. (1988). Micropropagation of sugar cane. Laboratory methodology: Annual Report by Experimental station, Hawaiian Sugar Planters' Association, A34-A37.

Nawaz, M., Ullah, I., Iqbal, N., Iqbal, M. Z., & Javed, M. A. (2013). Improving *in vitro* leaf disk regeneration system of sugarcane (*Saccharum officinarum* L.) with concurrent

shoot/root induction from somatic embryos. *Turkish Journal of Biology*, *37*, 726-732. doi: 10.3906/biy-1301-16

Naz, S. (2003). Micropropagation of promising varieties of sugarcane and their acclimatization response. Activities on Sugar Crops in Pakistan: Proceedings of the Fourth Workshop on Research & Development, 1-9.

Patel, D., & Patel, R. (2014). Influence of sett size, seed rate and sett treatment on yield and quality of sugarcane. *The Bioscan*, *9*(1), 55-57.

Rehman, A., Jingdong, L., Shahzad, B., Chandio, A. A., Hussain, I., Nabi, G., & Iqbal, M. S. (2015). Economic perspectives of major field crops of Pakistan: An empirical study. *Pacific Science Review B: Humanities and Social Sciences*, *1*(3), 145-158.

Rehman, Z. U., Wassan, I. A., & Dost, M. K. B. (2012). Socio-economic problems of farmers in Pakistan: Focused area Tauluka Pano Akil District Sukkur. *Kuwait Chapter of Arabian Journal of Business and Management Review*, *1*(5), 114-129.

Rithesh, S. (2013). Comparative economics of sugarcane processed for sugar jaggery. M. Sc. (Agri.) Thesis.

Ruchika, S., & Sushma, T. (2015). A review on red rot: the "Cancer" of sugarcane. *Journal of Plant Pathology and Microbiology*, S:1, doi: 10.4172/2157-7471.S1-003

Saunders, M., Lewis, P., & Thornhill, A. (2009). Research Methods for Business Students. Pearson Education.

Takahshi, W., & Takamizo, T. (2013). Plant regeneration from embryogenic calli of the wild sugarcane (*Saccharum spontaneum* L.) clone Glagah Kloet. *Bulletin of the National Institute of Livestock and Grassland Science*, *13*, 23-32.

Tiwari, A. K., Tripathi, S., Lal, M., & Mishra, S. (2012). Screening of some chemical disinfectants for media sterilization during in vitro micropropagation of sugarcane. *Sugar Tech*, *14*(4), 364-369.

Uzma, Khan, M. R., Muhammad, A., Hussain, I., Shah, S. H., Kumar, T., Inam, S., Zubair, M., Rehman, H. u. R., Sher, A., Rehman, N., Ahmed, S., & Ali, G. M. (2012). Rapid *in vitro* multiplication of sugarcane elite genotypes and detection of sugarcane mosaic virus through two steps RT-PCR. *International Journal of Agriculture & Biology*, *14*(6), 870–878.

Yadav, S., Ahmad, A., Rastogi, J., & Lal, M. (2012). Tissue culture strategies in sugarcane (*Saccharum officinarum* L.). *International Journal of Pharma and Bio Sciences*, *3*(2), B427–B441.

Yulianingtyas, A. Y. P., Sebayang, H. T., & Tyasmoro, S. Y. (2015). Pengaruh komposisi media tanam dan ukuran bibit pada pertumbuhan pembibitan tebu (*Saccharum officinarum* L.) (Doctoral dissertation, Brawijaya University).