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Research Paper

Optimizing tissue culture protocol for *in Vitro* shoots and roots development of sugarcane (*Saccharum officinarum*) under various concentration of hormones

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Abstract

Sugarcane (*Saccharum officinarum*) contributes 60–70% of annual sugar production in the world. In the present study, a complete optimization protocol of sugarcane cultivar HSF-240 was designed for *in-vitro* micro-propagation under different media concentrations. Young leaves were sterilized by using various concentrations of Clorox. The results revealed that almost all contamination was controlled by using antibiotic cerfotaxime (500mg-^{1}) in the initiation step of the culture medium. However, for callus induction, variable concentrations of 2,4-D were used. It was observed that a lower concentration of 3mgl^{-1} 2,4-D, showed better results than a higher concentration. The shoot proliferation and multiplication were obtained by using various hormonal blends. The results revealed that RM12 (BAP 1mgl^{-1} +KIN 0.5mgl^{-1}) exhibited good result. The highest shoot length and shoot number were obtained by BAP and KIN combination as compared to individual BAP and KIN. Similarly, root induction obtained by eight different hormonal applications of NAA and IBA. It was shown that the highest root length and root number were obtained at IBA (0.5mgl^{-1}) and by NAA (2mgl^{-1}). In conclusion, the current study revealed that diverse hormonal concentrations significantly affect the *in-vitro* growth of explants of sugarcane. It is suggested that further comparative investigations are required to understand the nutrient contents of sugarcane in a controlled environment and in soil for better acclimatization and the highest yield in a natural habitat.

Introduction

Sugarcane (*Saccharum officinarum* L.) is the chief shareholder in the economy of Pakistan as it is the second main cash crop of Pakistan (Saleem et al., 2022). In Pakistan, the industry of sugar uniquely depends on the production of sugar. It is used in agronomic as well as medicinal productions. Pakistan is fifth larger manufacturer of this crop around the globe (Khan et al., 2004). Sugarcane is cultivated in the tropical part of Sindh, the subtropical area of Punjab and Khyber Pakhtoon Khawa of Pakistan (Bashir et al., 2012). In Pakistan, the under-cultivation area was about 1.1288 million hectares that produced 63749.9 million tons raw material for eighty-four sugar-mills working in Pakistan (GOP, 2012). However, the due to transient in climatic conditions the flowering and seed setting of the crop make conventional breeding become problematic (Asad et al., 2009; Shakra et al., 2017).

Sugarcane accounts for 70% world's sugar production and it is mainly utilized for various purposes in the world (Gomathi et al., 2022). Brazil is the largest manufacturer of sugarcane followed by India, China, Thailand, Pakistan and Maxico (FAO). On the other hand, conventional breeding, emergence diseases, pests attack consequences in reduced yield of sugarcane (Sani & Mustafa, 2010). However, Plant tissue culture is paramount methodology which is widely used for mass propagation of disease-free plants in food crops and marketable horticulture (Salokhe, 2021). *In-vitro* micro-propagation method is a tool used for improvement of food crops including sugarcane as it offers large scale making of disease-free agricultural food crops in a very short time (Kumar et al., 2020). For these objectives, researchers have conducted studies for *in vitro* micro-propagation through callus culture, shoot tip culture and axillary bud culture (Khan et al., 2007; Hailu et al., 2018; Saleem et al., 2022). In addition, regeneration through callus induction is very initial and significant phase in tissue culture. A study revealed that the sugarcane tissue culture by callus induction using shoot tips, young inflorescences and young leaves as explants have been reported (Saleem et al., 2022). Nutrient medium containing various concentrations of 2-4, D showed call genesis (Tahir et al., 2011; Rashid et al., 2009). The maximum proportion of renewal has been showed in MS medium with varying applications of cytokinin (Zahra et al., 2010; Dibax et al., 2011). Another study reported that sugarcane showed significant *in vitro* rooting on MS medium using various concentrations of auxins (Ramnand et al., 2007 and Zahra et al., 2010). So, it has been validated that plant growth hormones regulates cell division, cell differentiation, growth of shoots and roots of explants (Kumar et al., 2020).

Furthermost micro-propagation investigations emphasis on the effect of auxin on callus induction and cytokinin on regeneration and development of food crops (Salokhe, 2021). However, synergetic and comparative effects of different combination of auxin and cytoknin have rarely been reported on HSF-240 sugarcane cultivar. There is limited information about root and soot induction and propagation in response to various combinations of auxin and cytonkinin. Moreover, callus, shoot and root induction and propagation are very key phases of plant profitable uses, genetic fidelity and acclimatization in natural environment (Hailu et al., 2018). However, diverse developmental stages of sugarcane in tissue culture are extremely dependent upon the interaction of various concentrations of plant hormones. Therefore, it has become a broad field of investigation to understand the improved protocols with always to be

present for significant crop improvement and yield production (Kumar et al., 2020). Keeping in view the importance of the sugarcane, the present study was designed to investigate and optimizing various compositions of growth regulators for *in Vitro* proliferation of shoots and roots of sugarcane.

Materials and methods

Plants materials and explant selection

The present study was carried out at Plant Tissue Culture laboratories in National Agriculture Research Center (NARC), Islamabad, Pakistan. Sugarcane indigenous cv. HSF-240 cultivar was chosen as a material for tissue culture and grown in the fields of crop sciences program (CSI, NARC, Islamabad). *Saccharum officinarum* L. belongs to the family Poaceae and the genus *Saccharum*. It is considered as the utmost widespread variety of Punjab and cultivated on a large scale in various area of Punjab; as it maturity early, generally resistant to pests due to firm rind, supplementary tillering, modest requirement of nourishment and upright percentage of sugar recovery.

The central aim of micro-propagation is to get identical copies of plants through using meristem as explants. For *in-vitro* proliferation of explants used should be *ex-vitro*. An old plant of (4-5 month) sugarcane HSF-240 cultivar was selected for the present study and young leaves were selected as explant for callus induction. For this purpose, leaf sheath was uncovered and interior soft portion. The media preparation and culture induction followed the protocol as described earlier with slight modification (Aftab et al., 1996).

Media preparation for callus induction

Murashige and Skoog (1962) salt media was employed throughout the study for shoot initiation, shoot proliferation and root initiation. A stock solution of MS medium is prepared and $3mgl^{-1}$ (w/v) phytagel was applied for solidification. In addition, the sucrose ($3\ mgl^{-1}$) solution was used for callus induction, proliferation and roots induction. Firstly, all the media was autoclaved at 121˚C at 30 psi pressure for 15 minutes. Afterward sterilization of the media it is standing to cool for solidification till it further uses. It is strongly opted to make the culture media one day prior to culturing in order to lessen the probabilities of contamination. All other materials were also sterilized and disinfectant by autoclaving them at 121˚C for 30 minutes. The working seat or laminar hood was also disinfected by carefully spraying the laminar flow hood with 70 % ethanol solution. The UV light might be switch on at least 30 minutes before culturing in order to diminish any microbial contamination in the hood.

For the surface sterilization various concentrations of Clorox was used (sodium hypochlorite 5.25%) for 15 minutes along with 3-4 drops of Tween-20 (Table 3.1). In addition, antibiotic cefotaxime was used for the remove bacterial contamination (500mg/L). Finally, in addition to surface sterilize, the explants were washed 4-5 times with deionized water and afterward filter dried for 12 minutes.

Callus induction media

For the tissue culturing, the explants were cut into 2-3 cm in length, then take out the outermost whorl of leaves, dried on sterilized filter paper for 12 minutes prior placing in the

test tubes containing MS media (Murashige and Skoog, 1962). Then the medium is supplemented with 3% sucrose and variable concentrations of 2, 4-D listed in table (Table 1). Finally, the pH was adjusted to range of 5.7-5.8 and the cultured racks were closed properly with cotton plugs and placed in the growth chamber in the dark room at 27°C±2°C for effective callus induction. As old media consumed, then the plants must be shifted onto the fresh media after every 15 days for efficient growth and development.

For sub-culturing, the friable calluses were transferred aseptically to the regeneration media. After that, the test tubes were re-shifted to the growth room at 27°C±2°C for the photoperiod of 16 hour and the intensity of light applied that is 2000 lux. It was observed green spots on the callus after 2 weeks. Subsequently the calli of explant were shifted to the multiplication media.

Table 1 Different combinations of media used for callus induction in sugarcane cv. HSF-240

Combinations	Medium	2,4-D (mgl^{1})	Sucrose (gl^{-1})	pH
C1M1	MS*	0	30	7.8
CIM2	MS	1	30	7.8
CIM3	MS	3	30	7.8
CIM4	MS	5	30	7.8
CIM5	MS	7	30	7.8
CIM6	MS	9	30	7.8

Note: *MS: Murashigue and Skoog; ©2, 4-D: 2, 4- dichlorophenoxy- acetic acid, CM: Callus medium

Shoot multiplication media

For shoot propagation, the young shoots emerging from the calli were transferred to the shoot multiplication media (SM). Murashige and Skoog (1962) media augmented with BAP and KIN individual and in various combinations were used for propagation (Table 2). A small cluster of 4-5 shoots were cut out and inoculated on the media in aseptic environment condition and kept in the presence of light for (16/8) hr. The brownish coloration of shoot portion was detached and subdivided clumps into the smaller 3 to 4 number of shoots by using sterilized scalpels. It was observed until long healthy shoots of sugarcane were developed. The shoot cultures media were retained in similar conditions above for the restoration of calli for efficient propagation. For sub-culturing, the young plants were transferred onto fresh media. The number and size of shoots were observed after regular intervals and recorded.

Table 2 Different combinations of media used for shoot induction and multiplication in sugarcane cv.HSF-240

Combinations	Medium	BAP (mgl^{-1})	KIN (mgl^{-1})	Sucrose (gl^{-1})	PH
SM1	MS	0.5	-	30	5.8
SM2	MS	1.0	-	30	5.8
SM3	MS	1.5	-	30	5.8
SM4	MS	2.0	-	30	5.8
SM5	MS	-	0.5	30	5.8
SM6	MS	-	1.0	30	5.8
SM7	MS	-	1.5	30	5.8
SM8	MS	-	2.0	30	5.8
SM9	MS	0.1	0.5	30	5.8
SM10	MS	0.2	0.5	30	5.8
SM11	MS	0.5	0.5	30	5.8
SM12	MS	1.0	0.5	30	5.8

Note: MS: Murashigue and Skoog; KIN: Kinetin; BAP: Benzyl amino purine; SM: Shooting medium

Root induction media

For root induction and propagation were done after maximum number of shoots attained in shoot induction media. For this purpose, the root induction media of various concentrations of auxins were used. The various combinations of IBA and NAA along with their concentration are given in the table (Table 3). A few healthy bunches of shoots were moved from culture tubes by using sterilized equipment and unwanted parts of the plantlets were excised under controlled condition. After shifting to the rooting medium jars were kept in growth chamber for 2-3 weeks under same environments condition as mentioned for shoot induction. The number of roots and root length (cm) was recorded afterward they emerge.

Table 3 Different combinations of media used for root induction in sugarcane cv. HSF-240

Combinations	Medium	NAA (mgl^{-1})	IBA(mgl^{-1})	Sucrose (gl^{-1})	PH
RM1	MS	0.5	-	30	5.8
RM2	MS	1.0	-	30	5.8
RM3	MS	1.5	-	30	5.8
RM4	MS	2.0	-	30	5.8
RM5	MS	-	0.5	30	5.8
RM6	MS	-	1.0	30	5.8
RM7	MS	-	1.5	30	5.8
RM8	MS	-	2.0	30	5.8

Note: MS: Murashigue and Skoog; NAA: Naphthalene acetic acid; IBA: Indole butyric; RM: Root medium

Results and discussion

In the present study, an efficient way of in vitro shoot, root induction and multiplication of sugarcane has been optimized and results compared to previous findings summarized here. Tissue culture technique of explants became most popular to produce multiple true-to-type virus free plants (Mustafa *et al*., 2020; Saleem et al., 2022).

The present finding revealed that the application of various concentrations of Clorox gave maximum (95%) healthy calli. However, the maximum browning appeared when explants were treated with Clorox 80%. On the other, it was observed that the higher concentration of Clorox inhibited the bacterial and fungal contaminations and also causes browning in media. But, before sub-culturing the brown part of explants was removed and shifted on fresh culture medium. It was observed that cefotaxime (500mg/L) assured 100% control of bacterial contamination. It became clear that the use of high concentration of Clorox and cefotaxime in medium provide an effective controlled condition. Research survey of previous findings reported that callus induction in sugarcane is influenced by numerous factors and genotypic interaction with plant growth regulators (Mustafa et al., 2020; Baday, 2020). Some other a few studies also revealed that the callus induction and propagation were also attained at 4 and 5 mg L^{-1} of 2, 4-D, but their higher concentrations inhibited callus induction in sugarcane (Tripathy et al., 2020; Saleem et al., 2022).

Moreover, healthy callus induction exclusively depends on good hormonal combination and concentration in the culture media. In addition, the Callus development also depends on the type of explant, the selected cultivar and proper hormonal balance (Dobhal et al., 2013). In the present study, young leaves of sugarcane were used as explant and obtained remarkable results. Tahir et al. (2011) reported that the young leaves as explants found to be good source for callus induction. Literature review revealed that the use of 2,4-D with MS media showed good callus induction (Tahir et al., 2011; Saleem et al., 2022), while the variable concentrations were also showed significant result for callus induction of sugarcane cultivars (Saleem et al., 2022). Findings from present study unveiled that Explants cultured without 2,4- D showed no response, turned brown and died after few days of callus culturing, while medium concentration of 2, 4-D showed bulge and callus induction after 2-3 weeks. However, the maximum callus induction was attained at combination of CIM3 followed by CIM4. The highest shoot proliferation was gained at SM12 and SM6 media nutrient combination, while maximum number of root and root length were recorded at root media culture combination of RM4 and RM4 and RM5 respectively (Figure 1). Our finding showed that the 95% callus induction were obtained in the media containing $3mgl^{-1}$ 2, 4-D. it is suggested for other food crop for better callus induction. It was also observed that by increasing the concentration of auxin in the culture media caused browning of calli and no healthy or friable callus were developed above $3mgl^{-1}$ of 2,4-D. However, similarly to our finding, Gopita et al. (2010) found better callus induction on MS medium supplemented with 3.0 mgl^{-1} 2, 4-D and 10% coconut. The result showed remarkable induction at concentration of $3mgl^{-1}$ 2,4-D. These finding also in good agreement with some other previous reported research for callus induction (Mustafa et al., 2020; Saleem et al., 2022).

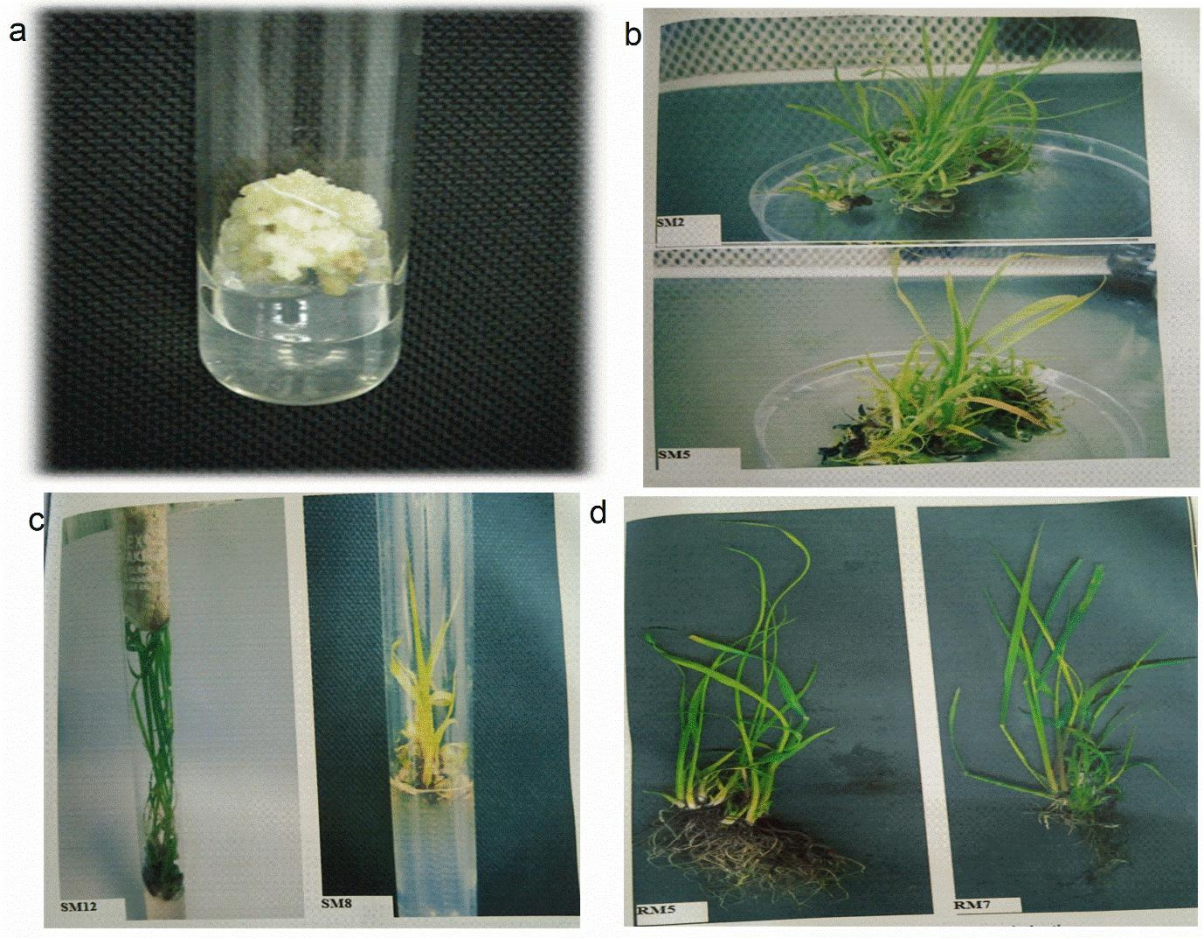

Figure 1 Pictorial presentations of the (a) in vitro callus induction, (b) shoot multiplication, (c) shoots length, (d) root length and number under different combination of media.

In vitro propagation of explants is very important step in mass propagation of any food crop for marketable economic benefits. The effect of various combinations of hormones on the regeneration of sugarcane from callus was carefully observed. The result shows that after third weeks green spots were observed on the calli which after 4th weeks were developed into small shoots in the culture media. HSF-240 cultivar of sugarcane gave maximum regeneration on shoot multiplication media of SM12 media followed by SM6 compared to other combination (Fig. 2a). On the other hand, the minimum shoot number was observed on shoot multiplication media at combination of SM2. The combination media of SM12 and SM6 are non-significant to each other but significantly different from others. From present findings it has been revealed that the hormonal combinations, the SM12 (BAP 1mgl-^{1} +KIN 0.5mgl-^{1}) showed highest number of shoots, while the medium SM2 (BAP Imgl-^{1}) exhibited minimum shoot number. Similarly, to our findings the Dibax et al. (2011) also reported that the various concentrations of BAP (0mgl^{-1}-0.25mgl^{-1}) showed highest (80%) regeneration on MS supplemented with BAP (0.25mgl^{-1}) at its higher concentration. The present study showed also good result as reported in a previous study show any shoot induction and number of shoots per vials varies with different treatment (Saleem et al., 2022). However, the remarkable regeneration responses was

observed on MS medium supplemented with BAP (1.0mg/L) + KIN (0.5mgl^{-1}) +IBA+ (3.0mgl^{-1}) at notable concentration.

On the other hand, maximum shoot length (8.27 cm) was noted at hormonal combinations of SM12 followed by medium SM4 (7.27 cm) as compared to SM2 (2.03 cm) and SM8 (1.76cm). It was recorded that SM12 is significantly different from other hormonal combination, while SM2 and SM8 showed non-significant to each other and significant to others (Fig. 2b). Hormonal combination of SM12 (BAP 1mgl-^{1} +KIN 0.5mgl-^{1}) showed maximum shoot length as compared to other medium while medium SM8 (KIN 2mgl-^{1}) showed minimum shoot length. It has been reported in a previous study that growth regulators (BA, KIN, NAA) individually and in combinations with others found optimized for regeneration, however, the BA showed notable root induction and root number (Rahman et al., 2018). Furthermore, among the all-culture media BA (1.5mgl^{-1}) and NAA (0.5mgl^{-1}) in combination exhibited highest percentage 90% of shoot length. The present study revealed that the hormonal combinations of BAP (2-4mgl^{-1}) and KIN (1mgl^{-1}) plus IAA(0.5mgl^{-1}) establishes to be best for maximum number of shoots and highest elongation of shoot test variety of sugarcane. It has been validated that the sugarcane cv.HSF-240 responds specifically to the variable hormonal combination in culture media. The hormonal combination of BAP (1mgl-^{1}) and KIN (0.5mgl^{-1}) showed remarkable results and could be choice for future study for sugarcane and other food crops. However, another previous study reported that only BAP @ 1mg/L alone was found optimum and revealed good result for shoot emergence from sugarcane (CV thatta-10) callus (Ather et al., 2009). Therefore, optimization of the suitable amount of growth regulators is the key step and have significant role for callus induction, shoots number and shoots length proliferation and development (Aslam et al., 2020; Mustafa et al., 2020).

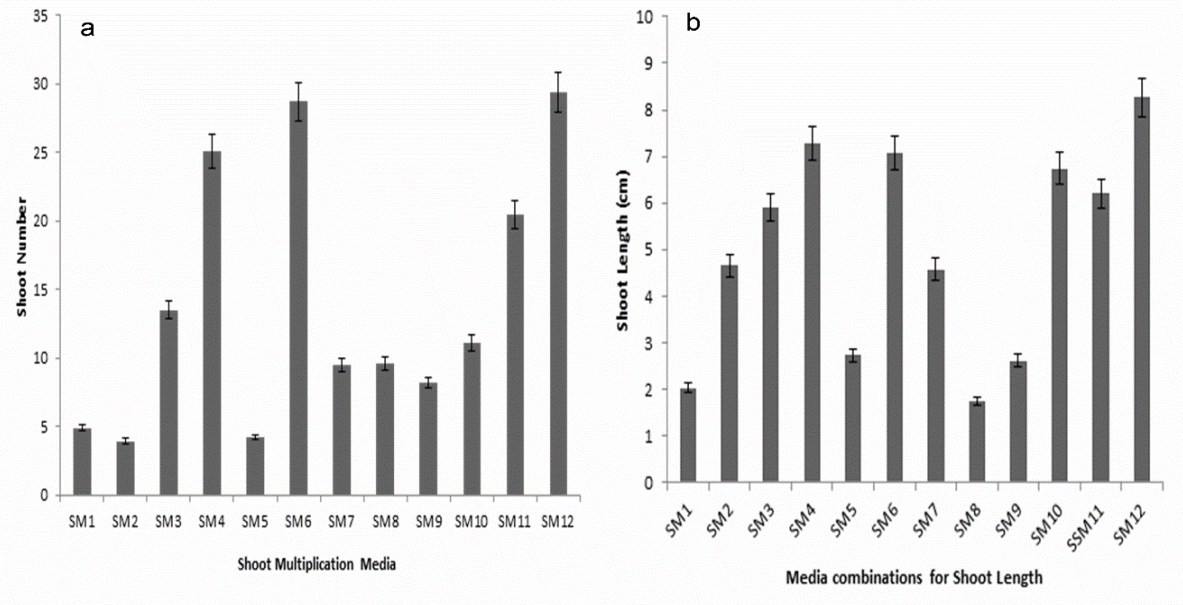

Figure 2 Shoot multiplication (a) and shoot length (b) response to different hormonal combinations

Root induction is also very crucial phase as plantlets of sugarcane often do not respond well, particularly in the lateral stages (Biswas et al., 2020). The present results show that the highest root elongation (8.72 cm) was observed in response to root media combination RM4 followed by RM5 (8.6 cm). Moreover, the minimum root length (2.68 cm) was recorded in response to medium RM3 (Fig. 3a) and highest number of roots (14.8 cm) was showed in response to hormonal combination of RM4. It is obvious from figure 2 that among all the hormonal combinations the RM4 (NAA 2mgl-^{1}) showed highest percentage of root elongation. Similarly, the number of root) was exhibited by RM4 at concentration of 2mgl-^{1} followed by and RM3 and least in RM7 hormonal combination (Figure 3b).

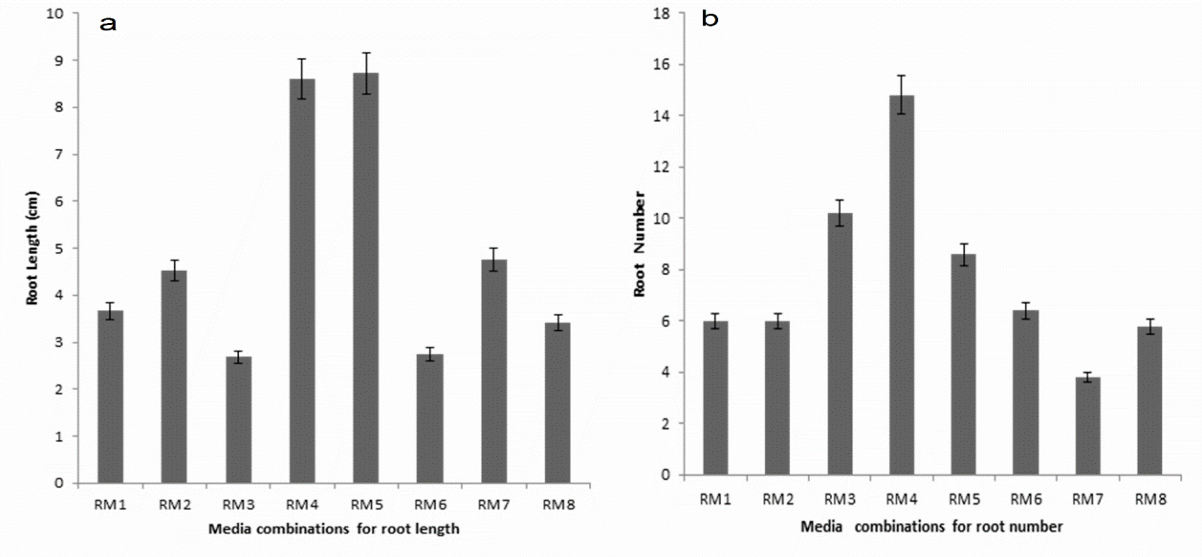

Figure 3: Root length (a) and root number (b) on different hormonal combinations

The results showed good agreement with previous reported findings obtained by Ramanand et al., (2007) to evaluate the rooting response in sugarcane cultivars COS- 96268 and COS-95255. The media MS supplemented with NAA (5.0mgl^{-1}) along with 50gm/L sugar was found to be most effective for maximum root elongation as compared to MS culture medium containing merely NAA (7.0mgl^{-1}) and (70gl^{-1}) sugar. Another study reported that the cultivars CP-77400 and BL-4 showed remarkable root elongation on MS medium fortified with 2.0mgl^{-1} NAA concentration (Ali et al., 2008).

The present study showed also good agreement with some other previous reported studies. It has been reported that variable concentration of hormone like NAA and sucrose in culture media prove to be paramount for maximum roots elongation and root number (Gopita et al., 2010). The present study well supported by previous findings that that the combine effect of 1 mg L^{-1} NAA and 1 mg L^{-1} IBA was also found to be significant effective for root induction in sugarcane cultivar (Tolera et al., 2016). In addition, it is confirmed that the NAA is alone be effective in root induction in some cultivar of sugarcane (Rahman et al., 2018; Biswas et al., 2020; Silva et al., 2020; Saleem et al., 2022). It is concluded from above results that medium with the growth regulators have pronounce effects on callus induction, shoot, root initiation and root- shoot proliferation.

Conclusions

Our findings disclosed that callus induction, plantlet regeneration, shoot-root induction and elongation in sugarcane cultivar are influenced by genotype and various concentrations of culture media combinations. The present study exposed that the sugarcane cultivar HSF-240 was best choice for *in-vitro* induction and utmost micro-propagation. A young leaf was a best choice as explant for tissue culture technique. Furthermore, bacterial and fungal contaminations in the culture initiation medium were 100 percent controlled by using Clorox and antibiotic cerfotaxime. The results disclosed that $3mgl^{-1}$ 2,4-D concentration showed good callus formation compared to higher concentration. However, in case of shoot proliferation and multiplication the BAP $1mgl^{-1}$ +KIN $0.5mgl^{-1}$ combination found to be best and recommended for future study. However, maximum root elongation was obtained at IBA ($0.5mgl^{-1}$) and maximum root length was observed at NAA ($2mgl^{-1}$). In conclusion, the present findings revealed that variable hormonal concentrations and combinations specifically influenced *in-vitro* tissue culture of plantlets of sugarcane cultivar. From the present findings, is suggested that response of sugarcane cultivar to different combination of hormones could be further screen for its genetic changes and acclimatization in natural habitat. It is further suggested that this protocol can be applicable for studying other cultivars of sugarcane and food crops.

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