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Genetic Response of Different Genotypes of Sugarcane at Different Tissue Culture Techniques Combinations

Muhammad Ijaz[a]*, Dr. Farooq Ahmad Khan[b], Zahid Mahmood[c], Aslam Javeed[d], Rameez Iftikhar[e], Muhammad Arfan[f]

[a]*Univerity of Agriculture faisalabad, Rom no 25 bachlor hostel AARI,Faisalabad, Pakistan*

[a]*Email: ijazkhan1901@gmail.com*

Abstract

Sugarcane is one of the major sugar crops of the world. Tissue culture study is mainly aimed to produce disease free plants. The study was carried out to find out the best combinations of tissue culture technique used for sugarcane to produce it asexually. It was also aimed to find out the best responsive genotype for tissue culture. From the five genotypes taken for the tissue culture study (HSF-242, CPF-246, CPF-247, S2002US-618, S2002US-718), Callogenesis study revealed significant differences for different genotypes and treatments. The genotype HSf-242 showed maximum callus formation at 3mg/l of 2, 4-D level but not best performance for organogenesis. For callus least performing genotype was S2002US-718. For organogenesis CPF-247 performed well at all treatments.

Keywords: Tissue culture; sugarcane culturing; media for sugarcane culturing

1. Introduction

Pakistan occupies a significant place in cane producing countries of the world. It ranks at the fifth position in cane acreage and production and almost 15th position in sugar recovery point of view. Plant tissue culture techniques are used worldwide by many researchers over the last two decades in the development of useful genetic variability in the economically important crops.

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* Corresponding author.

E-mail address: ijazkhan1901@gmail.com.

Initially attempt made for the regeneration of the plants through in vitro technique by [8]. Successful regeneration and somatic embryogenesis was further studied in sugar cane using different explants medium composition. Plant tissue culture (PTC) is a generic description which embraces plant protoplast, plant cell, plant tissue, plant organ and plant culture, where these various types of culture involve, as a common factor, the growth of microbe-free plant material in an aseptic (sterile) environment, such as sterilized nutrient medium in a test tube. This ability to generate any cells from such starting tissue is called cellular "totipotency". The technique of plant tissue culture may play a key role in the "Second Green Revolution'' in which biotechnology and gene modification are being used to improve crop yield and quality. Usually, the plant part (explant) is placed in a suitable tissue culture media, proliferation of the lateral buds or adventitious shoots or the differentiation of the shoots results in tremendous increase in the number of shoots available for rooting.

2. Material and methods

2.1 Plant Material:

For the proposed study to be carried out following material was needed.

2.2 Genetic material:

Five sugarcane (*Saccharum*spp.) accessions were evaluated for their callus induction capacity which was HSF-242, CPF-246, CPF-247, S2002US-618 and S2002US-718. These were collected from the field of Ayub Agriculture Research Centre Faisalabad in Pakistan. There was culture room, Air laminar flow cabinets with different scalpels, Air conditioned culture room, A pair scissors, Forceps and fire facility, For the preparation of media following chemicals were used M.S. chemical kit, Absolute alcohol, Distilled water, Gooch crucible, pH meter, Buffer for calibration, Solution for pH adjustment, Growth promoters, Growth regulators, 2, 4-D, Kinetin, Casein hydrolysate, Electronic balance, Hot plate and stirrer. Highly controlled condition of light temperature and humidity was present with in the incubation room. In order to wash glassware there was good water sink with in the washing room. Autoclave was essential part of the tissue culture laboratory. There were Newspaper, pair of scissor, muscan tape, aluminum foils rubber bands etc. Similarly callus induction of five varieties was performed by Gandonou [5].

2.3 Methods:

2.4 Explant sources:

Two kinds of explant sources were used from, the top portion, of the plant,

a) EX 1= Leaf cylinders were taken from innermost 1-5 leaves and was 2-3 mm in length.
b) EX 2= Pith explant was taken from 1-5 apical internodes.

2.5 Sterilization of the explant and culture:

The top portion of sugarcane plant was taken. Unnecessary portion of the cane top was removed and the remaining was surface sterilized [9]. These sterilized leaves were too removed and only the innermost leaves, which were infection free, were cut into 2-3 mm long pieces. These pieces were cultured aseptically into the test tubes containing 10 ml of the callus induction media.

2.6 Media used for callogenesis:

For callogenesis five media were using 5 repliation for each.

2.7 M.S. media with different Concentration. Of 2,4-D

2.7 Media with different concentration of 2,4-D

MEDIA CODE	M.S. + 2,4-D Concentration.
T1	M.S. + 0 mg/l 2,4 D
T2	M.S. + 1 mg/l 2,4 D
T3	M.S. + 3 mg/l 2,4 D
T4	M.S. + 5 mg/l 2,4 D
T5	M.S. + 7 mg/l 2,4 D

Fifty cultures of each explant were cultured at each of the five media so that five cultured infection free tubes were selected at randomly for data collection. Each cultured tube was kept in the dark at 28 $\pm$ 2 ^{o}C for the first two weeks and then was shifted under continuous florescent light of 2000 to 2500 lux intensity of light at the same temperature.similarly Behera and Sahoo [2] conducted an experiment in which the protocol for induction of callus and regeneration of plantlets was established through *in vitro* culture using young meristem of Sugarcane (*Saccharum officinarum* L. cv- Nayana) as an explant.

2.8 Sub-culturing of the callus produced:

The callus produced was sub-cultured at an interval of 15 to 20 days to increase the amount of callus. It was done on the same medium at which callus was produced initially. Data on the following characteristic of callus was recorded.

2.9 Callus proliferation/induction frequency (Callus scores)

++++, +++, ++, +, 0 were scored as best, better, good, satisfactory and no callus formation. Contaminated test tubes were counted and removed to maintain accuracy.

2.10 Regeneration

Regeneration media RM 1 (M.S. basal media), RM 2 (M.S. basal media + 480 mg/ l casein hydrolysate) RM 3

(M.S. basal media + 1 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 4 (M.S. basal media +1.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 5 (M.S. basal media + 2 mg/ l kinetin + 480 mg/ l casein hydrolysate + 0.5 mg/ l Nephthalene acetic acid) RM 6 (M.S. basal media + 2.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) was prepared for regeneration and percentage of regeneration was determined.

3. Statistical Analysis:

The data recorded for callogenesis was analysed statistically using complsetely randomized design. The differences among genotypes, explant sources, 2,4-D levels and their interaction was compared by Duncan's Multiple Range Test .

4. Result and discussion.

The present study was undertaken to test the response of five sugarcane accessions viz., HSF-242, CPF-246, CPF-247, S2002US-618 and S2002US-718 for callogenesis, organogenesis and treatment influences within *invitro*response in sugarcane.

4.1 Callogenesis studies: Callus induction is influenced a number of factors [7] some of which are evaluated below

4.2 Callus initiation frequency (CIF)

Callus formation is influenced by a number of factors some of which are discussed in the present study in the analysis of variance (table 1). The influential differences among genotypes, auxin (2, 4-D) levels and interaction of two factors i.e. genotypes x auxin levels were elucidated by analysis of variance. However, the interaction of genotypes x auxin levels was highly significant in the study.

Table 1: Analysis of Variance for Callus Initiation Frequency

Source	D.F	S.S	M.S	F- VALUE
Genotypes (G)	4	3.75	0.938	6.25**
2, 4-D levels (T)	3	30.6	10.2	68**
G X T	12	8.65	0.721	4.805**
Error	20	3	0.15	
Total	39	46		

Coefficient of Variation: 19.36%

**= Highly significant at $p<0.01$; *= Significant at $p<0.05$; ns= Non-significant

The result obtained from the analysis of variance it was elucidated that the effect of genotype and treatment is highly significant. It was also detected that the interaction of genotype and treatment of 2, 4-D levels were also

highly significant. The source that we took from the genotype effected highly significantly. As the explants taken from the inner most region of plant had less infected than upper region or pith. Different treatments level were showing different infection rate shown in figure 1 and 2.

4.3 Effects of genotypes on CIF (callus initiation frequency)

The response of genotypes for callus initiation frequency (callus score) were significant statistically Significant differences ($p<0.05$) was observed among the genotypes using DMR test. The genotypes showed high value of callus score ranging 2.375 to 1.5. Genotype CPF-247 was highest callus producer with an average of 2.375 callus score per test tube but genotype HSF-242 showed highest performance at 3mg/l of Auxin level (table 2). Genotypes HSF-242, HSF-246 and CPF-247 were statistically similar with an average 2.25, 2.00 and 2.357 respectively. The genotype S2002US-718 appeared to be least callus producer with the average of 1.5 as shown in figure 1 and 2.

These results revealed that callusing response is under the influenced of genotype [4] determined the response of three sugarcane varieties and found that callus induction ability is genotypes dependent. Burner [1] also studied the response of three sugarcane cultivars to callus induction using mature caryopses as explant and reached at the similar conclusions. Same results have also been reported in other members of the graminee family like on rice [6].

Table 2: Effects of genotypes on CIF

Genotypes	Infection rate score
V 1	2.25 AB
V 2	2.00 AB
V 3	2.357 A
V 4	1.875 BC
V 5	1.5 C

LSD VALUE= 0.4039

4.4 Effects of auxin (2, 4-D) levels on CIF (callus initiation frequency)

Satisfactory callus formation was noticed in all levels of 2, 4-D used in the study but the differences were significantly highly. Mean of the doses were analyzed using DMR test which showed that the T2 medium having 3mg/l of 2, 4-D with an average of 3.1 callus scores was the best callus producer (table 3). Callogenesis response seen at T1 and T4 media was not statistically different from each other; however, there performance was different from T2 and T3 media. The callus score of former and later media were 1.00, 1.3, 3.1 and 2.6 respectively (table 3). The poorest response was shown at T1 media (1mg/l) with average callus score of 1.00. The treatment $T_o$had no 2, 4-D level and there was no any callus formation.

It can be deduced from the results that concentration of 2, 4-D from 1-5 mg/l was considered to be the good for

callus induction with best performance at 3 mg/l. these results are consistent with Mamun*et al* who studied *invitro*micropropagation of two sugarcane varieties and found that 3 mg/l of 2, 4-D produced maximum callus and it was the confirmation of his study.

4.5 Response of genotype X regeneration media for CIF

Although all sugar cane genotypes showed response to callogenesis at all the four 2, 4-D levels. But there was marked differences in response of varieties at different 2, 4-D levels. By looking overall condition of interaction it became very clear that HSF-242 gave maximum response at 3mg/l of 2, 4-D level and CPF-247 at 5mg/l at 2, 4-D level with scores of 4 as shown in the (table 4). Least response was shown at 1mg/l of 2, 4-D level and 5mg/l of 2, 4-D level by S2002US-618 and S2002US-718 with score 1 as shown in the (table 4). Different reaction was shown by different genotypes at different levels of 2, 4-D and vice versa. The real picture was that HSF-242 showed maximum response at 3mg/l of 2, 4-D level with score 4. Least response was shown by S2002US-618 and S2002US-718 at 1 and 5mg/l of 2, 4-D levels. Similar kind of results was observed by Rehman et al.[10] in sugar cane variety SPF-238.

Table 3: Effect of Treatment on CIF

Treatment	Scores
T1	1.00 C
T2	3.1 A
T3	2.6 B
T4	1.3 C

LSD Value = 0.3613

Table 4: Response of Genotype X Regeneration Media for CIF

	V 1	V 2	V 3	V 4	V 5
T1	1 E	1.5 DE	1.5 DE	1 E	1 E
T2	4 A	2.5 BC	3 B	3 B	3 B
T3	2 CD	2.5 BC	4 A	2.5 BC	2 CD
T4	2 CD	1.5 DE	1 E	1 E	1 E

4.6 Organogenesis studies

The regeneration of callus depends upon a number of factors. The response under two factors was studied i.e., regeneration media and genotypes.

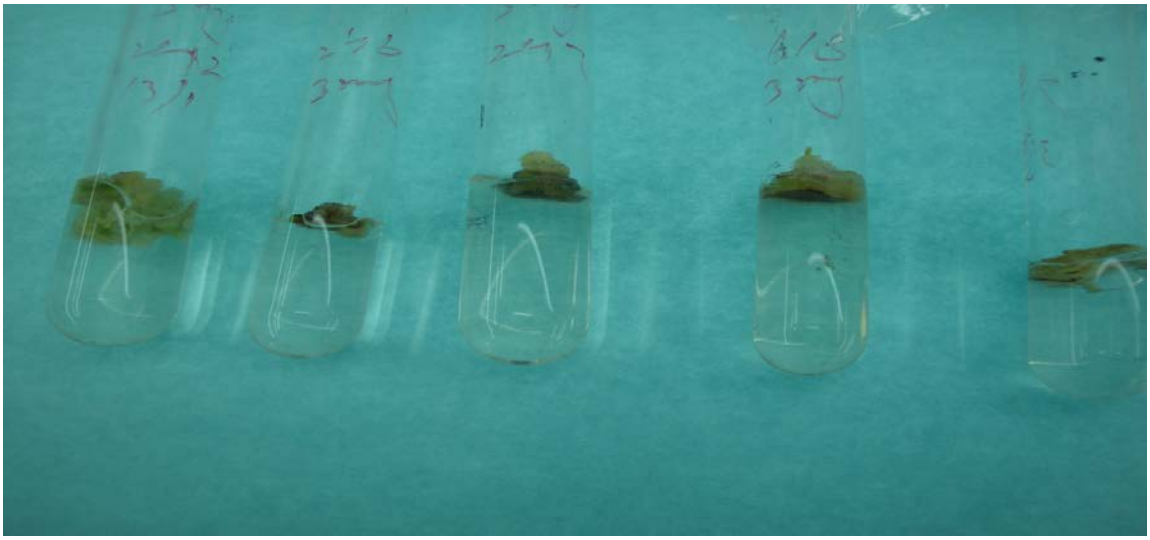

Figure 1: Effect of Genotypes for Callogenesis

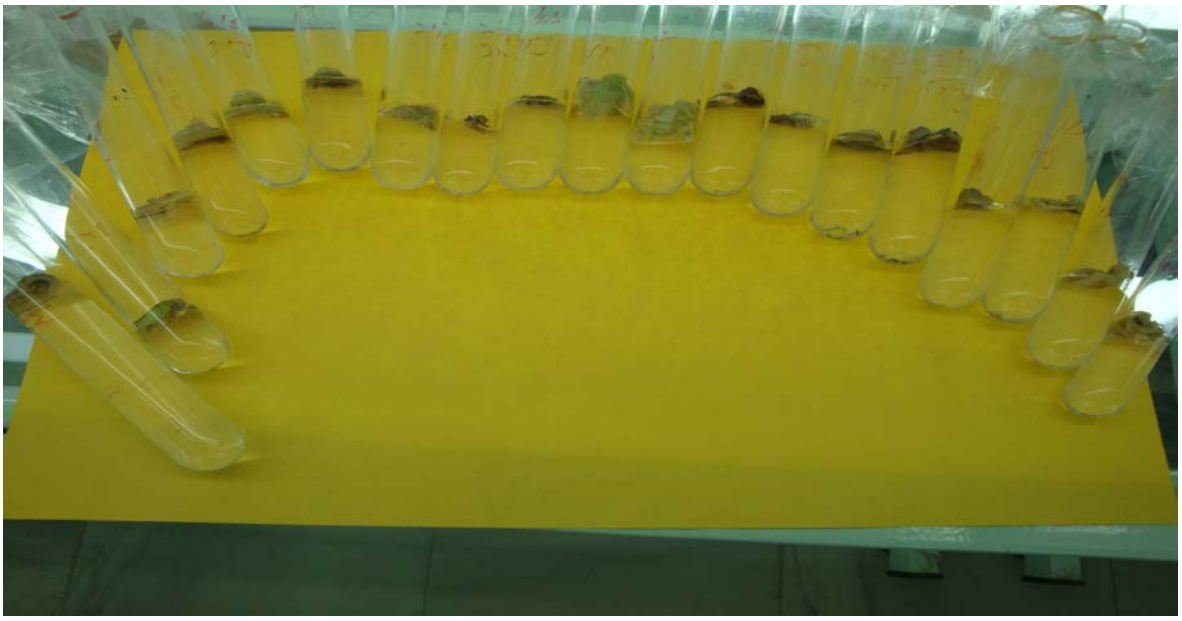

Figure 2: Response of different genotypes for callus formation at 3mg/l of 2, 4-D

Table 5: Analysis of Variance Table for Regeneration Percentage (%)

Source	D.F	S.S	M.S	F-VALUE
Genotypes (G)	4	64.93	16.23	139.142**
RM level (RM)	5	79.683	15.937	136.6**
G X RM	20	134.067	6.703	57.457**
Error	30	3.5	.117	
Total	59	282.183		

Coefficient of Variation: 8.80%

**= Highly significant at p<0.01; *= Significant at p <0.05; ns= Non-significant

The analysis of variance table 5 for percent regeneration showed that the differences among genotypes and regeneration media were highly significant. The interaction between genotype and regeneration media was also significant for non-regeneration percentage.

4.7 Genotypic response for regeneration

The analysis of variance table depicted that there were significant differences in the response of genotypes to the organogenesis in sugar cane.

Table 6: Response of Genotypes for Regeneration Percentage

Genotypes	Regeneration percentage
V 1	3.167 C
V 2	5.167 A
V 3	5 A
V 4	3.583 B
V 5	2.5 D

LSD value = 0.285

The response of genotypes showed that the genotype HSF-242 were highly responsive in callus formation but not responsive in the organogenesis. The genotype CPF-247 was highly responsive in regeneration followed by CPF-246. The genotype S2002US-618 was more responsive as compared to HSF-242 and S2002US-718. It was concluded that it is not compulsory that the more responsive to callus should be responsive to regeneration. The DMRT test also showed that all the genotypes response differently.

4.8 The effect of regeneration media on organogenesis

The analysis of variance table showed that regeneration media, RM 1 (M.S. basal media), RM 2 (M.S. basal media + 480 mg/ l casein hydrolysate) RM 3 (M.S. basal media + 1 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 4 (M.S. basal media +1.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 5 (M.S. basal media + 2 mg/ l kinetin + 480 mg/ l casein hydrolysate + 0.5 mg/ l Nephthaleneacetic acid) RM 6 (M.S. basal media + 2.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) were significantly different. The following DMRT table showed significant differences between regenerations media levels.

The results showed that RM 5 showed best in organogenesis. It proved that if we used Nephethalene acetic acids it will regenerate more the callus. It gained the score 5.3 which were high as compared to other like RM 2 on second position gained the scored 4.7 and later ranking were RM 1 and RM 3 which showed similar regeneration score 3.9 and 3.7. RM 6 showed poor response. RM 1 and RM 2 also showed similar response but different from others. It concluded that the treatment RM 5 effected significantly due to addition of NAA. Highest shoot regeneration was achieved on medium only with BAP (05 ml/L) and addition of ABA (02 mg/ L) also showed higher percent shoot regeneration [3].

Table 7: Effect of Regeneration Media on Organogenesis

Genotypes	Regeneration percentage
RM 1	3.9 CD
RM 2	4.7 B
RM 3	3.7 D
RM 4	4.1 C
RM 5	5.3 A
RM 6	1.6 E

LSD value = 0.312

***4.9 Interaction of genotype X regeneration media for regeneration percentage*:**

Analysis of variance table showed that the response of interaction of genotype to the regeneration media was statistically different. The interaction data is given below in the table.

Table 8: Response of Genotype X Regeneration Media for Organogenesis .

	V 1	V 2	V 3	V 4	V 5
RM 1	4	4	4	3	4
RM 2	4	4	6	5	4
RM 3	3	5	5	2	1
RM 4	3.5	6	5	2	1
RM 5	4	7	9	6	4
RM 6	2	1	1	3	1

It was conclude that genotype CPF-247 performed best at RM 5 and other genotype also performed well. The genotype CPF-246 was on second position in response and got 7 scores at RM5. The genotypes HSF-242 and S200US-718 responded similarly by getting 4 scores while S2002US-618 responded efficiently by getting 6 scores at RM 5. At the RM 1 all the genotypes responded poorly. All the genotypes responded efficiently at RM 5 for regeneration. It need further investigating for efficient regeneration.

4.10 Analysis of variance for non-regeneration percentage (%)

The response of genotypes toward non- regeneration were analyzed and analysis of variance proved it statistically significant. Interaction of non-regeneration of genotypes at different treatment was highly significant and genotypic response toward non regeneration was also highly significant. Table 9 showed the results.

Analysis of variance showed that response of genotype and treatment for non-regeneration was highly significant. The interaction was also highly significant for non-regeneration (table 9).

4.11 *Genotypic response for non-regeneration*

The analysis of variance table depicted that there were significant differences in the response of genotypes to the lack of organogenesis in sugar cane.

Table 9: Analysis of Variance Table for Non-Regeneration Percentage (%)

Source	D.F	S.S	M.S	F-VALUE
Genotypes (G)	4	110.60	27.65	57.2069**
RM level (RM)	5	29.283	5.857	12.1172**
G X RM	20	203.80	10.19	21.0828**
Error	30	14.5	0.483	
Total	59	358.183		

Coefficient of Variation: 19.22%

Table 10: Response of Genotypes for Non-Regeneration Percentage (%)

Genotypes	Non-Regeneration percentage scores
V 1	3.333 B
V 2	2.667 C
V 3	2.167 C
V 4	3.833 B
V 5	6.083 A

LSD value = 0.579

The results from DMRT depicted that the response of genotypes HSF-242 and S2002Us-618 were similar to each other but different from other genotypes. The response of CPF-246 and CPF-247 was alike but different from other and genotype S2002US-718 responded differently toward non-regeneration from other genotypes. It showed high percentage of non-regeneration least regeneration was in CPF-247 by scoring 2.1 (table 10).

4.12 The effect of regeneration media on non-organogenesis (%)

The analysis of variance table 10 showed that regeration media, RM 1 (M.S. basal media), RM 2 (M.S. basal media + 480 mg/ l casein hydrolysate) RM 3 (M.S. basal media + 1 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 4 (M.S. basal media +1.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) RM 5 (M.S. basal media + 2 mg/ l kinetin + 480 mg/ l casein hydrolysate + 0.5 mg/ l Nephthalene acetic acid) RM 6 (M.S. basal media + 2.5 mg/ l kinetin + 480 mg/ l casein hydrolysate) exhibited significant differences. The following DMRT table 11 significant differences between regenerations media levels.

The table 11 shows that all levels of treatment showed that RM1 and RM4 response towards non-generation

similarly but differently from other media levels. High level of non- regeneration was present in RM3 (4.4) and least was in RM5 (3.2)

Table 11: Response of regeneration media to non-regeneration percentage (%)

Genotypes	Non-Regeneration percentage score
RM 1	3.4 BC
RM 2	3.4 D
RM 3	4.4 A
RM 4	4 AB
RM 5	3.2 C
RM 6	4.3 A

4.13 Interaction of genotype X regeneration media for non-regeneration percentage

Analysis of variance table 12 showed that response of interaction of genotypes to non-regeneration media was statistically different. The interaction data was given below in the table.

Table 12: Response of Genotype X Regeneration Media for Non-Organogenesis.

	V 1	V 2	V 3	V 4	V 5
RM 1	2.5	3	3.5	4.5	4.5
RM 2	1.5	3.5	1.5	3.5	4
RM 3	4.5	1.5	1.5	6	8.5
RM 4	4	3.5	1.5	6	8.5
RM 5	5.5	1	0	2.5	2.5
RM 6	1	4.5	5	2.5	8.5

The interaction of genotypes and RM level were significant different showed from analysis of variance. The genotype S2002US-718 response toward regeneration was poor at all treatment except RM 5 and high non-regeneration percentage at RM 6. It showed some regeneration but not satisfactory. The non-regeneration of HSF-242 was also high at RM 5 and at other levels of media was also high. The genotype HSF-242 was less responsive for regeneration by application of NAA. The non-regeneration rate of HSF-242 was high at RM3 and RM5 by scoring 4.5 and 5.5. CPF-246 showed least non-regeneration at RM5 and highest at RM6 by scoring 1 and 4.5 respectively. CPF-247 showed no any non-regenerations at RM5 and highest at RM6 by scoring 5. S2002US-618 showed high non-regeneration at RM3 (6) and least at RM5 (2.5). S2002US-718 showed maximum non-regeneration at RM3 and RM4 (8.5) and lowest at RM5 2.5.

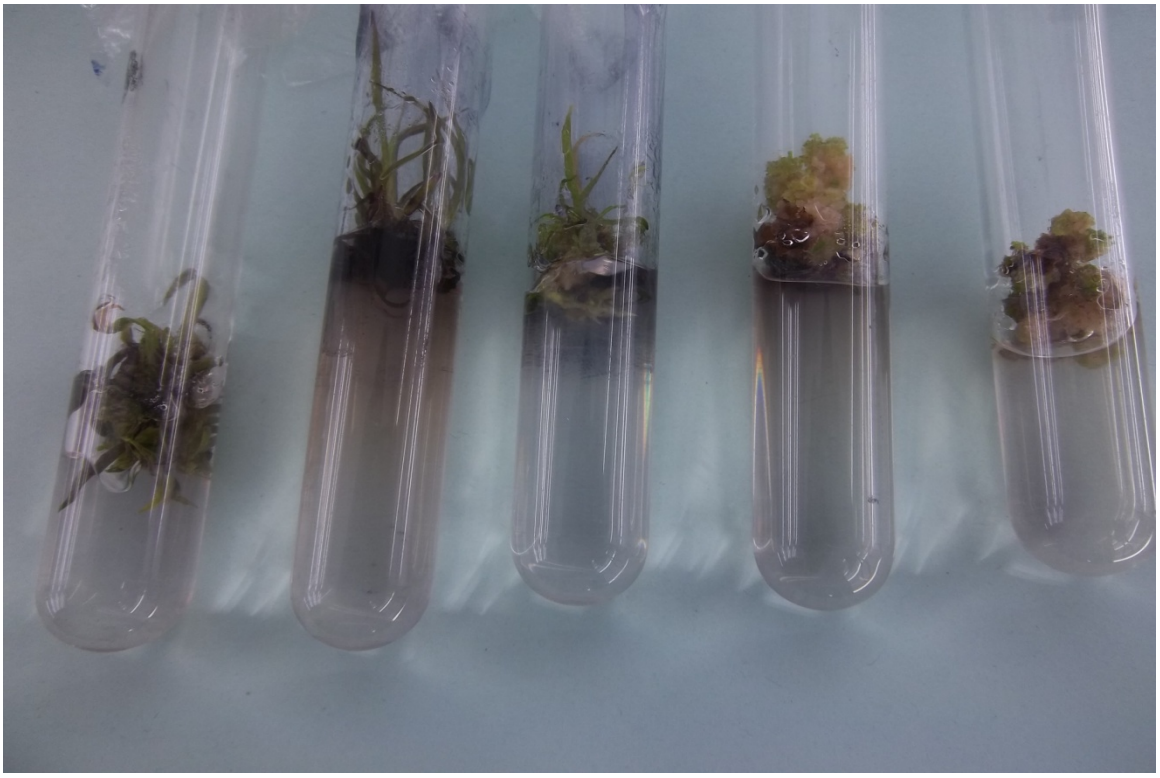

Figure 3: General Plantlet development process

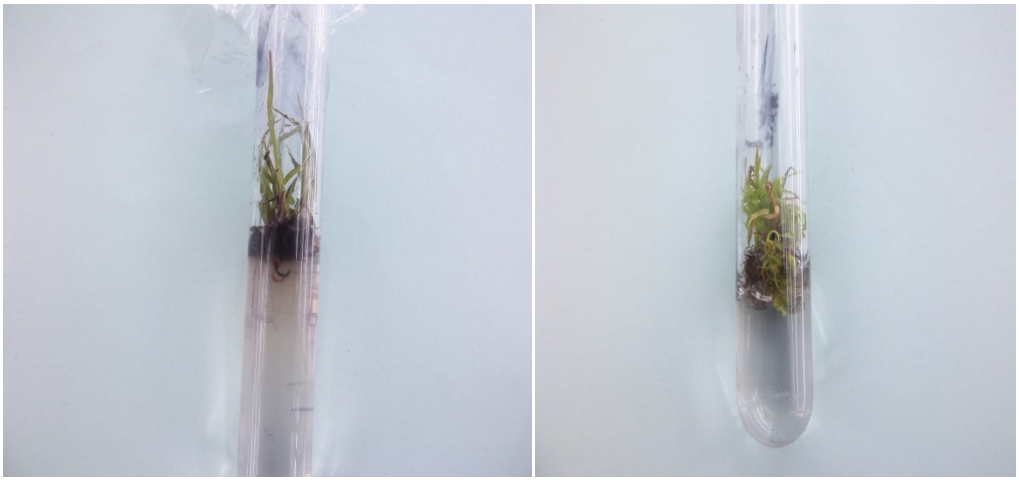

Figure 4: regeneration response of two different genotypes

5. Conclusions

The tissue culture study revealed that at different treatment overall performance of HSF-247 was better for callus induction and genotype HSF-242 performed best at 3mg/l of 2, 4-D level. The genotype S2002US-718

exhibited poor response at all levels of treatments. The analysis of variance revealed significant effect of genotype and treatment and their interaction. Response of different genotypes for infection rate was also significant for genotype and treatment both. The genotype CPF-246 showed maximum infection rate 7mg/l of 4-D level. Least infection rate was observed at 3mg/l of 2,4-D level. The analysis of variance showed that the response of genotype and treat and their interaction were significant. The genotype HSF-242 performed best in callus formation but not performed same way in regeneration process. The genotype CPF-247 performed best at RM 5 (M.S. basal media + 2 mg/ l kinetin + 480 mg/ l casein hydrolysate + 0.5 mg/ l Nephthalene acetic acid) from the analysis it is revealed that all the genotypes performed well at RM 5. CPF-246 and CPF-247 were best performing genotypes and S2002US-718 was least performing. The response of genotypes, treatment and their interaction for non-regeneration was also significant. The genotype S2002US-718 showed highest non-regeneration rate at all treatment except RM 5. Non regeneration rate after S2002US-718 was high in S2002US-618

6. Recommendations

These results revealed that callusing response is under the influenced of genotype [4] determined the response of three sugarcane varieties and found that callus induction ability is genotypes dependent. The genotype HSF-242 is good callus inducing genotype at 3mg/l of 2,4-D.

The study proved that all of genotypes performed best at RM 5 (M.S. basal media + 2 mg/ l kinetin + 480 mg/ l casein hydrolysate + 0.5 mg/ l Nephthalene acetic acid) and it is the best medium for the organogenesis.

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