Standardization of a genotype independent combination of growth regulators for axenic shoot tip culture of cotton

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ABSTRACT

A genotype independent media regime is optimized for clonal micro-propagation of selected cotton genotypes. The shoot tips of 7-days old seedlings of *G. arboreum*, *G. herbaceum* and *G. hirstum* var. S-12 and Sadori were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of benzyl-amino-purine (BAP) and kinetin (KIN) for shooting and Indol-butyric-acid (IBA) for rooting. Recoded observations revealed that 4.4 μM BAP and 5.75 μM KIN is the optimal combination of growth hormones to induce the highest number of shoots (5.92 shoots per explant) within minimum time (32.95 days) whereas rooting (3 roots per shoot) took minimally, 16-days at 3.75 μM IBA. The *in vitro* generated healthy plantlets were acclimatized for a month under high humidity with 91.75% survival rate. Additionally, cotton plantlets generated *in vitro* during this study were tested for the presence of CLCu-Virus (cotton leaf curl virus) by molecular diagnostics to be proved as virus free material.

1. Introduction

Cotton is grown throughout the world as a source of oil, animal feed and the most importantly fiber. Throughout the world, rural and urban labor is connected to this crop due to its raw material for textile mills, ginning factories and oil expellers. Cotton is the member of genus *Gossypium* that consists of fifty one species, out of which, four are cultivated species that include *Gossypium hirsutum* (allotetraploid, AD1), *G. barbadense* (allotetraploid, AD2), *G. arboreum* (diploid, A2) and *G. herbaceum* (diploid, A1) (Fryxell, 1992; Briddon et al., 2002). Both diploid and tetraploid cultivars are cultivated in different regions of Pakistan. It contributes over 10% to the national GDP (Syed et al., 2017).

Conventional breeding has made great progress in the improvement of cotton, however, breeding incompatibility among diploid and tetraploid cotton varieties have narrowed the genetic pool available for cotton breeding (Zhang and Stewart, 2000). Conventional breeding can be refreshed by utilizing the plant biotechnology techniques such as gene transfer and subsequent somatic embryogenesis and organ culture (Abdellatef and Khalafalla, 2007). There are the two main steps used for the morphogenesis of somatic cells of plants in plant tissue culture technology. One, cotton somatic embryogenesis (SE) was first demonstrated by Price and Smith (1979) since then much progress has been

done in the field (Finer, 1988; Firoozabady and DeBoer, 1993; Sun et al., 2005). SE is a crucial process of generating transgenic because a single cell can give rise to a somatic embryo, thus chimeric transformants are rare in this process (Pushpa and Raveendran, 2010). Nevertheless, the success of *in vitro* regeneration of cotton using SE is restricted to non-commercial Coker cultivars. Even in the case of Coker cultivars, SE has low efficiency, involve long time period for regeneration, low conversion rate of SE into plantlets, genotype dependent, high number of abnormal embryos, absence of shoot elongation, fatal browning, and issues in rooting. The loss of regeneration capacity of somatic embryos depends on plant genotype, type of explants, growth media and sub-culturing frequency (Pushpa and Raveendran, 2010). It is found that in long kept cultures some epigenetic modifications occur that affect the regenerative capacity of cultures. Thus, the progress of cotton tissue culture through SE that is indirect route like usage of leaf tissues, protoplasts or callus has hampered the improvement of commercial cotton utilizing genetic engineering (Gould et al., 1991; Agrawal et al., 1997).

On the other hand cotton direct organogenesis, using explants like cotyledonary nodes (whole or split; with or without cotyledons), shoot tips, hypocotyls sections and apical meristems to induce shooting and subsequent rooting is an alternative to obtain complete plantlets without facing the problems as described earlier (Hazra et al., 2001;

Gupta et al., 1997; Saeed et al., 1997). In this process different types and levels of phytohormones like cytokinins and auxin are used to facilitate shooting and subsequent rooting to get complete plantlets (Gupta et al., 1997; Saeed et al., 1997; Yasin and Yasmin, 2018). Organ cultures have low frequency of genetic modifications and somaclonal variations due to the absence of de-differentiation and re-differentiation phases during the induction of callus and somatic embryos (Gupta et al., 1997).

Hence, Due to the problems related to somatic embryogenesis of cotton tissue culture, it necessitates the development of an easy, reliable and efficient tissue culture protocols for cotton that can be used for the transformation and improvement of cotton, particularly in the Pakistani cultivars which are adapted to local conditions. The procedure presented here is simple and less expensive to regenerate cotton plantlets. The all methods described previously for regeneration of cotton were genotype dependent but our media regime is genotype independent. This direct organogenesis method can be coupled with plant transformation methods such as particle bombardment or Agrobacterium -mediated transformation to improve the prospect of genetic improvement of commercial cotton cultivars.

2. Material and methods

2.1. Plant material

The plant material used in the present study was cotton seeds of *G. herbaceum* L., *G. arboreum* and *G.hirsutum* (S-12 and Sadori). Germplasm was collected from Nuclear Institute of Agriculture, Tandojam.

2.2. Explant preparation for culture initiation

De-linted seeds of selected cotton varieties were prepared as described earlier in (Saeed et al., 1997; Yasin and Yasmin, 2018). Overnight soaked seeds were uncoated and inoculated in ½ MS media (Murashige, and Skoog, 1962) until germinated. 7-days old seedlings were washed in 10% bleach solution for 20 min and dipped in 70% ethanol for less than a minute. Further seedlings were rinsed 3-times in sterile distilled water and dried for a while. All procedures were carried out under clean bench. 0.5 cm long shoot tips without cotyledons were excised from sterilized samplings for culturing on MS basal medium (Yasmin et al., 2016) supplemented with various concentrations of BAP (0, 2.2, 4.4 μM) and/or KIN (0, 4.6, 5.75, 6.9 μM). The cultures were incubated at 22 ± 3 °C as 16-h photoperiod and 2000 lux luminous intensity.

2.3. Rooting of in vitro generated plantlets

Multiplied shoots were further cultured on the MS medium with numerous concentrations of IBA (Indol butyric acid; 0, 1.25, 2.5, 3.75, 4.9, 6.15, 7.4, 8.65, 9.8 μM) separately, for rooting. Rooted plantlets were hardened in greenhouse using an aseptic combination of soil and grinded coconut husk (1:1) for 4–5 weeks. The data was collected for shoots frequency (%), total shoots/explant, days taken to initiate shooting, root frequency (%), and days taken to initiate rooting.

2.4. Statistical analysis

The recorded data was analyzed through statistical methods like analysis of variance and Tukey's Multiple Range Test to observe the statistical differences within means of treatments as integrated in Microsoft excel.

2.5. DNA isolation and quantification

A DNA mini-preparation method for cotton was developed during this study based on general rules of DNA isolation. The DNA extraction buffer (EB) used was 100 mM Tris-base and 5 mM EDTA with 0.5 M glucose. Lysis buffer (LB) contains 0.2 M Tris-base (pH 8.0), 50 mM EDTA (pH 8.0), 2 M NaCl and 50 mMCTAB. 5% SDS (Zhang and Stewart, 2000). Isolated DNA quantified in agarose gel (0.8–1.0%). The quality of DNA depends on its purity that was evaluated by a spectrophotometer taking absorbance at 260 and 280 nm and calculating the ratios A_{260}/A_{280}. Enzymatic digestion was done by digesting 20 μg of DNA by 1 unit of *Hin*dIII and *EcoRI* (Femantas TM) according to the instructions of manufacturer's in the recommended buffer at 37 °C overnight. Digested DNA samples were visualized on 0.8% agarose gel after ½ to 1 h electrophoresis at 80 V in Tris-acetate-EDTA. The gel was photographed.

2.6. Molecular diagnosis of in vitro generated cotton plantlets for virus

Molecular diagnosis carried out to confirm the virus absence in *in vitro* generated cotton plantlets and the presence of virus in cotton plants with CLCu-disease infected scion grafts. PCRs were conducted using DNA of *in vitro* generated plantlets or disease grafted plantlets. DNA isolated by the above described optimized method and molecular diagnostics were done using primer pairs CLCV1/CLCV2 (5′- CCG TGCT GCT GCC CCC ATT GTC CGC GTC AC-3' & 5′- CTGCCACAACCATGGA TTCACGCACAGGG-3′) which can amplify approximately 1350bp long DNA fragment in PCR [2]. PCR assays performed using 12.5 μl of SYBR Green containing PCR Master Mix (Thermo Scientific, USA), 100–500 ng gDNA template, 1-μl MgCl₂, 2- μl of each primer and required molecular grade water to make up volume of 25 μl. The program profile was: (a) 95 °C for 5 min; (b) 25 cycles of 94 °C for 30 s; (c) 45 °C for 30 s; and (d) 72 °C for 1 min. Resultant PCR product was separated on the 0.8% agarose gel at 80 V for 45 min and the required bands visualized under UV-light of a trans-illuminator and photographed through gel documentation system.

3. Results

A genotype independent micro-propagation based media regime was standardized for four cotton varieties *G. arboreum* L. (2x), *G. herbaceum* L. (2x), S-12 (4x) and Sadori (4x).

3.1. Effect of BAP and KIN on shoot proliferation of cotton

The results of shoot proliferation frequency (%) of selected four cotton varieties in axenic culture are shown in Table 1. The effects of 12 treatments of BAP and KIN including control, on shoot proliferation was observed on 03 distant time intervals that were after 4, 8, and 12 weeks of explants in *in vitro* culture. Shoot proliferation frequency is a percent measure of explants revealing visible shoot growth. The first visible sign of shoot proliferation in selected cotton varieties was observed after 27-days of *in vitro* growth. Thus, there was no visible shoot induction during first three weeks of axenic culture that interval could be referred as lag phase prerequisite for explants to adopt the artificial environment and multiplication of new shoots on cell level that was visible after 27-days. The first sign of shoot proliferation was observed in treatment T10 (4.4 μM BAP and 5.75 μM KIN) after 27 days followed by in T11 (4.4 μM BAP+ 6.9 μM KIN) and T9 (4.4 μM BAP+ 4.6 μM KIN). According to the Table 1 the highest percentage of shoot proliferation (76%) was observed in Sadori (4x) at the end of 4 weeks of growth of shoot tips in MS media supplemented with 4.4 μM BAP and 5.75 μM KIN (T10) followed by S-12 (69.6%), *G. arboreum* L. (68%), and *G. herbaceum* L. (55%) respectively. Tetraploid varieties of cotton responded to the tested treatments of growth regulators first. During the same time frame, *G. arboreum* L. (2x) and *G. herbaceum* L. (2x) revealed the highest shooting in treatment 11 that was 72% and 60.8% respectively. The difference between these two treatments is the concentration of KIN. Negative control did not show any shoot proliferation until 10th week while during next two weeks only one to three explants out

Table 1

Shoot Frequency (%) of four cotton varieties after 4–12 weeks in *in vitro* growth.

Treatments MS media + growth regulators (μM)	Shoots proliferation frequency (%)											
	4 weeks				8 weeks				12 weeks			
	A	B	C	D	A	B	C	D	A	B	C	D
T0 = No BAP& KIN	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.8	2.4	1.6
T1 = 0.0 BAP+ 4.6 KIN	20	24.8	20.8	31.2	26.4	30.4	40.8	45.6	41.6	58.4	48.8	56
T2 = 0.0 BAP+ 5.75 KIN	13.6	16	17.6	15.2	23.2	32.8	37.6	42.4	58.4	61.6	60.8	61.6
T3 = 0.0 BAP+ 6.9 KIN	11.2	16.8	23.2	20	28.8	27.2	39.2	47.2	60	63.2	55.2	64
T4 = 2.2 BAP+ 0.0 KIN	28	23.2	33.6	31.2	56.8	54.4	62.4	57.6	83.2	79.2	81.6	85.6
T5 = 2.2 BAP+ 4.6 KIN	41.6	45.6	46.4	43.2	64.8	72.8	63.2	61.6	89.6	89.6	90.4	95.2
T6 = 2.2 BAP+ 5.75 KIN	44	47.2	40.8	48	68.8	65.6	60.8	64.8	91.2	92	97.6	94.4
T7 = 2.2 BAP+ 6.9 KIN	39.2	37.6	35.2	43.2	65.6	63.2	61.6	62.4	88.8	94.4	89.6	93.6
T8 = 4.4 BAP+ 0.0 KIN	34.4	36.8	39.2	36	63.2	64.8	71.2	65.6	88.8	92.8	90.4	95.2
T9 = 4.4 BAP+ 4.6 KIN	63.2	57.6	65.6	71.2	98.4	100	100	100	100	100	100	100
T10 = 4.4 BAP+ 5.75 KIN	68	55.2	69.6	76	100	99.2	100	100	100	100	100	100
T11 = 4.4 BAP+ 6.9 KIN	72	60.8	68.8	73.6	97.6	96.8	100	100	100	100	100	100

of 25 total explants showed shoot growth that resulted in a shoot proliferation frequency of 0.8–2.4%. Recorded data also revealed that the treatments without BAP (T0 = 0.8–2.4%; T1 = 20–56%; T2 = 14–61.6%; T3 = 11–64%) were least effective and demonstrated poor shoot proliferation i.e. in the range of 0.8–64% (Table 1). BAP is a cytokinin that promotes shoot growth and all the evaluated concentrations of BAP promoted shoot growth for example at the concentration of 2.2 μM BAP (T4 = 23–85.6%; T5 = 42–95%; T6 = 41–97.6%; T7 = 35–94.4%) revealed an increase in the rate of shoot proliferation as compared to treatments without BAP and when the concentration of BAP was increased to 4.4 μM the shoot proliferation rate increased (T8 = 34–39%; T9 = 58–100%; T10 = 55–100%; T11: 61–100%). Interestingly, the treatments containing only KIN, were also found least effective but better then control. BAP alone showed better shoot induction as compared to KIN alone. Nevertheless, the interaction of BAP and KIN is significant as compared to treatments containing BAP or KIN alone. BAP at the concentration of 4.4 μM with different concentrations of KIN (T9 = 4.6 μM KIN; T10 = 5.75 μM KIN; T11 = 6.9 μM KIN) showed the best shoot proliferation in both tetraploid and diploid cotton varieties with a rate of 100% after 8-weeks of *in vitro* growth (Table 1). In summary we can finalize that the treatment T9, T10, and T11 are equivalent in their effectiveness to induce *in vitro* shooting in cotton explants, nevertheless T10 revealed shoot growth within the shortest time period of 33-days followed by 35 days (T11) and 48 days (T9) on average (Insert Table 1 here).

3.2. Effect of BAP and KIN on days taken to initiate shoots per cotton explant

The analyzed data of days taken to initiate shoots per cotton explant in MS media supplemented with different concentrations of phytohormones (BAP + KIN), are presented in Table 2. Explants were shoot tips and data was recorded continuously during the first 3-months for this parameter. The statistical analysis of variance for the days taken to induce shoots of four cotton varieties demonstrated that the one of the main effects i.e. treatments (12 different concentrations of phyto-hormones (BAP + KIN) is highly significant (F = 110.49 @ p = 8.37E-77). However, second main effect (cotton varieties/genotypes) and interaction of these two factors is non-significant at the probability level of p = 0.05. The results revealed that the minimum days taken to initiate new shoots were 27.2-days that were observed in MS basal media T10 whereas maximum days (77.8-days) were taken by treatment-3 (containing KIN alone as 6.9 μM). The negative control induced shooting after 70.95 days. KIN alone in treatments T1, T2 and T3 revealed an interesting trend that as the concentration of KIN was increased the shoot induction is delayed and shoots took longer to be visible. KIN at low concentration showed shoot induction pattern equivalent to

Table 2

The effect of different concentrations of BAP and KIN on the *in vitro* growth of selected cotton varieties. Note: Means represent 5 replications with 25-explants in each. Tukey Multiple Range Test is applied to find the difference among means at p = 0.05. Means followed by same letters are not significantly.

Treatments (μM)	Days to shoot	Shoots per explant
T0 = No BAP & KIN	70.95abc	0.09j
T1 = 0.0 BAP+ 4.6 KIN	69.25abcd	0.79h
T2 = 0.0 BAP+ 5.75 KIN	73.3 ab	0.55h
T3 = 0.0 BAP+ 6.9 KIN	77.45a	0.34i
T4 = 2.2 BAP+ 0.0 KIN	62.55bcde	1.17g
T5 = 2.2 BAP+ 4.6 KIN	58.65def	1.67e
T6 = 2.2 BAP+ 5.75 KIN	54.5ef	2.06d
T7 = 2.2 BAP+ 6.9 KIN	69.45abcd	2.06d
T8 = 4.4 BAP+ 0.0 KIN	59.35cdef	1.40f
T9 = 4.4 BAP+ 4.6 KIN	48.1f	3.28c
T10 = 4.4 BAP+ 5.75 KIN	32.95g	5.92a
T11 = 4.4 BAP+ 6.9 KIN	34.7g	4.40b

negative control, however KIN at the concentration of 6.9 μM took the longest time (77.45 days) to induce shooting (Table 2). As the BAP was introduced in MS media like in T4 (BAP 2.2 μM alone) the number of days reduced to 62.6-days followed by T5 (58.65 days) and T6 (54.5 days) whereas T7 (2.2 BAP + 6.9 KIN) revealed an increase in the days taken to shoot induction that is up to 69.45-days (Table 2). Treatment-8 containing BAP at the concentration of 4.4 μM again boosted shoot induction and reduced the days to 59.35 days. The most efficient treatments were T9 to T11 which displayed shoot induction after 48, 32.95 and 34.7 days respectively. These all mean days are significantly different populations at confidence level of 95% in TMRT. Additionally, an increase in the concentration of KIN decreased the shoot proliferation and took more days to induce shooting. Negative control did not show any shoot induction during the first ten weeks (70.95 days) of observation. Nevertheless, on average treatment 10 is the optimal combination of hormones to induce shooing within minimum (32.95-days) time period, on average.

3.3. Effect of BAP and KIN on number of shoots per cotton explant

Data was recorded for the number of shoots per explant after 12-weeks of *in vitro* growth with regular subcultures within 20–15 days in the same treatment. The recorded data for this parameter is mean number of shoots per explant whereas each mean represents 5 replications with 25 total explants within each replication (Table 2). The statistical analysis of variance for the number of shoots of cotton showed that the one of the main effects i.e. treatments (12 different concentrations of phyto-hormones (BAP + KIN) are highly significant

Table 3

Root Frequency (%) of four cotton varieties after 4–8 weeks in axenic culture supplemented with different concentrations of IBA.

Treatments MS media with IBA (μM)	Roots proliferation frequency (%)											
	4 weeks				6 weeks				8 weeks			
	A	B	C	D	A	B	C	D	A	B	C	D
T0 = 0.00	0	0	0	0	0	0	0	0	0	0	0	0
T1 = 1.25	7.2	6.4	9.6	8.8	40.8	44	39.2	40.8	81.6	79.2	89.6	83.2
T2 = 2.50	10.4	8	8	7.2	31.2	39.2	46.4	48	78.4	85.6	83.2	81.6
T3 = 3.75	20.8	16.8	21.6	18.4	72	69.6	79.2	80.8	99.2	97.6	100	100
T4 = 4.90	14.4	17.6	19.2	17.6	71.2	70.4	78.4	73.6	95.2	96.8	100	100
T5 = 6.15	4.8	5.6	4	3.2	48.8	61.6	60.8	66.4	81.6	88	92.8	87.2
T6 = 7.40	7.2	8	6.4	7.2	47.2	56.8	56.8	64.8	85.6	79.2	80	95.2
T7 = 8.65	6.4	4.8	5.6	4	52	51.2	63.2	60	80.8	87.2	88.8	84
T8 = 9.80	5.6	5.6	8	8.8	49.6	52	55.2	62.4	78.4	84	80.8	87.2

Note: The total number of explants in each replication for this parameter was 25 and five replications were carried out to get the presented data. In table A, B, C, and D represent four cotton varieties *G. arboreum* L. (2x), *G. herbaceum* L. (2x), S-12 (4x) and Sadori (4x), respectively.

($F = 835.84$ @ $p = 1.1$E-155). However, second main effect (cotton varieties/genotypes) and interaction of these two factors is non-significant at the probability level of $p = 0.05$. The results presented in Table 2, revealed the maximum shoots per explant (6.0) in T10 and minimum shoots (0.34) were found in T3 while ignoring control readings. Thus, the phyto-hormones in combination were (KIN + BAP) found highly significant. Negative control showed very few shoot initiation during the whole course of study (0.09; Table 2). BAP (T4) and KIN (T1) alone showed few shoots per explant revealing these concentrations as inefficient. However, BAP alone performed better as compared to KIN alone. Based on the TMRT ten different mean populations were found showing significant differences among the effect of hormonal concentrations on the number of shoots. These different populations are indicated in Table 2 and means followed by the same letter are not significantly different. When BAP and KIN in combination added to media, they affected number of shoots positively and the shoot number was increased from 1.50 to 5.9. As the highest number of shoots are recorded in T10 therefore, it is the optimal treatment (Insert Table 2 here).

3.4. Effect of IBA on root frequency

The data for root frequency (%) of shoot tips of four cotton varieties in axenic culture are presented in Table 3. The effects of 9 treatments of IBA including control, on root proliferation was observed on 03 time intervals that were after 4, 6, and 8-weeks of *in vitro* culture. Root frequency is a percent measure of shoots revealing visible root induction out of the total shoots in the same treatments of growth regulators. The first visible sign of root proliferation in selected cotton varieties was observed after 16-days (T3 = 3.75 μM) after 16 days followed by 17.2 days (T4 = 4.90 μM) of *in vitro* culture media containing IBA. During this experiment the shootlets were sub-cultured after every 15 days on the same media regime. According to the Table 3 the highest percentage of root induction (21.6%) was observed in S-12 (4x) at the end of 4 weeks of growth of shootlets in MS media supplemented with 3.75 μM IBA (T3) followed by *G. arboreum* L. (20.8% in T3), S-12 (19.2% in T4), Sadori (18.4% in T3), *G. herbaceum* L./Sadori (17.6 in T4) and *G. arboreum* L. (14.4% in T4) respectively. After 6-weeks of *in vitro* growth, treatments T1 and T2 revealed variable root growth response ranging between 31 and 48%. However, treatments T5, T6, T7 and T8 revealed 49–66% root proliferation rate in all tested cotton varieties after 6-weeks of growth. Negative control did not show any root proliferation during the whole course of study that is about two months. IBA is an auxin that promotes root growth and all the evaluated concentrations of IBA promoted root growth. It is obvious from corresponding data table that as the concentration of IBA is increasing in MS media the root induction rate is also increasing; this trend is prevailing

un-till the concentration of IBA reached to T4 = 4.90 μM further increase in concentration showed a depression in the rate of root induction. At the end of 8 week (Table 3) of *in vitro* culture of all cotton varieties studied in the present investigation, the rate of root proliferation was increased to 95.2–100% in treatments T3 and T4. Treatment without IBA did not show any root induction whereas T1 and T2 revealed root induction up 90%. Furthermore, in T1 and T2 the diploid and tetraploid varieties demonstrated the root induction in the range of 78.4–85.6% and 81.6–89.6%, respectively. In summary T3 and T4 are equivalent in their effectiveness to induce *in vitro* rooting in cotton shootlets, nevertheless T3 used less concentration of growth regulator IBA thus will reduce the final cost of plantlets produced using this media regime (Insert Table 3 here).

3.5. Effect of IBA on the days taken to initiate rooting

The results for the day taken to initiate rooting on cotton shoots as affected by different concentrations of IBA are presented in Table 4. The ANOVA ($F = 1236.99$ @ $p = 2.57$E-69) for this parameter revealed that the main effect of treatments is highly significant. Treatments were 9-different concentrations of IBA. However, when TMRT ($p = 0.05$) was utilized to find out the significant mean populations it indicated the presence of 3-different mean populations. Population one represent T0, MS media without IBA, second population consists of T3 and T4 whereas third population consists of T1, T2, T5, T6, T7 and T8 (Table 4). Treatment 3 and 4, representing one population, took 15.95 and 17.15 days respectively to initiate rooting on shoots. Further table reflects that the negative control did not show any root induction during the whole course of this experiment that was 4-weeks. However,

Table 4

Effect of IBA on days taken to initiate rooting and survival rate of cotton plantlets generated through *in vitro* propagation.

Treatments (μM IBA)	Days to root	Survival rate
T0 = 0.00	0c	00c ± 0
T1 = 1.25	22.75a	78.00 ± 10.36
T2 = 2.50	22.35a	85.50b ± 7.59
T3 = 3.75	15.95b	86.75 ± 3.42
T4 = 4.90	17.15b	91.75 + 6.02
T5 = 6.15	23.65a	86.75 + 6.02
T6 = 7.40	24.2a	83.50 + 4.79
T7 = 8.65	23.95a	76.75 + 3.59
T8 = 9.80	23.85a	69.75 + 2.98

Note: Means represent 5 replications with 25-explants in each. Tukey Multiple Range Test is applied to find the difference among means at $p = 0.05$. Means followed by same letters are not significantly different. Percentages represent at least 20 explants in each.

as the concentration of IBA was increased in media to 2.0 mgL^{-1} the days taken to induce root were again increased. The comparison between negative control and other treatments showed that without IBA induction of roots is not possible during 4-weeks of *in vitro* culture. Although treatment 3 and 4 are optimal, not significantly different and took minimum days to induce roots T3 can be utilized for mass production of rooting in shoot tip cultures of cotton as this treatment utilizes less growth regulator which will affect the overall cost of *in vitro* generated plantlets.

3.6. Effect of IBA on survival rate (%) of in vitro generated plantlets of cotton

The survival rate (%) of cotton plantlets generated *in vitro* is presented in Table 4. After successful regeneration of *in vitro* plantlets, initial acclimatization took about a week and during that time bottles of plantlets were kept open for some time in growth room. Such hardened plantlets with well-developed roots were removed from the culture vessels without damaging the roots and agar was washed thoroughly from the roots in warm water. These plantlets transferred to small pots filled with soil and coir (1:1). The results revealed that the highest survival rate of *in vitro* generated cotton plantlets was 91.75% (Insert Table 4 here).

3.7. Genotype independent media regime

In all the studied parameters the main effect of genotypes and interaction of treatments and genotypes came out to be non-significant revealing the fact that this media regime is not affected by genotypes (Table 5). Thus a genotype independent media regime for tissue culture of cotton in selected varieties.is optimized in the present study (Insert Table 5 & Fig. 1 here).

3.8. Molecular diagnosis for CLCuV free plantlets

The isolated DNA yielded 310.00 ± 23.09 μg μl^{-1} gram^{-1} of tissues, DNA quality (A$_{260}$/A$_{280}$) was 1.91 ± 0.06, enzymatic digestion (EcoRI and HindIII) and PRC compatibility was also good (data not shown). In vitro generated plantlets of different varieties were randomly selected time to time and DNA was isolated. PCRs were conducted using primer pairs CLCV1/CLCV2 to get a 1350 bp long PCR product however, no such band was observed from the *in vitro* generated cotton plant material. Thus, molecular diagnostics proved that the tissue cultured material was CLCuV free ((Insert Fig. 2 here).

4. Discussion

The first study on *in vitro* morphogenesis of cotton through somatic embryogenesis was reported in early 1980 (Davidonis, and Hamilton, 1983), since then a range of explants is used to initiate cotton tissue culture. Thus, different tissue culture based protocols and regeneration methods of cotton are available but these are genotype dependent. Shoot regeneration from shoot tips/apex is quite simple as compared to somatic embryogenesis. It needs less time to regenerate plantlets and the plantlets regenerated through this methodology are true to type and have very low rate of soma-clonal variation (Ahsan et al., 2014).

Application of shoot tissues in cotton micropropagation is demonstrated by many researchers. Aslam et al. (2010) successfully developed the apical meristem culture of cotton cultivars CIM-443, CIM-446, NIAB-Karishma, FH-900 and NIAB-98. Shoot tips and cotyledonary nodes of G. arboreum were used as explants and micro-propagated plantlets were obtained (Laud et al., 2010). Embryonic axes of three days old *in vitro* germinated seeds of G. arboreum were utilized to get direct shoot organogenesis (Talegaokar and Dangat, 2010). Shoot apices of two cotton G. hirsutum cultivars, Nazilli 84S and Cukurova 1518, were micro-propagated to healthy plantlets (Ozyigit and Gozukirmizi, 2008). Shoot tips of 5–7 days old seedlings of G. arboreum and G. herbaceum were micro-propagated to get complete plantlets within 6–8 months (Saeed et al., 1997; Yasin and Yasmin, 2018). In present study shoot tips (without cotyledons) of 7–10 days old seedlings of selected genotypes were excised and micro-propagated. In accordance to (Bushra et al., 2004), it is observed that the shoot tips from seedling younger than 5 days and older than 10 days are difficult to isolate because of their tininess and hardness respectively whereas more mature explants produce phenolic compounds that hamper *in vitro* growth. In contrast, there are different investigations where researcher have used smaller shoot apex and got success (Gupta et al., 1997; Saeed et al., 1997). Ozyigit and Gozukirmizi (2008) demonstrated that the 7-days old explant discharge less phenolics in culture media and we also observed this phenomenon.

In cotton tissue culture mostly MS (Murashige and Skoog, 1962) medium is utilized as basic media to provide nutrients to explants (Saeed et al., 1997; Yasin and Yasmin, 2018). MS basal media is further supplemented with cytokinins like BAP and KIN or auxins like IBA, NAA etc. BAP and KIN are cytokinins that regenerate shooting and promote cell division while auxins induce rooting in *in vitro* cultures (Agrawal et al., 1997). Hereby different concentrations of BAP and KIN were evaluated for shooting. BAP 4.4 μM in combination with KIN 5.75 μM in MS medium gave 100% shooting within 8-weeks and 5.92 shoots per explant in 12-weeks. According to Bushra et al. (2004) there is no need to add hormones in basal media as plant tissues naturally have hormones however they observed this phenomenon in a different variety of G. hirsutum. In contrast we found very low shoot initiation when hormones were not added into media. The observed differences could be physiological state of explants, genotypic effects or seasonal variation. The results of present study are in agreement to Pushpa and Raveendran (2010) who demonstrated the efficiency of shoot apex in MS medium and 0.1 mgL^{-1} KIN irrespective of the genotype. During *in vitro* micro-propagation the induction of multiple shoots in explants varied with hormonal concentration and was also influenced by the age of explants (Ozyigit and Gozukirmizi, 2008). MS salts without growth hormones did not support the multiple shoots induction in present study. According to Satyavathi et al. (2002) cytokinins alone or in combination with auxins are very effective for shoot proliferation. It is also reported that the KIN alone in media only supports the induction of single shoot (Bazargani et al., 2011). In present research without or at low concentration of BAP shoot induction was minimum and KIN alone was not able to induce good shooting however combination of BAP and KIN was found more effective for shoot induction, in agreement with Bazargani et al. (2011). According to (Saeed et al., 1997) in higher concentrations of auxin or cytokinin callus is induced at the base of a shoot tip that promotes the death of tip. Similar observations are

Table 5
Genotype independent media regime based on insignificant interaction of genotypes and treatments. Note: Tukey Multiple Range Test is applied to find the difference among means at p = 0.05. Means followed by same letters are not significantly.

Parameters	*G. arboreum* L. (2x)	*G. herbaceum* L. (2x)	S-12 (4x)	Sadori (4x)
Number of shoots per explant	1.94a	1.99a	1.98a	2.00a
Days taken to shoot	60.07a	60.20a	57.22a	59.58a
Days taken to initiate rooting	19.56a	18.82a	19.22a	19.67a

Explant preparation

Multiplication of shoot tips of cotton *in vitro*

In vitro generated cotton plantlets with well-developed rooting system

Fig. 1. In vitro culture of cotton.

recoded by Bazargani et al. (2011) and during the present study. However the use of low concentration of BAP is cost-effective and also stimulates normal organogenesis. A report demonstrated that the BAP and KIN in combination negatively affect shooting of cotyledonary nodes of a Sudanese *G. hirsutum* L. cv Barac B- 67 (Abdellatef and Khalafalla, 2007) which is contrary to our observation of *in vitro* growth of *G. hirsutum, G. herbaceum* and *G. arboreum* nevertheless we used shoot tips as explant and different cotton varieties as compared to

Note: M-1kb DNA ladder; 1: Negative control. 2: S-12 infected plant (positive control); lane 3-7: Randomly selected in-vitro plantlets; lane 8-13

Fig. 2. Molecular diagnostics of CLCuV in *in vitro* generated plantlets.

Abdellatef and Khalafalla (2007). Hence, it is important to discover optimal concentrations and combinations of growth regulators to get better growth response of explants *in vitro*.

Shoot and root related parameters like shoot or root frequency, number of shoots per explants and days taken to shoot or root vary among different reports due to the genotype used and hormonal combination applied. 100% shoot induction with 17 shoots per explant in 50-days at 4 mgL^{-1} BAP and 0.1 mgL^{-1} Thidiazuron (TDZ) in MS salts with B_5 vitamins and 3–5 cm long roots in ½ MS medium were recorded in *G. bickii* by Yang et al. (2010). In *G. hirsutum* and *G. arboreum* 86 and 90% shoot regeneration, 83.8 and 85% shooting efficiency, respectively, was observed on medium containing BAP and KIN as 1 mgL^{-1} whereas rooting was recorded in hormone free MS media (Khatoon et al., 2014). Upland cotton revealed 3.43 shoots per cotyledonary nodes in MS media having KIN 0.25 mgL^{-1} while 93.3% rooting regeneration was recorded in MS media with 0.5 mgL^{-1} NAA and 0.1 mgL^{-1} KIN (Rauf et al., 2004). Root induction in cotton tissue cultures is reported as a major problem and to solve this problem Gould et al. (1991) transferred *in vitro* generated shoots directly to the soil for root initiation. In another study, Gould and Magallanes-Cedeno (1998) grafted *in vitro* generated shoots on cotton seedlings. In contrast we used 3.75 μM IBA in MS basal media and roots were induced within 16-days however 100% root induction was observed in 8-weeks. As soon as good rooting system developed, plantlets were acclimatized and transferred in pots which showed good plant vigor and 86.75–92% survival rate. DNA was isolated from randomly collected micro-propagated plantlets from growth unit and tested for the presence of CLCuV through PCR. Primer pairs CLCV1/CLCV2 (Briddon et al., 2002) were used for molecular diagnosis of CLCuV that can amplify a 1350 bp long DNA fragment. However, no PCR product was amplified during this process hence *in vitro* generated cotton plant material was found virus free. The same molecular diagnostic approach was applied by numerous cotton researchers to identify CLCuV in infected cotton plants (Ullah et al., 2014; Akhtar et al., 2014; Rashida et al., 2005).

5. Conclusion

It is very clear from the above mentioned studies that cotton tissue culture is genotype dependent and different explants exhibit different regeneration capacities hence the first step of cotton *in vitro* culturing is usually is to optimize the basal media and supplementation of growth regulators for proper micro-propagation of selected cultivars. Nonetheless, in present study we have standardized a genotype independent media for 4-cotton cultivars widely cultivated in Sindh, Pakistan. This effort will start a new search for genotype independent media to improve cotton tissue culture. The work on local cotton varieties and their regeneration abilities will be a valuable source to generate masses of off-season plantlets and genetically modified plants in the future.

Author contribution

SY conducted experiments and prepared the manuscript, AY conceptualized the experiments provided technical assistance, helped in experimental analysis, manuscript preparation and discussion.

Conflict of interest

There is no conflict of interest.

Note: The total number of explants in each replication for this parameter was 25 and five replications were carried out to get the presented data. In table A, B, C, and D represent four cotton varieties *G. arboreum* L. (2x), *G. herbaceum* L. (2x), S-12 (4x) and Sadori (4x), respectively.

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