Embryogenic Capabilities of Cotton through Tissue Culture and Expression Analysis of SERK3 for Regeneration Potential

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ABSTRACT
Plant tissue culture technique was used to develop a regeneration procedure in local Pakistani cotton varieties. Three cotton varieties Coker 312, CEMB66 and Klean cotton were cultured on MS media supplemented with vitamins and growth regulators. The results showed that the regeneration potential of Coker 312 was higher, CEMB 66 moderate while Klean cotton lacks the cotton regeneration potential. The molecular analysis identified the Somatic Embryogenesis Receptor Kinase3 (SERK3) gene and its expression analysis confirmed that it plays role for embryo formation through tissue culture. Its low expression resulted in calli to die rather than forming embryos. Therefore, SERK3 gene is considered important for callus induction and its development into primary embryogenic and embryogenic stage. Further investigations will be helpful to understand the mechanism of regeneration potential of cotton through tissue culture.

Key words: Callus induction, Cotton regeneration, Embryogenesis, Gene expression, Plant tissue culture.

INTRODUCTION
Plant tissue culture is the technique in which plant tissues, cells, organs or different parts of plants are grown on a media (solid or liquid) supplemented with all necessary nutrients under specific sterile condition (Kumar et al., 2019). It is an essential method for clonal propagation, transgenic plants production and somatic cell genetics. It brings the genetic improvement in crops at a faster pace in comparison to conventional breeding techniques.

As a source of natural fiber, cotton (Gossypium sp.) is a most important cash crop of the world. Trade of cotton reaches over 500 USD billion every year (Zhang et al., 2013). Pakistan is the 5th largest producer and 3rd largest exporter of cotton. It contributes 10% to GDP and 55% to Foreign Exchange Earnings of Pakistan. Thus, cotton is crucial to the economies of the countries throughout the world.

Somatic embryogenesis (SE) means transformation of somatic cells into embryogenic cells which is a cell differentiation process and involves de-differentiation and re-differentiation of plant tissue for reconstruction of somatic cells to generate embryogenic cells (EC) and it is also considered a classic example of cell-totipotency (Yang and Zhang, 2010). Somatic embryogenesis in cotton is challenging as it is recalcitrant. This limitation is due to long incubation time for callus induction, low frequency of formation of embryos, genotype dependence for regeneration, lack of information about inheritance and molecular mechanism during in vitro embryogenesis, somaclonal variations and tissue culture media composition (Ahsan et al., 2014). Therefore, only few cotton varieties belonging to Gossypium hirsutum has ability of regeneration like Coker (Khan et al., 2006), Sicala, Siokara (Cousins et al. 1991), Simian-3 (Zhang et al., 2001) and Acala varieties (Rangan and Rajasekaran 1996).
emergence of radicle, embryos were transferred to MS media (Murashige and Skoog, 1962).

**Callus initiation and maintenance**

Callus Induction media (CIM), Callus Maintenance media (CMM) and Somatic Embryogenesis Induction Media (SEIM) were utilized for callus initiation and maintenance. Composition of CIM is MS media, 100mg/l myo-inositol, B5 vitamins (Gamborg et al., 1968) and 30g/l glucose. Media was supplemented with the growth regulators such as: (CIM1) 0.1mg/l 2,4-D + 0.5mg/l kinetin (CIM2) 0.4mg/l NAA + 0.4mg/l kinetin. Composition of CMM is MS media containing 100mg/l myo-inositol, B5 vitamins and 30g/l glucose. The SEIM contained MS media without NH4NO3 and concentration of KNO3 was doubled, 100mg/l myo-inositol, B5 vitamins and 30g/l glucose. Media were solidified with 3g/l phytagel and 0.75g/l MgCl2. pH was adjusted to 5.8 and autoclaving for 20 minutes at 121°C.

Hypocotyl of 7 day old seedlings were sectioned into 4mm and placed on CIM. Four to six explants were placed on 20 x 100 mm culture plates and incubated at 16:8h light intensity was used for 24 hours as they grew better and retained their potential to grow in light conditions, as it was reported by Smith et al., (1977). Hypocotyls from 7 day old embryos of Coker 312, CEMB-66 and Klean cotton were collected at three stages of Coker 312 and CEMB-66 were used. Reaction conditions were: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec followed by annealing at 58°C for 35 sec and extension at 72°C for 40 sec and then the final Extension at 72°C for 10 min.

Gene Expression Analysis by Real Time Quantitative PCR (qRT PCR)

For gene expression analysis (Fernández et al., 2006), qRT PCR was performed with both the Primer sets in 96 well plate (BioRAD) with iQTM SYBER-Green Super mix (BioRAD) in iQ5 Cycler (BioRAD). For internal control, GAPDH was used. In each reaction 50 ng cDNA was collected at three stages of Coker 312 and CEMB-66 were used. Reaction conditions were: initial denaturation at 95°C for 5min, 30 cycles of denaturation at 95°C for 30 sec followed by annealing at 58°C for 35 sec and extension at 72°C for 40 sec and then Extension at 72°C for 10 min. For statistical analysis, iQ5 software version 1.0 was used which take into account CT values of the gene of different samples and normalized with GAPDH.

**RESULTS AND DISCUSSION**

**Seed germination**

Seed germination ratio has been assessed after 48 h. Germination ratio for Coker312 was 100% and around 70% germination for local varieties has been documented.

**Development of non-embryogenic calli**

For callus induction and later on for embryogenesis, high light intensity was used for 24 hours as they grew better and retained their potential to grow in light conditions, as it is reported by Smith et al., (1977). Hypocotyls from 7 day old embryos of Coker 312, CEMB-66 and Klean cotton were transferred to CIM. The callus induction was high and efficient in CIM1 containing 0.1mg/l 2,4-D + 0.5mg/l kinetin as growth regulators. Combination of 2,4-D and kinetin is considered as best for callus induction (Trolinder and Goodin, 1987). Hypocotyls cultured on media containing NAA produced considerable amount of calli in a longer period of time. The optimum concentration of NAA is good for rooting of the shoots generated through tissue culture (Anandan et al., 2019). This result was in accordance with Trolinder and Goodin (1987, 1988 a, b).

The cDNA was confirmed by GAPDH Primers which were designed for 200bp product of GAPDH as internal control. PCR reaction mixture 20µl consisted of 0.5µl cDNA, 2µl 10X PCR buffer, 2µl 25mM MgCl2, 2µl 1mM dNTPs, 0.5µl 10pmol each GAPDH F.P and R.P, 0.3µl 5U/µl Taq polymerase and 12.2µl Nuclease Free water. PCR cycling conditions were kept as initial denaturation was at 94°C for 2 min, followed by 25 cycles at 94°C for 30 sec, 55°C for 45 sec, 72°C for 50 sec and extension for 10 min at 72°C.

After confirmation, gene specific PCR was carried out. Reaction mixture 20µl consists of 2µl cDNA template, 2µl 10X PCR buffer, 2µl 25mM MgCl2, 2µl 1mM dNTPs, 2µl 10pmol each F.P and R.P, 0.5µl 5U/µl Taq polymerase and 7.5µl Nuclease Free water. Thermo cycler conditions were: initiation denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 sec followed by annealing at 58°C for 35 sec and extension at 72°C for 40 sec and then the final Extension at 72°C for 10 min.

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Calli from Coker 312 were growing faster and continuously proliferating and actively dividing as evident from their retention of greenish color. Calli from CEMB-66 and Klean cotton grew slowly and some of them turned brown and their growth became static. Browning of calli was due to the enzymatic reactions which results in the oxidation of phenolic compounds and chlorogenic acids (Cheng and Crisosto, 1995). This problem was solved by increasing the rate of subculturing within 2 to 3 weeks and discarded the brown portions which kept them fresh and growing condition (Chaudhary and Dantu, 2015). Subculturing the calli on fresh media is important for primary and mature embryo formation and keeping calli viable for a longer duration (Ahsan et al., 2014). It was observed that the calli was growing and attained non-embryogenic stage (Fig 1). Rate of callus formation was higher in Coker 312, moderate in CEMB-66 and lower in Klean cotton.

Formation of primary embryogenic calli

After six weeks, half of the calli from each species were shifted to CMM to initiate the embryo formation and the other half were kept on CIM for proliferation. The CMM lacks growth regulators as it is known that 2,4-D is not a promoter of differentiation and germination (Haq and Yusuf, 2004). Coker 312 calli began to form pro-embryogenic masses (PEM) as it was visible from the swollen portion that began to form on the surface of light greenish calli. In CEMB-66 only 60% calli formed PEM while none was observed in Klean cotton as all of them turned dark brown and died. The growing PEM calli were subcultured and observed with naked eye and under microscope (Fig 1). The combination of Auxin and Cytokinin is important for the regeneration of embryos and organs in tissue culture as Ashwini et al. (2018) also developed an efficient protocol for the regeneration of garlic from root meristem by using 2,4-D and BAP.

Embryogenic calli

As the swollen portion on the PEM calli increased, they were shifted to SEIM containing double the amount of KNO₃ and NH₄NO₃ was not added as the proliferation of calli was enhanced with KNO₃ (Haq and Yusuf, 2004). Proembryogenic calli forms the embryogenic calli (Fig 1a). Coker 312 calli showed active growth on SEIM but growth of calli from CEMB 66 was very slow. This may be due to media and genotype correlation as the correlation of media and genotypes is significant sometimes in the tissue culture procedure as observed by Sparjanbabu et al. (2019). The micrograph images of Coker 312 and CEMB 66 is given in Fig 2.

RNA estimation and cDNA confirmation

RNA was appeared as two distinct bands of ribosomal RNA, 28S and 18S on 0.8% agarose gel (Fig 3a). cDNA was confirmed by amplification of 200bp fragment of GAPDH primers in PCR (Fig 3b).
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Fig 2: Micrograph images of Embryogenic Calli in Coker 312 (A) and CEMB-66 (B).

Fig 3a: Qualitative analysis of Total RNA on 0.8% agarose gel electrophoresis isolated from Coker-312 at stage 1: non-embryogenic, Stage 2: Primary embryogenic and Stage 3: mature embryogenic.

Fig 3b: Qualitative analysis of cDNA of CEMB-66, Klean Cotton and Coker 312 at Stage 1: non-embryogenic, Stage 2: Primary embryogenic and Stage 3 Embryogenic

PCR for SERK3 gene identification in calli
Primer set 1 amplified the 203bp fragment while Primer set 2 amplified the 189bp fragment of SERK3. This gene belongs to the family SERK, which increase its expression during callus induction. They are Transcription factors which are involved in de-differentiation, trans-differentiation and cell totipotency (Pilarska et al., 2016).

The SERK3 was amplified in all the three stages of calli showing its importance in SE as it is required for the formation and maturation of embryos (Figure 4a, b). If the Phylogenetic analyses of the predicted sequences is performed, then that may be helpful to analyze or characterize the genetic role for accurate functioning of the gene for regeneration potential in the local varieties as has been observed by Dev et al. (2018) in Eggplant.

Gene expression analysis by qRT PCR
Real time PCR data analysis has shown that expression of SERK3 gene for Coker 312 was almost constant throughout the three phases as Cts < 29. The Ct values of non-embryogenic, primary embryogenic and embryogenic stages with primer set 1 were 22.96, 23.38 and 26.91 respectively while with Primer set 2 Ct values were 21.94, 22.33 and 24.13. As Ct value < 29 for primer set 1 and primer set 2, it shows a strong positive reaction which in turn indicates high expression of SERK3 during the three stages. The expression was highest during primary embryogenic stage (Fig 5a).

In CEMB66, Ct values in non-embryogenic, primary embryogenic and embryogenic stages with primer set 1 were 29.43, 23.34 and 28.64 respectively while with Primer set 2 Ct values are 25.24, 19.66 and 24.58 respectively. Ct value
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**Fig 5b:** SERK3 gene expression in CEMB-66 with Primer Set 1 and Primer Set 2. Expression of SERK3 drops down during primary embryogenic stage. Error bars indicate standard error.

**Fig 5a:** SERK3 gene expression in Coker 312 with Primer Set 1 and Primer Set 2. An increase in SERK3 gene expression is observed in chart. Error bars indicate standard error.

indicated a highest expression of SERK3 during primary embryogenic stage, thus indicating the fact that calli having the expression of SERK3 were able to transform into primary embryogenic stage and maintained the developmental process (Fig 5b).

**CONCLUSION**

It is concluded that Coker 312 is a standard for cotton regeneration through tissue culture. The calli remained alive during all the stages of somatic embryogenesis, showing its high efficiency for embryo formation. Local variety CEMB 66 has moderate and Klean cotton lacks the ability. Molecular analysis identified the SERK3 gene and its expression analysis confirmed that it plays role for embryogenesis. Its low expression resulted in calli to die. Therefore, SERK3 is considered important for callus induction and its development into primary embryogenic and embryogenic stage. Further investigations will lead the embryogenesis and organogenesis in local varieties of cotton.

**ACKNOWLEDGEMENT**

This work is financially supported by Higher Education Commission Pakistan (HEC).

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