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(RESEARCH ARTICLE)

An alternative cost effective method for diminishing contamination severity associated with plant cell and tissue culture

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Abstract

Contamination is one of the serious and major issues faced during the process of tissue culturing of any plants. Normally, in order to remediate and shorten the contamination problem; different percentage (%) of Sodium Hypochloride, common bleach and quantity of ethanol have been frequently utilized for surface sterilization to mitigate microbial contamination during tissue culture procedures. But in-spite of the use of these chemicals and its significant effect in remediating the contamination cruelty; the cost lead to the scientists are also focusing. The aimed of the present study was to find out an alternative safer and cost effective method for remediating contamination severity associated with tissue culture related aspects. Whereas, in present study we had cultured different crop species on different composition of nutrient medium as well applied various techniques of surface sterilization, and tested the plant viability through plant regenerations ability, performance under different concentrations, comparative analysis, gene expressions, sustain of chlorophyll content, ion membrane leakage, photochemical efficacy and microscopic analysis. However, it was interestingly noticed that through using sterilized surgical cotton instead of preparing media with solidifying agar; the results showed surprisingly innovative observation and showed almost zero contamination.

Keywords: Contamination; Sodium Hypochloride; Sterilization; Surgical Cotton; Tissue Culture

1. Introduction

Plant tissue culture is a tool for obtaining rapid, mass multiplication of disease free and true to type planting material (Singh, 2003). Thus, tissue culture has the benefits of rapid propagation of new varieties, regeneration of large number of true to type plantlets from a small tissue, elimination of pathogens and storage of plant germplasm under aseptic condition (Risher and Amler, 2005). On the other hand, *invitro* multiplication of plants is very useful because it provides a means of overcoming difficulties of producing large numbers of relatively homogenous seedlings and system extensively used in wide range of tissue culture investigations carried-out by many scientists. The findings of these techniques provided an argument for the use of tissue and cell culture techniques as tools for plants improvement. Recently tissue culture technologies are now extensively used as a biological tool for clonal propagation, disease elimination and mass propagation of several crop plants (Sharma et al., 2007; Durak et al., 2010).

Explants surface sterilization is one of the critical steps in plant tissue culture. During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; explants need to be surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period. However, there

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were various sterilizing agents used to remediate the pathogen issues; mercury chloride solution is utilized to mitigate microbial contamination in tissue culture (Ali et al., 2004; Tanaka and Makino, 2009). Though Sodium hypochloride, percentage of ethanol were also been used to kill the pathogen associating in tissue culture (Behera and Sahoo, 2009). Conversely, some micro-organisms are inhibited by acidification of culture media and by exudates from the plant tissues and may be weakly expressed or latent as endophytes in the tissues (Lal et al., 2009). As we described above that, micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods.

Micropropagation is a vegetative propagation of plant under aseptic conditions. The major advantage of micropropagation is the extremely high multiplication rates. Therefore, this technique is highly suited for rapid multiplication of rare genotypes, and of plants having rare genotypes. Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture and excess material produced can often be stored over long periods (Gosal et al., 2006). An often-cited disadvantage of modern plant tissue culture methods is the relatively higher costs involved as compared to other methods (Ali et al., 2004). The need for low-cost plant tissue culture systems, applicable for micropropagation and *in vitro* conservation of plant genetic resources, has been emphasized to allow the large-scale application of such technology in developing countries. The use of chemicals such as carbon sources, gelling agents, inorganic and organic supplements, and growth regulators in culture media, make this technique expensive. Sucrose is usually used as a source of carbon and agar as the gelling agent, and together they constitute the most expensive components of the culture media. The level of knowledge of cost-effective propagation of plant is not common and has slowed down domestication of many trees. The low cost propagation of plant is a solution for domestication and conservation of indigenous and threatened plant species. Therefore, the present hypothesized work has been planned to develop low cost technique for micropropagation of plant through tissue culture technique.

2. Material and methods

2.1. Collection of explants

The source of explants material was collected surrounding the area of Hyderabad district. The experiment was conducted at the Tissue Culture Laboratory, Department of Biotechnology, Faculty of Crop Production, Sindh Agriculture University, Tando Jam, Pakistan. The experiment was design through Completely Randomized Design (CRD) with four different biological replications.

2.2. Surface Sterilization

The seeds of different plants and explants (shoots) were thoroughly washed with sterilized distilled water followed by soaking 12hours in dark. However, on the next day the seeds were sterilized with 5% common domastic bleach solution followed by rinse three times under laminar airflow cabinet. The seeds were transferred to sterilized culture bottles containing MS-basal salts solution dipped with common surgical cotton. Although, for meristem culture the 5mm of bud section was used to regenerate the plants, though the basic procedure was followed same as described above for embryogenesis.

2.3. Preparation of nutrient media and culturing of explants

Different sources of plants (Explants) were cultured on MS medium (Supplementary Table 3) contaminating macro, micro nutrients, cystein (50 mg l^{-1}), Thiamine HcL (1.00 mg l^{-1}), Myo-insotol (100 mg l^{-1}) and 3g/L of to establish plantlets. After that MS medium with BAP (6.0 mg l^{-1}), cystein (50 mg l^{-1}), Thiamine HcL (1.00 mg l^{-1}), Myo-insotol (100 mg l^{-1}) and 30g/L sugar were used for multiplication of plantlets. Meanwhile there were no any ager quantity was used in the medium solution. Instead of agar we preferred to use surgical cotton into a medium. After two sub-culturing, the plantlets were transferred to new freshly media containing different concentrations BAP. The pH of the medium was adjusted to 5.6 to 5.8 with 0.1 N solution of NaOH or HCl.

2.4. Experimental Designs

Culturing of explants was performed according the culture technique as reported by Hussain *et al.* (2001), whereas; embryogenesis was performed with some modifications. The explants were cultured on Murashige and Skooge (MS, 1962) basal medium first for the formation of embryos. A total of 30 seedlings/explants were cultured on each of Basal medium supplemented with concentrations of 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L of BAP. The observations on the survival rate, days taken to initiate the shoots, total number of shoots and length of shoots, days taken to initiate the roots, total number of roots and length of roots were recorded.

2.5. Culture Maintenance

Cultures were maintained under incubation at 28°C for 24 hours in dark for embryogenesis and then transfer to growth room where fluorescent light having about 3000lux light intensity. The optimum temperature was determined under 25°C ±2 light and dark conditions in each 16/8 h cycle. After 3 to 4 weeks of shoot formation, actively growing cultures were transferred to fresh medium in jars for further growth and proliferations.

2.6. Phenotypic Analysis and observations

Observations were made at two days intervals; whereas the changes happen were measure routinely. The survival percentage, number explants regenerated, efficiency of embryogenesis, number of shoots, length of shoots, number of roots, length of roots, number of leaves, length of leaves, ion leakage, photochemical analysis, chlorophyll content, senescence of leaves and gene expression were observed.

2.7. DNA Extraction

For reverse transcriptase PCR analyses about one microgram of total RNA was used to synthesize complimentary DNA (cDNA) by using reverse transcriptase by following the manufacturer's procedures.

2.8. Quantitative PCR amplification

PCR reaction was carried out in a 25µl volume containing 1µL cDNA, 2.0µL of 10mM dNTP, 2.5µLPCR Buffer 1µL each primer (Supplementary Table 4), 0.25 µL Taq DNA polymerase. The PCR reaction was cycled in a GeneAmp PCR system. The PCR reactions were carried out by following cycle as: initial denaturation at 94 ºC for 5 min and 33 cycles of 30s at 94 ºC, 30 s at 57 ºC, 45s at 72ºC followed by incubation at 72ºC for 5 min (Hee et al., 2010).

2.9. Microscopic Examination

Encountering the contamination surveillances among the agar related cultures and surgical cotton related cultures were examined and then processed according to a standard protocol (Bowman et al., 1989). Scanning microscopy was performed using a Philip S3400N apparatus. AbsE cell death photography and cell counting were performed as described by Ryu et al., (2004). The slices were fixed in formaldehyde and then cleared in ethanol. The traces were drawn using Photoshop 7.0 software. Numbers of AbsE cells were counted using ImageJ software in a visual field.

2.10. Measurement of chlorophyll content

The fresh leaves of seedlings were processed immediately after collection. The collected leaves were chopped into fine pieces and a quantity weighing 0.5 g was measured off through an analytical balance. The quantified material was then homogenized by adding of 10ml of 80% acetone into the homogenizer. Since the concentration of pigments was too great for reading to be performed on a spectrophotometer, the obtained extract was diluted by adding 9 ml of 80% acetone ml-1 of extract (Guo et al., 2004).

2.11. Membrane Ion leakage

About 7 mm discs were cut from the upper part of both outer and inner leaves avoiding the central midrib. The discs were placed in distilled H_2O and washed by shaking for one hour to remove ions from the surface and the cut sections. Afterwards, 40 discs were placed in each Petri dish, 10 ml of dH_2O were added and the dishes were placed in the growth chamber. Every 24 hours the aqueous solution was collected, and the conductivity measured with a portable conductivity meter (Guo et al., 2004).

2.12. Photochemical Efficiency

The photochemical efficiency was deduced from chlorophyll fluorescence characteristics using a spectrophotometry efficiency analyzer. Further process was followed at described by Oh et al., (1997). Conductivity was expressed as the percentage of initial conductivity versus total conductivity.

2.13. Statistical Analysis

Data so obtained were statistically analyzed with independent experiment consisted of four different biological replications under Completely Randomized Design (CRD). Data were analyzed by one-way ANOVA and mean separation were performed by Duncan's multiple range tests. Statistics software SPSS version 16.0 (SPSS Inc., USA) was used to identify differences between observations. Data were been categorized as shown * and ** point out in significant differences at $P < 0.05$ and $P < 0.01$ according to Student's *t*-test respectively.

3. Results

3.1. Sustained existence of tissue cultured plants

The existence of tissue cultured plants is preferable than conventional breeding plants. Tissue cultured plants showed better yield, diseases resistance varieties, and stress tolerant varieties. Genetic engineering techniques are gaining popularity because the desired gene can be introduced from any source without species barrier in plant genome in short time to improve its characters. Consequently, establishment of reliable tissue culture protocols for shoot initiation, multiplication and regeneration is desired in order to improve plant yield through genetic transformation. In present study, the mass clonal production was obtained through the tissue culture technique. The explant mostly grown under the hormonal treatment without the presence of Agar in medium, instead of Agar; the common surgical cotton was used. The results on survival rate of plants were investigated and observed that mostly seedlings regenerated at different composition of media sustained the persistence (Figure 1E). Further it was interestingly noted that mostly seedlings survival percentage significantly greater that 80% of overall media, though length of shoots and number of shoots were also noted remarkable greater compared the plants grown on agar medium (Figure 1 B, D). Histological dimension represented that there was no significant effect of the cells size and number of cells encountered and noted normally plant possess (Figure 1C).

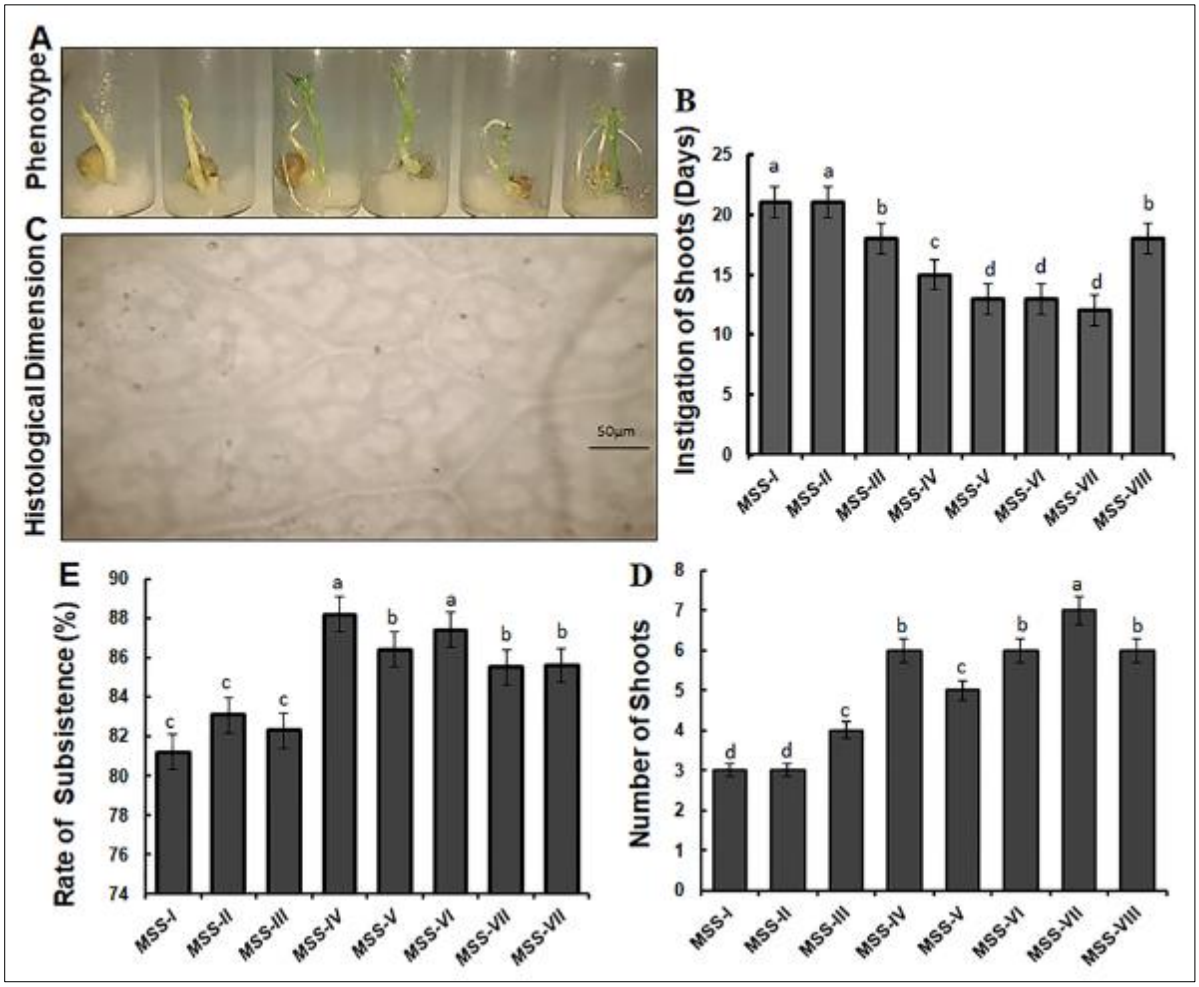

Figure 1 Sustained existence of tissue cultured plants. A) Phenotype of plants observed after sub-culturing. B) Instigation of shoots. C) Histological Dimensions. D) Encountering number of shoots. E) Rate of subsistence. Error bar represent the SD of the average from three different biological replicates. Asterisks denotes statistical significant differences using student's t-test ($P<0.05$, $P<0.01$)

3.2. Initiation, Proliferation and multiplication of shoot cultures

In order to further investigate and understand the mechanisms, the initiation and multiplication of shoots of fundamental steps of tissue culture observed. Results shows that shoot initiation and multiplication response of plant

were evaluated, that depend upon the type of plant that we grow in an aseptic artificial condition and the medium interaction that we used in different concentrations for the specific plant. For the shoots initiation we just only used MS media without agar having surgical cotton in base; that was observed after 16 days varying with the plant to plant at the specific temperature between 23- 25 ˚C and then observations was recorded. After initiation of shoots we add not only different types of plant growth regulators phytohormones for the multiplications of shoots but also added different concentrations of phytohormones observed in different types of plant for their better growth and development. After 18 days result revealed that, those plants with highest performance as well as those plants with lowest performance were recorded (Supplementary Figure S-1). The overall results were obtained remarkably well on medium MSS-V compared to that of other media (Figure 2A). Although, histological analyses proved that there were fewer changes happened inside the cells morphology (Figure 2A).

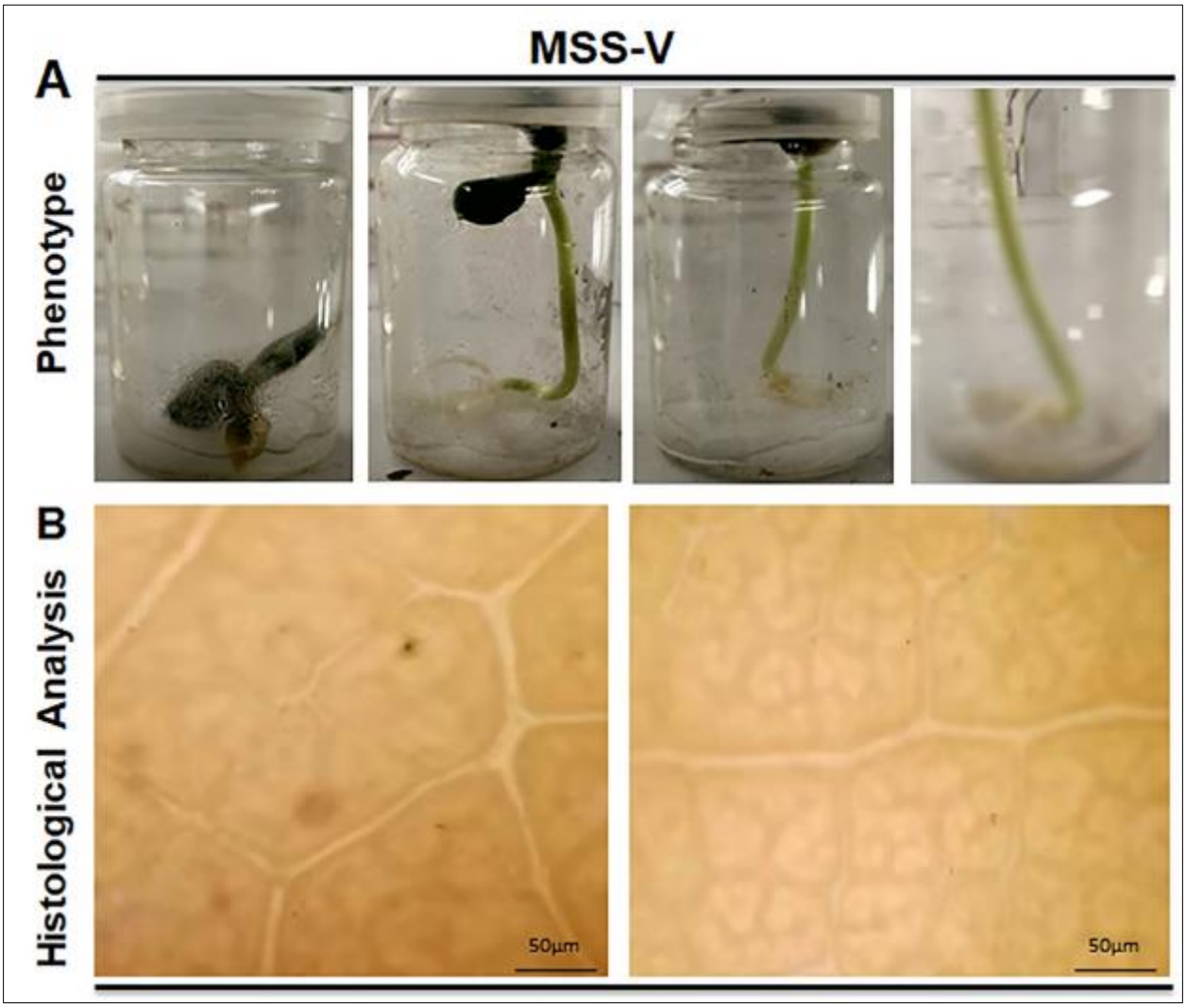

Figure 2 Mass Clonal Proliferation and multiplication of shoots. A) Phenotype of plants observed after three weeks of culturing. B) Histological analysis of each plants obtained though tissue culture. Error bar represent the SD of the average from three different biological replicates. Asterisks denotes statistical significant differences using student's t-test ($P<0.05$, $P<0.01$)

3.3. Hormonal competency regulates the shoot regeneration potential

Further we investigated that, the two different combinations of IAA and BAP and MS medium for shoot regeneration either has effect on mass production. The data revealed that shoot regeneration was affected by phytohormone combination/concentration in plant genotypes. The shoot regeneration was observed on different medium concentrations containing IAA 0.1, 0.5, 0.75, 1.0, 1.2, 1.5 1.75 and 2.0 mg/L followed by media containing BAP 0.5, 1.0, 2.0, 3.0, 4.0,5.0 and 6.0 mg/L. The best and the lowest performance were observed on the medium concentration where the best growth and development observed in specific plant (Figure 3 A, B, C). Further, the rate of sustained were also

observed and suggested that survival rate consistently higher among all the plant regenerated at various concentration of nutrient media (Figure 3D). Thus the maximum percentage of survival observed at higher concentration of BAP. The obtained results signifying that plant regenerated on surgical cotton instead of using costly agar grows well and sustained persistence among various tested nutrient media.

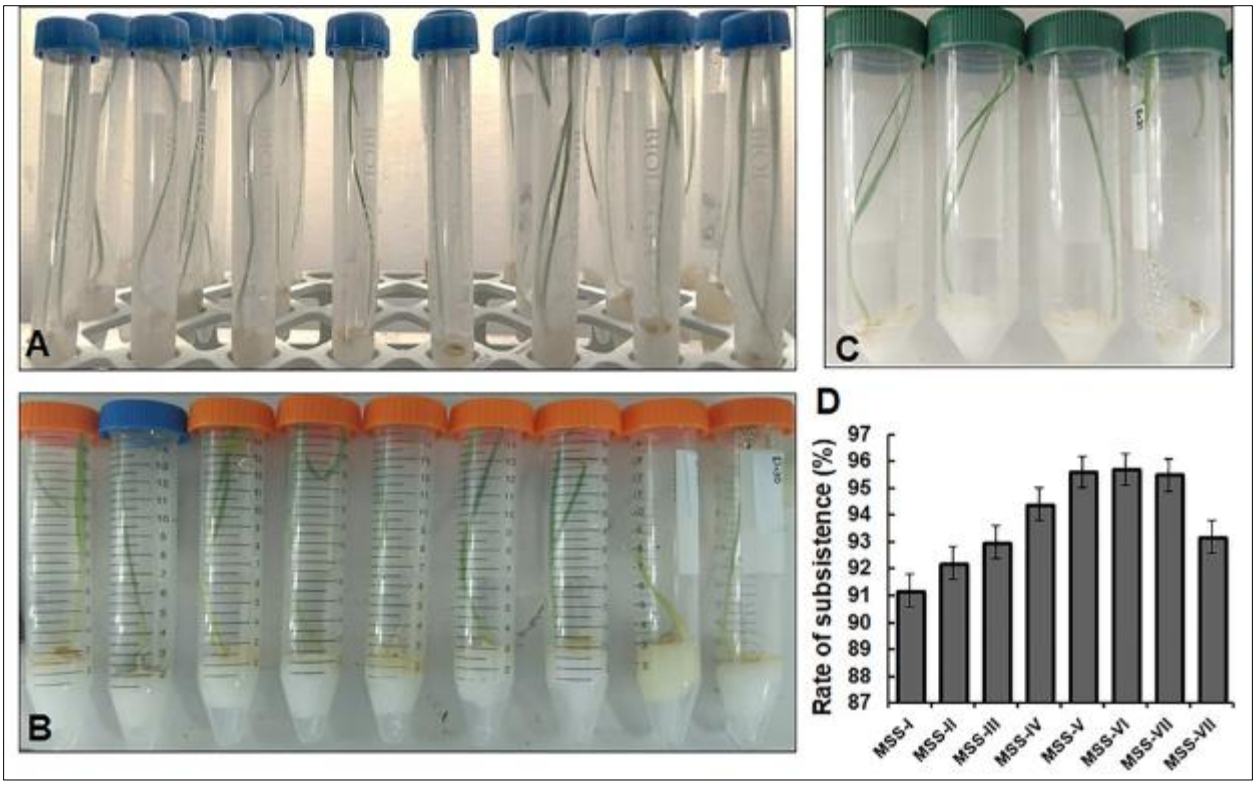

Figure 3 Hormonal competency regulates the shoot regeneration potential. A) Seedlings regenerated at the concentration of MS-Basal+3mg/L BAP+1mg/LNAA+0.5mg/L GA+ 30g Sugar. B) Seedlings regenerated at the concentration of MS-Basal+4mg/L BAP+1.2mg/LNAA+0.5mg/L GA+ 30g Sugar. C) MS-Basal+5mg/L BAP+ 1.2mg/LNAA +0.5mg/L GA+ 30g Sugar. D) Rate of Subsistence. Error bar represent the SD of the average from three different biological replicates. Asterisks denotes statistical significant differences using student's t-test ($P<0.05$, $P<0.01$)

3.4. Comparative analysis of surgical cotton and agar used in nutrient medium

Results showed that the growth of plants on cotton supported with MS medium per gram of cotton was superior to that on agar medium. The higher amounts of shoot regeneration of plants on cotton may be due to better nutrient diffusion on cotton than agar, and/or due to agar impurities. Further analysis revealed that shoot formation of plant was better on cotton than MS medium per gram than agar that was used as support for the explants. Plant was affected by the degree of cotton saturation. However, when MS medium was added to cotton, the number of shoots induced increased by more than half to the agar medium. Additionally, the length of leaves regenerated on various nutrient media consistently higher and showed remarkably better response (Figure 4A). Further, photochemical efficiency and ion leakage measurement also suggesting that there were no any significant loss observed amongst all the leaves regenerated on various media. Ion leakage noted normally in all leaves suggesting the existed level of plants physiology (Figure 4D). Also photosynthetic analysis via measurement of Chlorophyll content in all leaves of seedlings regenerated on various media, the overall percentage of chlorophyll content observed surprisingly higher more than 80% in all seedlings. Though, MSS-IV, MSS-V, MSS-VI and MSS-VII showed outstandingly higher percentage more than 90% in all leaves of regenerated leaves (Figure 4C). In order to further understand, the leaves were placed on medium having hormonal concentration for embryogenesis and senescence approaches. Phenotypic analysis of discs was measured at two days interval till the entire experiment (Figure 5). However, slight yellowing of leaves was observed at the beginning of infiltrations. Contrast to that, we had also treated leaves with normal water, and found that by using hormonal activity; senescence approach was accelerated and significantly increased the yellowing fading of leaves. After 20 days of infiltration of discs leaves, the leaves flattering to yellow faded was observed in treated of all treatments, instead using water, which slightly reduced senescence activity. Further, in order to confirm the efficiency and effectiveness, we

had also assessed the changes happened via expression, ion leakage measurement, chlorophyll content and PSA, SAG and CA gene expression (Figure 6 A,B,C,D).

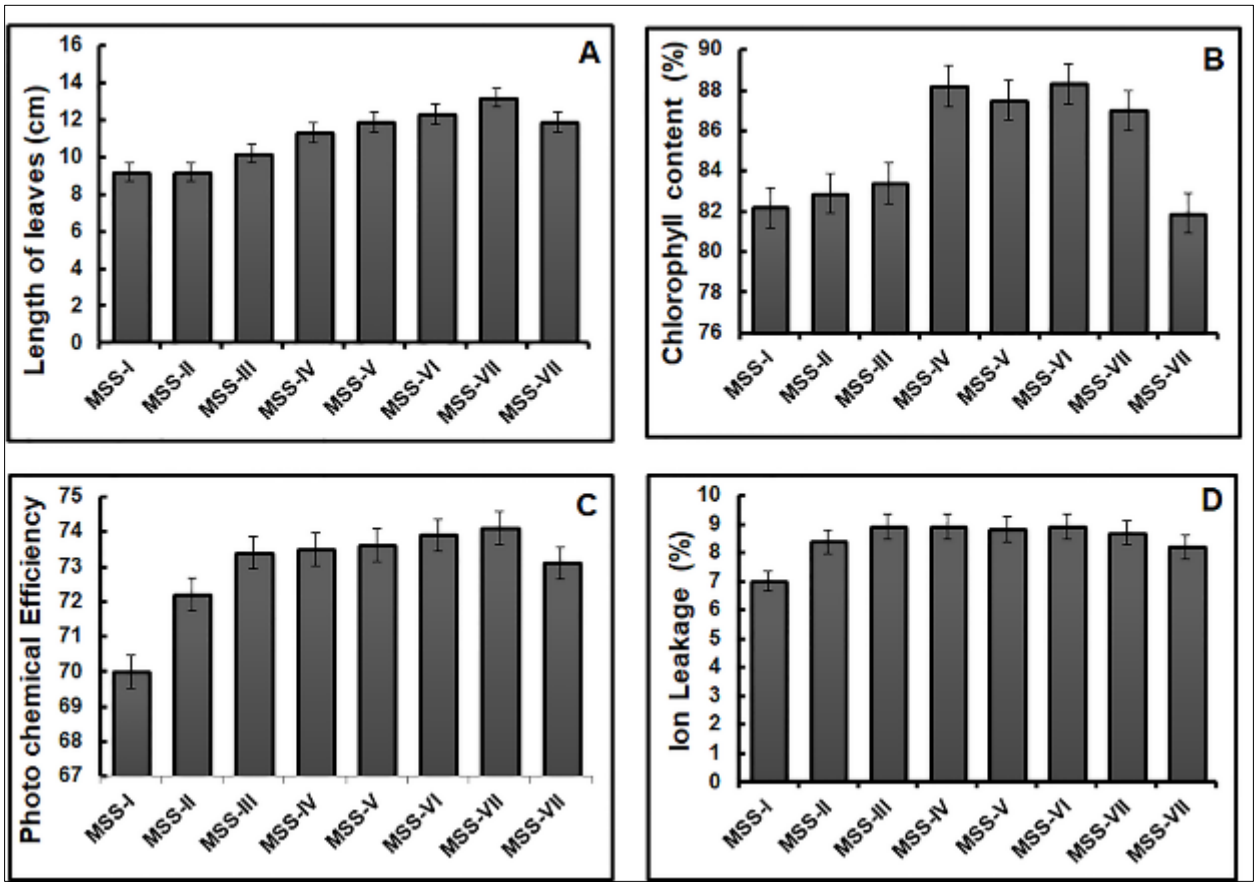

Figure 4 Regulation of media composition influence the behaviours of plants. A) Length of leaves. B) Chlorophyll Content. C) Photochemical Efficacy. D) Ion Leakage. Error bar represent the SD of the average from three different biological replicates. Asterisks denotes statistical significant differences using student's t-test ($P<0.05$, $P<0.01$)

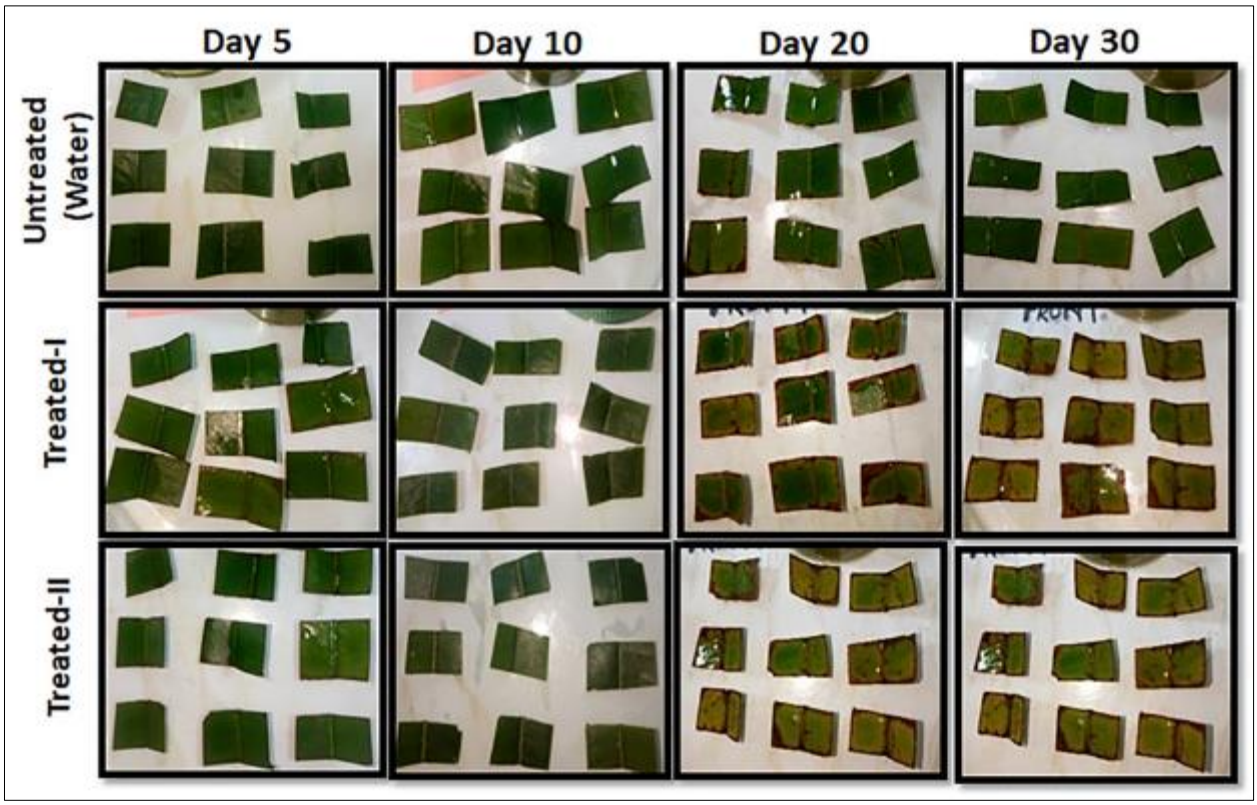

Figure 5 Phenotypic analysis of inoculated for somatic-embryogenesis. The sectioned leaves were placed surgical cotton with treated hormones about 25 days and zero contamination was found. Experimental data are average from three different biological replicates

3.5. Hardening and acclimatization of tissue culture regenerated plants

The plantlets with well-developed shoots and roots were transferred to pots having sterilized soil medium. After acclimatization where the plantlets moved to new environment were first transferred to the earthen pots for hardening in the green house and afterwards in the field. These plantlets are being evaluated for desired agronomic traits. Plantlets in the field for evaluation among the cytokinin and auxins showed best results for shooting and rooting purpose respectively.

3.6. Gene Expression Pattern and histological analysis

In order to further investigate the mechanism involved, the gene expression and histological analysis were measured. The photosynthetic associated gene (PSA) was used to measure the expressions in leaves. Almost in all leaves there were similar level of gene expression measured which proved that plant grows well on medium supplemented with various hormonal activity on surgical cotton used instead of agar (Figure 7). Histological measurement and analysis were also observed and resulted almost similar cellular activity that on control (Figure 8), the control samples were taken from the seedlings regenerated on agar medium. Amongst various medium, the plants were regenerated common surgical cotton showed better response.

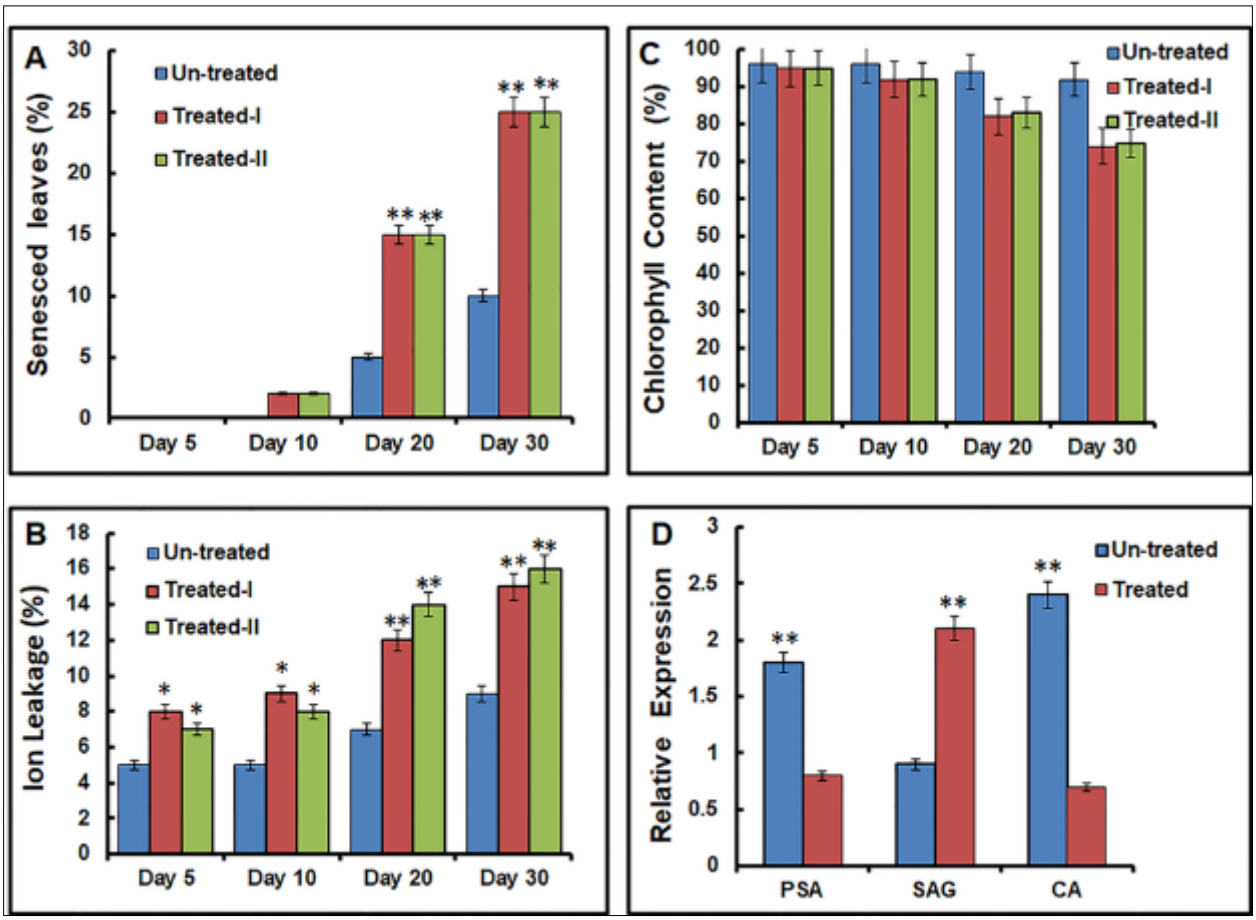

Figure 6 Senescence of leaves with minus contagion. A) Senesced leaves. B) Ion Leakage. C) Chlorophyll Content. D) Relative Expression. Error bar represent the SD of the average from three different biological replicates. Asterisks denotes statistical significant differences using student's t-test ($P<0.05$, $P<0.01$)

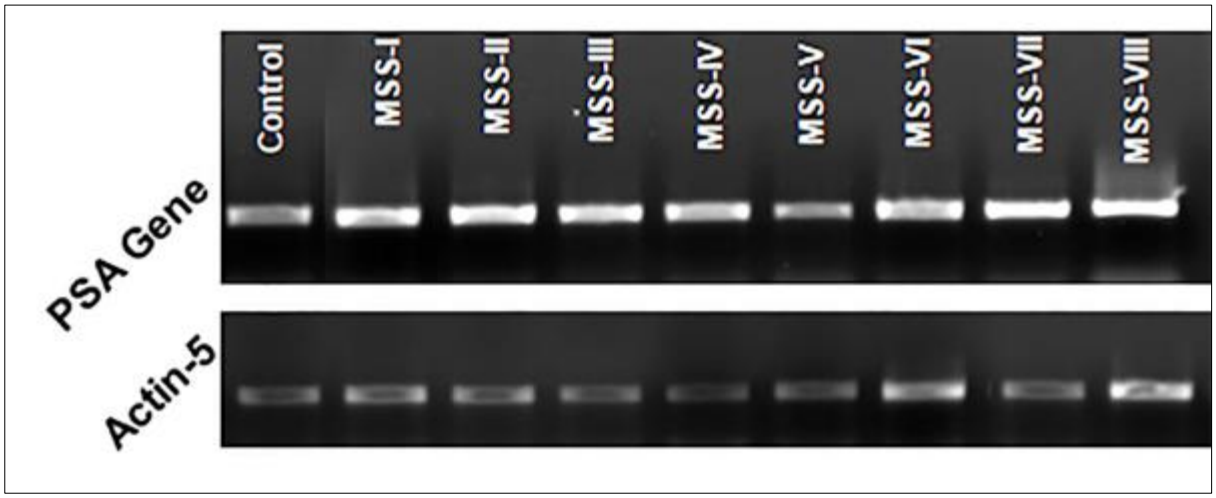

Figure 7 PSA Gene (a Photosynthetic Associated Gene) expression in seedling regenerated on different media. DNA was extracted after 12th day of regeneration and amplified though PCR. The total of 33 cycles were performed for all treated samples.

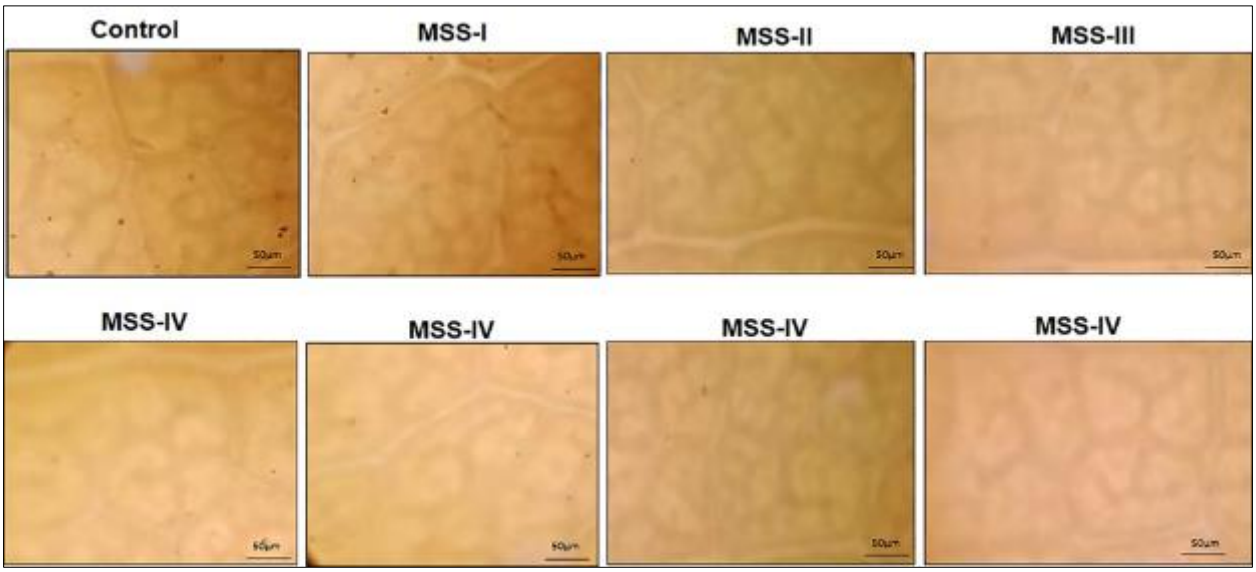

Figure 8 Histological analysis of seedling regenerated on different media. Scanning Electron Microscopy (SEM) was performed using a Philip S3400N apparatus. Photograph was performed using a Nikon IX-71 camera. Observation is the concise of three biological repeats.

4. Discussions

Conventional breeding methods in Pakistan are being used for enhancing the production and quality improvement of crop. However, limited gene pool availability and long duration of these methods are the major limitations for improvement of the crop through conventional methods. Genetic engineering techniques are gaining popularity because the desired gene can be introduced from any source without species barrier in plant genome in short time to improve its characters. Transformation of crop entirely depends upon regeneration of transformed explants through tissue culture. Consequently, establishment of reliable tissue culture protocols for shoot initiation, multiplication and regeneration is desired in order to improve crop yield through genetic transformation (Mehmood et al. 2013). The existence of tissue cultured plants is preferable than conventional breeding plants. Tissue cultured plants' showed better yield, diseases resistance varieties, and stress tolerant varieties for better development and growth of the plants.

Plants obtained through in vitro tissue cultures techniques such as micropropagation and shoot regeneration gave phenotypic variability that was due to true genetic changes. Chaleff & Keil, (1982), reported that some phenotypic variability was the result of physiological changes during In vitro conditions; such as use of different concentrations or levels of plant growth regulators BAP and IAA hence such plantlets normally revert to their parent type in field conditions and gave better result than their donor plant (Yasmin, et al. 2009). Tissue culture techniques for shooting purpose such as micropropagation and the different responses according to the treatments show an explant at the beginning of the culture. In this context, it is important to observe that the use of in vitro plants as explant source allows for more homogeneous material to begin testing the influence of different growth regulator combinations on the multiplication rate, avoiding the losses caused by contamination due to disinfection problems (Alderete, et.al. 2006). Although most plant tissues can grow well in liquid media, a semisolid medium is recommended for use under some circumstances. The contaminants present in agar can be released and affect the culture of plant tissues. Agar support is an expensive component of tissue culture medium. In the present study, rolled cotton fiber was tested as a possible substitute for agar because it is readily available and at a low cost. The use of fabric tissue (100% polyester) facilitates periodic changes of the medium without transfer of the cultured tissues, and this also may be true for cultures using cotton support Rita M. Moraes-Cerdeira. *et.al*. 1995).

5. Conclusion

The present study concludes that, use of surgical cotton in nutrient media instead of solidifying agar is the best option, as it is cost effective and showed low level of contaminations throughout the experiment.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors have read the manuscript and disclosed that they have no any conflict of interest.

Author's Contributions

AJK proposed the hypothesized work and wrote the manuscript. MA, MIK interpreted the results and suggested the line of work, MRR observed the parameters and SB and LZ revised the manuscript with critical opinions.

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