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Research Article

Somatic Embryogenesis and Regeneration in Chinese King Grass (*Pennisetum Purpureum* cv.)

Sadaf Younas[1], Plosha Khanum[1*], Ummara Waheed[1], Muhammad Zahid Rashid[2], Anum Masood Bokhari[3], Amina[2], Rao Wali Muhammad[4], Minahil Rashid[1], Fatima TU Zahra[5]

[1] *Institute of Plant Breeding and Biotechnology, MNS University of Agriculture Multan, Pakistan.*
[2] *Horticultural Research Institute, AARI Faisalabad Pakistan.*
[3] *Department of Horticulture, MNS University of Agriculture Multan, Pakistan.*
[4] *PARC Research and Training Station, BZU, Multan.*
[5] *Department of Plant pathology, University of Agriculture Faisalabad 38000, Pakistan.*

ABSTRACT

The demand for animal product is quickly expanding in Pakistan, due to increasing population which result in shortage of animal feed. Animal nutrition deficiency had negative impact on livestock productivity and production. Improving farm animal productivity is one of the major challenge nowadays. Therefore, we need to increase crop productivity by cultivating high yielding fodder varieties. King grass (*Pennisetum purpureum* cv.) belongs to family Poaceae. The shape is almost similar to sugar cane and very high in nutritional value. Freshly chopped king grass can be utilized as animal feed, hay, silage or feed pellets but serious problems such as pests and diseases, non-availability of good seed material affecting its nutritional value. Therefore, tissue culture technique provides an alternative method for producing disease-free and high-quality plant material at large scale. An efficient embryogenic callus of king grass was obtained which was further capable of plant regeneration. The MS medium combined with (MS + 2,4-D 4.0 mg/L) produced the best callus response in which callus was creamy yellow and nodular in shape and took minimum days for callus induction. The result shows that the king grass's new inner whorl of leaves and shoot apical meristem were very responsive to in-vitro somatic embryogenesis and plant regeneration by using hormones such as (BAP), (KIN), (IAA), (2 ,4- D) or (NAA). Regeneration under MS + BAP (1.5 mgL-1) was the best. The minimum days for rooting were taken by using MS + I A A (0. 5 m g L^{-1}) + N A A (1 .5 m g L^{-1}). While MS medium with BAP and NAA showed best response for shooting and rooting. In this study different parameters such as days taken to callus formation, callus color, shape, days taken to plant regeneration, shoot formation and root formation was observed. The study achieved efficient plant regeneration, shoot formation, and root formation, providing a viable method for large-scale production of high-quality King Grass for animal feed.

Keywords: Embryogenic callus, plant regeneration, plant growth regulators, morphogenic response.

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Correspondence
Plosha Khanum
Plosha.khanum@mnsuam.edu.pk

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INTRODUCTION

Forage grass is essential for successful livestock production as ruminants significantly depend on fodder for their feed and production (Chaudhury et al., 2000). King grass (*Pennisetum purpureum* cv.) belongs to the family Poaceae. It is a hybrid between elephant grass and pearl millet combining both for their advantageous traits. This grass is native to Africa, and it was brought centuries ago as animal feed in South America, Asia and Australia (Woodard, 1993). It is used in "cut and carry" systems all over the world in tropical and subtropical areas (Wadi et

al., 2004). This fast growing plant is a C4 grass that has high rate of photosynthetic production and possible to harvest the crop multiple times throughout the year, making it an important raw product (Zhou et al., 2020). It produces in large amounts, 1076 fresh tons per hectare each year (Anderson et al., 2008). The plant exhibits resistance to multiple cutting and regenerates rapidly, producing abundant edible green shoots. Therefore, enhancing the utilization and preservation of the king grass resources through knowledge-based approaches is predicted to have a significant positive impact on livestock value chains (Rego et al., 2010). This type of grass has great potential for generating dry matter per cultivated area and contains amount of soluble carbohydrates (De Jesus Ferreira et al., 2013). This by product is an alternative for use in ensilage of high moisture grasses because it has a high dry matter content as well as a great nutritional value, which minimizes effluent losses and improves the material quality (Licitra et al., 1996). The net energy content of king grass is 2070 kcal/kg, with 25.48% crude fiber, 11.88% crude protein, 0.7% calcium, 59.7% NDF, and 57% total digestible nutrition (Tuturoong et al., 2020). There are various factors that impact the composition of nutritive value of animal feed such as climate, agronomic techniques, feed processing method and genetic variability (Younas et al., 2005). It is frequently used as an alternative to conventional animal feed, mostly during the dry seasons, because of its high biomass output and use as a way to improve feeding in order to improve maximum weight and compensate for the lack of good quality pastures (Hendarto et al., 2022). The leaves and stems of the majority of king grass species were affected by a fungus that causes white mold (Farrell et al., 2002). Smutted heads is one of the disease which changes the plants shape instead of being fat and juicy as they normally are, the infected stems become rigid and shoot premature flowers that are thin and fibrous (Ratnadass et al., 2012).

The limitations of conventional breeding require the application of biotechnology (Cicerale et al., 2012). King Grass may become a very desirable crop for the production of fodder due to advancements in biotechnology. Improving the advantageous features of king grass can be achieved through genetic engineering or bioremediation and breeding. Making it more appealing as an animal feed includes tissue culture, metabolic engineering, and bioinformatics to improve its quality and taste. In the tropical regions, it's an indispensable feed option due to large production availability. It serves as an important feed resource in tropical areas due to its abundant availability. Due to its excellent agronomic characteristics as well as its suitable chemical composition, this grass has a strong potential to become one of the major energy crops. The problems of gene bank, limited genetic variation, and preservation of genetic resources can all be solved in several ways.

The issues of limited in vitro methods such as propagation, storage, and genetic diversity seem to be solvable in various ways (Snyman et al., 2011). To foster somatic embryogenesis, tissue culture from several explants of different grass species serves as a source (Vasil et al., 1982). On capable inflorescences and juvenile foliage, embryogenic callus forms rapidly (Taylor et al., 1992). Callus induction is a very important phase in evaluating the suitability of a genotype for tissue culture research and for plant tissue culture improvement programs (Visarada et al., 2002).

Forage grasses were cultured in callus tissue using excised embryos, shoot tips, as well as segments of immature inflorescences. Subsequently, through regeneration, a variety of plants with varying genetic composition were developed (Efferth, 2019). These methods, as pointed out by Vasil et al. (1983), make possible the successful cultivation of protoplasts, which are cell devoid of cell walls, essential for the regeneration of plants. It holds great potential for increasing plants due to the possibility these techniques have in generating maximum planting material and assuming a tissue-culture propagation system, provides a means of amplifying maximum plants (Barpete et al., 2014).

MATERIALS AND METHODS

Explants for culture were taken from different parts (meristem, leaf) of 4-5 months old healthy king grass cultivated in the field. Explants were rinsed under tap water for 10 to 15 minutes next to the collection of ex-plants, washed with d_2H2O, and then cut into small segments. All the instruments utilized in culture preparation having medium were autoclaved at 121^0C about 15 to 20 minutes for the purpose of disinfection. Finally, sterile and disinfected explant segments were placed over the medium. The explant like meristem of king grass was very useful for plant regeneration by using many hormones such as Benzylaminopurine (BAP), Kinetin (KIN), Indole acetic acid (IAA), (2,4-D), and Naphthalene acetic acid (NAA) . The treatments used for regeneration with three replications and one control for callus regeneration. The protocol optimized on (2,4-D) for somatic embryogenesis, BAP for shooting, regeneration, and NAA, IAA for root regeneration were used and monitored the results using CRD along with the parameters such as days taken to callus regeneration, callus color, shape, days for shoot and root regeneration. In laboratory-controlled conditions, CRD design under various parameters used to study the morphogenic response of explant to various

hormone treatments in king grass. Different treatments were used, and data was collected for various observations. The data that had been calculated were subsequently subjected to ANOVA. The means of the treatments were compared at $p < 0.01$ using HSD Tukey's test.

RESULTS

Fourteen treatments with one control were used and data was collected for various observations. The data that had been calculated were subsequently subjected to ANOVA. The means of the treatments were compared at $p<0.01$ using HSD Tukey's test.

Table 1. Analysis of variance for the effect of treatments on the number of days to callus formation.

Source	Degree of freedom	Sum of square	Mean square	F value	P value
Replication	2	47.51	23.756	7.75	0.0021**
Treatment	14	3906.58	279.041	91.04	0.0000**
Error	28	85.82	3.065		
Total	44	4039.91			

*indicate significant data at p≤0.05, ** indicate highly significant data at p≤0.01

Table 2. Effect of different treatments on the number of days to callus formation.

Treatments	Concentrations	Number of days to callus formation	Callus color	Callus shape
T_0	MS media (control)	No Callus formed	No color	No shape
T_1	MS + 2,4 D (1.0 mg L^{-1})	38.667 ^{ab}	Yellow	Friable
T_2	MS + 2,4 D (2.0 mg L^{-1})	33.667 ^{bcd}	Yellow	Friable
T_3	MS + 2,4 D (3.0 mg L^{-1})	29.000 ^{def}	Yellow	Friable
T_4	MS + 2,4 D (4.0 mg L^{-1})	23.667 ^{f}	Creamy yellow	Nodular
T_5	MS + Kin (0.01 mg L^{-1})	38.00 ^{ab}	Yellow	Friable
T_6	MS + Kin (0.05 mg L^{-1})	36.000 ^{abc}	Yellow	Friable
T_7	MS + Kin (0.1 mg L^{-1})	32.667 ^{bcd}	Yellow	Friable
T_8	MS + Kin (0.5 mg L^{-1})	31.000 ^{cde}	Yellow	Friable
T_9	M S + K i n (0.01 m g L^{-1}) + 2,4 D (2.0 mg L^{-1})	32.000 ^{cde}	Yellow	Friable
T_{10}	M S + K i n (0.5 m g L^{-1})+2, 4 D (2.0 mg L^{-1})	26.000 ^{ef}	Creamy yellow	Nodular
T_{11}	M S +K i n (0.01 m g L^{-1}) + 2, 4 D (3.0 mg L^{-1})	35.000 ^{abcd}	Brown	Semitranslucent
T_{12}	M S + K i n (0.5 m g L^{-1}) + 2, 4 D (3.0 mg L^{-1})	35.667 ^{abc}	Brown	Semitranslucent
T_{13}	M S + K i n (0.01 m g L^{-1}) + 2, 4 D (4.0 m g L^{-1})	34.000 ^{abcd}	Brown	Semitranslucent
T_{14}	M S + K i n (0.5 m g L^{-1}) + 2,4 D (4.0 m g L^{-1})	37.000 ^{abc}	Brown	Semitranslucent
				p≤0.01

The findings indicated that the different concentrations of auxin had a significant effect on callus induction. For callus induction 14 treatments were used which consist of various doses of auxins (2, 4- D, kinetin) and their combinations. Different treatments showed different variation in days for callus regeneration, callus color and shape. The best callus induced in treatment 4 (M S + 2, 4- D 4.0 m g/ L) creamy yellow and nodular in shape and took minimum days. This

means that this type of callus has the ability to regenerate. The most significant increase in callus formation was observed when using the growth regulator hormones at a (4.0 mg/L) concentration.

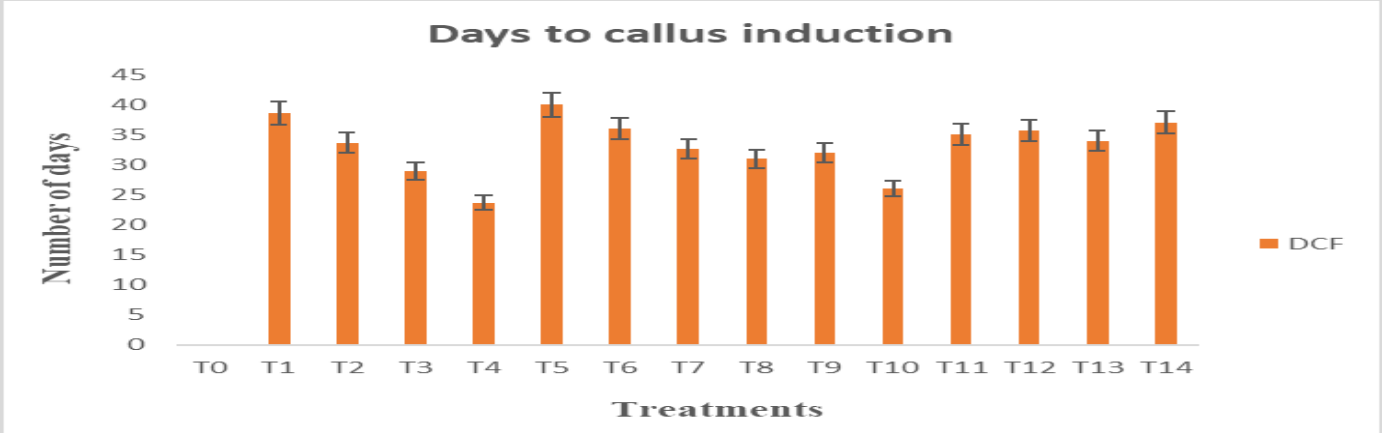

Figure 1. The average number of days taken for callus regeneration. The minimum number of days for callus regeneration were observed in T4. The maximum number of days for callus regeneration were observed in T5.

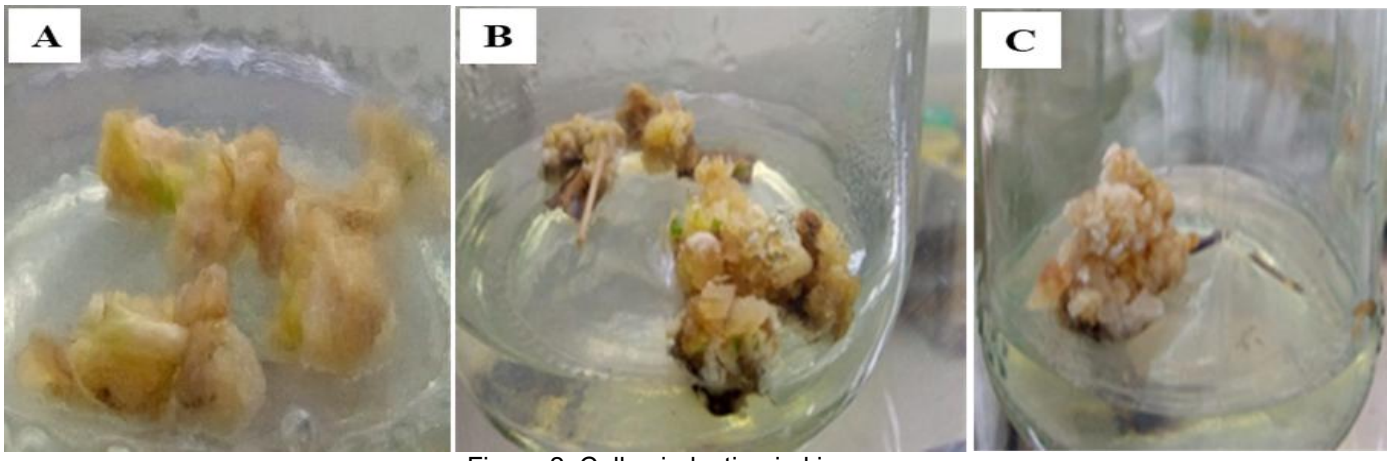

Figure 2. Callus induction in king grass

Table 3. Analysis of variance for the effect of treatments on the number of days to shoot regeneration

Source	Degree of Freedom	Sum of square	Mean square	F value	P value
Replication	2	9.50	4.750		
Treatment	3	12220.25	406.750	287.12	0.000**
Error	6	8.50	1.417		
Total	11	1238.25			

Four treatments were used and data was collected for various observations. The data that had been calculated were subsequently subjected to ANOVA. The means of the treatments were compared at $p < 0.01$ using HSD Tukey's test.* indicate Significant data at $p \leq 0.0\,5$, ** indicate data at $p \leq 0.0\,1$.

Table 4. Effect of different treatments on the number of days to shoot regeneration.

Treatments	Concentrations	Number of days to shoot formation
T_1	MS + BAP (0. 5 mgL^{-1})	26.000^a
T_2	M S + B A P (1. 0 m g L^{-1})	23.000^a
T_3	MS + BAP (1.5 mgL^{-1})	18.000^b
T_4	MS + BAP (2.0 mgL^{-1})	0.0000^c

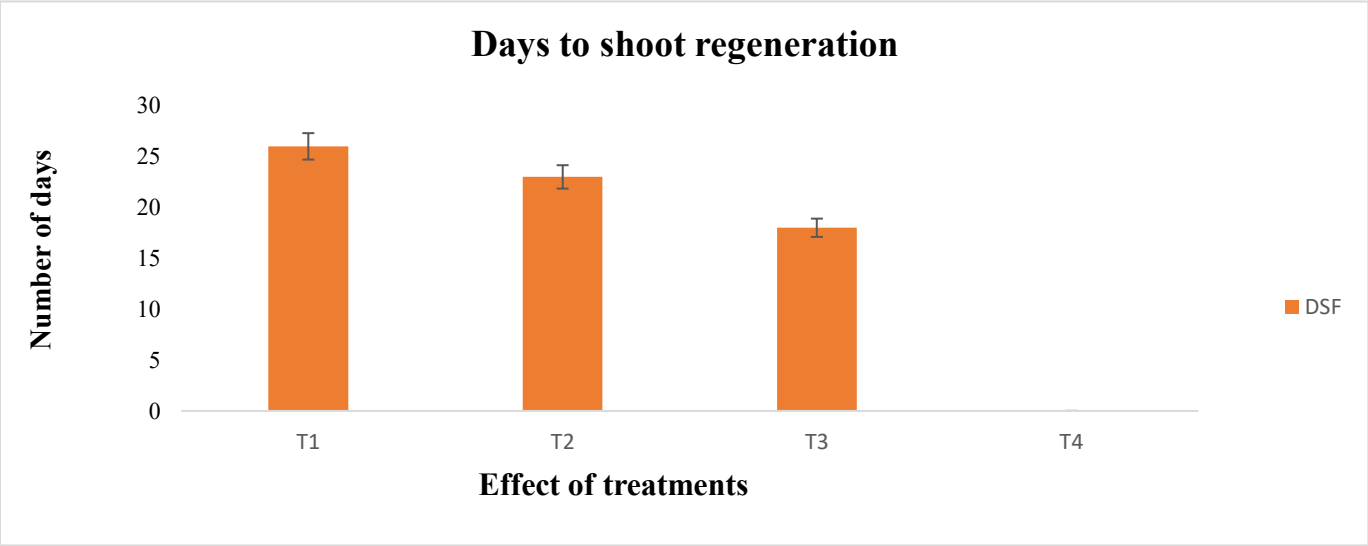

Figure 3. The average number of days taken for shoot regeneration. The minimum number of days for shoot regeneration were observed in T3. T1 showed the highest number of days for shoot regeneration.

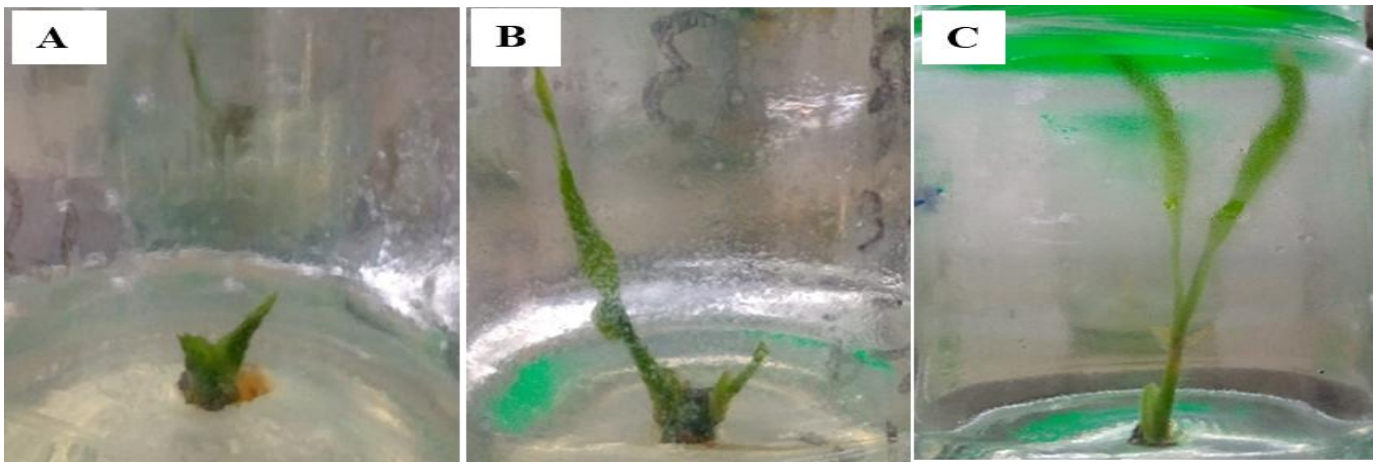

Figure 4. Shoot formation in king grass.

Table 5. Analysis of variance for the effect of treatments on the number of days to root formation

Source	Degree of Freedom	Sum of square	Mean square	F value	P value
Replication	2	9.00	4.500	3.05	0.0645*
Treatment	13	3495.81	268.908	2182.39	0.0000**
Error	26	38.33	1.474		
Total	41	3543.14			

Fourteen different treatments were used and data was collected for various observations. The data that had been calculated were subsequently subjected to ANOVA. The means of the treatments were compared at $p<0.01$ using HSD Tukey's test.

Table 6. Effect of different treatments on the number of days to root formation.

Treatments	Concentrations	Number of days to root formation
T_1	MS + NAA (0.1 mgL^{-1})	No root formation
T_2	MS + NAA (0.5 mgL^{-1})	No root formation

T_3	MS + NAA (1.0 mgL^{-1})	14.667 ^{c}
T_4	MS + IAA (0.1 mgL^{-1})	No root formation
T_5	MS + IAA (0.5 mgL^{-1})	No root formation
T_6	MS + IAA (1.0 mgL^{-1})	19.667 ^{ab}
T_7	MS + IAA (0.5 mgL^{-1}) + NAA (0.1 mgL^{-1})	No root formation
T_8	MS + IAA (0. 5 m gL^{-1}) + NA A (0. 5 m gL^{-1})	20.667 ^{a}
T_9	MS + IAA (0. 5 m gL^{-1}) + N A A (1 .0 m gL^{-1})	18.333 ^{ab}
T_{10}	MS + IAA (0. 5 m gL^{-1}) + N AA (1 .5 m gL^{-1})	13.667 ^{c}
T_{11}	MS + IAA (1.0 m gL^{-1}) + NAA (0.1 mgL^{-1})	No root formation
T_{12}	MS + IAA (1.0 mgL^{-1}) + NAA (0.5 mgL^{-1})	No root formation
T_{13}	MS + IAA (1.0 mgL^{-1}) + NAA (1.0 mgL^{-1})	22.000 ^{a}
T_{14}	MS + IAA (1.0 mgL^{-1}) + NAA (1.5 mgL^{-1})	16.000 ^{bc}

* indicate Significant data at p≤0.05, ** indicate highly significant data at p≤0.01.

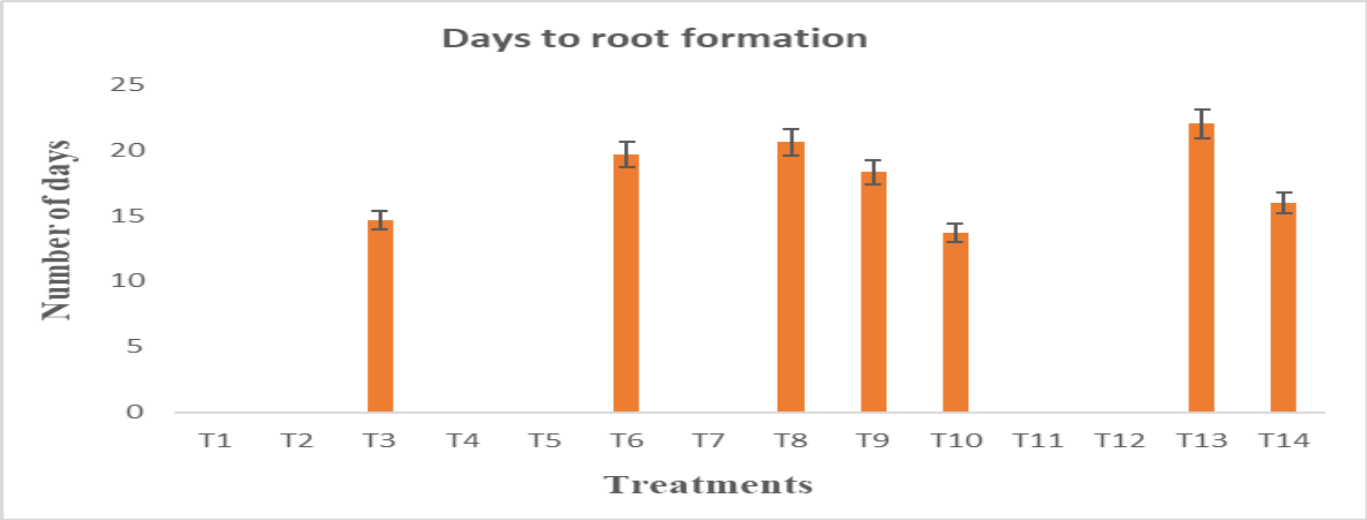

Figure 5. The minimum number of days for root formation were observed in T10 and maximum number of days for root regeneration were observed in T13.

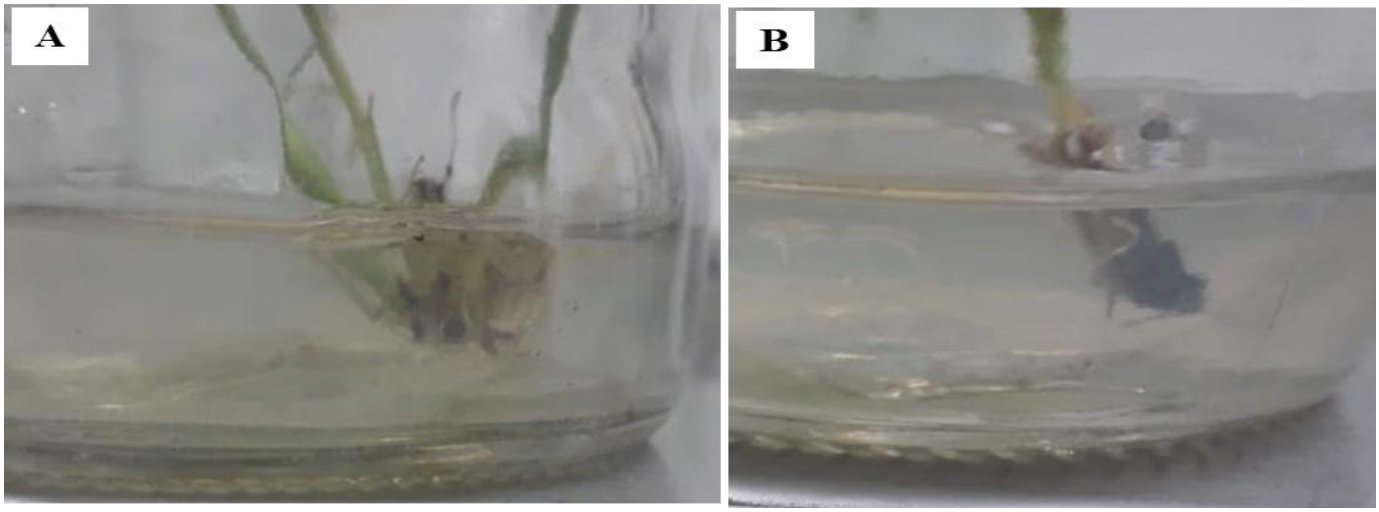

Figure 6. Root formation in king grass

DISCUSSION

King grass essential for sustainable agriculture since they help to conserve soil, prevent erosion and improve the nutrient quality of the soil. Tissue culturing is a field which is utilized to enhance the accessibility of recognized plant varieties exhibiting desirable characteristics, ultimately resulting in plant yield (Hanafi et al., 2014). By utilizing various plant parts like stem, nodes, buds, shoots, embryos, leaves and nodes *in-vitro* plantlets were cultured and investigated by various scientists. *In-vitro* culture techniques have various benefits for crop development, such as superior clones with high efficiency and create new diversity through culture-induced variation. (Umami et al., 2022). After two weeks of explants were cultured on modified media varying concentrations of PGRs (0.5 – 5.0 mg/L), callus induction was seen. Several plant growth regulators significantly altered the time for king grass to develop callus in the current study. Significantly, maximum days for callus formation were noted in T1 (M S + 2, 4- D @ 1.0 mg L^{-1}), T4 had the lowest, however (M S + 2, 4-D @ 4.0 mg L^{-1}), revealing a dose-dependent effect of 2, 4-D. These results are in line with previous studies that demonstrate that elevated 2,4-D levels up to a certain point encourage cell division and proliferation, which raises the rate of callogenesis (Ali et al., 2011; Shah et al., 2020). Cytokinins such as kinetin, which are mainly known to encourage shoot regeneration, can aid in the development of calluses when paired with auxins. This is demonstrated by the similar performance of T1 with T5 (MS + Kinetin @0.05 mg L^{-1}) and T14 (MS + Kinetin @0.5 mg L^{-1} + 2,4-D @4.0 mg L^{-1}) (Ramakrishnan et al., 2014). A balanced auxin-cytokinin ratio or higher 2,4-D concentrations may also considerably shorten the time needed for callus initiation, as indicated by the statistically similar of T4 and T10. Similar findings from studies on Gramineae species indicate that callogenesis efficiency was enhanced by better auxin and cytokinin combinations (Gandonou et al., 2005; Hoque and Mansfield, 2004). These findings highlight the significance of hormone levels and balance in creating effective tissue culture-based propagation methods for king grass and related crops. Auxins function best, according to a number of authors, when the callus is exposed to a higher 2,4-D concentration for a prolonged period of time (Ali et al*.,* 2007). A method for distinguishing between friable and nodular callus using various auxin types and concentrations. On the other hand, Gopitha et al. (2010) discovered that the best results for callus induction were obtained at lower levels 2, 4-D, and Kin. Consistent with previous findings in Triticum (Zale et al., 2004) and Oryza sativa (Schween and Schwenkel, 2003), these results suggest that the quantity of plant growth regulators used has a significant impact on the ability to produce callus. Begum et al. (1995) found that the largest percentage of callus induction occurred at 3.5 m g/l 2-4-D and that callus formation peaked at 85% 2,4-D. Treatments 4 and 10 produced the maximum callus in king grass, with creamy yellow, nodules as the callus's shape.

Research on somatic embryogenesis has produced a rapid technology for plant multiplication in contrast to the conventional approach. Therefore, callus induction presents a clear chance to further improve this altered genotype through the use of different biotechnological techniques. The most shoots and the ideal shoot length were produced by the in-vitro plantlet production system that used MS medium in conjunction with a synergistic combination of BAP and Kin (Baskaran, 2005). Barley plants were micropropagated using the successful meristematic shoot segments from mature seedlings created from embryos (Sharma et al*.,* 2004). A rapid, efficient, and genotype-independent method for plant regeneration was developed using pearl millet shoot tips (Mythili et al., 2001). According to the current study, there were significant differences in the number of days needed for king grass to form shoots under various hormonal treatments. While T2 (MS + BAP @1.0 mg L^{-1}) demonstrated delayed shoot formation, T4 (MS + BAP @2.0 mg L^{-1}) produced the earliest shoot initiation. These outcomes are in line with earlier research that found BAP to be a useful cytokinin for inducing shoots in a variety of grass species. The performance of T4 in the current study is supported by Khan et al. (2018), who found that increasing BAP concentration in Napier grass increases shoot proliferation and decreases the time needed for shoot initiation. Additionally, the shoot formation delay observed in T2 was statistically comparable to that of T3 (MS + Kinetin @0.01 mg L^{-1}), as demonstrated in the work of Goyal et al. (2015), where BAP with Kinetin in shoot induction efficiency in tropical forage grasses.

This implies that lower concentrations of kinetin might not have the same effect as higher BAP levels. Interactions with endogenous hormone levels or species-specific hormonal sensitivity could be the cause of the differences in cytokinin response (Murthy et al., 2012). These findings demonstrate how important it is to maximise the concentration of cytokinin in king grass micropropagation methods in order to achieve successful shoot induction. The current study revealed notable variations in the production of king grass roots under different auxin treatments, highlighting the crucial role that plant growth regulators play in *in vitro* root development. High auxin concentrations can inhibit optimal root biomass formation, as demonstrated by the fact that T3 (MS + NAA @1.0 mg L^{-1}) had the highest root development rate and T14 (MS + IAA @1.0 mg L^{-1} + NAA @1.5 mg L^{-1}) had the lowest rate. It is well known that the synthetic auxin

NAA (naphthalene acetic acid) has a potent root-inducing effect. The findings are in line with earlier research that discovered that even low levels of NAA significantly boosted root initiation and elongation (Gopi & Ponmurugan, 2006; Ahmad et al., 2012). A low dose of IAA (indole-3-acetic acid) combined with NAA may also result in beneficial rooting responses, according to the statistical similarity between T3 and T10 (MS + IAA @0.5 mg L^{-1} + NAA @1.5 mg L^{-1}). However, higher combined dosages, like T14, may cause feedback inhibition or hormonal imbalance, which would reduce root biomass. Similar findings are observed in the study of grain tissue culture and grass species, where achieving desired root symptoms requires the ideal auxine balance (Sabbir et al., 2019; Singh et al., 2020). These findings highlight the fact that root weight development *in vitro* settings is influenced by auxin type, concentration, and combination. The study of micropropagation has yielded a rapid technique for king grass reproduction that differs from traditional methods. Therefore, gene manipulation and other biotechnology techniques can further improve this grass by causing callus and encouraging the regeneration of shoots and roots.

CONCLUSIONS

This study demonstrates the successful establishment of an efficient tissue culture protocol for king grass using somatic embryogenesis. The optimization of callus induction, shooting, and rooting using different plant growth regulators (PGRs) provides a valuable tool for large-scale production of high-quality, disease-free king grass plants. The results of this study highlight the potential of tissue culture technology for improving the productivity and sustainability of king grass cultivation. Furthermore, this protocol can be used as a basis for future genetic improvement programs, such as breeding for desirable traits or genetic transformation. Overall, this research contributes significantly to the development of efficient micropropagation strategies for king grass, with implications for agricultural productivity and sustainability.

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AUTHOR CONTRIBUTIONS

SY and PK conceptualized the idea. SY did experimentation and wrote the manuscript. UW, MZR, AMB, RWM and MR reviewed the draft for improvements.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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