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Regulation of Callus Induction Efficiency in Banana Using Dichlorophenoxyacetic Acid in combination with Benzylaminopurine

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Abstract

The regulation of callus induction in banana (*Musa* spp.) was investigated using plant growth regulators, specifically 2,4-dichlorophenoxyacetic acid (2,4-D) combined with other growth regulators to enhance callus formation efficiency. Different concentrations of 2,4-D, indole-3-acetic acid (IAA), and benzylaminopurine (BAP) were added to Murashige and Skoog (MS) basal medium to evaluate their effects on callus induction frequency, initiation time, proliferation efficiency and the number of regenerated calli. Results demonstrated that callus induction was significantly modulated by hormone concentrations with the highest frequency observed at specific concentration of MS medium containing 4.0 mg/L 2,4-D, 0.5 mg/L IAA, and 0.5 mg/L BAP, yielding 95% induction frequency of robust, creamy white callus within two to three weeks of explant inoculation. The efficiency of callus proliferation measured as the percentage of explants producing calli, peaked at 87.5% on MS medium with 1.0 mg/L 2,4-D. This concentration also yielded the highest number of regenerated calli (16 ± 0.22), followed by treatments containing 2.0 mg/L and 3.0 mg/L 2,4-D, which produced 15 ± 0.21 and 12 ± 0.16 calli per explant, respectively. In contrast, the lowest regeneration rate (7 ± 0.11) was observed with 7.0 mg/L 2,4-D. These results underscore the importance of selecting precise hormone concentrations to achieve optimal callus induction and proliferation. Overall, this study reveals that 2,4-D, combined with low levels of IAA and BAP, effectively induces callus in banana tissue culture, with an optimal concentration range for balancing induction frequency, initiation time and proliferation. These findings offer insights for enhancing callus culture protocols for bananas, providing a foundation for future research on somatic embryogenesis and regeneration in climate-resilient banana cultivars.

Keywords: Banana, Callus, Dichlorophenoxyacetic Acid, Growth Hormones, Shoots Proliferation

INTRODUCTION

Banana (*Musa* spp.) is one of the most essential subsistence fruit crops globally and serves as a staple food for millions of people in developing countries. It is often referred to as "poor man's crop" due to its widespread cultivation in many tropical and subtropical regions of the world, where it supports food security and rural livelihoods (Khaldun et al., 2007). Bananas are the 4th most important and valuable food commodity in the whole world after wheat, rice, and milk/dairy products. It has much nutritive as well as medicinal importance, as every part of banana plant has its own importance to cure the many disorders and diseases such as the digestive, cardio-vascular, bronchitis, diabetics, hysteria, epilepsy, leprosy, haemorrhages, stroke, cancer, and hypertension (Mohapatra et al., 2010; Kumar et al., 2012; Sidhu and Zafar, 2018).

Meanwhile, it has been evidenced that conventional propagation of banana multiplied vegetatively with very low possibilities to germination, taking too much time and more possibilities to spread disease through suckers from one generation to another generation (Lakshmana et al., 2006). Due to diversity in climatic conditions create the more difficulties in banana improvement through traditional method of breeding programme (Raza et al., 2010). Which still a big problem in conventional breeding programme, development of elite banana cultivars with disease resistance and maximum yield are often overcome by close-fitting associations between fruit quality, yield and disease resistance. The germination via plant tissue culture techniques has opened up new possibilities to create more somaclones variation in banana as compared to traditional method of breeding programme (Sahijram et al., 2010). Now as day's, Plant Cell and Tissue Culture is a large paraphernalia and also has played an important role in the rapid increase of disease-free planting materials of recently added varieties through in vitro system technology on a continuous basis ((Dalvi et al., 2012). Shoot tip culture is a

moderately simple in vitro strategy for rapid propagation of certain banana materials and clean or pathogen-free plant materials (Singh et al., 2001). Since the plant is restored through tissue culture procedures, it may be a practical alternative to improve the quality and efficiency of bananas.

Consequently, in order to keep in mind the above facts and potential of biotechnological approaches, the objectives of this study aimed to generate genetic variability in banana cultivar through the techniques of soma clonal variations by callus culture, direct shoot regenerations and proliferation, also observed the variations through gene expressions, the technique is so far provides the opportunity to select plants with desirable traits such as early fruiting and short stature. The present study suggests the optimized protocol for determination of different consecutive concentrations of growth regulators such as 2,4-D, BAP, NAA and IAA on callus induction, shoot regeneration and proliferations, direct regenerations. Further, the present study also suggests optimized methods for the induction of somaclonal variations and variations obtained in tissue and cells through gene specific expressions.

MATERIALS AND METHODS

Collection of Explant Material: Offshoots of Banana (*Musa spp*.) plants collected from Agricultural Research Center and further Tissue Culture experiment conducted in the laboratory of plant tissue culture at department of Biotechnology, Sindh Agriculture University, Tandojam. The offshoots were selected based on specific criteria, including their age (4-6 months) and size (approximately 25-30 cm in height), to ensure optimal physiological maturity and viability for tissue culture.

Preparation of Explant: Young healthy suckers of banana plants selected and carefully removed using a sterilized scalpel to expose the inner meristematic tissues. The sucker's outer layer and cleaned them under running tap water to eliminate any dirt. Peeled off outer layers to expose the meristematic tissue. This step was performed under aseptic conditions in a laminar flow hood to minimize contamination.

Surface Sterilization: Explants were immersed in 70% ethanol for 30 seconds, followed by rinsing in sterile distilled water and transferred to 0.1% (w/v) mercuric chloride solution for 10–12 minutes under aseptic conditions. Rinsed the explants thoroughly (3–4 times) with sterile distilled water to remove any traces of the sterilizing agent.

Preparation of Nutrient Media

MS Basel Medium: Murashige and Skoog (MS) basal medium were prepared with added macro- and micronutrients, including 30 g/L sucrose as the carbon source. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15–20 minutes.

Hormonal Treatment: Various concentrations of 2,4-D (0, 1, 2, 3, 4, and 5 mg/L) were prepared by dissolving in a minimal amount of ethanol or NaOH as needed, then adding to the culture medium. Prepared control media without any plant growth regulators (PGR) to serve as a baseline.

Experimental Design: Under aseptic conditions, explants were placed on MS medium containing different concentrations of 2,4-D. Transferred three explants to each petri dish or culture container. Each treatment included 4 different replicates to ensure statistical significance. Cultures were placed in growth chamber at 25 ± 2°C with 16-hour photoperiod under fluorescent light (40µmol m^2/s). Humidity was maintained at level of 60 to 70%. For assessing the callus induction efficiency, cultured explants were monitored weekly for callus formation and noted the time of first callus initiation.

Collection of Data: Callus induction percentage was recorded by dividing the number of explants with callus through the total number of explants in each treatment and multiply by 100. Measure fresh weight of callus by carefully detaching the callus from the explant and weighing it using an analytical balance. Assess callus morphology (color, texture, compactness) under a stereomicroscope and categorize it as friable, compact, or embryogenic.

Statistical Analysis: Statistical analysis was conducted using analysis of variance (ANOVA) to assess significant differences between treatments. For comparisons of means where significant differences are found, apply post hoc tests (e.g., Tukey's HSD) to evaluate differences in callus induction percentage and fresh weight across various concentrations of 2,4-D. Set the significance level at $p < 0.05$.

Phenotypic analysis and documentation: photographic evidence of callus was captured at different stages of development for visual documentation. Include images of each treatment to highlight morphological differences induced by 2,4-D concentrations.

RESULTS AND DISCUSSIONS

Callus induction frequency modulated by hormones concentrations: The results showed a significant ($P<0.05$) increase in callus induction frequency when the appropriate concentrations of hormones were applied under laboratory conditions. No doubt, through the method of callus culture, the callus tissues can be formed from any part of plant such as roots, stem, leaves, meristems and mature embryos (Ryu et al., 2004; Raza et al., 2010). During callus formation there is some degree of de-differentiation formed both in morphology and metabolism resulting in lose the ability of photosynthesis (Khatri et al., 2002). The results further proposing that callus formation was significantly induced within two to three weeks after inoculation of the explants on MS basal medium containing various concentrations of supplemented hormones (i.e. MSC-I to MSC-X). While, in present work except the controlled media MS (Basal) + 0.0mg/L 2,4-D + 0.0mg/L IAA + 0.0mg/L BAP + 30g Sucrose (MSC-I), no growth was observed throughout the entire experiment across all four replications in treatments where no hormonal concentration was applied. Besides, in all other concentrations of MSC, the callus induction was triggered but more profuse callus as well as highest potential percentage (95% ± 0.10) induction frequency with healthy, creamy, and fine white callus were significantly ($P<0.05$) observed on MS (Basal) + 4.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-IV) medium followed by MS (Basal) + 3.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-V), MS (Basal) + 2.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-VI), respectively. Nevertheless, the development of callus induction in Musa found considerably recalcitrant by many researchers (Stroose et al., 2004). On another hand, the contrary lowest potential rate of callus (55% ±0.56) observed on MS (Basal) + 7.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-X) media. It has been interestingly noted that throughout the whole experiment as the concentration of supplemented hormone i.e. MS (Basal) + 2.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-IV) resulted significantly decrease frequency of regeneration of calli. In contrary, yellowish, weak, small, and fragile calli observed at high concentration from MS (Basal) + 5.0mg/L 2,4-D +0.5mg/L IAA.+0.5mg/L-BAP + 30gSucrose (MSC-VII) to MS (Basal) + 7.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-X). Besides, dissimilar concentrations of hormone expressed various regeneration of callus as shown in Figure 1.

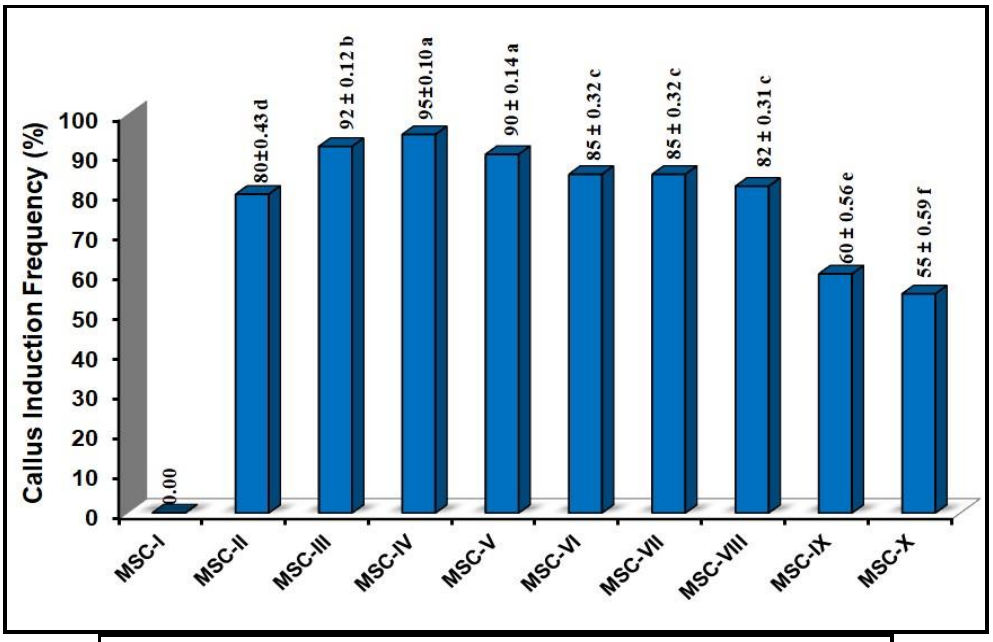
Days taken to callus initiate: In order to investigate the rate of callus initiation, the different concentrations of media were used, however some researchers used same for initiation and multiplications while different concentrations of hormones in different phases (Alango et al., 2018; Dalvi et al., 2012; Amin et al., 2009). In present results, the days taken to callus initiation were measured on different combinations of 2,4-D with IAA; the data so obtained are summarized in Figure 2. The results based on the duration of callus initiation varied significantly ($P<0.05$) from 35 to 58 days across different concentrations of media supplementations. During present investigation, the callus initiation was highly ($P<0.05$) regulated by different supplementation and type of the growth hormones used in the experiment. Among different tested concentrations for callus initiations, the minimum days (35±0.321) were taken significantly ($P<0.05$) on MS (basal) medium containing 3.0mg/L 2,4D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose (MSC-V). Interestingly, on MS (Basal) + 2.0mg/L 2, 4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-IV) media, the callus initiation was noticed after 37±0.29 days of inoculations (Figure 2). Besides, the antagonistic low response observed on the media concentration of MS (Basal) + 7.0mg/L 2,4-D +0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose, MS (Basal) + 7.0mg/L 2,4-D +0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-X) and MS (Basal) + 6.5mg/L 2,4-D +0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-X) which taken about 58±0.62 days for callus induction. Furthermore, various concentrations of media were found different level of competences of initiation of callus. The results described at particular concentration showed best result as comparison to the lowest or highest concentration of plant growth hormone for induction of callus takes various level competences of initiations.

Proliferation of calli numbers: Callus proliferation capability changed significantly between genotypes and diverse according to the different concentration of media along with supplemented hormones (Raza et al., 2010; Ryu et al., 2004). However, as reported that the lumps of cell obtained from suspensions cell which eventually form the calli and then organogenesis or somatic embryogenesis. Accordingly in present result, it has been found that comparatively ($P<0.05$) 2,4-D was better than IAA and IBA + BAP for profuse proliferating efficiency of calli. Thus, the best proliferation rate (18±0.56) was observed significantly ($P<0.05$) on MS (Basal)+2.0mg/L2,4D+0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-IV) media followed by proliferation appeared on MS (Basal) + 1.0mg/L 2,4-D + 0.5mg/L IAA + 0.5mg/L BAP + 30g Sucrose (MSC-III) media where 17±0.44 calli observed. Though there were various concentrations used which showed different numbers of proliferated calli throughout all four replications. The lowest (8±0.21) proliferated number on calli observed on MS (Basal) + 7.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose (MSC-X).

Proliferation efficiency (%): The seedlings regenerated on artificial nutrient media usually depends upon the different concentration of media, type of explant and variety of banana has great potential as a means of vegetative multiplication of economically important varieties, especially for those difficult to propagate by ancient farming methods like seeds or cuttings (Mohamed and Hussain, 2004. However, in present results it was observed that without use of any supplemented hormones, there was no any growth and sign of initiations and proliferation obtained (Figure 4). Nevertheless, the proliferation efficiency observed significantly ($P<0.05$) in a

wide range of 12.3% to 87.5% at various concentration of media. The highest proliferation percentage (87.59%) significantly (P<0.05) observed on MS (Basal) + 1.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose (MSC-III) media. As compared to highest percentage, the lowest percentage observed on MS (Basal) + 7.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose (MSC-X) media that was about 12.39%. Furthermore, the uppermost proliferation rate was also noticed at the media MS (Basal) + 3.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose showed remarkably (P<0.05) fine percentage around 87.49%. On the other hand, the lowest proliferation rate was noticed at the media containing MS (Basal)+6.5mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP + 30g Sucrose (MSC- IX) showed percentage about 15.9%. Along with these results the percentages depend upon standard concentrations of the media.

Number of regenerated calli: It was observed that results was remarkable significant (P<0.05) for the number of calli regenerated on various concentrations of nutrient media. The results suggesting that regenerated number of calli on MS medium supplemented with different concentration of phyto-hormones was highly regulated grown under aseptic artificial condition. The results of various concentrations expressed that the maximum number of regenerated calli were noticed (16±0.22) under the supplementation of MS (Basal)+1.0mg/L 2,4-D+0.5mg/L IAA + 0.5mg/L BAP+30g Sucrose followed by (15±0.21 and 12± 0.16) on the concentration of MS (Basal)+2.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose and MS (Basal)+3.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose respectively. While minimum number of regenerated calli (7±0.11) were obtained under the concentration of MS (Basal)+7.0mg/L 2,4-D+0.5mg/L IAA+0.5mg/L BAP+30g Sucrose.



MSC-I	= MS (Basal)+0.0mg/L BAP + 0.5mg/L IAA + 0.0mg/L 2,4-D+ 30g Sucrose
MSC-II	= MS (Basal)+0.5mg/L BAP + 0.5mg/L IAA + 0.5mg/L 2,4-D+ 30g Sucrose
MSC-III	= MS (Basal)+0.5mg/L BAP + 0.5mg/L IAA + 1.0mg/L 2,4-D+ 30g Sucrose
MSC-IV	= MS (Basal)+0.5mg/L BAP + 0.5mg/L IAA + 2.0mg/L 2,4-D+ 30g Sucrose
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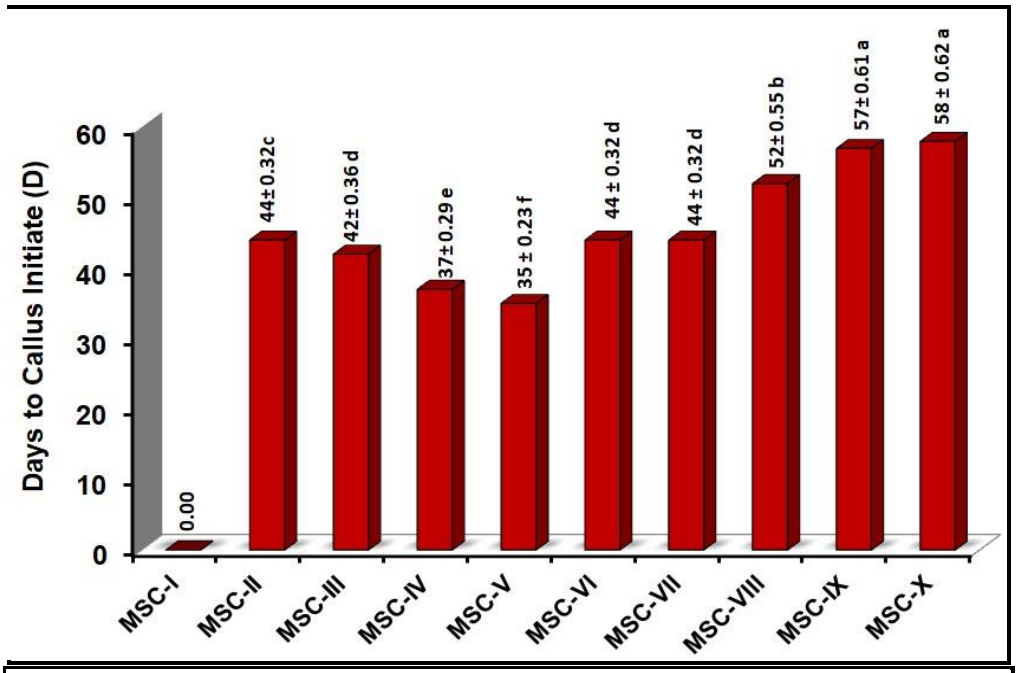
MSC-VII	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 5.0mg/L 2,4-D+ 30g Sucrose
MSC-VIII	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 6.0mg/L 2,4-D+ 30g Sucrose
MSC-IX	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 6.5mg/L 2,4-D+ 30g Sucrose
MSC-X	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 7.0mg/L 2,4-D+ 30g Sucrose

Figure 1. Frequency (%) of Callus Induction on MS basal medium containing various supplementations of 2,4-D, BAP and IAA.



MSC-I	= MS (Basal)+0.0mg/L BAP + 0.5mg/L IAA + 0.0mg/L 2,4-D+ 30g Sucrose
MSC-II	= MS (Basal)+0.5mg/L BAP + 0.5mg/L IAA + 0.5mg/L 2,4-D+ 30g Sucrose
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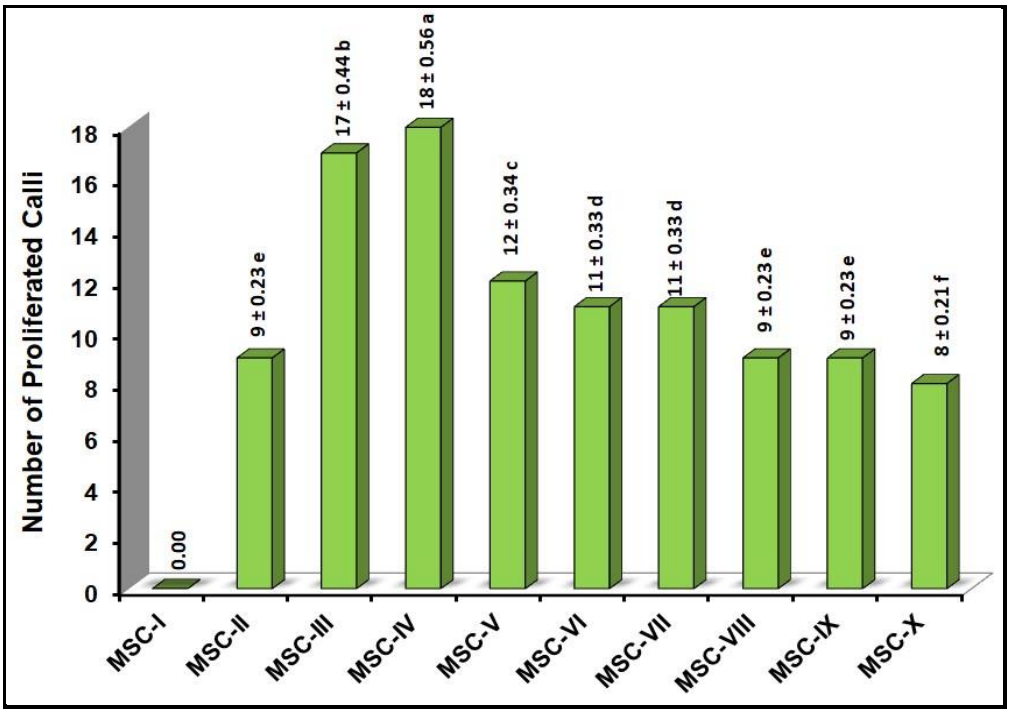
MSC-X	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 7.0mg/L 2,4-D+ 30g Sucrose

Figure 2 Days taken to initiate the callus on MS basal medium containing various supplementations of 2,4-D, BAP and IAA.



MSC-I	= MS (Basal)+0.0mg/L BAP + 0.5mg/L IAA + 0.0mg/L 2,4-D+ 30g Sucrose
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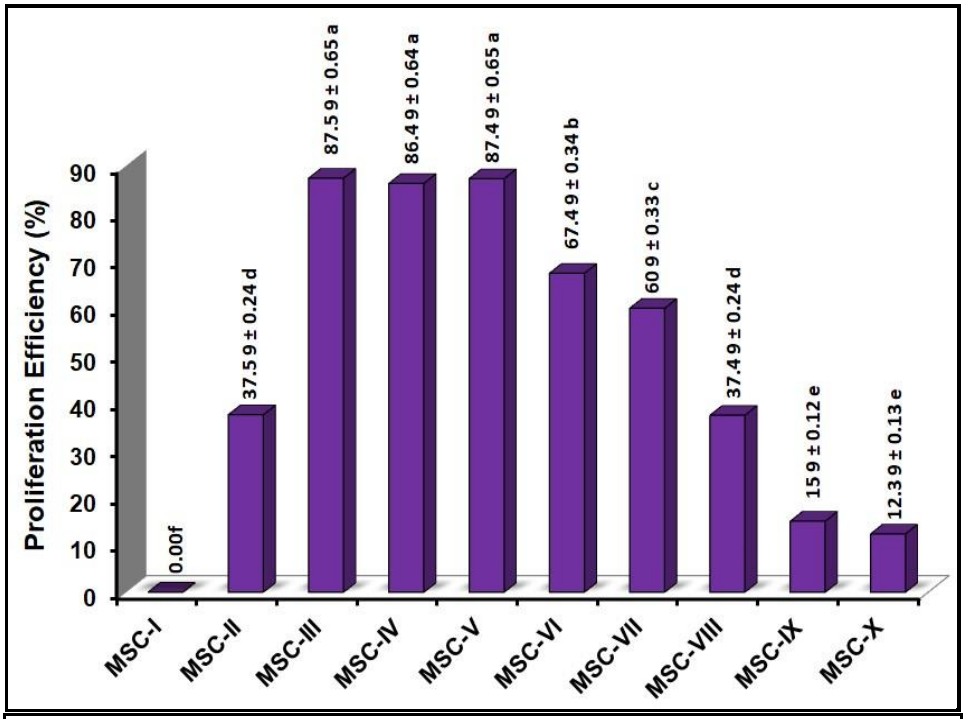
Figure 3 Number of Proliferated Calli regenerated on MS basal medium containing various supplementations of 2,4-D, BAP and IAA.



MSC-I	= MS (Basal)+0.0mg/L BAP + 0.5mg/L IAA + 0.0mg/L 2,4-D+ 30g Sucrose
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MSC-X	= MS (Basal)+0.5 mg/L BAP + 0.5mg/L IAA + 7.0mg/L 2,4-D+ 30g Sucrose

Figure 4. Proliferation Efficiency (%) Calli flourished on MS basal medium containing various supplementations of 2,4-D, BAP and IAA.

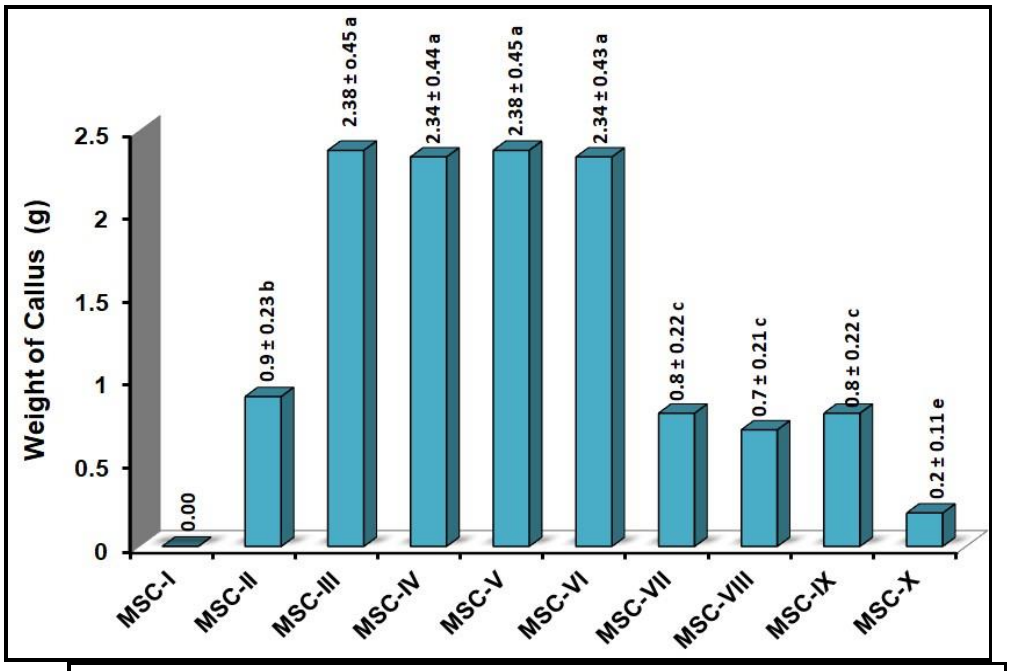

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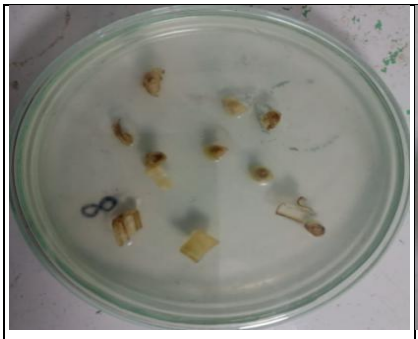
Figure 5. Weight of Callus observed on MS basal medium containing various supplementations of 2,4-D, BAP and IAA.

		MS (Basal) + 0.5mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose	MS (Basal) + 1.0mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose

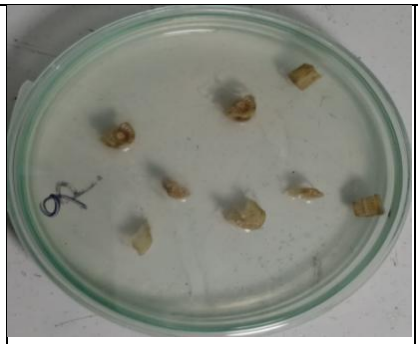
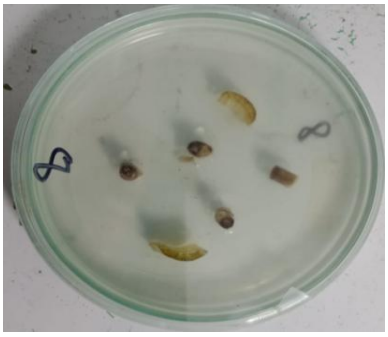
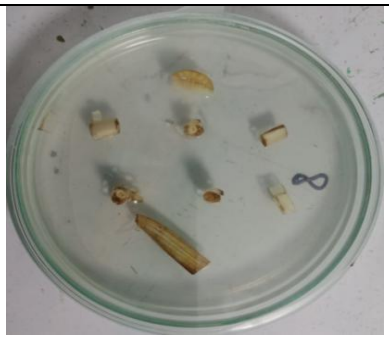
		MS (Basal) + 2.0mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose	MS (Basal) + 3.0mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose
		MS (Basal) + 4.0mg/L 2-4 D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose	MS (Basal) + 5.0mg/L 2-4 D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose
		MS (Basal) + 6.0mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose	MS (Basal) + 6.5mg/L 2,4-D +0.5mg/L IAA +0.5mg/L BAP +30g Sucrose

Figure 6. Excided pieces of banana offshoots inoculated for callus initiation on MS basal medium containing various supplementations of 2-4 D, BAP and IAA

CONCLUSION

The present study concluded that hormonal concentrations significantly impact callus induction and proliferation in banana tissue culture. Optimal callus induction was achieved with MS medium containing 4.0 mg/L 2,4-D, 0.5 mg/L IAA, and 0.5 mg/L BAP, yielding a high induction frequency of 95%. Whereas callus proliferation peaked at 87.5% on MS medium containing 1.0 mg/L 2,4-D, with the highest regenerated calli per explant (16 ± 0.22). Increasing 2,4-D concentration beyond 3.0 mg/L reduced the regeneration rate, with the lowest rate observed at 7.0 mg/L 2,4-D. These results emphasize the critical role of fine-tuning hormone concentrations for effective callus formation and proliferation. A combination of 2,4-D with low levels of IAA and BAP was optimal for callus induction, balancing initiation time and yield. This protocol offers a valuable foundation for improving callus culture methods in banana, especially under climate-resilient breeding programs. Future research can leverage these findings to enhance somatic embryogenesis and regeneration in banana cultivars for climate resilience.

CONFLICT OF INTEREST

All authors have read the manuscript and declared that they have no conflict of interest

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