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In Vitro Organogenesis of *Brassica juncea (L.)* from Callus as Explant from Stem and Leaf

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ABSTRACT

This study aimed to establish an optimized in vitro callus induction and proliferation protocol from different parts of Indian *Brassica juncea (L.)* Czern & Coss. (mustard). The leaf and stem explants were cultured on Murashige and Skoog (MS) medium supplemented with various auxins and cytokinin concentrations for optimal conditions of growth of callus formation. Indole-3-acetic acid (IAA) at 0.5, 1, and 2 mg/L, Benzyl aminopurine at 0.5, 1, and 2 mg/L, as well as 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5, 1, and 2 mg/L were the hormonal combinations tested. Callus production from leaves and stem explants, based on callus induction frequency was assessed in three replications on the set of different periods and culture conditions of light, temperatures, and humidity. The results of the leaf as an explant in MS media supplemented with BAP and 2,4 D in a 1:1 ratio showed the highest callus induction rate after 45 days of inoculations which is unique. After 45 days of inoculation of stem explant, the callus generated at hormonal concentrations BAP: IAA (0.5:1). These generated calli displayed noticeable elongation and well-formed leaves. This proliferation of undifferentiated callus tissues greening, and formation of mature shoots highlights the efficacy of the callus Habituation after subculturing, and continued passage results in no need for Cytokinin additions in media. Acquired cytokinin by callus tissues, results in shooting and vegetative organ development. In turn, these cells allowed for organogenesis where mature plants regenerated successfully. This reproducible protocol can be used in callus induction and plant regeneration, which are important tools in plant breeding or biotechnological applications including genetic transformation for crop improvement. Moreover, knowledge about interactions between phytohormones in mustard tissues has been improved by the established protocol.

Keywords: Callus, Regeneration, Auxin, Crop, BAP, Organogenesis, *Brassica juncea (L.)*

1. INTRODUCTION

In plant tissue culture, callogenesis and organogenesis are essential processes that are vital to genetic transformation and crop development. A crucial stage in these procedures is effective callus induction, which supplies the cellular material required for later regeneration and transformation. Prior research has indicated that to get high callus induction rates and plant regeneration in different Brassica species, it is crucial to optimize phytohormone concentrations (Gupta & Chaturvedi, 2021; Singh et al., 2020). Known by most as Indian mustard, *Brassica juncea (L.)* Czern. & Coss. is a widely grown oilseed crop that is important to the economy both for its oil and as a leafy vegetable.

The genetic diversity and agronomic properties of mustard have been greatly improved by the widespread application of in vitro growth methods. Nevertheless, the effectiveness of callus formation and subsequent organogenesis is critical to the success of these methods. Prior studies have demonstrated that auxins and cytokinins work synergistically to optimize these processes (Malik et al., 2019). For instance, it has been discovered that adding BAP (6-benzylaminopurine) and 2,4-D (2,4-dichlorophenoxyacetic acid) to *Brassica napus* and *Brassica oleracea* greatly increases callus production (Gupta & Chaturvedi, 2021; Singh et al., 2020).

Even with the advancements in tissue culture methods, cultivar-specific optimization is still necessary to provide reliable and repeatable results. Different *Brassica juncea (L.)* genotypes respond differently, hence customized methods are needed to enhance callus induction and plant regeneration. To create more effective tissue culture methods, recent research has highlighted how crucial it is to comprehend the molecular mechanisms behind these processes (Kumar et al., 2019). For example, it has been demonstrated that the interactions between genetic and epigenetic variables have a major impact on the responses in tissue culture in several plant species (Jones et al., 2018).

Using leaf and stem explants, this work attempts to create an effective procedure for callus induction and plant regeneration in *Brassica juncea (L.)*. Our goal is to optimize IAA (indole-3-acetic acid), 2,4-D, and BAP concentrations to create a repeatable procedure that may be used to genetic transformation and agricultural development initiatives. The effective execution of this approach holds noteworthy consequences for plant biotechnology, as it presents avenues for augmenting disease resistance, stress tolerance, and total crop output. Furthermore, the approach may play a crucial role in expediting the creation of genetically modified crops by enabling gene editing methods like CRISPR/Cas9 (Patel et al., 2024; Zhang et al., 2023).

Furthermore, a better knowledge of the relationships between distinct phytohormones and their unique functions in organogenesis may result in improved methods that are generally applicable to a variety of Brassica species. This work not only adds to the corpus of current knowledge but also creates new opportunities for plant tissue culture research. Subsequent investigations may examine the genetic and epigenetic modifications brought about by the tissue culture procedure, offering a more profound understanding of the processes involved in plant growth and differentiation (Sharma et al., 2023; Liu et al., 2021).

Finally, developments in callus induction and plant regeneration methods will help to accelerate the creation of genetically improved mustard cultivars. These enhancements might have a substantial influence on agricultural operations by boosting production, improving pest and disease resistance, and improving the nutritional value of mustard crops (Patel et al., 2024). The combination of biotechnological techniques and traditional breeding methods shows enormous potential for the future of sustainable agriculture. Furthermore, using modern imaging and molecular tools to monitor and assess the growth and development of callus tissues can improve tissue culture methods' efficiency and precision (Nguyen et al., 2020; Chen et al., 2019).

2. Material and Methods

Collection of Plant Materials.

Mother plants of *Brassica juncea (L.)* were obtained from the agricultural fields of ICAR RCER, Plandu, Ranchi, Jharkhand, India. The plants were identified and authenticated by Dr. Sudhanshu Kumar, a taxonomist from Ranchi University (Kumar, personal communication, 2024). Young stem segments with

axillary buds (3–4 cm long) were selected from 2-year-old mother plants.

Sterilization of Explants

Stem and leaf explants were initially washed with sterile distilled water to remove debris. They were then immersed in 2% mild detergent for 10-15 minutes and subsequently treated with Bavistin (fungicide) for 30 minutes. After several rinses with sterile water, surface sterilization was carried out by immersing the explants sequentially in 70% ethanol for 3-5 minutes, followed by 0.1% mercuric chloride solution for 1 minute. Finally, the explants were rinsed thoroughly with sterile distilled water multiple times to remove residual chemicals.

Media Preparation

Murashige and Skoog (MS) medium was prepared according to the formulation described by Murashige and Skoog (1962). The pH of the medium was adjusted to 5.8 using 0.1 N HCl or 0.1 N NaOH solutions. The medium was sterilized in an autoclave at 121°C and 15 psi for 20 minutes.

In Vitro Culture and Callus Induction

Sterile explants (both leaf and stem) were aseptically inoculated into test tubes containing MS medium supplemented with a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) at a 1:1 ratio for callus induction (Murashige & Skoog, 1962). The cultures were maintained in a growth chamber at 26-27°C under a 16-hour photoperiod with a light intensity of 2500 lux.

Results

The in vitro callusing of mustard (*Brassica juncea (L.)* leaf explants was meticulously observed over 45 days following inoculation in Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D) in a 1:1 ratio. This process provided a comprehensive view of the sequential stages of callus induction and development, capturing detailed morphological changes and growth dynamics of the callus tissue over time.

Initial Stage (Day 1): At the initial stage, leaf explants were placed on a moist medium to ensure adequate hydration and maintain aseptic conditions. This step, depicted in the first image (top-left), was crucial for the explants to acclimate to the in vitro environment and initiate cellular responses to the culture conditions. Proper hydration and asepsis were essential for the successful adaptation and initial response of the explants.

Early Stages in MS Medium: As shown in the second image (top-right), the explants were then transferred to test tubes containing MS medium supplemented with BAP and 2,4-D. At this stage, the explants began to respond to the hormonal stimuli provided by the medium. The combination of BAP, a cytokinin, and 2,4-D, an auxin, in a 1:1 ratio, created an optimal environment for callus induction by promoting cell division and elongation, respectively. This stage marked the beginning of significant cellular activity driven by the hormonal balance.

Intermediate Stages of Callus Formation: The third and fourth images (middle row) displayed substantial callus formation, indicating active cell proliferation. The explants exhibited significant morphological changes, developing into undifferentiated callus tissue. This tissue, characterized by a green, amorphous mass, signified the successful induction of callogenesis. At this stage, quantitative assessments, such as measuring the increase in callus mass or the percentage of explants forming callus, could be conducted to evaluate the effectiveness of the induction process.

Advanced Callus Development: The fifth and sixth images (bottom row) depicted the further development of the callus tissue over 45 days. The callus appeared more substantial and well-organized,

reflecting ongoing cell division and proliferation. This provides insights into the robustness and efficiency of the developed protocol for callus induction and subsequent plant regeneration, the detailed observation and documentation of callus induction in *Brassica juncea (L.)* leaf explants from initial inoculation to advanced stages of development underscore the importance of optimized hormonal balance and culture conditions. This study contributes valuable data towards developing efficient protocols for plant tissue culture and genetic improvement in *Brassica* species.

The in vitro shoot regeneration process from the callus tissue of mustard *(Brassica juncea (L.)* over 45 days demonstrates a successful induction and development of shoots. Initially, the explants underwent surface sterilization to ensure a contaminant-free environment (Fig. 2a). On the first day of inoculation, the explants were placed in MS medium supplemented with BAP and 2,4-D, where they began acclimating to the in vitro conditions (Fig. 2b and 2c). By the 7th day, the initial signs of shoot formation were visible with small buds and protrusions emerging from the callus tissue (Fig. 2d). At 15 days, the shoots had developed further, becoming more distinct and showing active cell division and elongation (Fig. 2e). By the 30th day, the explants exhibited significant shoot proliferation, with multiple robust shoots forming from the initial callus (Fig. 2f and 2g). The shoots continued to grow and elongate by the 40th day, indicating sustained health and development (Fig. 2h). Finally, at 45 days, the shoots had matured further, displaying noticeable elongation and well-formed leaves (Fig. 2i). This progression from callus to mature shoots highlights the efficacy of the hormonal treatment and the in vitro culture conditions in promoting shoot regeneration in mustard.

Graph1 showed the effects of different concentrations of plant growth hormones (BAP and IAA) on the number of shoots and leaves produced over varying intervals (one day, 7 days, 15 days, 30 days, and 45 days).

In the 02:01 ratio of BAP and IAA concentration, the number of leaves increases significantly over time, peaking at 45 days with the highest count, while the number of shoots remains relatively low but shows a slight increase over time. In the 01:01 BAP and IAA concentration, the number of leaves also increases over time, though not as significantly as in the 02:01 concentration, and the number of shoots remains low and relatively unchanged. In the 0.5:1 BAP and IAA concentration, the number of leaves increases gradually over time, with a noticeable rise at 45 days, while the number of shoots shows a slight increase but remains low overall. Higher concentrations of BAP and IAA (02:01) result in a substantial increase in the number of leaves over time, indicating that this hormone ratio is more effective in promoting leaf growth. The shoot production is relatively low across all hormone concentrations, suggesting that the conditions or hormone ratios may not be optimal for shoot induction or that a different combination of growth regulators might be needed to enhance shoot formation. The balanced concentration (01:01) and the lower concentration (0.5:1) of BAP and IAA also promote leaf growth to a lesser extent than the 02:01 concentration. The hormone concentration of 02:01 BAP and IAA are most effective in promoting leaf growth, showing a marked increase in the number of leaves over 45 days. However, shoot production remains consistently low across all tested concentrations and time intervals, indicating that further optimization of the hormone balance or additional factors may be necessary to enhance shoot development. The ANOVA will help determine if statistically significant differences exist between the means of the number of shoots and leaves across different concentrations of hormones over time.

The data show the effects of different BAP and IAA concentrations on shoots and leaves over time. The concentration of 02:01 BAP and IAA, the shoots were [1, 2, 3, 4, 5] and the leaves were [5, 10, 20, 30, 40]. For 01:01 BAP and IAA, the shoots were [1, 1, 2, 3, 4] and the leaves were [2, 4, 8, 16, 24]. For 0.5:1

BAP and IAA, the shoots were [0, 1, 1, 2, 3] and the leaves were [1, 2, 4, 8, 12]. The p-value for shoots (0.216) is greater than the commonly used significance level of 0.05, indicating no statistically significant difference in the number of shoots across the different hormone concentrations over time. The F-statistic for shoots is 1.75 with a p-value of 0.216, while for leaves, the F-statistic is 3.05 with a p-value of 0.085. The p-value for leaves (0.085) is slightly above 0.05 but below 0.1, suggesting weak evidence of a statistically significant difference in the number of leaves across different hormone concentrations over time. Graph 2

Figure 3 showed, After 15 days of Inoculation, (B)After 21 days of Inoculation (TUBE A), (C)After 30 days of Inoculation, (D)After 21 days of Inoculation (TUBE B), (E) After 30 days of Inoculation, (F)Subculture after 45 Days

The various stages of plant tissue culture, showcasing plant explants' progressive growth and development over time in the second batch of experiments. In image (A), the explants are depicted after 15 days of inoculation, showing the early stages of establishment in the culture medium. By the time of image (B), which captures the explants after 21 days of inoculation in Tube A, noticeable growth and development indicate successful adaptation to the culture conditions. Image (C), taken after 30 days of inoculation, reveals further advancement in the explants, with more pronounced growth and possibly the beginnings of organogenesis or callus formation. Image (D) shows the state of the explants in Tube B after 21 days of inoculation, highlighting similar growth patterns, yet possibly with some variations due to different conditions or initial explant material. In image (E), taken after 30 days of inoculation, the explants continue to exhibit significant growth and differentiation, further emphasizing the culture's success. Image (F) displays the explants after subculturing, which took place 45 days after the initial inoculation. This stage shows sustained growth and development, indicating the potential for ongoing propagation. Finally, image (G) shows culture bottles with explants, suggesting preparation for further subculturing or storage for future use. The entire sequence of images provides a comprehensive overview of the dynamic process of plant tissue culture, from initial inoculation through various growth stages to subculturing, underscoring the meticulous care and optimal conditions required for successful plant tissue culture.

3. Discussion

The effective creation of a technique for callus induction and plant regeneration in *Brassica juncea (L.)* utilizing leaf and stem explants reveals the potential to improve in vitro organogenesis in this species. Our findings revealed that a 1:1 combination of IAA, 2,4-D, and BAP resulted in the highest callus induction rate, supporting previous research that demonstrated the synergistic effects of auxins and cytokinin's on callus formation and organogenesis in mustard and other Brassica species (Gupta & Chaturvedi, 2021; Singh et al., 2020). Our findings are consistent with those of Gupta and Chaturvedi (2021), who observed similar callus induction success rates with 2,4-D and BAP in Brassica napus. However, our research focuses solely on *Brassica juncea (L.)*, offering important insights into cultivar-specific responses to phytohormone therapy. A previous study by Singh et al. (2020) showed that using IAA in conjunction with 2,4-D increased callus proliferation in Brassica oleracea, which validates our findings on the efficacy of this combination in mustard. Furthermore, Malik et al. (2019) found that varying cytokinin concentrations had a substantial effect on regeneration efficiency in Brassica species, emphasizing the need to adjust hormone dosages for individual cultivars. Our work verifies these findings and underlines the importance of personalized techniques to maximize organogenesis efficiency. The capacity to successfully induce callus and regenerate plants using *Brassica juncea (L.)* explants has important

implications for plant biotechnology and breeding programs. This technique can help in genetic transformation and the introduction of desirable features like disease resistance and stress tolerance (Yadav et al., 2022). Improved callus induction techniques, for example, can improve the efficiency of Agrobacterium-mediated transformation, a vital stage in transgenic crop development. The use of this approach in agricultural biotechnology may result in the generation of mustard varieties with increased characteristics. For example, the capacity to regenerate plants from tissue culture might be used to make mustard plants that are more resistant to biotic and abiotic stressors, resulting in higher agricultural yields and sustainability. This is especially important considering mustard's economic value as an oilseed crop and its involvement in traditional agriculture in many countries (Sharma et al., 2022). Furthermore, our findings imply that this approach might be applied to other economically significant Brassica crops, widening the scope of this study. Sharma et al. (2022) revealed that identical tissue culture procedures may be used to *Brassica rapa and Brassica nigra*, potentially leading to the production of better varieties of both crops. While our study establishes a strong procedure for callus formation and plant regeneration, it also identifies various topics for further investigation. One drawback is the diversity in response between various *Brassica juncea (L.)* cultivars, indicating the need for additional procedure improvement and customization for other genotypes. Furthermore, understanding the molecular mechanisms behind callus formation and organogenesis in mustard may give deeper insights and contribute to the development of more effective tissue culture techniques (Kumar et al., 2019). Future studies should look at the long-term stability and genetic integrity of regenerated plants to guarantee that desired qualities are passed down across generations (Sharma & Das, 2023). They are furthermore, using sophisticated biotechnological methods like as CRISPR/Cas9 may improve the accuracy and effectiveness of genetic alterations in mustard plants (Patel et al., 2024).

4. Conclusion

The creation of an effective methodology for callus induction and plant regeneration in *Brassica juncea (L.)* utilizing leaf and stem explants marks a significant step forward in plant tissue culture and biotechnology. Our study found that combining IAA, 2,4-D, and BAP in a 1:1 ratio resulted in the highest callus induction rates, emphasizing the relevance of optimum hormone concentrations for individual cultivars. These findings confirm earlier studies and give cultivar-specific insights, furthering our understanding of phytohormone interactions in mustard. The consequences of this discovery are far-reaching, providing useful tools for genetic transformation and agricultural improvement projects. By easing the introduction of desired characteristics such as disease resistance and stress tolerance, the procedure proposed in this work has the potential to dramatically enhance agricultural biotechnology. The flexibility to adapt this technique to other economically significant Brassica crops emphasizes its broader applicability and effect. However, the work also suggests opportunities for future research, such as better optimizing the procedure for different genotypes and investigating the molecular pathways behind callus development and organogenesis. Ensuring the long-term stability and genetic integrity of regenerated plants is critical to the success of tissue culture techniques. Integrating sophisticated biotechnological methods like as CRISPR/Cas9 might improve the accuracy and efficacy of genetic alterations in mustard plants. In conclusion, this study provides a solid framework for improving in vitro organogenesis in *Brassica juncea (L.)*, opening the way for future advances in plant biotechnology and agricultural development. Continued study and improvement will improve the efficacy and application of these strategies, promoting sustainable farming practices and food security.

Table and Figures

Figure1: - In vitro callusing of the leaf as an explant of mustard (*Brassica juncea (L.)* from the first day(B), 20 days (d) to 45 days(f) after inoculation in MS media supplemented with BAP and 2,4 D in 1:1 ratio.

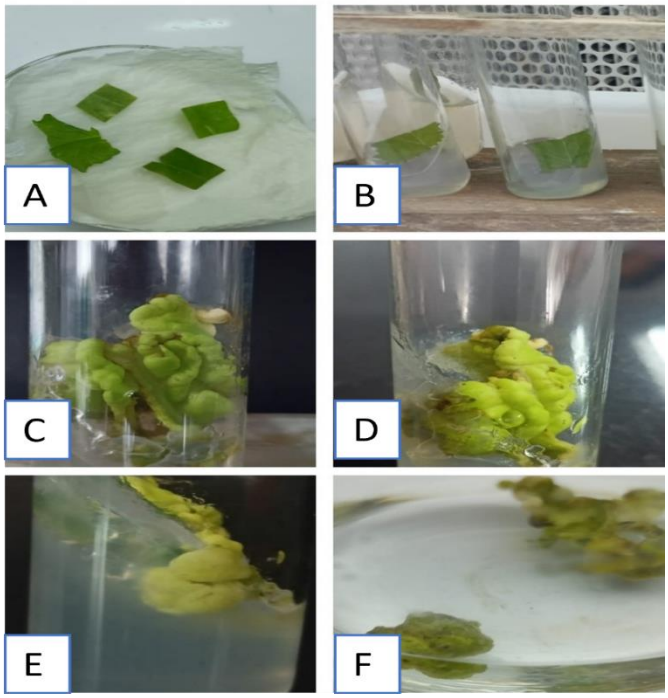


Figure. 1. a. Surface sterilization, b. First day of Inoculation c. First day of Inoculation d.7- days of Inoculation e. 15- days of Inoculation f. 30- days of Inoculation g. 30- days of Inoculation h. 40- days of Inoculation i.45- days of Inoculation.

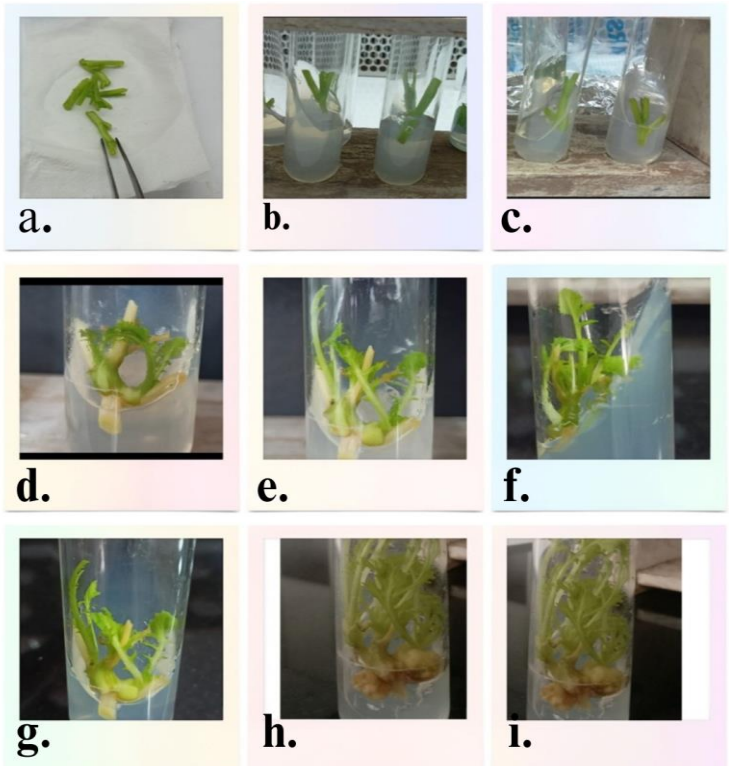
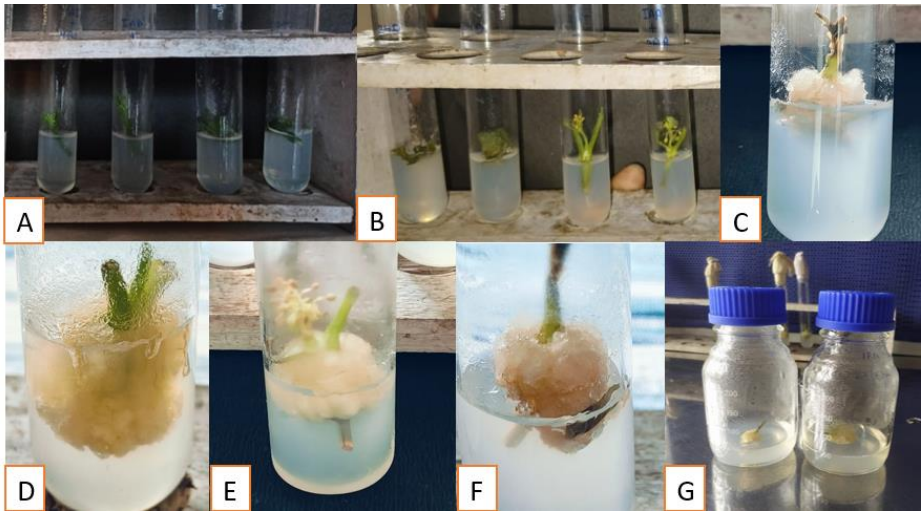
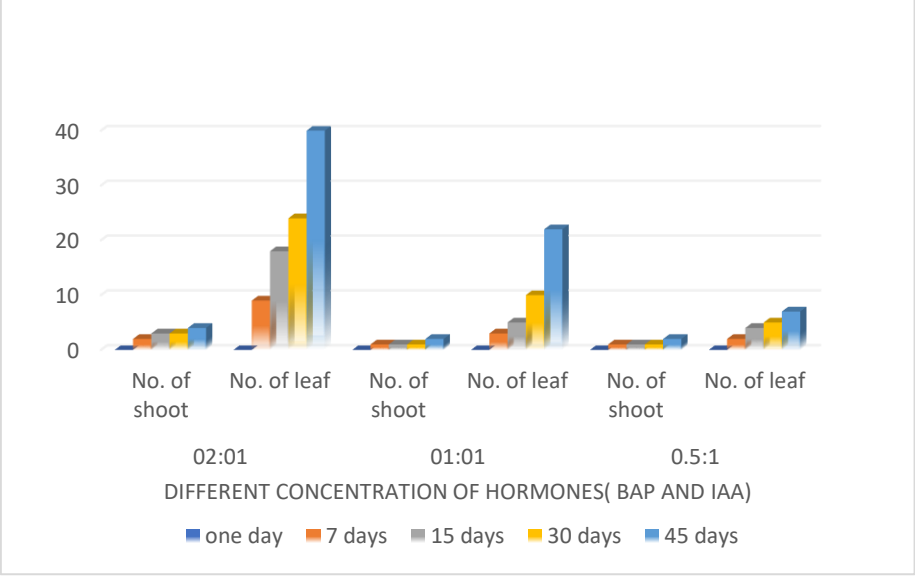


Figure 3: -(A) After 15 days of Inoculation, (B)After 21 days of Inoculation (TUBE A), (C)After 30 days of Inoculation, (D)After 21 days of Inoculation (TUBE B), (E) After 30 days of Inoculation, (F)Subculture after 45 Days



Graph 1: - the effects of different concentrations of plant growth hormones (BAP and IAA) on the number of shoots and leaves produced over varying intervals (one day, 7 days, 15 days, 30 days, and 45 days).



Graph 2: The F-statistic for shoots is 1.75 with a p-value of 0.216, while for leaves, the F-statistic is 3.05 with a p-value of 0.085.

Measurement	F-statistic	p-value
Shoots	1.75	0.216
Leaves	3.05	0.085

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