RESEARCH PAPER

In Vitro Callus Induction And Optimization Of *Convolvulus prostratus* Using In Vitro Leaf As Explant

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ABSTRACT
This study investigates the role of gibberellic acid (GA3) in enhancing seed germination and the use of the Plackett-Burman design to identify key factors influencing callus induction. GA3 significantly promoted seed germination in various species, with optimal concentrations varying by species—ranging from 1 mg/L in Prunus to over 5 mg/L in Penstemon digitalis, and 2.5–5.0 mg/L in Moringa oleifera. For callus induction, the Plackett-Burman design efficiently identified critical medium components with minimal experimental runs. Among tested factors, 2.0 mg/L 2,4- D was the most effective auxin, and its combination with 0.5 mg/L TDZ improved callus morphology and greening. Statistical analysis highlighted ADSO4 as the most significant factor influencing callus weight, supported by the highest effect (+1.32), mean square (1.74), and F-value (15.25), clearly exceeding the experimental error estimated from dummy variables. KH2PO4 (F = 7.87) and sucrose (F = 4.98) also showed significant impact. Conversely, 2,4-D, casein hydrolysate, and TDZ had minimal effects (F < 0.78). These findings emphasize the importance of hormone customization and statistical optimization in plant tissue culture and suggest prioritizing ADSO4, KH2PO4, and sucrose in future optimization studies..

Keywords: Placket Burman, Callus, Convolvulus prostratus
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INTRODUCTION

Convolvulus prostratus is a well-documented herb in Ayurvedic medicine for its use in treating neurological disorders, epilepsy, and anxiety. The plant exhibits significant antioxidant and nootropic activities, which are attributed to the presence of flavonoids, alkaloids, and glycosides (Dhingra & Valecha, 2007). The increasing demand for herbal-based cognitive enhancers has led to the overexploitation of this species, necessitating efficient propagation strategies for its sustainable use.

Callus induction is a critical step in plant tissue culture, serving as the foundation for various biotechnological applications such as secondary metabolite production, plant regeneration, and genetic transformation. The efficiency of callus induction is influenced by several factors, including plant growth regulators (PGRs), carbohydrates, and nitrogen sources. This review focuses on the effects of 2,4-D, TDZ, sucrose, casein hydrolysate, adenine sulfate, and KH_2PO_4 on callus induction in Plakcet burman, a member of the Convolvulaceae family. The optimization of in vitro callus induction is a complex process due to the numerous variables involved. Traditionally, researchers have relied on trial-and-error approaches to identify suitable conditions, but modern statistical methods offer a more systematic and efficient way to optimize the process. Techniques such as the Plackett-Burman Design (PBD) and Response Surface Methodology (RSM) have been successfully employed in optimizing tissue culture protocols by evaluating the influence of multiple factors simultaneously (Ali et al., 2014). The Plackett-Burman Design (PBD) is an experimental design method that helps identify the most significant factors affecting callus induction, thereby reducing the number of experimental runs required (Plackett & Burman, 1946).

To overcome the limitations of conventional methods, the present study employs the Plackett–Burman Design (PBD)—a statistical screening tool that allows for the rapid identification of significant variables using minimal experimental runs (George et al., 2008). In this research, leaf explants of C. prostratus were cultured on full-strength Murashige and Skoog (MS) medium supplemented with varying concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), Thidiazuron (TDZ), sucrose, casein hydrolysate,

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ammonium sulfate ($AdSO_4$), and potassium dihydrogen phosphate (KH_2PO_4) (Srivastava & Sharma, 2014). Cultures were maintained at 26°C under a photoperiod of 16 hours darkness and 8 hours light to promote optimal callus initiation (Mishra & Rathore, 2015).

The use of a statistically guided PBD approach in this context enables the evaluation of multiple factors simultaneously and facilitates the identification of those with the most significant influence on callus formation (Thakur & Thakur, 2016). This method ensures higher efficiency and reproducibility, paving the way for the development of a robust protocol for further in vitro applications (Singh & Dwivedi, 2016).

The discussion is supported by studies on related species and other plants, as direct research on Plakcet burman is limited. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin widely used for callus induction in various plant species. It promotes cell division and differentiation by mimicking the effects of natural auxins. In studies on related species, 2,4-D has been shown to significantly enhance callus formation when used alone or in combination with cytokinins. The effective concentration of 2,4-D for callus induction varies among species. For example, in Clitoria ternatea, the highest friable callus fresh weight was achieved with 0.25 mg/L 2,4-D (Zakaria et al., 2024). Similarly, in Talinum paniculatum, 1-3 mg/L 2,4-D combined with kinetin induced callus formation (Restiani et al., 2022). The combination of 2,4-D with cytokinins like BAP or TDZ has been found to enhance callus induction. For instance, in Coffea liberica, the combination of 2.0 ppm 2,4-D and 1.0 ppm BAP resulted in the fastest callus proliferation (Lizawati et al., 2020).

Thidiazuron (TDZ) is a cytokinin-like compound that has been increasingly used for callus induction due to its ability to stimulate both auxin- and cytokinin-like activities. TDZ has been particularly effective in recalcitrant species and has been shown to induce callus formation at lower concentrations compared to traditional cytokinin. In Ananas comosus, the combination of 6.0 mg/L 2,4-D and 2.0 mg/L TDZ resulted in the highest callus fresh weight (Salihan & Yusuf, 2020). Similarly, in Solanum virginianum, 5 mg/L TDZ was optimal for callus induction and biomass accumulation (Usman et al., 2022). TDZ has been reported to induce both somatic embryogenesis and shoot organogenesis, depending on the species and concentration used. In woody plants, TDZ has been particularly effective in overcoming recalcitrance (Novikova & Zaytseva, 2018).

Sucrose is a key carbohydrate source in tissue culture media, providing energy and carbon skeletons for cell growth. The concentration of sucrose can significantly influence callus induction and growth. Studies on Clitoria ternatea showed that 15 g/L sucrose was optimal for callus induction, while higher concentrations (30 g/L) were less effective (Zakaria et al., 2024). Similarly, in Wedelia biflora, 4% sucrose was found to be ideal for callus growth (Norayu, 2017). Sucrose not only supports callus growth but also influences the production of secondary metabolites. For example, in Wedelia biflora, 4% sucrose enhanced stigmasterol production in callus cultures (Norayu, 2017).

Casein hydrolysate is a nitrogen source commonly added to tissue culture media to enhance callus growth and plant regeneration. It provides amino acids, which are essential for protein synthesis and cell division. In Digitalis lanata, the addition of 500 mg/L casein hydrolysate to the medium containing 1.5 mg/L kinetin and 0.5 mg/L IAA significantly improved callus growth (Fatima et al., 2009). The response to casein hydrolysate can vary among species. For example, in Valeriana jatamansi, the addition of casein hydrolysate enhanced callus induction and valepotriate production (Das et al., 2013). Adenine sulfate is a nitrogen source that has been used in combination with other growth regulators to enhance callus induction and plant regeneration. It acts as a source of adenine, which is essential for nucleic acid synthesis.

In *Digitalis lanata*, the combination of 1.5 mg/L kinetin, 0.5 mg/L IAA, and 500 mg/L casein hydrolysate was found to be optimal for callus induction (Fatima et al., 2009). Adenine sulfate has been used in combination with other nitrogen sources like casein hydrolysate to enhance callus growth and regeneration. For example, in Passiflora quadrangularis, the combination of 2 mg/L 2,4-D and 0.5 mg/L BAP induced callus formation at a high rate (Oros et al., 2024).

KH_2PO_4 (monobasic potassium phosphate) is a source of phosphorus and potassium, which are essential for various cellular processes, including nucleic acid synthesis and energy production. The role of KH_2PO_4 in callus induction is less frequently studied, but it is often included in tissue culture media as a macronutrient. The MS medium, which is commonly used for plant tissue culture, contains 170 mg/L KH_2PO_4. This concentration has been found to support callus induction in various species, including Telfairia occidentalis (Sakpere et al., 2014).

The response to KH_2PO_4 can vary among species. For example, in *Piper betle*, the addition of KH_2PO_4 to the medium enhanced callus growth and secondary metabolite production (Junairiah et al., 2023).

Comparative Analysis of Key Factors

Factor	Optimal Concentration/Condition	Effect on Callus Induction	Citation
2,4-D	0.25-3.0 mg/L	Enhances callus formation	(Zakaria et al., 2024) (Restiani et al., 2022)

TDZ	0.25-5.0 mg/L	Induces callus and biomass	(Novikova & Zaytseva, 2018) (Usman et al., 2022)
Sucrose	3-4%	Supports callus growth	(Zakaria et al., 2024) (Norayu, 2017)
Casein Hydrolysate	500 mg/L	Enhances callus growth	(Fatima et al., 2009) (Das et al., 2013)
Adenine Sulfate	1.5 mg/L	Promotes callus induction	(Fatima et al., 2009) (Oros et al., 2024)
KH_2PO_4	170 mg/L	Supports nutrient uptake	(Sakpere et al., 2014) (Junairiah et al., 2023)

The efficiency of callus induction in Plakcet burman, a member of the Convolvulaceae family, can be optimized by carefully selecting the concentrations of 2,4-D, TDZ, sucrose, casein hydrolysate, adenine sulfate, and KH_2PO_4. Based on studies on related species, the following conclusions can be drawn:
2,4-D is a critical auxin for callus induction, with optimal concentrations ranging from 0.25 mg/L to 3.0 mg/L, depending on the species. TDZ is a potent cytokinin-like compound that can induce callus formation at lower concentrations compared to traditional cytokinins. Sucrose at 3-4% is ideal for supporting callus growth and secondary metabolite production. Casein hydrolysate and adenine sulfate are effective nitrogen sources that enhance callus growth and regeneration. KH_2PO_4 provides essential macronutrients for callus induction and growth. Further research is recommended to determine the specific optimal conditions for Plakcet burman, as responses to these factors can vary among species.

MATERIAL AND METHOD:

Plant Material and Surface Sterilization

Seeds of *Convolvulus prostratus* were obtained from a certified local herbal nursery and were used as explants for in vitro callus induction studies. Prior to culture, seeds were thoroughly washed under running tap water for 10 minutes to remove surface debris. They were then surface sterilized in a laminar airflow cabinet using 70% ethanol for 30 seconds followed by immersion in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. After sterilization, seeds were rinsed three to four times with sterile distilled water to eliminate any residual $HgCl_2$.

Culture Medium Preparation

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium for all experiments. The medium was supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 minutes at 15 psi. All culture media were prepared under sterile conditions and dispensed into sterile culture tubes or jars prior to inoculation.

Experimental Design for Optimization

A **Plackett–Burman Design (PBD)** was employed to screen and identify the most influential factors affecting callus induction from seed explants. Six independent variables were tested at two levels (high and low):

2, 4-Dichlorophenoxyacetic acid (2, 4-D): 0.5 mg/L and 2.0 mg/L
Thidiazuron (TDZ): 0.1 mg/L and 1.0 mg/L
Sucrose: 2% and 5%
Casein hydrolysate: 250 mg/L and 1000 mg/L
Adenine sulfate ($AdSO_4$): 25 mg/L and 100 mg/L
Monopotassium phosphate (KH_2PO_4): 50 mg/L and 200 mg/L

Experimental Design – Screening Using Plackett–Burman Design

A Plackett–Burman Design (PBD) was used to identify the most influential medium components for callus induction. The design allowed for the evaluation of six independent variables at two levels (high and low):

Factor	Low (-1)	High (+1)
2,4-D (mg/L)	0.5	5.0
TDZ (mg/L)	0.1	2.5
Sucrose (%)	2.0	5.0
Casein hydrolysate (mg/L)	250	1000
Adenine sulfate ($AdSO_4$) (mg/L)	25	100
KH_2PO_4 (mg/L)	50	200

The experimental matrix consisted of 12 treatment combinations arranged using a standard PBD matrix. Each treatment was replicated three times, with ten seeds per replicate.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2
	A:Sucrose	B:2 4 D	C:Casein hydrolysis	D:TDZ	E:KH2PO4	F:ADSO4	G:G	H:H	I:I	J:J	K:K	Callus	Callus
	gm/l	mg/l	mg/l	mg/l	mg/l	mg/l						Weight	Proliferation
1	2	5	250	0.1	250	10	1	1	1	-1	-1	0.331	50
2	2	0.5	250	2.5	250	100	-1	-1	-1	-1	1	1.214	60
3	5	5	250	2.5	50	10	-1	-1	1	1	-1	1.179	50

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4	5	5	1000	2.5	250	100	-1	-1	1	-1	1	0.657	30
5	5	0.5	250	0.1	50	100	1	1	-1	1	1	1.502	70
6	2	0.5	1000	2.5	50	10	-1	-1	1	1	1	2.318	100
7	2	5	1000	0.1	50	100	-1	-1	-1	1	-1	2.42	100
8	5	0.5	1000	0.1	250	10	1	1	1	-1	1	1.374	50
9	2	5	250	2.5	50	100	1	1	-1	1	1	2.322	50
10	2	5	1000	2.5	250	100	1	1	-1	-1	-1	0.964	40
11	5	0.5	1000	0.1	50	10	1	1	1	1	-1	0.216	10
12	5	0.5	250	0.1	250	100	-1	-1	-1	-1	-1	1.622	70

The MS medium was prepared as described earlier, and seed explants were inoculated onto this medium supplemented with the respective concentrations of **BAP**, **TDZ**, and **casein hydrolysate**. All media were prepared in a laminar flow cabinet and sterilized by autoclaving at 121°C for 20 minutes.

Culture Conditions

Cultures were incubated at 26 ± 2°C under a **16-hour dark / 8-hour light photoperiod**, with light provided by fluorescent lamps at 40 µmol m^{-2} s^{-1}. After inoculation, cultures were monitored for callus initiation, with data recorded after 4 weeks of culture.

Inoculation and Culture Conditions

Sterilized seed explants were aseptically inoculated onto MS media supplemented with respective PBD treatment combinations. Cultures were maintained in a growth chamber at 26 ± 2°C with a 16-hour dark / 8-hour light photoperiod. Illumination was provided by white fluorescent lights at an intensity of ~40 µmol m^{-2} s^{-1}. The cultures were observed over a period of four weeks for signs of callus initiation and development.

Statistical Analysis

The data obtained from the PBD experiments were subjected to analysis using **State ease software**. Analysis of variance (ANOVA) was performed to identify the significant factors influencing callus induction. A p-value ≤ 0.05 was considered statistically significant. Based on the results of PBD.

RESULTS AND DISCUSSION:

Overview of Leaf Induction in *Convolvulus Prostratus*

The present investigation aimed to optimize in vitro leaf regeneration in *Convolvulus prostratus* through the use of in vitro grown seedling as explants cultured on full-strength Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs) like Gibbrrellic acid and medium components.

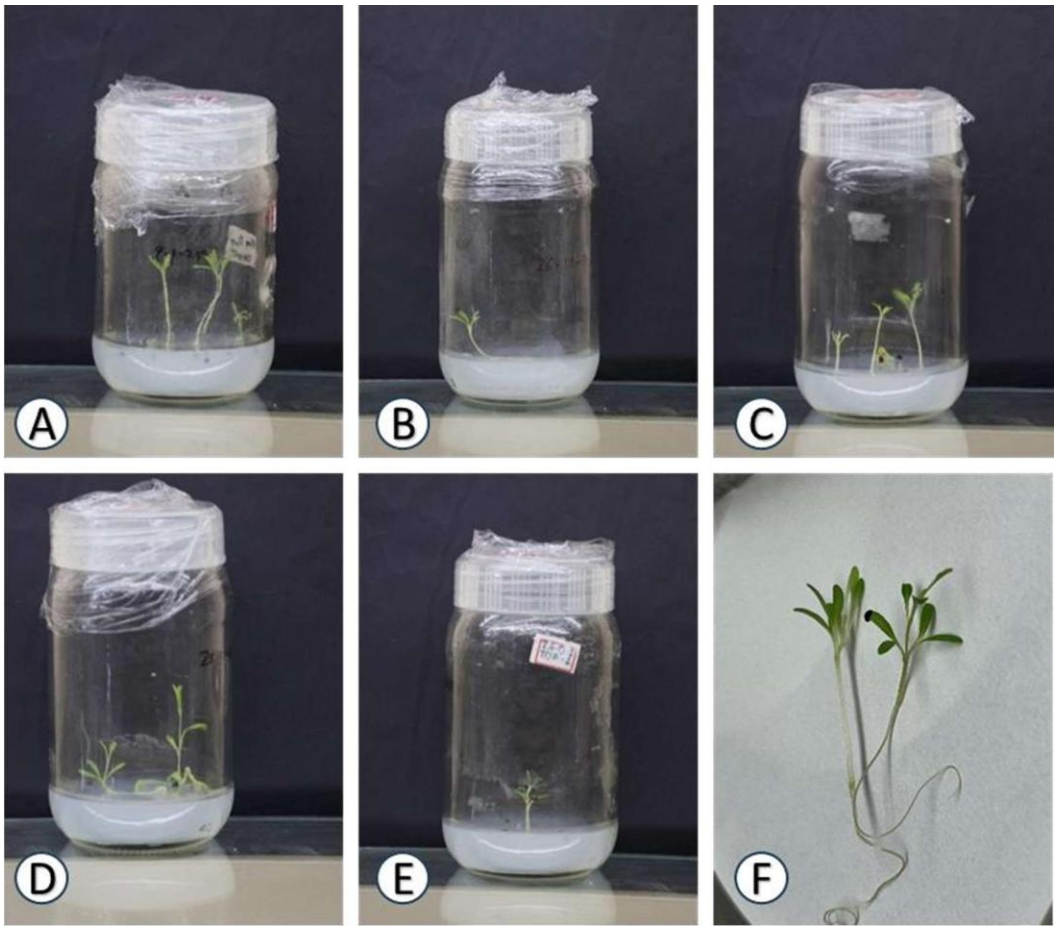


Figure 1. With the used of Gibberellin hormone in Picture A, C, D a clearly shown Shoots, Leaf and Root Formation. It shown the best growth-the seedlings in a look healthy and bigger. Picture B, E shown weaker growth, smaller seedling. Figure F Shown the proper growth of seeds.

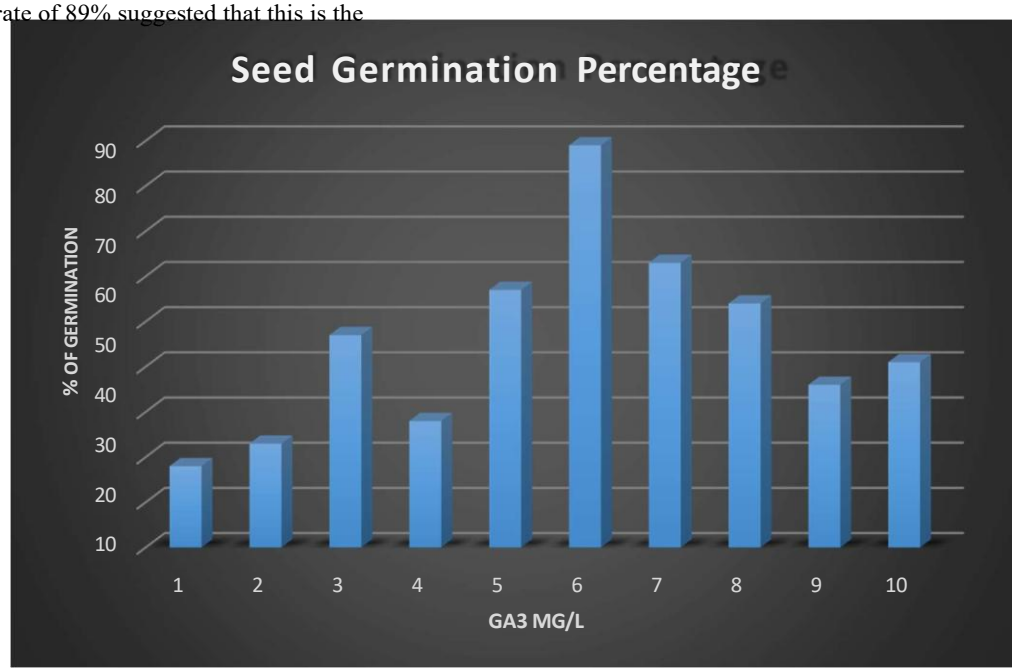
Table 2. Seed Germination using GA3

Sr. No	GA3 Concentration (mg/l)	Seed Germination %
1	0.5	18
2	1.0	23
3	1.5	47

4	2.0	28
5	2.5	57
6	3.0	89
7	3.5	63
8	4.0	54
9	4.5	36
10	5.0	41

***Seed germination % was recorded after the day.**

The table shows how different GA_3 (Gibberellic Acid) levels influence seed germination rate. GA_3, a plant hormone, promotes seed germination, breaks dormancy. The study show GA_3 at 0.5 mg/L to 5.0 mg/L seed germination rates were recorded. At the lowest concentrations of 0.5 and 1.0 mg/L, seed germination was somewhat low at 18% and 23%, respectively, indicating that such small dosages of GA3 are insufficient to encourage significant germination. As the concentration was increased to 1.5 mg/L, germination climbed to 47%; a further increase to 2.5 mg/L yielded 57% germination. At 3.0 mg/L, the maximum germination rate of 89% suggested that this is the optimal dose to promote germination in the given seeds. But as GA_3 concentration increased above this point, germination declined. Germination dropped to 63% at 3.5 mg/L, then to 54% at 4.0 mg/L, 36% at 4.5 mg/L, and 41% at 5.0 mg/L. Germination increases with increasing GA until a certain point; then, with higher increases, it declines. 3.0 mg/L is the most effective concentration for best seed germination.



Graph: 1 Effect of seed germination on the seed germination percentage

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In Moringa oleifera GA3 concentrations of 2.5 mg/L and 5.0 mg/L enhance the germination rate of *Moringa oleifera* seeds. Seeds treated with these concentrations showed a higher germination rate compared to untreated seeds, although the highest germination was observed at 7.5 mg/L, which is slightly above the specified range (Eghobor et al., 2015) (Santoso, 2022). In *Prunus* species, GA3 concentrations of 1 mg/L and 3 mg/L significantly improved germination rates. The highest germination percentage was observed at 1 mg/L without cold treatment, suggesting that lower concentrations within the specified range can be effective for certain species (Ghayyad, 2018). *Macrotyloma uniflorum* GA3 at 2 mg/L was found to be the most effective concentration, resulting in an 88% germination rate. This demonstrates that lower concentrations within the range can be optimal for certain legumes (Lalitha et al., 2016). Experiments with *Penstemon digitalis* showed that GA3 increased germination rates, although the most effective concentrations were higher than

5 mg/L. This suggests that while GA3 is beneficial, the optimal concentration may vary significantly between species (Mello et al., 2009).

Optimization of Plant Growth Regulators for Callus Induction

The Plackett-Burman design is highly efficient in screening a large number of factors with a limited number of experiments. This is achieved by evaluating the main effects of each factor while assuming that interactions between factors are negligible in the initial stages of experimentation (Gregor et al., 2024; Zaykov, 2023).

In the context of callus production, this design allows researchers to systematically investigate the effects of various media components and growth regulators, such as 2,4-D, putrescine, and glycine, on callus induction and growth (Keighobadi et al., 2020). The design has been successfully applied to screen factors affecting callus production in different plant species. For instance, in Allium cepa L., the Plackett-Burman design was used to identify that putrescine, glutamine, 2,4-D, and glycine significantly contribute to callus production, with putrescine having the most substantial positive effect (Keighobadi et al., 2020).Similarly, in cassava, the design helped identify that Gamborg B5 medium, sucrose, and 2,4-D were significant factors influencing callus formation frequency, demonstrating the design's utility in optimizing media composition for different plant species (Ubaidah et al., 2024). The Plackett-Burman design provides a robust statistical framework for identifying significant factors, which can then be further optimized using more detailed experimental designs like full factorial or response surface methodologies (Zaykov, 2023). It is particularly useful in situations where resources are limited, as it reduces the number of experimental runs required compared to traditional full factorial designs (Gregor et al., 2024).

The efficacy of various auxins and cytokinins, individually and in combination, was assessed for optimal callus induction. Among the auxins tested, 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 2.0 mg/L proved most effective, inducing friable, cream-colored callus with a high fresh weight yield. This aligns with findings in *Vanda* species, where 2.0 mg/L 2, 4-D resulted in an 83.3% callus induction rate (Budisantoso et al., 2017). The addition of cytokinins such as thidiazuron (TDZ) and kinetin (KIN) influenced the morphology and proliferation rate of the callus. Notably, the combination of 2.0 mg/L 2, 4-D with 0.5 mg/L TDZ enhanced callus compactness and greening, particularly in leaf explants. This synergistic effect of auxin-cytokinin combinations has been

documented in *Ipomoea mauritiana*, where 0.5 mg/L 2, 4-D combined with 1.0 mg/L benzylaminopurine (BAP) yielded optimal callus growth (Chakraborty et al., 2016).

Table 3: Placket Burman

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2
	A:Sucrose	B:2 4 D	C:Casein hydrolysis	D:TDZ	E:KH2PO4	F:ADSO4	G:G	H:H	I:I	J:J	K:K	Callus	Callus
	gm/l	mg/l	mg/l	mg/l	mg/l	mg/l						Weight	Proliferation
1	2	5	250	0.1	250	10	1	1	1	-1	-1	0.331	50

2	2	0.5	250	2.5	250	100	-1	-1	-1	-1	1	1.214	60
3	5	5	250	2.5	50	10	-1	-1	1	1	-1	1.179	50
4	5	5	1000	2.5	250	100	-1	-1	1	-1	1	0.657	30
5	5	0.5	250	0.1	50	100	1	1	-1	1	1	1.502	70
6	2	0.5	1000	2.5	50	10	-1	-1	1	1	1	2.318	100
7	2	5	1000	0.1	50	100	-1	-1	-1	1	-1	2.42	100
8	5	0.5	1000	0.1	250	10	1	1	1	-1	1	1.374	50
9	2	5	250	2.5	50	100	1	1	-1	1	1	2.322	50
10	2	5	1000	2.5	250	100	1	1	-1	-1	-1	0.964	40
11	5	0.5	1000	0.1	50	10	1	1	1	1	-1	0.216	10
12	5	0.5	250	0.1	250	100	-1	-1	-1	-1	-1	1.622	70

Table 4: Callus Percentage:

	FACTORS										
	Sucrose	**2 ,4 -D**	**C:Casein hydrolysis**	**TDZ**	**$KH2PO_4$**	**$ADSO_4$**	**G:G**	**H:H**	**I:I**	**J:J**	**K:K**
Σ(H)	280	360	330	330	300	420	270	270	290	380	360
Σ(L)	400	320	350	350	380	260	410	410	390	300	320
Differenc e	-120	40	-20	-20	-80	160	-140	-140	-100	80	40
EFFECT	-30	10	-5	-5	-20	40	-35	-35	-25	20	10
MEAN SQUARE	900	100	25	25	400	1600	1225	1225	625	400	100

EXP ERROR	-13										
	Sucrose	**2 4 D**	**C:Casein hydrolysis**	**TDZ**	**KH2PO4**	**ADSO4**	**G:G**	**H:H**	**I:I**	**J:J**	**K:K**
F TEST	69.23077	7.692307692	1.923076923	1.923077	30.76923	123.0769	94.23077	94.23077	48.07692	30.76923	7.692308

The results clearly show that $ADSO_4$ (with an effect of +40) had significant influence. The MEAN SQUARE values, where $ADSO_4$ has the highest (1600), support this. The F-test indicates that the actual variables with notable effects are ADSO4 (F = 123.08), Sucrose (F = 69.23), and KH2PO4 (F = 30.77). These numbers are far more than the dummy-based error variance, implying that these elements really and significantly affect the result. By contrast, 2,4-D, Casein hydrolysis, and TDZ had extremely low F-values (7.69 or less), suggesting a non-significant impact under the conditions examined.

Finally, the study reveals that $ADSO_4$, Sucrose, and KH_2PO_4 are the most important and statistically relevant variables in this experiment after using the dummy runs (G:G, H:H, I:I) as the foundation for calculating experimental error. Future optimization research should give top

priority these. While their impacts are not to be read biologically, dummy factors fulfill their function in evaluating the design and calculating experimental error.

Table 5: Callus Weight:

	FACTORS										
	Sucrose	**2 4 D**	**C:Casein hydrolysis**	**TDZ**	**KH2PO4**	**ADSO4**	**G:G**	**H:H**	**I:I**	**J:J**	**K:K**
Σ(H)	6.55	7.873	7.949	8.654	6.162	10.701	6.709	6.709	6.709	9.957	9.387
Σ(L)	9.569	8.246	8.17	7.465	9.957	5.418	9.41	9.41	10.044	6.162	6.732
Difference	-3.019	-0.373	-0.221	1.189	-3.795	5.283	-2.701	-2.701	-3.335	3.795	2.655
EFFECT	- 0.75475	- 0.09325	- 0.05525	0.29725	- 0.94875	1.32075	- 0.67525	- 0.67525	- 0.83375	0.94875	0.66375
MEAN SQUARE	0.569648	0.008696	0.003053	0.088358	0.900127	1.744381	0.455963	0.455963	0.695139	0.900127	0.440564

EXP ERROR	- 0.114 35										
	Sucrose	**2 4 D**	**C:Casein hydrolysis**	**TDZ**	**KH2PO4**	**ADSO4**	**G:G**	**H:H**	**I:I**	**J:J**	**K:K**
F Test	4.981 614	0.076 043	0.026 695	0.772 694	7.871 68	15.25 475	3.987 429	3.987 429	6.079 047	7.871 68	3.852 768

The F-test results clearly show that $ADSO_4$ (F = 15.25) and KH_2PO_4 (F = 7.87) have the most important effect on the result. Estimated from the dummy variables, these F-values are far higher than the background variance (G:G = 3.99, H:H = 3.99, I:I = 6.08). With an F-value of 4.98, sucrose also indicates a significant contribution to the reaction. Conversely, elements such 2,4-D (F = 0.076), Casein hydrolysis (F = 0.027), and TDZ (F = 0.77) exhibit extremely low F-values, implying they have little or non-significant effect under the experimental settings. Averaging F- values between 3.98 and 6.08, the dummy variables (G: G, H:H, and I: I), non-biological and included just for error estimation, help provide a standard for differentiating actual effects from background noise. Any experimental variable exhibiting an F-value far higher than these dummy runs can be deemed really significant.

Ultimately, this study shows clearly that $ADSO_4$ is the most important element affecting the reaction; next comes KH_2PO_4 and Sucrose. Future optimization tests should give this top priority. Dummy variables did their job well in calculating experimental error and establishing a statistical significance threshold; their impact should not be read biologically. In this situation, factors such 2,4-D, Casein hydrolysis, and TDZ are statistically non-significant and might not deserve more attention under comparable circumstances.

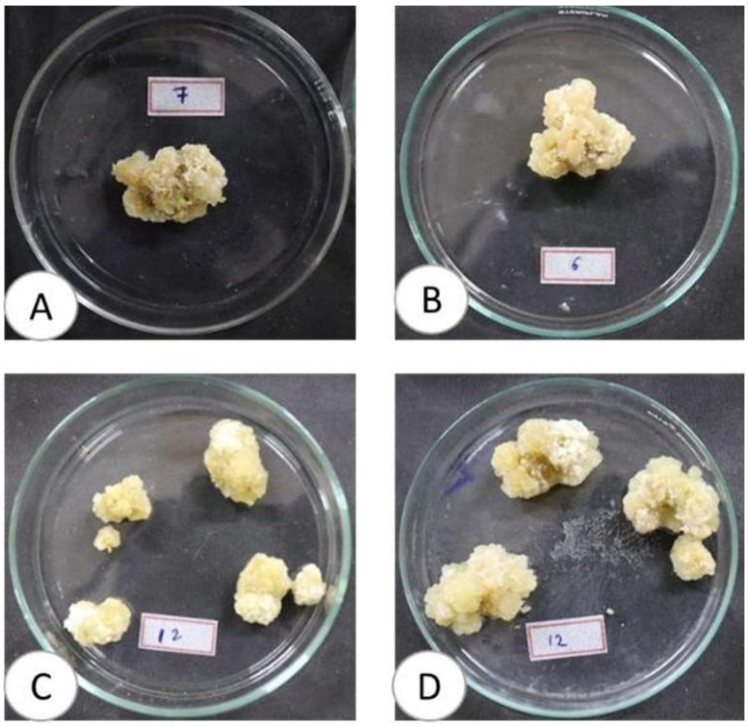

Figure 2: Picture A: Callus weight was 2.420 gm.

Picture B: callus weight was 4.798 gm.

Picture C Callus 1 weight was 0.964, Callus 2 weight was 0.916, Callus weight was 1.502 gm, and callus

weight was 1.374 gm.

Picture D: Callus 1 weight was 3.049 gm, callus 2 weight was 3.301 gm, callus 3 weight was 3.071 gm.

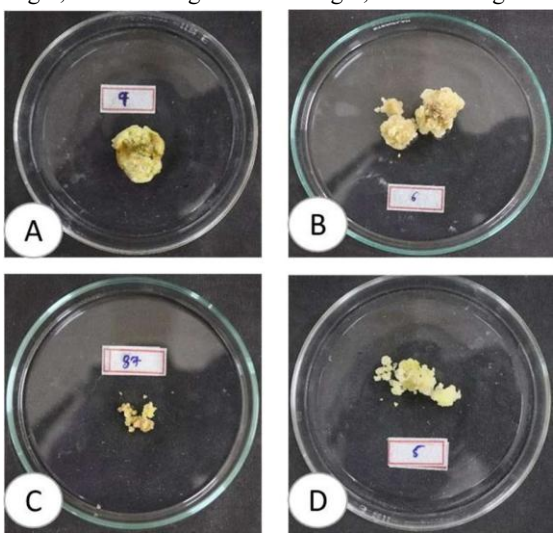

Figure 3:Picture A callus weight was2.322 gm. Picture B: callus weight was 2.218 gm. Picture C: Callus weight was 0.183 gm. Picture D: Callus weight was 0.700 gm

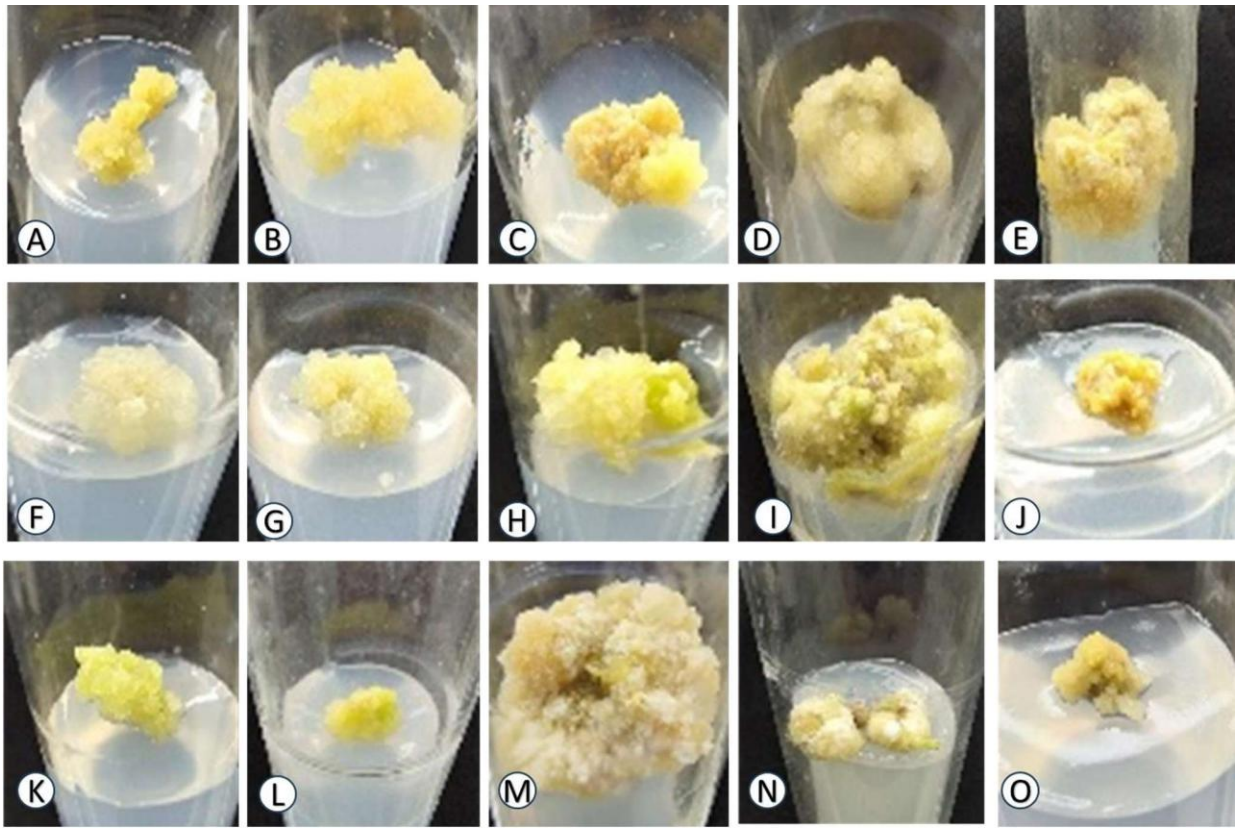

Figure 4: These pictures were shown the callus multiplication that induced from leaf explant. Picture A, B, C multiplication stage of callus induction after first subculture; Yellow, Small and compact callus forming. Figure D, E show better development, callus becoming more nodular and larger. Picture F, G, H White, soft callus formation. Picture I large, vigorously

growing callus possibly ready for generation. Picture J, K, Moderate callus, slightly yellowish-white might still be developing. Picture L very little callus or delayed induction. Picture M Healthy, friable white callus- ideal for further organogenesis. Picture N, O Minimal to weak callus formation, indicating poor response at this treatment.

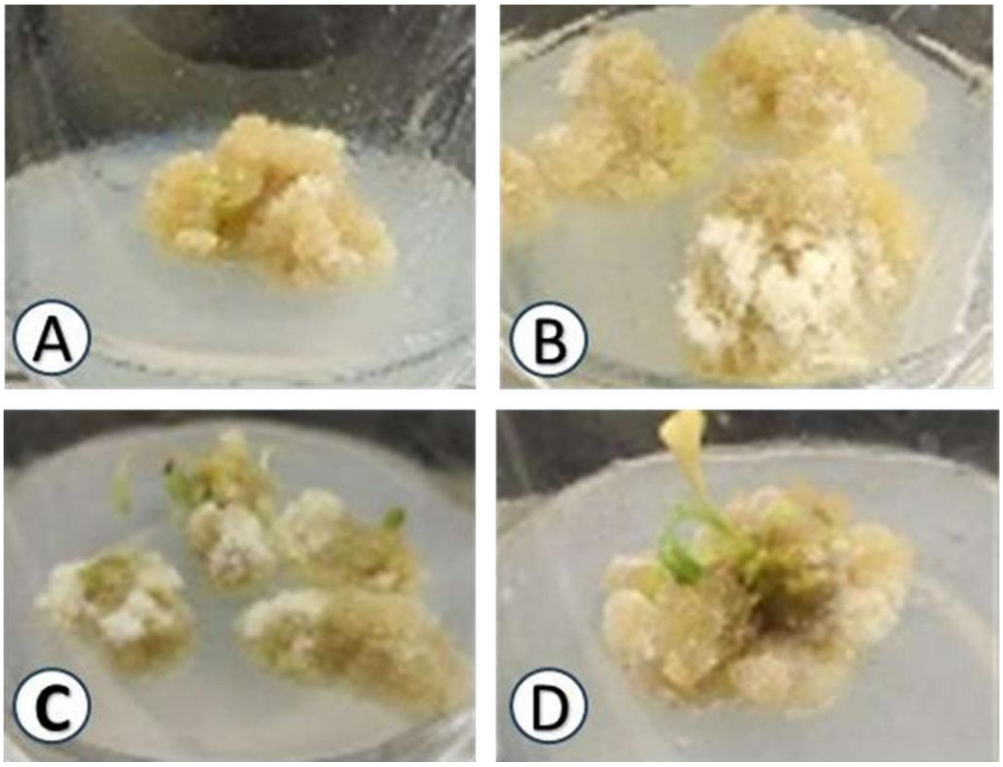

Figure 5: These is shown callus multiplication induced from the leaf after second subculture . Picture A: A fairly healthy-looking callus, creamy- white to yellowish in color, compact texture, good callus formation. Picture B: Prolific callus Growth, larger amount callus, still yellowish but starting to show some friable structure. Slightly white patches were seen. Picture C: The callus is whiter, softer and has some greenish spots, indicating possible start of shoot or root formation, the green spot indicates photosynthetic activity starting. Picture D: the callus looking like a bit darker, maybe browning and there are green spot visible, a brown color shown the callus indicate stress and aging.

For potato (*Solanum tuberosum*), 2.0-4.0 mg/l of 2,4-D was optimal for callus formation, while TDZ was used in subsequent stages for shoot regeneration (D et al., 2014). In tea clone UPASI 9, a combination of 1.1 mg/l of both 2,4-D and TDZ was found to be most effective for callus proliferation, reducing the time required for initiation and proliferation (M et al.,2023). For the panda plant (*Kalanchoe tomentosa*), higher concentrations of 2,4-D promoted callus proliferation, while TDZ at higher concentrations increased callogenesis but inhibited root production (Pakum et al., 2021). In Jatropha curcas, combinations of 5.0 ppm 2,4-D with 1.0 ppm TDZ and 7.5 ppm 2,4-D with 1.5 ppm TDZ resulted in the fastest callus formation, with the highest percentage of callus formation observed at 2.5 ppm 2,4-D with 1.0 ppm TDZ (Lizawati, 2012).

Casein hydrolysate provides a rich source of amino acids and peptides, which are essential for cellular growth and development. These components serve as building blocks for proteins and other macromolecules necessary for cell proliferation and differentiation (Cogan et al., 1981) (Grozeva et al., 1994).In the study of *Dendrobium* sp. plantlets, the combination of sucrose and casein hydrolysate significantly improved plantlet growth, indicating the importance of casein hydrolysate as a nutritional supplement in culture media (Arif, 2025). In the case of *Convolvulus pluricaulis,* a higher concentration of

casein hydrolysate in the culture medium resulted in maximum callus growth and enhanced hypotensive activity of the callus tissue (Mudgal, 2024). The effects of KH_2PO_4 (potassium dihydrogen phosphate) and ammonium sulfate on plant tissue growth and development have been explored in various studies, highlighting their roles in optimizing plant growth conditions. In wheat, KH2PO4 application increased enzyme activities and reduced oxidative damage under low-temperature stress, enhancing photosynthetic capacity and reducing yield loss (Chen et al., 2024). The concentration of sucrose in the culture medium can significantly impact callus growth. For instance, in *Coleus blumei*, a higher sucrose concentration (50 g/l) resulted in the highest callus fresh weight, indicating enhanced growth. In tobacco callus cultures, a 3% sucrose concentration was found to be optimal for growth and shoot formation, with deviations from this concentration leading to reduced growth rates (Brown et al., 1979).

CONCLUSION:

Gibberellic acid (GA_3) significantly promotes seed germination in several plant species. With the highest rate at 7.5 mg/L, 2.5–5.0 mg/L GA_3 in *Moringa oleifera* increased germination (Eghobor et al., 2015; Santoso, 2022). While 2 mg/L was ideal for Macrotyloma uniflorum, Prunus species reacted best to 1 mg/L (Ghayyad, 2018; Lalitha et al., 2016). Effective doses in *Penstemon digitalis* surpassed 5 mg/L, suggesting species-specific reactions (Mello et al., 2009). The Plackett-Burman design efficiently finds important media components for callus induction by means of major effects with few experiments (Gregor et al., 2024; Zaykov, 2023). In Allium cepa and cassava, 2,4-D, putrescine, and glycine had major effects on callus generation (Keighobadi et al., 2020; Ubaidah et al., 2024). Among auxins, 2.0 mg/L 2,4-D produced the most effective callus; combining it with 0.5 mg/L TDZ improved quality and greening, as also seen *in Ipomoea mauritiana* (Budisantoso et al., 2017; Chakraborty et al., 2016).

These findings underline the need of customized hormone combinations and statistical optimization. Supported by its high effect value (+1.32), the highest mean square (1.74), and an F-value of 15.25, all of which greatly exceed the background variance set by dummy variables, the experimental data clearly show $ADSO_4$ as the most important factor affecting callus weight. Under the tested conditions, KH_2PO_4 (F = 7.87) and sucrose (F = 4.98) also showed statistically significant effects, suggesting their powerful influence in callus development. By comparison, 2,4-D, casein hydrolysate, and TDZ showed very low F-values all under 0.78—indicating they have little to no effect under this experimental setup. With their mean F-values (3.98–6.08) offering a benchmark for significance, dummy variables G:G, H:H, and I:I worked well in predicting experimental error. Any variable with an F-value much above this range, such as $ADSO_4$, KH_2PO_4, and sucrose, can be deemed really important. While suggesting that other examined variables would not need more attention under comparable experimental settings, our results underline the need of giving these three elements top priority in future optimization research.

These hormone customization and statistical optimization. Test results indicate that $ADSO_4$ significantly affects callus weight, with a high effect value (+1.32), greatest mean square (1.74), and F-value (15.25), outperforming background variation from dummy factors. KH_2PO_4 (F = 7.87) and sucrose (F = 4.98) also significantly impacted callus formation throughout testing. The F-values for 2,4-D, casein hydrolysate, and TDZ were < 0.78, indicating minimal impact in this experiment. With significance-level mean F-values (3.98–6.08), dummy variables G:G, H:H, and I:I predicted experimental error. A high F-value for variables like $ADSO_4$, KH_2PO_4, and sucrose indicates significance. Our findings suggest that comparable experimental settings would not require greater attention to other parameters, but future optimization research should prioritize these three elements..

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