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Synergistic influence of growth regulators, micronutrients, and precursors on colchicine production in *Gloriosa superba* callus

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Abstract
The main origin of colchicine, a key medicinal alkaloid, is the temperate plant known as Colchicum autumnale. Given its rarity in India, the tropical species *Gloriosa superba* L., which is prevalent throughout India, and synthesizes colchicine to a considerable degree, gains substantial relevance. Callus cultures of *Gloriosa superba*, developed on Murashige & Skoog (MS) medium produced higher levels of colchicine in corm as compared to other explants. The amount of colchicine showed differences in relation to the array of supplements used in the culture medium. The biosynthesis of colchicine was observed to be maximized in callus tissue cultivated on MS medium augmented with 2, 4-D91.5 mg/l) and kinetin (0.4 mg/l) and 100 µMof Manganese, 30 µM of Zinc, 0.5% µM of Copper optimally synergized with 0.5 µM of Tyrosine.

Keywords: *Gloriosa superba*, Cochicine, Callus cultures, Precursors

Introduction
Gloriosa superba is prevalently distributed in the low scrub jungles across the Indian subcontinent. This particular type is grouped under the Colchicaceae family. The morphology of the plants stems is slender, reaching impressive lengths of nearly 20 feet, originating from a durable, fleshy, creamy-white tuberous rhizome. The rizhomes are c characterized by a cylindrical shape, exhibiting bifurcation, and displaying a V-shaped configuration with limbs that may be equal or in equal in length. The leaves display an alternating, opposite, or whorled configuration, are attached without a stalk, and possess an ovate-lanceolate form with pointed ends, indicating a spiral twist that promotes their role as tendrils. The tubers of *Gloriosa superba* are recognized for their tonic and stomachic properties ((Brossi, *et al*., 1988) [4], as well as their anthelminthic effects when utilized for various medicinal applications. This flora behaves as a digestive system irritant and might bring about purging. It is prescribed to trigger labour pains and serves in the treatment of gonorrhoea and dermatological issues, particularly vasculitis (Yadav *et al*., 2012; Yadav *et al*., 2013) [22, 23]. Colchicine, an important alkaloid, affects cellular division by interfering with the correct construction of microtubules in the fibers of the mitotic spindle.
The entire plant, especially the tubers are extremely poisonous. The toxic properties of the plant are essentially due to the presence of a highly active alkaloid colchicine. Colchicine occurs as a pale yellow to greenish yellow in colour as odourless crystals or amorphous scales or powder (Fig-1).

- **Colchicine:** $C_{22}H_{25}NO_6$
- **Molecular weight:** 399.44 g/mol

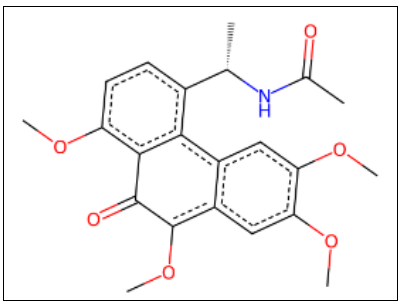


Fig-1

Growing interest in the practical aspects of plant tissue culture lead to the exploration of cell culture system for biosynthesis of a variety of secondary metabolites particularly pharmaceutically important compounds. Recent studies confirm that callus cultures and *in vitro* tubers of *G. superba* retain the capacity to synthesize colchicine in yields comparable to or higher than intact plants (Vismaya *et al*., 2021; Mahendran *et al*., 2018) [21, 15]. Novel strategies such as elicitor treatment, precursor feeding, and microbial associations have further improved yields (Jawahar *et al*., 2018; Das *et al*., 2020) [12, 6].

The increasing scholarly interest in the pragmatic dimensions of plant tissue culture has prompted the investigation of cell culture systems for the biosynthesis of an array of secondary metabolites particularly those compounds of significant pharmaceutical value. Recent empirical studies substantiate that callus cultures and *in vitro* tubers of *G. superba* preserve the capability to produce colchicine in yields that are comparable to or exceed those of intact plants (Vismaya *et al*., 2021; Mahendran *et al*., 2018) [21, 15]. Innovative methodologies, including elicitor treatment, precursor feeding, and microbial symbioses, have further enhanced these yields (Jawahar *et al*., 2018; Das *et al*., 2020) [12, 6].

The yield of secondary metabolites from intact plants is generally higher than that of derived from tissue cultures of the same (Flower, 1986). However in *Gloriosa superba*, the callus derived from plants has been found to retain the capacity to synthesise product identical to the one produced from intact plants in increased quantities. Sometimes it has been observed that a spectrum of novel compounds not found in intact plant may also be produced while using tissue culturing (Bohm, 1980). Apart from that, the desired secondary metabolite could also be enhanced by elicitors, precursor feeding and changes in phytohormones and carbon source.

Keeping this in view, the present work has been taken up to enhance the colchicine content in *Gloriosa superba* under *in vitro* conditions by adopting various chemical manipulations.

The production of secondary metabolites from whole plants is typically more substantial than that obtained from tissue cultures of the same species (Flower, 1986). Nevertheless, in the case of *Gloriosa superba*, the callus derived from these plants has demonstrated the ability to synthesize compounds that are analogous to those produced by intact plants, albeit in greater quantities. It has also been noted that a range of novel compounds, which are not present in intact plants, may be generated through the application of tissue culture techniques (Bohm, 1980). In addition, the accumulation of the desired secondary metabolite can be further augmented through the use of elicitors, precursor feeding, modifications in phytohormone levels, and alterations in carbon sources.

In light of this knowledge, the current study has been initiated to augment the colchicine content in *Gloriosa superba* under *in vitro* conditions by employing various chemical interventions.

Material and Methods

The plant material was systematically gathered during the months of September through November from the forests adjacent to Shadnagar (Mahabubnagar district, Telangana) and subsequently cultivated in pots. The corns were held at -20 ˚C and thawed quickly right before culturing started. Once the outer integument was removed, the corm was washed thoroughly under a constant stream of water before being sliced nearly into uniform discs. The resultant discs were exposed to a series of sterilization treatments prior to their utilization for inoculation, specifically comprising 70% ethanol for 1 minute, teepol for 10 minutes, a further rinse in running water for 1minute, and a 0.01% $HgCl_2$ solution for a duration of 5 minutes, followed by three washings with distilled water in order to eliminate any residual sterilants from the explant.

Among the extensive variety of synthetic media for plant cell culturing, four fundamental media, namely, Murashige & Skoog (MS, 1962) [16], Gamborgs-B5 (1976), and Blaydes (1966) [2] were evaluated. Consistent with previous findings, the MS medium demonstrated the highest efficacy for biomass and metabolite accumulation in cultures of *G. superba* cultures (Nikhila *et al*., 2017; Vismaya *et al*., 2021) [17, 21].

The corms were subjected to inoculation onto the aforementioned sterilized nutrient media contained within the culture vessels, the cultures were maintained in a meticulously overseen setting that included illumination ranging from 1500 to 2000 Lux, a regimen of 14 hours of light succeeded by 10 hours of darkness, and a stable temperature of 22±3 °C. The growth of calli was quantitatively assessed as a function of the increase in both fresh and dry weights, which were recorded at the conclusion of the 20th and 45th days. The cultures were examined to gauge their ability to felicitate callus formation, with evaluations performed via the Callus Induction Frequency (CIF) at the termination of the day 20.

Subsequent to the selection of the basal medium and the optimization of the carbon source characteristics, the effects of auxins (De-Eknamkul & Ellis, 1985), cytokinins, micronutrients (Mn, Zn, Cu) (Cheniae, 1970; Udvardy, 1970) [20], and the responses of amino acid precursors (Yeoman, 1987), such as phenylalanine and tyrosine, on the production of colchicine (mg/g D.W.) in callus cultures of *Gloriosa superba* were investigated (Seabrook, 1980) [18].

The callus tissue, which had been desiccated, received treatment with both 10% acetic acid and ethanol for a total of four hours. The resulting extract was concentrated to one quarter of its original volume, and the total alkaloids were precipitated through a gradual addition of concentrated ammonium hydroxide. Subsequent to that, the residue was integrated into a scant amount of chloroform.

Ashley and Harris's 1944 description of the chromatographic absorption technique was applied to isolate colchicine. An aliquot of the aforementioned solution was subjected to chromatography on silica gel-G plates utilizing a methanol-ammonium hydroxide mixture (200:30). The identification of alkaloids was accomplished through fluorescence observation under ultraviolet light, in addition to the application of the Marquis reagent (1ml formaldehyde+ 10ml concentrated sulfuric acid). The quantification of colchicine content was performed in accordance with the methodology established by King, J.S. (1951) [13].

Results and Discussion

Selection of media and optimisation of its composition for growth and secondary metabolite yield are fundamental prerequisites for establishing stable invitro cultures. MS

medium was found to be the most effective, corroborating findings from recent suspension culture and tuberization studies (Nikhila *et al*., 2017; Vismaya *et al*., 2021) [17, 21].

Composition of the culture media used for establishing callus showed an influence on the biomass and also on the synthetic pathways for secondary metabolite production (Flower, 1982). Out of all the media tried. MS was found suitable as it gave rise to a maximum CIF of 62% with a biomass yield of 1.089 gm (table-1 & graph-1).

The synergistic effect of auxins and cytokinins on the biomass yield has been studied (Table, graph-2&3). Similarly, precursor supplementation significantly enhanced colchicine accumulation in morphogenic cultures (Jawahar *et al*., 2018) [12].

The combined effect of auxins and cytokinins have responded favourably in inducing colchicine biosynthesis as reported by Shargool (1982) [19]. Varying concentrations of kinetin along with NAA responded positively. At a fixed concentration of 0.5 mg/l of NAA, addition of 0.4mg/l of kinetin yielded a biomass of 1.82 gm. Similar yield in biomass was obtained with the combination of 2, 4-D and kinetin as well. Callus grown in MS medium supplemented with 1.5 mg/l 2, 4-D and 0.4 mg/l of kinetin gave rise to a maximum yield of 4.35 mg/g D.W. of colchicine (Table, graph-5). The combination of NAA and kinetin proved to be of importance in terms of biomass, though this combination could not influence sustainability on the colchicine yield (Table, graph-4).

The supplementation of Manganese at 100µM gave maximum biomass of 1.76 gm with a yield of 3.75 mg/g D.W. of colchicine, while Zinc at 30 µM resulted in 1.81 gm of biomass and an yield of 2.845 mg/g D.W. of colchicine and Copper at 0.05 um showed a biomass of 1.84 gm with a colchicine yield of 2.05 mg/g D.W. (Table, graph 6 & 7).

Phenylalanine at 2 um gave a maximum colchicine yield of 4.2 mg/g D.W. whereas tyrosine at 0.5µM yielded a maximum of 4.265 mg/g D.W. (Table, graph-8).

Thus, *in vitro* systems enhanced with elicitors, precursors, microbial associations, and *in vitro* tuberization represent efficient platforms for sustainable and large-scale production of colchicine (Frontiers in Plant Science, 2017).

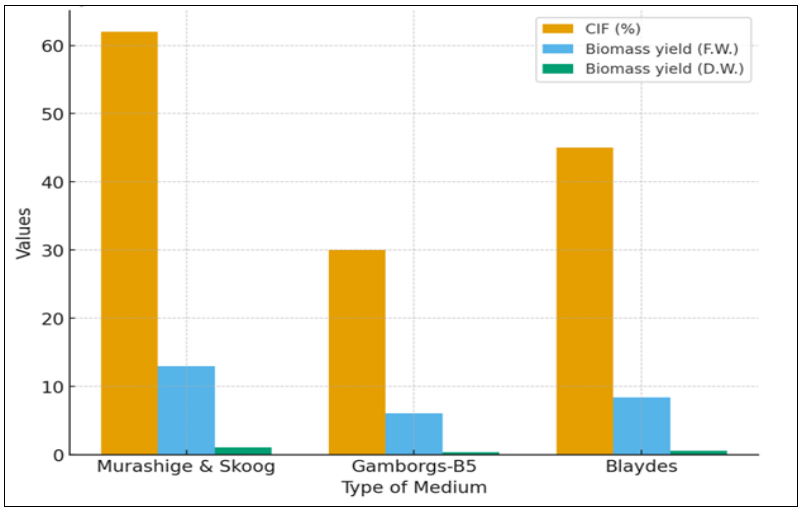
Conclusion

The evaluation of multiple culture media that generated significant amounts of colchicine implies that the Murashige and Skoog (MS) medium stands out as the most productive selection. The callus cultivated on MS medium enriched with 1.5 mg/l of 2,4-D,0.4 mg/l of kinetin and 100 µM of manganese (Mn), 30 µM of zinc (Zn) and 0.5 µM of copper (Cu), 0.5 mM of tyrosine culminated in the optimal synthesis of colchicine.

It is maintained that with the prudent fusion of phytohormones, carbon sources, micronutrients, precursors and elicitors, one can successfully boost the generation of noteworthy pharmaceutical compounds *in vitro*.

Table 1: Influence of different culture media on CIF and Biomass yield.

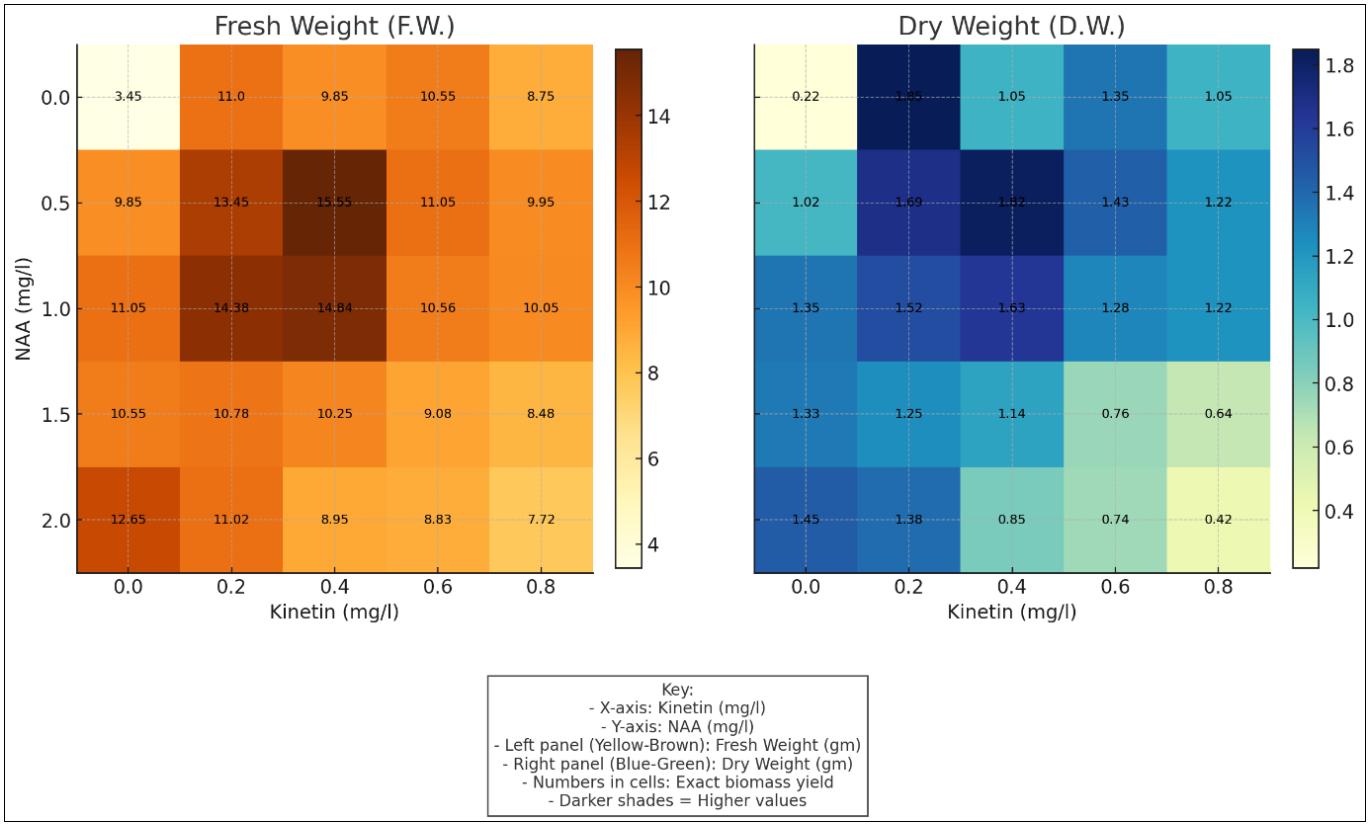
Type of Medium	CIF(%)	Biomass yield(gm)	
		F.W.	D.W.
Murashige & Skoog	62	13.0	1.08
Gamborgs-B5	30	6.05	0.38
Blaydes	45	8.42	0.60



Graph 1: Influence of Different Culture Media on CIF and Biomass Yield

Table 2: Influence of Auxins and Cytokinins on Biomass yield (gm).

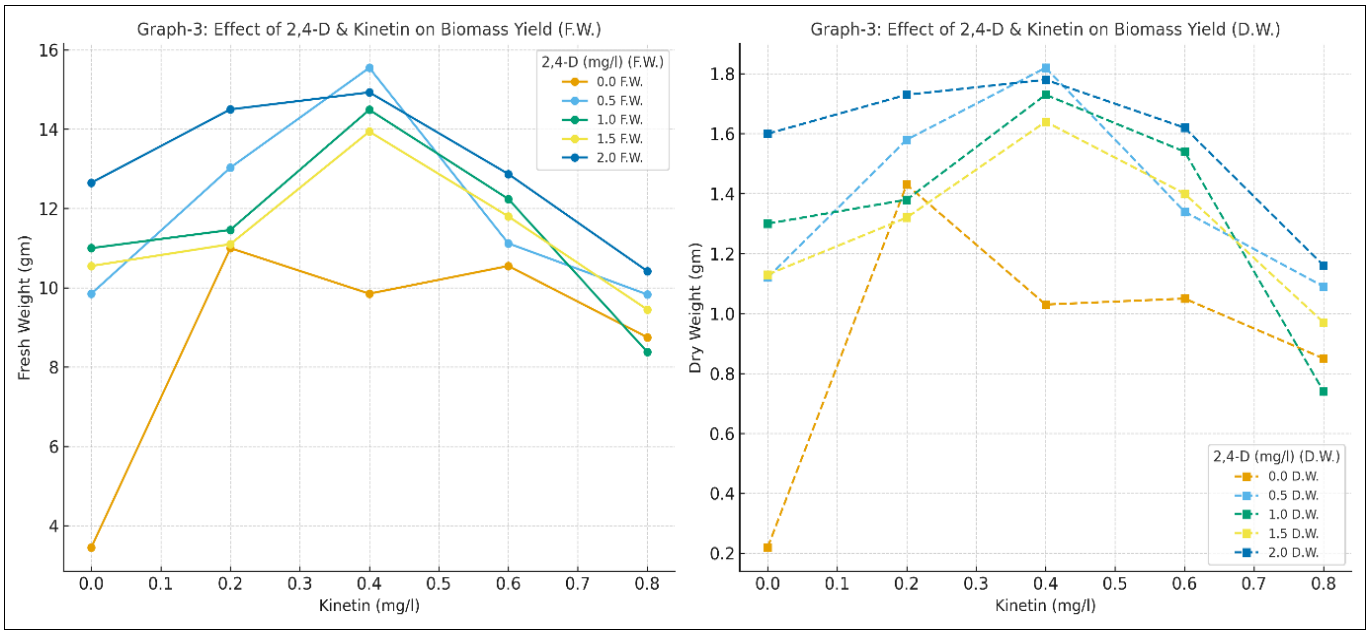
NAA(mg/l)	Kinetin(mg/l)				
	0.0	0.2	0.4	0.6	0.8
F.W.	3.45	11.0	9.85	10.55	8.75
D.W.	0.22	1.85	1.05	1.35	1.05
0.5 F.W.	9.85	13.45	15.55	11.05	9.95
D.W.	1.02	1.69	1.82	1.43	1.22
1.0 F.W.	11.05	14.38	14.84	10.56	10.05
D.W.	1.35	1.52	1.63	1.28	1.22
1.5 F.W.	10.55	10.78	10.25	9.08	8.48
D.W.	1.33	1.25	1.14	0.76	0.64
2.0 F.W.	12.65	11.02	8.95	8.83	7.72
D.W	1.45	1.38	0.85	0.74	0.42



Graph 2: Influence of Auxins and Cytokinins on Biomass Yield

Table 3: Influence of Auxins and Cytokinins on Biomass yield (gm).

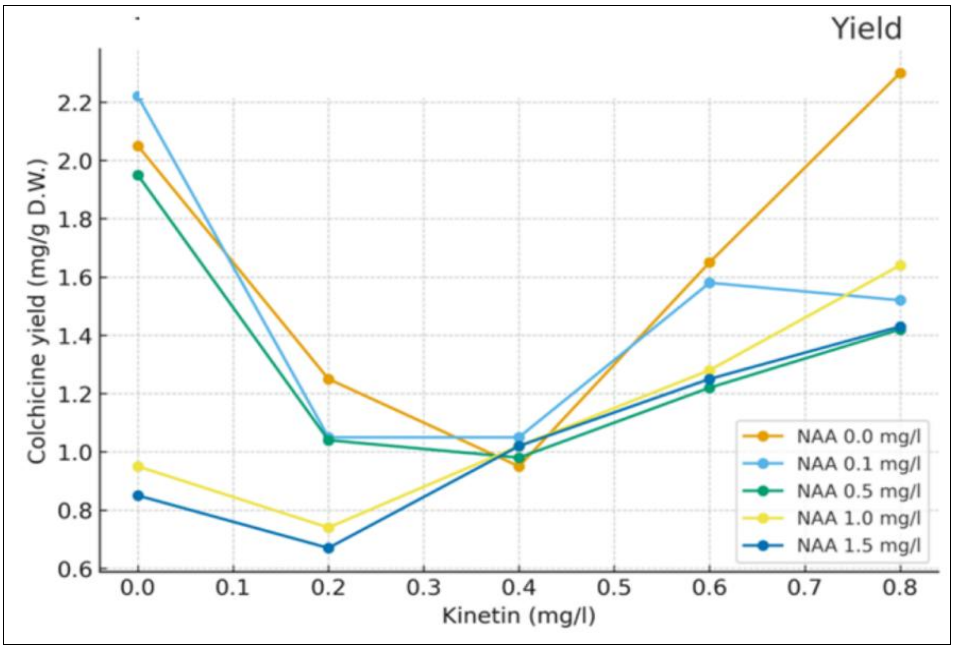
2,4-D(mg/l)	Kinetin(mg/l)				
	0.0	**0.2**	**0.4**	**0.6**	**0.8**
F.W.	3.45	11.0	9.85	10.55	8.75
D.W.	0.22	1.43	1.03	1.05	0.85
0.5 F.W.	9.85	13.03	15.55	11.12	9.83
D.W.	1.12	1.58	1.82	1.34	1.09
1.0 F.W.	11.00	11.46	14.49	12.24	8.38
D.W.	1.30	1.38	1.73	1.54	0.74
1.5 F.W.	10.55	11.10	13.94	11.80	9.45
D.W.	1.33	1.32	1.64	1.40	0.97
2.0 F.W.	12.65	14.50	14.93	12.87	10.42
D.W	1.60	1.73	1.78	1.62	1.16



Graph 3: Effect of 2,4-D & Kinetin on Biomass Yield (F.W.) and (D.W.)

Table 4: Influence of Auxins and Cytokinins on Colchicine yield (mg/g D.W.).

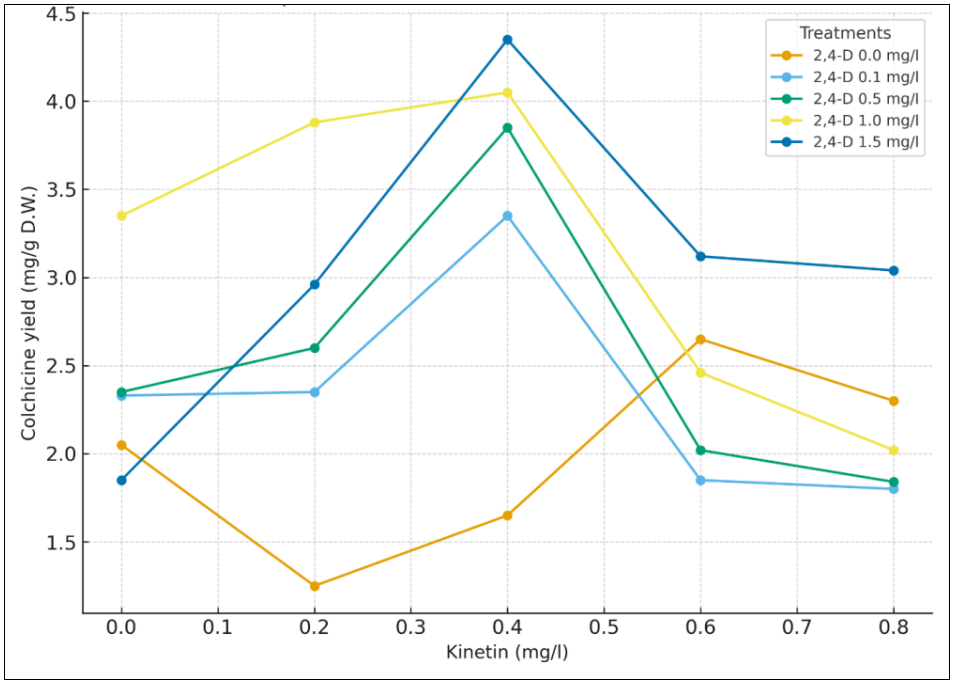
NAA (mg/l)	Kinetin(mg/l)				
	0.0	**0.2**	**0.4**	**0.6**	**0.8**
0.0	2.05	1.25	1.65	2.30	2.65
0.1	2.22	1.05	1.25	1.38	1.52
0.5	1.95	1.35	1.93	2.32	2.46
1.0	0.80	0.74	0.98	1.45	1.64
1.5	0.85	0.67	1.02	1.28	1.43



Graph 4: Effect of NAA & Kinetin on Colchicine Yield

Table 5: Influence of Auxins and Cytokinins on Colchicine yield (mg/g D.W.).

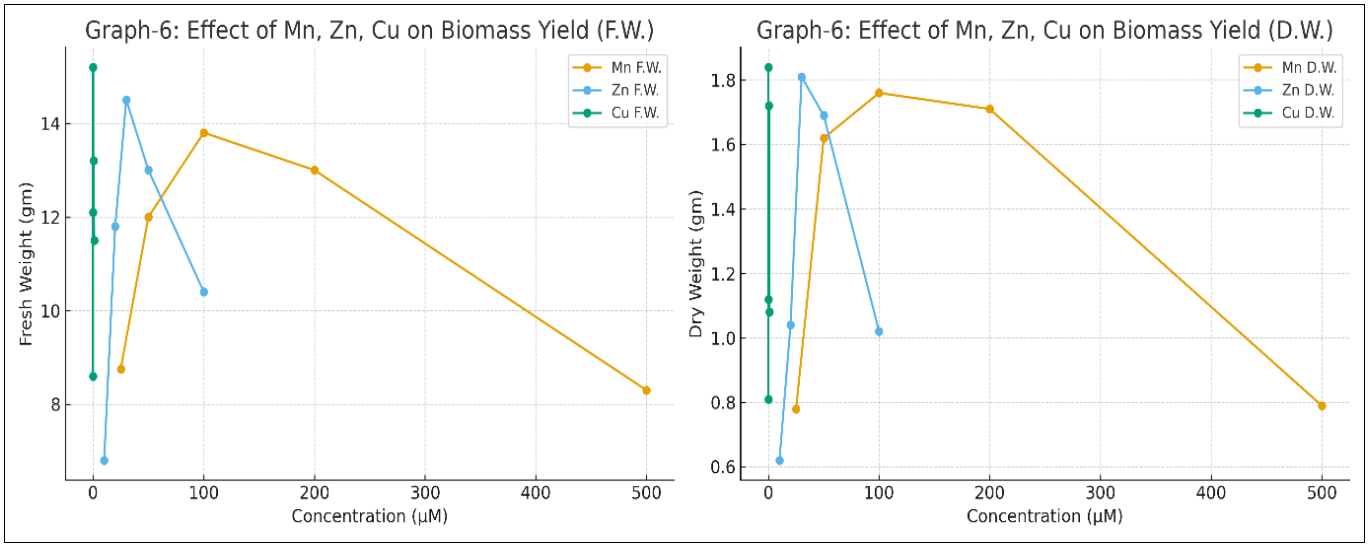
2,4-D(mg/l)	Kinetin(mg/l)				
	0.0	**0.2**	**0.4**	**0.6**	**0.8**
0.0	2.05	1.25	1.65	2.65	2.30
0.1	2.33	2.35	3.35	1.85	1.80
0.5	2.35	2.60	3.85	2.02	1.84
1.0	3.35	3.88	4.05	2.46	2.02
1.5	1.85	2.96	4.35	3.12	3.04



Graph 5: Effect of 2,4-D & Kinetin on Colchicine Yield

Table 6: Influence of Mn, Zn and Cu on the Biomass yield (gm)

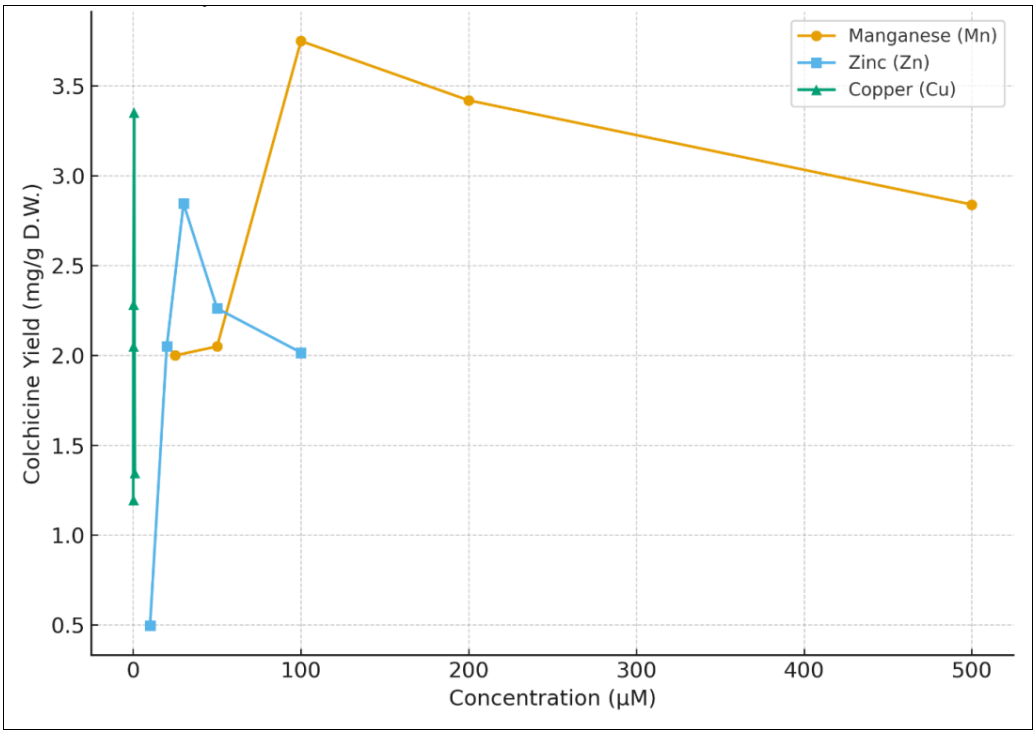
Manganese(µM)	**25**	**50**	**100**	**200**	**500**
F.W.	8.75	12.00	13.80	13.00	8.30
D.W.	0.78	1.62	1.76	1.71	0.79
Zinc(µM)	10	20	30	50	100
F.W.	6.8	11.80	14.50	13.00	10.40
D.W.	0.62	1.04	1.81	1.69	1.02
Copper(µM)	0.01	0.05	0.10	0.5	1.0
F.W.	8.60	15.20	12.10	13.20	11.50
D.W.	0.81	1.84	1.12	1.72	1.08



Graph 6: Effect of Mn, Zn, Cu on Biomass Yield (F.W.) and (D.W.)

Table 7: Influence of Mn,Zn and Cu on Colchicine yield (mg/g D.W.).

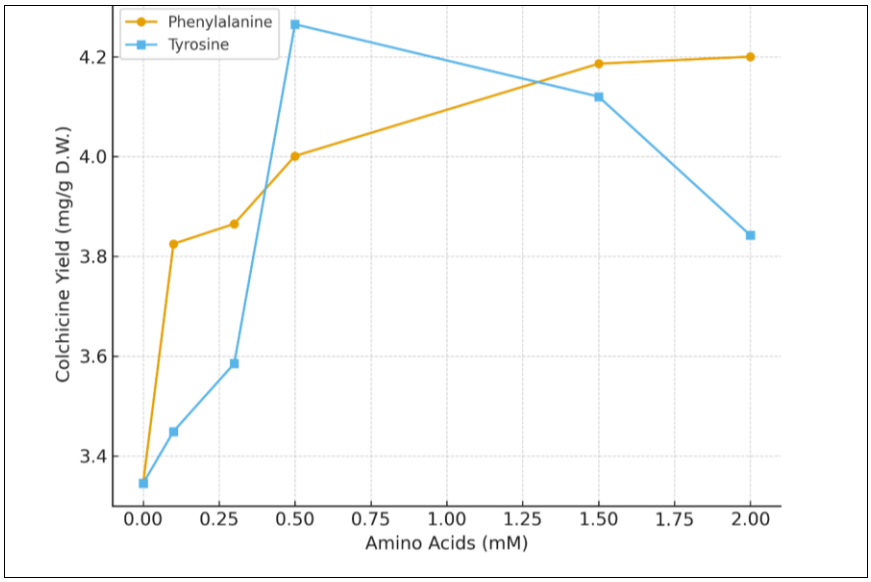
Manganese(µM)	**25**	**50**	**100**	**200**	**500**
	2.00	2.05	3.75	3.42	2.84
Zinc(µM)	10	20	30	50	100
	0.495	2.05	2.845	2.262	2.016
Copper(µM)	0.01	0.05	0.10	0.5	1.0
	1.195	2.05	2.285	3.354	1.345



Graph 7: Influence of Mn, Zn, and Cu on Colchicine Yield

Table 8: Response of Phenylalanine and Tyrosine on Colchicine yield (mg/g D.W.).

Amino Acids (mM)	0.0	0.1	0.3	0.5	1.5	2.0
Phenylalanine	3.345	3.825	3.865	4.001	4.186	4.200
Tyrosine	3.345	3.449	3.585	4.265	4.120	3.842



Graph 8: Response of Phenylalanine and Tyrosine on Colchicine Yield (mg/g D.W.)

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