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Development Of Optimized Liquid Culture System For *Hybanthus Enneaspermus* (L.) F. Muell Using Response Surface Methodology

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ABSTRACT
Hybanthus enneaspermus (L.) F. Muell. is a pharmacologically important ethnobotanical herb. Over exploitation from its natural habitats has led to its scarcity. To fulfil the growing pharmaceutical demands, development of novel *in vitro* culture methods for *H. enneaspermus*is necessary. In the present study, the central composite design (CCD) of response surface methodology (RSM) has been applied for liquid media component optimization for shoot regeneration from leaf explants.
Research Method: An experimental model comprising of four independent variables of growth regulators (Kinetin (KN), 6-benzylamonipurine (BAP), indole acetic acid (IAA), and Salicylic acid (SA) and their two dependents responses (shoot regeneration percentage (Y1) and the average number of shoots per explant (Y2) were calculated.
Findings: The maximum responses for Y1 and Y2, was 88.33 ± 0.75 and 72.5 ± 0.84 respectively were obtained with the growth regulator concentrations - KN2.31µM, BAP 4.44 µM, IAA 2.84 µM, and SA 100mg.MS salt at full strength and salicylic acid at concentration 100 mg/l were found to be most suitable for shoot regeneration in liquid culture media. *H. enneaspermus* plants with high aurantiamide acetate and coumarin levels were obtained using SA containing liquid culture media.
Limitation: Large scale application of this procedure needs to be cost-effective owing to the cost involved in designing the same.
Value: total flavonoids and phenols content along with chlorophyll accumulation were found to increase in plants cultured on liquid MS media.

Keywords: *Hybanthus enneaspermus*, Media optimization, Response surface methodology, Central composite design.

Introduction

Hybanthusenneaspermus (L.) F. Muell., commonly known as 'Humpback flower' is an important plant of medicinal value from Violaceae family. The plant is a small perennial suffrutescent herb, mostly found in tropical and subtropical regions of the world, including countries like India, Sri Lanka, Africa, and Australia (Ramya & Devika, 2022). In India, the herbis traditionally used for healing wound? The plant bears several other important medicinal characteristics like antimicrobial, antiplasmodial, antiarthritic, antimalarial, antirheumatic, emmenagogic, sedative, antispasmodic, antiasthmatic, anti-infertility, antibacterial, anticonvulsant, antidiabetic, antifungal, anti-allergic analgesic, and antioxidant properties. It is used to cure differentailments such as diarrhea, dysentery, strangury, burning sensations, urinary infections, leucorrhea, dysuria, and sterility (Rajsekhar et al., 2016). The *H. enneaspermus* plant produces a variety of phytochemicals such as alkaloids, steroids, aurantiamide acetate, phenols, flavones, catechins, tannins, anthraquinones, amino acids, isoarborinol, β-sitosterol, flavonoids, triterpenes, and glycosides(Patel et al., 2013) which has medicinal properties (cite reference).Two important phytochemicals, aurantiamide hasanti-inflammatory properties and, coumarins are heterocyclic compounds used as drugs to treat asthma, lymphoedema, Alzheimer's disease and cancer(Behera et al., 2016) are found in *H. enneaspermus*.
Due to excessive utilization, *H. enneaspermus* which are not commercially cultivated, become scare in natural habitats. The plant population is also being affected by overgrazing of animals, and increased human activities (cite reference). Growing plants commercially on a large scale is challenging as the plant has poor germination rates (General statement or particular to this type of plant?). The conventional propagation methods are proving to be insufficient to suffice the rising demands of pharmaceutical industries. Developing innovative methods to propagatethe *H. enneaspermus* plant is thus necessary (Karthi & Velayutham, 2017).
Propagating plants in liquid culture is considered a more advantageous technique as it has higher multiplication rate, greater plantlet survival, and easier transfer to *ex-vitro* conditions. Liquid culture system is also considerably a cost-efficient approach to generate large number of plants (Nirmal et al., 2023).*in vitro* Automation of liquid cultureprocess not only enhance the efficiency of plant propagation, but also improve the reliability of the plant propagation including nutrient delivery and monitoring, environmental control, aeration and gas exchange.

Multiple *invitro* propagation studies have been documented for *H. enneaspermus* using different explants as a starting material for propagation.Studies so far includecallus culture(Velayutham et al., 2012), nodal explants (Karthi&Velayutham, 2017), shoot tips, stem explants (Murugan& Kamaraj, 2018), young leaves(Premkumar et al., 2013), and somatic embryogenesisin *H. enneaspermus*.
Response Surface Methodology (RSM) is a statistical approach to analyze the relationship between multiple variables and their resulting effects on a system's response. RSM was successfully employed in optimization of elicitors for enhanced production of therapeutic metabolites from root cultures of *Swertiapaniculata* (Kaur et al., 2020).In the current study, central composite design (CCD) of RSM was used to construct predicted models of four independent variables of growth regulators (Kn, BAP, IAA and SA) and their two dependent responses Y1(shoot regeneration percentage) and Y2(average number of shoots per explant).

Materials and methods

Plant material and sterilization of explants

Health young leaves were collected from field grown *H. enneaspermus*in the botanical garden of VHN Senthikumara Nadar College, Virudhunagar, Tamilnadu. The collected leaves were initially washed under running tap water to get rid of soil particles. Then the washed leaves were surface sterilized by treating with 70% alcohol for 1 minute, followed by submerging in the solution containing 20% Clorox with 2 drops of Tween20 for 20 minutes under aseptic condition. After surface sterilization, the leaves are washed vigorously with sterile distilled water to remove Clorox solution.

Optimization of MS in liquid media

The sterilized *H. enneaspermus* leaf explants were initially cultured on a solid media for multiple shoot induction. The media composition used is as follows - MS media, with 30g/L sucrose, along with growth regulators –IAA (5.71 µM), KI (2.32 µM) and BAP (2.22 µM). The pH of the medium was adjusted to 5.7-5.8. The cultured explants were incubated in growth chambers for 4 weeks under 24 hrs light setting at (25±1) ºC.
To optimize MS salt concentration for shoot propagation, healthy explants were cultured on liquid shoot proliferation media with five different MS salt concentrations., Quarter strength (0.25), half strength (0.5), three quarters strength (0.75), full strength (1), one and quarter strength (1.25) and one and half strength (1.50). The liquid media was supplemented with growth regulators IAA (5.71 µM), KI (2.32 µM) and BAP (2.22 µM). Plantlets were cultured for a period of 4weeks. Average numbers of shoots per plantlet were calculated and the fresh and dry weights of the shoots were recorded on 4 weeks culture. Each experiment was done at least twice using triplicate.

Assessment of suitable plant elicitors for multiple shoot induction

Leaves explants from *invitro* cultured plant was transferred to liquid shoot regeneration media containing full-strength MS salts, supplemented with IAA (5.71µM), KI (2.32 µM), and BAP (2.22 µM) with different elicitors namely Yeast extracts (YE), Methyl jasmonic acid (MeJA), and salicylic acid (SA) each at three different concentrations - 50mg/ml, 100mg/ml, and 200mg/ml. Three replicates were used per treatment. The plantlets were grown for 28days.The average number of shoots per explant were counted and the shoot regeneration percentage was calculated.

Design of experimental model for optimization of growth medium components using RSM

The experimental design was constructed using the 'rsm' package (Version2.10.4) in the R programming language. Various functions offered in the package were used to determine the optimal concentrations of MS liquid media components. To design the experimental model, four growth elicitors KN, BAP, IAA, and SA were considered as independent variables and were coded as X1, X2, X3, and X4 respectively. The central composite design (CCD) function in the rsm package was used to form a response surface design. To limit the experimental procedures and variations resulting from experimental data, the block design strategy was employed. The values of coefficients for independent variables and their subsequent responses were analyzed using the second-order (SO) polynomial tool. The efficiency of the design was determined by the coefficient of determination (R2) value. Analysis of variance (ANOVA) was performed using 'rsm' function to identify the prominent interactions between variables, falling within the range of the generated model. The stepwise regression function was used to further refine the predicted model. Using the contour function, two-dimensional pareto plots were generated to help predict the most effective interactions amongst variables that lead enhanced responses (Y1 and Y2).

Experimental validation of CCD-RSM model

To validate the CCD-RSM model, the *invitro* cultured leaves explants of *H. enneaspermus* were transferred to 100ml flasks containing full-strength liquid MS medium containing different concentrations of IAA, KI, BAP, and SA (Table 3). The pH of the medium was adjusted to 5.6-5.7. The cultures were incubated in an incubator shaker at 25 ºC. The plants were sub-cultured every 2 weeks. After four weeks of incubation, the shoot regeneration percentage and average number of shoots per leaf explants were calculated. The Quantile-Quantile (Q-Q) plots were generated by plotting experimental response against predicted response of Y1 and Y2.

Quantification of secondary metabolites

Secondary metabolite quantification was performed using six-week-old *in vitro* regenerated plants. For quantification of total phenols and flavonoids, the regenerated shoots were dried at 30±2 °C and ground to form a powder. Samples were prepared by dissolving 500mg of prepared powder in 10 ml of 100% methanol. The mixtures were allowed to dry up to 5ml and centrifuged at 10,000 rpm. The supernatant was recovered and was used for further analysis.

Estimation of total phenol content:

The total phenol content was determined by method described by Singleton et al., (1999). For analysis 100 µl of prepared extract were mixed with 100 µl Folin – Ciocaliteu reagent. After 5 minutes, 150 µl of 20% sodium carbonate solution was added to mixture. After 30 minutes at room temperature, the total amount of phenol content was estimated

Estimation of total flavonoid content:

Analysis of the total flavonoid content was done as per Chang et al., 2002. In a 25 ml flask, 1.5 ml of prepared methanolic extract was added along with 4.5 ml distilled water and 1 ml of 5% sodium nitrate solution. The mixture was incubated at room temperature for six minutes, followed by addition of 10ml aluminium chloride (10%). The mixture was again incubated for 6 minutes, before adding 10 ml of 4% sodium hydroxide solution. The final volume was adjusted to 25 ml with 60% aqueous ethanol. The contents were allowed to react for 15 minutes. Absorbance was recorded at 510 nm using spectrophotometrically.

Quantification of accumulated chlorophyll:

Chlorophyll accumulation was measured spectrophotometrically, as described by Vyas et al., 2008. From the regenerated shoots, the leaves were immersed in chilled acetone (80%). The homogenized mixture was filtered with the help of filter paper and absorbance was recorded at three different wavelengths – 645 nm, 654 nm, and 663 nm. Following the formula given below, the determination of the total chlorophyll content was calculated.

Total chlorophyll (mg/g) = $\frac{20.0\times A_{645}+8.02\times A_{663}}{a\times 1000\times W}\times v$

Where,

a = length of the path of light in the cell

V = volume of the extract in ml

W = fresh weight of the sample in g.

Auratiamide acetate estimation:

Extraction of aurantiamide acetate was performed as per protocol developed by Banerji and Das (1975). The collected leaves/callus were dried and 5 gm of the sample was used for extraction with50ml chloroform in room temperature for 12 hrs and the extract was made to evaporated under room temperature and concentrated into dryness. The concentrated extracts were dissolved to 15 ml methanol and subjected to chromatography. Silica gel60 (230–400 mesh, Merck) used as a solid phase and benzene-chloroform (1:3) used as mobile phase. The first elute is collected as fractions and subjected to FTIR and HPLC analyses.

FT-IR analysis was done by using Perkin Elmer Fourier transform infrared (FT-IR) spectrophotometer and spectrum was recorded range from 400 to 4000 cm^{-1}. HPLC analyses were also performed using the collected elute. The mobile phase used for HPLC analysis was Acetonitrile-water (3:1) and 0.01% orthophosphoric acid. The oven temperature was maintained at 300 ºC and the total run time was 20 minutes. The flow rate of 1.0ml/min was maintained and detection was carried out on PDA at 220nm.

Coumarin estimation:

Coumarin detection was adopted from Behera et al. (2016).The collected leaves/callus were dried and 5 gm of the sample was used for extraction with 50 ml methanol in room temperature for 12 hrs, concentrated to dryness and the concentrate dissolved in HPLC grade acetonitrile, filtered for HPLC analysis. To perform HPLC, Shimazu CBM-20A HPLC with C18 Reverse Phase Column (250 X 4.6mm, 5µM) was used. The following gradient was used for separation: 0 min, 20:80 (A:B); 20 min, 80:20 (A:B), with flow rate of 1 ml min-1 (A, acetonitrile; B, water containing 0.01 % orthophosphoric acid). The detection was performed at 220 nm.

Results

MS salt effect

Significant effect of MS salt strength was observed on percentage of shoot regeneration. The percentage on shoot regeneration varied from 14.75% to 98.25% (Table 1). Maximum shoot regeneration (98.25%) was observed at 1 MS salt strength. On average, 68.25 shoots were observed per leaf explant at 1 MS salt strength. Least shoot regeneration (11.25%) was observed at 1.50 MS salt strength. However, the minimum number of average shoots per leaf explants (8.5) were recorded for 0.25 MS salt strength. The MS salt strength was found to have significant effect on the fresh and dry weights of the regenerated shoots. At MS strength 1, the regenerated shoots had highest, 28.50 gm/l fresh weight

and 23.75 gm/l dry weight. The maximum dry weight percentage of 83.33% was also observed in the shoots regenerated at 1 MS salt strength, followed by 63.64% at 1.50 MS strength. Lowest dry weight percentage (34.43%) was recorded for 0.25 MS salt strength. The growth curves of *H. enneaspermus* plants culture on liquid media show increase in fresh and dry weights with increase in growth period

Table 1. Effects of MS salt strength on direct multiple shoot induction using leaf as explant of *H. enneaspermus* (L.) F. Muell

MS Salt strength	Shoot regeneration (%)	Average number of shoots per leaf explant	Fresh weight (g l^{-1})	Dry weight (g l^{-1})	% Dry weight
Quarter strength (0.25)	14.75	8.5	15.25	7.25	34.43
Half strength (0.5)	38.36	12.25	18.75	8.25	45.33
Three quarters strength (0.75)	60.78	25.25	22.75	12.75	56.04
Full (1)	**98.25**	**68.25**	**28.50**	**23.75**	**83.33**
One and quarter strength (1.25)	50.25	32.25	12.25	6.75	55.10
One and half strength (1.50)	11.25	12.25	08.25	5.25	63.64

Mean separation within columns by Duncan's multiple range test at 5% level
Shoots were cultured in MS medium with different salt strength supplemented with 5.71µM IAA, 2.32 µM KI and 2.22 µM BAP using 250ml flask containing 100ml medium.

Effect of plant elicitors:
All three plant elicitors (YE, MeJA, and SA) showed a positive effect on the induction of shoot regeneration (Table 2). The shoot regeneration after treatment with growth elicitors ranged between 62.17 ± 1.6% and 73.5 ± 1.87%. The highest shoot regeneration was recorded for salicylic acid, especially at the concentration of 100mg/L. The same treatment also produced the highest number of shoots per leaf i.e., 60.2. Yeast extracts at a concentration of 50 mg/L showed significant shoot regeneration of 70.33%. MeJA showed the maximum regeneration of 69.17% at a concentration of 100mg/L.

Table 2. Effect of different types of elicitors on direct multiple shoot induction using leaf as explant of *H. enneaspermus* (L.) F. Muell

Elicitors	Conc. of elictors (mg/L)	Shoot regeneration (%)	Average number of shoots per leaf explant
YE	50	70.33 ± 1.37	53.8 ± 2.14
	100	62.67 ± 3.14	53.4 ± 3.16
	200	56.5 ± 1.87	46.8 ± 2.59
MejA	50	66.33 ± 1.75	51.2 ± 1.52
	100	69.17 ± 1.47	44.8 ± 2.4
	200	62.17 ± 1.6	42.2 ± 3.78
SA	50	71.17 ± 1.72	55.2 ± 2.1
	100	**73.5 ± 1.87**	**60.2 ± 2.58**
	200	64.17 ± 2.32	53.8 ± 1.75

Explants were cultured on full strength MS medium supplemented with 5.71µM IAA, 2.32 µM KI and 2.22 µM BAP. Triplicates were used per treatment. Variability around the mean was represented as ± SD. Data having the same letter in a column were not significantly different by Duncan's multiple comparison test ($P < 0.05$).

Experimental design using RSM and its experimental validation:
The experimental design generated using RSM predicted the effect of independent variables on the dependent responses with high accuracy. The CCD-RSM model is thus adequate to predict the values of variables and interpret their results. Following the model development, the experiments were performed according to the specifications mentioned in (Table 3). In the series of 30 experiments, variable concentrations of growth regulators (KN - 0.64 to 7.32 µM, BAP - 2.22 to 11.10 µM, IAA - X3; 2.84 to 7.13 µM, and SA - 50 to 125 mg) were used as per prediction. Each variable was analyzed at five different concentration levels to induce shoot proliferation in *H. enneaspermus* (Y1 and Y2).

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Table 3. A Central Composite Designwith different concentrations of Kinetin (KN), 6- benzyladenine (BAP), 3-Indoleacetic acid (IAA) and Salicylic acid (SA) for shoot regeneration percentage(y1) and average number of shoots per leaf explant (y2) of *H. enneaspermus* (L.) F. Muell.

Expt.No	KN (µM)	BAP (µM)	IAA (µM)	SA (mg)	y1*	y2*	Block
1	2.31	4.44	2.84	50	76.67 ± 1.37	52.83 ± 1.72	1
2	5.65	4.44	2.84	50	57.67 ± 1.49	37.17 ± 0.75	1
3	2.31	8.88	2.84	50	58.17 ± 1.95	39.33 ± 1.03	1
4	5.65	8.88	2.84	50	34.50 ± 1.38	10.17 ± 0.75	1
5	2.31	4.44	7.13	50	34.83 ± 1.57	6.83 ± 1.17	1
6	5.65	4.44	7.13	50	30.83 ± 1.21	4.33 ± 0.82	1
7	2.31	8.88	7.13	50	31.83 ± 1.07	5.67 ± 0.82	1
8	5.65	8.88	7.13	50	55.33 ± 1.25	35.5 ± 0.84	1
9	**2.31**	**4.44**	**2.84**	**100**	**88.33 ± 0.75**	**72.5 ± 0.84**	1
10	5.65	4.44	2.84	100	87.00 ± 1.63	68.83 ± 0.75	1
11	2.31	8.88	2.84	100	86.50 ± 1.26	67.67 ± 1.21	1
12	5.65	8.88	2.84	100	82.33 ± 0.75	63.33 ± 1.03	1
13	2.31	4.44	7.13	100	41.50 ± 0.96	19.33 ± 1.03	1
14	5.65	4.44	7.13	100	63.33 ± 0.47	45.5 ± 1.05	1
15	2.31	8.88	7.13	100	69.17 ± 1.07	46.83 ± 0.75	1
16	5.65	8.88	7.13	100	38.33 ± 1.60	12.83 ± 0.75	1
17	3.98	6.66	4.98	75	63.33 ± 0.94	72.67 ± 1.97	1
18	3.98	6.66	4.98	75	72.83 ± 1.07	45.83 ± 1.17	1
19	3.98	6.66	4.98	75	73.83 ± 1.34	47.33 ± 0.52	1
20	0.64	6.66	4.98	75	13.83 ± 1.07	11.33 ± 0.52	2
21	7.32	6.66	4.98	75	15.83 ± 1.34	12.33 ± 0.82	2
22	3.98	2.22	4.98	75	17.67 ± 1.70	17.83 ± 1.47	2
23	3.98	11.1	4.98	75	15.83 ± 1.34	19.33 ± 1.51	2
24	3.98	6.66	0.68	75	23.83 ± 1.34	6.83 ± 0.98	2
25	3.98	6.66	9.25	75	26.17 ± 0.69	26.83 ± 0.75	2
26	3.98	6.66	4.98	25	20.83 ± 1.67	6.83 ± 1.83	2
27	3.98	6.66	4.98	125	20.50 ± 1.38	7.17 ± 0.98	2
28	3.98	6.66	4.98	75	50.33 ± 1.80	54.17 ± 0.41	2
29	3.98	6.66	4.98	75	72.67 ± 0.94	47.67 ± 1.03	2
30	3.98	6.66	4.98	75	73.83 ± 1.34	48.17 ± 0.75	2

* Each represented value is means ± SE
Shoots were cultured on full strength MS medium with supplemented with different concentration of IAA, KI, BAP and SA

The significance of the generated model was assessed by performing regression analysis on variables and responses using SO polynomial functions. During analysis, the insignificant values of the blocks were skipped. The obtained R2 and p values were considered for detecting the significance. Only the variables X3 and X4 in the model showed high significance. The variable combination X2:X3 also showed significance to some extent. The interactions ofX1:X2, X1: X4, X2:X4, X1:X3, and X3:X4 were found to be insignificant for Y1 and Y2. The R2 values for responses, Y1 and Y2, were 0.734 and 0.639, respectively (Table 4).

Parameters	y1				y2			
	Coefficient estimate	Standard error	t value	p Value	Coefficient estimate	Standard error	t value	p value
Intercept	115.15065	13.02404	8.8414	4.197e-07	81.57317	13.9994	5.8269	4.392e-5
Block	-31.56488	7.07484	-4.4616	0.0005373	-19.28878	7.60471	-2.5364	0.02373
X_1	-1.40333	3.77509	-0.3717	0.7156561	-1.30542	4.05783	-0.3217	0.75243
X_2	-1.15333	3.77509	-0.3055	0.7644721	-0.95792	4.05783	-0.2361	0.81680
X_3	-8.38917	3.77509	-2.2222	0.0432563	-8.56958	4.05783	-2.0024	0.06500
X_4	7.33333	3.77509	1.9426	0.0724607	8.56958	4.05783	2.1119	0.05315
X_1: X_2	-2.04250	4.62353	-0.4416	0.6654081	-2.62562	4.96980	-0.5283	0.60555
X_1:X_3	3.66625	4.62353	0.7930	0.4410356	4.52063	4.96980	0.9096	0.37842
X_1: X_4	0.54125	4.62353	0.1171	0.9084719	0.10313	4.96980	0.0208	0.98374
X_2: X_3	4.52125	4.62353	0.9779	0.3447302	4.72938	4.96980	0.9516	0.35743
X_2: X_4	1.02125	4.62353	0.2209	0.8283738	-0.31312	4.96980	-0.0630	0.95065
X_3 :X_4	-3.60250	4.62353	-0.7792	0.4488522	-3.79187	4.96980	-0.7630	0.45814
X_1 ^ 2	-8.02374	3.54356	-2.2643	0.0399594	-7.13443	3.80895	-1.8731	0.08209
X_2 ^ 2	-7.54374	3.54356	-2.1289	0.0515027	-5.44693	3.80895	-1.4300	0.17464
X_3 ^ 2	-5.48124	3.54356	-1.5468	0.1442089	-5.88443	3.80895	-1.5449	0.14467
X_4 ^ 2	-6.56499	3.54356	-1.8527	0.0851288	-8.34193	3.80895	-2.1901	0.04595
Multiple R^2: 0.7342		Adjusted R^2: 0.4494			Multiple R2: 0.6395		Adjusted R2: 0.2533	
ANOVA:					ANOVA:			
Response	Df	Sum Sq	Mean Sq	F value	Pr > F	Df	Sum Sq	Mean Sq	F value	Pr > F
Block	1	5616.6	5616.6	16.4214	0.001188	1	1830.7	1830.68	4.6325	0.04930
FO(X_1 X_2 X_3 X_4)	2	3058.9	764.7	2.2359	0.117446	4	3410.0	852.49	2.1572	0.12730
TWI(X_1 X_2 X_3 X_4)	6	837.9	139.7	0.4083	0.861439	6	1026.9	171.16	0.4331	0.84484
PQ(X_1 X_2 X_3 X_4)	4	3711.7	927.9	2.7130	0.073037	4	3547.4	886.84	2.2441	0.11646
Residual	14	4788.4	342.0			14	5532.6	395.18		
Lack of fit	10	4370.4	437.0	4.1816	0.090210	10	5051.5	505.15	4.2001	0.08957
Pure error	4	418.1	104.5			4	481.1	120.27		

The result of ANOVA stated the two-way interaction (TWI) to be insignificant, whereas pure quadratic (PQ) and first-order (FO) were found to affect the CCD-RSM design significantly. The obtained F values for Y1 (16.42) and Y2 (4.63) designate the model to be highly significant. The significance is further validated by low p-values (Pr> F) obtained for

Y1 (0.001) and Y2 (0.049). The results of ANOVA suggest the model to be a good fit with experimental data. (Figure 1, 2).

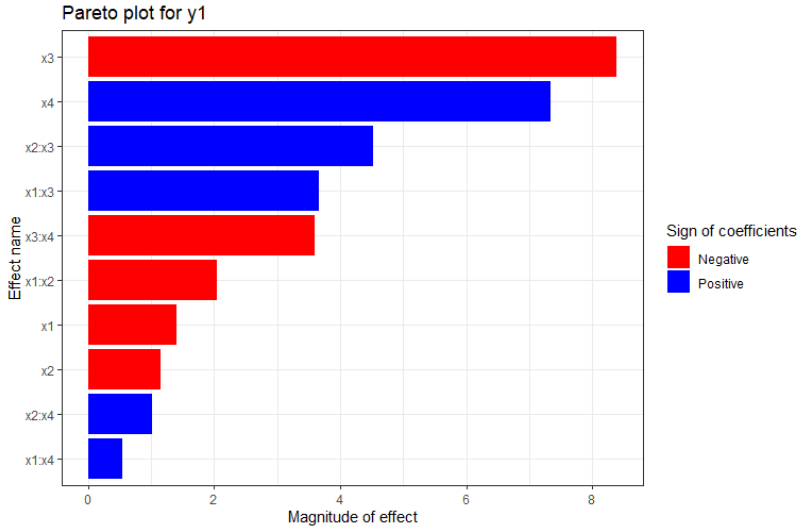


Figure 1. Pareto chart showing the effect of different media components for shoot regeneration percentage (y1).

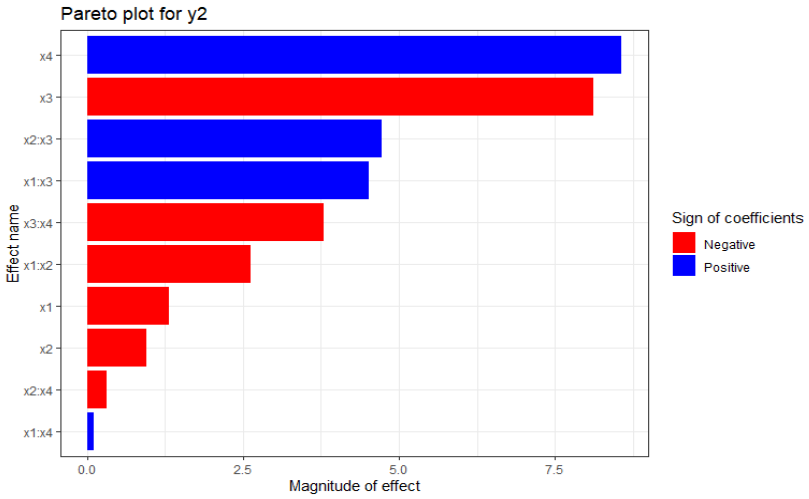
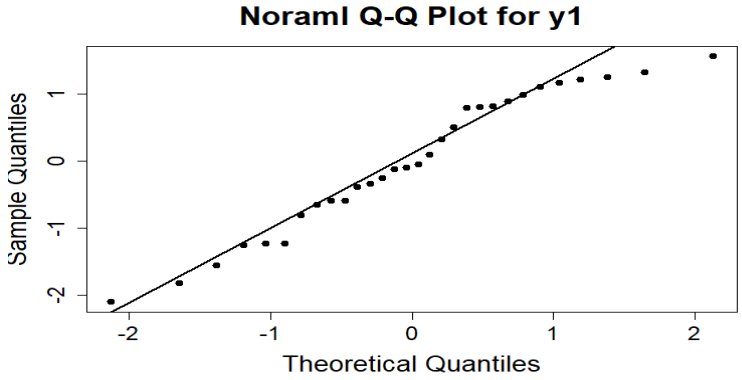


Figure 2. Pareto chart showing the effect of different media components for average number of shoots per leaf explant.

The Quantile-Quantile plot was constructed by plotting experimental responses against the predicted responses for Y1 and Y2 (Figure 3,4). The quantiles in the plots consistently showed placement within close proximity. A linear relationship was observed between the quantiles and their predicted values. The developed model has greatly improved the prediction of conditions suitable for the regeneration of shoots in *H. enneaspermus* leaf explants grown on liquid culture media.



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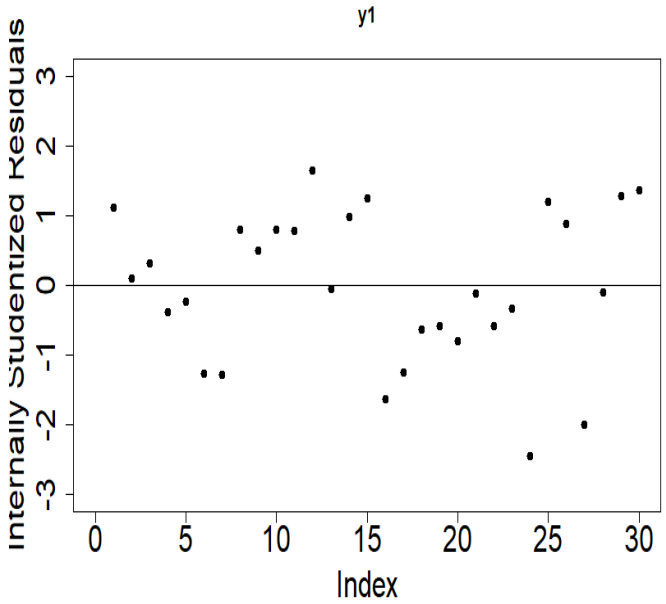

Figure 3. Quantile-Quantile (Q-Q) plot displaying the predicted response (black line) versus observed response (black dots) of the shoot regeneration percentage (Y1) and Plot of internally studentized residuals (black line) against predicted response (black dots) of Y1.

The validation of the designed RSM model was performed by experimental confirmation of the predicted values ofY1 and Y2. The maximum responses predicted for Y1 and Y2were 88.33 ± 0.75 and 72.5 ± 0.84, respectively.
These experimentally (Table 5) validated response values were found to be in close agreement with predicted values. The maximum values obtained experimentally are87.45 ± 0.57 (Y1) and 78.5± 0.58 (Y2) (). The following growth regulator concentrations were used for the experiments - KN 2.32 µM, BAP 3.30 µM, IAA 5.70 µM, and SA 80 mg. These results highlight the effectiveness of the CCD-RSM model in predicting the ideal combinations of variables eventually producing desired outcomes in *H. enneaspermus*.

Table 5. Optimized medium composition for shoot regeneration percentageand average number of shoots per leaf explant (y2) of *H. enneaspermus* (L.) F. Muell.

No.	Component Concentration				Shoot regeneration (%) - y1	Average number of shoots per leaf explant - y2
	KN (µM)	BAP (µM)	IAA (µM)	SA (mg)		
1[a]	2.32	2.22	5.71	100	**73.5 ± 1.87**	**60.2 ± 2.58**
2[b]	4.60	3.30	5.70	80	88.33 ± 0.75	72.5 ± 0.84
3[c]					87.45 ± 0.57	78.5± 0.58

[a]the values before optimization
[b]the values predicted by desirability method
[c]the values validated by experiment.

Secondary metabolites and chlorophyll estimation:
The total phenols, flavonoids, and chlorophyll accumulation on liquid culture media during the growth period was recorded the contents were measured in mg/g FW (Figure 5). A significant increase in the levels was observed during the growth period (Figure 6). The maximum amounts of phenols, flavonoids and chlorophyll were recorded on the 4[th]point. The contents were measured in mg/g FW.

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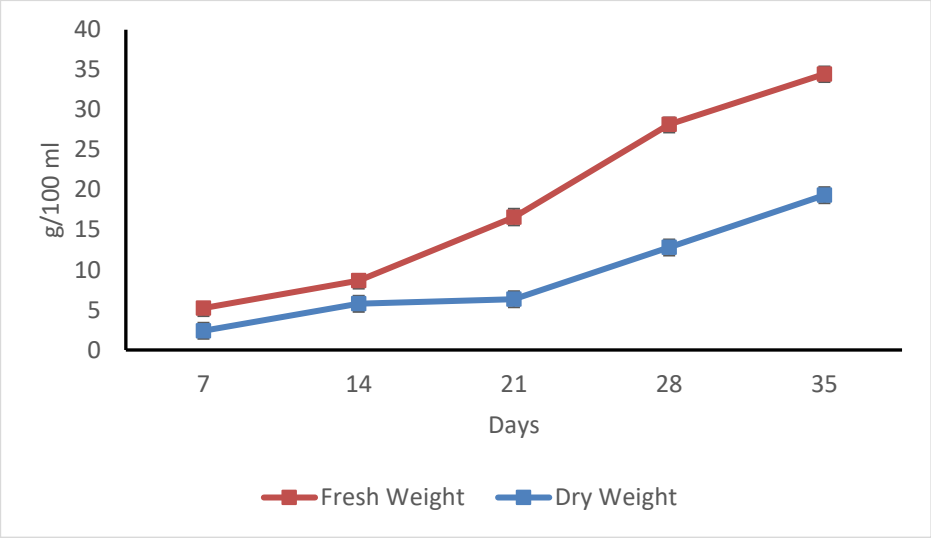
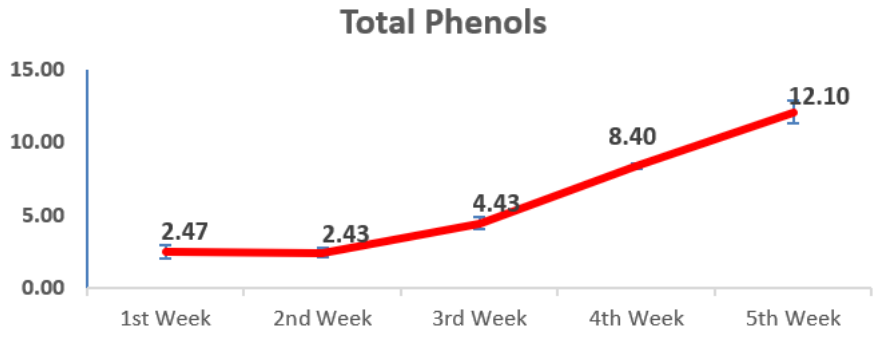
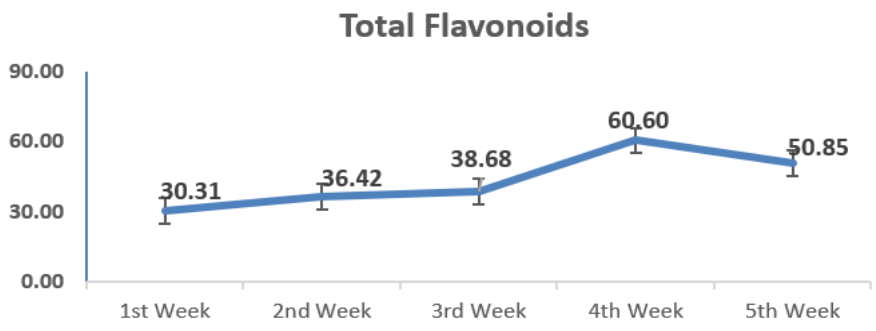


Figure 5. Growth curves of *H. enneaspermus* (L.) F. Muell explant on liquid medium. Total flavonoids, Total phenols and Total chlorophyll accumulation during growth period of *H. enneaspermus* (L.) F. Muell on liquid medium.

Aurantiamide acetate and coumarin quantification:
Plants regenerated on liquid media containing elicitor (SA) were found to have the highest levels of Coumarin (8.8) and aurantiamide acetate (7.8). The lowest levels of coumarin (1.8) were observed in plants regenerated on solid media and the lowest levels of aurantiamide acetate (2.5) were observed in callus generated from leaf explants. The plant elicitors thus show a great effect on aurantiamide acetate and coumarin levels in the liquid media.

Discussion
The secondary metabolites derived from medicinal plants are economically important. These natural phytochemicals have multiple applications in pharmacology. Due to climate change and human activities, medicinal plants declined in natural habitat. The development of *in vitro* cultivation methods for such plants would be beneficial for increasing the production of secondary metabolites(Parthasarathy et al., 2024). As discussed, the *H. enneaspermus* is a rich source of a variety of phytochemicals. Multiple methods for *invitro* propagation of the *H. enneaspermus*. Murugan and Kamaraj (Murugan & Kamaraj, 2018)achieved high frequency (88%) of callus regeneration from stem explants of *H.*





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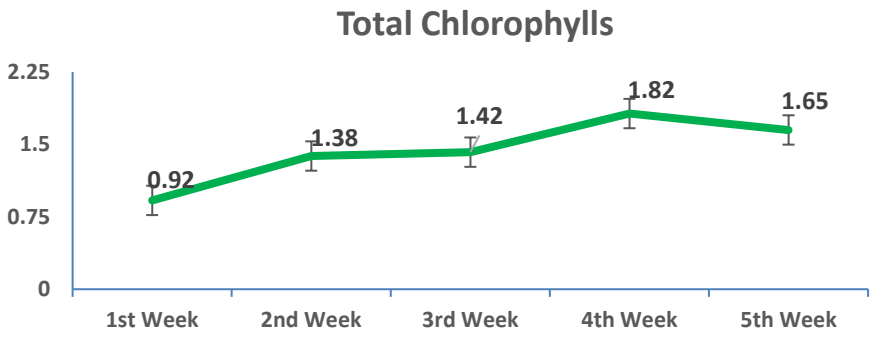

Figure 6. Total flavonoids, Total phenols and chlorophyll accumulation during growth period of *H. enneaspermus* (L.) F. Muell on liquid medium. Vertical bars represent the means of triplicates with ±SE

enneaspermus using MS media supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8 mg/l BAP, 2 mg/l α-naphthaleneacetic acid (NAA), and 0.8 mg/l Kinetin (Kn). Another *H. enneaspermus* propagation study performed using stem explants revealed that the cytokinins BAP and KIN have significantly positive effects on shoot regeneration(Velayutham et al., 2012). For somatic embryogenesis in *H. enneaspermus*, half-strength MS medium containing 0.5 mg/l BAP turned out to be most effective, as it led to the germination of 207.0 ± 4.2 explants have reported that medium containing KI (2.32 µM/l) and BAP (2.22 µM/l) induced maximum shoot regeneration with the highest average number of shoots (Velayutham et al., 2012). The present study has obtained comparable results showing enhanced shoot regeneration responses with the maximum of 87.45 ± 0.57 (Y1) and 78.5± 0.58 (Y2) shoots regenerated per explant in the media fortified with KN 2.32 µM, BAP 3.30 µM, IAA 5.70 µM, and SA 80 mg. Also, the MS media of strength 1 was found to be highly effective for shoot regeneration and produced shoots had the maximum (83.33%) percentage of dry weight in *H. enneaspermus*.

The role of plant elicitors in shoot induction has been explored with salicylic acid showing abundant shoot regeneration of 73.5 ± 1.87% at 100 mg/l concentration. SA resulted in the development of a 60.2 ± 2.58 average number of shoots per leaf explant. (Jeyasri et al., 2023) have documented the role of SA and MeJA in enhancing the production of phytochemicals in medicinal plants cultured *in vitro*. Enhanced L-DOPA production in *H. enneaspermus* plants was reported by with the use of SA (100 µM) treatment for 6 hours. The increased content of phytochemicals in plants could efficiently supply the needs of the pharmaceutical industry. In the present study, the plants cultured on liquid media containing plant elicitor (SA) showed increased production of pharmacologically important secondary metabolites - aurantiamide acetate and coumarin. Also, the highlights the increase in the total flavonoids, total phenols and total chlorophyll accumulation during the growth period of *H. enneaspermus* (L.) F. Muell on a liquid medium. The liquid culture media has been proven to be highly effective in the accumulation of secondary metabolites (Figure 7). The addition of growth regulators (SA) further enhanced their synthesis.

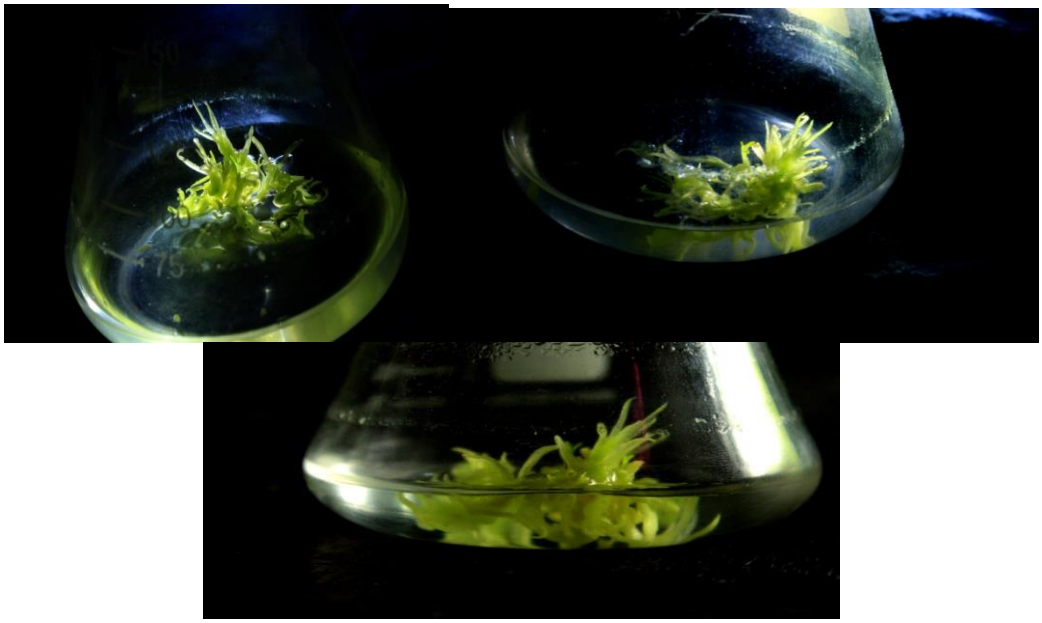

Figure 7. Liquid culture system on direct multiple shoot induction using leaf as explant of *H. enneaspermus* (L.) F. Muell

Optimization of media components is essential to obtain the desired results. The CCD function of RSM has been proven to be highly useful for the optimization of various experimental parameters. The model consisting 30 experimental variations was constructed for the study incorporating four independent variables. After experimental validation, regression analysis was performed, followed by analysis of variance (ANOVA). Performing ANOVA helps in comprehending the aptness of the model. Coefficients for each linear, and related quadratic term, as well as interactions between factors could be derived using ANOVA. The higher R2 values signify higher correlation amongst variables and better predictability of the designed model. The responses generated from such a model tend to show accord between predicted and experimental values. The R2 values obtained from the statistical analysis are0.7342and 0.6395. In a similar study performed on *S. dulcis* by (Premkumar et al., 2020) for media optimization, R2 values greater than 0.8 were obtained for both responses, indicating adequacy of the designed CCD-RSM model.
The high R2 values obtained in the present study were supported by the predictability of the design and the correlation between the variables, and their responses are designated to a positive relationship between the predicted and experimental values. The p values lesser than 0.05 authenticate the significance of the corresponding coefficient in the CCD-RSM model. The obtained p values, 0.001 and 0.049denote perfectly significant coefficients in the model. The Pareto plot displayed the significant effect of the variables X3 and X4 on Y1 and Y2. The quantile-quantile plots generated by plotting the internal studentized residuals against predicted response were helpful in recognizing the constant error in the model. The random distribution of the residuals within the specified range signaled absence of outliers. The data generated through statistical and experimental analysis during this study has high significance from the perspective of plant conservation, *invitro* propagation, and availing essential phytochemicals for pharmaceutical industry.

Conclusion

The experimental validation of the predicted model resulted in enhanced shoot regeneration. The maximum shoot regeneration was achieved on liquid MS media containing KN 2.32 µM, BAP 3.30 µM, IAA 5.70 µM, and SA 80 mg. The use of liquid MS media during growth period also enriched *H, enneaspermus* plants with phenols, flavonoids and increased chlorophyll accumulation. The use of growth elicitor SA (100 mg/l) in the liquid MS media caused increase in aurantiamide acetate and coumarin levels. The study will be helpful in future for *H. enneaspermus* plant propagation using leaf explants and also for the mass production of various secondary metabolites.

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