

OPTIMIZATION OF HAIRY ROOT PROLIFERATION IN LIQUID MEDIUM USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

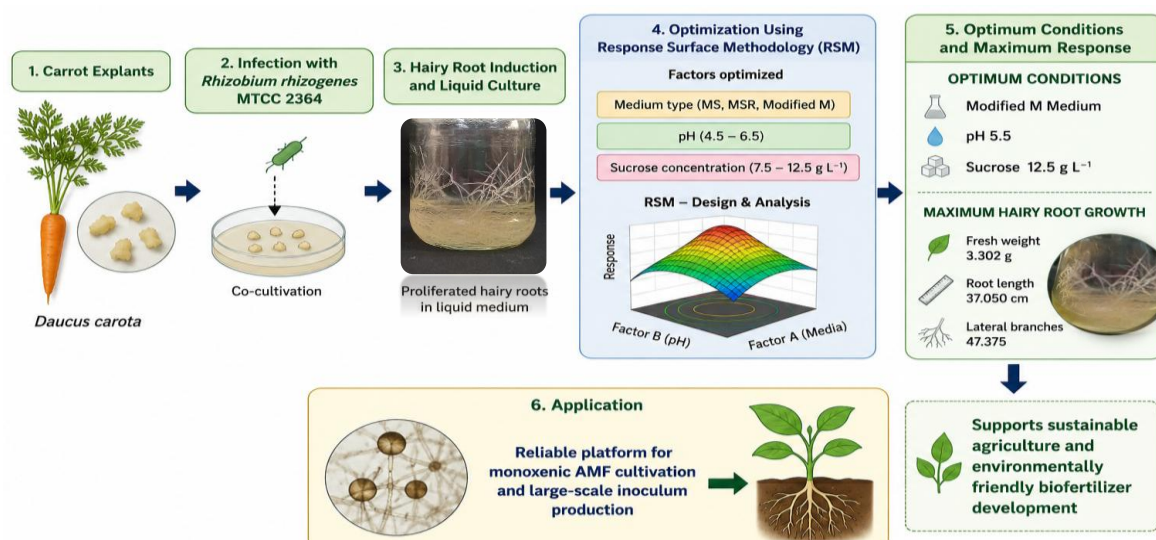
Aim: Arbuscular mycorrhizal (AM) fungi are valuable tools for sustainable agriculture, but their large-scale production is limited by the lack of optimized *in vitro* host root systems; therefore, this study used Response Surface Methodology (RSM) to optimize key physicochemical factors affecting hairy root proliferation in liquid culture for efficient AMF multiplication.

Methodology: *Rhizobium rhizogenes* strain MTCC 2364 was used to generate hairy roots in carrot (*Daucus carota*) explants. The effects of culture medium, pH, and sucrose content on fresh weight, root length, and number of lateral branches were assessed using a Box-Behnken experimental design. ANOVA and second-order polynomial regression were used to analyze the data.

Results: Hairy root induction occurred within 16–17 days after inoculation, achieving a high transformation efficiency of 94%. RSM analysis identified culture medium, pH, and sucrose concentration as key factors influencing root growth, with optimized conditions (Modified M medium, pH 5.5, and 12.5 g L⁻¹ sucrose) predicting 3.30 g fresh weight, 37.05 cm root length, and 47.38 lateral branches (R² > 0.88).

Interpretation: The resulting methodology contributes to the optimization of hairy root growth which can be used for a scalable, repeatable method of AMF inoculum production.

KEYWORDS: Arbuscular mycorrhizal fungi, Hairy root culture, Response surface methodology, *In vitro* optimization.



INTRODUCTION

Sustainable agricultural production is essential to meet the food demands of a rapidly growing global population. According to the United Nations World Population Prospects, the world population is projected to increase from 8.2 billion in 2024 to approximately 9.7 billion by 2050 (INED, 2024). However, expansion of cultivable land is increasingly constrained by soil degradation, biodiversity loss, and climate change, necessitating improved productivity through environmentally sustainable approaches.

Although chemical fertilizers have substantially increased crop productivity, their excessive use has caused environmental problems such as greenhouse gas emissions, nutrient leaching, eutrophication, and soil degradation. As a sustainable alternative, biofertilizers containing beneficial microorganisms, including nitrogen-fixing bacteria, phosphate-solubilizing microbes, and arbuscular mycorrhizal fungi (AMF), are gaining importance. These microbial inoculants improve nutrient availability, enhance soil health, and reduce dependence on chemical fertilizers while maintaining crop productivity (Samantaray *et al.*, 2024).

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with nearly 80% of terrestrial plant species and play a vital role in improving phosphorus uptake, water-use efficiency, and soil health. They also enhance plant tolerance to biotic and abiotic stresses, including drought, salinity, and temperature extremes. Consequently, AMF are recognized as key biofertilizers for sustainable agriculture and reduced fertilizer dependency (Formenti *et al.*, 2026; Choudhary and Mathur, 2026).

Large-scale utilization of AMF is constrained by their obligate biotrophic nature and the limitations of conventional pot culture methods, which are laborious and often yield inconsistent inoculum. Root organ culture (ROC) using *Rhizobium rhizogenes*-induced hairy roots provides an efficient alternative due to their rapid growth, extensive branching, and genetic stability, making them ideal hosts for AMF multiplication (Declerck *et al.*, 2005; Fortin *et al.*, 2002).

Hairy root cultures have become valuable biotechnological platforms because of their high growth rates, stable genetic characteristics, and suitability for large-scale cultivation. Recent advances demonstrate that hairy root systems can be effectively optimized for enhanced biomass production and bioprocess scalability through controlled culture conditions and bioreactor technologies (Elbouzidi *et al.*, 2026). Such systems provide a promising foundation for the development of efficient AMF inoculum production technologies.

Hairy root growth is influenced by several interacting factors such as culture medium, sucrose concentration, pH, and environmental conditions. Response Surface Methodology (RSM) is an efficient statistical tool that evaluates these factors simultaneously, identifies their interactions, and determines optimal culture conditions with fewer experimental trials (Box and Behnken, 1960).

Therefore, the present study was undertaken to develop an efficient *in vitro* system for carrot hairy root proliferation using *Rhizobium rhizogenes*. The effects of culture medium, sucrose concentration, and pH on hairy root growth were investigated and optimized using Response Surface Methodology. Hairy root fresh weight, root length, and number of lateral branches were used as response variables. The optimized protocol developed in this study is expected to provide a reliable platform for large-scale hairy root production and facilitate future applications in arbuscular mycorrhizal fungal inoculum development and other plant biotechnology programmes.

MATERIALS AND METHODS

Bacterial strain and explant preparation

Rhizobium rhizogenes strain MTCC 2364 obtained from the Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, Tamil Nadu, India, was used in this study for the induction of hairy roots.

In this study, carrot (*Daucus carota*) was selected as the explant for root induction. Healthy, disease-free, and fully matured medium-sized carrots were freshly harvested from agricultural fields located in the lower hills of Kodaikanal, Tamil Nadu, India, and utilized on the same day of collection.

The selected carrots were thoroughly washed, peeled, and subjected to surface sterilization using 0.1% mercuric chloride (Hg Cl₂) for 15 min. with continuous stirring, and then washed with sterilized water for 4 to 5 min. The surface-sterilized carrots were further washed with 70% ethanol for 3 seconds. Each carrot was sliced into 1 cm thick discs and used as explants for hairy root induction.

Hairy root induction

A loopful of *R. rhizogenes* culture was inoculated into the 20 ml of yeast extract mannitol (YEM) broth in a sterile culture tube. The inoculated cultures were grown overnight at 25°C in orbital shaker at 80 rpm. A 24-hour-old culture of *R. rhizogenes* was uniformly inoculated onto the surface of the carrot discs. The explants were placed into hormone-free basal Murashige and Skoog (MS) medium with acetosyringone at 100 µM concentration without antibiotics and incubated in dark conditions at 26°C. After 48 h, the explants were checked for the production of hairy roots and growth of bacteria on the media. If the growth were traced, then immediately the explants were transferred to a half-strength MS medium containing cefotaxime 500 ppm. The explants were transferred to the fresh medium as above till the bacterial growth was completely arrested. The concentration of cefotaxime was reduced in every transfer to fresh medium from 500 ppm to 100 ppm.

The transformation rate was calculated as below

$$\text{Transformation efficiency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A. \text{rhizogenes}} \times 100$$

Hairy root multiplication

The transformed hairy roots (minimum 3 cm long) free of contamination were excised from the explants using a sterile scalpel blade and transferred to a Petri plate containing MSR medium devoid of hormones and antibiotics. After subculturing, the plates were incubated in the dark at 26°C.

Production of hairy roots in liquid medium

In a liquid medium, the protocol for hairy root multiplication was standardized. Culture medium, sucrose concentration, and medium pH were identified as key factors influencing hairy root multiplication, growth, and lateral branch formation in liquid culture. Therefore, these critical parameters were selected and optimized to develop an efficient protocol for rapid hairy root proliferation.

The transformed hairy roots were subcultured in liquid media. Three different liquid media: MS (1), MSR (2), and Modified M medium (3) were used.

S. No	Factors	Range
1.	Media	1,2,3
2.	pH	4.5, 5.5, 6.5
3.	Sucrose concentration (g/L)	7.5, 10.0, 12.5

Statistical optimization using RSM

Response Surface Methodology (RSM) is a statistical and mathematical approach used to optimize multiple independent variables by modelling their effect on a desired response through a developed regression equation. It enables the prediction of the experimental outcome based on variable interactions and identifies the most influential factors affecting the response by analyzing variance (ANOVA).

Response Surface Methodology (RSM) was applied to optimize culture conditions influencing hairy root growth. Experimental design and statistical analyses were performed using Design-Expert® software (version 10.0, Stat-Ease Inc., Minneapolis, MN, USA). A three-factor Box–Behnken design (BBD) was employed to evaluate the effects of carbon source concentration (sucrose, g L⁻¹), culture medium pH, and culture medium type on hairy root development. The response variables included mean daily root elongation rate and mean number of lateral branches formed per day. A total of 17 experimental runs were generated according to the BBD matrix.

A second-order polynomial model was used to describe the relationship between independent variables and responses:

$$Y_i = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ij} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ii} x_i x_j + \varepsilon$$

Where Y_i denotes the response variable (e.g., fresh weight of the root, average root length and number of side branches); x refers to the independent variables (i.e., phytagel concentration, sucrose level, and pH of the medium); β_0 is the intercept term; β_i , β_{ii} , and β_{ij} represent the coefficients of the linear, quadratic, and interaction terms, respectively. Here, k is the total number of variables considered. The coefficients were estimated using the ordinary least squares (OLS) method. Model adequacy and statistical significance were evaluated using analysis of variance (ANOVA). Response surface plots were generated to visualize interaction effects and determine optimal culture conditions.

RESULTS AND DISCUSSION

Establishment of hairy root culture system

In the present study, carrot explants inoculated with *R. rhizogenes* strain MTCC 2364 successfully induced hairy roots under *in vitro* conditions, whereas uninoculated controls showed no root formation. Initial swelling and cellular proliferation were observed at wounded sites within two weeks, followed by visible hairy root initiation after 17 days on hormone-free MS medium supplemented with acetosyringone under dark incubation. The induced roots were thin, highly branched, and displayed characteristic plagiotropic growth typical of *R. rhizogenes*-mediated transformation.

Bacterial overgrowth following co-cultivation was effectively controlled using cefotaxime. A gradual reduction of antibiotic concentration from 500 ppm to 100 ppm effectively eliminated bacterial contamination without negatively affecting root growth. A transformation efficiency of 94% was achieved, demonstrating the high susceptibility of carrot explants to *R. rhizogenes* infection and confirming their suitability as a host system for monoxenic cultures.

Arbuscular mycorrhizal fungal (AMF) inoculants are increasingly utilized in agriculture, horticulture, and environmental remediation due to their ability to enhance nutrient acquisition, improve soil health, and reduce chemical inputs (Priyadharshini *et al.*, 2025).

Various *in vitro* cultivation systems have been developed for AMF propagation, including autotrophic culture systems, liquid culture systems, and solid agar-based root organ cultures (Declerck *et al.*, 2005; IJdo *et al.*, 2011). Among these, liquid culture systems are particularly advantageous because they provide uniform nutrient availability, improved aeration, and rapid hairy root proliferation, thereby facilitating large-scale inoculum production (Cardillo *et al.*, 2010; Ghorui *et al.*, 2023).

Various host plants, including tobacco, tomato, chicory, cucumber, grapevine, and carrot, have been employed for hairy root culture and AMF propagation studies (Fortin *et al.*, 2002; Declerck *et al.*, 2005). However, carrot

has become the most widely adopted host because of its high transformation efficiency, rapid hairy root growth, extensive branching pattern, and proven ability to support the complete life cycle of numerous AMF species under monoxenic conditions (Declerck *et al.*, 1996; Fortin *et al.*, 2002; Bidondo *et al.*, 2012). The present findings agree with earlier reports indicating that hairy root induction efficiency is strongly influenced by bacterial strain, explant type, culture medium, and environmental conditions (Priya *et al.*, 2024). Similarly, Radha *et al.*, (2014) reported high transformation efficiency in *Daucus carota* cultured on half-strength MS medium supplemented with 150 μ M acetosyringone and 48 h co-cultivation.

Growth characteristics and culture stabilization

Excised hairy roots (>3 cm length) established rapidly upon transfer to liquid medium and showed active elongation within 7–10 days. Subcultures maintained under dark conditions produced fast-growing and highly branched root systems. The root lines remained morphologically stable across successive subcultures, indicating successful establishment of transformed cultures.

Bacterial-free transformed hairy root bits from cowpea and tomato have previously been cultured in both solid and liquid media without growth regulators, with profuse root proliferation observed in liquid medium under shaking conditions (Prabakaran, 2008). Similar growth behaviour has been reported in several hairy root systems, where transformed roots proliferated efficiently in hormone-free media and exhibited enhanced branching and growth rates. Hairy root line 30 showed a lag phase during the first 10 days, followed by rapid exponential growth from day 10 to 25 and a stationary phase from day 25 to 40 due to nutrient limitation. The biomass doubling time was approximately 6.5 days (Mai *et al.*, 2022). Similar growth patterns have also been reported in *Fagopyrum tataricum* (Zhao *et al.*, 2014), cucurbits (Rekha and Thiruvengadam, 2017), and *Vitis vinifera* cv. Pinot (Tisserant *et al.*, 2016).

Effect of culture medium on hairy root proliferation

Hairy root growth varied significantly among the tested liquid media. MSR medium supported vigorous growth and biomass accumulation, followed by Modified M medium, whereas standard MS medium resulted in comparatively slower development. Roots grown in optimized media exhibited elongated primary axes, dense lateral branching, and healthy creamy-white morphology, indicating improved nutrient utilization and physiological activity.

Hairy root growth and biomass accumulation are strongly influenced by basal medium composition, pH, and carbon source (Guillon *et al.*, 2006). Previous studies have demonstrated that modified White's medium supports superior hairy root proliferation compared with Murashige and Skoog medium due to its favourable nitrogen composition and improved pH stability (Devikrishna *et al.*, 2010). Similarly, Cardillo *et al.*, (2010) highlighted the importance of medium composition in determining growth performance in liquid culture systems. Devikrishna *et al.*, (2010) further reported higher hairy root induction and transformation frequencies in tomato and cowpea explants cultured on modified White's medium than on diluted MS medium.

Influence of pH and carbon source on hairy root multiplication

The medium pH had a marked influence on hairy root proliferation. Maximum growth of 3.5 g fresh weight with 37.8 cm length, 48 lateral branches at 60 days after inoculation, occurred at pH 5.5, characterized by rapid elongation and extensive lateral branching. Reduced growth at pH 4.5 and moderate performance at pH 6.5 suggest that mild acidic conditions favour nutrient uptake and metabolic activity in transformed roots.

Sucrose concentration also significantly affected growth responses. Optimal proliferation occurred at 12.5 g L⁻¹ sucrose, while lower concentrations (7.5 g/L) limited branching and higher osmotic conditions reduced elongation. These findings confirm that balanced carbon availability is essential for sustaining rapid root metabolism and morphogenesis. Balanced carbon availability is essential because sucrose provides the energy required for cell growth and metabolism, while excess sucrose may cause osmotic stress that inhibits root development and low levels may limit energy supply.

Response Surface Methodology identified Modified M medium supplemented with 12.5 g L⁻¹ sucrose at pH 5.5 as the optimum combination for hairy root multiplication, producing maximum biomass, root elongation, and lateral branching (Table 1). Hairy root proliferation under the optimized liquid culture conditions is illustrated in Figure 1.

These findings confirm that balanced carbon availability is essential because sucrose serves as the primary carbon and energy source for cell growth and metabolism, while excessive sucrose can impose osmotic stress and lower concentrations may limit energy availability for root proliferation. The superior growth observed at pH 5.5 further indicates that mildly acidic conditions favour nutrient uptake and metabolic activity in transformed roots, supporting efficient elongation and lateral branch formation (Guillon *et al.*, 2006; Priya *et al.*, 2024).

Statistical optimization of hairy root proliferation using RSM

The optimization of culture conditions for enhanced hairy root proliferation was conducted using Response Surface Methodology (RSM) based on a Box-Behnken Design (BBD), focusing on three independent variables: media type (A), pH (B), and sucrose concentration (C). These parameters were evaluated for their effects on three major response variables: fresh weight (FW), root length (RL), and number of side branches (SB), with ANOVA in order to determine the significance of each factor and their interactions. Table 2 presents observed data for fresh weight, root length, and number of side branches at 30 and 60 days under different treatment combinations. It clearly shows that treatments with optimized media (Modified M), pH 5.5, and higher sucrose levels resulted in enhanced root growth and branching.

The ANOVA results indicated that the quadratic model adequately fitted the response data. The model F-values of 43.67, 11.03, and 5.80 for fresh weight, root length, and number of side branches, respectively, confirmed the significance of the model. Based on the P-values, factors A, B, C, AB, A², and B² significantly affected fresh weight; A, B, C, and B² influenced root length; while A, C, BC, A², and B² significantly affected side branching. The R² values of 0.9825, 0.9341, and 0.8818 were in close agreement with the adjusted R² values of 0.9600, 0.8494, and 0.8818 for fresh weight, root length, and side branches, respectively. Similarly, the predicted R² values (0.7637, 0.7859, and 0.7978) showed good agreement with the adjusted R² values, with differences less than 0.2, indicating good model adequacy and reliability.

For fresh weight (FW), the model is found to be highly significant, with an F-value of 43.67 and a p-value of <0.0001, indicating an excellent fit. Table 2 depicts the ANOVA results, which indicates that the quadratic model for fresh weight is statistically significant, with key contributions from media type, pH, and sucrose concentration. Interaction and quadratic effects also play an important role in influencing biomass production. Among the tested variables, media (A) emerged as the most influential factor, showing a high mean square value of 9.46, an F-value of 150.6, and a p-value <0.0001. pH (B) also contributed significantly to FW, with a mean square of 6.09, an F-value of 96.94, and a p-value <0.0001. Sucrose concentration (C) showed a moderate effect on fresh weight yet significant impact, with a mean square of 0.6238, an F-value of 9.93, and a p-value of 0.0161. The interaction between media and pH (AB) is also statistically significant (F = 42.9, p = 0.0003), demonstrating a synergistic influence on FW. Other interaction terms (AC and BC) as well as the second-order term for sucrose (C²) are not significant. However, the quadratic terms for media (A²) and pH (B²) showed significant non-linear effects, with p-values <0.05. The model displayed high reliability and predictive power, with an R² of 0.9825, an adjusted R² of 0.9600, and a predicted R² of 0.7637. Figure 2 indicates a strong agreement between predicted and actual values, confirming model reliability. Response surface plots revealed that media and pH had a major interactive effect on fresh weight, with sucrose showing a comparatively moderate influence.

The predicted second-order polynomial equation for fresh weight (FW):

$$FW = 1.56 + 0.5450 A + 0.4388 B + 0.1400 C + 0.5800 AB + 0.1510 AC + 0.2030 BC - 0.2331 A^2 - 0.7171 B^2 - 0.2294 C^2$$

In the case of root length (RL), the model is statistically significant with an F-value of 11.03 and a p-value of 0.0023. Media (A) was again the most critical variable, with a mean square of 1.44, an F-value of 28.52, and a p-value of 0.0011. pH (B) also showed a significant impact, with a mean square of 0.65, an F-value of 12.87, and a p-value of 0.0089. Sucrose concentration (C) had a relatively mild effect, with a mean square of 0.30, an F-value of 5.89, and a p-value of 0.0456. None of the interaction terms (AB, AC, or BC) was statistically significant. However, the second-order term for pH (B²) demonstrated a strong non-linear effect, with a mean square of 1.78, an F-value of 35.27, and a p-value of 0.0006. The R² value of the model was 0.9341, with an adjusted R² of 0.8494 and a predicted R² of 0.7859, indicating a high level of model adequacy. Figure 3 shows that the model adequately predicted root length as evidenced by the close alignment of predicted and observed values. Among the variables, media and pH significantly influenced elongation, while interaction effects were less pronounced.

The equation for root length (RL):

$$RL = 22.15 + 6.72 A + 4.53 B + 2.17 C + 3.80 AB + 2.14 AC + 1.59 BC - 1.64 A^2 - 11.53 B^2 - 2.65 C^2$$

Table 3 shows that the model for root length is significant, showing that all three factors and their interactions affect root elongation. The high model fit confirms its reliability in predicting optimal conditions for maximum root growth.

For side branch emergence (SB), the model is significant with an F-value of 5.80 and a p-value of 0.0151. Media (A) and sucrose (C) are the key influencing variables, with A showing a mean square of 9.27, an F-value of 8.10, and a p-value of 0.0249, and C displaying a mean square of 7.84, an F-value of 6.84, and a p-value of 0.0346. While pH (B) did not show a significant direct effect, its interaction with sucrose (BC) is statistically significant, with an F-value of 8.16 and a p-value of 0.0245. Other interaction terms (AB and AC) are not significant. Among the quadratic terms, both A² and B² are significant, confirming the presence of non-linear relationships, whereas C² is not. The model showed good consistency, with an R² value of 0.8818, an adjusted R² of 0.7297, and a predicted R² of 0.7978, though slightly lower than those observed for FW and RL. Figure 4 shows that the number of side branches was moderately predicted by the model with an acceptable fit. The interaction between sucrose concentration and media showed a noticeable effect, while pH had a comparatively lesser influence.

The predicted second-order polynomial equation:

$$SB = 36.29 + 6.13 A + 3.27 B + 5.02 C - 0.8750 AB - 0.2500 AC + 5.50 BC - 6.70 A^2 - 6.42 B^2 - 3.41 C^2$$

Table 4 indicates that the ANOVA analysis reveals that the number of side branches is significantly influenced by individual and combined effects of the variables. The quadratic model effectively explains variation in branching patterns under different treatments.

RSM analysis revealed that medium composition, pH, and sucrose concentration significantly affected hairy root proliferation, with medium type exerting the greatest influence on fresh weight, root length, and lateral branching. Significant medium and pH interactions for fresh weight and the quadratic effect of pH on root length indicate the strong sensitivity of transformed roots to culture conditions. In contrast to the present optimum conditions (pH 5.5 and 12.5 g L⁻¹ sucrose), Priya *et al.*, (2024) reported optimum values at pH 4.0 and approximately 11 g L⁻¹ sucrose, indicating system-specific variation. The significant interaction between pH and sucrose affecting side branching further confirms the combined regulatory influence of these factors.

The optimized conditions (Modified M medium, pH 5.5, and 12.5 g L⁻¹ sucrose) predicted a fresh weight of 3.302 g, root length of 37.050 cm, and 47.375 lateral branches, resulting in maximum hairy root proliferation. Enhanced growth under these conditions provides a robust platform for monoxenic AMF cultivation and demonstrates the effectiveness of RSM in optimizing hairy root production for large-scale inoculum development. Similar RSM-based optimization approaches have proven effective in enhancing hairy root biomass and metabolite production in other systems (Amdoun *et al.*, 2020), supporting the applicability of statistical modelling for biotechnological scale-up.

CONCLUSION

An efficient carrot hairy root culture system was successfully established using *Rhizobium rhizogenes* MTCC 2364, and Response Surface Methodology identified Modified M medium supplemented with 12.5 g L⁻¹ sucrose at pH 5.5 as the optimum condition for maximum root proliferation. The optimized protocol provides a reliable and scalable platform for monoxenic AMF inoculum production, contributing to the development of sustainable biofertilizer technologies.

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Authorship & Contributorship

Authors' contributions: KK conceptualized the study. MP wrote the manuscript. KK, RTV, RS, RA, SMPK, IYR and PVP reviewed and edited the manuscript. All authors read and approved the final manuscript for publication

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Table 1: Effect of medium, pH and sucrose concentration on hairy root multiplication

Factor	Levels tested	Response of hairy roots	Best performing level
Liquid medium	MS	Moderate biomass, less branching	Modified M medium
	MSR	Improved growth compared to MS, moderate lateral roots	
	Modified M	Maximum biomass, dense and highly branching	
pH	4.5	Slow growth, reduced elongation, fewer lateral roots	5.5
	5.5	Rapid elongation and high lateral branching, optimal growth	
	6.5	Slightly reduction in growth, moderate elongation and branching	
Sucrose (%)	7.5	Lower growth, less developed roots	12.5
	10.0	Moderate growth and better root thickness and branching	
	12.5	Highest growth rate and healthy roots	

Run	Factors			Fresh weight of the hairy roots (gm)		Length of the hairy roots (cm)		No. of side branches (Nos.)	
						Over on 0 th day			
	Media	pH	Conc. of sucrose (gL ⁻¹)	30 th day	0 th day	30 th day	60 th day	30 th day	60 th day
1	MS	5.5	12.5	0.197	0.476	7.7	18.9	15	32
2	MS	4.5	10.0	0.030	0.040	6.1	9.5	9	13
3	MSR	4.5	7.5	0.053	0.184	6.4	11.5	12	32
4	MSR	5.5	10.0	0.987	2.360	10.5	23.5	25	38
5	MSR	5.5	10.0	1.205	2.066	12.4	28.4	33	47
6	M	4.5	10.0	0.098	0.156	7.4	16	14	31
7	M	5.5	7.5	0.792	2.540	9.8	23.5	21	32
8	M	5.5	12.5	1.145	3.495	12.8	37.8	30	48
9	MS	6.5	10.0	0.027	0.061	5.6	11.5	9	24
10	M	6.5	10.0	1.030	3.460	8.3	33.2	19	37
11	MSR	4.5	12.5	0.025	0.197	5.5	11	10	23
12	MSR	5.5	10.0	0.723	2.320	10.4	35	32	62
13	MSR	5.5	10.0	0.980	2.415	11.8	32.5	30	60
14	MS	5.5	7.50	0.105	0.375	8.7	16.7	10	17
15	MSR	5.5	10.0	0.975	2.365	10	28	27	46
16	MSR	6.5	7.5	0.144	1.435	6.4	15.5	7	20
17	MSR	6.5	12.5	0.692	2.600	6.8	24	22	55

Table 2: Experimental values of fresh weight, root length and emergence of side branches in hairy root multiplication

Table 3: ANOVA table for the quadratic model of efficient hairy root proliferation conditions on fresh weight of the root

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	24.68	9	2.74	43.67	< 0.0001	Significant
A-Media	9.46	1	9.46	150.6	< 0.0001	Significant
B-pH	6.09	1	6.09	96.94	< 0.0001	Significant
C-Sucrose concentration	0.6238	1	0.6238	9.93	0.0161	Significant
AB	2.69	1	2.69	42.9	0.0003	Significant
AC	0.1823	1	0.1823	2.9	0.1322	not significant
BC	0.3318	1	0.3318	5.28	0.0551	not significant
A ²	0.6055	1	0.6055	9.64	0.0172	Significant
B ²	4.18	1	4.18	66.6	< 0.0001	Significant
C ²	0.176	1	0.176	2.8	0.138	not significant
Residual	0.4397	7	0.0628			
Lack of Fit	0.3636	3	0.1212	6.37	0.0528	not significant
Pue Error	0.0761	4	0.019			
Cor Total	25.12	16				
Std. Dev.	0.2506			R²	0.9825	
Mean	1.56			Adjusted R²	0.96	
C.V. %	16.05			Predicted R²	0.7637	
				Adeq Precision	20.3914	

Table 4: ANOVA table for the quadratic model of efficient hairy root proliferation conditions on length of the hairy roots

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1264	9	140.44	11.03	0.0023	Significant
A-Media	363.15	1	363.15	28.52	0.0011	Significant
B-pH	163.81	1	163.81	12.87	0.0089	Significant
C-Sucrose concentration	75.03	1	75.03	5.89	0.0456	Significant
AB	57.76	1	57.76	4.54	0.0707	not significant
AC	36.6	1	36.6	2.87	0.1338	not significant
BC	20.25	1	20.25	1.59	0.2477	not significant
A ²	10.81	1	10.81	0.8492	0.3874	not significant
B ²	449.08	1	449.08	35.27	0.0006	Significant
C ²	56.17	1	56.17	4.41	0.0738	not significant
Residual	89.13	7	12.73			
Lack of Fit	10.42	3	3.47	0.1765	0.9071	not significant
Pure Error	78.71	4	19.68			
Cor Total	1353.12	16				
Std. Dev.	3.57			R²	0.9341	
Mean	22.15			Adjusted R²	0.8494	
C.V. %	16.11			Predicted R²	0.7859	
				Adeq Precision	9.8522	

Table 5: ANOVA table for the quadratic model of efficient hairy root proliferation conditions on the number of side branches

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3098.08	9	344.23	5.8	0.0151	Significant
A-Media	480.5	1	480.5	8.1	0.0249	Significant
B-pH	171.13	1	171.13	2.88	0.1333	not significant
C-Sucrose concentration	406.13	1	406.13	6.84	0.0346	Significant
B	6.25	1	6.25	0.1053	0.755	not significant
AC	0.25	1	0.25	0.0042	0.9501	not significant
BC	484	1	484	8.16	0.0245	Significant
A ²	637.01	1	637.01	10.73	0.0136	Significant
B ²	611.38	1	611.38	10.3	0.0149	Significant
C ²	154.12	1	154.12	2.6	0.1511	not significant
Residual	415.45	7	59.35			
Lack of Fit	4.25	3	1.42	0.0138	0.9974	not significant
Pure Error	411.2	4	102.8			
Cor Total	3513.53	16				
Std. Dev.	7.7			R²	0.8818	
Mean	36.29			Adjusted R²	0.7297	
C.V. %	21.23			Predicted R²	0.7978	

				Adeq Precision	7.214	
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Fig 1: Hairy root culture in liquid medium

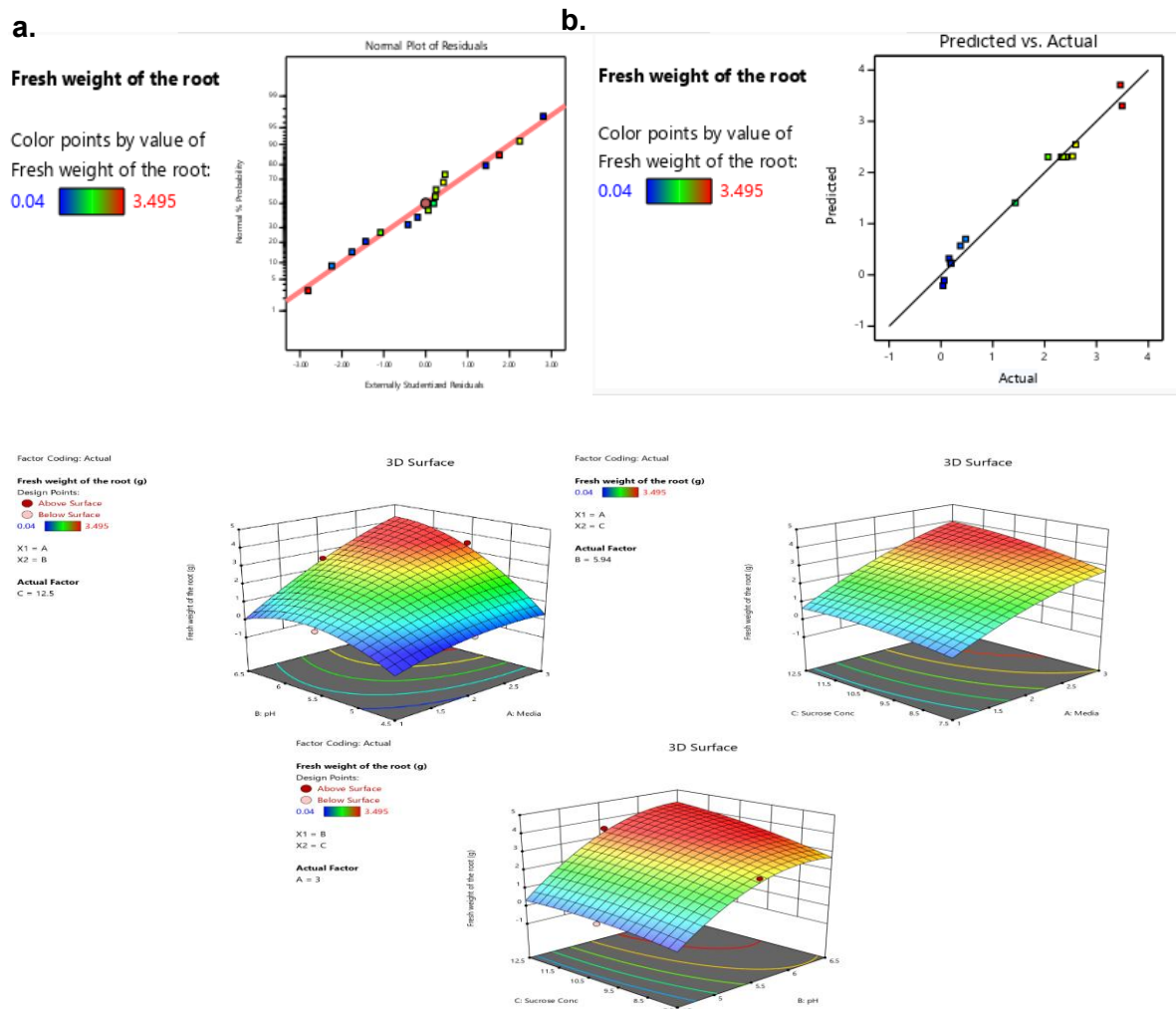
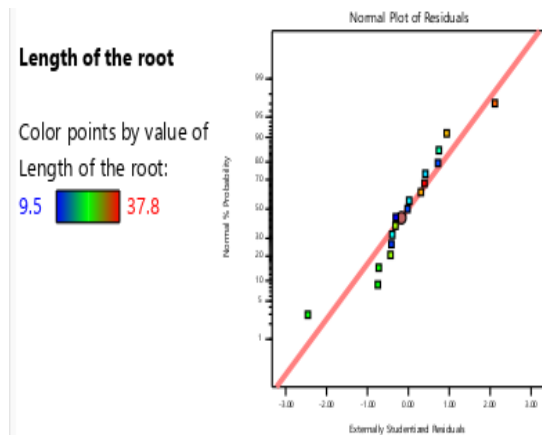
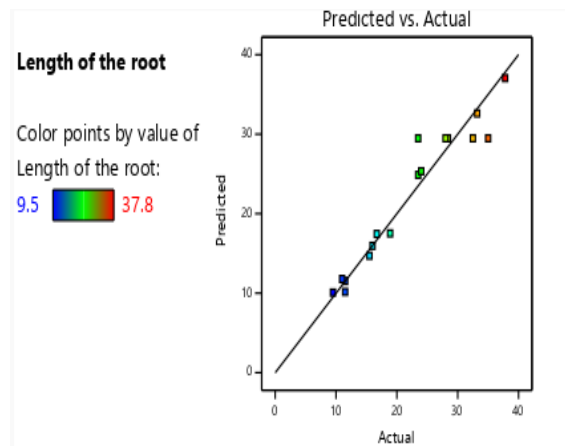


Fig 2: Graphical representation of the influence of input parameters on fresh weight of the root as response variable. a. Normal plot of residuals b. predicted vs actual weight of root c. 2D contour and 3D graphs on the influence of Media, pH and sucrose concentration on the fresh weight of the root. Residual and response surface analysis confirming the adequacy of the quadratic model for fresh weight. Normal probability plot shows random distribution, while predicted vs actual values indicate good model fit. Contour and 3D plots illustrate the combined influence of media, pH, and sucrose concentration on fresh weight.

a.



b.



Factor Coding: Actual

Length of the root (cm)

Design Points:

● Above Surface

○ Below Surface

9.5  37.8

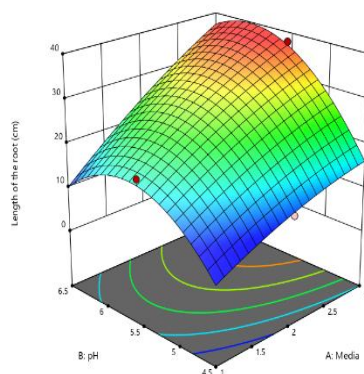
X1 = A

X2 = B

Actual Factor

C = 12.5

3D Surface



Factor Coding: Actual

Length of the root (cm)

Design Points:

● Above Surface

○ Below Surface

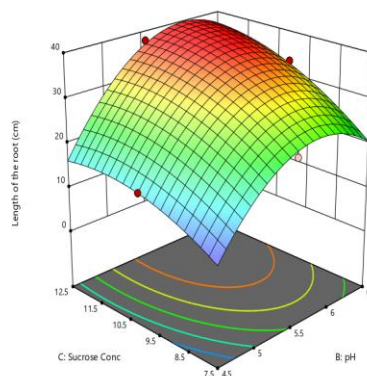
9.5  37.8

X1 = B

X2 = C

Actual Factor

A = 3



3D Surface

Factor Coding: Actual

Length of the root (cm)

Design Points:

● Above Surface

○ Below Surface

9.5  37.8

X1 = A

X2 = C

Actual Factor

B = 5.5

3D Surface

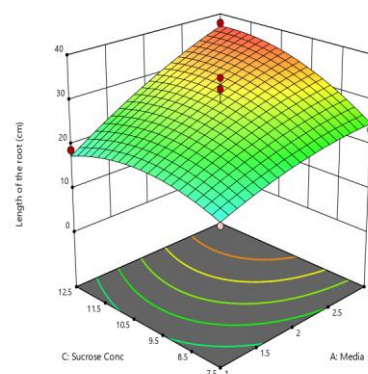


Fig 3: Graphical representation of the influence of input parameters on the length of the root as the response variable. a. Normal plot of residuals b. predicted vs actual length of root c. 2D contour and 3D graphs on the influence of Media, pH and sucrose concentration on length of the root. Diagnostic plots validating the model for root length. Residuals follow normal distribution and predicted values closely match actual observations. Surface plots demonstrate the interaction effects of media, pH, and sucrose on root elongation

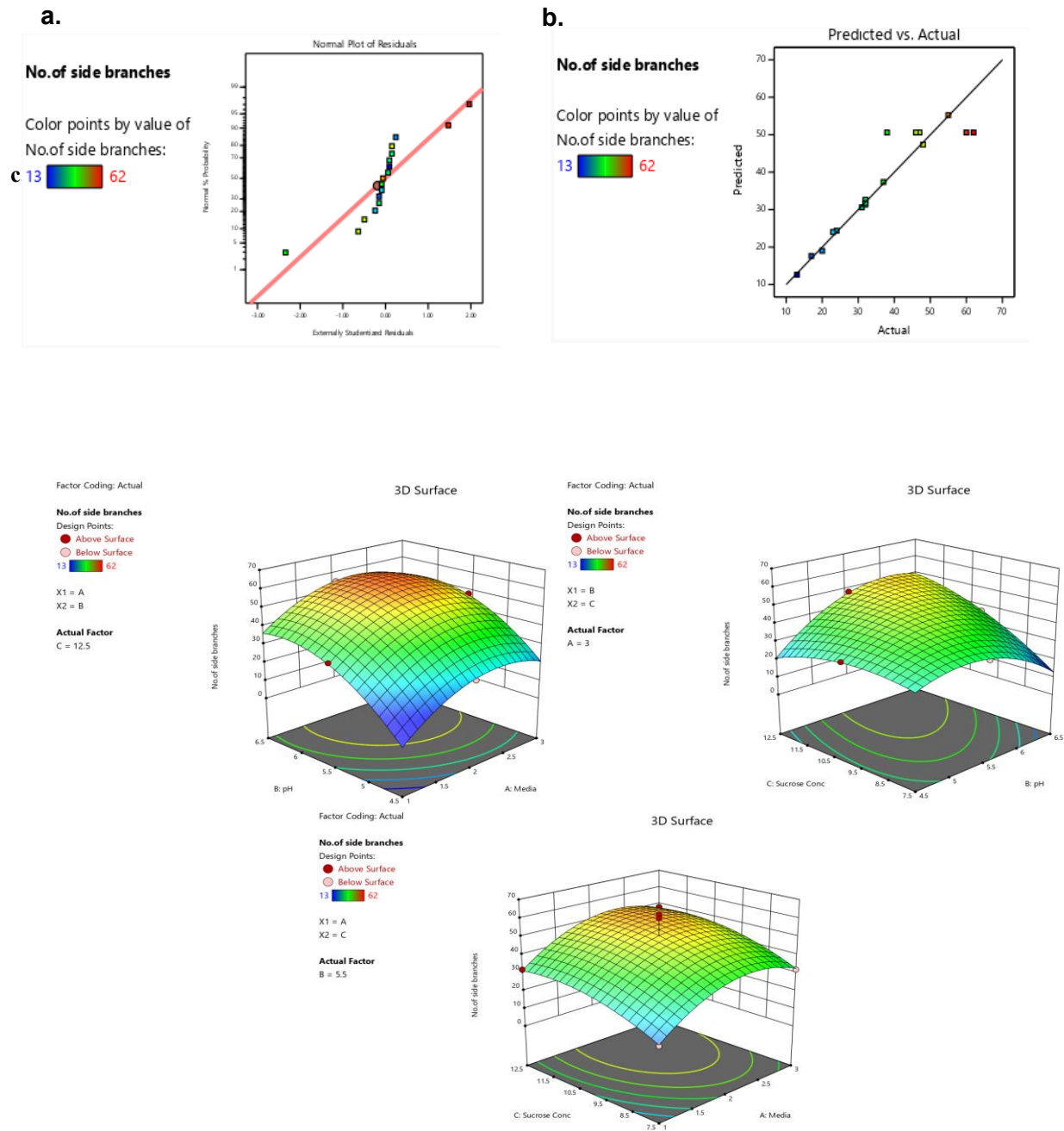


Fig 4: Graphical representation of the influence of input parameters on no of side branches of the root as the response variable. a. Normal plot of residuals b. predicted vs actual no. of side branches of root c. 2D contour and 3D graphs on the influence of Media, pH and sucrose concentration on no. of side branches. Model validation and interaction effects for number of side branches. Residual plots confirm normality and independence, while predicted vs actual plots show acceptable agreement. Response surface plots depict the influence of culture conditions on branching pattern.