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RESEARCH ARTICLE

Response surface methodology-based optimization of hairy roots cultures for *in vitro* AM production

P. Vijaya Priya[1], K. Kumutha[1*], R. Subhashini[2], R. Renuka[3], T. Sivakumar[4] & P. Kannan[5]

[1] Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, India

[2] Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, India

[3] Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore 641 003, India

[4] Department of Crop Physiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, India

[5] Department of Soil Science and Agricultural Chemistry, Tamil Nadu Agricultural University, Coimbatore 641 003, India

*Email: kkumutha@tnau.ac.in

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Abstract

Arbuscular mycorrhizal fungi can do wonders in promoting the crop growth as well maintaining soil health. *In vitro* root organ culture technology is an exciting avenue for AM biofertilizer production. This study aims to optimize the key influencing factors for enhancing the hairy root production used for *in vitro* AMF culturing using response surface methodology, a statistical and mathematical tool used for designing optimization studies. Study uses *Rhizobium rhizogenes* MTCC 2364 for transformation in carrot explant. Factors considered for optimization are concentration of gelling agent (phytagel), carbon source (sucrose) and pH of the Modified Strullu and Romand medium (MSR). Design Expert software uses second order polynomial regression equation for predicting the outcome of each experiment. Totally, 17 experiments were run following Box-Behnken design and average hairy root length and average side branch emergence were taken as response. ANOVA analysis reveals that the concentration of phytagel and sucrose had a strong influence on root length, while the phytagel and pH of the medium had a strong effect on side branch emergence. Overall, taking into account the both responses, concentration of phytagel had a significant impact on hairy root production. The maximum average hairy root length obtained was 1.12 cm and number of side branches emerged were 6.78 per day. Based on these results, the optimal parameters were MSR medium with 3gL^{-1} phytagel, 11gL^{-1} sucrose and a pH of 4 for boosting hairy root development. This study is a cost-effective approach and minimizes the time taken for establishing the hairy root technology.

Keywords

Arbuscular mycorrhizal fungi (AMF); Hairy root production; Response surface methodology (RSM); Carrot root organ culture; *Rhizobium rhizogenes*

Introduction

The world population has been significantly expanding during the precedin g century and expected to increase in the near future. According to the United Nations, present world population is around 8 billion which is most likely to exceed by 2025 and by 2045 it will further move across 9 billion people. The demographic trends showed the population increase of 2 billion per year from 1998 to 2010 and 1 billion per year since 2010 (1). The world's ever- increasing population has exacerbated the overuse of

agrochemicals to nourish the mankind. Recent intensive agricultural operations like excess application of inorganic fertilizers and pesticides are harming the natural environment leading to loss of soil fertility and productivity. To overcome the threat to food security, usage of biofertilizers is a greener approach for increasing production without compromising the soil environment. Biofertilizers are expected to provide long-term benefits in promoting sustainable agriculture and minimizing the harmful effects of chemical inputs by reducing their need and improving the soil fertility. Biofertilizers are economically viable, ecologically sound, biologically feasible and widely recognized by farmers as a supplement to commercial chemical fertilizers (2).

These beneficial microorganisms once applied colonize the rhizosphere region or other plant parts during seed treatment or foliar application and stimulates the plant growth and development and reported to enhance the crop yield by 10-40 % (3). In addition to provide nutrients to the soil and plants, they also shield plants from insects and diseases (4). Biofertilizers are classified into several groups based on their mode of action and function. They include N fixers, P solubilizers, P mobilizers, K solubilizers, Zn solubilizers, Plant growth promoting bacteria (PGPR), Arbuscular Mycorrhizal Fungi (AM) and algal biofertilizers. Among all, AM fungal biofertilizers are of greater importance as their application has numerous benefits and also minimizes the use of chemical fertilizers up to 50 %, though this calculation is dependent on the variety of plant species and strain of arbuscular mycorrhizal fungi (AMF) used (5).

AM biofertilizers have been reported to increase 12-15 % of the yield in various crops (6) as it enhances the uptake of phosphorous and several other nutrients like Fe, Mg, S, Cu, Ca, Zn, Mn, etc., along with water and minerals. Fungal hyphae get attached to the plant root and act as water channel, resulting in a twofold increase in the acquisition of less mobile nutrients (7). In addition, it also enhances the plant resistance to various biotic and abiotic stressors and improves the soil physiochemical properties via various mechanisms. Though AMF application has enormous benefits to plants the major drawback is that the production process is highly complicated.

As AMF is an obligate symbiont it requires a live host for its propagation. Conventionally, AMF is propagated by inoculating it in cereal crop mostly maize or sorghum using a sterile carrier likes vermiculite and allowing it to grow for a period of 50 to 60 days. After which the colonized root along with the vermiculite are directly used as inoculum. The major drawback of this system is that there are more chances of contamination, time consuming and low yield of the spores or infective propagules. To address this, scientists have devised a variety of strategies for large-scale AMF production, including aeroponic, hydroponic, bioreactors and Root-organ culture (ROC) methods. However, ROC propagation technique has been found to be the most effective method for large-scale AM multiplication in a short period of time and space (8). *In vitro* AMF propagation through ROC i.e. Monoxenic AM production method is highly efficient as it multiplies AM spore in dual culture plates along with Ri-transformed hairy roots (9). In this method, *Rhizobium rhizogenes* mediated transformation is brought out in any suitable explant and the hairy roots (HR) are developed. The developed HR cultures are multiplied and the AMF of interest is cocultured with these HR lines for infection. Once the AMF infects the hairy root it starts to multiply in the Petriplates leaving behind an enormous count of visible AMF spores. Hence using this technique, contamination free AMF spores and hyphae can be prepared in higher concentration and comparatively lesser time. The major lacuna of this system is that the technique needs further optimization to increase the productivity of AMF propagation. The major step to be standardized is the hairy root multiplication process. If we are able to maximize the HR production process and minimize the time needed for HR multiplication, this technology will be more feasible for large scale production. Although there are numerous publications emphasizing the use of MSR media for HR multiplication, there are no studies focused at optimizing the influencing factors in the media. Hence through this study we have aimed in optimizing the key influencing factors to increase the hairy root multiplication. The present study uses response surface methodology (RSM), a mathematical and statistically tool to standardize the key influencing factors for hairy root multiplication. The key factors influencing the HR multiplication are concentration of the gelling agent (phytagel), pH of the media and the concentration of the carbon source used (sucrose).

Materials and Methods

Biological materials

Rhizobium rhizogenes strain MTCC 2364 obtained from culture collection centre of Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, Tamil Nadu, India was used in this study. The bacterial culture was revived in yeast extract mannitol broth and incubated at 27 °C on rotary shaker at 100 rpm for 24 h. For this study, carrot (*Daucus carota*) was taken as explant for root induction. Disease free and fully matured medium sized carrots were collected directly from the fields of lower hills of Kodaikanal, Tamil Nadu, India and used on the same day of collection.

Explant preparation and hairy root induction

Carrots were washed, scrapped and surface sterilized using 0.1 % $HgCl_2$. Carrots were then flame sterilized using ethanol. The carrots were horizontally sliced into several discs each of approximately 2 cm thickness.

Using sterile needle, the carrot discs were pricked at 10 to 15 spots. Freshly grown 24 h old culture of *R. rhizogenes* (OD 0.5, A_{600}) was spread over carrot disc surfaces. Inoculated discs were carefully transferred to Petriplate containing half strength Murashige and Skoog (MS) medium () supplemented with acetosyringone (100 μM). The plates were placed in upright position under dark

condition at 27 °C for root induction. After 1 week the carrots were transferred to Petriplate containing half strength MS medium supplemented with cefotaxime (500 ppm). The antibiotic concentration was gradually reduced from 500 ppm to 100 ppm in subsequent weeks till bacteria free hairy root cultures were obtained.

Hairy root cultivation

Once hairy roots of 5 to 6 cm length were induced from the callus, it is detached using sterile forceps. The detached root bits were transferred to Petriplates containing MSR medium devoid of antibiotic and hormones (10). In each Petriplate, 2 hairy root bits were placed in opposite direction to enhance the growth and easy proliferation in the plates. The inoculated plates were inverted and incubated under dark condition at 27 °C for further root multiplication (Fig. 1).

Optimization of culture media for enhanced root multiplication

The concentration of the gelling agent (phytagel) and sucrose along with the pH of the growth medium were considered to highly influence the process of hairy root multiplication. Earlier studies suggest that the gelling agent, carbon source and pH of the medium are the influential factors of hairy root production. These three factors have interaction effect on the strength of the medium, nutritional need and proliferation of the hairy roots and hence selected for this study. The growth rate of hairy roots and induction of side branches highly depend on the foresaid factors. Hence, in this study with the aspect of fastening the root multiplication process the 3 key influencing factors in culture media were standardized. Three levels of each factor i.e. the concentration of the phytagel (3 to 5 gL^{-1}), the pH of the growth media (4 to 6) and concentration of sucrose (7.5 to 12.5 gL^{-1}) were taken for optimization using RSM.

Experimental design

Response surface methodology is a mathematical and statistical tool applied to optimize various independent factors of the process by predicting the response of the experiment using the developed regression equation. The factors that highly influence the output responses are identified by analysing the analysis of variance (ANOVA). This technique is highly advantageous as it predicts the output of the process before hand and provides an experimental design with a minimal combination of factors under various levels. Hence, it avoids the need to carry out a laborious experiment involving all the combination of factors which is usually done in process optimization experiments. The major advantage includes limited number of experiments thereby minimizing the amount of time, resources and cost. In addition, it allows effective detection of the relationship between independent variables (11).

The experiment was designed following Box Behnken design (BBD) using Design-Expert software 10.0 (Stat-Ease, Inc., USA) (12). In this study, three independent variables, concentration of the gelling agent *i.e.,* phytagel (gL^{-1}), pH of the medium and the concentration of the carbon source *i.e.,* sucrose (gL^{-1}) were taken for process optimization. The average hairy root length per day and average number of side branches emerged per day were taken as output responses.

With these three factors and by using the following second order polynomial regression equation the outcome of each experiment was predicted.

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

Where Y_i is the outcome variable (average root length and average side branch); x represents the independent variable (concentration of the phytagel, the sucrose and the pH of the growth media); β_0 is the constant term; β_i, β_{ii}, β_{ij} are regression coefficients of the linear, second order polynomial and interaction parameters respectively and k is the number of covariance. The regression coefficients are calculated using the ordinary least-squares method. Totally in this study, 17 experiments were designed using 3 independent variables by following BBD as in Table 2.

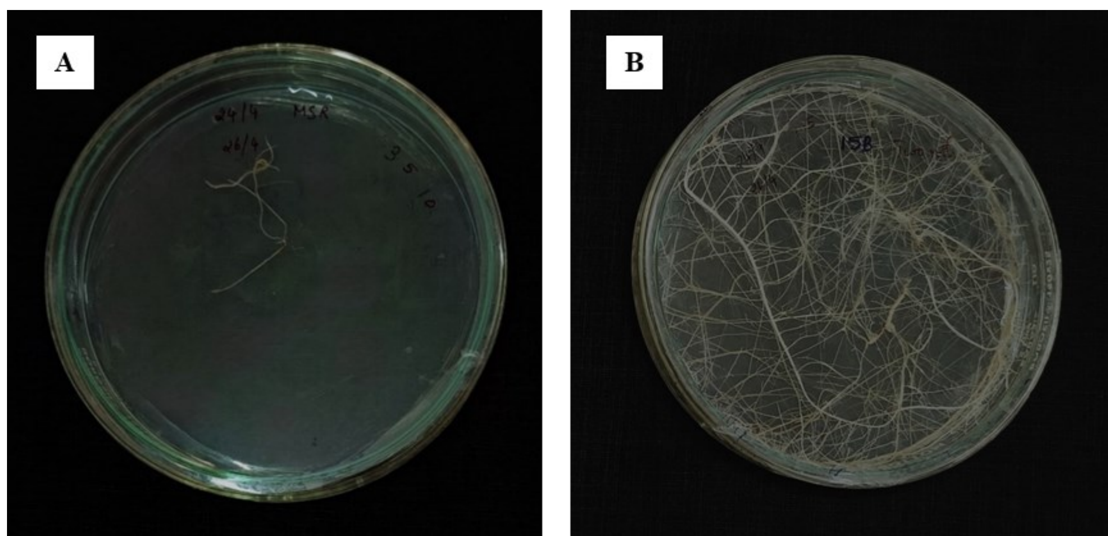

Fig. 1. Hairy root multiplication in optimized MSR medium. A: Hairy roots placed at 0 day; B: Hairy roots produced at 30th day.

Results and Discussion

The mycorrhizal inoculant market has grown dramatically over the last 2 decades, with AMF dominating the industry. The need for AM inoculants, particularly in the field of agriculture and horticulture, has exceeded the availability status. To overcome this issue, it is critical to know about various mass cultivation strategies capable of producing a huge number of sterile AM inoculum at low cost (8). The best method to bridge this gap is the production of AMF through *in vitro* ROC technique.

Carrots have been reported as an excellent host for monoxenic AMF production **(**13). Even though carrot has been used for monoxenic AMF production since 1996 (14), there are very minimum reports on optimization of growth conditions for increased hairy root production.

It had studied the standardization process for monoxenic AMF production wherein they had optimized the type of culture media to be used (MS medium, modified white and minimal medium) along with the incubation time (28, 42 and 72 h) and at both light and dark incubation conditions using 2 *R. rhizogenes* strains (532 and 2364) in 2 different explants (cowpea and tomato) (15). Upon infection in both the explants under modified white media, the strain 532 performed well with incubation period of 48 h under dark condition and produced numerous fine hairy roots. Thus, confirming that the source of explant, strain used, along with the growth media and conditions are highly influencing the growth and development of hairy roots.

In the present study, carrot (*Daucus carota*) was selected as the explant and MSR as the medium for root multiplication based on our previous optimization experiments (Data yet to be published). Some of the pioneer works used carrot as suitable explant (16, 17) while it was designed MSR medium and proposed its significance in monoxenic culturing of AMF (9) from then several researchers used carrot and MSR medium for *in vitro* culturing of AMF (14, 18-21). Even though there are many works suggesting the use of carrot and MSR medium for monoxenic culturing of AMF these protocols lack the optimization of key influencing factors in the media for maximized HR yield. The key influencing factors optimized in this study were the concentration of gelling agent (phytagel), pH of the medium and concentration of sucrose. In this study, freshly harvested fully matured carrots were taken as explant for HR production as reported, who compared 3 sources of carrot *viz*., matured cool stored, matured fresh and spring carrots as explant for HR production. The results of his study revealed that fresh source of mature carrots produced higher hairy roots than the other treatments showing the significance of using fresh carrots for study (22).

Gelling agents are one of the influencing factors in HR production technology. The strength of the media plays a crucial role in root penetration and multiplication process and it depends on the concentration of the gelling agent added. Increase in the concentration of the gelling agent increases the strength of the medium (23). In this study, concentration of phytagel was standardized using RSM, results revealed that 3 gL^{-1} concentration of phytagel was highly effective in maximizing the HR production. This may be because at lower concentrations of gelling agents the strength of the medium will be lesser making it easier for the hairy roots to penetrate and proliferate. The mechanical properties of the growth medium were particularly important for successful plant tissue culture. Phytagel is a complicated fluid, thus precise evaluation of its rheological qualities is essential for a correct application in plant or tissue culture (24).

These results were in contrary with a study in which they had reported that gellan gum performed well than phytagel in HR production and spore multiplication process. They also reported that phytagel forms a transparent media with poor gel strength, eventually leading to the formation of cracks during handling (8). Various gelling agents have been demonstrated to influence plant growth *in vitro*, depending on the type, manufacturer and concentration used. The use of gellan gum and phytagel in ROC technology instead of bacto-agar as reported by the original authors Becard and Fortin, was brought about mainly due to monetary constraints (16). Both phytagel and gellan gum are ten times less expensive than bacto-agar (25). Apparently, there are no studies on the influence of various concentrations of phytagel on hairy root production.

The next crucial factor to concentrate is the pH of the culture medium. Tissue culture medium usually requires adjustment to pH levels ranging from 5.5 to 5.9 before to autoclaving because lower pH levels during autoclaving prevent the gelling agent from solidifying (26). The availability of hydrogen ions in acidic medium solution is also influenced by the buffering abilities of the nutrient components (27). Furthermore, carbon sources, carbohydrate concentration and gelling agents all contribute to the amount of sucrose hydrolysis and pH of the medium following autoclaving. As a result, the medium with a lower original pH may rise higher, while the medium with a higher original pH may become lower in order to achieve solution equilibrium.

Hence optimizing the initial pH of the media is very much important to maintain the appropriate pH for hairy root growth. The pH of the medium is critical, particularly in liquid *in vitro* systems, where pH has been found to influence nutrient intake such as nitrogen ultimately influencing the plant growth rates (28). In plant tissue culture, lowering the pH of culture media to less than 4.5 has been found to alter tissue morphogenesis (29) and decrease bacterial contamination (30).

Previous work on optimization of the hairy root production in different explants using varying pH levels (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 6.5) revealed that higher root production of 12.1 gL^{-1} and 11.52 gL^{-1} dry weight was achieved at pH 5.8 and 6.0 respectively in *Withania somnifera* (31) and *Gymnema sylvestre* (32). Similarly, another experiment investigated the influence of pH (4, 5, 6, 7, 8) on hairy root formation in *Panax ginseng*. The pH range of 6.0 to 6.5 was shown to be ideal, resulting in a higher

growth rate (7.44) and increased ginsenoside production (239.68 mgL^{-1}) (33). However, these results contradicted that pH levels (5.0, 5.5 and 5.9) have no effect on the growth of hairy root cultures produced from *Trigonella foenumgraecum* in WP 3 liquid medium (34). This could be due to the components present in the media as the media composition has a greater influence on the pH. This study is noteworthy since it is the first to examine the impact of varying pH levels on the formation of hairy roots in AMF cultivation, especially when MSR medium is used. In this investigation, the optimum pH for enhanced hairy root production using MSR medium was found to be 4.0. This pH could be optimum to buffer the medium and provide the necessary culture conditions for hairy root production even after autoclaving. Additionally, most of the AMF strains prefer low pH for sporulation hence this could be optimal for AM spore as well as hairy roots production.

The third key parameter to consider is the sucrose concentration in the medium. Carbohydrates should be added to the culture media as they provide energy necessary for the growth and development of root cultures under laboratory conditions (35). Concentration of sucrose in the culture media has been shown to greatly influence the growth and development of transformed roots (36). When comparing the impact of various carbohydrate sources on the development of hairy roots, sucrose was shown to be the best (37-40). There are several studies employing different sucrose concentrations for inducing hairy roots. Hence, experiment to optimize the sucrose concentration is of greater importance. In our study, sucrose (11.0 gL^{-1}) in MSR medium was shown to boost the growth rate and side branch emergence of hairy roots in carrots.

Previous studies have also highlighted the significance of optimizing the sucrose concentration for hairy root production. Earlier an investigation on optimizing the sucrose concentration (10, 20, 30, 40 and 50 gL^{-1}) for the growth and synthesis of pyranocoumarins from an *Angelica gigas* hairy root culture showed that optimum concentration of sucrose as 40 gL^{-1} (41). This work is also supported by another study wherein the optimum sucrose concentration for higher hairy root production in *Bupleurum falcatum* was observed as 40 gL^{-1} and further increase in sucrose concentrations caused necrosis and inhibited lateral root growth (42). Hinderance to lateral root growth due to increasing sugar concentrations was also noted in untransformed root cultures of *B. falcatum* (43). Highest number of hairy roots as well as the highest root rate, fresh weight and root length were obtained from quinoa explants when the MS medium was supplemented with 30 gL^{-1} sucrose and a significant decrease in root growth was noticed at lower concentration (44). It was standardized 3 % sucrose in MS medium for higher hairy root production and high biomass accumulation (11.92 gL^{-1} dry weight) in *Withania somnifera* than the other levels of sucrose (1 %, 2 %, 4 %, 5 %, 6 % and 8 %) (31).

All these above studies are aimed to produce HR for metabolite production and hence higher sucrose concentration has been used and optimized. Wherein in our case we intended produce HR that can survive for a longer period of time without browning or necrosis as AMF spores require longer time for colonization. Accordingly, without compromising the growth as well the quality of the roots the sucrose range has been fixed from 7.5 to 12.5 gL^{-1} for this optimization experiment. Among this range we obtained the best results with 11.0 gL^{-1} of sucrose.

Statistical optimization of hairy root production through RSM

The predicted second order equation with coded factors generated by BBD design with average root length as a response variable for optimizing the process of HR multiplication is as follows:

Average root length = 3.13018 - 0.997587 A - 0.693118 B + 0.235856 C + 0.029692 AB - 0.0706856 AC-0.00311538 BC + 0.171753 A^2 + 0.0583614 B^2 + 0.00634499 C^2-----------(Eqn. 1)

Wherein the polynomial equation generated with coded factors for optimizing the process of HR multiplication with average side branches as response variable is as follows,

Average side branched = 14.2975 - 4.07503 A + 1.51181 B - 1.49584 C + 0.0808333 AB - 0.819111 AC - 0.394 BC + 1.39658 A^2 + 0.279361 B^2 + 0.340387 C^2------------------(Eqn. 2)

In both the equations (Eqn. 1) and (Eqn. 2), A is the concentration of phytagel, B is the pH of the media, C is the concentration of the sucrose, AB, BC, AC are the interaction variables of the former; A^2, B^2, C^2 are squared values of the independent variables.

This analysis shows the effects of different concentrations of phytagel (A in gL^{-1}), pH levels (B), and sucrose concentrations (C in gL^{-1}) on root length (RL in cm) and emergence of side branches (SB) in hairy root cultures. List of the independent variables with their varying levels selected for optimization in this study are given in the Table 1 and the experimental design generated using BBD along with their predicted and experimented values are reported in the Table 2. The ANOVA table of polynomial regression shows that the model fits the data well for optimizing the hairy root multiplication (Table 3 and 4). All the independent variables in this model exhibits synergistic effect on outcome. The ANOVA Table 3 shows that the most significant factor affecting the RL is the concentration of phytagel (A), which has the highest mean square value (0.2649), along with high F-value (218.39) and significant p-value (<0.0001), indicating the significance of this factor on root length (RL). Similarly, sucrose concentration (C) is found to be another critical factor with a second highest mean square (0.2076) along with F-value of 171.17, and p-value <0.0001, depicting that it has substantial effect on the

Table 1. List of independent variables and their levels used for optimization of hairy root multiplication.

Independent variable	Symbol	Coded values		
		-1	0	+1
Concentration of phytagel (gL^{-1})	X_1	3	4	5
pH of the medium	X_2	4	5	6
Concentration of sucrose (gL^{-1})	X_3	7.5	10	12.5

Table 2. Predicted and experimental values of average root length and emergence of side branches in hairy root multiplication.

Run	Independent variables			Response values			
				Average root length (cm/day)		Average number of side branches emerged (per day)	
	A: Gelling agent (gL^{-1})	B: pH	C: Sucrose (gL^{-1})	Experimented value	Predicted Value	Experimented value	Predicted Value
1	4	4	12.5	0.798	0.774	6.140	5.980
2	3	5	12.5	1.200	1.220	9.180	9.540
3	5	5	12.5	0.475	0.499	3.989	4.070
4	4	6	7.5	0.383	0.408	6.880	7.040
5	4	4	7.5	0.421	0.436	3.400	3.690
6	3	6	10.0	0.846	0.846	6.167	6.090
7	5	6	10.0	0.549	0.541	4.667	4.870
8	3	4	10.0	0.940	0.949	5.083	4.880
9	4	5	10.0	0.520	0.485	2.980	3.120
10	4	6	12.5	0.730	0.715	5.680	5.390
11	4	5	10.0	0.484	0.485	3.500	3.120
12	4	5	10.0	0.507	0.485	3.400	3.120
13	5	5	7.5	0.546	0.530	8.200	7.840
14	3	5	7.5	0.564	0.541	5.200	5.120
15	5	4	10.0	0.525	0.525	3.260	3.340
16	4	5	10.0	0.428	0.485	2.211	3.120
17	4	5	10.0	0.486	0.485	3.500	3.120

Table 3. ANOVA table depicting the effects of the independent variables on the average root length.

Source	Sum of Squares	df	Mean square	F-value	p-value	
Model	0.7600	9	0.0844	69.63	< 0.0001	Significant
A-Gelling agent	0.2649	1	0.2649	218.39	< 0.0001	
B-pH	0.0038	1	0.0038	3.16	0.1187	
C-Sucrose	0.2076	1	0.2076	171.17	< 0.0001	
AB	0.0035	1	0.0035	2.91	0.1319	
AC	0.1249	1	0.1249	102.99	< 0.0001	
BC	0.0002	1	0.0002	0.20	0.6682	
A^2	0.1242	1	0.1242	102.41	< 0.0001	
B^2	0.0143	1	0.0143	11.82	0.0109	
C^2	0.0066	1	0.0066	5.46	0.0521	
Residual	0.0085	7	0.0012			
Lack of Fit	0.0035	3	0.0013	0.93	0.5041	not significant
Pure Error	0.0050	4	0.0010			
Cor Total	0.7685	16				

RL. The interaction between the phytagel (A) and sucrose (C) also exhibits a notable mean square value of 0.1249, F-value (102.99) and a significant p-value (<0.0001), indicating that these two factors together also have a greater influence on the outcome. Wherein the factor pH (B) alone has a low mean square (0.0038) and the p-value is not significant (0.1187) making it a less significant factor. Additionally, the second order term for the phytagel (A^2) presents a mean square of 0.1242, F-value of 102.41, and a p-value of less than 0.0001, showing a significant non-linear effect on response. Whereas, the lowest mean square value (0.0002) is noted in the interaction term (BC), with F-value of 0.20 and a non-significant p-value of 0.6682, indicating it as the least most significant variable. Overall, the maximum response of average root length was seen in coefficient of phytagel (A) and sucrose (C) wherein pH (B), AB, BC, B^2 and C^2 turned out to be a least significant factors or non-significant factors.

The ANOVA (Table 4) depicts the effects of phytagel concentration (A), pH (B) and sucrose concentration (C) on the emergence of side branches (SB). Phytagel concentration as well as pH of the media exhibited the highest mean square value of 3.8 with F value of 14.78 (A) and 14.76 (B) along with significant p-values. Here in this response, the concentration of sucrose (C) exhibited a low mean square value of 0.2141 with F-value (0.84) and p-value (0.39) thereby having a least significant effect on SB. But the second order term of sucrose (C^2) reported the highest mean square value 19.06 with the F-value (74.10) and a significant p-value (<0.0001), depicting the importance of this factor over the response variable. The interaction between the phytagel and sucrose (AC) showed significant effect than the other interaction variable with a mean square of 16.77, F-value (65.22) and a significant p-value (<0.0001) suggesting a strong combined effect of these two factors. The higher order of the phytagel factor (A^2) also exhibited a higher mean square value of 8.21, indicating a significant non-linear impact over response. In contrast, the lowest mean square value is for the interaction between phytagel and pH (AB), with mean square value of 0.0261 and a non-significant F-value (0.10) and p-value (0.7592), reporting it as the least effective factor on the SB. The coefficient of mean square terms in emergence of side branches (SB) shows the maximum response for phytagel (A) and pH (B) with highest mean square values while the C, B^2 and BC showed least effect over response.

Table 4. ANOVA table depicting the effects of the independent variables on the emergence of side branches.

Source	Sum of Squares	df	Mean square	F-value	p-value	
Model	58.11	9	6.4600	25.11	0.0002	Significant
A-Gelling agent	3.80	1	3.8000	14.78	0.0063	
B-pH	3.80	1	3.8000	14.76	0.0064	
C-Sucrose	0.21	1	0.2141	0.84	0.3918	
AB	0.03	1	0.0261	0.10	0.7592	
AC	16.77	1	16.7700	65.22	< 0.0001	
BC	3.88	1	3.8800	15.09	0.0060	
A^2	8.21	1	8.2100	31.93	0.0008	
B^2	0.33	1	0.3286	1.28	0.2956	
C^2	19.06	1	19.0600	74.10	< 0.0001	
Residual	1.80	7	0.2572			
Lack of Fit	0.59	3	0.1958	0.65	0.6252	not significant
Pure Error	1.21	4	0.3032			
Cor Total	59.91	16				

The F value of RL is 69 and SB is 25 along with significant p-value (0.0001) shown in the Table 3 and Table 4 indicating the overall significance of the variables selected for models with least noise. The maximum significant F value (218.39 and 14.78) was observed with concentration of phytagel (A) for both RL and SB indicating that the concentration of the gelling agent as the most influencing factor for optimizing the root multiplication. Sucrose concentration (171.17) was found to be most influencing factor for RL while pH (14.76) was found to be highly important in case of response SB.

As the p-value of the variables A, C, AC, A^2, B^2 are less than 0.0001 they are significant for RL similarly in case of SB significant variables are A, B, AC, BC, A^2, C^2. The R^2 value represents the goodness of fit in the model, which explains to what extent the independent variable influences the dependent variable. From the Table 5, the R^2 value of 0.989 for root length (RL) is found to be consistent with adjusted R^2 value of 0.975. Similarly, in case of side branch (SB) R^2 value of 0.970 is found to be consistent with adjusted R^2 value of 0.931. There is also consistency found between the predicted R^2 (0.917) and adjusted R^2 (0.975) of RL. Also, the predicted R^2 of SB (0.812) was found to be consistent with adjusted R^2 (0.931).

RSM generates hyperbolic response surface graphs which exhibits the interactions between the different factors against the response variable. Since the interactions between the factors are non linear hyperbolic graphs are obtained indicating that the coordinates in the peak represent a maximum response wherein the coordinates in the valley represent the minimum response. The correlation of variable factors with each other in optimizing the root multiplication process using average root length and average of side branch emergence as responses was given in Fig. 2 and Fig. 3 respectively. Fig. 2A, 2B, 2C are displaying hyperbolic surface graphs in 3D of pH vs. gelling agent; sucrose vs. gelling agent; sucrose vs. pH respectively. Fig. 3A, 3B, 3C are displaying hyperbolic surface graphs in 3D of pH vs. gelling agent; sucrose vs. gelling agent; sucrose vs. pH respectively.

Table 5. Goodness of fit for average root length and average side branches emerged.

Variables	Average root length (cm/day)	Average side branches emerged (No./day)
R^2	0.989	0.970
Adjusted R^2	0.975	0.931
Predicted R^2	0.917	0.812

The root length and emergence of side branch was found to be increased with increase in concentration of gelling agent (phytagel) upto $3gL^{-1}$, pH of 4 and sucrose of $11gL^{-1}$, the values less than the former result in decrease in root length and emergence of side branch. Under the above optimized conditions, the average root length observed was 1.12 cm per day and average side branch emerged was 6.78 per day.

Conclusion

Considering the importance and need for AMF biofertilizers, optimizing the *in vitro* production technique for higher yield is the need of the hour. One of the key limiting factors for *in vitro* AMF production is hairy root multiplication. In this research, three key influencing factors in HR production *i.e.,* concentration of the gelling agent (phytagel), pH of the MSR medium and the concentration of the carbon source (sucrose) were optimized using RSM. This optimized data will be useful for enhancing the production of hairy root which paves the way for multiplication of AM spore enormously.

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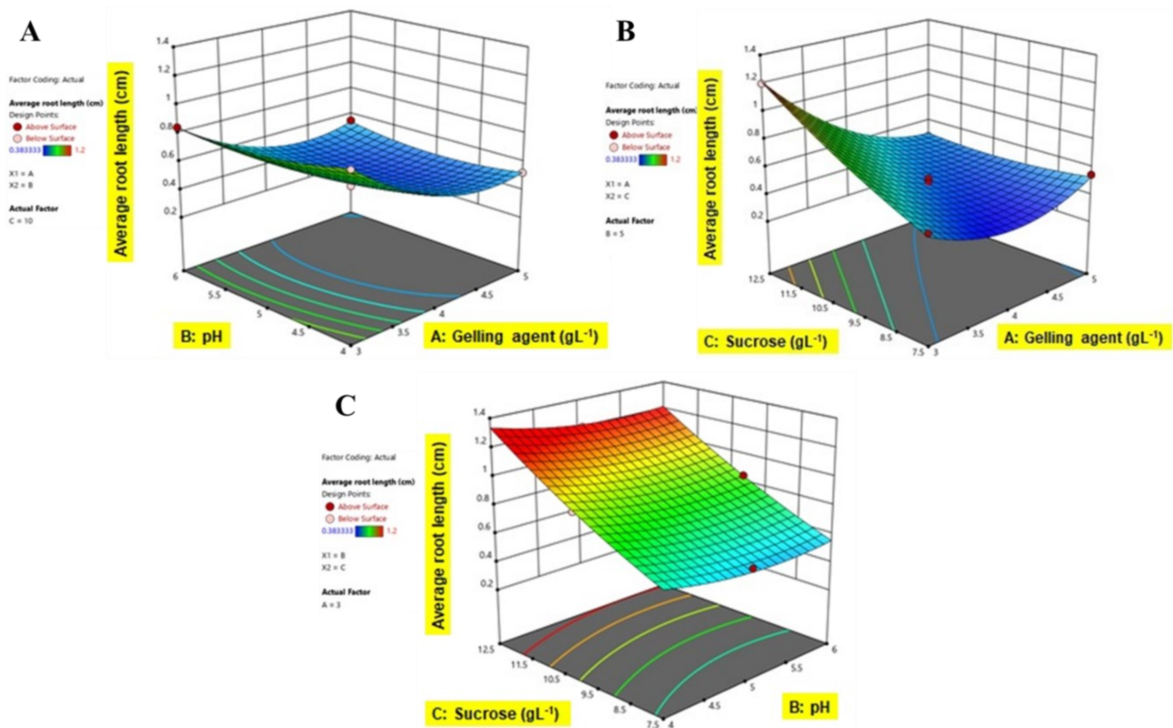

Fig. 2. Hyperbolic 3D surface graphs for the response factor average hairy root length; A. Effect of pH vs. phytagel B. Effect of sucrose vs. phytagel; C. Effect of sucrose vs. pH.

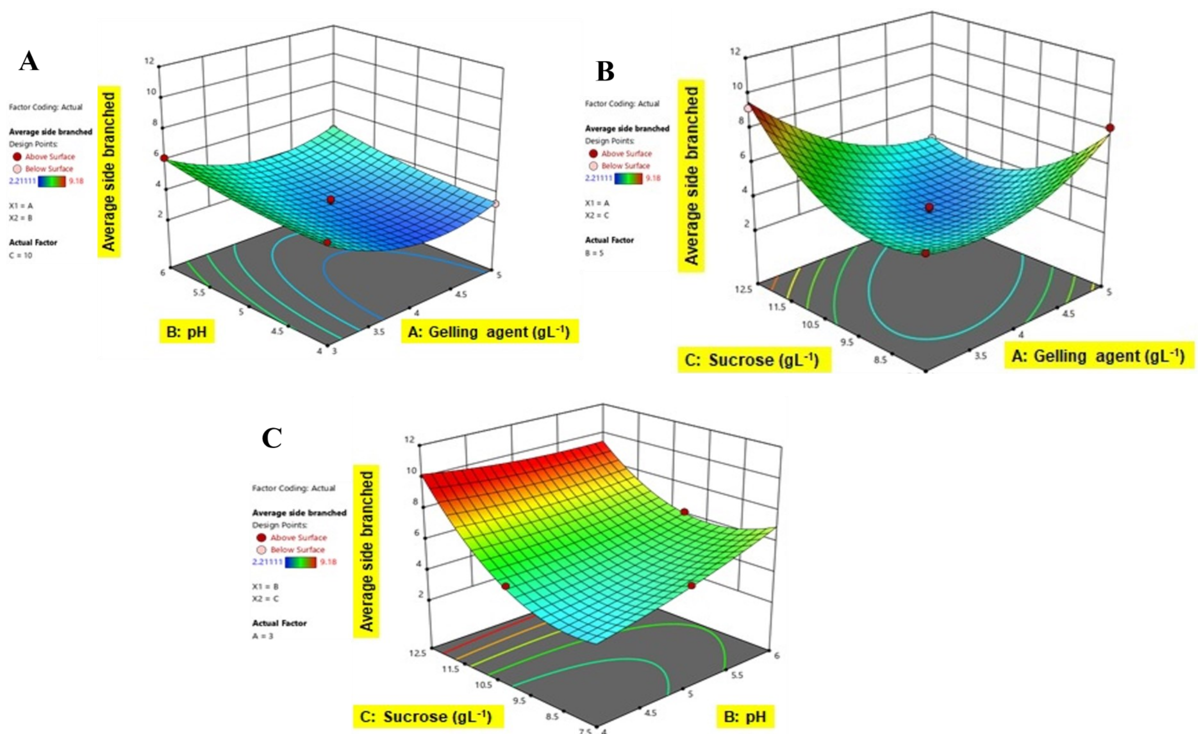

Fig. 3. Hyperbolic 3D surface graphs for the response factor average side branch emergence; A. Effect of pH vs. phytagel agent; B. Effect of sucrose vs. phytagel agent; C. Effect of sucrose vs. pH.

Authors' contributions

PVP carried out the experiment, took observations, analysed the data and writing of the original draft. KK guided the research by formulating the research concept and approved the final manuscript. RS participated in summarizing and revising the manuscript. RR participated in summarizing and revising the manuscript. TS participated in summarizing and revising the manuscript. PK participated in summarizing and revising the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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