Efficient plant regeneration through callus culture in Hedychium spicatum Buch.Ham. ex. D. Don using response surface methodology

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Abstract

Hedychium spicatum(Family-Zingiberaceae), commonly known as Kapoor Kachri is widely known for its medicinal properties and high market demand. The species is harvested mainly from the wild to meet the raw material requirements for the pharmaceutical and cosmaceutical industries; therefore, it needs urgent attention for its conservation and mass production. The present study developed an efficient *in vitro* propagation protocol for large-scale species production. The central composite design- response surface methodology (CCD-RSM) experiment was designed to optimize the plant growth regulators (PGRs) concentration for maximum callus production, shoot regeneration and rooting. Seed radicle was used as explants in Murashige and Skoog (MS) medium supplemented with different concentrations of naphthalene acetic acid (NAA; 2.5-5.0 µM) in combination with thidiazuron (TDZ; 2.5-5.0 µM) for callus induction. TDZ (5µM) with NAA (2.5µM) showed maximum callus induction (98%) after 6 weeks of incubation. Callus pieces were transferred to MS medium supplemented with different concentrations of TDZ, NAA and Indole-3-butyric acid (IBA) for shoot regeneration. The highest regeneration frequency (100 %) was observed on MS medium enriched with 2.5 µM TDZ and 3.5 µM NAA that showed a maximum number of shoots/explants (16.19 no.). Regenerated shoots were rooted better (average number of roots/shoot - 11.71) on MS medium supplemented with 2 µM NAA and 1.5 µM IBA in combination. After subsequent acclimatization and hardening process in the greenhouse, the plantlets were planted in the experimental field with a survival rate of 83% after 4 months. The protocol established in the present study has prospects to meet the challenges of quality planting material for large-scale cultivation and raw material sources for industrial utilization.

Introduction

The Himalayan Mountains are known for the richness and uniqueness of medicinal and aromatic plants (MAPs). A large number of MAPs of this region are used in traditional systems of medicine (Ayurveda, Siddha, and Unani, etc.). The congenial environment of the region supports about 1748 species of MAPs (Samant et al. 1998); however, Indian pharmaceutical industries utilize only 700 species, of which 50% are from the Indian Himalayan Region (Purohit 1997; Ved et al. 1998).

Among others, *Hedychium spicatum* (Zingiberaceae) is known for its medicinal importance. The species is distributed in Bhutan, Nepal, Myanmar, northern Thailand and some parts of China. In India, it is found in the temperate and subtropical range of the Himalayan region at an altitude of 1200–2800 m asl (Kritikar and Basu 1975; Wu and Raven 2000). The rhizomes of the species are known for analgesic, anti-inflammatory, anticancer, antioxidant, hypoglycemic, tranquillizing and many other biological activities (Rawat et al. 2011; Samuel and Tripathi 1994; Prakash et al. 2010; Joshi et al. 2008; Dixit and Varma 1975; Kaundal et al. 2015). Earlier studies on essential oil obtained from the rhizome revealed that 1,8-cineole (4.3–72%) is a marker compound of the species. Similarly, linalool, 10-epi-γ-eudesmol, α-terpineol, β-eudesmol and β-selinene have been reported in significant amounts in the essential oil of the rhizome (Mishra et al.2016; Raina and Negi 2015; Verma and Padalia 2010; Rawat et al.2018). Over the years, the species has gained popularity considering its market value; the estimated annual demand is around 400 tons annually and is increasing yearly (Sharma et al. 2008). Pharmaceutical industries receive significantly fewer amounts of raw material, and that is from the wild; therefore, the species is continuously depleting from its natural habitat. Under such

circumstances, refining or scaling up the existing propagation techniques and using high-end technologies to fulfill the raw material need is highly desirable.

Among others, plant tissue culture is considered a successful method for large-scale clonal propagation of plants. This technique requires less space, a limited amount of propagules (explants) and time compared to conventional methods. In addition, this technique contributes to the efficient and continuous production of active ingredients via callus and suspension culture (Petersen and Alfermann 2001). Thus, the method can be used for continuous, sustainable, economical and viable production of natural compounds, regardless of environmental conditions (Karuppusamy 2009). The *in vitro* propagation methods are available for the species (Badoni et al. 2010; Giri and Tamta, 2011; 2013); however, the propagation protocol of the species at the commercial level has yet to be developed.

Response surface methodology (RSM) is a mathematically approved method for designing experiments and statistical modeling to assess the relative significance and interactive effect between different variables for the optimal response. In this study a class of three level complete factorial designs (central composite design) was used to determine the optimization values of the operating variables. Although, the tissue culture medium and PGRs concentration for micropropagation and callus induction have been successfully optimized using RSM in different medicinal plant species. For instance, the media optimization study for shoot induction in *C. asiatica* using RSM suggested that this method helps to study parameters in concentration ranges, along with studying the interactive effect of two factors and makes the optimization process less time consuming and cost-effective with minimal use of resources (Katoch et al. 2022; Gururajan et al. 2021). The data from the CCD-RSM model appropriately defines the optimal conditions for *in vitro* plant regeneration via growth regulators in lettuce and other crops (Gomez et al. 2015). This method is useful for statistically designing experiments, developing models, evaluating the interactive effect of multiple independent variables, and determining the optimum concentration for a desirable response. The present study aims to identify the optimum concentration of PGRs for callus induction and plant regeneration in *H. spicatum* using *a* central composite design (CCD-RSM).

Materials and methods

Plant material and surface disinfection

The fully matured fruits of *Hedychium spicatum* were collected from Sri Narayan Ashram nursery (2550 m asl; 29˚59'01" N; 80˚38'06"E) District Pithoragarh, Uttarakhand, India. The fruits were brought to the G.B. Pant National Institute of Himalayan Environment (NIHE) laboratory, Kosi-Katarmal, Almora, Uttarakhand, India (1150 m asl; 29˚38'22.54" N; 79˚ 37' 24.87") in polythene bags. The seeds were separated from the fruit pulp, washed with distilled water, and air-dried for a week at room temperature (25 ± 2^{o}C). Seeds were stored at 4^{o}C before the onset of the experiment. For *in vitro* germination, seeds were washed with an aqueous solution of liquid detergent containing 0.2% (w/v) Tween-20 (HiMedia, India) for 20 min followed by distilled water (4 times). Then, the seeds were treated with 2% (w/v) Bavistin (BASF India Ltd), kept on a shaker for 30 min, and rinsed thoroughly with sterilized distilled water (4 times). Subsequently, seeds were treated with 0.1% (w/v) $HgCl_2$ solution containing two drops of Tween-20 for 5 min, and constant shaking was done under a laminar

airflow cabinet (Thermadyne, India). These seeds were finally washed 4–5 times with sterile distilled water to remove all traces of sterilant.

Medium Composition and culture condition

The culture medium consisted of Murashige and Skoog (MS; 1962) basal medium containing 3% sucrose (w/v), 0.1% myoinositol, 0.2% phytagel (w/v) with various concentrations and a combination of plant growth regulators [thidiazuron (TDZ); α-naphthalene acetic acid (NAA); indole-3-butyric acid (IBA)]. All chemicals were procured from Himedia, Mumbai (India). The pH of the medium was adjusted to 5.7 ± 0.1 with 1 N NaOH or 1 N HCl before autoclaving (1.05 kg/cm^2, 121˚C for 20 min). Cultures were incubated at 25 ± 1˚C in 16 h light and 8 h dark cycle, with irradiance (42 µmol /m^2/s) by cool fluorescent tubes (Philips T1 40 W/54, India).

In vitro seed germination

Seeds of *H. spicatum* were inoculated on ½ MS basal medium without PGRs in petriplates for germination. Each treatment considered 5 replicates (petriplates), and each replicate contained 10 seeds. After four weeks of culturing, the germination percentage (GP) was calculated using the following formula:

GP % = (No. of germinated seeds /Total no. of seeds sown) x100

Callus induction

The radicle of germinated seeds was used as an explant for callus induction. Small pieces of radicle were inoculated on MS medium supplemented with different concentrations of TDZ (0.5, 2.5, 5, 7.5 and 10 µM) in combination with NAA (0.5, 2.5, 5, 7.5 and 10 µM) in test tubes and incubated in culture room. The frequency of callus induction was recorded after 6 weeks of culture. The frequency of callus induction was calculated using the following formula:

$$\text{Frequency of callus induction (\%)} = \frac{\text{Number of explants induced callus}}{\text{Total number of explants inoculated}} \times 100$$

Shoots regeneration

For the regeneration of shoots, organogenic calli were inoculated on MS medium supplemented with different concentrations of TDZ (0.5, 1.5, 2.5, 3.5 5µM) in combination with NAA (0.5, 1.5, 2.5, 3.5 5 µM) and IBA (0.5, 1.5, 2.5, 3.5 5 µM). The culture medium without any PGRs was used as a control. Each treatment consisted of 5 replicates (Conical flask), and each replicate contained 3 calli (0.5 g each). The percentage of calli responses and the average no. of shoots per 1.5 g of calli were recorded after 28 days of culture and calculated using the following formula:

$$\text{Explants induced shoots (\%)} = \frac{\text{Number of culture induced shoots}}{\text{Total number of explants inoculated}} \text{ x } 100$$

Root induction and plantlet acclimatization

When regenerated shoots elongated to 3–4 cm, they were separated into single ones from clumps and inoculated on MS medium supplemented with different concentrations of auxins NAA (0.5, 1, 2, and 4 µM) and

IBA (0.5, 1, 2, and 4 µM). The culture medium without any PGRs was used as a control. The healthy plantlets with well-developed roots were removed from the medium, washed with tap water to remove agar, and transferred into small plastic cups containing sterile nursery soil. Pots were covered with transparent polythene bags with small perforations to maintain high humidity and placed in the culture room. After 4 weeks, polythene was removed, and plants were transferred to polybags containing soil and FYM (3:1 ratio) and kept inside the greenhouse for further hardening.

Experimental design and statistical analysis

MINITAB software 19.0 was used to design the experiments and interpret the data. In Response surface methodology (RSM), full factorial Central Composite Design (CCD) was used to optimize two independent factors viz. TDZ and NAA are used for callus induction, NAA and IBA are used for rooting, and TDZ, NAA and IBA are used for shoot regeneration. These factors were tested at lower (− 1), middle (0), and upper (1) levels. Upper and lower concentrations of these variables: TDZ (2.5–5.0 µM), NAA (2.5–5.0 µM) for callus induction, TDZ (1.5–3.5 µM), NAA (0–3.5 µM), IBA (0–3.5 µM) for shoot regeneration and NAA (1.0–3.0 µM), IBA (0–3.0 µM) for rooting were selected based on preliminary experimentation. There were 14 experimental runs for callus induction and rooting, whereas 20 runs for shoot regeneration were conducted to evaluate each variable's linear, square and interactive effects for the maximum callus formation, shoot regeneration and rooting.

The following second-order quadratic equation was used to calculate the response surface of shoot induction and rooting:

$$Yi = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB \ldots \text{(Eq. 1)}$$

Where Yi is the predicted response; A and B denotes independent variables; β_0 - constant response value; β_1 and β_2 - linear coefficients of variable A and B; β_{11} and β_{22} - quadratic coefficients and β_{12} - interaction coefficient of two variables.

The second-order quadratic equation for shoot regeneration was:

$$Yi = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} BC + \beta_{14} CA \ldots \text{(Eq. 2)}$$

Where Yi is the predicted response; A, B and C denote independent variables; β_0 denotes constant response value; β_1,β_2 and $\beta_3$depict linear coefficients of variables A, B and C; β_{11}, β_{22}, and β_{33} showed quadratic coefficients and β_{12}, β_{13}, and β_{14} represents interaction coefficient of three variables.

Regression Coefficient estimation and Analysis of Variance (ANOVA) for callus induction, shoot regeneration, and rooting were analyzed using coded units. A p-value of less than 0.001 was considered the most significant.

Results

In vitro seed germination

The disinfection treatment was efficient for 92% *in vitro* seed germination. The seeds started germination after 2 weeks of culture up to 4 weeks with 84.42% germination in ½ MS medium.

Model fitting and statistical analysis

The effect of optimizing independent variables (TDZ and NAA) on the responses (percentage of callus induction) was tested using CCD-RSM design at three levels (− 1, 0, +1) full factorial experiments with 14 experimental runs including 4 cube points, 3 centre points in a cube, 4 axial point and 3 centre points in axial. Second-order regression analysis (Eq. 1) was used to model the dataset obtained with the CCD design matrix. As shown in Table 1, the response values, experimental (10% to 100%) and predicted values (13 % to 95%) obtained from this experimental design infer that the callus induction was strongly influenced by the linear and quadratic models of TDZ and NAA.

From quadratic Eq. (1), the following response values were calculated for the frequency of callus induction:

$Y_1 = -153.8 + 62.7733\ (A) + 62.6400(B) -8.6187(A^2) -14.0587(B^2) + 6.40\ (AB)\ldots$ (2)

Where Y_1 (Frequency of callus induction %) represent the response coefficients of the TDZ (A) and NAA (B) variables. Response surface regression and analysis of variance (ANOVA) were studied to estimate the significance of the quadratic model (Eq. 2). The P- values were utilized as a tool to test the importance of each of the coefficients, which in turn are required to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant the corresponding coefficient. At a 95 % confidence level, P-value less than 0.001 were considered most significant, whereas P-value less than 0.05 were considered significant. The fit of the model was also expressed by the coefficient of determination (R^2).

Optimization of callus induction

The proposed models exhibited a high R^2 coefficient (97.6 %), indicating the model's adequacy. It suggests that it is a reliable model and that 97.6 % of the sample variation of callus induction is attributed to a high correlation between the independent variables. The model does not explain only 2.4 % of the total variation of callus induction. The relatively high-adjusted determination coefficient (R^2_{Adj} = 95.6%) accounts for a high significance of the model and indicates good agreement between the experimental and the predicted values. As shown in Table 2, the linear coefficient of TDZ (27.667) shows a greater impact on callus induction % in contrast to the NAA (-23.500).

The interaction effects of TDZ and NAA also represented the highest impact on callus induction % (10.00 %) (Table 2). In addition, the VIF values in Table 2 are equal to one for the linear and interactive effects of both model variables (A^2, B^2, and A*B), which indicates the factors had a non-correlated orthogonal relationship with other factors in the model. The square effect of both factors had a Variance Inflation Factor (VIF) value of 1.26, suggesting that it was moderately correlated with other factors. Thus, there was no case of multicollinearity in the data. These statistics signify that the fitted model was good because the model captures most of the variation. The ANOVA analysis, through the quadratic regression model, revealed that the linear and square effect of factor A (TDZ) and B (NAA) is highly significant, having a high model F-values (103.02 and 36, respectively). A very low probability value ($p<0.001$) as compared to interaction effects was

found to have a significant (F-value=10.42; $p < 0.05$) positive effect over the response variable (Table 3). The RSM-generated 3-D plots (Fig 1) were used to analyze the effect of interactions of the variables, and plots were generated with a response on the z-axis against two independent variables. The curvature effect of the response surface interaction plot of TDZ and NAA resulted in maximum callus induction (98.98 %) in response to the optimum concentrations for both the hormones (5 µM TDZ, 2.5 µM NAA) in MS medium.

Optimization of PGRs concentration for Shoot regeneration

The effect of optimizing independent variables (TDZ, NAA and IBA) on the responses (percentage of shoot induction and average no. of shoots) was tested using CCD-RSM design at three levels (− 1, 0, +1) full factorial experiments with 20 experimental runs including 8 cube points, 4 centre points in a cube, 6 axial point and 2 centre points in axial. Second-order regression analysis (Eq. 1) was used to model the dataset obtained with the CCD design matrix.

From quadratic Eq. (1), the following response values were calculated for the percentage induction of shoots and an average number of shoots per 1.5 g of callus:

Shoot induction %

$Y_2 = -71.39 + 97.33\,(A) + 22.33\,(B) + 4.28\,(C) - 19.64\,(A^2) - 3.95\,(B^2) - 0.57\,(C^2) + 2.85\,(AB) + 3.14(AC) - 6.19\,(BC)\ldots$ (Eq. 3)

Average no. of shoots

$Y_3 = -23.61 + 24.11\,(A) + 2.22\,(B) + 0.79\,(C) - 4.90\,(A^2) - 0.34\,(B^2) + 0.06\,(C^2) + 0.74\,(AB) + 0.42\,(AC) - 1.07\,(BC)\ldots$ (Eq. 4)

Where Y_2 (Shoot induction %) and Y_3 (average number of shoots per 1.5 gm of callus) represent response coefficients of the TDZ (A), NAA (B), IBA (C) variables.

Response surface regression and ANOVA were used to estimate the significance of the quadratic model (Eq. 3-4) using coded units. Regression analysis of the full quadratic model equation for percentage induction of shoots and average no. of shoots showed the high coefficient of determination (R^2) values (98.6 and 97.2 %, respectively), indicating that the present model is significant under a given experimental range. R^2_{Adj} values (96.7 and 93.3 %) also showed good agreement between the experimental and the predicted values (Table 5). In addition, a high R^2 pre-value (85.39% for shoot induction and 74.86 % for avg. no. of shoots) suggests an acceptable correlation between the observed and the predicted values. Central composite factor experimental design and experimental and predicted values are shown in Table 4. The terms A, A*B and A*C had VIF values of one, indicating that variables are not correlated and multicollinearity does not exist in the regression model. However, VIF values of linear effect (factor B and C (1.09), square effect (factor A*A=1.61, B*B=3.11 and C*C=3.11) and interactive effect (factor B*C=1.12) of all the variables of the model indicating that it was moderately correlated with other factors. Thus, the data had no multicollinearity, and the fitted model was significantly good (Table 5). The ANOVA analysis of the models indicated above revealed the relationship between the effects of independent variables on the percentage of induction of shoots and the average

number of shoots. The significance of each term was evaluated by the high Fisher's test (F model = mean square regression/ mean square residual) and the low probability value (P<0.05). The results showed that the linear, quadratic and interactive effects of TDZ, NAA and IBA were highly significant (P<0.001) with a 95% confidence level (Table 6). Hence, the model proved suitable for predicting shoot regeneration under experimental conditions.

The 3-D response surface plots (Fig 2-5) for predicting shoot induction percentage and average no. of shoots in response to the interactive effect of different concentrations of TDZ and NAA are depicted in Fig. 2-3. As shown in Fig. 2 and 3, 2.5 µM of TDZ and 3.5 µM of NAA in MS medium resulted in maximum shoot induction (100 %) and average no. of shoots per 1.5 g of callus (16.19).

Optimization of PGRs concentration for rooting

The two independent variables (NAA and IBA) were tested for optimization of rooting medium using CCD design at three levels (− 1, 0, +1) involving 14 experimental runs including 4 cube points, 3 centre points in a cube, 4 axial point and 3 centre points in axial. Second-order regression analysis (Eq. 1) was used to model the dataset obtained with the CCD design matrix. From quadratic Eq. (1), the following response values were calculated for the average number of roots per shoot:

$Y_4 = -8.6905 + 16.55\ (A) + 3.72\ (B) -3.55\ (A^2) -1.13\ (B^2) -0.60\ (AB)$… (5)

Where Y_4 (Avg. no of roots/shoot) represent response coefficients of the NAA (A) and IBA (B) variables.

Response surface regression analysis indicated that the proposed models exhibited a high R^2 coefficient (97.6 %) with a 95 % confidence level, indicating the model's adequacy. It also suggests that it is a reliable model and that 97.6 % of the sample variation of rooting is attributed to a high correlation between the independent variables. In addition, the high-adjusted determination coefficient (R^2_{Adj} = 95.5%) and high R^2 pre value (78.28%) account for a high significance of the model and indicated good agreement between the experimental and the predicted values (Table 8). CCD design, experimental and predicted values are shown in Table 7. The VIF values in Table 8 are equal to one for the linear and interactive effects of both model variables (A2, B2, and A*B), indicating that variables are not correlated and multicollinearity does not exist in the regression model. The square effect of both factors had a VIF value of 1.26, suggesting that it is moderately correlated with other factors. Thus, the data had no multicollinearity, and the fitted model was significantly good. The ANOVA analysis of the proposed model revealed that the linear and square effects of NAA and IBA were highly significant (F= 28.81 and 101.06, respectively; p<0.001) as compared to interaction effects which were found to have a significant (F= 8.11; p < 0.05) positive effect over the response variable (Table 9). The 3-D response surface interaction plot (Fig 6) of NAA and IBA showed the highest no. of rooting (11.71) in response to the optimal concentrations for both the PGRs (2 µM NAA, 1.5 µM IBA) in MS medium. The linear effect of optimal concentration of NAA (2 µM) also revealed the maximum no. of root/shoot (9.93), which was highly significant.

Hardening and field transfer of plantlets

After 4 weeks of acclimatization in the culture room, plants were transferred to polybags containing soil and FYM (3:1 ratio) and kept inside the greenhouse for hardening.After4 weeks of hardening, 91% of plants survived. Further, hardened plants were transferred to the experimental field with a survival rate of 83 % after 2 months.

Discussion

An attempt was made to optimize PGRs (TDZ, NAA and IBA) concentrations at three different levels for maximum callus induction, shoot regeneration and rooting using RSM, a collection of statistical and mathematical methods, with a central composite design in *H.spicatum*. This efficient method tests the effect of interactions among the variables with the minimum number of experiments. A high similarity was observed between the predicted and reflected accuracy and accountability of the RSM to optimize the PGRs concentration for the production of *in vitro* plants. From the coefficient regression analysis, the positive value of the regression coefficient always favours the optimization techniques (Kaur et al., 2020; Sharma et al., 2016). In general, the R^2adj and R^2 pre should be within approximately 20% of each other to be in reasonable agreement; otherwise, there may be a problem with the data or the model (Montgomery 2007). Significant interactions between the three variables were observed and analyzed from the 3D surface plots. Similar statistical methods have been used for optimizing PGRs concentration in different medicinal plant species (Bagherieh and Nezamdoost2016; Tandon et al. 2021). In most plant regeneration protocols, the composition of plant growth regulators is significantly different for both the callus induction and shoot regeneration (Gómez-Montes et al. 2015). We have used CCD-RSM full factorial experimental designing to optimize different independent PGRs concentrations for callus induction, shoot regeneration and rooting using only one cytokine (TDZ) and two auxins (NAA and IBA).

In general, *H. spicatum* is propagated through seed and rhizome; both processes have limitations. Seeds have poor germination rates, taking longer for germination, and propagation through rhizomes is season dependent. Plant tissue culture techniques (using various pathways) can provide sufficient plants for commercial use in various pharmaceutical industries (Bisht et al. 2012; Giri and Tamta 2012; Bisht et al. 2015; Bisht et al. 2017). Successful establishment of the explant in a culture medium depends on different factors, and the selection of suitable explants is important for maximum response. Young tissue explants are preferred as they respond highly to callus initiation and reduce microbial contamination (Nurwahyuni et al. 2020). Hence, we used explants from germinated seed radicles. In *Curcuma longa* L. (family-Zingibaraceae), the root explant was more suitable for callus induction, with an 85% frequency of callus formation compared to the leaf explant with 25% (Jie et al. 2019). From the preliminary experimentation data (not shown), the radicle explants of germinated seed were better at inducing callus and shoot regeneration than the shoot tip and rhizome bud explants. It was suggested that different cellular dedifferentiation capabilities between the various explants have different callusing potentials (Sujatha and Mukta, 1996).

Based on the literature, it is observed that multiple shoot regeneration was best on TDZ (1.0 mg L-1 TDZ) containing MS medium in *H.coronarium*. To promote rooting, regenerated shoots from TDZ containing media were transferred to the NAA supplemented medium and both PGRs act as potent regulators for *in vitro* propagation systems (Verma and Bansal 2014). We used a different combination of TDZ and NAA for the callus induction and shoot regeneration. As a result, the higher concentration of TDZ (5 µM) with a lower

concentration of NAA (2.5 µM) promotes callus induction. In contrast, a slightly higher concentration of NAA (3.5 µM) compared to TDZ (2.5 µM) promotes maximum shoot regeneration. TDZ induces the expression of specific genes and transcription regulatory sequences that are either directly responsible for callus formation or induce other auxins or cytokinins for callus induction (Galán-Ávila et al. 2020; Ali et al. 2022).The higher concentration of NAA (20 µM) showed a better response to induce direct embryogenesis from the seed radicle of the zygotic embryo (Giri and Tamta 2013).However, a commercial-scale propagation protocol has not been developed so far. Previous studies attempted to optimize PGRs concentration for micropropagation of *H.spicatum* through shoot tip and rhizome bud explants (Koul et al. 2005; Badoni et al. 2010). A previous study suggested that the lower concentration of TDZ was the most effective treatment in relation to the induction of high-frequency shoot multiplication, the number of shoots per explant and the average number of shoots in *H.spicatum*. (Giri and Tamta, 2011).They also suggested that *in-vitro* rooting was observed using a lower concentration of indole-3-butyric acid (2.5 µM).These findings suggest that statistical modeling using RSM provides more efficient experiments to determine complex interactions than the traditional studies using comparisons of existing PGRs concentrations in growth media or factorial experiments that require large amounts of plant materials. This optimization should be carried out for each plant examined, as it has been shown that the impact of PGRs is highly dependent on the nature of the plant. For the determination of PGRs associations and PGRs concentrations, the use of the factorial test design proved to be quite sufficient(Niedz and Evens 2016). Therefore, the success achieved with the increased production of callus and *in vitro* plantlets makes this protocol a suitable and potentially biotechnologically reliable strategy for the production of large number of plantlets.

Conclusion

In this study, the PGRs concentration in MS medium for callus induction, plant regeneration and rooting of *H.spicatum* was optimized. Further, the CCD-RSM experiment was designed to analyze each variable's linear model and quadratic model (including pair-wise and interactive effects) to study the maximum callus growth and plant production from seed radicle explants. From the results obtained, it can be concluded that different concentrations of TDZ and NAA in combination promote callus induction and shoot regeneration, while NAA alone promotes rooting. Our data may encourage the application of RSM in future tissue culture optimization in which an understanding of the complex interactions between plant growth regulators (PGRs) is sought. The present study may further be exploited for large-scale *in vitro* propagation and industrial utilization.

Declarations

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Author contributions VD performed all the experiments, data analysis, and interpretation, conceptualization and wrote the original and revised draft manuscript. IDB supervised tissue culture experiments, conceptualization, review, and correction of the manuscript. VP supervised data analysis, review, and correction of the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Data availability

Data are available with the authors and can be made available on a request basis.

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Tables

Table 1. Central composite design matrix of independent variables (TDZ and NAA) with corresponding experimental and predicted value for callus induction.

Run order	Treatments		Frequency of callus induction (%)	
	TDZ	NAA	Experimental	Prediction
1	2.5	2.5	68	63.6476
2	5	2.5	100	98.981
3	2.5	5	0	0
4	5	5	72	71.981
5	3.75	3.75	88	93.2476
6	3.75	3.75	91	93.2476
7	3.75	3.75	92	93.2476
8	2.5	3.75	33	40.7048
9	5	3.75	95	96.0381
10	3.75	2.5	78	83.3714
11	3.75	5	33	36.3714
12	3.75	3.75	88	81.8381
13	3.75	3.75	87	81.8381
14	3.75	3.75	88	81.8381

Table 2.Regression coefficient analysis for frequency of callus induction (%), using coded units

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	87.54	2.46	35.65	0.000	
Blocks 1	5.70	1.76	3.24	0.014	1.13
TDZ (A)	27.67	2.53	10.94	0.000	1.00
NAA (B)	-23.50	2.53	-9.29	0.000	1.00
TDZ*TDZ (A*A)	-13.47	3.75	-3.59	0.009	1.26
NAA*NAA (B*B)	-21.97	3.75	-5.86	0.001	1.26
TDZ*NAA (A*B)	10.00	3.10	3.23	0.014	1.00
R^2	97.63%				
R^2 (adj)	95.60%				
R^2(pre)	85.21%				

SE Coef- standard error of the coefficient.

VIF-Variance Inflation Factor

** Very significant, where $p < 0.001$.

* Significant, where $p < 0.05$.

Table 3 Summary of the ANOVA analysis for the models indicated in Eqs. 2.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	11074.6	1845.77	48.10	0.000
Blocks	1	402.0	402.02	10.48	0.014
Linear	2	7906.2	3953.08	103.02	0.000
TDZ	1	4592.7	4592.67	119.69	0.000
NAA	1	3313.5	3313.50	86.35	0.000
Square	2	2762.7	1381.33	36.00	0.000
TDZ*TDZ	1	494.6	494.59	12.89	0.009
NAA*NAA	1	1316.0	1316.00	34.30	0.001
2-Way Interaction	1	400.0	400.00	10.42	0.014
TDZ*NAA	1	400.0	400.00	10.42	0.014

DF-Degrees of Freedom

Table 4. Central composite design matrix of independent variables (TDZ, NAA and IBA) with corresponding experimental and predicted value for Shoot regeneration.

Run order	Treatments			Shoot induction %		Avg. No. of shoots	
	TDZ	NAA	IBA	Experimental	Prediction	Experimental	Prediction
1	1.5	0	0	33	33.958	1	1.3797
2	3.5	3.5	0	100	96.898	12.8	13.1951
3	3.5	0	3.5	82	78.565	10.1	9.4284
4	1.5	3.5	3.5	27	27.214	1	1.5068
5	2.5	1.75	1.75	90	92.683	12.4	12.145
6	2.5	1.75	1.75	90	92.683	12.5	12.145
7	3.5	0	0	27	27.158	1.3	0.7297
8	1.5	3.5	0	72	73.698	8	8.9451
9	1.5	0	3.5	52	53.365	7.5	7.3784
10	3.5	3.5	3.5	63	62.414	9.5	9.0568
11	2.5	1.75	1.75	90	87.683	12.5	12.295
12	2.5	1.75	1.75	88	87.683	11.9	12.295
13	1.5	1.75	1.75	62	57.765	7.6	5.89
14	3.5	1.75	1.75	70	76.965	7.9	9.19
15	2.5	0	1.75	68	66.511	9.4	9.6541
16	2.5	3.5	0	100	100	17.6	16.1963
17	2.5	1.75	0	88	86.511	12	12.2541
18	2.5	0	3.5	80	82.443	12.8	13.5296
19	2.5	1.75	1.75	90	87.013	12.4	12.443
20	2.5	1.75	1.75	88	87.013	11.9	12.443

Table 5. Regression coefficient analysis for Shoot induction (%) and avg. no. of shoots, using coded units

Term	Shoot induction %					Average no. of shoots				
	Coef	SE Coef	T-Value	P-Value	VIF	Coef	SE Coef	T-Value	P-Value	VIF
Constant	89.13	1.62	55.17	0.000		12.294	0.443	27.72	0.000	
Blocks 1	3.56	1.39	2.56	0.034	1.55	-0.149	0.382	-0.39	0.706	1.55
Blocks 2	-1.44	1.39	-1.04	0.329	1.55	0.001	0.382	0.00	0.999	1.55
TDZ (A)	9.60	1.31	7.33	0.000	1.00	1.650	0.360	4.59	0.002	1.00
NAA (B)	8.40	1.31	6.43	0.000	1.09	1.723	0.359	4.81	0.001	1.09
IBA (C)	-1.27	1.31	-0.97	0.360	1.09	0.390	0.359	1.09	0.308	1.09
TDZ*TDZ (A*A)	-19.65	2.35	-8.35	0.000	1.61	-4.903	0.646	-7.59	0.000	1.61
NAA*NAA (B*B)	-12.10	3.28	-3.69	0.006	3.11	-1.066	0.901	-1.18	0.271	3.11
IBA*IBA (C*C)	-1.77	3.28	-0.54	0.604	3.11	0.201	0.901	0.22	0.829	3.11
TDZ*NAA (A*B)	5.00	1.46	3.41	0.009	1.00	1.300	0.402	3.23	0.012	1.00
TDZ*IBA (A*C)	5.50	1.46	3.76	0.006	1.00	0.750	0.402	1.87	0.099	1.00
NAA*IBA (B*C)	-18.97	1.40	-13.52	0.000	1.12	-3.284	0.385	-8.52	0.000	1.12
R^2	98.62 %					97.16%				
R^2(adj)	96.72 %					93.26%				
R^2(pre)	85.39 %					74.86%				

SE Coef- standard error of the coefficient.

VIF-Variance Inflation Factor

** Very significant, where $p < 0.001$.

* Significant, where $p < 0.05$.

Table 6. Summary of the ANOVA analysis for the models indicated in Eqs. 3-4.

Source	Shoot induction %					Average no. of shoots				
	DF	Adj SS	Adj MS	F-Value	P-Value	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	9810.73	891.88	51.98	0.000	11	353.946	32.1769	24.89	0.000
Blocks	2	113.14	56.57	3.30	0.090	2	0.256	0.1282	0.10	0.907
Linear	3	1701.96	567.32	33.06	0.000	3	57.208	19.0692	14.75	0.001
TDZ	1	921.60	921.60	53.71	0.000	1	27.225	27.2250	21.06	0.002
NAA	1	708.82	708.82	41.31	0.000	1	29.857	29.8569	23.09	0.001
IBA	1	16.20	16.20	0.94	0.360	1	1.530	1.5297	1.18	0.308
Square	3	3473.13	1157.71	67.47	0.000	3	119.671	39.8905	30.85	0.000
TDZ*TDZ	1	1196.57	1196.57	69.74	0.000	1	74.515	74.5146	57.63	0.000
NAA*NAA	1	233.47	233.47	13.61	0.006	1	1.809	1.8091	1.40	0.271
IBA*IBA	1	5.00	5.00	0.29	0.604	1	0.064	0.0645	0.05	0.829
2-Way Interaction	3	3577.73	1192.58	69.50	0.000	3	111.985	37.3282	28.87	0.000
TDZ*NAA	1	200.00	200.00	11.66	0.009	1	13.520	13.5200	10.46	0.012
TDZ*IBA	1	242.00	242.00	14.10	0.006	1	4.500	4.5000	3.48	0.099
NAA*IBA	1	3135.73	3135.73	182.75	0.000	1	93.965	93.9646	72.67	0.000

DF-Degrees of Freedom

Table 7. Central composite design matrix of independent variables (NAA and IBA) with corresponding experimental and predicted value for rooting.

Run order	Treatments		Avg. no. of roots/shoot	
	NAA	IBA	Experimental	Prediction
1	1	0	4.2	4.581
2	3	0	9.2	9.281
3	1	3	4.1	3.7476
4	3	3	5.5	4.8476
5	2	1.5	11.6	11.7143
6	2	1.5	11.6	11.7143
7	2	1.5	11.4	11.7143
8	1	1.5	6.2	6.1714
9	3	1.5	8.5	9.0714
10	2	0	10.4	9.9381
11	2	3	6.3	7.3048
12	2	1.5	11.4	11.1714
13	2	1.5	11.6	11.1714
14	2	1.5	11.6	11.1714

Table 8 Regression coefficient analysis for average number of roots/ shoot (using coded units)

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	11.443	0.251	45.67	0.000	
Blocks 1	0.271	0.180	1.51	0.175	1.13
NAA (A)	1.450	0.258	5.62	0.001	1.00
IBA (B)	-1.317	0.258	-5.10	0.001	1.00
NAA*NAA (A*A)	-3.550	0.383	-9.28	0.000	1.26
IBA*IBA (B*B)	-2.550	0.383	-6.66	0.000	1.26
NAA*IBA (A*B)	-0.900	0.316	-2.85	0.025	1.00
R^2	97.56%				
R^2(adj)	95.48%				
R^2(pre)	78.28%				

SE Coef- standard error of the coefficient.

VIF-Variance Inflation Factor

** Very significant, where $p < 0.001$.

* Significant, where $p < 0.05$.

Table 9. Summary of the ANOVA analysis for the models indicated in Eqs. 5.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	112.032	18.6721	46.74	0.000
Blocks	1	0.910	0.9101	2.28	0.175
Linear	2	23.017	11.5083	28.81	0.000
NAA	1	12.615	12.6150	31.58	0.001
IBA	1	10.402	10.4017	26.04	0.001
Square	2	80.736	40.3679	101.06	0.000
NAA*NAA	1	34.370	34.3705	86.04	0.000
IBA*IBA	1	17.734	17.7341	44.40	0.000
2-Way Interaction	1	3.240	3.2400	8.11	0.025
NAA*IBA	1	3.240	3.2400	8.11	0.025

DF-Degrees of Freedom

Figures

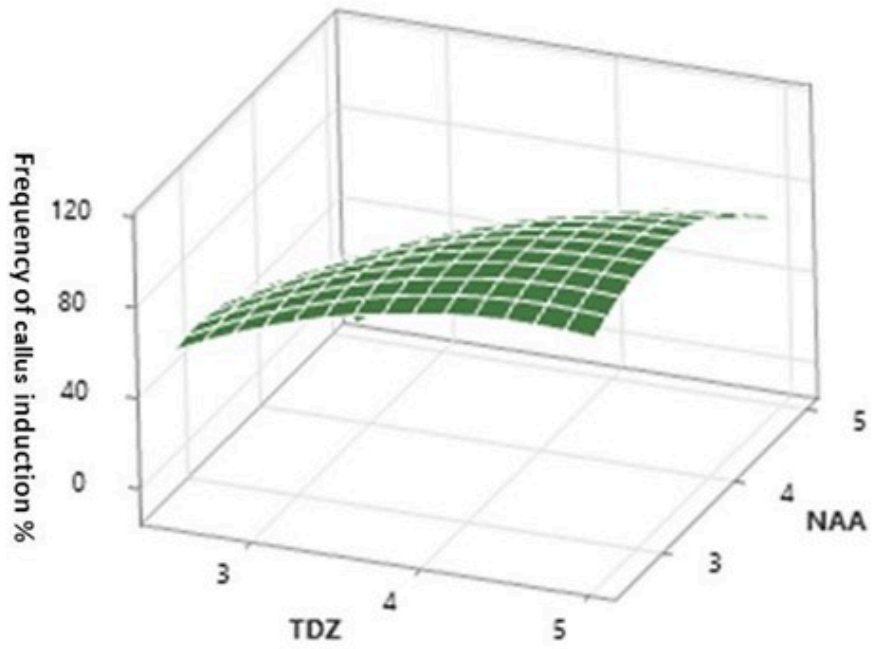

Figure 1

Surface Plot of Frequency of callus induction vs NAA, TDZ

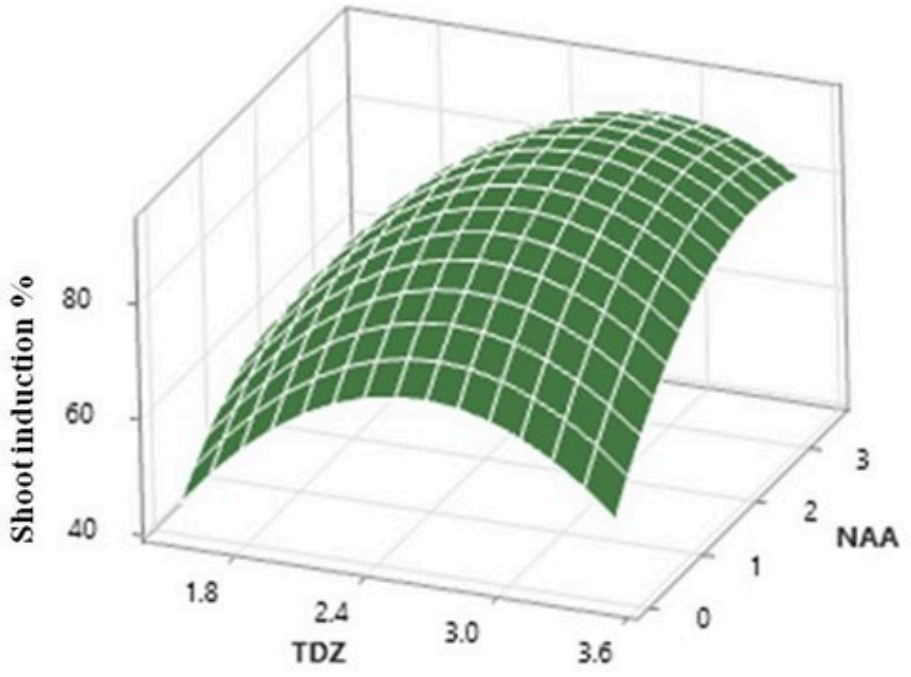

Figure 2

Surface Plot of Frequency of Shoot induction vs NAA, TDZ

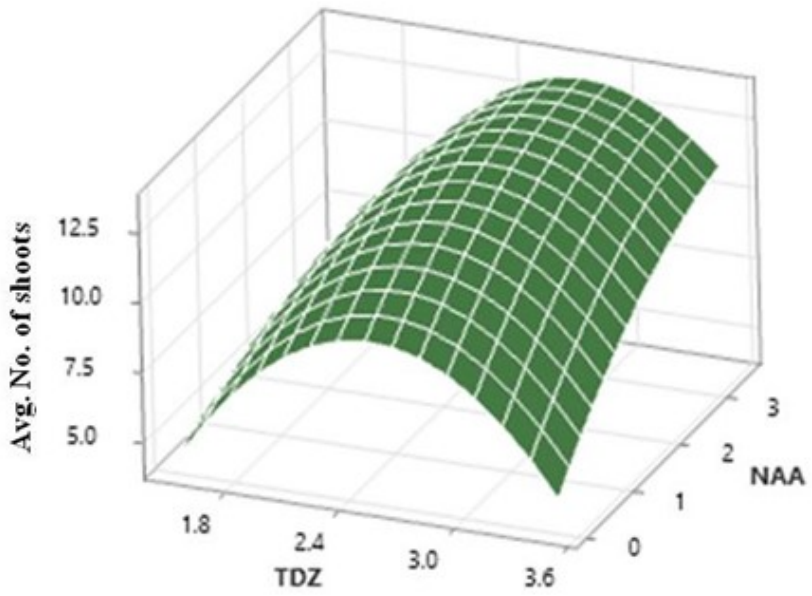

Figure 3

Surface Plot of Avg. no. of shoots vs NAA, TDZ

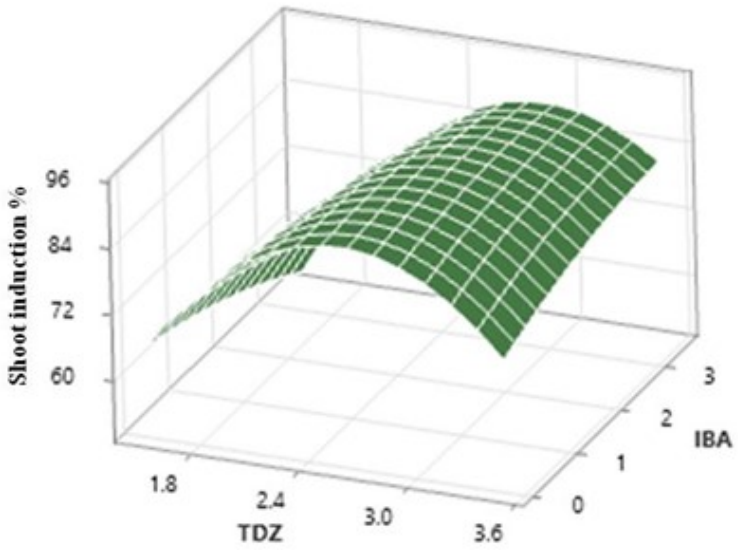

Figure 4

Surface Plot of Frequency of Shoot induction vs IBA, TDZ

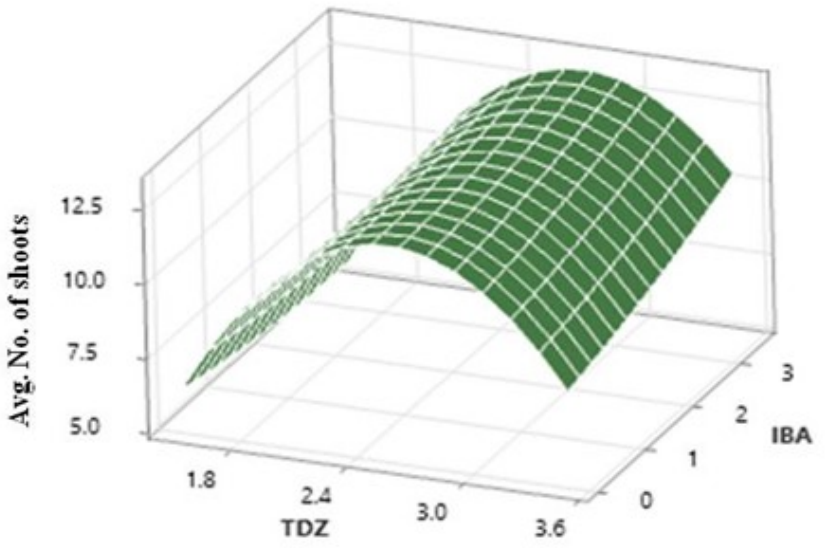

Figure 5

Surface Plot of Avg. no. of shoots vs IBA, TDZ

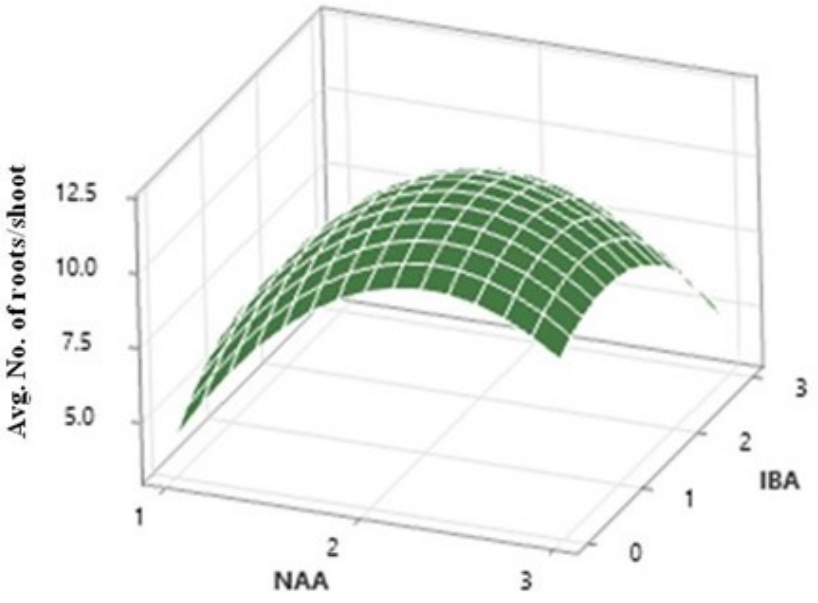

Figure 6

Surface Plot of Avg. no. of roots/shoot vs NAA, IBA

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