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Research Article

Response Surface Optimization Of *In Vitro* Culture Medium For Enhanced Production Of The Therapeutically Important Secondary Metabolite – Withaferin A

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ABSTRACT

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In vitro plant cultures are an alternative to traditional farming of important medicinal plants. Optimization of the media components is the main criterion in establishing successful *in vitro* cultures that aim at producing increased secondary metabolites for therapeutic purposes. The present study depicts the use of Response Surface Methodology (RSM) in *in vitro* culture media optimization of *Withania somnifera*. Three individual factors were evaluated in effecting secondary metabolite production. Sucrose concentration in Murashige and skoog's (MS) media, the cytokinin (BAP) concentration and the pH range was optimized for enhancing the production of pharmaceutically important secondary metabolite Withaferin A. Optimization was carried out using a Central Composite Factorial (CCF) method using MODDE software version 9.0. The optimized media yielded a maximum of 3.5mg/g DW Withaferin A when compared to 1.25mg/g DW of unoptimized media. This is an overall 2.8 fold increase in concentration of withaferin A when compared to the control MS media. This optimized media can be utilized to produce increased levels of Withaferin A from *in vitro* cultures of *Withania somnifera*.

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INTRODUCTION:

For many centuries, plants have been extensively studied for their medicinal and pharmacological properties. These pharmacological properties of the medicinal plants have been attributed to the major class of plant products called secondary metabolites. Plants synthesize numerous secondary metabolites with enormous applications in medicinal, healthcare, bio-pesticide and several other industries.[1]

Withania somnifera, (Indian Ginseng) is one such plant, whose secondary metabolites have tremendous pharmacological and medicinal properties. It belongs to the family solanaceae and contains various withanolides as major secondary metabolites that attribute to its anti-bacterial, anti-inflammatory, anti-tumor and many other defensive properties.[2-6] Withanolide production from the field grown plants is limited due to the effect of various biotic and abiotic factors. Moreover, the spatial and temporal regulation of the plant secondary metabolites limits their production in plant tissues. [7] *In vitro* cell culture technology is the field of interest in producing these commercially important plant products.[8] This plant with immense therapeutic potential is now red listed and considered as one of the rare species due to its unavailability in natural conditions.[9] Hence, to meet the increasing market demand, biotechnological interventions for enhancement of yield through cell and organ culture is the need of the hour.

For establishing an efficient secondary metabolite producing system for Withaferin A, it is necessary to optimize the media components and associated culture conditions. Interaction between different media components is complex and there needs to be an optimized, methodological in house study to standardize the conditions for enhanced production of secondary metabolites. The traditional "one factor at a time" approach for optimizing various conditions for secondary metabolite production is laborious and time consuming.[10] The statistical method of optimization is always advantageous than the traditional method. [11] Between the classical

one factor at a time method and the statistical method, the latter uses fewer experiments to analyze optimal conditions for generating the desired response.[12] Response surface methodology (RSM) is one such approach that overcomes the limitations of a conventional one factor at a time approach by statistically optimizing all the parameters at the same time and also by reducing the number of experiments in comparison to the traditional experimental design.[13] RSM quantifies the relationship between controllable input parameters and obtained response.[14] RSM is an extremely powerful statistical approach to carry out experiments that is used in mathematical modeling. It is also an ideal process for standardizing the variables in optimization of target metabolite production and also in simultaneously evaluating the interaction and significance among the variables.[15-18] Using RSM, effect of individual variables can be identified, their relative significance can be studied and the optimum constituents can be evaluated.[19,20] Central composite factorial design is an optimization strategy in RSM that can be used to screen wide range of parameters and simultaneously assess the role of each parameter.[21] This approach includes a program of statistical optimization with factorial design and centre points that are supplemented with a group of star points for estimation of curvature. Here CCF program of RSM was used for optimizing media components that would provide enhanced yield of this highly commercially valuable secondary metabolite - Withaferin A. This paper reports the first study using RSM to optimize the cytokinin BAP, pH and sucrose concentrations in the culture medium for developing an efficient system that would enhance the production of Withaferin A in shoot cultures of *W.somnifera*.

MATERIALS AND METHODS:

Materials

W.somnifera seeds were obtained from CIMAP (Hyderabad). Withaferin A standard (>95% purity) was procured from Natural Laboratories,

Bengaluru, MS media from HI media, BAP from Duchefa, Netherlands and HPLC grade solvents were purchased from Merck Ltd.

Methods:

Establishment of aseptic cultures of *W.somnifera*

W.somnifera was propagated *in vitro* from seeds. The surface sterilized seeds (0.1% Mercuric Chloride) were germinated on MS media with different concentrations of GA₃ (0.25%, 0.5%, 1.0% and 2.0%). [22] Aseptically germinated plantlets were cultured on sterile MS basal media and incubated under standard culture conditions (25± 2°C, 16h light and 8h dark cycle with 40-50mol m⁻² s⁻¹ light). The plants were maintained by periodic subculturing every 21 days.

Media optimization

Optimization of media components is an important criterion in any tissue culture study. *In vitro* maintained plantlets were transferred to different strengths of MS (¾, ½, ¼ and 1 MS) and B5 media to optimize the media that would generate maximum biomass. The change in biomass and Withaferin A content was assessed periodically (4 days interval).

With optimization of the plant tissue culture media, standardization of appropriate phytohormone concentration (NAA, IBA, 2,4D, BAP, Kn and IAA) is necessary to achieve increased biomass and secondary metabolite content. Shoots measuring 2-3cm were subcultured on to MS media containing different concentrations of each phytohormones (0.25, 0.5, 1.0, 2.0, 3.0 mg/l). Biomass and Withaferin A content were evaluated for every 4 days. The media combination yielding the maximum biomass and secondary metabolite content was selected for plant maintenance.

Growth Curve

28 days old plants maintained on MS media served as the starting material for the study of growth kinetics. 10% standardized inoculum was used to inoculate the required number of culture bottles and were then incubated at standard culture conditions in an orbital shaker at 120 rpm. Studying the patterns of Withaferin

A expression in concurrence with change in biomass was the objective of the study. Samples were analyzed for 28 days maintaining a 4 day interval. The explants were weighed and then oven dried at 65°C for 30 minutes to obtain constant weight. 1g of each sample was processed to quantify the Withaferin A content. The experiment was repeated in triplicates to validate the result.

Quantitation of Withaferin A

The quantitation of Withaferin A content of *W.somnifera* from different media composition was carried out by HPLC. [23, 24] The plantlets were air dried to obtain constant weight for uniform analysis. One gram of air dried and *in vitro* grown plantlets were completely macerated with 30ml of HPLC grade methanol. The sample was desiccated at room temperature for one week. The residue was suspended in 1 ml of HPLC grade methanol. The extract was then filtered by passing through 0.22µm membrane filter and the filtrate was used for further HPLC analysis. The analysis was carried out using Shimadzu High Performance Liquid Chromatography and LC6AD system (Japan) using SUPELCOSIL column C18 (25cmX 4.6mm, 5µm particle) at 230nm. This analysis employed isocratic elution with methanol: water (65:35) as the mobile phase and a flow rate of 2ml/min with injection volume of 20µl. The pure Withaferin A was used as standard for comparison.

RSM for Withaferin A production

RSM is a tool used for bioprocess optimization and is a collection of statistical techniques that is used to design experiments and evaluate factors for optimization of *in vitro* conditions. [25] A central composite factorial design for three independent variables was employed to optimize biomass and Withaferin A production. The experiments were designed based on the output from software MODDE version 9.0, Umetrics.

In the present study, experiments were designed to optimize the best media combination with appropriate cytokinin (BAP) and sucrose concentration and an optimum pH in yielding enhanced levels of withaferin A from

W.somnifera. Sucrose is the widely used source of carbon in plant tissue culture media. In addition to being the main energy source for plants, it also influences secondary metabolite biosynthesis.[26] Sucrose concentration is one of the major factors affecting secondary metabolite biosynthesis as are the concentrations of other auxins and cytokinins.[27] Sucrose concentration was studied in the range of 2-4% (2%, 3% and 4%) along with 0-3mg/l BAP (0, 0.5, 1.0, 2.0 and 3.0 mg/l). BAP was considered based on our initial experiments where BAP containing media yielded increased biomass compared to MS basal media. Our finding was also supported by the report of Fatima and Anis on the study of the role of phytohormones on *W. somnifera* propagation, where of all the different cytokinins studied, BAP was found to be comparatively more efficient and induced better shoot proliferation.[28] The H⁺ ion concentration plays a significant role in nutrient uptake and secondary metabolite synthesis.[29] Change in medium pH results in alteration of cell membrane permeability in turn affecting secondary metabolite release.[30, 31] The standard pH conditions of any growth medium are maintained at 5.88 and hence both the top and bottom ranges on either side were taken into account. A pH range from 4 to 7 was considered as variables in the optimization studies for designing the optimum media yielding highest amount of Withaferin A.

The concentration of other media components and culture conditions were maintained unaltered. 10% weight of explants was cultured on each media combination and incubated for various time periods at standard culture conditions. The CCF is split into two blocks, the cube portion and the star portion and the condition gets satisfied when α (the distance of the star points to the centre) is equal to

$$\alpha = \left[\frac{k(1+P_s)}{1+P_c} \right]^{1/2} \quad (1)$$

Where K= number of factor

N_s = Number of star point runs

N_c = Number of runs from cube portion

P_s= N_{so}/N_s – proportion of centre points in the star portion P_c= N_{co}/N_c – proportion of centre points in the cube portion.

Modde software version 9.0 (Umetrics) was used to calculate the polynomial coefficients and to determine the responses of the dependent variables. All the experiments were repeated thrice to estimate variability in measurements. The experimental data received after many experiments were analyzed based on the second order polynomial equation given by Maran et al.,

$$Y = \beta_0 + \sum_{a=1}^4 \beta_a X_a + \sum_{a=1}^4 \sum_{b=1}^4 \beta_{ab} X_a X_b + \sum_{a=1}^4 \beta_a^2 X_a^2$$

(2)

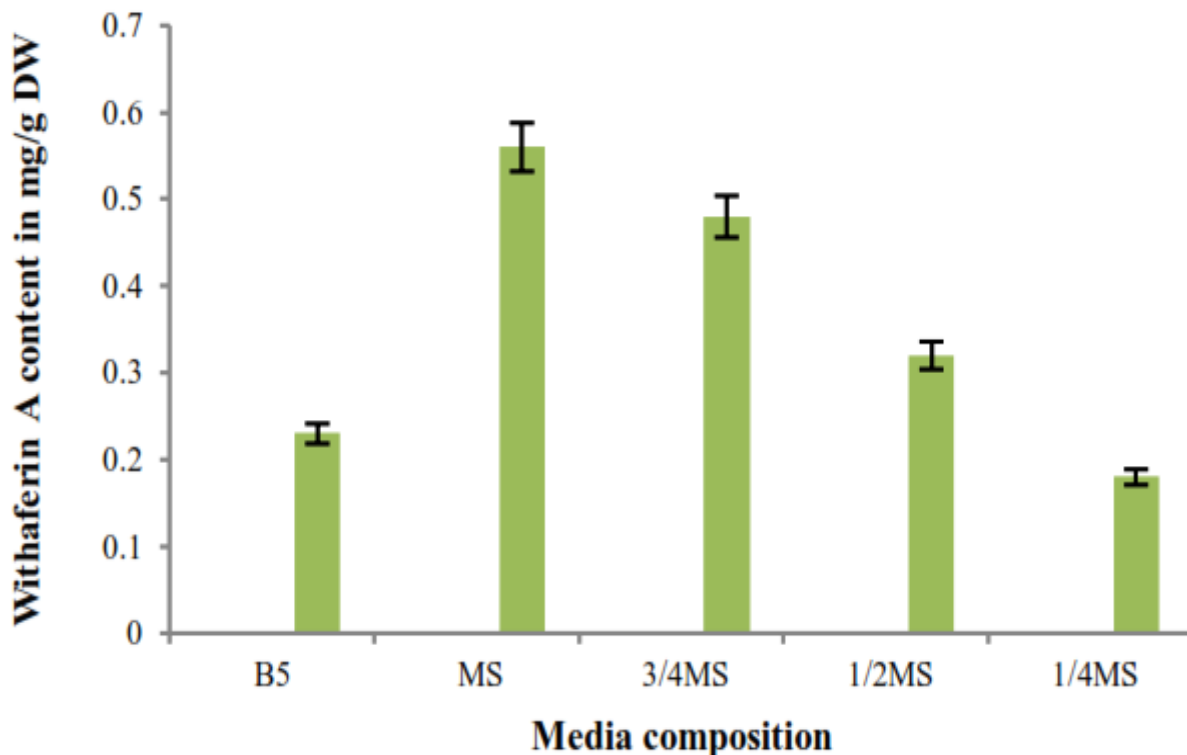
Y is the independent response, β_0 is a constants, β_a is the linear coefficient, β_b is the squared coefficient, β_{ab} is the cross product coefficient. [32]

RESULTS:

In vitro propagation

Seeds of *W. somnifera* showed maximum germination in MS media supplemented with 0.5% GA₃. Majority of the seeds germinated after 8-12 days with a germination frequency of 82±0.08%. In the study for choosing the optimum media that supports increased growth and secondary metabolite accumulation in *W.somnifera*, full strength MS was found to be better than B5, ½, ¼ and ¾ MS (Fig. 1).

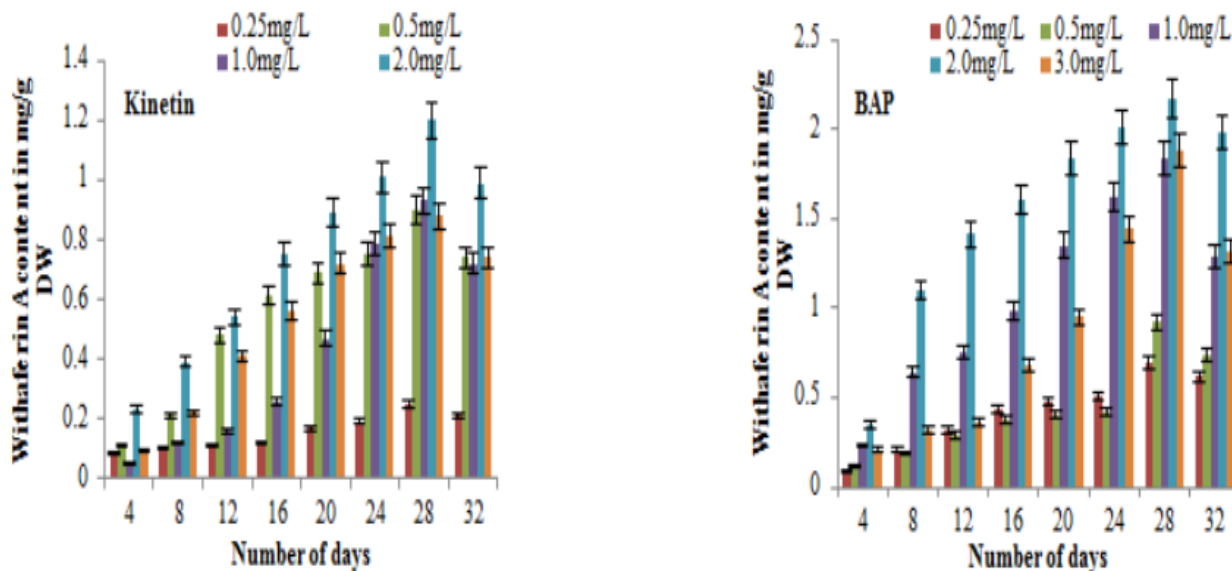
Fig. 1: Plot of Biomass versus Withaferin A content in different media composition.

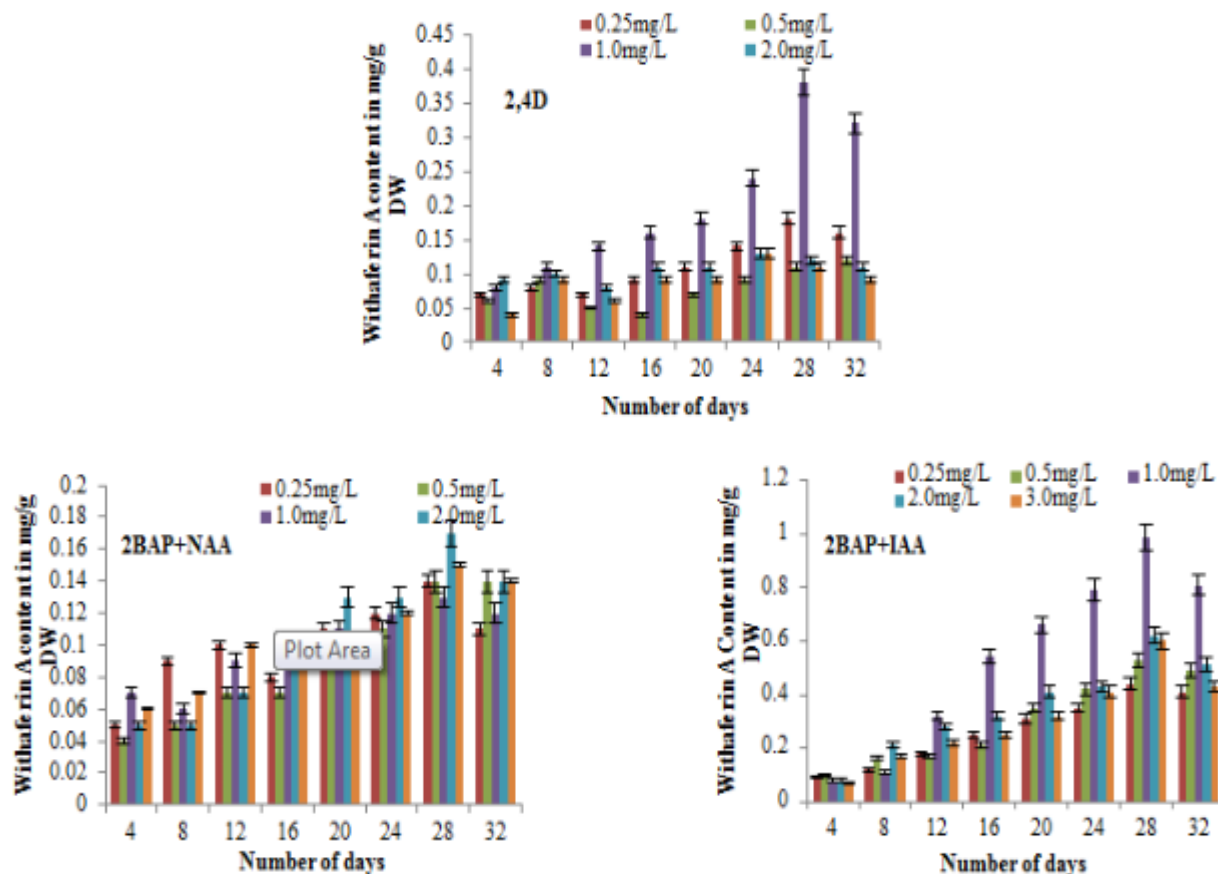


Explants grown on MS media, when subcultured onto plant growth media containing different concentrations of phytohormones showed MS media supplemented with 2mg/l BAP to be the best media combination in

generating increased biomass along with maximum multiple shoot growth. This media combination also yielded the maximum Withaferin A content of 2.7 ± 0.12 mg/g DW (Fig. 2).

Fig. 2: Growth kinetics study of Withaferin A from *W.somnifera* on MS media fortified with different concentrations of phytohormones.





Impact of medium components on Withaferin A production

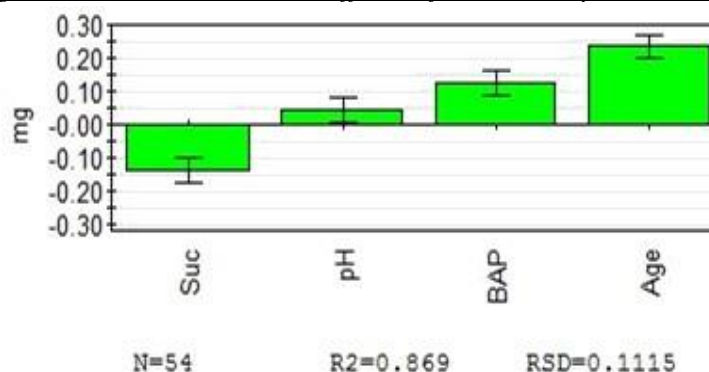
The amount of Withaferin A accumulated during all the 54 runs with 3 centre points varied from 0.13 to 3.56 mg/g DW. The objective of this experiment was to screen for optimum interactions among the independent variables in yielding maximum Withaferin A content. During the analysis it was observed that the variation among the repeated experiments is much less than the overall variation, proving our experimental conditions and data to be ideal.

The model efficiency was analyzed using four basic parameters. R2 value is greater than 0.8 showing the model to be of high significance. Q2

value corresponds to 0.8 showing that the model is good. A very good replicate model and high sensitivity yielded model validity less than 0.25. Reproducibility value is close to 1 showing that the model is 100% reproducible and there is no variability among the duplicate sets of experimental data.

The coefficient plot was used to display the regression coefficients with confidence intervals. From the plot (Fig. 4) it is clearly evident that all the independent variables sucrose, pH, BAP concentration and age have a significant impact in deciding the concentration of Withaferin A content.

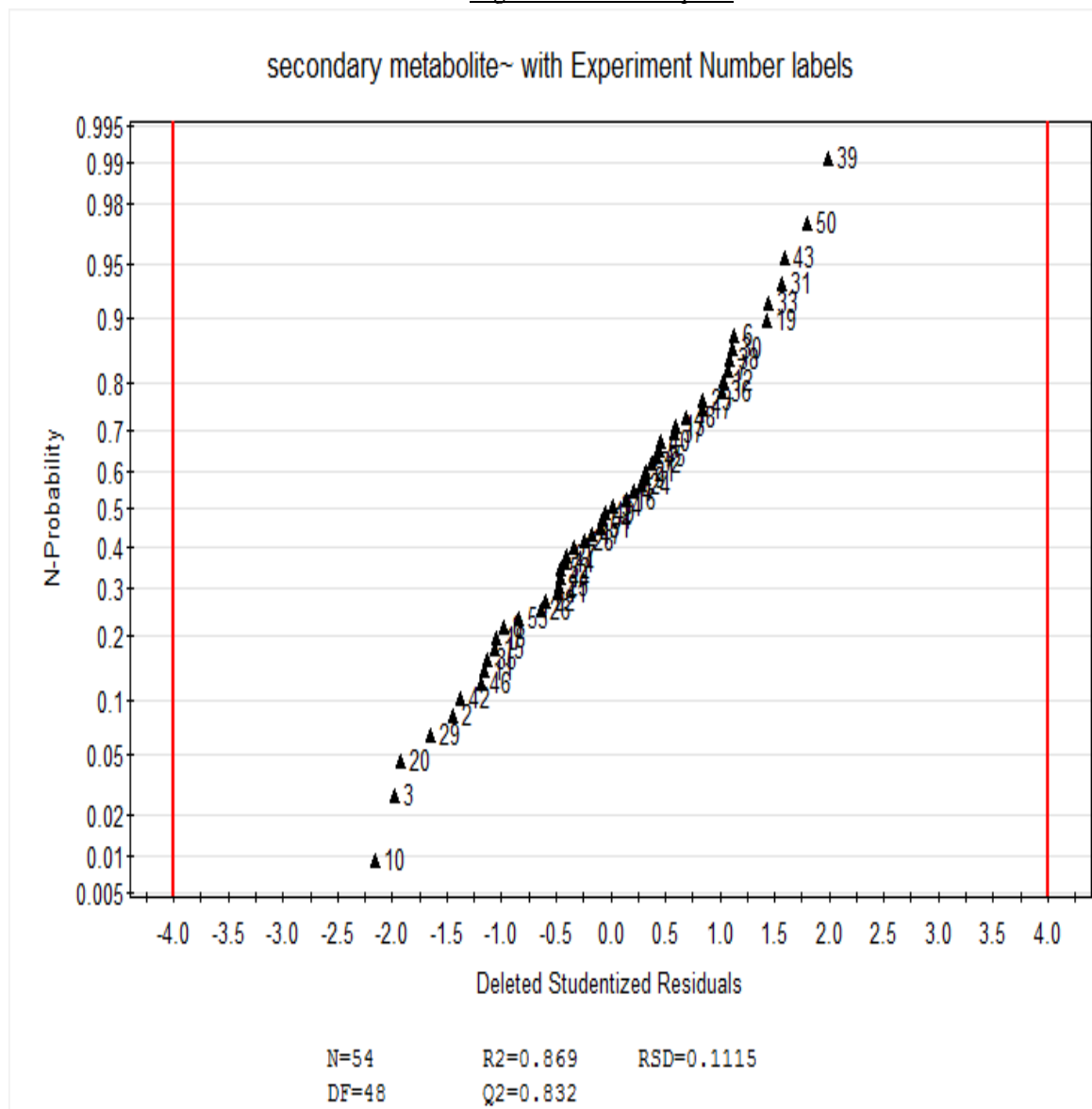
Fig. 3: Scaled and centered coefficient for secondary metabolite.



Sucrose is having negative impact on the overall Withaferin A content, which is inferred from our observation that as sucrose concentration increases more than 3%, the secondary metabolite content is observed to decrease. Fig.4

shows that there are no outliers in the experimental data. All the observed data were located very close to the straight line indicating that there is not much significant difference between the observed and predicted result.

Fig. 4: Residual N-plot.



The overlay prediction plot (Fig. 6) shows evidently that as sucrose concentration increases, the metabolite content decreases. BAP concentration has a linear effect where maximum Withaferin A was produced in 2mg/l BAP containing medium which is clearly depicted in figure 7 showing the sweet spot area of maximum Withaferin A production. The 4D contour plot and the surface plot (Fig.6) comparing the combined effect of all the three individual

parameters shows the region coloured in red corresponding to high withaferin A content. This maximum yield of the secondary metabolite was achieved using sucrose concentration of 2% and 2mg/l BAP at mid range pH. pH has a hyperbolic effect, indicating that ranges from 5.0-6.0 are significant in altering the metabolite content and a maximum is achieved at pH 5.62

Fig. 5: Overlay Prediction plot showing role of independent variables

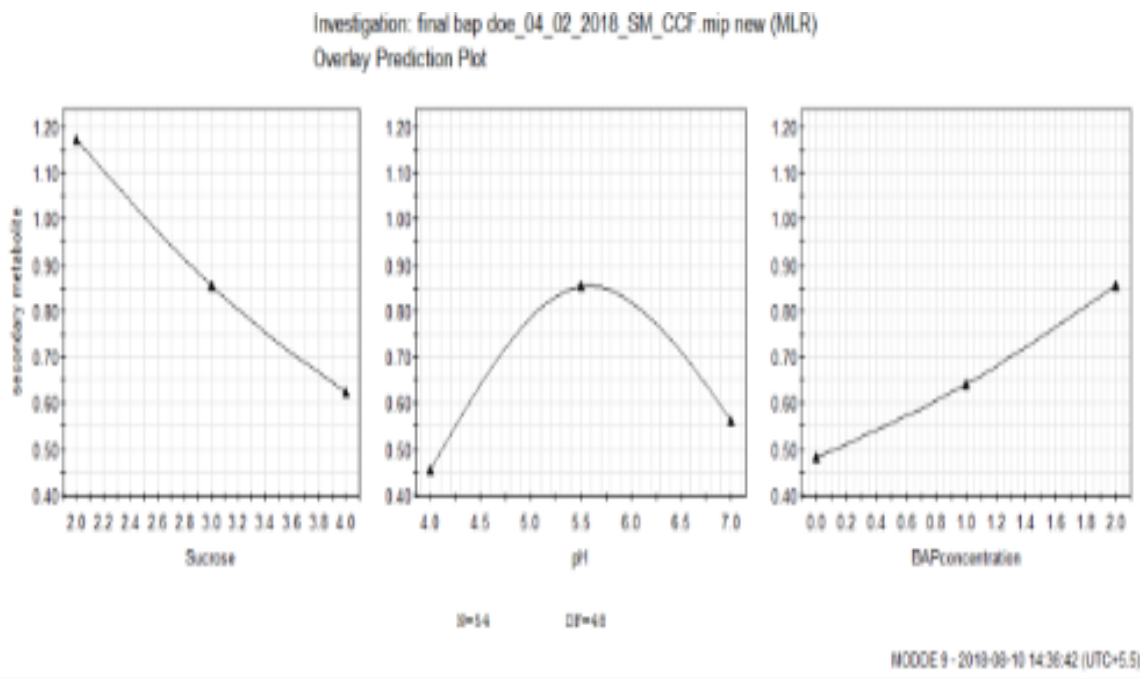


Fig. 6: 4D Contour plot for maximum Withaferin A production.

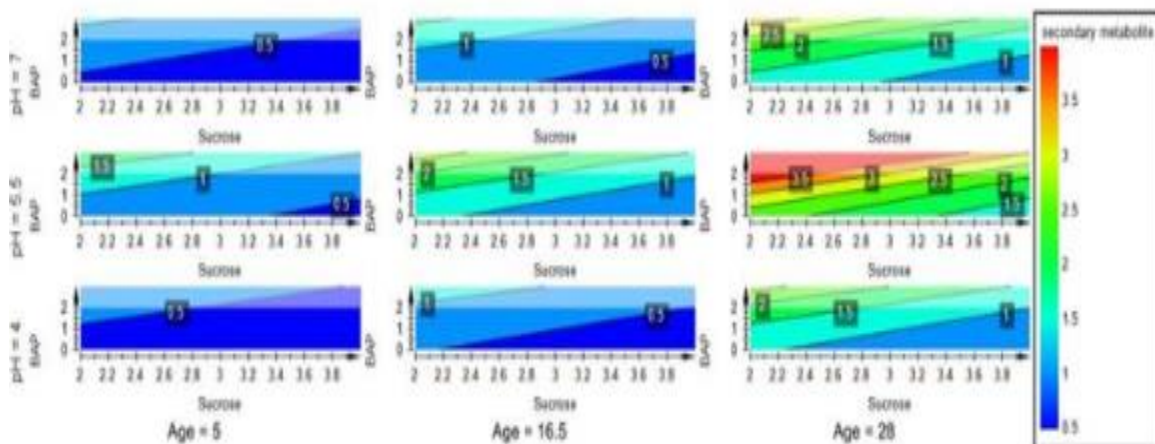


Table 1: ANOVA table to show model significance

Secondary Metabolite	DF	SS	MS (Variance)	F value	P value	SD
Total	54	5.199	0.0962922	-	-	-
Constant	1	0.633	0.631943	-	-	-
Total Corrected	53	4.567	0.0861855	-	-	0.293574
Regression	5	3.971	0.79432	63.9473	0.000	0.891247

Residual	48	0.595	0.0124215	-	-	0.111452
Lack of fit	19	0.356	0.0187888	2.27748	0.022	0.137072
Pure error	29	0.239	0.00824982	-	-	0.0908285

This model is based on quadratic regression and is proved to be significant using analysis of variance (Table 1). This model has a significant F value of 63.94. This value proves the model to be significant. P value of the model, which is the deciding factor for model significance, is 0.000 indicating this model to be statistically significant. The R² value was 0.869; which indicates that 86.9% of the variability in the experimental design could be explained statistically. By substituting the values in the regression equation, the optimized media component for maximum production of Withaferin A was being obtained and the same was also experimentally checked. The maximum Withaferin A concentration was achieved at 2% sucrose, pH 5.62 and 2mg/l BAP. This concentration was 3.5mg/g DW.

DISCUSSION:

Plants have always been the fore runners in any potential drug discovery. Withaferin A is one such secondary metabolite from *W. somnifera*, which is been widely exploited for its anti-stress and anti-cancer properties.[33] The enormous medicinal properties of Withaferin A have increased its market demand. Hence, there is a necessity to develop an efficient *in vitro* procedure for increasing both the biomass and the secondary metabolite content. Therefore a response surface methodology study was done to optimize the media that would help achieve maximum withaferin A concentration.

From our studies on seed germination it is evident that GA₃ has a significant role in breaking dormancy and inducing germination. The positive role of GA₃ in increasing germination frequency and reducing germination time has also been studied by many other researchers.[33- 36] The self sufficiency of MS full strength media to support growth and shoot generation was supported by our studies and other researchers too. [9, 37] Supplementation of MS basal media with small molecules that have a significant role in regulating growth and combatting stress, help in promoting greater plant survival rate. These molecules are called phytohormones and play a major role in *in vitro* plant tissue culture. Of the different classes of phytohormones, cytokinins are well known for their activity in promoting growth and accumulating plant secondary metabolites.

Our results are in corroboration to other similar studies where 2 BAP has been reported as the best regeneration media.[38-40] Several studies in other plants species like *Trifolium pratense*, *Bacopa monnieri*, *Holarrhena pubescens* are also in agreement to our statement that BAP at the concentration of 2mg/l is the best cytokinin for multiple shoot induction.[41-43]

It is a well known fact that the nature and the amount of the carbon source have a profound effect in deciding the biomass and secondary metabolite concentration. Previous studies have already reported that highest biomass and secondary metabolite content was achieved when sucrose was used as the carbon source.[44] But our results showed 2% sucrose to be optimum in contrast to the observations by other researchers, where 4% sucrose enhanced the accumulation of withanolides *in vitro*. [45,46] pH of the plant tissue culture medium play a significant role in making the macro and micro nutrients available to the plants. The uptake of nutrients by the plants is directly dependent on the pH. The role of pH was also assessed by Murthy and Praveen 2012, who observed that the optimum pH of 5.88 favoured biomass accumulation where as a slightly alkaline pH of 6.0 favoured withanolideA accumulation.[47]

The combined role of sucrose, BAP and pH in increasing the secondary metabolite yield is evident from the fact that 3.5mg/g DW Withaferin A was obtained by optimizing the culture conditions using design of experiments and this concentration is much higher than the concentration reported by other researchers without elicitation. [48, 49, 50]

CONCLUSION:

The quantity and the quality of major withanolides differ with different tissue type and the various growth conditions in the field.[51] This proves to be a major difficulty in standardizing the plant formulations for their commercial exploitation.[52] Optimization of various culture parameters is of utmost need in achieving increased biomass and secondary metabolite content. Response surface methodology was used to optimize the concentrations of sucrose, BAP and pH. The synergistic effect of 2% sucrose as carbon source, 2mg/l BAP as the cytokinin phytohormone in combination with an optimum pH of 5.62 proved to be the best combination

for *in vitro* establishment of *W.somnifera* cultures for enhanced Withaferin A production. The media that was optimized using RSM yielded nearly one and a half times more withaferin A than the unoptimized media. In our study we have optimized the *in vitro* growth media composition for producing increased Withaferin A content from our local cultivar type. This media combination standardized using statistical design of various experiments could be exploited to commercially produce the highly valuable secondary metabolite Withaferin A in large quantities. Production of increased Withaferin A through *in vitro* cultures is highly necessary to meet the ever increasing market demand.

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