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Analysis of Influence of Environmental Conditions on Ganoderic Acid Content in *Ganoderma lucidum* Using Orthogonal Design

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Abstract The influence of environmental conditions on the ganoderic acid (GA) content in the fungus *Ganoderma lucidum* was investigated using a one-factor-at-a-time design and orthogonal design. Among the various medium components examined, sucrose, soybean powder or peptone, ferrous sulfate, and pH 6.0 were the most suitable carbon source (factor A), nitrogen source (factor B), mineral source (factor C), and initial pH (factor D), respectively, for the GA content in the one-factor-at-a-time design. According to the orthogonal design, the order of effect for the four factors on the GA content was A>C>D>B. The best level of factor A was A_2 (sucrose) with a value of +0.34 mg/100 mg DW. The optimal treatment combination was $A_2B_1C_3D_1$ with which the GA content reached up to 2.63±0.011 mg/100 mg DW. The interactions between the mineral ion and the nitrogen source, and the mineral ion and the pH were both highly significant ($P<0.01$). The highest interaction effect was ($B_2 \times D_2$) with a value of +0.19 mg/100 mg DW, which was higher than the level effect value for B_2 (peptone) and D_2 (pH 5.0). Therefore, the results proved that interactions between factors cannot be ignored. The results also indicated the importance of the interactions between the factors, which may help to understand the metabolic pathway leading to triterpene biosynthesis and the expression and regulation of the key enzymes involved.

Key words: *Ganoderma lucidum*, ganoderic acid content, orthogonal design, statistical analysis

Ganoderma lucidum (Leyss.: Fr.) Karst is a medicinal mushroom widely used in China (named Ling Zhi) for many centuries as a folklore medicine for the treatment of diseases, such as hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancers, and gastric ulcer [11, 17]. Many analogs of the parental oxygenated triterpenes (especially ganoderic acid) have already been isolated from this mushroom, modified by enzymatic reactions, and found to exhibit various biological functions. In particular, ganoderic acid (GA) has been shown to help alleviate common allergies by inhibiting the chemical mediators of inflammation, including histamines [14, 18].

Most reports on the GA from *G. lucidum* have been concerned with its isolation and pharmaceutical mechanisms [2, 9]. Thus, little is known about the molecular mechanisms of triterpene biosynthesis. As a secondary metabolite, GA is synthesized via the mevalonate pathway, where farnesyl diphosphate is converted to squalene, then to 2,3-oxidosqualene, and finally undergoes a series of cyclization, oxidation, and reduction reactions. Every reaction is catalyzed by a corresponding enzyme [10, 26], and a positive correlation has been noted between the expression levels of these enzymes and the amount of triterpenes produced [27]. In particular, the expression level of squalene synthase (SQS) has been found to be correlated with the triterpene content in *G. lucidum* [28].

Thus, a detailed study of the growth parameters that affect the production of GA may help to elucidate the expression and regulation of the key enzymes and biosynthetic steps of the end product.

Previous studies on the effect of environmental factors on the GA content [4–7, 20–23] have mainly been concerned with the effect of single factors on the GA product. Thus, there has been no comparison of the order of effect for these factors, or analysis of the interactions between such factors. Accordingly, this study used an orthogonal design to investigate the influence of environmental conditions on the GA content in a liquid culture. The order of effect for the factors was analyzed, and effect sizes for the interactions were established using a variance analysis.

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MATERIALS AND METHODS

Mushroom Strain and Growth Conditions

The strain of *G. lucidum* HG was obtained from the culture collection of the Edible Fungi Institute, Shanghai Academy of Agricultural Science. Unless otherwise stated, the incubation temperature was 28°C. The stock culture was maintained on potato dextrose agar (PDA) slants that were incubated for 6 days, then stored at 4°C. The inoculum was prepared by transferring the mycelium of *G. lucidum* to petri-dishes containing a PDA medium and incubated for 6 days. Mycelial agar discs (0.5 cm) were then cut and transferred into the seed culture medium. The seed culture was grown in 250-ml flasks containing 100 ml of a PD-broth and placed on a rotary shaker incubator (150 rev/min) for 6 days. The basal medium included the following components: 32 g/l glucose, 10 g/l peptone, 3 g/l $KH_2PO_4 \cdot H_2O$, and 1.5 g/l $MgSO_4 \cdot 7H_2O$. The second set of flask culture experiments were performed in 250-ml flasks containing 100 ml of the basal medium after incubating with 5% (v/v) of the seed culture.

Measurement of Ganoderic Acid

The specific production of ganoderic acid (GA) was assayed using the procedure described by Tsujikura *et al.* [24]. The ethanol-soluble compounds from the dried and ground mycelia (100 mg) were solubilized by 50% (v/v) ethanol (3 ml) for 1 week (twice). After removing the mycelia by centrifugation, the supernatants were dried at 50°C under a vacuum. The residues were then resuspended in water, and later extracted with chloroform. The GA in the chloroform extract was further extracted with 5% (w/v) $NaHCO_3$. After adding 2 N HCl to adjust the pH of the $NaHCO_3$ phase below 3.0, the GA in the $NaHCO_3$ phase was again extracted with chloroform. After removing the chloroform by evaporation at 40°C, the GA was dissolved in absolute ethanol, and its absorbency measured at 245 nm.

One-Factor-at-a-Time Design

To optimize the medium composition, different kinds of carbon source (sucrose, glucose, maltose, mannose, lactose, and soluble starch) and nitrogen source (soybean powder, peptone, yeast extract, and ammonium sulfate) were individually added to the basal medium. Various mineral sources (ferrous sulfate, zinc sulfate, calcium chloride, and sodium chloride) were also added to the basal medium, respectively. The pH of the medium was adjusted to the desired value (pH values of 4.0, 5.0, 6.0, and 7.0) by the addition of either 1 N HCl or 1 N NaOH. All the experiments were performed at least in triplicate. Three optimal factor levels were chosen for the orthogonal design.

Orthogonal Design

An orthogonal design table, L_{27} (3^{13}), was used to determine the optimal conditions for the GA content. Four factors and three levels were selected: maltose, sucrose, and glucose as the carbon sources; soybean powder, peptone, and yeast extract as the nitrogen sources; and ferrous sulfate, zinc sulfate, and calcium chloride as the mineral ions at an initial pH of 4.0, 5.0, and 6.0. The design contained 27 treatments and the experimental conditions for each treatment combination are listed in Table 1. All the experiments were carried out at least in triplicate.

Statistical Analysis Method

Variance Analysis Method. The data were first considered based on an intuitive analysis, followed by a variance

Table 1. Experimental factors and levels for orthogonal design.

Treatment combination	Carbon source	Nitrogen source	Mineral ions	pH	Treatment combination	Carbon source	Nitrogen source	Mineral ions	pH
1	A1	B1	C1	D1	15	A2	B2	C3	D2
2	A1	B1	C2	D2	16	A2	B3	C1	D1
3	A1	B1	C3	D3	17	A2	B3	C2	D2
4	A1	B2	C1	D2	18	A2	B3	C3	D3
5	A1	B2	C2	D3	19	A3	B1	C1	D3
6	A1	B2	C3	D1	20	A3	B1	C2	D1
7	A1	B3	C1	D3	21	A3	B1	C3	D2
8	A1	B3	C2	D1	22	A3	B2	C1	D1
9	A1	B3	C3	D2	23	A3	B2	C2	D2
10	A2	B1	C1	D2	24	A3	B2	C3	D3
11	A2	B1	C2	D3	25	A3	B3	C1	D2
12	A2	B1	C3	D1	26	A3	B3	C2	D3
13	A2	B2	C1	D3	27	A3	B3	C3	D1
14	A2	B2	C2	D1					

A1, maltose; A2, sucrose; A3, glucose; B1, soybean powder; B2, peptone; B3, yeast extract; C1, ferrous sulfate; C2, zinc sulfate; C3, calcium chloride; D1, pH 4.0; D2, pH 5.0; D3, pH 6.0.

analysis [8]. The general linear model (GLM), which underlies most statistical analyses, was used, as denoted by

$$y_{ijklh}=\mu+A_i+B_j+C_k+D_l+(A\times B)_{ij}+(A\times C)_{ik}+(A\times D)_{il}+(B\times C)_{jk}+(B\times D)_{jl}+(C\times D)_{kl}+e_{ijklh}$$

where symbols y_{ijklh} and μ represent the sum and average of the GA content for the 27 combination treatments, respectively, symbols A, B, C, D, and (A×B) *etc.* represent the carbon source, nitrogen source, mineral ion, pH value, and different interactions (i=1, 2, 3; j=1, 2, 3, k=1, 2, 3, l=1, 2, 3), respectively, and symbol e_{ijklh} represents the random error.

Calculation of Effect Value. The effect value was denoted as the form of deviation from the mean.

Effect value of level [19]: $a_i=k_i-\bar{y}$

Effect value of interaction: $(ab)_{ij}=[ab]_{ij}-a_i-b_j$

where symbols k_i and $\bar{y}$ represent the average for every factor level and average for the 27 combination treatments, respectively, symbol $[ab]_{ij}$ represents the combination effect values for a_i and b_j, and symbols a_i and b_j represent the level values for the factors.

Results

Results of One-Factor-at-a-Time Design

This experiment investigated the effects of the carbon sources (factor A), nitrogen sources (factor B), mineral sources (factor C), and initial pHs (factor D) and the results are shown in Table 2. The results revealed that the best carbon source (factor A) was sucrose, with which the GA content reached up to 2.43±0.036 mg/100 mg DW, followed by maltose and glucose. Therefore, sucrose, maltose, and glucose were selected as the carbon source levels for the orthogonal design. Meanwhile, soybean powder and peptone were both efficient nitrogen sources (factor B), with which the GA content reached up to 1.27±0.012 and 1.27±0.047 mg/100 mg DW, respectively, followed by yeast extract. Therefore, soybean powder, peptone, and yeast extract were selected as the nitrogen source levels for the orthogonal design. For the mineral ion test, the maximum GA content of up to 1.35±0.047 mg/100 mg DW was achieved with ferrous sulfate in the medium, followed by zinc sulfate and calcium chloride. Therefore, ferrous sulfate, zinc sulfate, and calcium chloride were selected as the mineral ion levels for the orthogonal design. The optimum pH (factor D) was 6.0, which achieved a GA content of up to 1.62±0.078 mg/100 mg DW, followed by pH values of 5.0 and 4.0. Therefore, 6.0, 5.0, and 4.0 were selected as the initial pH levels for the orthogonal design.

Results of Orthogonal Design

GA Content with Different Combination Treatments. The interaction results of assigning the three levels of the four factors are shown in Table 3, according to L_{27} (3^{13}). The results revealed great differences between the combination treatments. The best combination for the GA content was $A_2B_1C_3D_1$: sucrose, soybean powder, calcium chloride, and an initial pH of 4.0, where the maximum GA content was 2.63±0.011 mg/100 mg DW, followed by $A_2B_2C_3D_2$, where the GA content was 2.60±0.031 mg/100 mg DW. Eight of the combination treatments exceeded 2.0 mg/100 mg DW, and the worst combination was $A_1B_2C_2D_3$, where the GA content was only 1.18± 0.008 mg/100 mg DW.

Results of Intuitive Analysis. To determine the optimum level for each factor, an intuitive analysis was conducted based on a statistical calculation using the data in Table 4. It was found that the GA content reached the maximum with level II (sucrose) of factor A, level I (soybean powder) of factor B, level I (ferrous sulfate) of factor C, and level I (pH 4.0) of factor D, according to the k value. The order of effect on the GA content was then determined according to the order of magnitude of R in Table 4, resulting in A>C>D>B, which means that for the GA content, the carbon source (A) was the most important among the four factors.

Table 2. Effect of factors on GA content in shake-flask culture of *G. lucidum* using one-factor-at-a-time design.

Factors	GA content (mg/100 mg DW)	Factors	GA content (mg/100 mg DW)
Carbon sources (Factor A)		**Nitrogen sources (Factor B)**	
Sucrose	2.43±0.036	Soybean powder	1.27±0.012
Glucose	1.37±0.026	Peptone	1.27±0.047
Maltose	1.24±0.033	Yeast extract	0.95±0.103
Mannose	1.23±0.002	Ammonium sulfate	0.42±0.064
Lactose	1.04±0.020		
Soluble starch	0.97±0.024		
Mineral ions (Factor C)		**Initial pH Value (Factor D)**	
Ferrous sulfate	1.35±0.047	4.0	1.22±0.053
Zinc sulfate	1.28±0.012	5.0	1.52±0.012
Calcium chloride	1.08±0.103	6.0	1.62±0.078
Sodium chloride	1.07±0.064	7.0	1.02±0.053

Table 3. Assignment of factors and levels in shake-flask culture of *G. lucidum* using orthogonal design.

Run	A	B		A×B	C		A×C		D	A×D	B×C	B×D	C×D	Mycelial dry weight (g/l)	GA content (mg/100 mg DW)
	1	2	3	4	5	6	7	8	9	10	11	12	13		
1	1	1	1	1	1	1	1	1	1	1	1	1	1	8.54±0.91	1.35±0.037
2	1	1	1	1	2	2	2	2	2	2	2	2	2	8.94±0.58	1.49±0.028
3	1	1	1	1	3	3	3	3	3	3	3	3	3	15.33±0.79	1.31±0.053
4	1	2	2	2	1	1	1	2	2	2	3	3	3	3.94±0.79	1.83±0.024
5	1	2	2	2	2	2	2	3	3	3	1	1	1	4.65±0.15	1.18±0.008
6	1	2	2	2	3	3	3	1	1	1	2	2	2	3.07±0.28	1.46±0.031
7	1	3	3	3	1	1	1	3	3	3	2	2	2	5.97±0.25	1.52±0.045
8	1	3	3	3	2	2	2	1	1	1	3	3	3	9.59±0.17	1.28±0.068
9	1	3	3	3	3	3	3	2	2	2	1	1	1	9.36± 0.78	1.23±0.020
10	2	1	2	3	1	2	3	1	2	3	1	2	3	12.65±0.26	1.82±0.019
11	2	1	2	3	2	3	1	2	3	1	2	3	1	9.55±0.15	2.22±0.016
12	2	1	2	3	3	1	2	3	1	2	3	1	2	13.15±0.94	2.63±0.011
13	2	2	3	1	1	2	3	2	3	1	3	1	2	4.49±0.44	2.23±0.031
14	2	2	3	1	2	3	1	3	1	2	1	2	3	4.67±0.29	1.76±0.022
15	2	2	3	1	3	1	2	1	2	3	2	3	1	3.90±0.67	2.60±0.031
16	2	3	1	2	1	2	3	3	1	2	2	3	1	4.60±0.54	2.52±0.022
17	2	3	1	2	2	3	1	1	2	3	3	1	2	14.01±0.71	1.88±0.038
18	2	3	1	2	3	1	2	2	3	1	1	2	3	9.57±0.79	1.85±0.021
19	3	1	3	2	1	3	2	1	3	2	1	3	2	11.23±0.42	1.85±0.033
20	3	1	3	2	2	1	3	2	1	3	2	1	3	12.04±0.61	1.84±0.032
21	3	1	3	2	3	2	1	3	2	1	3	2	1	11.74±0.62	2.12±0.033
22	3	2	1	3	1	3	2	2	1	3	3	2	1	3.17±0.47	2.11±0.030
23	3	2	1	3	2	1	3	3	2	1	1	3	2	3.53±0.42	1.68±0.045
24	3	2	1	3	3	2	1	1	3	2	2	1	3	3.84±0.54	1.71±0.024
25	3	3	2	1	1	3	2	3	2	1	2	1	3	7.18±0.49	1.94±0.047
26	3	3	2	1	2	1	3	1	3	2	3	2	1	9.66±0.46	2.14±0.032
27	3	3	2	1	3	2	1	2	1	3	1	3	2	9.71±0.52	1.86±0.065

The column arrangement of A, B, C, and D was decided by the orthogonal design L_{27} (3^{13}) for 4 (factors) ×27 (run numbers), where every row of run numbers represents the average of triplicate experimental replicates. Values are mean±SD of triplicate determinations.

Table 4. Intuitive analysis of factor effect on GA content in shake-flask culture of *G. lucidum* using orthogonal design.

	GA content (mg/100 mg DW)												
	A	B		A×B	C		A×C		D	A×D	B×C	B×D	C×D
	1	2	3	4	5	6	7	8	9	10	11	12	13
K_1	12.66 ±0.31[a]	16.63 ±0.26	15.91 ±0.30	16.68 ±0.35	17.17 ±0.32	17.44 ±0.28	16.25 ±0.30	16.09 ±0.31	16.81 ±0.32	16.13 ±0.33	14.58 ±0.27	15.99 ±0.25	17.47 ±0.23
K_2	19.51 ±0.21	16.56 ±0.25	17.08 ±0.25	16.53 ±0.24	15.47 ±0.29	16.21 ±0.32	16.93 ±0.28	16.66 ±0.27	16.59 ±0.29	17.16 ±0.22	17.30 ±0.28	16.27 ±0.26	16.60 ±0.33
K_3	17.25 ±0.34	16.22 ±0.36	16.43 ±0.32	16.20 ±0.28	16.77 ±0.29	15.76 ±0.29	16.23 ±0.30	16.66 ±0.27	16.01 ±0.26	16.13 ±0.32	17.53 ±0.32	17.15 ±0.36	15.34 ±0.31
k_1	1.41 ±0.035[b]	1.85 ±0.029	1.77 ±0.033	1.85 ±0.039	1.91 ±0.036	1.94 ±0.031	1.81 ±0.033	1.79 ±0.034	1.87 ±0.036	1.79 ±0.037	1.62 ±0.030	1.78 ±0.028	1.94 ±0.025
k_2	2.17 ±0.023	1.84 ±0.027	1.90 ±0.028	1.84 ±0.027	1.72 ±0.032	1.80 ±0.036	1.88 ±0.031	1.85 ±0.030	1.84 ±0.032	1.91 ±0.024	1.92 ±0.031	1.81 ±0.029	1.84 ±0.036
k_3	1.92 ±0.038	1.80 ±0.040	1.83 ±0.036	1.80 ±0.031	1.86 ±0.032	1.75 ±0.032	1.80 ±0.033	1.85 ±0.030	1.78 ±0.029	1.79 ±0.036	1.95 ±0.035	1.91 ±0.040	1.70 ±0.034
R	0.76[c] ±0.015	0.050 ±0.013	0.13 ±0.0080	0.050 ±0.012	0.19 ±0.0040	0.19 ±0.0050	0.080 ±0.0020	0.060 ±0.0040	0.090 ±0.0070	0.12 ±0.013	0.33 ±0.0050	0.13 ±0.012	0.24 ±0.011

[a] K_i^A=ΣGA content at A_i. Values are the mean±SD of triplicate determinations.
[b] $k_i^A=K_i^A/9$. Values are the mean±SD of triplicate determinations.
[c] $Ri^A=\max\{K_i^A\}-\min\{k_i^A\}$. Values are the mean±SD of triplicate determinations.

Table 5. Variance analysis of media effect on GA content in shake-flask culture of *G. lucidum* using orthogonal design.

Source of variation	df	SS	MS	F-value	Significance
Total	80	12.81			
A	2	8.093	4.047	207.498	**
B	2	0.033	0.016	0.837	$F_{0.05}$ (2,48)=3.19
C	2	0.527	0.263	13.508	** $F_{0.01}$ (2,48)=5.08
D	2	0.114	0.057	2.911	
A×B	4	0.039	0.010	0.505	$F_{0.05}$ (4,48)=2.57
A×C	4	0.169	0.042	2.166	$F_{0.01}$ (4,48)=3.74
A×D	4	0.100	0.025	1.283	
B×C	4	1.787	0.447	22.903	**
B×D	4	0.237	0.059	3.033	*
C×D	4	0.774	0.193	9.917	**
Error	48	0.936	0.020		

df, SS, and MS represent degree of freedom, sum of squares, and mean square, respectively.

Meanwhile, the best level of factor A was A_2 (sucrose), with which the average GA content reached 2.17±0.023 mg/100 mg DW.

Results of Variance Analysis. The statistical analysis results shown in Table 5 were obtained using the analysis of variance (ANOVA) technique. The results showed that factors A and C were both highly significant ($P<0.01$) and that the different factor levels greatly affected the GA content. Two interactions, (B×C) and (C×D), were found to be highly significant ($P<0.01$), followed by (A×D) and (B×D) ($P<0.05$). The results also indicated that the arrangement of the different level interactions greatly affected the GA content.

Results of Effect Size Analysis. Effect sizes were calculated for the factors and interactions that were significant in the variance analysis using the formula of 2.4.2, ignoring any three or more variable interactions. The average of all 27 combination treatments was 1.83 mg/100 mg DW. The results in Table 6 show that the effect size for A_2 was +0.34 mg/100 mg DW, which was the highest for all the levels. Among the thirty-six interaction effect sizes that were significant in the variance analysis, the highest positive effect was (B_2×D_2) with a value of +0.19 mg/100 mg DW, whereas the highest negative effect was (B_1×C_1) with a value of −0.26 mg/100 mg DW.

Discussion

Examining the effect of environmental factors on the GA content is the first step to understanding the molecular mechanisms of GA biosynthesis in *G. lucidum*. Although several previous reports have already investigated the effect of factors on GA content, the analyses have only compared different levels of factors, without considering their interactions. Therefore, this study used an orthogonal design to analyze the effects of four factors (carbon source, nitrogen source, mineral ion, and pH) on the GA content produced by *G. lucidum* in a submerged culture. Whereas an orthogonal design usually requires 3^4×3 replicates, *i.e.*, 243 experiments, to achieve the experimental goals for full-factor experimental projects, the L_{27} (3^{13}) orthogonal design only requires 27×3 replicates (=81) to provide an effective response, thereby saving a lot of time and resources. This is because one of the most important properties of the design is comparability and orthogonality, *i.e.*, the ability to separate the individual effects of several variables in an experiment. Thus, a large number of factors can be tested simultaneously with a relatively small number of experimental runs, and the effects of each parameter can be estimated separately. This method has already been successfully applied to the improvement of culture media for the production of primary and secondary metabolites in fermentation processes

Table 6. Effect sizes for levels and interactions.

		A			B			C			D		
		A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	D_1	D_2	D_3
	A_1	−0.42									−0.08	+0.10	−0.02
A	A_2		+0.34								−0.12	−0.08	−0.02
	A_3			+0.09							−0.02	−0.02	+0.03
	B_1				+0.02			−0.26	+0.11	+0.14	+0.05	−0.05	−0.01
B	B_2					+0.01		+0.14	−0.19	+0.05	−0.10	+0.19	−0.08
	B_3						−0.03	+0.11	+0.08	−0.18	+0.04	−0.13	+0.09
	C_1							+0.08			+0.04	−0.06	+0.01
C	C_2								−0.11		−0.13	−0.04	+0.18
	C_3									+0.03	+0.08	+0.11	−0.19
	D_1										+0.04		
D	D_2											+0.01	
	D_3												−0.05

The diagonal indicates the effects of every level, whereas the triangle indicates the effects of the interactions that were dominant.

involving many factors [3, 16, 25]. Satisfactory results were also obtained in the present study. First, the most important factor among the four factors employed was the carbon source (factor A) and the best level was A_2 (sucrose), with which the effect value reached up to +0.34 mg/100 mg DW. The three levels of nitrogen source, mineral ion, and pH had relatively lower values. Second, the results showed that the interactions should not be neglected, as several interaction effect values were higher than the level effect values. Third, the optimum media was a combination treatment of $A_2B_1C_3D_1$ with which the GA content reached up to 2.63 mg/100 mg DW. With the combination of $A_2B_1C_3D_1$, the interaction effect size ($B_1 \times C_3$) was 0.14 mg/100 mg DW, which was just lower than that for A_2, indicating that the optimum combination was different from the result of the one-factor-at-a-time experiment because of the interaction effect. The effects of the factors and their interactions on the GA content were confirmed in this experiment.

According to previous papers, the supplementation of certain mineral ions to the medium can accelerate the polysaccharide product [1, 12, 13, 15]. This is because some mineral ions recognized as an enzyme cofactor or favorable bioelement may be involved in the metabolism and synthesis, thereby affecting the biosynthesis of secondary metabolites. However, since there have been no previous reports on whether or not GA is affected by mineral ions, the present study added several mineral ions to the media to investigate their effect on the GA content. From an intuitive analysis, it was found that the mineral ions had a significant effect on the GA content. The best level of mineral ion was C_1 (ferrous sulfate), with which the effect size was +0.08 mg/100 mg DW. The next level was C_3 (calcium chloride) and the worst level was C_2 (zinc sulfate), with respective values of +0.03 and −0.11 mg/100 mg DW. The results from a variance analysis showed that the interactions between the nitrogen source and mineral ion, and pH and mineral ion were both significant, where the interaction effect sizes for ($B_2 \times C_1$) and ($B_1 \times C_3$) were both +0.14 mg/100 mg DW, and the effect size for ($C_2 \times D_3$) was +0.18 mg/100 mg DW, which were higher than the effect sizes for C_1, C_2, and C_3. Therefore, these results show that the effect of the mineral ions was mainly interaction related.

The interactions occurring in media with multiple components can have an ultimate affect on the product. Thus, it is very important to study both the function of factors and their interactions. In the present experiment, thirty-six out of fifty-four pair interactions were found to be significant, where seventeen had positive effects and nineteen had negative effects on the GA content. The highest positive effect was ($B_2 \times D_2$) with a value of +0.19 mg/100 mg DW, which was lower than the level effect size of A_2. The next highest positive interaction effect was ($C_2 \times D_3$) with a value of +0.18 mg/100 mg DW. The highest negative effect was ($B_1 \times C_1$) with a value of −0.26 mg/100 mg DW, which was lower than the level effect size of A_1. Therefore, these results prove that interactions should not be neglected.

On the basis of confirming the optimal cultural conditions for the GA content and cloning the key enzymes, this will provide a good knowledge of the molecular mechanisms involved in the biosynthesis of GA in *G. lucidum*.

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