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Research article

Enhancement of botrallin and TMC-264 production in liquid culture of endophytic fungus *Hyalodendriella* sp. Ponipodef12 after treatments with metal ions

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ABSTRACT

Background: *Hyalodendriella* sp. Ponipodef12, an endophytic fungus from a poplar hybrid, was a high producer of botrallin and TMC-264 with various bioactivities. In this study, the influences of eight metal ions (*i.e.*, Mn^{2+}, Na^{+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+}) on botrallin and TMC-264 production in liquid culture of the endophytic fungus *Hyalodendriella* sp. Ponipodef12 were investigated.
Results: Three most effective metal ions (Zn^{2+}, Cu^{2+} and Mg^{2+}) along with their optimum concentrations were screened. The optimum addition time and concentrations of Zn^{2+}, Cu^{2+} and Mg^{2+} were further obtained respectively for improving botrallin and TMC-264 production. The combination effects of Zn^{2+}, Cu^{2+} and Mg^{2+} on the production of botrallin and TMC-264 by employing statistical method based on the central composite design (CCD) and response surface methodology (RSM) were evaluated, and two quadratic predictive models were developed for botrallin and TMC-264 production. The yields of botrallin and TMC-264, which were predicted as 144.12 mg/L and 36.04 mg/L respectively, were validated to be 146.51 mg/L and 36.63 mg/L accordingly with the optimum concentrations of Zn^{2+} at 0.81 mmol/L, Cu^{2+} at 0.20 mmol/L, and Mg^{2+} at 0.13 mmol/L in medium.
Conclusion: The results indicated that the enhancement of botrallin and TMC-264 accumulation in liquid culture of the endophytic fungus *Hyalodendriella* sp. Ponipodef12 by the metal ions and their combination should be an effective strategy.

1. Introduction

Endophytic fungi are eukaryotic organisms that inhabit intra- and/or inter-cellular healthy tissues of the plants without causing discernible symptoms of plant disease [1]. These endophytes have proved to be a promising source of natural products with novel structures and/or strong bioactivities to show their applications in pharmaceutical, agricultural and food industry [2,3].

Hyalodendriella sp. Ponipodef12 was an endophytic fungus derived from the healthy stems of poplar hybrid 'Neva' of *Populus deltoides* Marsh × *P. nigra* L. [4]. In our previous studies, four dibenzo-α-pyrones namely botrallin, TMC-264, palmariol B, and alternariol 9-methyl ether were obtained from *Hyalodendriella* sp. Ponipodef12. Among them, botrallin and TMC-264 (Fig. 1) were found to be the predominant bioactive components [5,6,7]. Botrallin has been isolated from the fungi *Botrytis allii* [8], *Microsphaeropsis olivacea* [9] and *Hyalodendriella* sp. [5], and exhibited antimicrobial, antinematodal and acetylcholinesterase inhibitory activities [5,9]. TMC-264 has been isolated from the fungi *Phoma* sp. [10] and *Hyalodendriella* sp. [6]. It also showed antimicrobial and antinematodal activities [6]. In addition, TMC-264 selectively inhibited interleukin-4 (IL-4) signaling by interfering with phosphorylation of the signal transducer and activator of transcription 6 (STAT6), as well as binding of the phosphorylated STAT6 to the recognition sequence, so it might be an inhibitor of IL-4 signaling for treatment of allergic diseases [11].

Various strategies have been developed to increase metabolite yield in microorganism or plant cultures, which include optimization of medium, utilization of two-phase culture systems, addition of precursors and metal ions, as well as application of elicitation by using polysaccharides and oligosaccharides [12,13,14,15,16,17,18,19,20]. Many metal ions (*i.e.*, K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+} and Mo^{5+}), which played important roles

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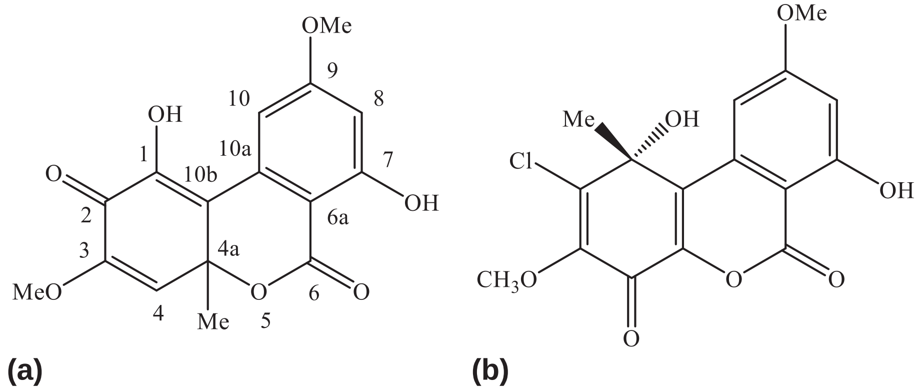

Fig. 1. Structures of botrallin (a) and TMC-264 (b).

in cell growth and metabolism, were essential for microorganisms [21,22,23]. In order to speed up application of botrallin and TMC-264, one of the most important approaches is to increase the yields of botrallin and TMC-264 in fermentation culture of *Hyalodendriella* sp. Ponipodef12. In our previous studies, obvious enhancement of botrallin and TMC-264 production in the liquid culture of *Hyalodendriella* sp. Ponipodef12 was achieved by *in situ* resin adsorption [24]. In this study, the effects of eight metal ions on the production of botrallin and TMC-264 in liquid culture of *Hyalodendriella* sp. Ponipodef12 were investigated. Firstly, the single metal ion at its different concentrations was added in medium to screen its enhancing effect by "one-factor-at-a-time (OFAT)", and three most effective metal ions (Zn^{2+}, Cu^{2+} and Mg^{2+}) with their optimum concentrations were screened. Secondly, three effective metal ions (Zn^{2+}, Cu^{2+} and Mg^{2+}) along with their addition time were studied to obtain the appropriate combination of addition time and concentration for each ion for improving botrallin and TMC-264 production. Lastly, the combination effects of Zn^{2+}, Cu^{2+} and Mg^{2+} on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12 were studied by employing statistical method based on the central composite design (CCD) and response surface methodology (RSM) analysis to develop the quadratic predictive models and obtain the maximal yields of botrallin and TMC-264. To the best of our knowledge, the effects of metal ions on botrallin and TMC-264 production of *Hyalodendriella* sp. Ponipodef12 have not yet been reported. The purpose was to investigate the enhancing effects of the metal ions for botrallin and TMC-264 biosynthesis in liquid culture of *Hyalodendriella* sp. Ponipodef12, as well as to provide data supporting dibenzo-α-pyrone production on a large scale.

2. Materials and methods

2.1. Endophytic fungus and culture conditions

Endophytic fungus *Hyalodendriella* sp. Ponipodef12 (GenBank accession number HQ731647) was isolated from the healthy stems of the 'Neva' hybrid of *P. deltoides* Marsh x *P. nigra* L in our previous study [4]. It was stored both on PDA (potato 200 g/L, dextrose 20 g/L and agar 20 g/L) slants at 4°C and in 40% glycerol at −70°C in the Herbarium of the College of Plant Protection, China Agricultural University (Beijing, China). The fungus was cultured on PDA medium in Petri dishes at 25°C for 10 d. For seed culture, four plugs of agar medium (0.4 × 0.4 cm) with fungal cultures were inoculated in each 250-mL Erlenmeyer flask containing 100 mL potato dextrose broth (PDB) medium, and incubated on a rotary shaker at 150 rpm and 25°C for 5 d. For fermentation culture, about 14 mycelia pellets were inoculated in each 250-mL Erlenmeyer flask containing 80 mL PDB medium, and incubated on a rotary shaker at 150 rpm and 25°C.

2.2. Preparation of the media containing metal ions

The cultures were supplemented with $MnSO_4 \times H_2O$, $MgSO_4 \times 7H_2O$, Na_2SO_4, $FeSO_4 \times 7H_2O$, $ZnSO_4 \times 7H_2O$, $CuSO_4 \times 5H_2O$, $FeCl_3 \times 6H_2O$, $AlCl_3 \times 6H_2O$ and $Al(NO_3)_3 \times 9H_2O$, for Mn^{2+}, Mg^{2+}, Na^{+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+} and Al^{3+} on day 10 of culture, respectively. Each inorganic salt was dissolved with sterile water, filtrated through a microfilter (pore size, 0.22 μm). The levels considered for the metal ions Mn^{2+}, Mg^{2+}, Na^{+}, Fe^{2+}, Zn^{2+} and Cu^{2+} were set at 0.1, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 mmol/L and the levels considered for Fe^{3+} and Al^{3+} were set at 0.1, 0.5, 1.0, 2.0, and 4.0 mmol/L based on the gradient dilution method. Each experiment had three replicates including the control cultures without addition of metal ions. As Zn^{2+}, Cu^{2+} and Mg^{2+} were found to be the most effective metal ions (Tables S1, S2, and S3), they were applied in the next experiments at different concentrations (0.25, 0.50, 0.75, 1.0 and 1.25 mmol/L for Zn^{2+}; 0.125, 0.25, 0.50, 0.75 and 1.0 mmol/L for Cu^{2+}; 0.0625, 0.125, 0.25, 0.50, 1.0 and 2.0 mmol/L for Mg^{2+}) on days 0, 5, 10, 15 and 20 of culture, respectively. For investigating the combination effects of Zn^{2+}, Cu^{2+} and Mg^{2+} on the botrallin and TMC-264 production, the final concentrations of the metal ions were set by the software of Design Expert 8.0 (Stat-Ease, USA).

2.3. Analytical procedures

The mycelia of the endophytic fungus *Hyalodendriella* sp. Ponipodef12 were separated from the fermentation broth by filtration under vacuum through a pre-weighed filter paper. It was then rinsed three times with distilled water, dried in an oven at 60°C to a constant dry weight (dw), and then the mycelia dry weight was obtained.

Botrallin and TMC-264 extraction and quantification in the samples were based on the methods as described previously [6,24]. For botrallin and TMC-264 analysis in mycelia, 100 mg of dry mycelia powder was deposited into a vial with 10 mL of ethyl acetate, and then subjected to ultrasonic treatment (three times, 60 min each). After removal of the solid by filtration, the filtrate was evaporated to dryness and redissolved in 1 mL of methanol. For analysis of botrallin and TMC-264 yield in medium, 5 mL of the culture broth was evaporated to dryness and extracted with 5 mL of ethyl acetate, and the liquid extraction was then evaporated to dryness and redissolved in 1 mL of methanol.

The content of botrallin and TMC-264 was analyzed by a high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan), which consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, a SPD-M20A photodiode array detector, and CBM-20Alite system controller. A reversed-phase Ultimate TM XB C_{18} column (250 mm × 4.6 mm, 5 μm, Welch Materials, Inc., Ellicott, MD, USA) was used for separation by using a mobile phase of methanol-H_2O

(60:40, v/v) at a flow rate of 1 mL/min. The temperature was maintained at 40°C, and UV detection at 234 nm. The sample injection volume was 10 μL. The LC-solution multi-PDA workstation was employed to acquire and process chromatographic data. Botrallin and TMC-264 were detected and quantified with the standards prepared according to the previous method [5,6]. The linear equation of botrallin by HPLC analysis was $Y = 5.71711\ 9\ 106X - 85{,}792.5$ ($R^2 = 0.9997$), and that of TMC-264 was $Y = 1.62393\ 9\ 107X - 137{,}986$ ($R^2 = 0.9981$), where Y was the peak area, X was quality (μg) of the sample injected for each time, and R was the correlation coefficient.

2.4. Methodology and design of experiments

The optimal concentrations of Zn^{2+}, Cu^{2+} and Mg^{2+} for the enhancement of botrallin and TMC-264 production by *Hyalodendriella* sp. Ponipodef12 were determined by means of CCD and RSM using Design Expert 8.0 (Stat-Ease, USA). 100 μL of metal ion solutions was added to 80 mL medium in the experiments with different concentrations of $ZnSO_4$, $CuSO_4$ and $MgSO_4$ on day 15, respectively. The three factors were designated as X_1, X_2, X_3, and each independent variable (final metal ion concentration) in the CCD experiments was studied at five coded levels (−1.682, −1, 0, +1, +1.682) (Table 1) and a set of 20 experiments were carried out (Table 2). The factors were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X, i = 1, 2, 3 \quad \text{[Equation 1]}$$

where x_i was the coded value of the variable X_i, while X_0 was the value of X_i at the center point, and ΔX was the step change of an independent variable.

The response variable (botrallin and TMC-264 production) was explained by the following second-order polynomial equation:

$$Y = a_0 + a_1 x_1 + a_2 x_2 + a_3 x_3 + a_{12} x_1 x_2 + a_{13} x_1 x_3 + a_{23} x_2 x_3 + a_{11} x_1^2 + a_{22} x_2^2 + a_{33} x_3^2 \quad \text{[Equation 2]}$$

where Y was the predicted response value; a_0 was the intercept term; x_1, x_2 and x_3 were coded independent variables; a_1, a_2 and a_3 were linear coefficients; a_{12}, a_{13} and a_{23} were interaction coefficients; and a_{11}, a_{22} and a_{33} were the quadratic term coefficients. All of the coefficients of the second polynomial model and the responses obtained from the experimental design were subjected to multiple nonlinear regression analyses.

The fitness of the second-order polynomial model equation was evaluated by the coefficient (R^2) of determination. The analysis of variance (ANOVA) and test of significance for regression coefficients were conducted by F-test. In order to visualize the relationship between the response values and test independent variables, the fitted polynomial equation was separately expressed as 3D response surfaces and 2D contour plots by the software of Design Expert [25].

2.5. Statistical analysis

All tests were carried out in triplicate, and the results were represented by their mean values and the standard deviations (SD).

Table 1
Coded values (x) and uncoded values (X) of variables in the CCD experiments.

Variable (mmol/L)	Symbol		Coded level				
	Uncoded	Coded	−1.682	−1	0	+1	+1.682
Zn^{2+}	X_1	x_1	0.1591	0.5000	1.0000	1.5000	1.8409
Cu^{2+}	X_2	x_2	0.0398	0.1250	0.2500	0.3750	0.4602
Mg^{2+}	X_3	x_3	0.0199	0.0625	0.1250	0.1875	0.2301

Table 2
Central composite design matrix for the experimental design and predicted responses for botrallin and TMC-264 production.

Run	Coded level			Botrallin yield (mg/L)		TMC-264 yield (mg/L)	
	x_1	x_2	x_3	Observed	Predicted	Observed	Predicted
1	−1	1	−1	60.00	60.47	15.00	14.34
2	1	−1	−1	57.80	56.77	8.40	7.40
3	−1	−1	−1	100.00	100.17	25.50	25.14
4	1	−1	1	68.10	67.33	15.00	15.02
5	0	1.682	0	59.20	58.97	12.30	12.28
6	1	1	1	40.10	39.62	12.50	12.21
7	0	0	−1.682	47.50	47.93	10.40	11.24
8	−1	1	1	50.70	51.42	7.70	8.06
9	0	0	0	135.40	137.95	33.75	34.77
10	1.682	0	0	39.90	41.46	11.00	11.39
11	−1	−1	1	93.00	93.28	24.70	23.96
12	0	0	0	140.00	137.95	35.50	34.77
13	−1.682	0	0	89.00	87.88	22.30	22.82
14	0	0	1.682	49.20	49.20	12.30	12.37
15	0	−1.682	0	115.00	115.66	22.80	23.73
16	0	0	0	138.00	137.95	36.00	34.77
17	0	0	0	138.40	137.95	34.50	34.77
18	0	0	0	136.00	137.95	34.00	34.77
19	0	0	0	140.00	137.95	35.00	34.77
20	1	1	−1	31.80	31.22	9.60	9.69

The data were submitted to analysis of variance (one-way ANOVA) to detect significant differences by PROC ANOVA of SAS version 9.1. The term significant has been used to denote the differences for which $p \leq 0.05$.

3. Results

3.1. Effects of metal ions on botrallin and TMC-264 production

Eight metal ions (*i.e.*, Mn^{2+}, Na^{+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+}) were separately added in medium on day 10 of culture. The effects of the metal ions on mycelia growth and production of botrallin and TMC-264 in liquid culture of *Hyalodendriella* sp. Ponipodef12 were presented in Tables S1, S2, S3, S4, S5, S6, S7, S8, and S9. The enhanced capacity of the metal ions for botrallin and TMC-264 production was in order of $Zn^{2+} > Cu^{2+} > Mg^{2+} > Al^{3+}$ (in the form of $AlCl_3$) $> Na^{+} > Fe^{3+} > Fe^{2+} > Mn^{2+}$ respectively at their appropriate concentrations. Three metal ions, Zn^{2+}, Cu^{2+} and Mg^{2+} were the most effective to enhance production of botrallin and TMC-264. They were selected for further enhancing experiments for botrallin and TMC-264 production in liquid culture of endophytic fungus *Hyalodendriella* sp. Ponipodef12.

Addition time of the metal ions has been considered as a main factor to affect biosynthesis of fungal metabolites [14]. Zn^{2+}, Cu^{2+} and Mg^{2+} were found to be effective to improve botrallin and TMC-264 biosynthesis in liquid culture of *Hyalodendriella* sp. Ponipodef12 in this study. Hence, their concentrations in combination with addition time were further optimized. As the ten-day-old cultures treated with 0.5–1.0 mmol/L of Zn^{2+} reached ideal botrallin and TMC-264 production, the highest concentration of Zn^{2+} in subsequent studies was limited at 1.25 mmol/L. Similarly, the highest concentrations of Cu^{2+} and Mg^{2+} were set at 1.00 mmol/L and 2.00 mmol/L, respectively. Fig. 2 showed the effects of Zn^{2+}, Cu^{2+} and Mg^{2+} on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12, which were dependent on both their concentrations and addition time (added on days 0, 5, 10, 15 and 20).

The effects of Zn^{2+} and its addition time on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12 were shown in Fig. 2a and Table S10. When the cultures were fed with

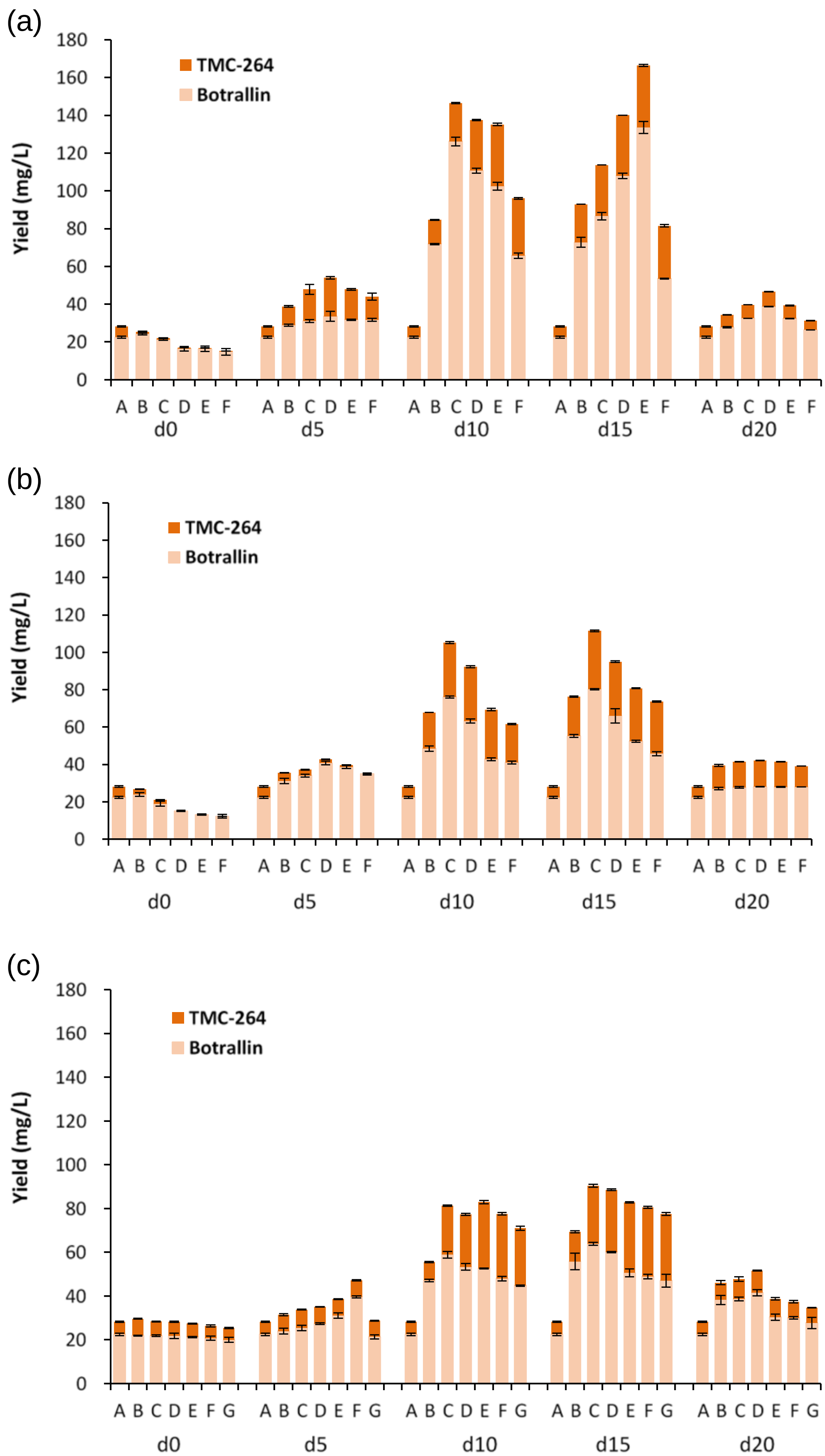

Fig. 2. Effects of Zn^{2+} (a), Cu^{2+} (b) and Mg^{2+} (c) and their addition time on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12. Zn^{2+}, Cu^{2+} and Mg^{2+} were separately in the form of $ZnSO_4$, $CuSO_4$ and $MgSO_4$ added in medium. In Fig. 2a, the letters A—F meant Zn^{2+} concentrations in medium as 0.00, 0.25, 0.50, 0.75, 1.00 and 1.25 mmol/L, respectively. In panel b, the letters A—F meant Cu^{2+} concentrations in medium as 0.00, 0.125, 0.25, 0.50, 0.75 and 1.00 mmol/L, respectively. In panel c, the letters A—G meant Mg^{2+} concentrations in medium as 0.00, 0.0625, 0.125, 0.25, 0.50, 1.00 and 2.00 mmol/L, respectively. The codes d0, d5, d10, d15 and d20 meant the metal ions with their concentrations were added in medium on days 0, 5, 10, 15 and 20 of culture, respectively.

0.25–1.25 mmol/L of Zn^{2+} on days 10 and 15, the production of botrallin and TMC-264 was improved obviously. With 1.00 mmol/L of Zn^{2+} added on day 15, the highest yields of botrallin (133.54 mg/L) and TMC-264 (32.83 mg/L) were obtained. The total yield (botrallin plus TMC-264) was improved to reach 166.37 mg/L, which was about 5.90-fold of the control yield (28.22 mg/L).

Fig. 2b and Table S11 showed the effects of Cu^{2+} on botrallin and TMC-264 production in *Hyalodendriella* sp. Ponipodef12 liquid cultures, which were dependent on both Cu^{2+} concentrations (0.125 to 1.00 mmol/L) and its addition time (added on days 5, 10, 15 and 20). As shown in Fig. 2b, with 0.25 mmol/L of Cu^{2+} fed on day 15, the highest yields of botrallin (80.20 mg/L) and TMC-264 (31.29 mg/L) were obtained. The total yield of botrallin plus TMC-264 was improved to reach 111.50 mg/L, which was about 3.95-fold of the control yield (28.22 mg/L).

The further optimized concentrations of Mg^{2+} were set as 0.0625, 0.125, 0.25, 0.50, 1.00 and 2.00 mmol/L, and were added on days 0, 5, 10, 15 and 20, respectively. The effects of Mg^{2+} on mycelia growth and production of botrallin and TMC-264 were showed in Fig. 2c and Table S12. When the cultures were fed with 0.0625–2.00 mmol/L of Mg^{2+} on days 10 and 15, the production of botrallin and TMC-264 was improved obviously. The highest yields of botrallin (63.85 mg/L) and TMC-264 (32.21 mg/L) were obtained when Mg^{2+} was added on day 15 at 0.125 mmol/L and 0.50 mmol/L, respectively. The total yield of botrallin plus TMC-264 was improved to reach 90.34 mg/L, which was about 3.20-fold of the control yield (28.22 mg/L).

3.2. Combination effects of copper, zinc and magnesium ions on botrallin and TMC-264 production

According to the above single-factor experiments, Zn^{2+}, Cu^{2+} and Mg^{2+} all showed their obvious effects on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12 separately at their most suitable concentrations and addition time which were 1.00 mmol/L of Zn^{2+} added on day 15, 0.25 mmol/L of Cu^{2+} added on day 15, and 0.125 mmol/L of Mg^{2+} added on day 15. In order to study the combination effects of Zn^{2+}, Cu^{2+} and Mg^{2+}, the suitable concentrations of Zn^{2+}, Cu^{2+} and Mg^{2+} in medium for botrallin and TMC-264 production were determined using CCD experiments and RSM analysis. Three ions were all added on day 15, and five levels of each variable (metal ion concentration, mmol/L) were set by the software of Design Expert 8.0 (Stat-Ease, USA), which are presented in Table 1. Subsequently, 20 trials of CCD were carried out to optimize the production of botrallin and TMC-264. The values of response Y_1 (botrallin yield) and Y_2 (TMC-264 yield) under the different ions combinations were presented in Table 2. There was a considerable variation of botrallin and TMC-264 yields depending upon the different ions combination. Botrallin and TMC-264 yields ranged from 31.80–140.00 mg/L, and 7.70–36.00 mg/L, respectively.

The empirical relationships between botrallin and TMC-264 yields (Y_1 and Y_2) and the tested variables (metal ion concentrations) were obtained by application of RSM. By employing multiple regression analysis on the experimental data, the response variables (Y_1 and Y_2) and the tested variables were related by the following second-order polynomial equations [Equation 3 and Equation 4]:

$$Y_1\ (\text{Botrallin yield}) = 137.95 - 13.80\,x_1 - 16.85\,x_2 + 0.38\,x_3 + 3.54\,x_1x_2 + 4.36\,x_1x_3 - 0.54\,x_2x_3 - 25.91\,x_1^2 - 17.90\,x_2^2 - 31.60\,x_3^2 \quad \text{[Equation 3]}$$

$$Y_2\ (\text{TMC-264 yield}) = 34.77 - 3.40\,x_1 - 3.40\,x_2 + 0.34\,x_3 + 3.28\,x_1x_2 + 2.20\,x_1x_3 - 1.27\,x_2x_3 - 6.24\,x_1^2 - 5.93\,x_2^2 - 8.12\,x_3^2 \quad \text{[Equation 4]}$$

Statistical testing of the model was performed with Fisher's *F*-test to obtain the mathematical relationship between response and process variables. In order to ensure a good model, the test for significance of regression model was performed and applied with the ANOVA. Table 3 showed the results of ANOVA for production of botrallin and TMC-264. Values of prob. (p) > F less than 0.05 indicated model terms are significant for production of botrallin and TMC-264. The non-significant lack-of-fit (more than 0.05) showed that each quadratic model was valid for present study. Non-significant lack-of-fit was good for data fitness in the model of this study. The predicted R^2 of 0.9973 and adjusted R^2 of 0.9984 for botrallin, and predicted R^2 of 0.9815 and determination coefficients adjusted R^2 of 0.9928 for TMC-264 were reasonable agreement with their values of R^2 (0.9992 and 0.9962), which were closer to 1.0, indicated the better fitness of model in the experimental data. In addition, low variation coefficient (CV) of models for botrallin and TMC-264 indicated that the quadratic multinomial regression models for botrallin and TMC-264 had high reliability.

There were no significant differences between predicted and actual values in 20 trials of CCD for botrallin and TMC-264 yields shown in Table 2, which indicated that the models had high degree of fit. Furthermore, we also analyzed for the significance of each secondary coefficient of the quadratic multinomial regression model with the results shown in Table 4. Statistical testing of the model was performed with Fisher's *F*-test. Greater *F*-value and smaller *p*-value indicated that second item had more significant effect on *Y* values. Table 4 showed that most regression coefficients, especially all quadratic term coefficients of the two models were very significant, and demonstrated the research variables (*i.e.*, Zn^{2+}, Cu^{2+}, Mg^{2+} concentrations) and their interactions had significant roles in the formation of botrallin and TMC-264.

The three-dimensional (3D) response surface and two-dimensional (2D) contour plots were the graphical representation of the regression equation used to determine the optimum values of the variables within the ranges considered [26]. The entire relationships between

Table 3
ANOVA for the second-order polynomial models.

Compound	Source	SS	DF	MS	*F*-value	Prob. (p) > F	
Botrallin	Model	31,048.77	9	3449.86	1309.18	<0.0001	Significant
	Residual	26.34	10	2.63			
	Lack of fit	7.43	5	1.49	0.39	0.8360	Not significant
	Pure error	18.91	5	3.78			
	Corrected total	31,075.11	19				
$R^2 = 0.9992$; Adj-$R^2 = 0.9984$; Predicted $R^2 = 0.9973$; CV = 1.88%							
TMC-264	Model	2149.25	9	238.81	293.61	<0.0001	Significant
	Residual	8.13	10	0.81			
	Lack of fit	4.33	5	0.87	1.14	0.4449	Not significant
	Pure error	3.80	5	0.76			
	Corrected total	2157.38	19				
$R^2 = 0.9962$; Adj-$R^2 = 0.9928$; Predicted $R^2 = 0.9815$; CV = 4.31%							

SS, sum of squares of model parameters; *DF*, degree of freedom; *MS*, mean square of model parameters, *CV*, coefficient of variance; $PRESS_1 = 83.63$; $PRESS_2 = 39.81$.

Table 4
Regression results of the central composite design.

Compound	Factor	Coefficient	$p-$Value
Botrallin	Intercept	137.95	
	x_1	-13.80	<0.0001
	x_2	-16.85	<0.0001
	x_3	0.38	0.4098
	$x_1 x_2$	3.54	0.0001
	$x_1 x_3$	4.36	<0.0001
	$x_2 x_3$	-0.54	0.3710
	x_1^2	-25.91	<0.0001
	x_2^2	-17.90	<0.0001
	x_3^2	-31.60	<0.0001
$TMC-264$	Intercept	34.77	
	x_1	-3.40	<0.0001
	x_2	-3.40	<0.0001
	x_3	0.34	01980
	$x_1 x_2$	3.28	<0.0001
	$x_1 x_3$	2.20	<0.0001
	$x_2 x_3$	-1.27	0.0025
	x_1^2	-6.24	<0.0001
	x_2^2	-5.93	<0.0001
	x_3^2	-8.12	<0.0001

reaction factors and responses could be better understood by examining the planned series of response surface plots generated from the predicted models [Equation 3 and Equation 4] by using the Design Expert software 8.0. The interactive effects among the three independent variables being at fixed level on the production of botrallin and TMC-264 were shown in 3D surface and 2D contour plots (Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8), respectively. The maximum predicted value was identified by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours could be obtained when there was a perfect interaction between the independent variables.

An elliptical response surface in the entire region was found from the second order quadratic equation for the botrallin production with the interaction of Zn^{2+} and Cu^{2+} concentrations (Fig. 3). The results showed that botrallin production was considerably affected by varying the concentrations of Zn^{2+} and Cu^{2+}. The maximum production of botrallin was predicted at the given ranges of both Zn^{2+} and Cu^{2+} concentrations. The production decreased at the maximum and minimum values of ranges considered in both parameters.

Fig. 4 shows the response surface plots for variation in the botrallin production, as a function of Cu^{2+} and Mg^{2+} concentration by keeping the concentration of Zn^{2+} at 1.0 mmol/L. The production of botrallin was affected by the concentrations of Cu^{2+} and Mg^{2+}. The production of botrallin increased with increasing of Cu^{2+} concentration at a certain level. The effect of Mg^{2+} on botrallin production was similarly with Cu^{2+}.

Fig. 5 also showed the elliptical response surface plots of botrallin production as a function of Zn^{2+} and Mg^{2+} concentration. The predicted botrallin production decreased at the higher and lower values of ranges for both Zn^{2+} and Mg^{2+} concentrations. Maximum production was obtained near the center points of the response surface.

Among Fig. 6, Fig. 7 and Fig. 8, all were found an elliptical response surface in the entire region from the second order quadratic equation for interactions cyclically on the yields of TMC-264 between two variables among three variables. The TMC-264 production was considerably affected by varying the concentrations of Zn^{2+}, Cu^{2+} and Mg^{2+}. The maximum production of TMC-264 was predicted at given ranges of Zn^{2+}, Cu^{2+} and Mg^{2+}concentration. The TMC-264 production decreased at the maximum and minimum values of ranges considered among three parameters.

The highest yields of botrallin and TMC-264 were predicted as 144.12 mg/L and 36.04 mg/L by solving the quadratic regression equations [Equation 3 and Equation 4] between botrallin and TMC-265 yields and three metal ions at the optimal concentrations of Zn^{2+} at 0.81 mmol/L; Cu^{2+} at 0.20 mmol/L; Mg^{2+} at 0.13 mmol/L in medium. In order to verify the optimization results as well as to validate the model developed, a set of experiment with five replications was performed according to the media constituent presented in Table 5. Under the determined conditions, the mean yield values of botrallin (146.51 mg/L) and TMC-264 (36.63 mg/L) were obtained from the actual experiments, which were individually 6.84-fold and 6.44-fold of those (21.41 mg/L and 5.69 mg/L) in the original basal medium. Based on the Student *t*-test, the above model was satisfactory and adequate for reflecting the expected optimization as no significant differences were observed between the predicted maximum yields of botrallin and TMC-264 and the experimental ones.

4. Discussion

Metal ions belong to the abiotic elicitors and play an integral role in the growth and metabolite production of microorganisms, and the addition of metal ions to enhance secondary metabolite production of fungi has been widely reported [27]. The mycelia pellet formation and fumaric acid production were significantly affected by the trace metal ions Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} in fermentation culture of *Rhizopus oryzae* ATCC 20344 [28]. The mycelia growth and polysaccharide production were obviously enhanced by the metal ions Zn^{2+}, Se^{2+} and Fe^{2+} in submerged culture of *Ganoderma lucidum* [29]. Versicolorin production of *Aspergillus parasiticus* was completely dependent on

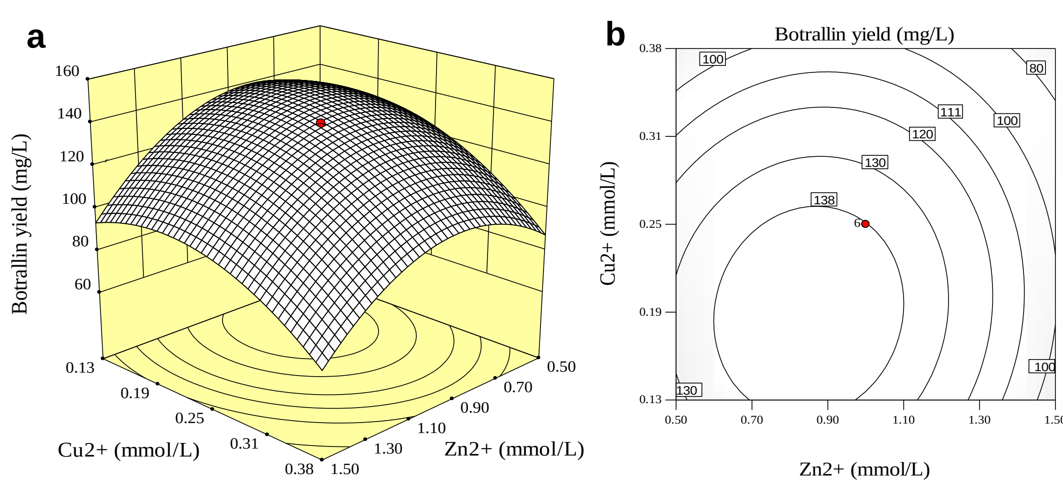

Fig. 3. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of botrallin yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables Zn^{2+} and Cu^{2+} concentration (mmol/L).

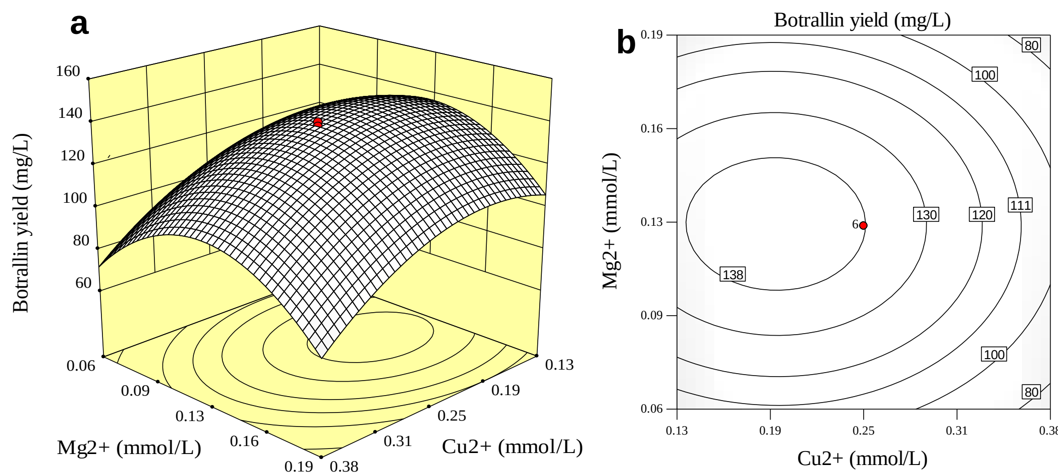

Fig. 4. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of botrallin yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables Cu^{2+} and Mg^{2+} concentration (mmol/L).

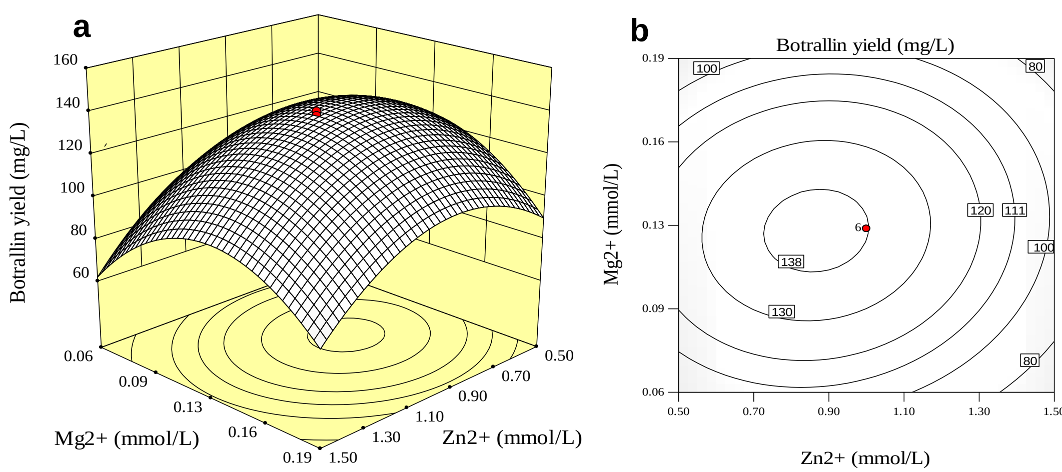

Fig. 5. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of botrallin yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables: Zn^{2+} and Mg^{2+} (mmol/L).

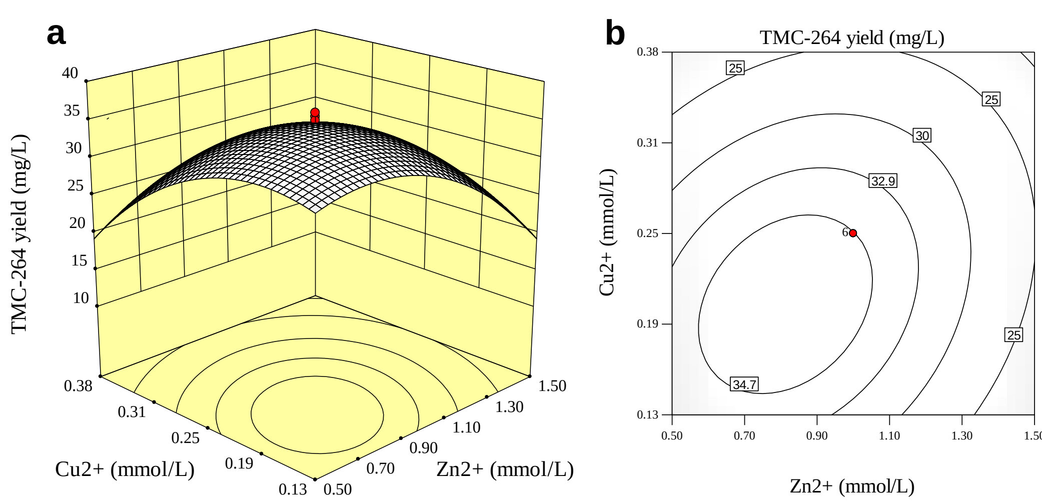

Fig. 6. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of TMC-264 yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables: Zn^{2+} and Cu^{2+} (mmol/L).

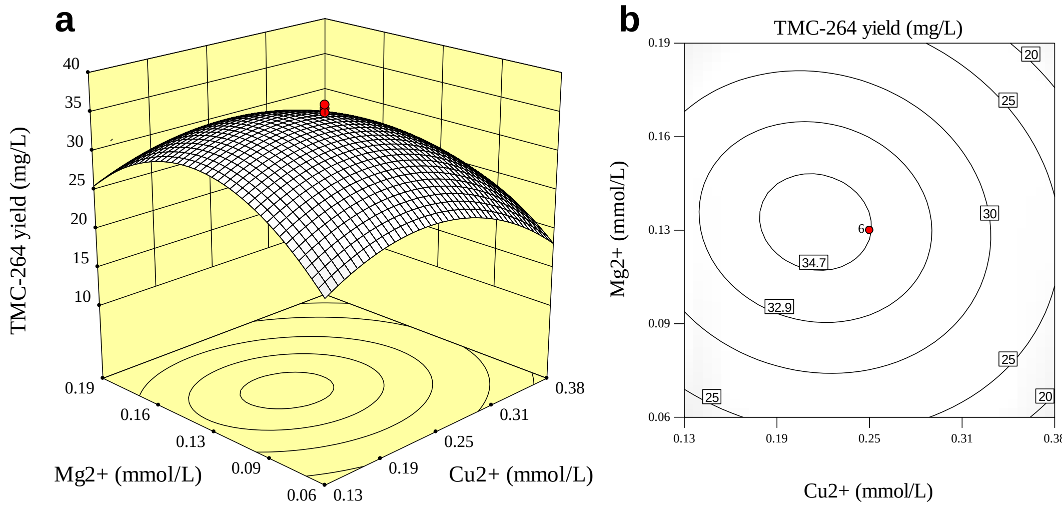

Fig. 7. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of TMC-264 yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables: Cu^{2+} and Mg^{2+} (mmol/L).

Zn^{2+} [30]. Cu^{2+} stimulated dipicolinic acid synthesis in *Penicillium citreoviride* strain 3114 [31], and improved the production of laccase by the white-rot fungus *Pleurotus pulmonarius* in solid state fermentation [32].

Addition of two or more metal ions in medium could synergistically enhance or inhibit metabolite production of fungi. Zinc ion (Zn^{2+}) promoted the production of alternariol, and Mn^{2+} acted synergistically with Zn^{2+} to provide a further three fold stimulation of alternariol synthesis in *Alternaria alternata* [33]. Zn^{2+} enhanced the production of ergot and quinoline alkaloids, and both Fe^{2+} and Cu^{2+} synergistically inhibited alkaloid biosynthesis in the cultures of *Penicillium citrinum* [34]. Ca^{2+}, Cu^{2+} and Al^{3+} synergistically enhanced palmarumycin production in liquid culture of endophytic fungus *Berkleasmium* sp. Dzf12. When Al^{3+} concentration was higher than 4 mmol/L, palmarumycin C_{12} production was favored, and palmarumycin C_{13} production was suppressed [23].

5. Conclusion

In this work, the metal ions were first studied for their stimulatory effects on botrallin and TMC-264 production in liquid culture of *Hyalodendriella* sp. Ponipodef12. Based on the one-factor-at-a-time (OFAT) experiments, three metal ions Zn^{2+}, Cu^{2+} and Mg^{2+} at their appropriate concentrations showed the most significant enhancing effects on the production of botrallin and TMC-264, and were selected for the further study for the combination of their addition time and concentrations. When the cultures were respectively fed with 0.25–1.25 mmol/L of Zn^{2+}, 0.125–1.00 mmol/L of Cu^{2+}, and 0.0625–2.00 mmol/L of Mg^{2+} on days 10 and 15 of culture, the yields of botrallin and TMC-264 were improved obviously. The combination interactions of Zn^{2+}, Cu^{2+} and Mg^{2+} for botrallin and TMC-264 production were further optimized by employing a statistical method based on CCD and RSM. The yields of botrallin and TMC-264, which were predicted as 144.12 mg/L and 36.04 mg/L respectively, were validated to be 146.51 mg/L and 36.63 mg/L accordingly with the optimum concentrations of Zn^{2+} at 0.81 mmol/L, Cu^{2+} at 0.20 mmol/L, and Mg^{2+} at 0.13 mmol/L in medium. There were no significant differences between the experimental and predicted values, which indicated that the models had high degree of fitting, and could guide the production of botrallin and TMC-264 in liquid culture of the endophytic fungus *Hyalodendriella* sp. Ponipodef12. The results indicated that enhancement of botrallin and TMC-264 accumulation in

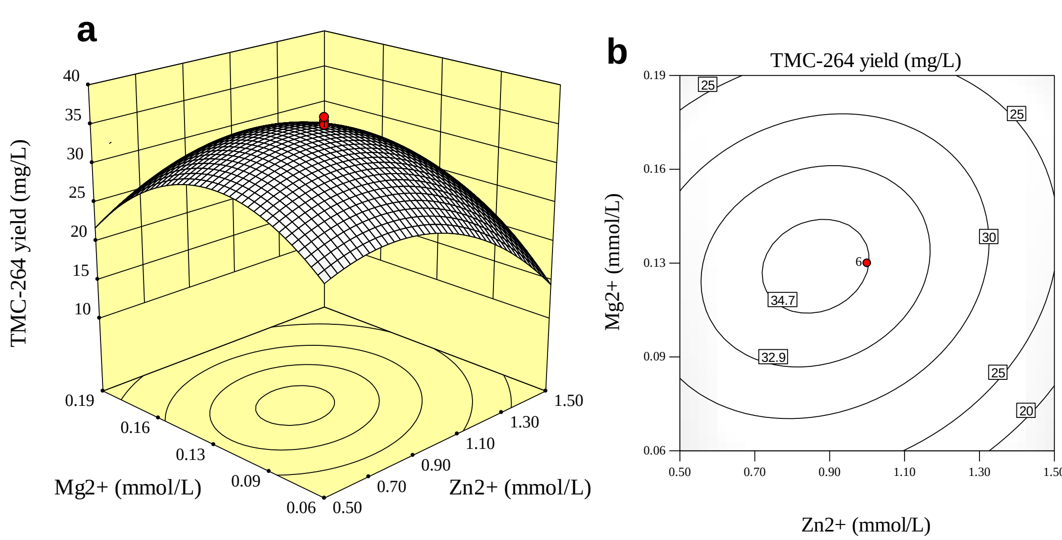

Fig. 8. Three-dimensional response surfaces (a) and two-dimensional contour plots (b) of TMC-264 yield (mg/L) of *Hyalodendriella* sp. Ponipodef12 *versus* the tested variables: Zn^{2+} and Mg^{2+} (mmol/L).

Table 5
The optimal culture conditions for botrallin and TMC-264 production of *Hyalodendriella* sp. Ponipodef12.

Tested variable and its concentration (mmol/L)	Botrallin yield (mg/L)		TMC−264 yield (mg/L)	
	Predicted	Experimental	Predicted	Experimental
Zn^{2+},0.81 Cu^{2+},0.20 Mg^{2+},0.13	144.12	146.51	36.04	36.63

liquid culture of *Hyalodendriella* sp. Ponipodef12 by the metal ions should be an effective strategy for large-scale production of botrallin and TMC-264 in the future. As the combination effects of only three metal ions (*i.e.*, Zn^{2+}, Cu^{2+} and Mg^{2+}) have been studied for their enhancing effects on botrallin and TMC-264 production in this work, more metal ions in the medium, as well as other parameters like pH, temperature, oxygen supply, precursors, should be considered in the future work. Furthermore, the mechanism of action of the metal ions on botrallin and TMC-264 biosynthesis also need to be studied in detail. After a series of optimization for their biosynthesis conditions, we could obtain the final medium for maximum production of botrallin and TMC-264 by natural fermentation of the endophytic fungus *Hyalodendriella* sp. Ponipodef12.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2016.09.002.

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