

## **Environmental stress and elicitors enhance taxol production by endophytic strains of *Paraconiothyrium variable* and *Epicoccum nigrum***

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### **ABSTRACT**

This study examined the effect of different elicitors (seven, different concentrations) and environmental factors (water activity ( $a_w$ ), pH) on taxol production by strains of two endophytic fungi, *Paraconiothyrium variable* and *Epicoccum nigrum*, isolated from temperate yew trees. A defined liquid broth medium was modified with elicitors, solute  $a_w$  depressors at different pH values. For *P. variable*, the best elicitor was salicylic acid at 50 mg/l which gave a taxol yield of  $14.7 \pm 4.8$   $\mu\text{g/l}$ . The study of synergistic effects between elicitor,  $a_w$  and pH on taxol production showed that the highest yield of taxol ( $68.9 \pm 11.9$   $\mu\text{g/l}$ ) was produced under modified ionic stress of 0.98  $a_w$  (KCl) at pH 5 when supplemented with 20 mg/l of salicylic acid. For *E. nigrum*, serine was the best elicitor which increased yield significantly (29.6 fold) when KCL was used as the  $a_w$  depressor (0.98  $a_w$ ) at pH 5.0 with 30 mg/l of serine. The maximum taxol yield produced by *E. nigrum* was  $57.1 \pm 11.8$   $\mu\text{g/l}$ . Surface response models were used to build contour maps to determine the conditions for maximum and marginal conditions for taxol yield in relation to the best elicitor and  $a_w$ , and the best pH for the first time. This will be beneficial for identifying key parameters for improvement of taxol yields by endophytic fungi.

## 1. Introduction

There has been significant interest in the potential for using endophytic fungi isolated from both tropical and temperate yew trees (*Taxus* species) for the production of the diterpenoid anti-cancer drug taxol. Taxol has also been shown to have potential therapeutic properties against non-cancerous diseases, including neurodegenerative diseases and polycystic kidney disease [1, 2]. The global market for taxol is large, with Bristol-Myers Squibb having a turnover of \$1.5 billion in revenue per annum in 2015. Taxol is normally extracted from the bark of the yew tree with >20kg required for every mg of taxol. Thus alternative sources have been sought for the production of taxol, including endophytic fungi.

We recently isolated endophytic strains of two species, *Paraconiothyrium variable* and *Epicoccum nigrum*, from temperate yew trees in the UK which could produce taxol [3]. The strains of *P. variable* and *E. nigrum* were found to produce 1.75 and 1.32  $\mu\text{g/l}$  taxol respectively in defined media. The previous study identified the optimum temperature, water activity ( $a_w$ ) and solute type for the taxol yield by the *P. variable* strain. For this strain optimum growth was at 0.99  $a_w$  and 25°C. However, optimum taxol yield was 7  $\mu\text{g/l}$  at 0.98  $a_w$  and 20-25°C.

Because the yields of taxol were in the low  $\mu\text{g/l}$  range, there was interest in evaluating approaches to enhance or stimulate production. For taxol and other secondary metabolites studies have been focused on whether precursors, carbon:nitrogen ratio and C-source, phosphate, trace elements, elicitors or environmental stress may stimulate production [4, 5, 6, 7]. Previously, taxol production was increased 8-fold in a *Nigrospora* species with elevated concentrations of the basal defined MID medium in a solid substrate fermentation system [8, 9]. Aldred et al. [9] demonstrated that solid substrate fermentation and environmental manipulation could significantly enhance the production of cholesterol lowering drugs such as the squalistatins produced by a *Phoma* species.

There have been very few detailed studies on the ecophysiology of taxol producing fungi and on optimization conditions for production. This is surprising as this product can be produced via fermentation in the same way as other secondary metabolite products from fungi, such as cyclosporins, statins and indeed penicillin. However, the ecological niches in which fungal groups grow need to be considered in trying to optimize production systems using fungi to try and simulate the conditions in nature [10]. It has also been shown that interacting conditions of water stress, temperature and pH and even light can impact on both growth and yield of secondary metabolites by filamentous fungi [6, 11, 12, 13]. Recent studies with tissue cultures of *Taxus chinensis* plants revealed that a temperature shift from the optimum for growth enhanced taxol yield. Culturing of such plant cells under water stress with mannitol, sorbitol and polyethylene glycol modified media, resulted in an enhancement of taxol yield [14, 15].

Other approaches including elicitation have been examined to try and stimulate biosynthesis of useful metabolites. Xu et al. [7] used elicitors such as sodium acetate to significantly increase taxol yield from a *Fusarium* strain. Supplementation of benzoate was also shown to affect the accumulation of taxol by *Pestalotiopsis microspore* when lowering inorganic phosphate in the culture media [5]. Furthermore, benzoate at 0.01 mM also activated taxol yield by *Periconia* sp., with an 8-fold yield increase over the control [6]. Other studies have indicated the ability of other elicitors such as phenylalanine and salicylic acid to improve the productivity of fungal taxol or that of plant cell tissue cultures of *Taxus* species [16, 17].

The objectives of this study were to (a) study the effect of seven different elicitors on taxol yield, (b) examine the interactions between water stress, pH and the best elicitors on taxol yield by the strains of *P. variable* and *E. nigrum* and (c) build models relating the key parameters to predict optimum and marginal conditions for taxol yield for the first time.

## 2. Materials and Methods

## 2.1 Fungal strains

Fungal strains used in this study were the taxol-producing *P. variabile* and *E. nigrum* isolated from English yew trees (*Taxus baccata*) [3].

## 2.2 Preparation of mycelial inoculum

The inoculum was prepared from 7-days-old cultures of both strains (*P. variabile* and *E. nigrum*) grown on a 2% milled yew leaf agar medium at 25°C. Mycelial agar plugs (5 mm diameter) were cut from the margin of the growing colony using a sterilized cork borer. Three agar plugs were used as the inoculum in 250 ml of culture broth in 1 litre flasks.

## 2.3 Use of elicitors to stimulate taxol yield

Ammonium acetate, jasmonic acid, phenyl alanine, salicylic acid, serine, silver nitrate and sodium acetate were used as elicitors in this study. Stock solutions were prepared for dissolving each elicitor in water, except for salicylic acid which was dissolved in ethanol. These were then sterilized by filtration through a 0.22 µm filter. Each inducer was added to the MID broth (Ca(NO<sub>3</sub>)<sub>2</sub>, 1.20 mM; KNO<sub>3</sub>, 0.79 mM; KCl, 0.87 mM; MgSO<sub>4</sub>, 3.0 mM; NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O, 0.14 mM; Sucrose, 87.60 mM; Ammonium tartrate, 2.10 mM; FeCl<sub>3</sub> H<sub>2</sub>O, 7.4 µM; MnSO<sub>4</sub>, 30.0 µM; ZnSO<sub>4</sub> 7H<sub>2</sub>O, 8.7 µM; H<sub>3</sub>PO<sub>4</sub>, 2.2 µM; KI, 4.5 µM; yeast extract 0.25 g/l, soytone (1g/l), pH 5.5) (Pinkerton and Strobel, 1976) at the different concentration levels shown in Table 1. The cultures were grown in static culture for 21 days at 25°C. All experiments were repeated once. Three weeks were chosen based on previous temporal studies with *Pestalotiopsis microspore* which suggested that taxol yield peaked after 2-3 weeks and then declined rapidly [18].

## 2.4. Effect of elicitor type, water activity and pH on taxol yield

The effects of the best elicitors,  $a_w$  and pH on taxol yield were studied for both *P. variabile* and *E. nigrum*. The design consisted of two  $a_w$  levels (0.995 and 0.98  $a_w$ ), and two pH levels (5 and 6). The inducers selected from the previous experiment were salicylic acid (20 and 50 mg/l) for *P. variabile* and serine (30 and 85 mg/l) for *E. nigrum*. The defined MID base medium detailed previously was used in this study. The  $a_w$  of this medium was adjusted by adding either KCl or glucose to obtain the target 0.98  $a_w$  level which was checked with the  $a_w$  meter (AquaLab, Decagon Devices, Inc., USA). The initial pH of the medium was adjusted by using 1 N HCl or 1N NaOH to give the desired pH. Stock solutions of each inducer were prepared by dissolving each one in water or ethanol (salicylic acid only) and sterilizing by filtration through a 0.22 µm filter. Each inducer was added to the MID broth (250 ml) at the tested concentration. The treatments were incubated at 25°C for 21 days. All experiments were repeated once. The cultured media were then extracted for taxol quantification by HPLC and confirmation using LC/MS [3].

## 2.5 Fungal taxol extraction

After 21 days incubation the fungal biomass was separated from the culture broth by filtration. The filtered culture broth was subsequently extracted by adding dichloromethane in two equal volumes to the culture broth [18]. The extracted solvent was evaporated by using the rotary evaporator to dryness at 35°C (Eyela, Tokyo, Japan). The dry residue was re-dissolved in 5 ml of 100% dichloromethane and then passed through a Solid Phase Extraction (SPE) column (silica gel; 15 ml with a bed weight 2 g; Thermo Scientific, UK) which was eluted in a stepwise manner. The elution was carried out starting with 15 ml of 100% dichloromethane and then continued with 15 ml

of dichloromethane:ethyl acetate at 1:1 v/v and 100% ethyl acetate. The last two eluents were collected, combined and then evaporated to dryness. The dry residue was dissolved in 1 ml of 100 % methanol. All samples were filtered through a 0.22  $\mu\text{m}$  nylon filter before analysis with HPLC and LC/MS. The standard taxol (Paclitaxel) was purchased from SIGMA and used for comparison in all experiments.

## 2.6 Quantification of taxol

Taxol in samples were analyzed by HPLC (Agilent 1200, Agilent Technologies, USA) with a C18 analysis column (Agilent Zorbax Eclipse, Part No. 990967-902). Fifty microlitres of sample was injected each time and detected at 230 nm. The mobile phase was methanol:water (80:20 v/v) at a flow rate of 1.0 ml/min. LC/MS was used for confirmation of both standards and treatment samples as described in Somjaipeng et al. [3].

## 2.7 Statistical analysis

The effect of the experimental factors on the taxol yield was analysed statistically by using MINITAB version 16.0 (Minitab Inc., USA). When assumptions of normality and equal variance were met, ANOVA was adopted to test for significant differences in response among the studied treatments. Post-hoc inter factor differences were calculated with Tukey multiple comparison tests. If these assumptions of a parametric test were violated, the significant differences were assessed using Kruskal-Wallis nonparametric ANOVA at statistical significant level of  $P < 0.05$ . Post-hoc inter factor differences were calculated with Dunn's multiple comparison nonparametric tests. Bonferroni correction was used to keep the Bonferroni individual  $\alpha = 0.05$ .

## 3. Results

### 3.1 Effect of different elicitors on taxol production by the two endophytic fungi

Figure 1 shows the effect of the different elicitors, at different concentrations, on the taxol yield by *P. variable*. There was a significant increase in taxol production in all the treatments when compared to the control. Supplementing the media with salicylic acid gave the most significant increase in yield by this endophytic strain. The highest taxol production ( $14.7 \pm 4.8 \mu\text{g/l}$ ) was obtained after induction with salicylic acid at 50 mg/l. Phenyl alanine-supplemented media had least effect on taxol yield by *P. variable*. The increase or decrease in taxol yield varied with type of elicitor used. For some there was an increase in taxol yield as concentration was increased (e.g. jasmonic acid), while for many of the others there was a decrease (e.g. salicylic acid, serine, sodium acetate).

Figure 2 shows the effect on taxol yield by the endophytic strain of *E. nigrum*. For this strain maximum taxol yield was obtained with serine ( $39.8 \pm 18.6 \mu\text{g/l}$ ) elicitor at 85 mg/l, while silver nitrate and acetic ammonium increased yields to a lesser extent. Again, threshold concentrations of different elicitors for maximum taxol yield varied.

### 3.2 Effect of the best elicitors, water activity and pH interactions on taxol production by the two endophytic strains

Further studies were carried out to examine the interaction between the impact of the best elicitor identified, combined with changes in water stress and pH values on taxol yield. Figure 3 shows the effect of these three-way interactions on taxol production. There was a significant improvement in taxol yield by *P. variable* when using the ionic solute KCl as the  $a_w$  depressor

combined with salicylic acid at 20 mg/l. This gave the highest taxol yield regardless of the cultivation pH used. However, pH 5 was more favourable with a maximum yield of  $68.9 \pm 11.9 \mu\text{g/l}$  (see Figure 3a). This was a 10.3 fold increase when compared with the non-modified  $a_w$  conditions. The difference in taxol yield was significant with different concentrations of salicylic acid under acidic condition at pH 5, whereas there was no significant effect at pH 6 (Figure 3b). When the non-ionic solute glucose was used as the  $a_w$  depressor, practically no taxol production was observed. The dry weights of mycelium were also examined as an indicator of the ability to grow in the presence of the inducer. There was a noticeable reduction in the mycelial biomass when taxol yield increased.

Figure 4 shows the effect of these interacting treatment conditions on taxol production by *E. nigrum*. The higher taxol yield was obtained at pH 5 ( $57.1 \pm 11.8 \mu\text{g/l}$ ) in the medium modified to 0.98  $a_w$  with the ionic solute KCl + 30 mg/l of serine as the elicitor (Figure 4a). The difference in the taxol production between this and the non-modified  $a_w$  condition was approx. 8.5 times. Taxol yields were again not significantly improved when a non-ionic solute such as glucose or sorbitol were used to modify the  $a_w$  of the media. Within the different concentrations of serine used at pH 6 there was no significant effects on taxol yield (Figure 4b). The different concentrations of serine had no influence on the fungal biomass dry weight of *E. nigrum*. However, cultures with sorbitol-modified media had more mycelial biomass.

### 3.3 Modelling the effect of salicylic acid, pH and $a_w$ on taxol production in liquid cultures of *P. variable*

Statistical analysis was extended to evaluate the effect of salicylic acid, pH and  $a_w$  on taxol yield in the defined liquid MID cultures of *P. variable* when KCl was used to modify  $a_w$ . An ANOVA analysis was carried out on the experimental data (Table 2). Among the quantitative variables examined, all three individually, and their interactions had a significant effect on taxol yield within the range used in this study ( $P < 0.05$ ). After the collected data were fitted, a first-order model with interaction in terms of the actual factors for taxol yield was developed as shown below:

$$\begin{aligned} \text{Taxol amount} = & 43122.4 - 5443.54\text{pH} - 222.997\text{salicylic acid} - 43771.2a_w & 1 \\ & + 5525.63\text{pH} * a_w + 226.48\text{salicylic acid} * a_w \end{aligned}$$

The main effects plot is useful in the practical interpretation of the results. In terms of the screening experiment, these plots can be used to compare the changes in the mean level to examine the direction of the response in relation to the tested level of factors providing the information for the subsequent experiment. Figure 5 represents the main effect plots of pH,  $a_w$  and salicylic acid concentration in term of experimental data. A recommended setting of factor levels to achieve a high response was obtained. Better yield could be achieved by choosing a relatively more acidic pH level, setting the salicylic acid concentration at a lower level and modifying the  $a_w$  of the culture broth.

Figure 6a presents contour plots of the interaction between pH x  $a_w$  on taxol yield when the level of salicylic concentration was held at a fixed level (20 mg/l). An increasing trend in yield with a slight increase in water stress was observed. Moreover, when the water stress is increased, the impact of pH on responses became more significant. It seemed that the response depended almost entirely on  $a_w$ . This finding is in good agreement with the high F value of  $a_w$  (F value=34.94). The higher the F value the more impact of the particular parameter. **Error! Reference source not found.** Changing pH from 5.0 to 6.0 produced more change in taxol yield when  $a_w=0.980$  than when  $a_w=0.985$ .

Figure 6b shows the interaction between increasing water stress and salicylic acid concentrations. It appeared that the effect of  $a_w$  as a factor was clearly predominant and impacted on the relative taxol production response. Under water stress, the concentration of salicylic acid had a

more important impact on the response. This was confirmed with the higher F value of  $a_w$ . Changing the concentration of salicylic acid from 20 to 50 mg/l resulted in a much smaller change in taxol yield when  $a_w$  was 0.984 than when  $a_w$  was 0.98.

### 3.4 Modelling the effect of serine, pH and $a_w$ on taxol production in liquid cultures of *E. nigrum*.

Evaluation of the effect of serine, pH and  $a_w$  on taxol production in M1D liquid cultures of *E. nigrum* was also investigated when KCl was used to modify the  $a_w$ . Normality of the distribution of the population was tested using the Kolmogorov–Smirnov test. The test revealed that the response variable was not normally distributed and needed to be normalized by transformation prior to analyses. The data sets were transformed by the Johnson transformations ( $P > 0.05$ ). The original data sets of taxol yields were thus transformed according to the following equation:

$$Y^* = -0.786458 + 0.502649 \times \operatorname{Asinh}\left(\frac{(x - 4.74745)}{0.690764}\right) \quad 2$$

In the equation,  $Y^*$  represents the transformed taxol yield and  $x$  represents the original data.

The back-transformed data were calculated from the equation below:

$$y = 4.74745 + \left(0.345382 e^{\left(\frac{(Y^* + 0.786458)}{0.502649}\right)}\right) - \left(0.345382 e^{\left(-\frac{(Y^* + 0.786458)}{0.502649}\right)}\right) \quad 3$$

where  $y$  represents back-transformed data,  $Y^*$  represents the transformed taxol yield.

An ANOVA analysis was carried out on the experimental data (Table 2). All three of the studied factors including pH, serine concentration and  $a_w$  had a significant effect on taxol yield within the range used in this study ( $P < 0.05$ ). Moreover, there was a significant effect of the interaction between serine concentration and  $a_w$  ( $P < 0.05$ ).

The first-order model with interaction in terms of the actual factors for taxol yield was developed as shown below:

$$Y^* = 548.304 - 0.648795pH - 7.79362serine + 7.903652a_w + 7.90365serine * a_w \quad 4$$

In the equation,  $Y^*$  represents the transformed taxol amount.

The main effects plot of pH,  $a_w$  and serine concentration in term of back-transformed data is shown in Figure 7. The results showed that with the decrease in pH level, a slight increase in water stress and a lower serine concentration provided the increasing trend in taxol yield. pH did not appear to be a major factor in taxol production in this case.

Figure 8 presents the contour plot of the effect of serine concentration x  $a_w$  on taxol yield when pH was held at 5.0 for *E. nigrum*. There was a small change in taxol yield when cultured under more freely available water conditions ( $>0.984 a_w$ ) than at  $0.98 a_w$ . However, an increasing trend in yield with a decrease in serine concentration stress was observed (from 85 to 30 mg/l). Thus, at lower levels of serine, the impact of water stress was more significant. The use of an elicitor had a stronger effect with *E. nigrum* than with *P. variable*. This was confirmed with the high F value of serine concentration (F value=41.25).

## 4. Discussion

The interaction between environmental factors is one of the critical considerations to enhance the production of secondary metabolites by fungi [19, 20, 21]. This study has provided some approaches which can be used for increasing taxol yield by examining interactions between temperature,  $a_w$ , solute type, pH and elicitor.

Previously Somjaipeeng et al. [3] showed that the optimum environmental conditions for growth of *P. variable* and that of taxol production were slightly different. Generally, the  $a_w$  range for production was from 0.96 to 0.995  $a_w$  and 25°C. However, taxol yield was increased under slight water stress (0.98  $a_w$ ) when compared with the treatment where water was freely available (0.99  $a_w$ ), regardless of temperature. This suggests that some slight ecophysiological manipulation of growth conditions may stimulate secondary metabolite production, including taxol. Previously, Baxter et al. [11] found that the optimum  $a_w$  for growth of a *Phoma* sp. which produced the cholesterol lowering metabolite squalistatin S1, was at 0.995  $a_w$ , while 0.990–0.980  $a_w$  was optimum for S. In addition, Aldred et al. [9] used solid substrate systems and obtained similar results with the *Phoma* species. However, they found best S1 production at 0.96  $a_w$ . In contrast, the maximum growth rate was found at 0.998–0.99  $a_w$ . These studies were in natural heterogeneous cereal-based substrates and not defined media.

The problems of using endophytic fungi to produce taxol under optimized fermentation processes are the low and unstable productivity. This was also observed for the endophytic strains isolated from the temperate yew trees which produced taxol [3]. The productivity issues found for these endophytic fungi *in vitro* may be independent of the natural habitat which they occupy in their host plants, where they are more protected from environmental fluxes [22]. There was certainly thus a need to examine whether more stable enhanced production could be achieved using elicitors and interacting environmental conditions.

It has been suggested previously that addition of low concentrations of elicitor compounds such as carbohydrates can bring about physiological changes in microorganisms resulting an increased production of secondary metabolites, enzymes or anti-microbials [23, 24, 25, 26]. Studies with *Penicillium chrysogenum* and oligosaccharide elicitors resulted in significant increases in penicillin production [23, 27]. Increases in bacitracin A production was also observed with *Bacillus licheniformis* oligoguluronate and mannan oligosaccharides [25]. These studies suggested that the elicitation mechanism may be operative at a transcription level, although the metabolic pathways and cascade of events, both intra- and extra-cellular levels, have not yet been determined. However, Reffatti et al. [26] found significant proteomic shifts in response to these elicitors.

Some of the elicitors used in the present study were able to increase taxol yield by *P. variable* when compared with the untreated control cultures. Salicylic acid affected the production of taxol most significantly. Salicylic acid has been previously shown to elicit an increase in taxol yield in suspension plant cell culture of *Taxus* sp. [1, 18, 28, 29, 30]. Recently, Soliman and Raizada [17] reported the significant enhancement in taxol yield (2x) from *Praconiothyrium* SSM001 when supplemented with salicylic acid. The biosynthesis of secondary metabolites in plants, including taxol, is widely accepted as the chemical defence responses to counter infection by plant pathogens. The infection by plant pathogens was reported to activate signalling molecules including salicylic acid [31]. This then activates the plant defence system by accumulating the anti-plant pathogen agents. This could be another explanation for the elicitation properties of salicylic acid increasing taxol yield obtained from endophytic fungi in this study. The threshold concentrations of different elicitors necessary may differ based on the results obtained in the present studies. For some compounds, as the concentration was increased the taxol yield was increased. For others, higher concentrations decreased taxol yield. More studies may be required to better understand the threshold range for different elicitors as well as the temporal effect on secondary metabolite production,

including taxol. Some studies have suggested that maximum taxol yield occurs after 14-21 days for endophytes before decreasing markedly [18].

For *E. nigrum*, a different elicitor was found to increase taxol production in the defined MID broth medium. The serine-induced conditions stimulated the most taxol biosynthesis and yield. In the presence of serine, previous reports have shown a significant increase in taxol yield, but only in plant cell cultures of *Taxus cuspidate* [32]. However, limited information on the influence of such amino acids on taxol production by endophytic fungi is available.

Under modified  $a_w$  conditions with KCl (0.98  $a_w$ ) at pH 5, the taxol yield by *P. variable* and *E. nigrum* was significantly increased when decreasing the concentration of salicylic acid to 20 mg/l and serine to 30 mg/l, respectively. The induction under non-modified  $a_w$  conditions resulted in very low production, although the elicitor was used. This finding is similar to some previous findings that taxol production by endophytic fungi was significantly reduced by repeated sub-culturing on defined artificial media *in vitro*.

Similar optimum pH conditions of 5.0 for taxol production and growth of *P. variable* and *E. nigrum* was found in this study. Interestingly, with water stress effects on mycelial growth, there was a slight shift in taxol yield in the presence of the elicitor. Fungal biomass was optimum at 0.99-0.98  $a_w$  while taxol production was at 0.98  $a_w$ . This suggests that there may be an interaction effect of slight modifications of growth due to water stress with the presence of an elicitor which may stimulate taxol biosynthesis. Good agreement was obtained between the observed and predicted results when evaluating the effect of elicitor, pH and  $a_w$  on taxol yield from both strains. This study showed that the elicitors combined with mild water stress conditions (0.98  $a_w$ ), enhanced taxol yield significantly. It has been previously shown that taxol is very stable over the pH range of 3-5 [33]. Studies with toxic secondary metabolites (mycotoxins) by Sanchis et al. [34] found that when examining optimum and marginal conditions of  $a_w$  and temperature for growth, secondary metabolite production optima were very different. The optimum conditions for fumonisin production by *Fusarium verticillioides* were found at 0.97  $a_w$ , while the most favourable conditions for growth of this strain was at 0.98-0.96  $a_w$ .

## 5. Conclusions

In summary, this study has shown that elicitors can be utilised to stimulate and enhance fungal taxol yield successfully for *P. variable* by adding salicylic acid (20 mg/l) and for *E. nigrum* by the addition of serine (30 mg/l). By combining modified  $a_w$  stress with the ionic solute KCl (0.98  $a_w$ ) at pH 5 and supplementation with these concentrations respectively, resulted in a significant stimulation in taxol yield by these two strains isolated from temperate yew trees. This suggests that it is possible to improve production of plant-derived bioactive compounds such as taxol by endophytic fungi using these three-way interacting conditions. The modelling also shows the relative ecological conditions for optimum and marginal taxol biosynthesis for the first time.

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Table 1. The different concentration levels of elicitors used in the experiments.

Types of elicitors	Levels (mg/l)		
Acetic ammonium	0.5	1	5
Jasmonic acid	210	1051	3154
Phenylalanine	1	5	10
Salicylic acid	50	100	150
Serine	85	100	150
Silver nitrate	1	2	3
Sodium acetate	4	123	410

Table 2. Statistical analyses of the experimental data using analysis of variance for the individual and two-way interacting factor on taxol production by *P. variabile*

Source	DF	Mean square	F value	P value
<b>Main effects</b>	3	2328.9	18.24	0.000
pH	1	1281.2	10.04	0.005
Salicylic acid	1	1244.3	9.75	0.006
a <sub>w</sub>	1	4461.0	34.94	0.000
<b>2-Way interactions</b>	2	1438.1	11.26	0.001
pH*a <sub>w</sub>	1	1145.0	8.97	0.001
Salicylic acid*a <sub>w</sub>	1	1731.2	13.56	0.008
Lack of fit	2	177.6	1.46	0.261

Table 3. Statistical analyses of the experimental data using analysis of variance for the transformed taxol yield data by *E. nigrum* on a define medium with different individual or two-way interactions.

Source	DF	Mean square	F value	P value
<b>Main effects</b>	3	6.1660	17.53	0.000
pH	1	2.5256	7.18	0.015
Serine	1	14.5249	41.28	0.000
a <sub>w</sub>	1	1.4474	4.11	0.055
<b>2-Way interactions</b>	2	7.0862	20.14	0.000
Serine*a <sub>w</sub>	1	7.0862	201.4	0.000
Lack of fit	2	0.6727	2.31	0.116

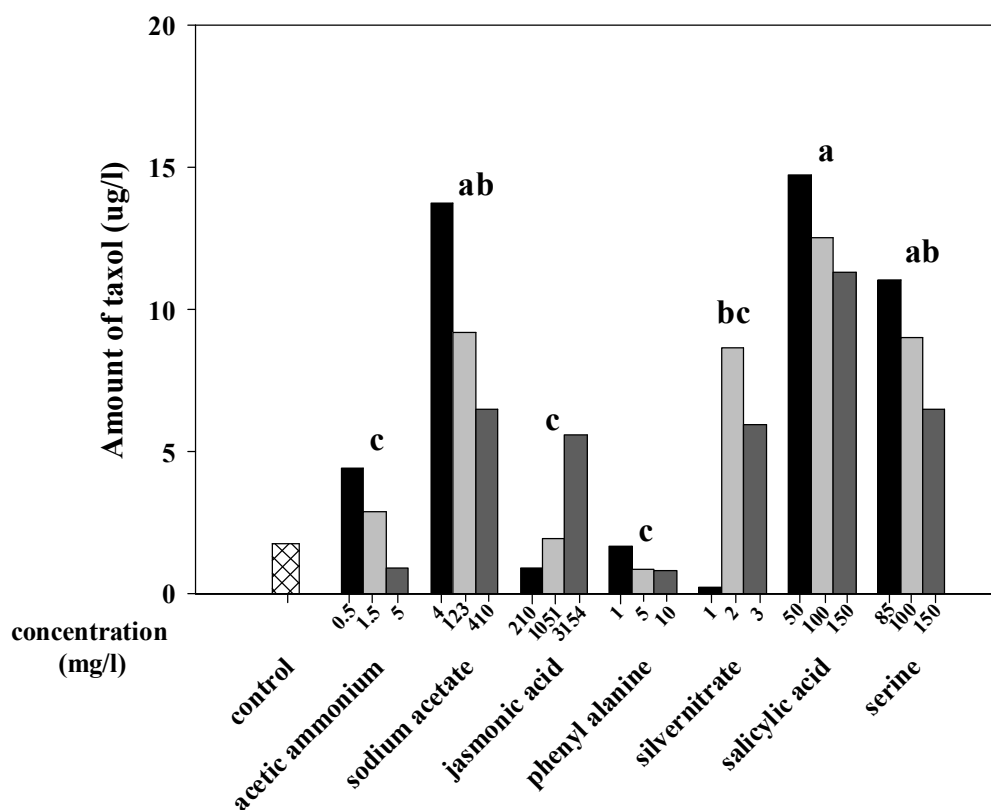


Figure 1 Effect of different concentrations of elicitors on taxol yield ( $\mu\text{g/l}$ ) by *P. variable* in a defined medium. Treatments with different letters are significantly different. Taxol yield in the control defined medium (M1D) alone is included for comparison.

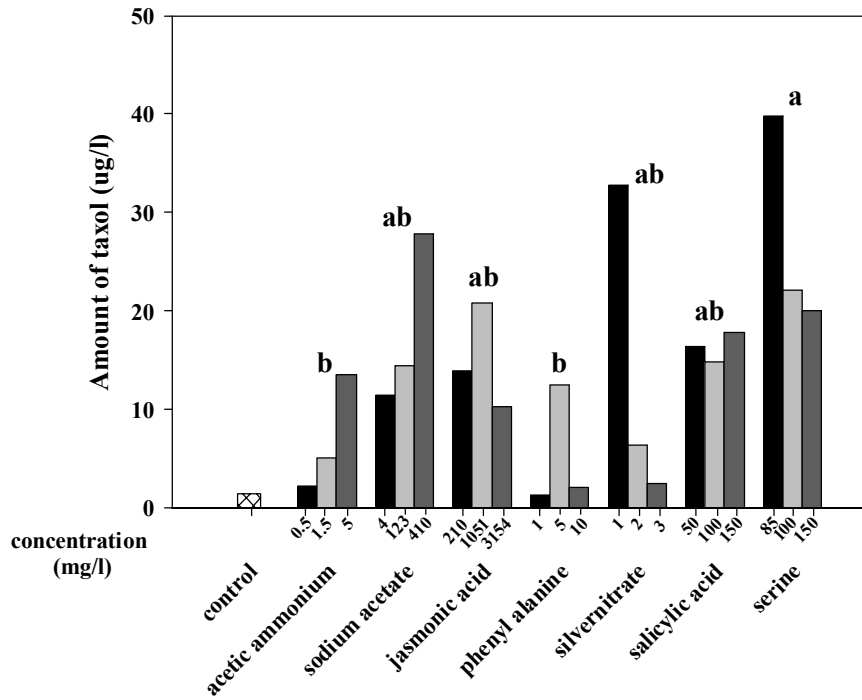
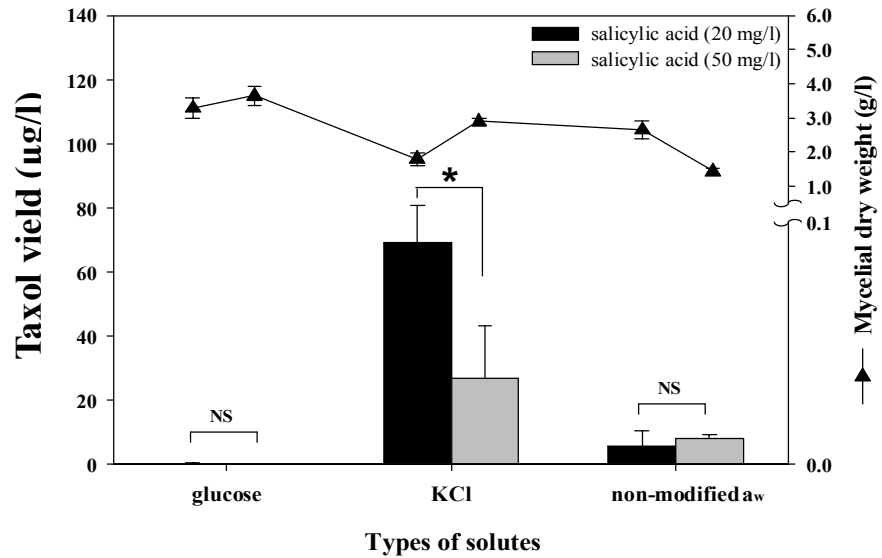


Figure 2 Effect of different concentrations of elicitors on taxol yield ( $\mu\text{g/l}$ ) by *E. nigrum* on a defined medium. Treatments with different letters are significantly different. Taxol yield in the control defined medium (M1D) alone is included for comparison.

(a) pH



(b) pH

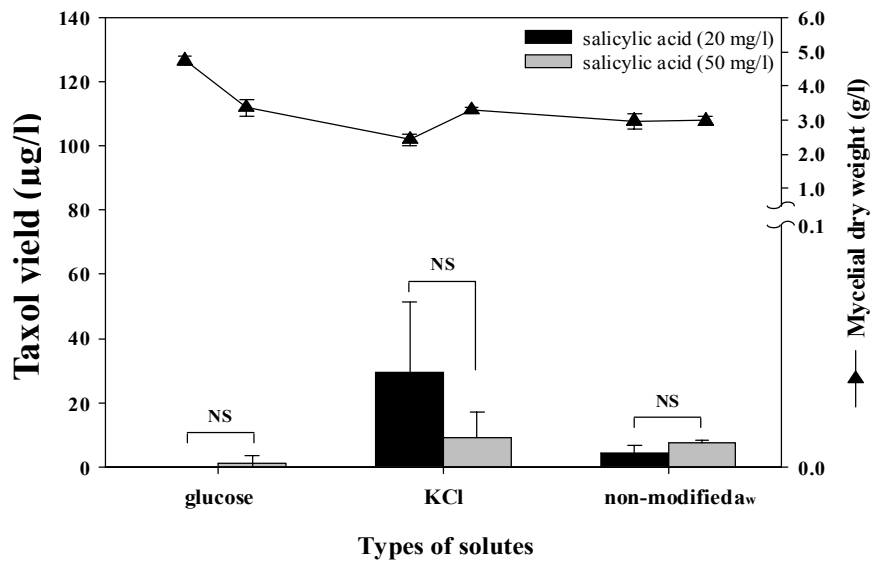


Figure 3 The effect of the different concentrations of salicylic acid, water activity ( $a_w$ ), solutes and pH on taxol production and mycelial dry weights of *P. variable*. Two different pH levels were used: (a) pH 5, (b) pH 6. The  $a_w$  of media were modified with glucose and KCl to 0.98. Data are presented as the mean  $\pm$  S.D. from three replicates. An asterisk indicates significant differences ( $P < 0.05$ , Student's t-test); NS, no significant difference.

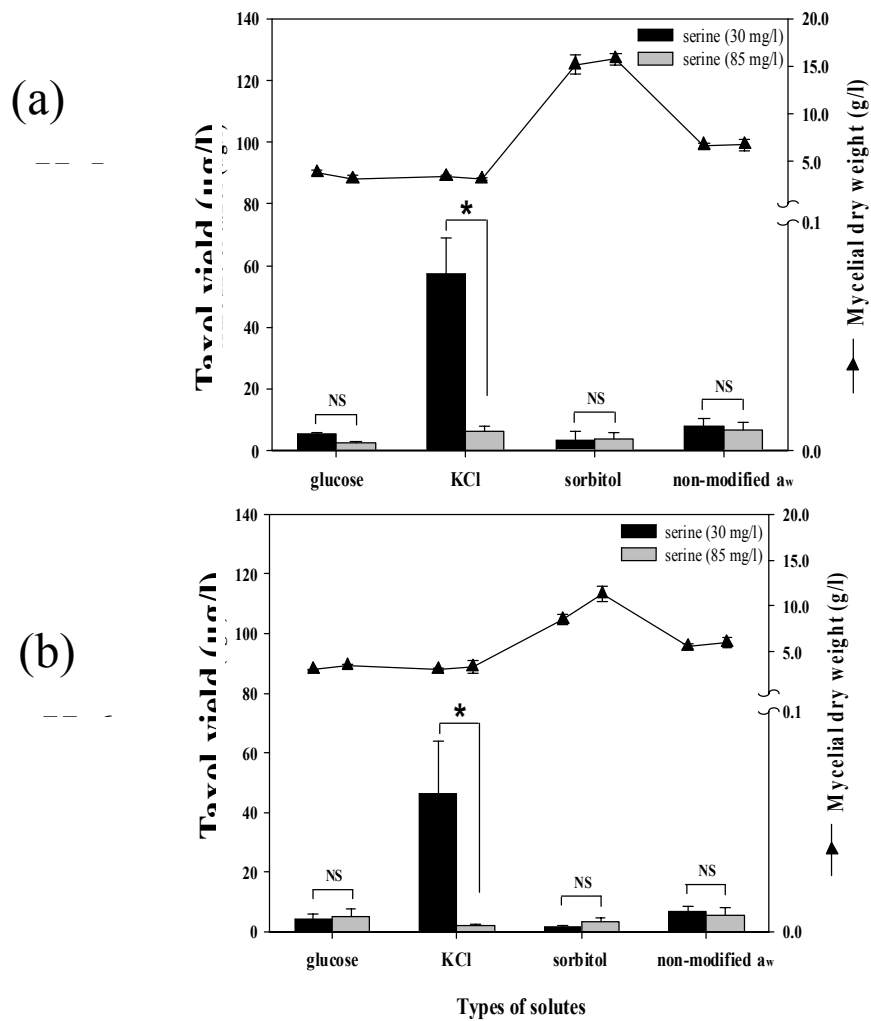


Figure 4. The effect of the different concentrations of salicylic acid, water activity ( $a_w$ ), solutes and pH on taxol production and mycelial dry weight of *E. nigrum*. Two different pH levels were used: (a) pH 5, and (b) pH 6. The  $a_w$  of media were modified with glucose, KCl and sorbitol to 0.98. Data are presented as the mean  $\pm$  SD from three replicates. An asterisk indicates significant differences ( $P < 0.05$ , Student's t-test); NS, no significant difference.



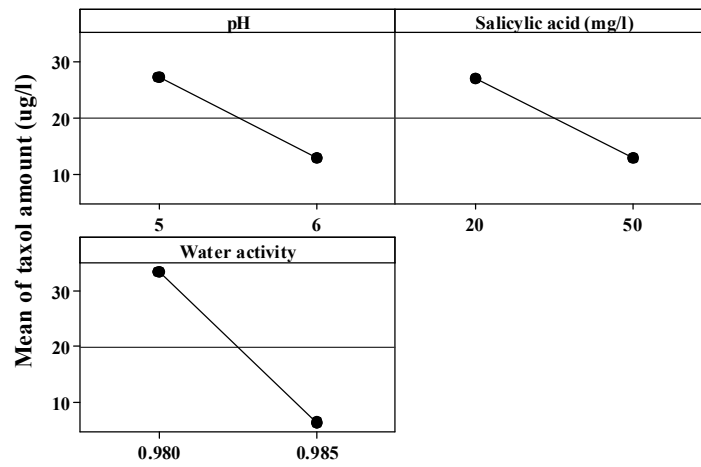


Figure 5. Main effects plot for (a) pH, (b) salicylic acid and (c) water activity on the mean taxol yield ( $\mu\text{g/l}$ ) in liquid cultures of *P. variable*.

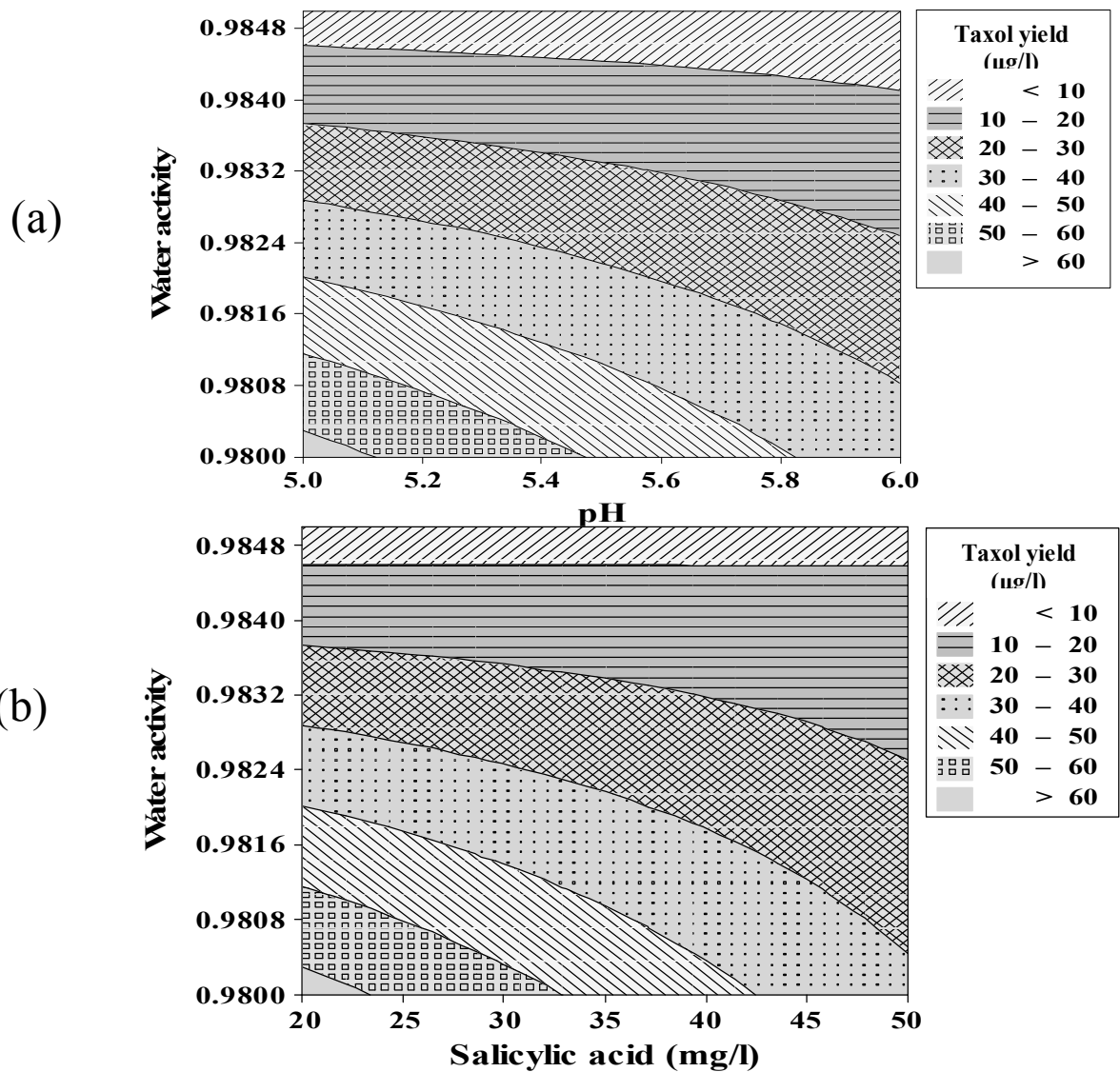


Figure 6. Somjaipeng et al.

Figure 6. The contour plot for (a) taxol yield as a function of pH and water activity ( $a_w$ ) with a fixed factor of the elicitor salicylic acid concentration at 20 mg/l and (b) for taxol yield as a function of  $a_w$  and salicylic acid concentration with pH 5 as the fixed factor for *P. variabile*.

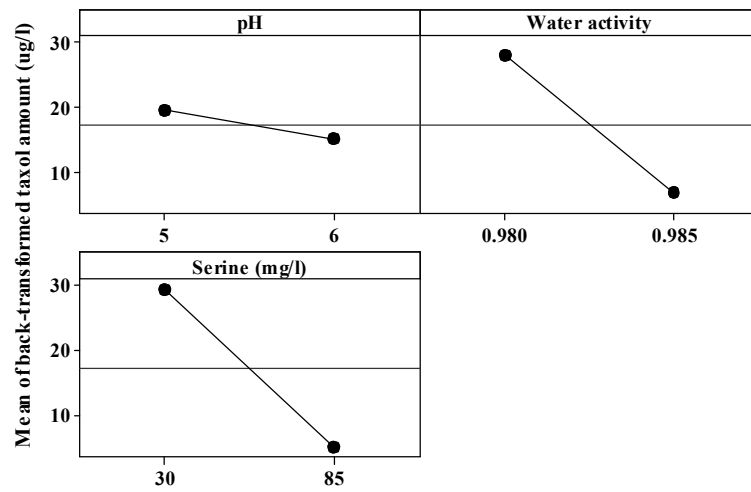


Figure 7. Main effects plot for (a) pH, (b) water activity and (c) serine on the mean taxol yield ( $\mu\text{g/l}$ ) in liquid cultures of *E. nigrum*.

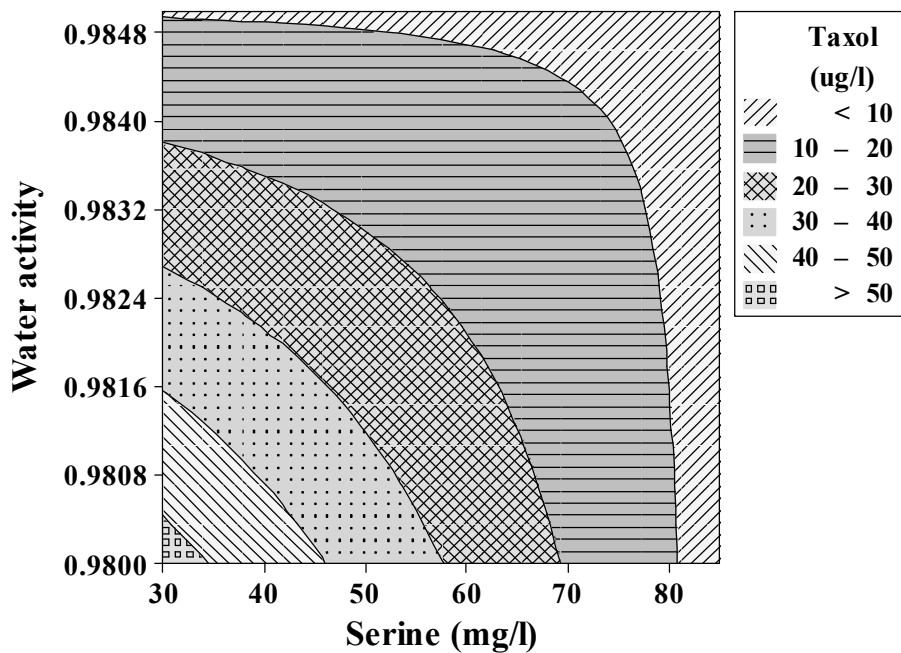


Figure 8. The contour plot for the effect of the interaction between water activity ( $a_w$ ) and serine concentration on back-transformed taxol yield ( $\mu\text{g/l}$ ) for *E. nigrum*. The pH was held at 5.0. The original statistical analysis was performed on the transformed data sets.