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

Supunnika Somjaipeng[1], Angel Medina, Naresh Magan*

Applied Mycology Group, AgriFood Theme, Cranfield University, Cranfield, Beds MK43 0AL, UK

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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 variabile* 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. variabile*, the best elicitor was salicylic acid at 50 mg/l which gave a taxol yield of 14.7 ± 4.8 μg/l. The study of synergistic effects between elicitor, a_w and pH on taxol production showed that the highest yield of taxol (68.9 ± 11.9 μg/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 ± 11.8 μg/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 >20 kg 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 variabile* and *Epicoccum nigrum*, from temperate yew trees in the UK which could produce taxol [3]. The strains of *P. variabile* and *E. nigrum* were found to produce 1.75 and 1.32 μg/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. variabile* strain. For this strain optimum growth was at 0.99 a_w and 25 °C. However, optimum taxol yield was 7 μg/l at 0.98 a_w and 20–25 °C.

Because the yields of taxol were in the low μg/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 M1D 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

* Corresponding author at: Applied Mycology Group, Vincent Building, Cranfield University, Cranfield, Beds MK43 0AL, UK.
E-mail address: n.magan@cranfield.ac.uk (N. Magan).
[1] Division of Agricultural Technology, Faculty of Science and Arts, Burapha University, Chanthaburi Campus, Chanthaburi 22170, Thailand.

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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 [6,11–13]. 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. variabile* 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 l 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 salicyclic acid which was dissolved in ethanol. These were then sterilized by filtration through a 0.22 μm filter. Each inducer was added to the M1D broth ($Ca(NO_3)_2$,1.20 mM; KNO_3, 0.79 mM; KCl, 0.87 mM; $MgSO_4$, 3.0 mM; NaH_2PO_4 H_2O, 0.14 mM; Sucrose, 87.60 mM; Ammonium tartrate, 2.10 mM; $FeCl_3$ H_2O, 7.4 μM; $MnSO_4$, 30.0 μM; $ZnSO_4$ $7H_2O$, 8.7 μM; H_3PO_4, 2.2 μM; KI, 4.5 μM; yeast extract 0.25 g/l, soytone (1 g/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].

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

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 1 N NaOH to give the desired pH. Stock solutions of each inducer were prepared by dissolving each one in water or ethanol (salicyclic acid only) and sterilizing by filtration through a 0.22 μm filter. Each inducer was added to the M1D 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 μm 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 analysed by HPLC (Algilent 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.,