



Research Article

Production, bioprocess optimization and γ -irradiation of *Penicillium polonicum*, as a new Taxol producing endophyte from *Ginkgo biloba*

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ABSTRACT

Twenty-eight fungal endophytes were recovered from the different parts of *Ginkgo biloba* and screened for their Taxol producing potency. Among these isolates, *Penicillium polonicum* AUMC14487 was reported as the potent Taxol producer (90.53 $\mu\text{g/l}$). The chemical identity of the extracted Taxol was verified from the TLC, HPLC, NMR, EDX, and FTIR analyses. The extracted Taxol displayed a strong antiproliferative activity against HEPG2 (IC₅₀ 4.06 μM) and MCF7 (IC₅₀ 6.07 μM). The yield of Taxol by *P. polonicum* was optimized by nutritional optimization with the Response Surface Methodology (RSM) using Plackett-Burman and Central Composite Designs. In addition to nutritional optimization, the effect of γ -irradiation of the spores of *P. polonicum* on its Taxol producing potency was evaluated. The yield of Taxol by *P. polonicum* was increased via nutritional optimization by response surface methodology with Plackett-Burman and FCCD designs, and γ -irradiation by about 4.5 folds, comparing to the control culture.

The yield of Taxol was increased by about 1.2 folds (401.2 $\mu\text{g/l}$) by γ -irradiation of the isolates at 0.5–0.75 kGy, comparing to the control cultures (332.2 $\mu\text{g/l}$).

The highest Taxol yield was obtained by growing *P. polonicum* on modified Czapek's-Dox medium (sucrose 40.0 g/l, malt extract 20.0 g/l, peptone 2.0 g/l, K₂PO₄ 2.0 g/l, KCl 1.0 g/l, NaNO₃ 2.0 g/l, MgSO₄·5H₂O 1.0 g/l) of pH 7.0 at 30.0 °C for 7.0 days. From the FCCD design, sucrose, malt extract and incubation time being the highest significant variables medium components affecting the Taxol production by *P. polonicum*.

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1. Introduction

Taxol is one of the most commercial anticancer drugs, with broad spectrum towards different types of cancer cell [1–3], it has been approved by USA FDA in 1992. The activity of Taxol elaborates from its unique specificity for binding with tubulin β -subunits heterodimer, promoting tubulin polymerization, disrupting the mitotic division of tumor cells [4,5]. The powerful activity of Taxol has been emphasized towards breast, lung, head, neck, uterine cancers and advanced forms of Kaposi's sarcoma, as reviewed in [6,7]. Taxol has been firstly isolated and characterized from the

bark of Yew tree *Taxus brevifolia* [8]. However, the tiny yield and vulnerability of this plant to unpredicted fluctuation with the ecological conditions were the major challenges for this source [9,10]. Semisynthetic process using 10-deacetylbaccatin III (DAB) intermediates extracted from the needles of *Taxus baccata* is the present technology for commercial Taxol production, however, the strong heterogeneity of DAB yield with environmental conditions of *T. baccata* is the major additional challenge [11].

Recently, exploring the Taxol producing potency of endophytic fungi isolated from the different medicinal plants raising the hope for using this technique as novel platform for commercial Taxol production [6,12,13]. The rationality of endophytic fungi as unique approach elaborates from their natural physiological identities such as fast growth, cost effective process, independent on climatic changes, feasibility of genetic manipulation, metabolic engineering and mass production [14]. Endophytic fungi are a group of fungi

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that internally colonize the different plant tissues without causing any apparent symptoms to their host plants [12,13,15]. Endophytic fungi especially inhabiting medicinal plants have been considered as a repertoire of plethora of novel secondary metabolites with the main chemical scaffold of terpenoids, steroids, quinones and polyketides [6,12,16–18]. Massive number of plants from different families were used as source for isolation of endophytic fungi and their Taxol producing potency were screened and determined as reviewed by [6,12,16,19]. Although, the promising implementation of the endophytic fungi for Taxol production, however, their lower reproducible yield and drastic loss of Taxol productivity with repeated subculturing and storage is the main practical challenge that hamper the commercial anticipation of these fungal for Taxol production [20,6,12,19]. Thus, searching for novel fungal endophytes from different medicinal plant hosts with promising, sustainable Taxol yield is the ultimate objective.

Ginkgo biloba has a great ethnopharmacological relevance, such as antibacterial, antioxidant, and anticancer, and anticardiovascular activities [21]. Several endophytic fungal isolates of *G. biloba* were collected and identified as potential producers to various flavonoids, alkaloids, terpenoids and miscellaneous compounds with diverse biological activities [21]. However, isolation and characterization of fungal endophytes with Taxol producing potency from *G. biloba* as family other than Taxaceae was the objective of this study. With the argument about the Taxol biosynthetic machineries, if it is derived from *Taxus brevifolia* "Taxol producing plant" via horizontal gene transfer, or naturally present on the genome of Taxol producing endophytic fungi [22,23], isolation of endophytic fungi with Taxol producing potency from family outside Taxaceae could be participate on unraveling this hypothesis. Thus, isolation of Taxol producing endophytic fungi from *G. biloba* in addition to assessment of the Taxol biosynthetic dependency on the plant host was the objective of the current study.

2. Material and methods

2.1. Isolation of the endophytic fungi dwelling *Ginkgo biloba*

Different parts of *Ginkgo biloba* (leaves, barks and twigs) were collected from Orman Garden (Cairo, Egypt) in spring 2017, and used as source for isolation of endophytic fungi. The different plant parts were surface sterilized with 70 % ethanol for 2 min, then by 2.5 % sodium hypochlorite for 2 min, then rinsing with sterile distilled water. The surface sterilized plant parts were sectioned into small pieces with sterile sharp blade, then placed on the surface of potato dextrose agar (PDA) with ampicillin (1 µg/mL) [24], incubated for 7 days at 30 °C. The effectiveness of surface sterilization of the plant parts was assessed by centrifuging the rinsing water, then 500 µl sterile water was added to the precipitate and plated into potato dextrose agar [25–27]. The developed hyphae of the endophytic fungal colonies were purified by subculturing on PDA and stored as slope and plate cultures at 4 °C. Control plate media were used to check the sterility of working area, while, positive control of plant parts without sterilization were used to check the epiphytic fungal flora.

2.2. Morphological and molecular identification of the recovered endophytic fungi

The recovered fungal isolates were identified to their species levels based on their micro and micro-morphological features by growing on Czapek's-Dox and PDA media for 10 days at 30 °C, according to the references key [28,29]. The highest potential Taxol producing fungal isolates were further molecularly identified based on the sequence of internal transcribed spacer (ITS) flanking

the 5.8S region (ITS1–5.8S–ITS2 rDNA) [16,30,31]. Fungal genomic DNA (gDNA) was extracted by pulverizing the mycelia (~0.2 g) in liquid nitrogen then adding 1 ml cetyltrimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 2% PVP40, 0.2 % 2-mercaptoethanol, 20 mM EDTA, 1.4 M NaCl in 100 mM Tris-HCl, pH 8.0). The gDNA was used as template for PCR with the primer set ITS4 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS5 5'-TCCTCCGCTTATT GATATGC-3'. The PCR reaction contains 10 µl of 2 × PCR master mixture (i-Taq™, Cat. No. 25027, iNTRON Biotech.), 2 µl of fungal gDNA, 1 µl of each primer (10 pmol/µl), completed to 20 µl with sterile distilled water [32]. The PCR was programmed to initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 10 s, extension at 72 °C for 30 s for 35 cycles, and final extension at 72 °C for 2 min. The PCR amplicon was analyzed by 1.5 % agarose gel in 1 × TBE buffer (Ambion Cat# AM9864), using DNA ladder (1 kb Nex-gene Ladder, Puregene, Cat. # PG010-55DI) and visualized by gel documentation system (Vilber Lourmat, France). The amplicons were purified and sequenced by Applied Biosystems Sequencer, HiSQV Bases, Version 6.0 using the same primers set. The obtained ITS sequences were BLAST searched non-redundantly on the NCBI database. For the multiple sequence alignments, the sequences were imported into MEGA 6.0 software and aligned with Clustal W muscle algorithm [33] and the phylogenetic tree was constructed with neighbor-joining method of MEGA 6.0 with 1000 bootstrap replication [34].

2.3. Screening for Taxol producing endophytic fungi, Taxol extraction and quantification

The recovered endophytic fungi inhabiting *Ginkgo biloba* were screened for Taxol production using potato dextrose broth (PDB) (Cat# DF0549-17-9) [22]. The fungal isolates were grown on PDA media for 7 days at 30 °C. One plug of each fungal isolate was inoculated into 100 mL of the PDB per 250 mL Erlenmeyer flasks, the cultures were incubated at 30 ± 1 °C under shaking conditions for 15 days. After incubation, the cultures were filtered by sterile cheesecloth, the filtrates were amended with 0.2 % sodium bicarbonate for fatty acids precipitation. Taxol has been extracted with double volume of dichloromethane (DCM), and the organic phase was collected, solvent was evaporated to dryness, and the residues were re-dissolved in 3 mL of methanol [13,27,35]. Taxol was fractionated and identified by TLC analysis using Merck 1 mm (20 × 20 cm) pre-coated silica gel plates (TLC Silica gel 60 F254, Merck KGaA, Darmstadt, Germany). After running, Taxol was detected by UV illumination at 254 nm (Min-UVIS, DESAGA, Heidelberg, Germany) [13,27]. From the TLC silica gel plates, the putative spots of Taxol were scraped-off and dissolved in DCM, vortex vigorously for 10 min and centrifuged at 1000 rpm for 5 min. The precipitated silica particles were removed and the supernatant was taken for Taxol quantification and purity checking by HPLC (Agilent Technology, G1315D) of C18 reverse phase column (Eclipse Plus C18 4.6 × 150 mm, 3.5 µm, Cat.# 959963-902). The mobile phase was methanol/ acetonitrile/water (25:35:40, v/v/v) at flow rate 1.0 mL/min for 20 min [36]. Taxol fractions were scanned from 200 to 400 nm by photodiode array detector (DAD), their chemical identity and concentrations were confirmed from the retention time and absorption peak area comparing to authentic sample (Sigma-Aldrich, Cat. # T7402).

2.4. Spectroscopic analysis for Taxol authentication

The UV spectroscopic analyses of Taxol samples were measured at λ 227 nm (RIGOL, Ultra-3000 Series) comparing to authentic Taxol [13,30,35,37]. Blank media was used as negative baseline for all spectrophotometric analyses.

FT-IR spectrum of the purified Taxol samples was analyzed by JASCO FT-IR 3600 Spectrophotometer. The purified Taxol sample was grinded with KBr pellets, pressed into discs under vacuum, comparing to authentic one. The absorption was measured in the region 500 to 4000 cm^{-1} [3]. The chemical structure of extracted Taxol was confirmed from the H and C NMR spectroscopy (JEOL, ECA-500II, 500 MHz NMR) comparing to authentic Taxol. The samples were dissolved in CDCl_3 , chemical shifts are given in ppm (δ -scale) and the coupling constants are expressed in hertz (Hz).

2.5. Bioprocess optimization of the selected fungal isolates to maximize their Taxol yield

The chemical components of Taxol production medium by selected fungal isolate were optimized to maximize their yield of Taxol, using the different designs of Response Surface Methodology such as Plackett-Burman design and Faced Central Composite design by the Design-Expert 7.0 statistical software package (Stat Ease Inc., Minneapolis, USA). Each experiment was run in three biological replicates and the mean values were considered. After incubation at the desired conditions, fungal biomass was filtrated, and the Taxol was extracted, purified and quantified by TLC and HPLC comparing to authentic sample as described above.

2.5.1. Plackett-Burman design

Plackett-Burman design has been used frequently for optimization of media component for fungal growth and production of desired compounds [38]. The optimum conditions for production of Taxol have been assessed *in silico* based on the media components that have been used in qualitative and quantitative screening [39]. Eleven factors have been included namely; sucrose, sodium nitrate, dipotassium phosphate, magnesium sulfate, potassium chloride, temperature, pH, incubation time, shaking speed, malt extract and peptone. The parameters were varied over two levels, the minimum and maximum ranges were selected. The statistical Design-Expert 7.0 was used to generate a set of 12 experimental designs. For each experiment, Taxol production was expressed by $\mu\text{g/l}$, trials were carried out in triplicates, and the average of Taxol yield was considered. Regression analysis of the trial data was conducted using statistical software. The effect of each variable was calculated [40] using the following equation

$$E = \left(\sum M_+ - \sum \frac{M_-}{N} \right)$$

Where, E is the effect of a testing variable, M_+ and M_- are taxol concentration of trials at the parameter of higher and lower levels, respectively, and N is the number of experiments was carried out. The effect of each variable on the production was determining by calculating their respective E-values.

$$E = \frac{\text{Tot}_{\text{high}} - \text{Tot}_{\text{low}}}{N_0}$$

Where Tot_{high} is the total responses at the high level, Tot_{low} is the total responses at the low level, and N_0 is the number of trials.

2.5.2. Faced central composite design

The most significant positive independent variables affecting Taxol production by the selected fungal isolates were optimized using FCCD to determine the individual and mutual interactions among the tested variables. FCCD is a statistical experimental design, in which each variable is represented by three different levels low (-1), medium (0), and high (+1) [41].

The linear, quadratic and interaction coefficients, regression model was illustrated by the following second-ordered polynomial equation,

$$Y = \beta_0 + \sum_f \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

Y, is the predicted response, β_0 is the regression coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, X_i is the coded level of independent variable.

To determine the optimal levels of the variables for Taxol production, three-dimensional (3D) response surface curves were plotted to study the interaction between the various factors, and to determine the variable condition of each factor for maximum Taxol production. 3D graphs were carried out by holding three factors constants in an ideal level and plotting the obtained response (Taxol conc.) for varying levels of the other two factors.

2.6. Gamma irradiation effect on Taxol yield by the target fungal isolate

The selected potent fungal isolates were γ -irradiated with ^{60}Co source (Gamma cell 4000-A-India) at different doses (0.25–3.0 kGy) comparing to the control non-irradiated cultures. The dose rate was 1.2 kGy/h at the time of the experiments. The irradiated spores were inoculated into the optimal media under standard cultural conditions, comparing to the non-irradiated spores as control. The cultures were incubated for 15 days at 30.0 °C on a rotary shaker (120.0 rpm), filtered, and Taxol was extracted, purified and quantified by TLC and HPLC as described above.

2.7. Antimicrobial activity of Taxol against different microorganism

The antimicrobial activity of extracted fungal Taxol was assessed against different types of wound infectious bacterial isolates namely; *Bacillus subtilis* and *Staphylococcus epidermidis* (Gram positive) and *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter agglomerans* (Gram negative), in addition to the antifungal activity against *Candida albicans*. These organisms were freshly streaked on nutrient agar medium and incubated at 37 °C for 24 h. After incubation, bacteria were suspended in sterile peptone water to obtained standard inocula of ~ 0.5 McFarland ($1-1.5$) $\times 10^8$ CFU/mL with UV-vis spectrophotometer (at 600 nm). The examined *Candida albicans* inocula were set at $(1-3) \times 10^6$ CFU/mL. The growth inhibition (mm) of microbial pathogens was assessed by agar disc diffusion method. Sterile standard antibiotic disks with the diameter of 6.0 mm were used to positive controls. Sterile paper discs (6.0 mm) were loaded with 20 μl of methanol as negative control and amoxicillin clavulanic acid (AMC) as positive control. Discs were loaded with different concentration of Taxol (0.1, 0.5 and 1.0 $\mu\text{g/ml}$). Three biological replicates were prepared. The plates were incubated at 37 °C for 24 h, and the resulting zones of inhibition were measured. Amoxicillin clavulanic acid (AMC); 30 $\mu\text{g/ml}$ and Nystatin 100 $\mu\text{g/ml}$ (6.0 mm diameter) were used to normalize the antimicrobial activity of Taxol. The inhibition Zone of growth was determined by a Vernier caliper (mm).

2.8. Antiproliferative activity

The antiproliferative activity of purified fungal Taxol has been evaluated against liver carcinoma (HPG2) and breast carcinoma (MCF7) (VACERA Institute, Cairo, Egypt). The viability of tumor cells was assessed by MTT assay [42]. The 96-well plate was seeded with 10^3 cells/ well, incubated overnight, then amended with different concentrations of fungal Taxol and re-incubated for 24 h. The MTT dye (20 μl) was added to each well, incubated for 2 h, and the developed formazan complex was dissolved by 100 μl DMSO, and the purple color was measured at λ_{570} nm. The IC_{50} value was expressed by the Taxol concentration reducing the growth of 50 % of initial number of tumor cells, normalizing to negative control.

2.9. Statistical analyses

The experiments were conducted in three biological replicates, and the results were expressed by mean \pm STDV. The significance was calculated by one-way ANOVA with Fisher's Least Significant Difference of post hoc test (<https://www.easycalculation.com/statistics/fishers-bsd-calculator.php>).

2.10. Fungal deposition

The ITS sequence of *Penicillium polonicum* was deposited at genbank with accession #MK128497.1, as well as, the isolate was physically deposited at Assiut University Mycological Centre (AUMC), Egypt, with accession # AUMC14487.

3. Results

3.1. Endophytic fungi of *Ginkgo biloba*; morphological, molecular identification and Taxol producing potency

Twenty-eight endophyte fungal isolates were recovered from the barks, twigs and leaves of *Ginkgo biloba* on PDA agar medium (Table 1), these fungal isolates were allocated in twigs (8 fungal), barks (7 isolates) and leaves (13 f isolates) of plant. These fungal isolates were initially identified to the species level based on their morphological features according to the universal keys and were reported to belongs to four genera namely; *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium*. Among these isolates, the prevalence of genus *Aspergillus* was reported to be > 75 %, followed by *Fusarium* that represented by 25 %, while *Penicillium* and *Cladosporium* were represented by about 7%. The genus *Aspergillus* was represented by five species namely; *A. flavus* (four isolates), *A. oryzae* (five isolates), *A. niger* (five isolates), *A. fumigatus* (three isolates), and *A. terreus* (four isolates). Two species of *Penicillium* (*P. polonicum* and *P. egyptiacum*) and one isolate of *Cladosporium* (*C. herbarum*) were recovered. The productivity of Taxol by the collected fungal isolates was assessed, by growing on PDB, incubation at the standard conditions, extraction and

Table 1
Screening for Taxol producing endophytic fungi of *Ginkgo biloba*.

Plant parts	Isolate No.	Fungal Isolate	TLC	HPLC ($\mu\text{g/L}$)
Twigs Barks	1	<i>Aspergillus flavus</i>T1	++	40.29
	2	<i>Penicillium polonicum</i> T1	+++	90.53
	3	<i>Aspergillus oryzae</i> 1	+	–
	4	<i>Aspergillus oryzae</i> 2	–	–
	5	<i>Cladosporium herbarum</i>	++	10.02
	6	<i>Aspergillus fumigatus</i> T1	+	–
	7	<i>Fusarium</i> sp. 2	–	–
	8	<i>Aspergillus niger</i> 4	+	–
Bark	9	<i>Aspergillus oryzae</i> 1	–	–
	10	<i>Aspergillus terreus</i> 1	–	–
	11	<i>Fusarium</i> sp. 1	–	–
	12	<i>Aspergillus flavus</i> 3	+	–
	13	<i>Aspergillus terreus</i> 3	–	–
	14	<i>Fusarium</i> sp. 4	–	–
	15	<i>Aspergillus niger</i>	+	–
Leaves	16	<i>Aspergillus terreus</i>L1	++	10.76
	17	<i>Aspergillus niger</i> 1	+	–
	18	<i>Aspergillus fumigatus</i> 1	–	–
	19	<i>Aspergillus terreus</i> L4	++	10.34
	20	<i>Fusarium</i> sp. 3	–	–
	21	<i>Penicillium egyptiacum</i>	–	–
	22	<i>Aspergillus niger</i> 5	–	–
	23	<i>Aspergillus flavus</i> 2	+	–
	24	<i>Aspergillus oryzae</i> 3	–	–
	25	<i>Aspergillus niger</i> 2	+	–
	26	<i>Aspergillus flavus</i> L4	++	10.19
	27	<i>Aspergillus fumigatus</i> 3	+	–
	28	<i>Aspergillus oryzae</i> 5	–	–

quantification of Taxol by TLC and HPLC. From the HPLC and TLC analyses (Fig. 1), the highest yield of Taxol has been reported by *P. polonicum* (90.53 $\mu\text{g/l}$), followed by *A. flavus* T1 (40.29 $\mu\text{g/l}$), while *C. herbarum*, *A. terreus* L1, *A. terreus* L4 and *A. flavus* L4 displayed a similar yield of Taxol (10.0–10.8 $\mu\text{g/l}$). However, the other fungal endophytes did not display any yield of Taxol based on the TLC and HPLC analyses. Among the recovered fungi, six isolates gave the highest potency for Taxol production, four of them were belong to genus *Aspergillus* (*A. flavus* T1, *A. flavus* L4, *A. terreus* L1, and *A. terreus* L4), in addition to *P. polonicum* and *Cladosporium herbarum* as revealed from the HPLC analysis. *Aspergillus flavus* T1, *P. polonicum* and *C. herbarum* have been isolated from the twigs, while, *A. terreus* L1, *A. terreus* L4 and *A. flavus* L4 have been isolated from leaves of *G. biloba*.

The morphological features of the potent six endophytic fungal isolates (*A. flavus* T1, *P. polonicum*, *C. herbarum*, *A. terreus* L1, *A. terreus* L4 and *A. flavus* L4) producing Taxol has been shown in Fig. 2. These fungal isolates were identified morphologically according to the universal morphological keys (Raper and Fennell, 1965; Pitt, 1980). *P. polonicum* growing optimally at 28 °C at Czapek's-Dox agar, producing blue green conidia with a granular colony surface, with exudate droplets (Fig. 2). The conidiophores are terverticillate, slightly roughened on Czapek's with definite phialides and metula, with smooth-walled and globose conidia (Frisvad, 1995) (Fig. 2). The colony reverse was cream yellow color pigment diffused into the agar medium. The microscopical and macroscopical features of this isolate typically follow the description of *P. polonicum* Westling by Frisvad and Samson [43]. Based on the Taxol yield, *P. polonicum* has been selected for further experimentation.

The morphological identification of *P. polonicum* has been validated by the molecular analysis of ITS region sequence (Cakir and Maden, 2015; Khalil et al., 2019). Using gDNA as template, the PCR amplicon (~550 bp) of *P. polonicum* was resolved, purified and sequenced. From the non-redundant blast search on NCBI database, the ITS sequence gave 99 % similarity with *P. polonicum*, with zero E values and 95 % query coverage. The isolate displayed 99 % similarity with *P. polonicum* isolates MH854992.1, NR103687.1, AF033475, MH862450.1, MH856411.1, NR119496.1, NR077206.1, JN942697.1, MH861768.1, MH856134.1, JN942742.1, NR138271.1, NR160219.1, MH862986.1 and MH856088.1 with zero E value and 95 % query coverage. The phylogenetic relatedness of *P. polonicum* with the database deposited isolates were constructed (Fig. 2). Based on the ITS sequence, three phylogenetic clades of *P. polonicum* were recovered with a strong sequence similarity as revealed from the root value 0.001, the target isolate belongs to *P. polonicum* clade I. Thus, from the microscopical and molecular analyses, the target isolate has been confirmed as *P. polonicum*, and deposited to genbank with accession number MK128497.1, as well as, the isolate has been deposited at Assiut University Mycological Centre (AUMC) under deposition # AUMC 14487.

3.2. Chemical identity, antimicrobial and anticancer activity of *P. polonicum* Taxol

The chemical identity of *P. polonicum* Taxol has been validated by ¹HNMR, ¹³CNMR, EDX and FT-IR analyses. After incubation of the fungal culture, Taxol was extracted by methylene chloride, initially checked by TLC and further purified by preparative TLC. Comparing to authentic Taxol, the putative Taxol samples were scrapped-off from the silica particles and further chemically analyzed. The resolved signals of ¹HNMR and ¹³CNMR for *P. polonicum* extracted Taxol were identical to the standard Taxol, that distributed between 1.0 and 8.0 ppm (Fig. 3). Three proton signals were resolved at 1.0–3.0 ppm corresponding to methyl, acetate and acetylene groups, while the signals for aromatic

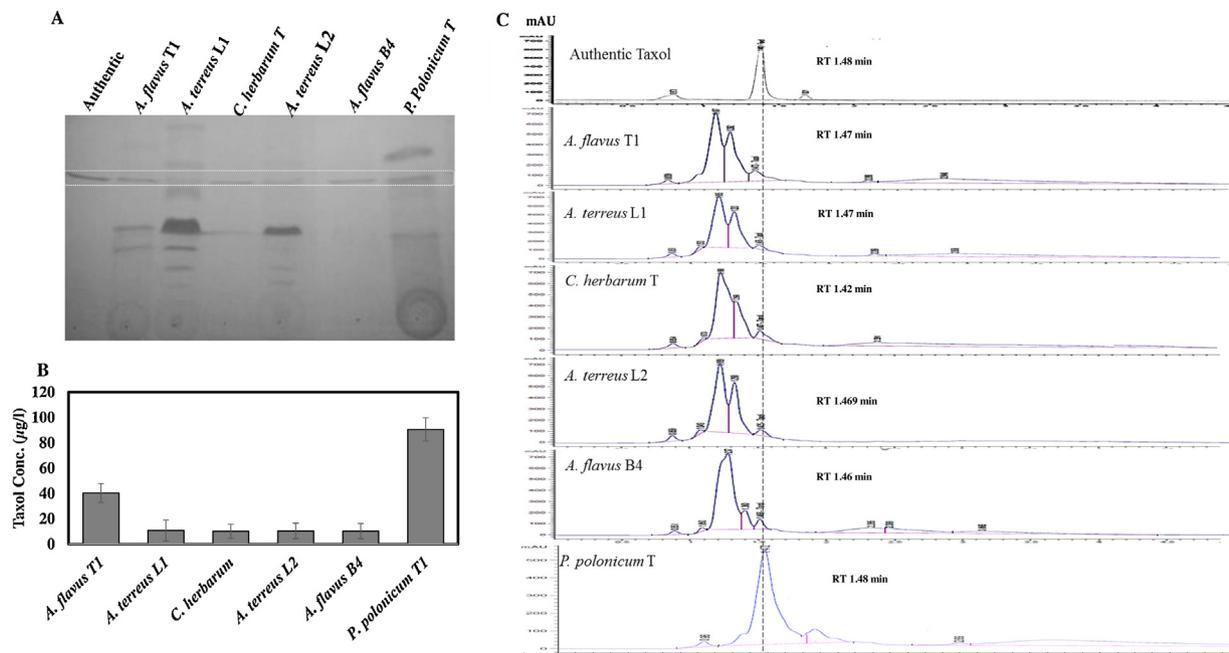


Fig. 1. Chromatographic analysis of the extracted Taxol and PCR screening for Taxol rate-limiting enzymes from the potent fungal endophytes of *P. gracilior*. The 24 fungal isolates were grown on PDB under standard cultural conditions, Taxol was extracted and checked by UV-absorbance at λ 227 nm and TLC analysis comparing to authentic Taxol. The highest Taxol producers (six isolates) were selected for further HPLC analysis and PCR amplification of Taxol biosynthetic marker genes. A, TLC chromatogram of extracted Taxol from the six fungal isolates (1, 59, 108, 4, 14 and 20). B, PCR amplicon of 10-deacetylbaconin III-O-acetyltransferase (dbat). C, Overall Taxol yield from the selected fungal isolates from HPLC analysis. D, HPLC chromatogram from the selected six fungal isolates comparing to authentic Taxol. The retention time of authentic Taxol was 6.2 min, 1 μ g/mL was injected into HPLC column giving peak area 1817.2 mAU that used for calculating the corresponding.

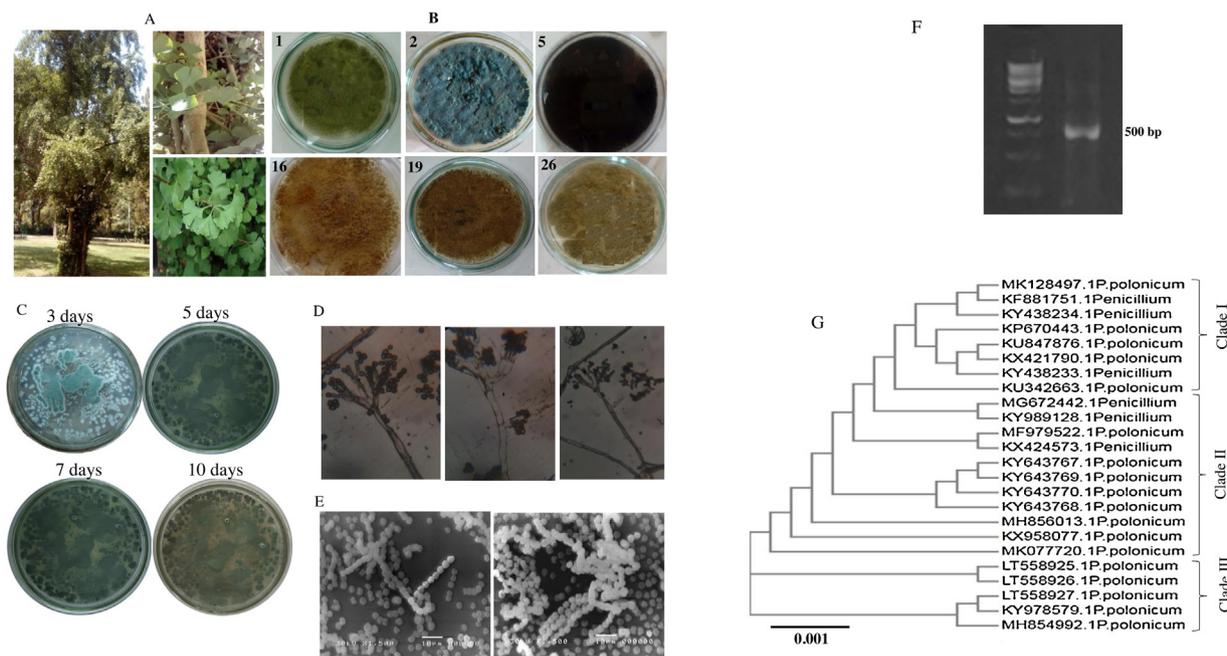


Fig. 2. Taxol producing fungal endophytes of *G. biloba*. A; Morphological views of *G. biloba* twigs, cork and leaves. B; Potent Taxol producing endophytes; *Aspergillus flavus* (1), *Penicillium polonicum* (2), *C. herbarum* (5) from twigs; *A. terreus* 1 (16), *A. terreus* 2 (19) and *A. flavus* (26) from leaves. C; Microscopical views of isolate #2 conidial heads (C) and conidiophores (D) at 1000x magnification by light microscope. Molecular identification of *P. polonicum*; PCR amplification of ITS region of *P. polonicum* (E), normalizing to 250-10000bp ladder (Cat.#. SM0312), Phylogenetic analysis of *P. polonicum* by Maximum Likelihood method (F).

moieties were resolved at 6.5–9.0 ppm. The FTIR spectra of *P. polonicum* Taxol were identical to authentic one. The purified Taxol had a representative peak at 3325.82 cm^{-1} that designated for the hydroxyl group (OH), peak at 2943.7 cm^{-1} that assigned to aliphatic CH stretch, peak at 1113.7 cm^{-1} corresponding to COO stretching

frequency, and peak at 1019.8 cm^{-1} assigned for the aromatic C–H. The aliphatic C–H stretch was observed at 2943.7 cm^{-1} , the C=O stretch was positioned at 1657.3 cm^{-1} , the peak at 1451.2 cm^{-1} due to NH stretching frequency. The carbonyl-oxygen stretching frequency was observed at 1413.1 and 1113.7 cm^{-1} .

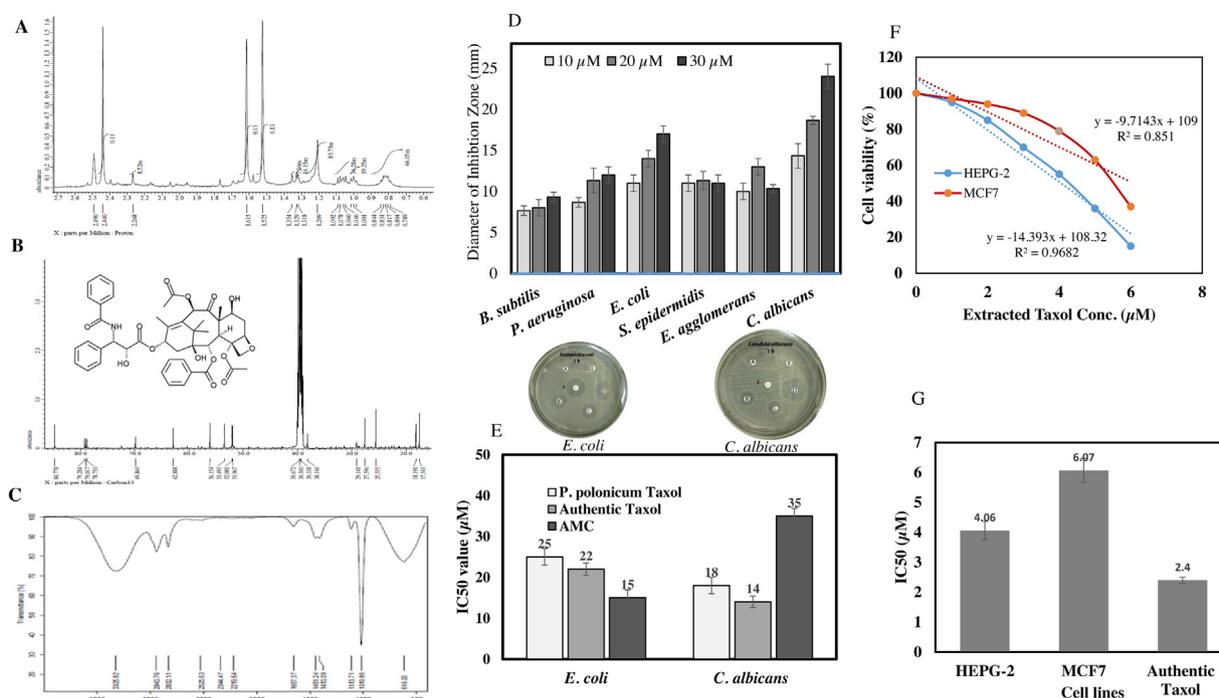


Fig. 3. Chemical analysis and antiproliferative activity of extracted Taxol from *P. polonicum*. Taxol was extracted, fractionated by TLC. The putative spots of Taxol were scraped-off from the TLC plates and spectroscopically analyzed. The spectra of ^1H NMR (A), ^{13}C NMR (B) and FT-IR analysis (C) for the extracted Taxol of *P. polonicum*. D, Antimicrobial activity of extracted Taxol against *B. subtilis*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, *E. agglomerans* and *C. albicans*. E, The IC_{50} values of extracted Taxol towards *E. coli* and *C. albicans*. Antiproliferative activity of *P. polonicum* Taxol against liver tumor cells (HEPG2) and breast adenocarcinoma (MCF7) (F) and their IC_{50} values (G).

The antiproliferative activity of purified Taxol from *P. polonicum* was evaluated towards liver carcinoma (HEPG2) and breast carcinoma (MCF7). From the viability assessment, the purified *P. polonicum* Taxol had IC_{50} values 4.06 μM and 6.07 μM towards HEPG2 and MCF7, respectively (Fig. 3). The IC_{50} values of Taxol from different sources was summarized in Table 2.

The antimicrobial activity of the extracted Taxol from *P. polonicum* has been evaluated against various multidrug resistant bacteria such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus epidermidis*, *Enterobacter agglomerans* in addition to fungal pathogen *Candida albicans* by the disc diffusion assay. Authentic Taxol and AMC were used as positive controls. From the results (Fig. 3), the antimicrobial activity of Taxol was noticeably fluctuated among the tested microbial isolates. Taxol had a significant antibacterial activity against *E. coli*, unlike to the

mild activity towards *B. subtilis*, *P. aeruginosa*, *S. epidermidis* and *E. agglomerans*. Obviously, the extracted Taxol displayed a significant antifungal activity against *C. albicans* in a concentration dependent manner as revealed from the visual observation and diameter of inhibition zone with Taxol concentration, comparing to authentic Taxol (Fig. 3). The IC_{50} values of *P. polonicum* extracted Taxol towards *E. coli* and *C. albicans* were 25 and 18 μM , while, it was 22 and 14 μM for authentic Taxol, respectively. While, the IC_{50} value for *E. coli* and *C. albicans* were 15 and 35 μM , respectively (Table 3).

3.3. Optimization of bioprocess variables using Response Surface Methodology

3.3.1. Plackett-Burman design screening for critical factors

The media composition for Taxol production by *P. polonicum* were optimized by the statistical experimental design of Plackett-Burman. As revealed from the results (Fig. 4), sucrose, dipotassium phosphate, potassium chloride and temperature displayed a significant effect on Taxol production by *P. polonicum*. This design was used to draw up the media components and the cultural conditions that affect taxol production. The response was calculated as the concentration of Taxol by TLC and HPLC. All the experiments were performed in triplicates and the average concentration was considered. Taxol production response in the 12

Table 2
 IC_{50} values of Taxol from different sources towards HEPG2 and MCF7 tumor cell lines.

Taxol Source	Tumor cell line	IC_{50} (μM)	Reference
<i>Penicillium polonicum</i>	HEPG2	4.06	This study
	MCF7	6.07	
<i>Aspergillus terreus</i>	HEPG2	4.6	[7]
	MCF7	5.4	
<i>Aspergillus flavipes</i>	HEPG2	6.0	[13]
	MCF7	7.4	
<i>Taxus brevifolia</i>	HEPG2	600	[12]
	MCF7	762	
	HEPG2	100	
	HEPG2	610	
	MCF7	590	
<i>Nodulisporium sylviforme</i>	MCF7	2.5	[55]
	HeLa	2.6	
	A549	4.1	
	HT-29	2.8	
	MCF7	12	
<i>Cladosporium oxysporum</i>	HCT15	430	[58]

Table 3
Antimicrobial activity of Taxol against different pathogenic microorganisms represented by diameter of inhibition zone (mm).

	Authentic Taxol	AMC	<i>P. polonicum</i> Taxol
<i>Bacillus subtilis</i>	8.6 \pm 0.5	8.0 \pm 1.00	11.33 \pm 0.577
<i>Pseudomonas aeruginosa</i>	8.6 \pm 0.5	6.0 \pm 0.00	14.00 \pm 1.00
<i>Escherichia coli</i>	11.0 \pm 1.0	22.0 \pm 1.0	22.00 \pm 1.00
<i>Staphylococcus epidermidis</i>	9.5 \pm 0.5	26.0 \pm 1.0	11.00 \pm 0.00
<i>Enterobacter agglomerans</i>	9.0 \pm 0.0	19.0 \pm 1.0	10.33 \pm 0.577
<i>Candida albicans</i>	9.3 \pm 1.5	6.00 \pm 0.00	24.00 \pm 1.52

The concentration of authentic Taxol and *P. polonicum* Taxol was is 20 μM .

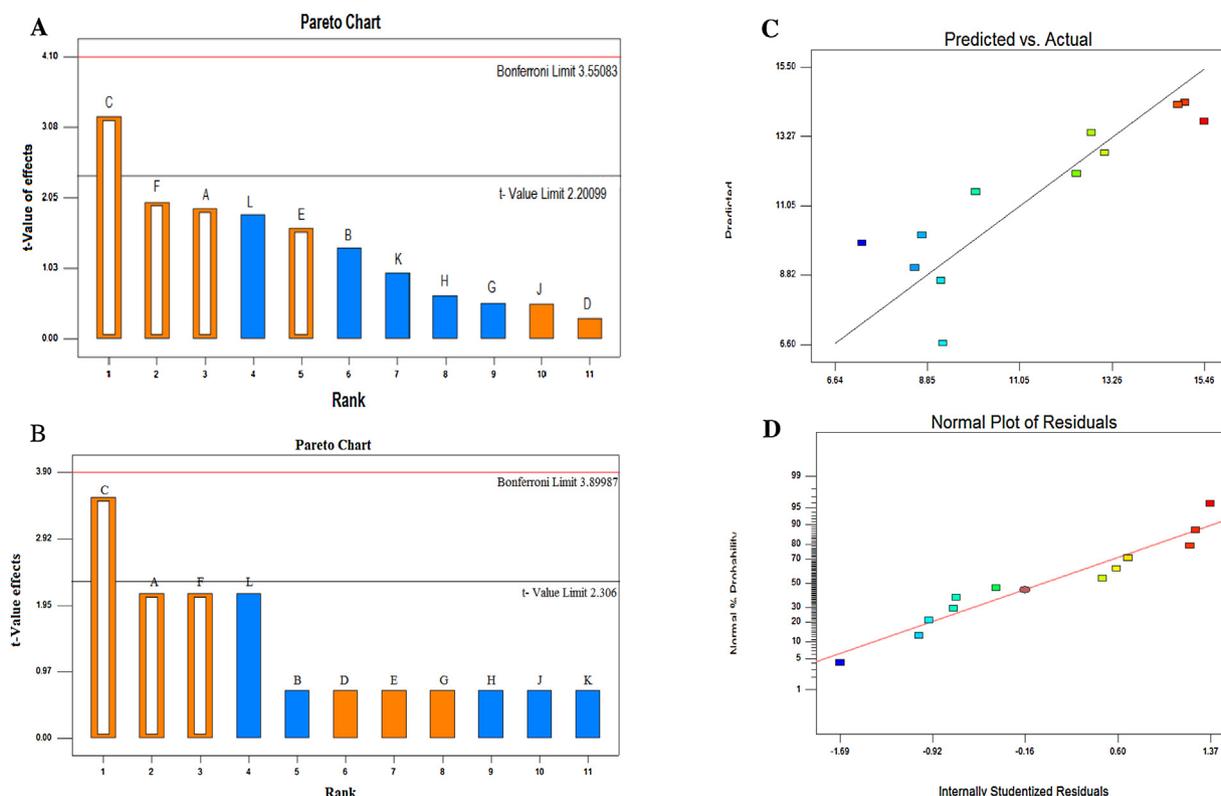


Fig. 4. Blackett-Burman design of the different variables for production of Taxol by *P. polonicum*. Pareto Charts showing the effect of individual factors on Taxol production where: C- Dipotassium phosphate, F- Temperature, A- Sucrose, L- Peptone, E- Potassium chloride, B- Sodium nitrate, K- Malt extract, H- Incubation time, G- pH, J- shaking speed, D- Magnesium sulfate (A, B). The normal probability plots of the variables for Taxol production by *P. polonicum* as determined by the first order polynomial equation. C, Plot of correlation between predicted and actual Taxol yield by *P. polonicum*. D, Normal probability plots of residuals.

Table 4
 Analysis of variance (ANOVA) for Taxol production.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance	R-Squared	Std. Dev.
Model	6.40	4	1.60	5.16	0.0296	Significant	0.7466	0.56
A- Sucrose.	1.12	1	1.12	3.62	0.0989	Significant		
C-Dipotassium phosphate	3.23	1	3.23	10.43	0.0145	Significant		
E- Potassium chloride	0.81	1	0.81	2.60	0.1510	Significant		
F- Temperature	1.24	1	1.24	3.98	0.0862	Significant		
Residual	2.17	7	0.31					
Cor Total	8.57	11						

Table 5
 Minimum and maximum ranges of the selected parameters in PBD for optimization of Taxol production.

No.	Factors	Levels	
		Minimum (-1)	Maximum (1)
1	Sucrose (g /L)	30.0	40.0
2	Malt Extract (g /L)	20.0	30.0
3	Peptone (g /L)	1.0	2.0
4	Sodium nitrate (g /L)	2.0	4.0
5	Dipotassium phosphate (g /L)	1.0	2.0
6	Magnesium sulfate (g /L)	0.5	1.0
7	Potassium chloride (g /L)	0.5	1.0
8	Temperature	25.0	30.0
9	pH	6.0	7.0
10	Incubation time (days)	7.0	12.0
11	Shaking speed (rpm)	120.0	150.0

runs experimental set-up was represented in Tables 4,5 . Statistical analysis of this design illustrates that the Model F-value of 5.16 implies their significant and Values of "p > F" fewer 0.050. The "Predicted R-Squared" of 0.2553 is in reasonable agreement with the "Adj. R- Squared" of 0.6018. "Adeq. Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The "Adeq. Precision" ratio of 6.374 indicates an adequate signal [44]. This model can be used to navigate the design space as mentioned in Table 4. Based on parameter estimated and by applying multiple regression analysis on the experimental data, the test variables, and the response variable were relating by the following model equation: First-order model equation Terms of Coded Factors: Taxol Conc. =+1.81 + 0.31A+0.52C + 0.26E+0.32 F. The actual and predicted levels of Taxol by *P. polonicum* upon Plackett-Burman optimization bioprocess are summarized in Table 6 and Fig. 4. The highest actual (204 µg/l) and predicted (239 µg/l) yield of Taxol by

Table 6
Plackett-Burman design to evaluate factors affecting Taxol production by *P. polonicum*.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Taxol		
												Experimental	Predicted	Residuals
1	1	-1	1	1	1	-1	-1	-1	1	-1	1	193.4	220.0	-26.6
2	1	-1	-1	-1	1	-1	1	1	-1	1	1	115.9	76.0	39.9
3	1	1	-1	-1	-1	1	-1	1	1	-1	1	126.6	100.0	26.6
4	1	1	-1	1	1	1	-1	-1	-1	1	-1	165.7	171.0	-5.3
5	-1	1	1	1	-1	-1	-1	1	-1	1	1	108.1	53.0	55.1
6	-1	-1	1	-1	1	1	-1	1	1	1	-1	197.0	225.0	-28.0
7	-1	1	1	-1	1	1	1	-1	-1	-1	1	197.1	163.0	34.1
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	69.7	84.0	-14.3
9	1	-1	1	1	-1	1	1	1	-1	-1	-1	204.0	239.0	-35.0
10	1	1	1	-1	-1	-1	1	-1	1	1	-1	154.2	154.0	0.2
11	-1	-1	-1	1	-1	1	1	-1	1	1	1	80.4	73.0	7.4
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	30.6	85.0	-54.4

P. polonicum upon factorial design by Plackett-Burman design was obtained at run # 9 at medium component; sucrose (40 g/l), malt extract (20 g/l), peptone (2 g/l), sodium nitrate (4 g/l), K₂HPO₄ (1 g/l), MgSO₄ (1 g/l), KCl (1 g/l) of initial pH 6.0, and the cultures were incubated at 30 °C for 7 days at 120 rpm shaking speed. The effect of individual parameters studied in Plackett-Burman design was represented in Pareto Chart depicting the order of significance of variables involved in Taxol production (Fig. 4). Thus, the most significant variables affecting Taxol productivity by *P. polonicum* were dipotassium phosphate, temperature, and sucrose as revealed from the Plackett-Burman design.

3.3.2. Face Centered Central Composite Design (FCCD) bioprocess optimization

Face Centered Central Composite Design was adopted for the choice of optimal level of the significant variable that was identified based on the Plackett-Burman design. The most

significant variables “dipotassium phosphate, potassium chloride, sucrose and temperature” have been selected for further optimization using FCCD design. The results of 30 runs of four variables with positive effects reveals the significant effect of the tested variables on the yield of Taxol by *P. polonicum* (Table 7). The yield of Taxol was increased by about 1.6 folds (332.08 µg/l) upon optimization with FCCD at the run #1 which the medium contains sucrose (40 g/l), dipotassium phosphate (2 g/l), potassium chloride (1 g/l), at incubation temperature 30 °C for 7 days incubation comparing to the basal medium at run #14 (204.1 µg/l). Upon FCCD optimization, the predicted Taxol yield was increased from 239 µg/l at run # 14 into 371.0 µg/l at run# 1, by about 1.55 folds increment. The three-dimensional response surface curve showing the effect of interactions of the most significant parameters; sucrose, dipotassium phosphate, potassium chloride and incubation temperature were shown in Fig. 5. The statistical analysis of the Linear Model design demonstrates that the F-value is 28.06

Table 7
Face Centered Central Composite design of the variables; sucrose, dipotassium phosphate, potassium chloride and temperature on the yield of Taxol by *P. polonicum*.

Runs	Sucrose (g/L)	Dipotassium phosphate (g/L)	Potassium chloride (g/L)	Temperature (°C)	Taxol yield (µg/l)		
					Experimental	Predicted	Residuals
1	40	2	1	30	332.08	371.0	-38.89
2	40	2	0.5	25	313.76	355.1	-41.38
3	35	1.50	0.75	22.50	238.14	262.0	-23.81
4	30	2	1	25	180.24	144.2	36.05
5	40	2	1	25	229.2	206.3	22.92
6	40	2	0.5	30	239.04	234.3	4.78
7	30	1	0.5	25	55.68	54.6	1.11
8	30	2	1	30	231.6	254.8	-23.16
9	25	1.5	0.75	27.5	143.28	107.5	35.82
10	40	1	1	25	146.16	127.2	19.00
11	35	2.5	0.75	27.5	211.44	232.6	-21.14
12	35	1.5	0.75	32.5	194.96	191.1	3.90
13	35	1.5	0.75	27.5	169.92	186.9	-16.99
14	40	1	1	30	204.0	239.0	-35.0
15	35	1.5	1.25	27.5	189.5	208.5	-18.95
16	35	1.5	0.75	27.5	172.2	146.3	25.82
17	35	1.5	0.75	27.5	174.6	144.9	29.68
18	30	1	1	30	151.9	127.6	24.31
19	45	1.5	0.75	27.5	194.6	190.7	3.89
20	30	2	0.50	25	138.8	138.8	0.00
21	35	1.5	0.75	27.5	169.3	147.3	22.01
22	35	1.5	0.75	27.5	169.4	203.3	-33.89
23	40	1	0.50	30	156.1	187.3	-31.22
24	35	0.5	0.75	27.5	151.8	182.1	-30.35
25	40	1	0.50	25	104.6	125.6	-20.93
26	35	1.5	0.75	27.5	169.0	202.8	-33.81
27	30	1	0.50	30	107.0	107.0	0.00
28	30	1	1	25	99.4	119.3	-19.89
29	35	1.5	0.25	27.50	150.8	165.9	-15.08
30	30	2	0.50	30	221.3	190.3	30.98

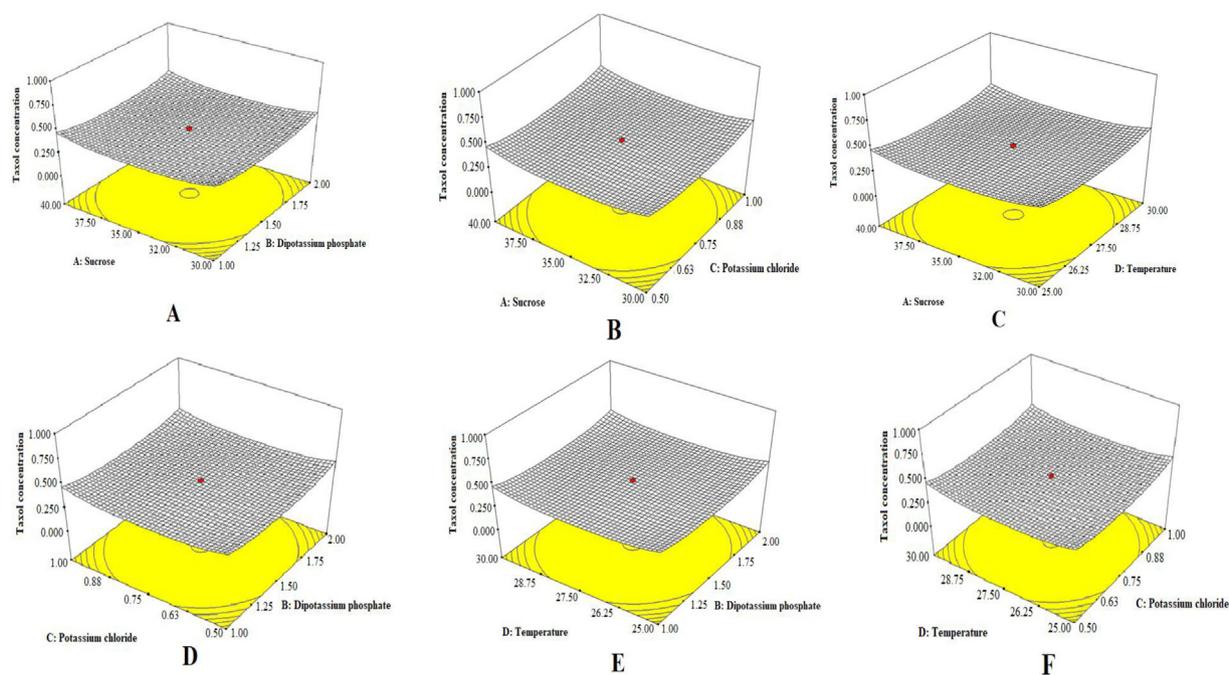


Fig. 5. Three-dimensional response surface curve showing the effect of interactions of a) Sucrose and Dipotassium phosphate b) Sucrose and Potassium chloride c) Sucrose and Temperature d) Potassium chloride and Dipotassium phosphate e) Temperature and Dipotassium phosphate f) Temperature and Potassium chloride from the Plackett-Burman design.

Table 8

ANOVA for response surface linear model for the experiments with CCD.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance	R-Squared	Std. Dev.
Model	9.02	4	2.26	28.06	< 0.0001	Significant	0.8178	0.28
A-Sucrose	1.88	1	1.88	23.37	< 0.0001	Significant		
B-Dipotassium phosphate	5.15	1	5.15	64.03	< 0.0001	Significant		
C-Potassium chloride	0.60	1	0.60	7.47	0.0114	Significant		
D-Temperature	1.40	1	1.40	17.36	0.0003	Significant		
Residual	2.01	25	0.080					
Lack of Fit	2.01	20	0.10	133.8	< 0.0001	Significant		
Pure Error	3.749	5	7.498					
Cor Total	11.03	29						

implying the significance of the model, and the values of "P > F" lower by about 0.050 reveals the significance of the model term. In this case sucrose, dipotassium phosphate, potassium chloride and temperature are significant model terms as shown in Table 8. The analysis revealed that the "Pred. R-Squared" of 0.7159 is in reasonable agreement with the "Adj. R-Squared" of 0.7887. Adeq. Precision" ratio of 19.736 measures the signal to noise ratio, as the ratio greater than four that being desirable, the Adeq. Precision ratio of 23.874 indicates the adequacy of signals. The results obtained from the CCD experiment were analyzed by ANOVA that yielded the following regression equation at the level of Taxol production: First-order model equation Terms of Coded Factors: Taxol Conc. = +2.17 + 0.28A+0.46B + 0.16C+0.24D.

3.4. Effect of Gamma irradiation and types of medium on Taxol production by *P. polonicum*

The yield of Taxol by *P. polonicum* has been further optimized with irradiation of the isolate with gamma-rays, in an endeavor to triggers the biosynthetic machinery of Taxol. The spores of *P. polonicum* was irradiated by gamma rays at different doses and

grown on the optimized Czapek's-Dox medium by response surface methodology with Plackett-Burman and FCCD designs. The cultures were incubated under standard conditions, and Taxol was extracted and quantified by TLC and HPLC. Noticeably, the yield of Taxol was increased by about 1.2 folds (401.2 $\mu\text{g/l}$) by γ -irradiation of the isolates at 0.5–0.75 kGy, comparing to the control cultures (332.2 $\mu\text{g/l}$) (Fig. 6). However, a significant reduction on the yield as well as fungal growth was observed upon further irradiation doses.

The influence of type of medium on Taxol production by *P. polonicum* has been assessed by growing the isolate on PDB, MID, modified Czapek's-Dox and malt extract. The cultures were incubated, Taxol was extracted and quantified. From the results (Fig. 6), the highest Taxol yield by *P. polonicum* has recovered by growing on modified Czapek's-Dox medium (401.2 $\mu\text{g/l}$), however, the yield of Taxol by the isolate upon growing on PDB, MID and Malt extract media was about 190–240 $\mu\text{g/l}$). Thus, upon nutritional optimization bioprocessing, and γ -irradiation, the yield of Taxol by *P. polonicum* has been strongly enhanced by about 4.4 folds (401.2 $\mu\text{g/l}$) comparing to the basal medium (90.5 $\mu\text{g/l}$).

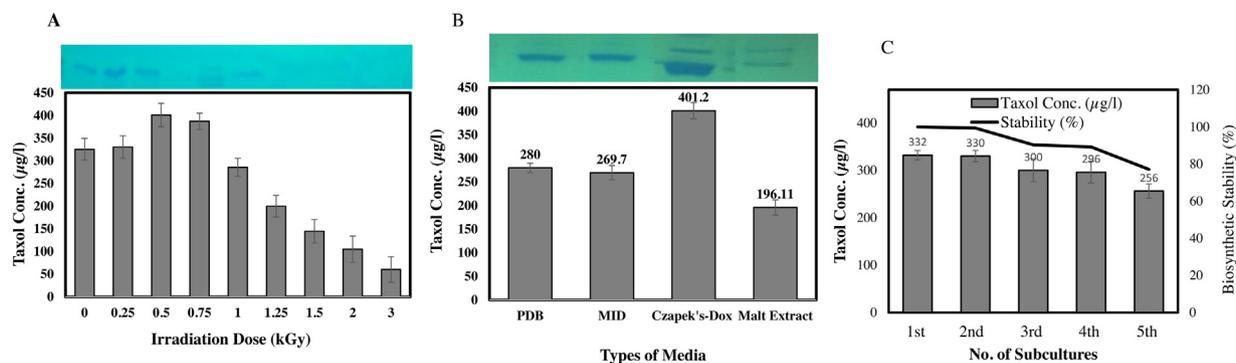


Fig. 6. Effect of Gamma irradiation dose and types of media on Taxol yield by *Penicillium polonicum*. A, The fungal cultures were irradiated with different doses of gamma rays, Taxol was extracted and quantified. B, The isolate of *P. polonicum* was grown on different types of media regarding to the optimized Czapek's-Dox medium as control, cultures were incubated at the desired conditions, and Taxol was extracted and quantified as described in Materials and Methods. The upper panel is the TLC plate with Taxol spots for each corresponding sample.

3.5. Effect of subculturing of *P. polonicum* on its Taxol biosynthetic stability

The effect of subculturing of *P. polonicum* on their Taxol biosynthetic stability has been evaluated, by the growing the different generations on the modified Czapek's-Dox media, incubated at standard conditions, Taxol was extracted and quantified as described above. From the results (Fig. 6), the biosynthetic stability of Taxol by *P. polonicum* was reduced by about 20 % at the fifth cultural generation, comparing to the original culture (zero culture).

4. Discussion

Fungal endophytes of with Taxol producing potency raised the hope for mass production of Taxol due to their fast growth, cost effective fermentation process, independence on climatic changes, resistance to shearing and feasibility of genetic manipulation [7,13,16,19,30,45,46]. However, the anticipation of fungi for industrial production of Taxol has been challenged by their lower reproducible yield and drastic loss of Taxol productivity with repeated subculturing [20,22]. Most of the endophytic Taxol-producing fungi were isolated from *Taxus* sp and *Podocarpus* sp which are belonging to family Taxaceae [6,12,20,22,35]. Exploring of the Taxol producing potency by endophytic fungi from plants outside Taxaceae family with sustainable Taxol productivity is the main objective by biotechnologists. *Ginkgo biloba* is one of the most traditionally recognized medicinal plants for its ethnopharmacological relevance such as anticancer activity, anti-inflammatory and antimicrobial activities [21], however, the Taxol producing potency of the endophytic fungi inhabiting this plant remain obscure. Thus, the objective of this study was to isolate and assess the Taxol producing potency of the endophytic fungi from *G. biloba*, as well as to assess the molecular biosynthetic stability of Taxol from the potent fungal isolate with storage and subcultures.

Twenty-eight endophyte fungal isolates were recovered from the different parts of *Ginkgo biloba*. Among the recovered fungi, the endophytic isolate *P. polonicum* gave the highest Taxol yield (90.53 µg/l), as authenticated from the TLC, UV- absorption and HPLC analysis. Similar screening paradigm for Taxol production has been reported for endophytes from *Podocarpus gracilior* [7] and other plants [13,16]. The Taxol yield by *P. polonicum* has been coincide with *A. flavipes* [13], *A. terreus* [27,37] an endophytes of *P. gracilior*, as well as for endophytes of *Taxus* spp such as *A. candidus* [47], *Fusarium solani* [48], *A. niger* [49] and *A. fumigatus* [50]. Consistently, the yield of Taxol by *P. polonicum* was coincident

with *A. flavipes* "saprophytic isolate" [13]. The identity of *P. polonicum* the potent Taxol producing fungal isolate was confirmed from the molecular analysis of the ITS region, the sequence was deposited on Genbank with accession #MK128497.1, as well as at Assiut University Mycological Centre (AUMC) under deposition # AUMC14487. Similarly, the identity of *P. polonicum* was confirmed based on the sequence of ITS region (Khalil et al., 2019, Çakir and Maden, 2015). Visagie et al., 2014. The isolate had 99 % similarity with *P. polonicum* isolates MH854992, NR103687, AF033475, MH862450, MH856411, NR119496, NR077206, JN942697, MH861768, MH856134, JN942742, NR138271, with zero E. value and 95 % query coverage.

The chemical identity of *P. polonicum* Taxol has been validated by ¹HNMR, ¹³CNMR, EDX and FT-IR analyses. The resolved signals of ¹HNMR and ¹³CNMR for *P. polonicum* Taxol were identical to the standard Taxol, that distributed between 1.0 and 8.0 ppm. Three proton signals were resolved at 1.0–3.0 ppm corresponding to methyl, acetate and acetylene groups, while the signals for aromatic moieties were resolved at 6.5–9.0 ppm [35]. Consistently, for all Taxane scaffolds, signals for their side chains protons were resolved at 2.0–7.0 ppm, while those for benzoate (C2), phenyl (C3) and benzamide (C3) groups were resolved at 7.0 and 8.4 ppm [51,52]. The FTIR spectra of *P. polonicum* Taxol were identical to authentic one, represented by a peak at 3325.82 cm⁻¹ for the hydroxyl group (OH), peak at 2943.7 cm⁻¹ to the aliphatic CH stretch, peak at 1113.7 cm⁻¹ to carboxyl stretching frequency, and peak at 1019.8 cm⁻¹ for the aromatic C–H. The aliphatic C–H stretch was observed at 2943.7 cm⁻¹, the C=O stretch was positioned at 1657.3 cm⁻¹, the peak at 1451.2 cm⁻¹ due to NH stretching frequency.

From the antimicrobial profile, Taxol had a significant antifungal activity against *C. albicans*, with mild antibacterial activity against *E. coli*. The extracted Taxol displayed a significant antifungal activity against *C. albicans* in a concentration dependent manner. The IC₅₀ values of *P. polonicum* extracted Taxol towards *E. coli* and *C. albicans* were 25 and 18 µM, while, it was 22 and 14 µM for authentic Taxol, respectively. The fungicidal activity of Taxol is one of the most characteristic biological features that has been observed towards a plethora of pathogenic fungi [53,23]. Taxol production by endophytes could be one of the main defense mechanisms of plant against different phytopathogens attacking the host plant. The antiproliferative activity of Taxol from *P. polonicum* has been evaluated towards various types of tumor including liver carcinoma (HPG2) and breast carcinoma (MCF7). From the viability assessment, *P. polonicum* Taxol had IC₅₀ values 4.06 µM and 6.07 µM towards HEPG2 and MCF7, respectively.

Similar IC₅₀ values for Taxol purified from *A. terreus* [7], *A. flavipes* [13], *N. sylviforme*, *C. oxysporum* [51,54], *T. brevifolia* [47,55] towards HEPG2 and MCF7 tumor cells.

The Taxol yield by *P. polonicum* has been optimized by modification of the chemical components of Czapek's-Dox as basal screening medium. The effect of eleven independent nutritional variables were assessed on productivity of Taxol by *P. polonicum* with the multiple response surface methodology. Plackett-Burman design and Centered Central Composite design have been used successfully for optimization of multiple variables by the microorganisms [44,56,57]. The predicted and actual yields of Taxol by *P. polonicum* as resolved from Plackett-Burman design were ranged from 30.6 and 239.0 µg/l, reflecting the significance of optimization process and efficiency of Plackett-Burman design. The highest actual Taxol yield by *P. polonicum* was 204.0 µg/l with the optimized variables sucrose (40 g/l), malt extract (20 g/l), peptone (2 g/l), NaNO₃ (4 g/l), K₂HPO₄ (1 g/l), MgSO₄·5H₂O (1 g/l), KCl (1 g/l), temperature 30 °C, pH 6.0, and incubated for 7 days at 120 rpm shaking. From the normal probability plot, the points are arranged near the diagonal line, revealing the adequate independent distribution of the variables. Thus, the expected yield of Taxol by *P. polonicum* was fitted maximally with the experimental results. From the Plackett-Burman design, the most significant variables affecting Taxol yield were sucrose (*p*-value 0.0989), K₂HPO₄ (*p*-value 0.0145), KCl (*p*-value 0.151) and incubation temperature (*p*-value 0.0862). The highly significant variables affecting Taxol yield by *P. polonicum* was further optimized with the FCCD design, giving the maximum yield (332.08 µg/l) with 40 g/l sucrose, 2 g/LK₂HPO₄, 1 g/l KCl and 30 °C incubation temperature. From the FCCD, sucrose (*p*-value <0.0001) and K₂HPO₄ (*p*-value <0.0001) were the most significant variable affecting Taxol yield by *P. polonicum* as revealed from the highest *f*-value 23.37 and 64.03, respectively. Thus, using the surface response methodology bioprocessing with Plackett-Burman and FCCD designs, the yield of Taxol by *P. polonicum* was increased into 401.25 µg/l comparing to 90.53 µg/l of control, by about 4.5 folds increment.

In an endeavor to maximize the yield of Taxol by *P. polonicum*, the fungal spores were irradiated by γ-rays, and the spores were grown on the modified Czapek's-Dox medium, Taxol was extracted and quantified. Upon γ-rays irradiation, the Taxol yield by *P. polonicum* was increased by about 1.2 folds comparing to the control cultures, suggesting the induction of biosynthetic gene cluster of Taxol. Similar results have been reported for Taxol biosynthesis by *Fusarium maire* and *Nodulisporium sylviforme* that increased by about 8.2 and 2.5 folds upon irradiation [58,6,12].

The molecular biosynthetic stability of Taxol by *P. polonium* has been evaluated with the fungal subculturing. The Taxol yield by *P. polonicum* was reduced by about 20% upon subculturing till the 5th subcultural generation. Attenuation of the biosynthetic machinery of Taxol by the fungi is the common metabolic practical challenge that limits the anticipation of fungi to be an industrial platform for commercial production of Taxol [6,12,14,22]. Noticeably, the attenuation of the biosynthetic potency of Taxol from *P. polonicum* have the same pattern to other endophytic fungi isolated from family *Taxaceae*.

In conclusion, the endophytic fungi inhabiting *G. biloba* were recovered and identified to the species level based on their morphological and molecular traits. The isolate *P. polonicum* was recovered from twigs of *G. biloba* and recognized as potent Taxol producer, based on the metabolic and molecular biomarker for Taxol synthesis. The yield of Taxol by *P. polonicum* was maximized via nutritional optimization of the isolate by response surface methodology with Plackett-Burman and FCCD designs, in addition to γ-irradiation by about 4.5 folds, comparing to the control isolate. The biosynthetic potency of Taxol by *P. polonicum* is relatively more

stable with the fungal multiple subculturing, comparing to other endophytes. Convincingly, the biosynthetic machinery of Taxol from endophytes inhabiting plants outside *Taxaceae* family, could be have an unique molecular regulators and expressing signals independent on their host plants.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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