Chemical Inhibitors Suggest Endophytic Fungal Paclitaxel is Derived from Both Mevalonate and Non-Mevalonate-Like Pathways

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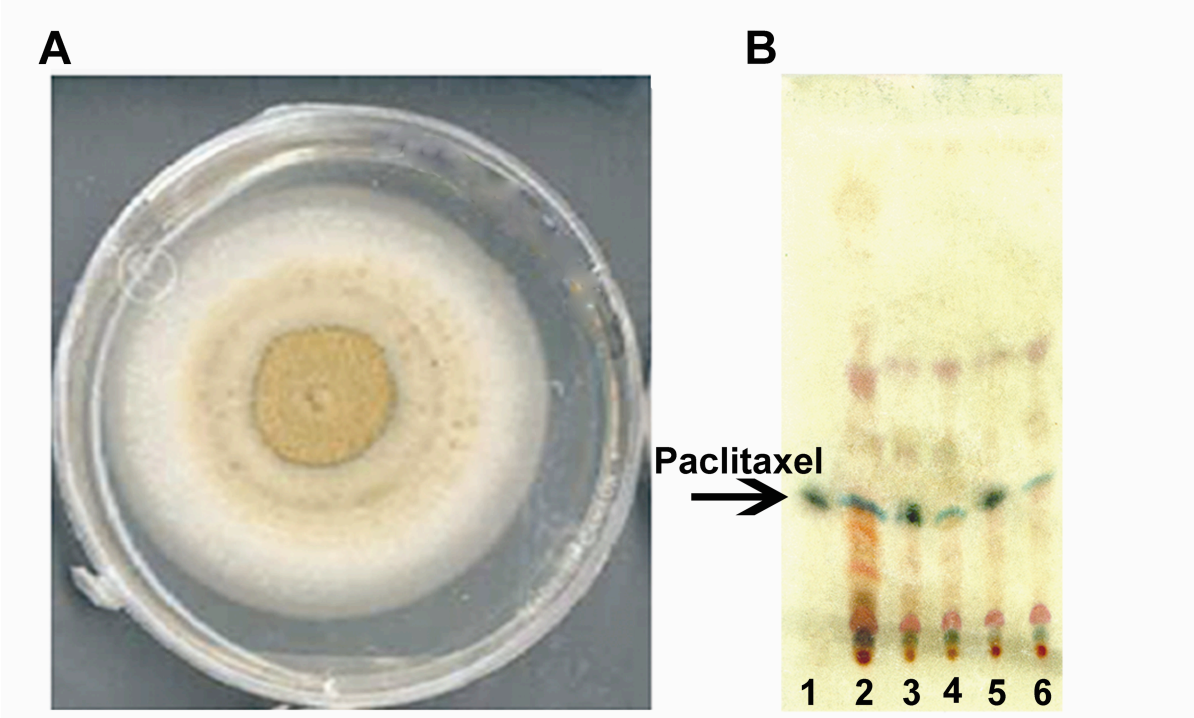

Figure S1. Culturing of paclitaxel-producing endophytic fungus. (A) Two week old pure fungal endophyte culture (SSM001) on PDA media isolated from T. media branches. (B) TLC of chloroform: methanol (9:1) extracts from five different fungal *Paraconiothyrium* SSM001 cultures (lanes 2-6) compared to authentic paclitaxel (lane 1).

Figure S1

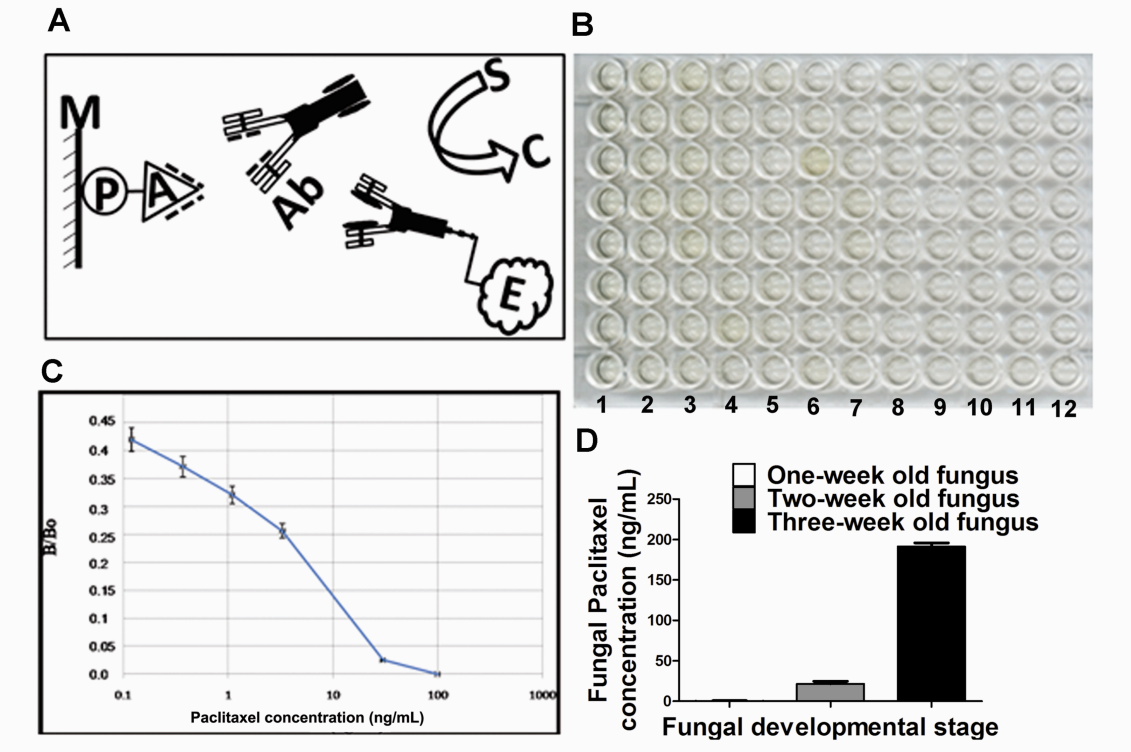

Figure S2. Quantification of paclitaxel using a competitive immunoassay with a commercial paclitaxel monoclonal antibody. (A) A schematic representation of the method. The surface of a microtiter plate (M symbol) is coated with the paclitaxel antigen (A triangle) via a protein linker (P circle). In the absence of exogenous paclitaxel, the paclitaxel monoclonal antibody (Ab symbol) binds to the plate-linked paclitaxel. Exogenous paclitaxel (sample) competes with the solid-phase antigen-antibody interaction and is washed away, thus reducing the final colorimetric signal. The solid-phase antigen-antibody interaction is detected with a secondary goat antibody linked to alkaline phosphatase enzyme (E symbol) which binds to the primary paclitaxel antibody (Ab symbol). Upon binding of the secondary antibody to the primary antibody, the alkaline phosphatase enzyme (E symbol) is activated, which cleaves the substrate p-nitrophenyl phosphate (S symbol), resulting in a yellow colour (C symbol). Exogenous sample paclitaxel thus reduces the yellow colour. (B) Example of the 96-well Paclitaxel competitive colorimetric immunoassay showing a paclitaxel standard curve on the left column, as well as blanks (reagents, but no exogenous paclitaxel) and samples in other rows. A blank corresponds to a yellow well, whereas a well containing a concentrated liquid paclitaxel standard or sample becomes more colourless. (C) Resulting paclitaxel standard curve from the competitive immunoassay demonstrating the linearity of the assay when plotted on a Log scale. The Y-axis (B/Bo) is the mean absorbance for each paclitaxel standard dilution (Abs B) added to non-paclitaxel *Fusarium* extract divided by the maximum absorbance in the absence of exogenous (aqueous-phase) paclitaxel (Abs Bo). All values were first subtracted from blank wells containing all reagents except exogenous paclitaxel. (D) Example of direct quantification of paclitaxel content (ng/mL) in the liquid cultures of fungus SSM001 at one, two and three week ages using the competitive paclitaxel immunoassay (raw data, diluted).

Figure S2

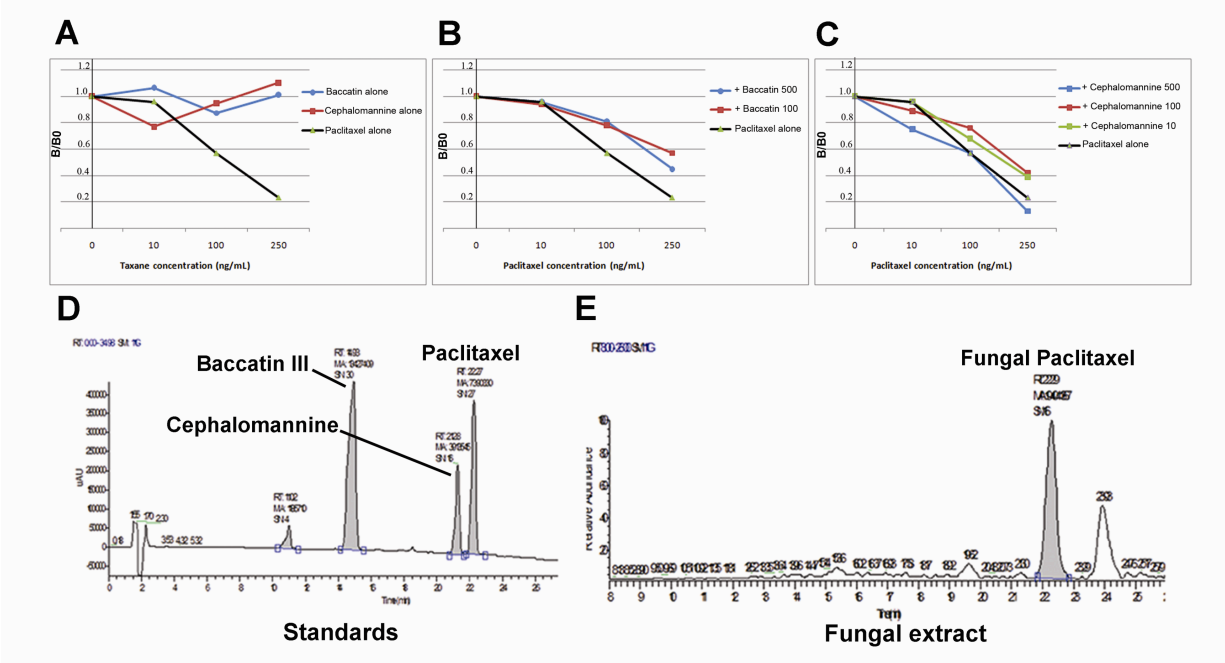

Figure S3. Determination of anti-paclitaxel antibody specificity. (A-C) Analysis of the sensitivity of the anti-paclitaxel antibody against the taxanes baccatin III and cephalomannine either (A) alone or (B,C) in combination with paclitaxel compared to paclitaxel alone. Shown are the means of three replicates. (D,E) HPLC analysis of (D) three different taxane standards, paclitaxel, baccatin III and cephalomannine, compared to (E) fungal SSM001 extract which showed only one peak corresponding to paclitaxel. This figure indicates that anti-paclitaxel antibody is specific to paclitaxel and not to related taxanes at the relative concentrations expected to occur in the fungal extract.

Figure S3

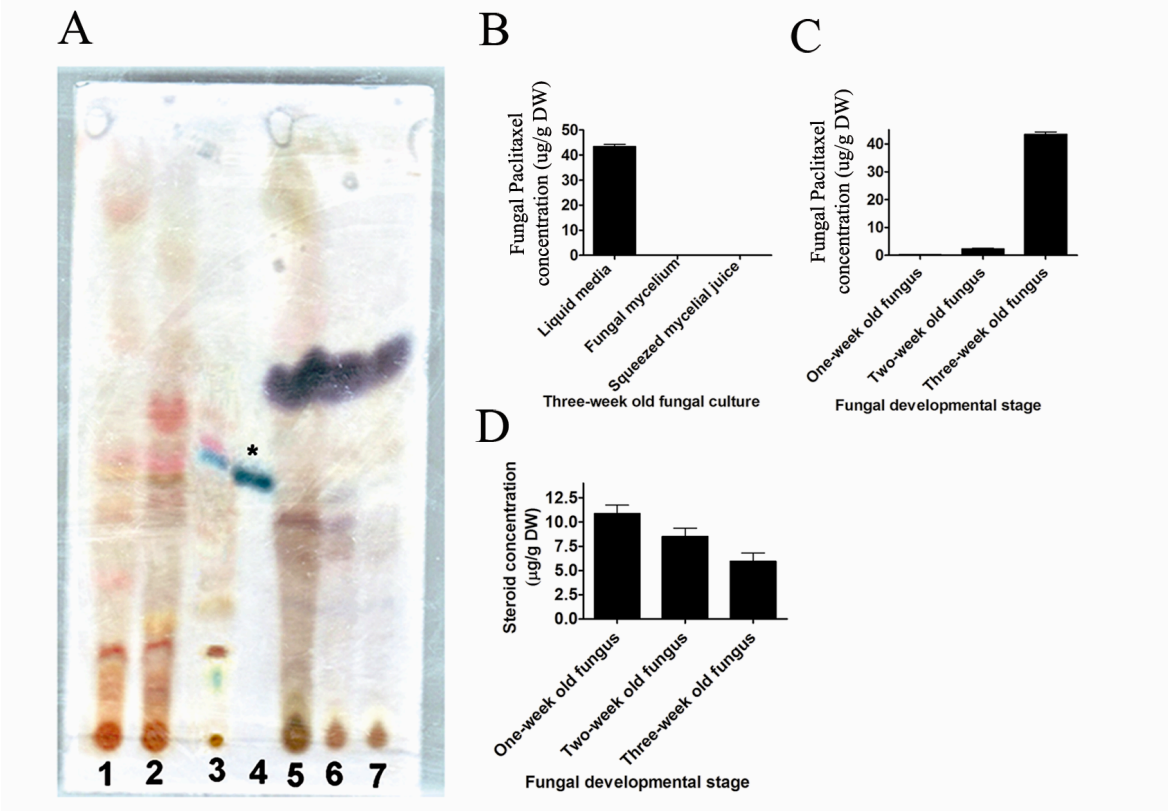

Figure S4. Quantification of fungal paclitaxel. (A) TLC of chloroform: methanol (9:1) extracts from different fungal culture samples at different developmental stages. Lane 1: one-week old liquid media fraction; Lane 2: two-week old liquid fraction; Lane 3: three-week old liquid fraction; Lane 4: paclitaxel standard (black asterisk); Lane 5: three-week old fungal mycelium fraction; Lane 6: two-week old mycelium fraction; Lane 7: one-week old mycelium fraction. The blue spot in the three-week old culture (liquid media fraction, Lane 3) matched the paclitaxel standard (Lane 4) and with an R_f value (relative to solvent front) of 0.56 and R_{st} value (relative to standard front) of 1.0. The blue spot was not present in any other lanes. (B) Quantification of paclitaxel concentration (µg paclitaxel/g mycelia dry weight, D.W.) in the liquid media fraction compared to mycelia and juice squeezed from the mycelia of fungus SSM001. Paclitaxel quantification was performed using a competitive immunoassay with a commercial paclitaxel monoclonal antibody (see Figure S2 for details). (C) Quantification of paclitaxel concentration (µg/g D.W.) in the liquid cultures of fungus SSM001 at different time points after inoculation. (D) Corresponding quantification of steroids from fungus SSM001 mycelia using the Liebermann-Burchard assay.

Figure S4

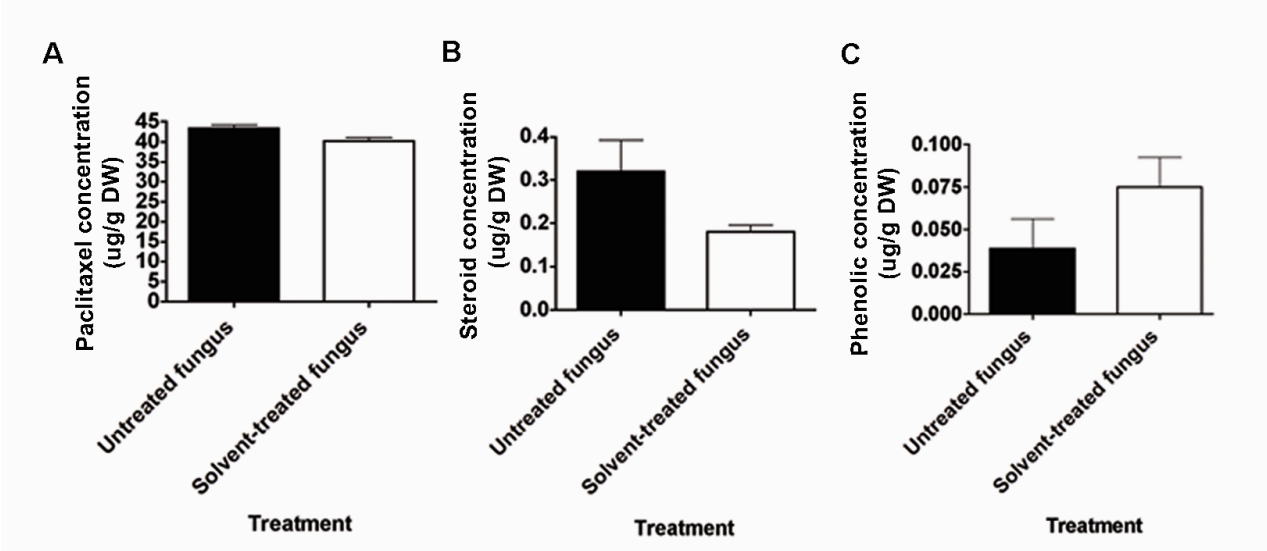

Figure S5. Effect of the DMSO: ethanol solvent system used in this study on fungal metabolism, three weeks after inoculation into liquid media cultures. (A) Effect on fungal paclitaxel accumulation [µg/g Dry Weight (D.W.), competitive immunoassay, liquid media fraction]. (B) Effect on fungal steroid levels (µg/g D.W., Liebermann-Burchard assay, fungal mycelia fraction). (C) Effect on accumulation of fungal phenolics (µg/g D.W., p-hydroxybenzaldehyde and Folin-Ciocalteu assay, liquid media fraction).

Figure S5

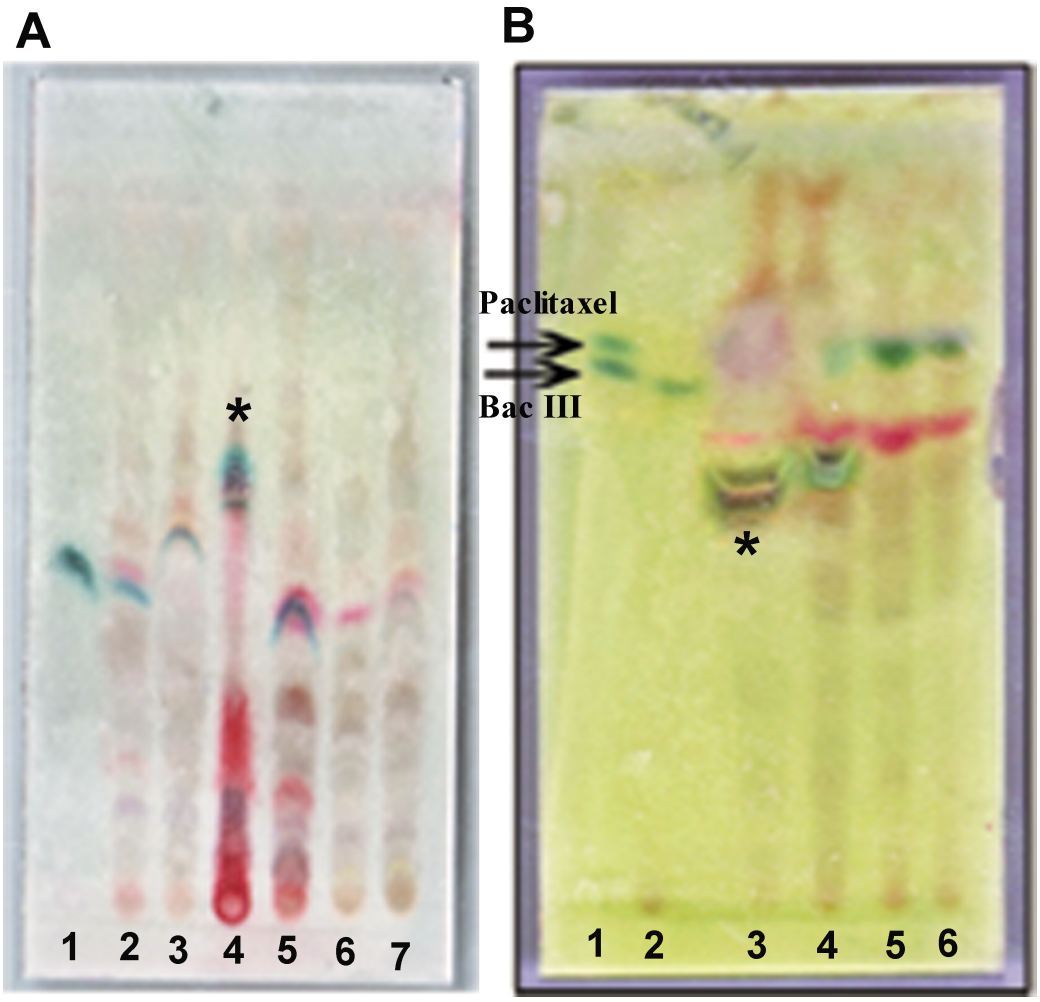

Figure S6. Effects on fungal paclitaxel accumulation of chemical inhibitors previously shown to target enzymes in the MVA and MEP terpenoid precursor or PAL phenolic pathways. (A) TLC showing the effects of compactin (mevastatin), a cytosolic MVA pathway inhibitor, and fosmidomycin, a plastid/bacterial MEP pathway inhibitor, on fungal paclitaxel in the liquid media fraction of three-week old fungal cultures. Lane 1: paclitaxel standard; Lane 2: untreated fungus; Lane 3: solvent-treated fungus; Lane 4: compactin at 50 nM, showing the appearance of novel blue spots (black asterisk); Lane 5: compactin at 5.0 nM; Lane 6: fosmidomycin at 3 µM, showing the disappearance of paclitaxel standard-equivalent-blue spot; Lane 7: fosmidomycin at 0.5 µM. (B) TLC showing the effects of cinnamic acid, an inhibitor of phenylalanine ammonia lyase (PAL), critical to the phenylisoserine side chain of plant paclitaxel. Cinnamic acid was applied to three-week old pure liquid cultures of fungal strain SSM001. Lane 1: pooled paclitaxel standard (upper blue spot) and baccatin III standard (Bac III, lower blue spot). Baccatin III is a paclitaxel intermediate in plants which lacks the phenylisoserine side chain; Lane 2: baccatin III standard; Lane 3: cinnamic acid at 1.26 mM, showing a novel blue spot (black asterisk); Lane 4: cinnamic acid at 0.42 mM, showing the partial re-appearance of the paclitaxel standard-equivalent blue spot; Lane 5: solvent-treated fungus; Lane 6: untreated fungus.

Figure S6

A

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AspergillusHMGS             KPDLTSEYPVVDG-HFSLRCYTEAVDACYKAYNAREKTLKEKV-QNGTNG
ArthrodermaHMGS             KPDLTSEYPYVDG-HYSIRCYTEAVDACYKAYNAREKVLKG---QNGDSN
LeptosphaeriaHMGS           KPDFTSEYPIVDG-HFSVRCYTEAVDACYKAYNAREETLKS---QQNGAN
PyrenophoraHMGS             KPDLTSEYPIVDG-HFSIRCYTEAVDACYKAYNEREKTLKA---QQN-GH
Paraconiothyrium_HMGS       LQNSPFEYPVVDGGHFSIRCYTEAVDACYKAYNAREQTLKSQANGSNGVN
                              : . *** *** *:*:*************** **:.**      ..

AspergillusHMGS             TAQDDSKTALDRFDYLCYHAPTC
ArthrodermaHMGS             GIVDESKTPLDRFDHILFHAPTC
LeptosphaeriaHMGS           GHAQGQETPLDRFDYMAFHAPTC
PyrenophoraHMGS             AVHQDVETPLDRFDYMAFHAPTC
Paraconiothyrium_HMGS       GHSQEQETPLDRFDYMCFHAPTC
                               :  :*.*****:: :*****
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B

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RhizobiumispDF                            QMPSKQPISAGIVIVAAGRGERAGSPTEGPKQYRPIG 39
AgrobacteriumDXS                          QMHSTQPMSIGIVIVAAGRGERAGSPEEGPKQYRAIG 39
SinorhizobiumDXS                          KMQGEEQFSCGVVIVAAGRGERAGQSAEGPKQYRTIG 60
OchrobactrumDXS                           LAEATTITKIAAVIVAAGRGERAGQSVEGPKQYRRIG 55
AspergillusDXS                            MVVSPNPPTIGIVVPAAGRGTRAGQG--CQKAYRRIA 35
                                             .    . . *: ***** ***.     * ** *.

RhizobiumispDF        GKPVIVHTLENFMTWEPATAIVVVIHPDDEALFAKAFRHIISATPIETVHGGPTRQRSVL 99
AgrobacteriumDXS      GRPVIAHTLEKFVTWPQTTKIVIVIHRDDEKLLRSAQETIVDSSGVEIAFGGTTRQQSVL 99
SinorhizobiumDXS      DRPVIAHTLDIFATWPGAGPVVVVIHPDDEELFAAARKRMAGALELTVVHGGATRQLSVL 120
OchrobactrumDXS       GEAVLARTLRVFIDCPLVDSIAVVIHPDDHALYERALP--ENRSNILVVNGGPTRQESTR 113
AspergillusDXS        GDTVINRVLKLFRSWHADCPIVIVHHADDTSLLEASID---RDQKIYTTTGGVERQASVL 92
                      . .*: :.*  *        :.:* * **  *   :         :  . **  ** *.

RhizobiumispDF        AGLRYLK--DKHVSHVLIHDAVRPFFDHTLLDRIAENLANG-ALAVLPAMPVTDTLKRAD 156
AgrobacteriumDXS      AGVRQLE--KTGVSHVMIHDAVRPFFDHDLLDRVAAALAAG-APAVLPAMPVTDTLKRAD 156
SinorhizobiumDXS      AGLEAVA--ATGVEYVMIHDAVRPFFDHTLLDRCGAALRDG-AQALLPAIAVADTLKRTK 177
OchrobactrumDXS       LGLRALQ--DSAPQYVMIHDGVRPFVGQDLLKRIVENLAP--DEGVLPALAVSDTLKQSS 169
AspergillusDXS        QGLRLLSTLDCVPSHVFIHDAARPFASHALLDSVLESLSTDPSIGVIPAIAVSDTLKKTD 152
                       *:. :       .:*:***..*** .: **.     *      .::**:.*:****::.

RhizobiumispDF        GAGTVLTTVSREQLFAAQTPQSFAFETILDAHEKAAASGRSDFTDDASIAEWLGIPVTIV 216
AgrobacteriumDXS      TDALVTETVPRAGLYAAQTPQSFRLADILAAHEKAAADNKTDFTDDAAIAEWAGLPVTLV 216
SinorhizobiumDXS      AAGLVAETVPRTDLYAAQTPQCFRLEPILSAHRRAAASGRSDFTDDASIAEWAGIPVLLV 237
OchrobactrumDXS       TDGTVKTTVPRAGLFAAQTPQAFPYAPILNAHEKAFAINRSDFTDDAAIAEWQGIAVRII 229
AspergillusDXS        SNGLITATVPRDGLFRAQTPQAFEFWSILAAHERAAKSG-SNYTDDASLFEQAGLPVRVV 211
                        . :  **.*  *: *****.*    ** **.:*   . :::****:: *  *:.* ::

RhizobiumispDF        EGTGDNVKLTVKKDIAMADDKLSA-------SLLPDVRTGNGYDVHQLEAGDGVTLCGVF 269
AgrobacteriumDXS      AGSADNVKLTIKRDIAMADEKLSA-------GLLPDVRTGNGYDVHQLEPGDGVTLCGVF 269
SinorhizobiumDXS      EGAVDNFKLTLRRDLSMADEKLTR-------VAIPDVRTGNGYDVHQLVDGDGVTLCGVF 290
OchrobactrumDXS       EGSADNTKLTWAKDIEMADKRLRQDH-----VSFPDIRTGNGYDVHSFEPGDHVTLCGVK 284
AspergillusDXS        PGDPQNIKLTFSVDFEEAERFLQARNSPIAPPTIPDVRVGHGYDTHRLVPGEGVILCGVK 271
                       *  :* ***   *:  *:  *           :**:*.*:***.* :  *: * ****

RhizobiumispDF        IPHDQKLKGHSDADVALHALTDALLATCGAGDIGDHFPPSDPQWKGAASRIFIEHAARIV 329
AgrobacteriumDXS      IPHDQTLKGHSDADVALHALTDALLATCGAGDIGDHFPPSDPQWRGAPSRIFIEHAARIV 329
SinorhizobiumDXS      IPHGRKLSGHSDADVALHALTDALLATCGAGDIGDHFPPSDPQWKGAPSRIFLEHAARIV 350
OchrobactrumDXS       IPHEAKLNGHSDADVGLHALTDALLATRGAGDIGTHFPPSDPQWKGAASRIFVEHAANIV 344
AspergillusDXS        IPHSASLLGHSDADVGLHALTNAFLGTIGADDIGSHFSPSDPQWKGASSDQFLRHAARLV 331
                       ***  .* *******.*****:*:*.* **.*** **.******:**.*  *:.***.:*

RhizobiumispDF        RERGGTIMNADVSLIAEAPKVGPHREAMRANLSEYLGIDLERCSVKATTNETIGFVGRRE 389
AgrobacteriumDXS      RDHGGTIMNADVSLIAEAPKVGPHREAMRAKLSEFLGISLERCSVKATTNEQIGFVGRRE 389
SinorhizobiumDXS      RERGGTVTNADISLIAEAPKVGPHRQQMRENLAAILGISLDRCSIKATTNEKLGFVGRNE 410
OchrobactrumDXS       REAGGRIANVDVTFISEAPKIGPHRIAMTEALCDMLGIAADRVSVKATTNEKLGFVGRRE 404
AspergillusDXS        VEAGGTITHCDVSFVCEKPRVSPYRDAMRDSVAGIVGLDRGRVSVKAGTNEKNGFL--IL 389
                       : ** : : *::::.* *::.*:*  *   :.  :*:   * *:** ***  **:

RhizobiumispDF        GIAAIATATVVYRGRT  405
AgrobacteriumDXS      GIAAIATATVVYRGGR  406
SinorhizobiumDXS      GIAAIATATVVYASRS  434
OchrobactrumDXS       GIAAIATATVIYPGEV  422
AspergillusDXS        GLIEIESRKIHYTCRA  449
                      *:  * : .: *
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C

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ParaconiothyriumDHQS      LHELTPGVPLRHGHAISIDMAYS ATLANGRKLMSDAEHHRILNLFSRAGLSMDHELFNEEI
AspergillusDHQS           LHELTPPVPLRHGHAISIDMAYS ATLANNRGLLSDDEHRRLLNLFSRAGLSMDHDLFNEEI
GlomerellaDHQS            LHELSPDVPLRHGHAISIDMAFS ATLAHVLGKLSSEEHRRLLNLFSRAGLSMDHPLFDEDM
PseudonocardiaDHQS        TLELAPPTPMLHGHAIAIDMAFS ATLAARRGDITTGERDRIHRLFSGLGLSVDSTYLTEQI
CyanotheceDHQS            TLELAPRVPMYHGHAVNIDMALS ATLAAKRGYISSTDRDRILGLMSRIGLALDHPLLESDI
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Figure S7

Figure S7. Identification of a *Paraconiothyrium* SSM001 gene encoding 3-hydroxymethyl glutyryl CoA synthase (HMGS), the enzyme preceding HMGR in the mevalonate pathway, and 3-dehydroquinate synthase (DHQS), a rate-limiting enzyme in the shikimate pathway, and identification of fungal *Aspergillus* DXS, the enzyme preceding DXR, in the bacterial/plant MEP pathway. (A) HMGS sequence similarity with other fungi using Clustal W. (B) Multiple sequence alignments of fungal *Aspergillus* DXS to bacterial and other fungal orthologs using Clustal W. (C) DHQS sequence similarity with other fungi using Clustal W. Highly conserved and similar amino acid residues are shown with an asterisk and dots, respectively.

Figure S7 continued

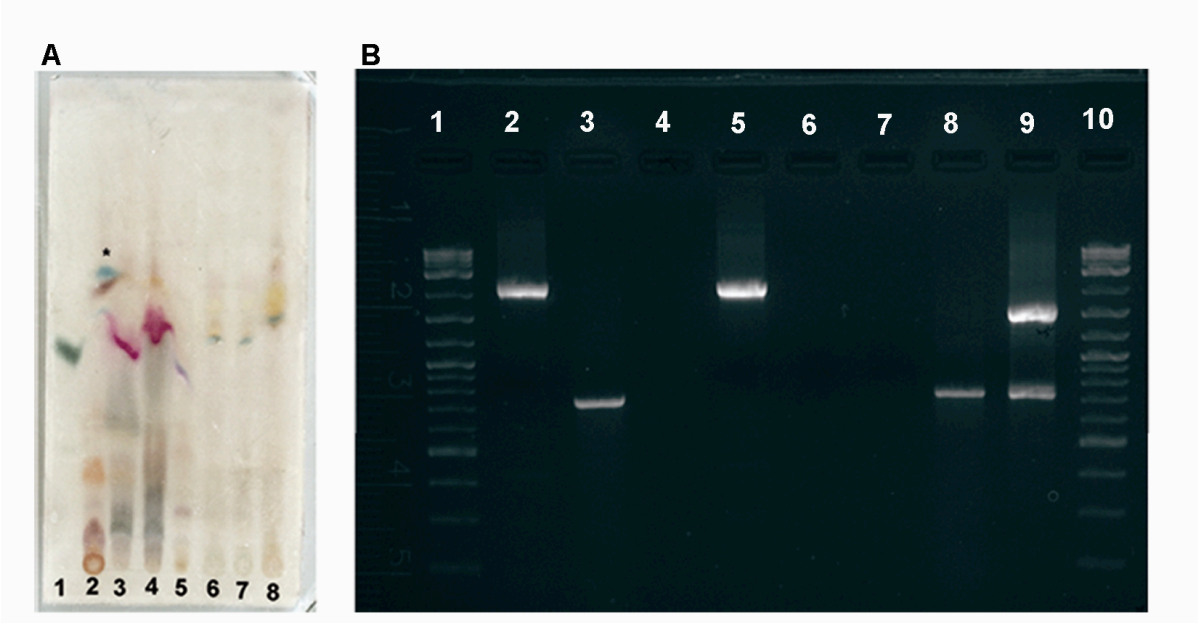

Figure S8. Determining whether the DXR-like enzyme observed in SSM001 fungus is due to a bacterial endophyte inhabiting the fungus. (A) TLC of SSM001 fungal liquid extract showing effects of chemical inhibitors. Lane 1: paclitaxel standard; Lane 2: 50 nM compactin (mevalonate inhibitor) confirming the disappearance of paclitaxel (blue spot) and the appearance of novel blue spots (black asterisk); Lane 3: 0.7 µM cycloheximide (eukaryotic cytosolic protein synthesis inhibitor) showing the disappearance of the paclitaxel spot, thus confirming the eukarotic origin of fungal paclitaxel; Lane 4: 0.5 µM cycloheximide; Lane 5: 7.0 nM cycloheximide; Lane 6: 5.0 mM aminooxyacetic acid (AOA, PAL inhibitor) showing that paclitaxel was unaffected; Lane 7: 2 mM AOA; Lane 8: 1.0 mM AOA. (B) Agarose gel electrophoresis to determine if *Paraconiothyrium* SSM001 fungus possesses bacterial endophytes. Shown are products of PCR amplification using primers specific for bacterial and plastidic 16S rDNA. Lanes 1 and 10: DNA marker (100 bp to 10 Kb); Lanes 2 and 5: SSM001 fungal genome template; Lanes 3 and 8: *E. coli* bacteria DNA template (positive control); Lanes 4 and 7: water template (negative control); Lane 6: fungal liquid media; Lane 9: *Taxus* plant genome template (positive control). The expected size of the bacterial/plastid 16S rDNA amplicon was 720 bp, equal to the lower band in lanes 3, 8 and 9. The large upper bands in lanes 2 and 5 (from the fungal genome) were cloned and sequenced and were found to be false-positives bearing no significant similarity to bacterial 16S rDNA.

Figure S8

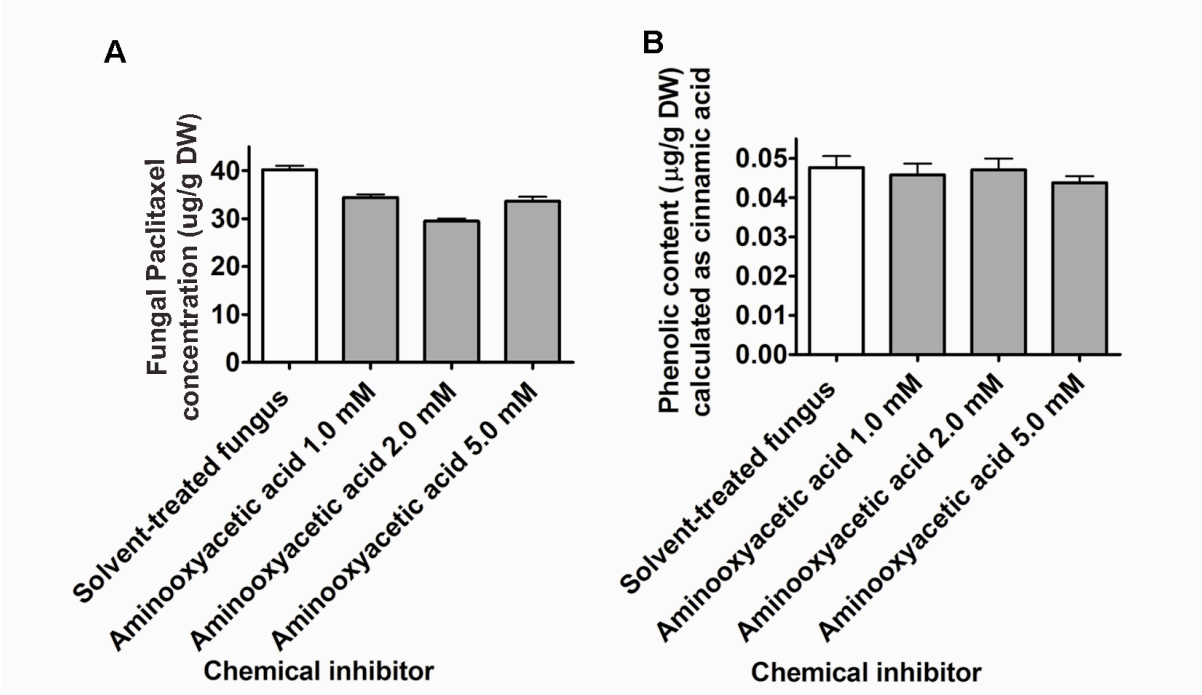

Figure S9. Effects of PAL inhibitor aminooxyacetic acid (AOA) on (A) fungal paclitaxel [µg paclitaxel/gram mycelia dry weight (D.W.), competitive immunoassay, liquid media fraction] and (B) fungal phenolics, calculated as cinnamic acid, a product of PAL in plants (µg/g D.W., P-hydroxybenzaldehyde and Folin-Ciocalteu assay, liquid media fraction).

Figure S9

Table *S*1 Effect of chemical inhibitors on fungal paclitaxel accumulation.

Chemical inhibitors and concentrations	Fungal Dry Weight (DW) g/L	Steroid content µg/L	Paclitaxel content µg/L	Steroid µg/gm DW	Paclitaxel µg/g DW
Solvent control (1 ml DMSO + 4 ml 70% ethanol)	11	2.03	80.3332 ± 0.83[1]	0.1846 (100%)[2]	7.3030(100%)[2]
Fosmidomycin 0.5 µM	14	2.35	43.533 ± 0.9	0.16858 (91.3%)	3.109 (42.6%)
Fosmidomycin 1.0 µM	14	1.82	30 ± 0.68	0.1302 (70.5%)	2.142 (29.3%)
Fosmidomycin 3.0 µM	12.2	1.41	0.8666 ± 0.18	0.1157 (62.7%)	0.071 (0.97%)
Compactin (mevastatin) 5.0 nM	10.7	0.963	80.333 ± 0.95	0.09042(49.0%)	7.50 (102.7%)
Compactin (mevastatin) 50.0 nM	11	1.449	0.066 ± 0.008	0.1318 (71.4%)	0.006 (0.08%)
Cycloheximide 7.0 nM	8.4	0.450	26.8 ± 0.4	0.05363(29.1%)	3.190 (43.6%)
Cycloheximide 0.5 µM	6.9	0.683	17.7332 ± 0.69	0.0996 (54.0%)	2.57 (35.2%)
Cycloheximide 0.7 µM	4	0.444	8.6 ± 0.88	0.1118 (60.6%)	2.15 (29.4%)
(N-Boc-aminooxy)acetic acid 1.0 µM	10.5	0.884	68.7933 ± 0.65	0.08429(45.7%)	6.551 (89.7%)
(N-Boc-aminooxy)acetic acid 2.0 µM	11	0.707	59 ± 0.5	0.06436(34.9%)	5.36 (73.4%)
(N-Boc-aminooxy)acetic acid 5.0 µM	10.9	0.834	67.2 ± 0.97	0.0766 (41.5%)	6.165 (84.4%)

[1]All concentrations were calculated as an average of three experiments and ± stands for the standard error of the mean.

[2]Bracket notes percentage relative to solvent control.

Table *S*2 Effect of cinnamic acid on fungal paclitaxel accumulation.

Chemical inhibitors and concentrations	**Fungal Dry Weight (DW) g/L**	**Paclitaxel content µg/L**	**Paclitaxel µg/g DW**
Solvent control (1 ml DMSO + 4 ml 70% ethanol)	12.4	193.44 ± 1.32[1]	15.60 (100%)[2]
dl-Cinnamic acid 0.42 mM	15.86	10.35 ± 0.46	0.66 (4.23%)
dl-Cinnamic acid 1.26 mM	14.378	0.151 ± 1.10	0.01 (0.064%)

[1]All concentrations were calculated as an average of three experiments and ± stands for the standard error of the mean.

[2]Bracket notes percentage relative to solvent control.