Screening of Taxol-producing Endophytic Fungi from *Ginkgo biloba* and *Taxus cuspidata* in Korea

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Received May 31, 1999

Endophytic fungi from *Ginkgo biloba* and *Taxus cuspidata* in Korea were screened for production of taxol. Eighteen and twelve fungal isolates from *G. biloba* and *T. cuspidata*, respectively, were shown to produce immunologically detectable amount of taxol. The highest production of taxol at 260 ng/*l* was achieved by stationary culture of an *Alternaria* isolate from *G. biloba*. The strain also produced unidentified antifungal agent(s) against *Pythium ultimum*. However, the activity gradually decreased when the strain was stored at 4°C for 6 months.

Key words : Ginkgo biloba, Taxus cuspidata, *taxol*, Alternaria, *endophyte*.

Plants and microbes are in various relationships in terms of their reliance to each other. Microbes sometimes invade plants causing disease in the host plants, whereas in other cases they co-exist peacefully in a symbiotic manner. Recent studies are revealing that such endophytic microbes can produce an array of complex secondary metabolites.[1,2] In this regard, endophytic microbes could be an ideal source for biologically active agents as the chemical inventory from the soil microorganisms becomes exhausted.

Furthermore, the endophytic relationships provide interesting insight into the entwined evolution of secondary metabolism between plants and microbes. For example, *Taxomyces andreanae*, a fungus, produces taxol to supplement the very same taxol production of the host *Taxus* plant.[2]

It is possible that medicinal woody plants provide unique environments for endophytic microflora to experience selective pressure exerted by the medicinal phytochemicals present in the plants. Therefore, occurrence of microbes with interesting chemotype from the medicinal plants could be more frequent than those from common plants. In this regard, endophytic fungi were isolated from Korean native *Taxus cuspidata*, *Ginkgo biloba*, containing potent cytotoxic principles ginkgolides and bilobalides, was also chosen for microbial screening in hopes of finding taxol producing microorganisms.

Materials and Methods

Plant materials. The twigs of *Ginkgo biloba* and *Taxus cuspidata* (about 1 cm in diameter) were collected from the garden of College of Agriculture and Life Sciences, Seoul National University, Suwon, Korea.

Isolation and culture of microorganisms. The twigs were surface-sterilized by soaking in 70% ethanol for 15 sec and brief flaming. The plant was cut longitudinally to separate bark, phloem and xylem tissues with a sterilized knife. The cuttings were placed on the top of water agar amended with streptomycin (10 ppm) and tetracycline (3 ppm) and incubated at 25°C. After several days, the plates were examined under a dissecting microscope, and young hyphal tip was cut with a Pasteur pipette to be transferred onto a PDA plate. For identification, hyphal tip was transferred onto a water agar plate with several pieces of γ-irradiated carnation leaves to allow fungal fruiting structures.[3] Liquid medium for surface culture (500 m*l* per 2*l*-Erlenmeyer flask, or 250 m*l* per 1-*l* Roux bottle) was M1D.[4] The medium was inoculated with several pieces of fungal plug and allowed to stand for 20 days at 25°C before harvest. The fungal plug was suspended in distilled water for storage at 4°C for later use.

Extraction and determination taxol. The fungal mat on M1D was removed by filtration through several layers of cheese cloth. The remaining broth was extracted twice each with one part of $MeCl_2$. The extract was combined, dried over anhydrous Na_2SO_4, and finally evaporated on a rotary evaporator. The dried material was weighed and subjected to taxol immunoassay.[5]

Identification of fungi and anti-fungal bioassay. The fungi, grown on carnation leaves on water agar plate, was examined under the microscope with or without Amann's mounting fluid and identified according to Barnet.[6] *Pythium ultimum* was inoculated on PDA plate with 5-day-old culture

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Abbreviation: PDA, potato-dextrose agar.

of the fungal isolates, and the bioactivity was judged by the diameter of the inhibition zone.

Results and Discussion

Fungal isolate. Total of 110 fungal isolates were obtained from each three samples of ginkgo and *T. cuspidata* (Table 1). Though each ginkgo twig was collected from three trees at nearby locations, the biodiversity of the fungi was considerable from tree to tree. From the *T. cuspidata*, about half of the isolates failed to produce spores when grown on carnation leaves. Overall, *Phoma* accounted for about a third

Table 1. Summary of fungal isolates from *G. biloba* and *T. cuspidata*.

	Number of Fungal Isolates							Total
	Sample of *G. biloba*				Sample of *T. cuspidata*			
	G(D)	G(L)	G(M)	subtotal	T(I)	T(W)	subtotal	
Alternaria	1	3		4				4
Ascophyta			1	1				1
Cephalosporium	2			2				2
Cladosporium		4		4				4
Epicoccum		1		1				1
Fusarium	1	2		3				3
Microphaeropsis	1		5	6				6
Papularia	1			1				1
Pestalotiopsis		2		2	1		1	3
Pennicilium					1		1	1
Phoma	1	8	9	18	19	6	25	43
unidentified		1	5	6	11	24	35	41
Total	7	21	20	48	32	30	62	110

Table 2. Taxol production from fungi isolated from *G. biloba* and *T. cuspidata*.

Sample ID[a]	Dry wt of Extract in mg/L	Taxol ng/mg Extract	Taxol ng/L	Producer
G(D)B2	6.40	0.69	4.42	UI[b]
G(D)B3	7.20	0.81	5.83	UI[b]
G(L)B1	8.6	ND[d]		*Pestalotiopsis*
G(L)B5	8.20	ND[d]		UI[b]
G(M)B5	4.2	ND[d]		UI[b]
G(D)X1	9.68	ND[d]		UI[b]
G(D)X3	7.88	0.59	4.65	UI[b]
G(D)X7	24.64	0.78	19.22	UI[b]
G(L)X2	6.52	0.78	5.09	UI[b]
G(L)X3	93.2	1.73	161.2	*Alternaria*
G(L)X4	76.0	1.51	114.8	*Alternaria*
G(L)X5	38.2	ND[d]		UI[b]
G(L)X6	16.2	1.01	16.4	*Pestalotiopsis*
G(L)X7	5.6	ND[d]		UI[b]
G(L)X8	70.0	2.15	150.5	*Alternaria*
G(L)X9	44.6	1.10	49.06	UI[b]
G(M)X1	15.44	0.88	13.59	UI[b]
G(M)X2	88.32	2.94	259.7	*Alternaria*
T(I)B2	9.80	1.01	9.90	UI[b]
T(I)B23	28.76	ND[d]		UI[b]
T(I)B24	13.12	0.51	6.69	UI[b]
T(I)B29	6.00	0.79	4.74	*Pestalotiopsis*
T(I)B34	12.40	0.49	6.07	UI[b]
T(I)B36	11.6	0.73	8.47	*Phoma*
T(I)B44	10.28	0.57	5.86	UI[b]
T(I)X1	7.40	0.56	4.14	UI[b]
T(W)B1	5.76	1.02	5.88	UI[b]
T(W)B8	5.44	ND[d]		UI[b]
T(W)B13	8.96	0.82	7.35	UI[b]
T(W)B18	9.12	0.71	6.48	UI[b]

[a]G, *G. biloba*; T, *T. cuspidata*. Characters in parenthesis are samlpling location code. B and X stand for bark and xylem.
[b]Unidentified. [c]Not detected.

of the isolates.

Antifungal activity against *P. ultimum*. *P. ultimum* was used for initial bioactivity test on PDA with 5-day-old culture. For the fastidious isolates, the fungi were allowed to grow until the diameter of mat became ca. 4~5 cm. *P. ultimum* was used as a primary test organism for antifungal activity. It is interesting that the frequency of the active isolates from ginkgo was higher than from *T. cuspidata* (data not shown). *Alternaria*, later shown to produce taxol, showed the most pronounced growth inhibition against *P. ultimum*. However, the growth inhibition did not seem to be due to taxol because *P. ultimum* was sensitive at only above 30 ppb of taxol. The fungus gradually lost the activity in 6 months during preservation at 4°C. Addition of ginkgo bark extract to the culture did not restore the activity against *P. ultimum*.

Taxol production. The fungi showing bioactivity against *Pythium* on PDA plate and representatives of morphological type-strains without activity were grown on M1D for 20 days, and the extracts were subjected to taxol analysis (Table 2). Production of taxol by the isolated *Pestalotiopsis* strains were in the range of 0~16 ng/*l*, although a number of high taxol producers are known in this genus.[4] However, four *Alternaria* strains from xylem of *Ginkgo* were found to produce taxol up to 260 ng/*l*. The highest taxol production from *Pestalotiopsis* is known to be up to 1,000 ng/*l*. Further screening is thus necessary to obtain a commercially viable taxol producer. It is interesting that the taxol-producing *Alternaria* strains were isolated from twigs of two ginkgo trees, but a nearby tree did not harbor the microorganism.

Taxol and related compounds were shown to be biosynthesized by various microorganisms. Fungi, such as *T. andreanae* from *Taxus brevifolia*[2] and *Pestalotiopsis microspora* from *Taxus wallachiana*,[7] are recorded to produce up to 1 μg of taxol. A bacterium, *Brevibacterium* sp. TA519, is claimed to produce 400~600 μg of taxol per liter of culture.[8] Our finding of taxol production by an *Alternaria* species isolated from *G. biloba* confirms that the biosynthesis of taxol by microorganism could be a widespread phenomena, not con-fined to a narrow spectrum of microorganism. Therefore, further search for higher taxol-producing microorganism which could produce commercially viable amount is warranted.

Acknowledgments. SUK thanks National Instrumantation Center for Enviromental Management, Seoul National University for the travel grant.

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