

Aspergillus terreus Yua-6 菌株對草莓灰黴病菌與木瓜炭疽病菌之拮抗能力分析

曾敏華、江主惠

E-mail: 345432@mail.dyu.edu.tw

摘要

植物的根圈附近存在許多能和植物相互作用的微生物，其中有些能幫助植物對抗病原菌。本實驗從田間土壤分離微生物，並以對峙培養與玻璃紙抗生法篩選出對草莓灰黴病菌(*Botrytis cinerea*)及木瓜炭疽病菌(*Colletotrichum gloeosporioides*)有拮抗能力之菌株，其中Yua-6菌株對此兩種病原真菌具最佳拮抗能力。利用適當引子對及聚合酶連鎖反應(polymerase chain reaction, PCR)，可擴增出Yua-6菌株之DNA片段約1.6 kb，經由DNA選殖、核醱酸解序及進行NCBI基因庫序列比對，得知Yua-6菌株和土麴菌(*Aspergillus terreus*)在此1.6 kb的序列上有100%的相同度。於PDB(potato dextrose broth)培養液培養6天的Yua-6發酵濾液經冷凍乾燥後添加於PDA培養基，結果發現含1 mg/ml及5 mg/ml的Yua-6冷凍乾燥液成分對木瓜炭疽病分別具19.29%及70.63%之抑制率，對草莓灰黴病菌則分別具4.93%及51.73%抑制率。另外，當噴灑10倍稀釋之Yua-6菌株發酵液到已接種木瓜炭疽菌孢子懸浮液(濃度 4×10^5 /ml)之成熟木瓜果實時，亦具有明顯保護效果。以Yua-6菌株發酵液之液相-液相萃取後得到乙酸乙酯層，於2 mg/ml濃度時對木瓜炭疽病具81.92%抑制率，對草莓灰黴病菌則具54.11%抑制率。此乙酸乙酯層再經矽膠管柱層析分離，當中以正己烷:乙酸乙酯 = 55:45所得之沖提液，調製成300 μ g/ml濃度時，對木瓜炭疽病有抑制效果。將此沖提液再以高壓液相層析儀(high pressure liquid chromatography, HPLC)分析，在滯留時間5分鐘時所收集之化合物，為具較佳抗菌效果之活性成分。

關鍵詞：拮抗菌、草莓灰黴病菌、木瓜炭疽病菌、土麴菌、生物防治

目錄

目錄	封面內頁	簽名頁	中文摘要	iii	英文摘要	v	誌謝	vi	目錄	vii	圖目錄	ix	表目錄	x																																																																															
1. 前言	1.1	1.1.1 草莓灰黴病菌與木瓜炭疽病菌的發生與防治	1	1.2 植物病害之生物防治	3	1.3 拮抗微生物及其在病害上的應用	3	1.4 土麴菌(<i>Aspergillus terreus</i>)之特性及應用	6	1.5 研究目的	7	2. 材料與方法	9	2.1 植物根圈土壤微生物之分離	9	2.2 土壤微生物拮抗能力之測試	9	2.3 土壤分離菌之菌種鑑定	10	2.3.1 Yua-6菌株DNA片段構築至TA載體	11	2.3.2 勝任細胞製備及質體轉型作用	12	2.3.3 質體DNA之快速篩選(Quick screening)	13	2.3.4 質體(plasmid) DNA抽取	14	2.4 Yua-6菌株最佳培養天數之測試	14	2.5 Yua-6菌株冷凍乾燥菌液之抑菌活性測試	15	2.6 木瓜果實炭疽病的防治試驗	16	2.7 Yua-6菌株發酵液之液相-液相萃取	16	2.8 抑菌活性成分分離與分析	17	3. 結果	19	3.1 土壤微生物之拮抗能力	19	3.2 Yua-6菌株之菌種鑑定	19	3.3 Yua-6菌株最佳培養天數之測試	20	3.4 Yua-6菌株冷凍乾燥液抑菌活性測試	21	3.5 木瓜果實炭疽病的防治試驗	21	3.6 Yua-6菌株發酵液之液相-液相萃取	22	3.7 抑菌活性成分分離與分析	23	4. 討論	24	5. 結論	29	參考文獻	48	附錄	55	圖目錄	圖1. 利用對峙培養法於PDA平板上測試田間土壤分離Yua-6	30	圖2. 利用玻璃紙抗生法於PDA平板上測試Yua-6菌株對植物病原真菌生長之拮抗作用	31	圖3. 聚合酶連鎖反應(polymerase chain reaction, PCR)進行Yua-6菌株基因體序列之鑑定	32	圖4. Yua-6菌株與其他6種 <i>Aspergillus</i> spp.進行核醱酸序列比對	36	圖5. 含不同濃度之Yua-6發酵濾液冷凍乾燥物對草莓灰黴病菌及木瓜炭疽病之菌絲生長抑制效果	37	圖6. 不同濃度之冷凍乾燥Yua-6發酵濾液對草莓灰黴病菌及木瓜炭疽病菌之菌絲生長抑制情形	38	圖7. Yua-6菌株發酵液進行木瓜果實炭疽病防治試驗。	39	圖8. Yua-6菌株發酵液冷凍乾燥以乙酸乙酯及水層分別萃取成分	40	圖9. 不同濃度之乙酸乙酯層經矽膠管柱層析的沖提部對草莓灰黴病菌及木瓜炭疽病菌之生長抑制效果。	41	圖10. Yua-6菌株之高壓液相層析儀(high pressure liquid chromatography, HPLC)分析	42	圖11. HPLC分析於所得之收集液	43	表目錄	表1. Yua-6菌株於PDB培養基最佳培養天數之測試	44	表2. 不同濃度之Yua-6冷凍乾燥發酵濾液	45	表3. 不同濃度之乙酸乙酯萃取物	46	表4. 乙酸乙酯層經矽膠管柱層析	47

參考文獻

- 行政院農委會農業統計年報。(2010)。作物生產-果品, 98. 彭淑貞, 黃勝泉, 張廣森。(2008)。草莓產業的發展及展望。苗栗區農業專訊 48期。
- Ahmed, A.S., Pe ' rez-Sa ' nchez, C., Egea, C., and Candela, M.E. (1999). Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathol.* 48, 58-65.
- Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980). Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci U S A* 77, 3957-3961.
- Alstrom, S. (1991). Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizosphere pseudomonads. *J. Gen. Appl. Microbiol.* 37, 495-501.

Bertagnolli, B.L., Daly, S., and Sinclair, J.B. (1998). Antimycotic compounds from the plant pathogen *rhizoctonia solani* and its antagonist *Trichoderma harzianum*. *J. Phytopathol.* 146, 131-135. Bonnarme, P., Gillet, B., Sepulchre, A.M., Role, C., Beloeil, J.C., and Ducrocq, C. (1995). Itaconate biosynthesis in *Aspergillus terreus*. *J. Bacteriol.* 177, 3573-3578. Bowers, J.H., Kinkel, L.L., and Jones, R.K. (1996). Influence of disease-suppressive strains of *Streptomyces* on the native *Streptomyces* community in soil as determined by the analysis of cellular fatty acids. *Can J Microbiol* 42, 27-37. Braun, P.G., and Sutton, J.C. (1988). Infection cycles and population dynamics of *botrytis cinerea* in strawberry leaves. *Can. J. Plant Pathol.* 10, 133-141. Chen, Z.G., Fujii, I., Ebizuka, Y., and Sankawa, U. (1992). Emodin O-methyltransferase from *Aspergillus terreus*. *Arch Microbiol* 158, 29-34. Chet, I., Inbar, J., and Hadar, I. (1997). Fungal antagonists and mycoparasites. *Environ. Microbiol.* IV, 165-184. Couey, H.M., Alvarez, A.M., and Nelson, M.G. (1984). Comparison of hot-water spray and immersion treatments for control of postharvest decay of papaya. *Plant Dis.* 68, 436-437. Curtis, R.F., Hassall, C.H., Jones, D.W., and Williams, T.W. (1960). The biosynthesis of phenols. Part II. Asteric acid, a metabolic product of *aspergillus terreus* thom. *J. Chem. Soc.*, 4838-4842. Dias, M.A., Lacerda, I.C., Pimentel, P.F., de Castro, H.F., and Rosa, C.A. (2002). Removal of heavy metals by an *Aspergillus terreus* strain immobilized in a polyurethane matrix. *Lett. Appl. Microbiol.* 34, 46-50. El-Abyad, M.S., El-Sayed, M.A., El-Shanshoury, A.R., and El-Sabbagh, S.M. (1996). Antimicrobial activities of *Streptomyces pulcher*, *S. canescens* and *S. citreofluorescens* against fungal and bacterial pathogens of tomato in vitro. *Folia Microbiol (Praha)* 41, 321-328. Elad, Y., Freeman, S., Minz, D., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., and Shafir, S. (2004). *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *Eur. J. plant path.* 110, 361-370. Fourati-Ben Fguira, L., Fotso, S., Ben Ameer-Mehdi, R., Mellouli, L., and Laatsch, H. (2005). Purification and structure elucidation of antifungal and antibacterial activities of newly isolated *Streptomyces* sp. strain US80. *Res Microbiol* 156, 341-347. Greenspan, M.D., and Yudkovitz, J.B. (1985). Mevinolinic acid biosynthesis by *Aspergillus terreus* and its relationship to fatty acid biosynthesis. *J. Bacteriol* 162, 704-707. Haas, D., and Defago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307-319. Hajjaj, H., Niederberger, P., and Duboc, P. (2001). Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Appl Environ Microbiol.* 67, 2596-2602. Islam, M.T., Hashidoko, Y., Deora, A., Ito, T., and Tahara, S. (2005). Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne *Peronosporomycetes*. *Appl Environ Microbiol.* 71, 3786-3796. Jarvis, W.R. (1962). Infection of strawberry and raspberry fruit by *Botrytis cinerea* Pers. *Ann Appl Biol.* 50, 569-575. Jarvis, W.R. (1964). The effect of some climatic factors on the incidence of grey-mould of strawberry and raspberry fruit. *Hortic. Res.* 3, 65-71. Jarvis, W.R. (1969). The phenology of flowering in strawberry and raspberry in relation to grey-mould control. *Hortic. Res.* 9, 8-17. Jarvis, W.R., and Borecka, H. (1968). The susceptibility of strawberry flowers to infection by *Botrytis cinerea* Pers. *Hortic. Res.* 8, 147-154. Jordan, V.W.L. (1978). Epidemiology and control of fruit rot caused by *Botrytis cinerea* on strawberry. *Pflanzenschutz.-Nachr. Bayer Kaji, A., Iwata, T., Kiriya, N., Wakusawa, S., and Miyamoto, K. (1994). Four new metabolites of Aspergillus terreus. Chem Pharm Bull (Tokyo) 42, 1682-1684.* Kerr, A. (1980). Biological control of crown gall through production of agrocin 84. *Plant Dis.* 64, 25-30. Kim, J.H., Lee, S.H., Kim, C.S., Lim, E.K., Choi, K.H., Kong, H.G., Kim, D.W., Lee, S.W., and Moon, B.J. (2007). Biological control of strawberry gray mold caused by *Botrytis cinerea* using *Bacillus licheniformis* N1 formulation. *J. Microbiol Biotechnol.* 17, 438-444. Koumoutsis, A., Chen, X.-H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., and Borriss, R. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J. Bacteriol.* 186, 1084-1096. Lay-Yee, M., Clare, G.K., Petry, R.J., Fullerton, R.A., and Gunson, A. (1998). Quality and disease incidence of 'Waimanalo Solo' papaya following forced-air heat treatments. *Hort Science* 33, 878-880. Leclere, V., Bechet, M., Adam, A., Guez, J.S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M., and Jacques, P. (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Appl Environ Microbiol* 71, 4577-4584. Melo, I.S., Faull, J.L., and Nascimento, R.S. (2006). Antagonism of *Aspergillus terreus* to *Sclerotinia Sclerotiorum*. *Braz. J. Microbiol.* 37, 417-419. Moyne, A.L., Shelby, R., Cleveland, T.E., and Tuzun, S. (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J Appl Microbiol.* 90, 622-629. Nakagawa, M., Hirota, A., Sakai, H., and Isogai, A. (1982). Terrecyclic acid A, a new antibiotic from *Aspergillus terreus*. I. Taxonomy, production, and chemical and biological properties. *J. Antibiot (Tokyo)* 35, 778-782. Neeno-Eckwall, E.C., Kinkel, L.L., and Schottel, J.L. (2001). Competition and antibiosis in the biological control of potato scab. *Can J Microbiol.* 47, 332-340. Nitta, K., Kiriya, N., Sakaguchi, Y., Tagushi, Y., and Yamamoto, Y. (1977). Studies on the metabolic products of *Aspergillus terreus*. III. metabolites of the strain IFO 8835. *Chem. pharm. bull. (Tokyo)* 25, 2593-2601. Nitta, K., Fujita, N., Yoshimura, T., Arai, K., and Yamamoto, U. (1983). Metabolic products of *Aspergillus terreus*. IX. Biosynthesis of butyrolactone derivatives isolated from strain IFO 8835 and 4100. *Chem Pharm Bull (Tokyo)* 31, 1528-1533. Pal, K.K., and McSpadden Gardener, B. (2006). Biological control of plant pathogens. *The Plant Health Instructor.* 10, 1-25. Paull, R.E., Nishijima, W., Reyes, M., and Cavaletto, C. (1997). Postharvest handling and losses during marketing of papaya (*Carica papaya* L.). *Postharvest Biology and Technology* 11, 165-179. Powelson, R.L. (1960). Initiation of Strawberry fruit rot caused by *Botrytis cinerea*. *Phytopathology* 50, 491-494. Rahman, M.A., Kadir, J., Mahmud, T.M.M., Abdul Rahman, R., and Begum, M.M. (2007). Screening of antagonistic bacteria for biocontrol activities on *Colletotrichum gloeosporioides* in papaya. *Asian journal of plant sciences.* 6, 12-20. Rahman, M.A., Mahmud, T.M.M., Kadir, J., Rahman, R.A., and Begum, M.M. (2009). Enhancing the efficacy of *Burkholderia cepacia* B23 with calcium chloride and chitosan to control Anthracnose of papaya during storage. *The plant pathology Journal.* 25, 361-368. Rojo, F.G., Reynoso, M.M., Ferez, M., Chulze, S.a.N., and Torres, A.M. (2007). Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Protection.* 26, 549-555. Sabaratnam, S., and Traquair, J.A. (2002). Formulation of a *Streptomyces*

biocontrol agent for the suppression of Rhizoctonia damping-off in tomato transplants. *Biological Control* .23, 245-253. Sankawa, U., Ebizuka, Y., Noguchi, H., Isikawa, Y., Kitagawa, S., Yamamoto, Y., Kobayashi, T., Iitak, Y., and Seto, H. (1983). Biosynthesis of citrinin in *Aspergillus terreus* : Incorporation studies with [2-¹³C, 2-²H₃], [1-¹³C, 18O₂] and [1-¹³C, 17O]-acetate. *Tetrahedron* 39, 3583-3591. Schimmel, T.G., Coffman, A.D., and Parsons, S.J. (1998). Effect of Butyrolactone I on the producing fungus, *Aspergillus terreus*. *Appl. Environ. Microbiol.* 64, 3707-3712. Schisler, D.A., Slininger, P.J., Behle, R.W., and Jackson, M.A. (2004). Formulation of *Bacillus* spp. for Biological Control of Plant Diseases. *Phytopathology* 94, 1267-1271. Schottel, J.L., Shimizu, K., and Kinkel, L.L. (2001). Relationships of in Vitro Pathogen Inhibition and Soil Colonization to Potato Scab Biocontrol by Antagonistic *Streptomyces* spp. *Biol Control*. 20, 102-112. Shanahan, P., O'Sullivan, D.J., Simpson, P., Glennon, J.D., and O'Gara, F. (1992). Isolation of 2,4-Diacetylphloroglucinol from a Fluorescent *Pseudomonad* and investigation of physiological parameters influencing Its production. *Appl Environ Microbiol.* 58, 353-358. Shi, J., Liu, A., Li, X., Feng, S., and Chen, W. (2010). Identification of endophytic bacterial strain MGP1 selected from papaya and its biocontrol effects on pathogens infecting harvested papaya fruit. *J Sci Food Agric* 90, 227-232. Slagg, C.M., and Fellows, H. (1947). Effects of certain soil fungi and their by products on *Ophiobolus graminis*. *J. Agr Res.* 75, 275-293. Takahashi, I., Ojima, N., Ogura, K., and Seto, S. (1978). Purification and characterization of dimethylallyl pyrophosphate:aspuvinone dimethylallyltransferase from *Aspergillus terreus*. *Biochemistry* 17, 2696-2702. Trillas, M.I., Casanova, E., Cotxarrera, L., Ordovas, J., Borrero, C., and Aviles, M. (2006). Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. *Biol Control*. 39, 32-38. Vagelas, I., Papachatzis, A., Kalorizou, H., and Wogiatzi, E. (2009). Biological control of *Botrytis* fruit rot (gray mold) on strawberry and red pepper fruits by olive oil mill wastewater. *Biotechnol Biotechnol Equip.* 23, 1489-1491. Vinci, V.A., Hoerner, T.D., Coffman, A.D., Schimmel, T.G., Dabora, R.L., Kirpekar, A.C., Ruby, C.L., and Stieber, R.W. (1991). Mutants of a lovastatin-hyperproducing *Aspergillus terreus* deficient in the production of sulochrin. *J. Ind Microbiol Biotechnol.* 8. Wilhite, S.E., Lumsden, R.D., and Straney, D.C. (2001). Peptide synthetase gene in *Trichoderma virens*. *Appl Environ Microbiol.* 67, 5055-5062. Wright, S.A., Zumoff, C.H., Schneider, L., and Beer, S.V. (2001). *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovora* in vitro. *Appl Environ Microbiol* 67, 284-292. Yoshihisa, H., Zenji, S., Fukushi, H., Katsuhiko, K., Haruhisa, S., and Takahito, S. (1989). Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. *Soil Biology and Biochemistry.* 21, 723-728. Yuan, W.M., and Crawford, D.L. (1995). Characterization of *streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl Environ Microbiol.* 61, 3119-3128.