CHAPTER IV

RESULTS AND DISCUSSION

1. Isolation of the endophytic fungus ARE-1

Only one isolate of the endophytic fungus was germinated from healthy leaf of *Annona reticulata* L. (Annonaceae) (Figure 1). It was designated as ARE-1. Its colony morphology on five different culture media, *i.e.* CzYA, MCzA, MEA, SDA and YES, is shown in Figure 2.

2. Classification of the endophytic fungus isolate ARE-1

2.1 Conventional method

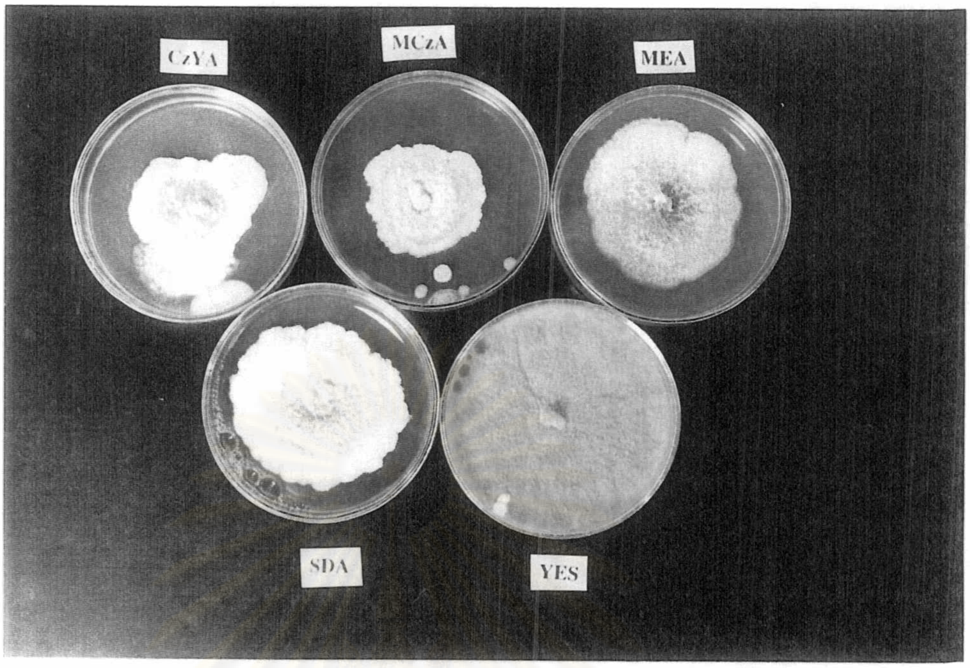
Isolate ARE-1 did not produce conidium or spore on common mycological media including PDA. In addition, it did not sporulate when grown on banana leaf agar, a nutritionally weak medium, under near UV light (a 12 h on/off cycle) for 2 months. This condition is suggested for promoting sporulation (Smith and Onions 1994). Therefore, ARE-1 was classified as Mycelia sterilia.

2.2 Molecular method

In attempt to classify the endophytic fungus isolate ARE-1, molecular method determining the nucleotide sequence of ITS1-5.8S-ITS2 region of rRNA gene was applied. Nucleotide sequence of ITS region can be used to separate taxa from class to species (Mitchell *et al.*, 1995).

Figure 1 Leaf of *Annona reticulata* L. (Annonaceae)

A



B

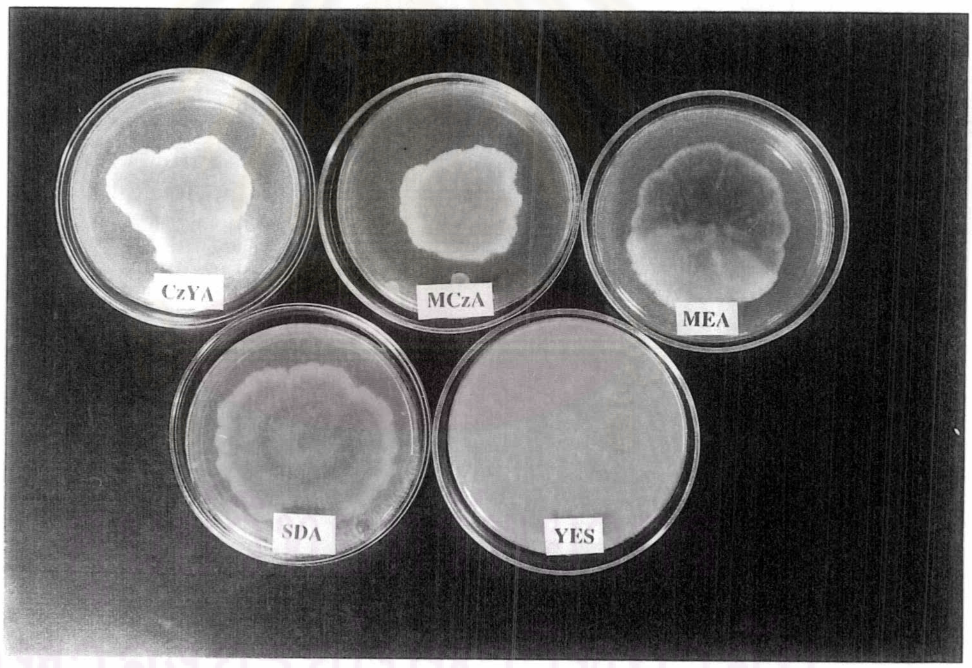


Figure 2 Colony morphology of the endophytic fungus isolate ARE-1 on five different mycological media (CzYA, MCzA, MEA, SDA and YES) after cultivation for 7 days at 30 °C. Appearance on the obverse side (**A**) and on the reverse side (**B**).

2.2.1 The PCR product of ITS1- 5.8S-ITS2 region of ribosomal DNA

PCR conditions were optimized to amplify a rDNA gene of the isolate ARE-1. The oligonucleotide primers ITS4 and ITS5 were used to amplify a DNA fragment at 3′ end of 18S, ITS1-5.8S-ITS2 and 5′ end of 28S rDNA. The PCR product with expected size was obtained, as shown in Figure 3.

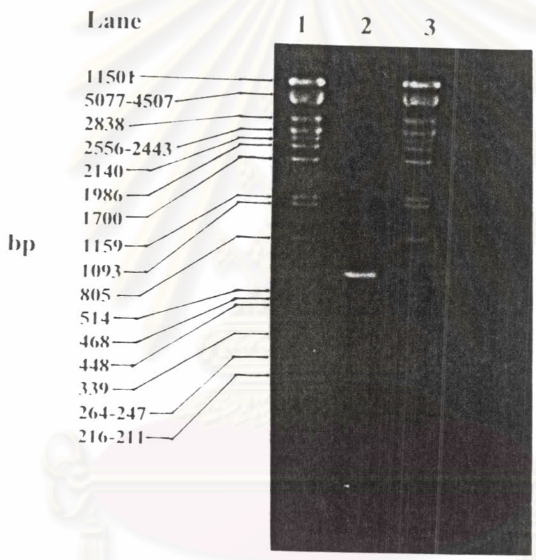


Figure 3 Agarose gel electrophoresis of PCR product from amplification of 3′end of 18S, ITS1-5.8S-ITS2 and 5′ end of 28S rDNA. Lanes 1 and 3 were λ*Pst*I marker, and Lane 2 was the PCR product of ARE-1.

2.2.2 Nucleotide sequences of partial 18S sequence, complete ITS1-5.8S-ITS2 sequences and partial 28S sequence of the isolate ARE-1

Sequencing of the PCR product resulted in a 583 bp fragment. It comprised 3′ end of 18S sequence, complete ITS1-5.8S-ITS2 sequences and 5′ end of 28S sequence, as shown in Figure 4.

1 TTGGA AGTAA AAGTC GTAAC AAGGT CTCCG TTGGT GAACC 40

18S ←|→ ITS1
41 AGCGG AGGGA TCATT G|CTGG AACGC GCCCC AGGCG CACCC 80

81 AGAAA CCCTT TGTGA ACTTA TACCT TACTG TTGCC TCGGC 120

121 GTACG CTGGC CCCTA GGGGT CCCTC TGTCT ACAGA GGAGC 160

161 AGGCG CGCCG GCGGC CAAGT TAACT CTTGT TTTTA CACTG 200

ITS1 ←|→ 5.8S
201 AAACT CTGAG AAAAA AACAA AAATG AATCA|AAACT TTCAA 240

241 CAACG GATCT CTTGG TTCTG GCATC GATGA AGAAC GCAGC 280

281 GAAAT GCGAT AAGTA ATGTG AATTG CAGAA TTCAG TGAAT 320

321 CATCG AATCT TTGAA CGCAC ATTGC GCCCT CCGGT ATTCC 360

5.8S ←|→ ITS2
361 GGAGG GCATG CCTGT TCGAG CGTCA TTT|CA ACCCT CAAGC 400

401 CTGGC TTGGT GTTGG GGCAC TGCCT GTAAA AGGGC AGGCC 440

441 CTCAA ATCTA GTGGC GAGCT CGCCA GGACC CCGAG CGTAG 480

481 TAGTT AAACC CTCGC TTTGG AAGGC CCTGG CGGTG CCCTG 520

ITS2 ←|→ 28S
521 CCGTT AAACC CCCAA CTCTT TGAAA AT|TTG ACCTC GGATC 560

561 AGGTA GGAAT ACCCG CTGAA CTT

Figure 4 Nucleotide sequences of the partial 18S sequence, complete ITS1-5.8S-ITS2 sequences and partial 28S sequence of the isolate ARE-1.

	10	20	30	40
Chaenothecopsis pusilla	-----TCGAG	TGAGGGTCCT	CGTGG--CCC	AACCTCCAA-
ARE1	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
Fungal endophyte MUT 2715	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
Diaporthe phaseolorum	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
D.meridionalis	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
D.caulivora	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
D.helianthi	----CTGGAA	CGCGCCCC--	CGGCGCACCC	AG-----AAA
D.vaccinii	----CTGGAA	-GCCCCCC--	AGAAGCACCC	AG-----AAA
Phomopsis vaccinii	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
P.oryzae	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
P.amygdali	----CTGGAA	CGCG---CCT	CGGCGCACCC	AG-----AAA
P.quercina	----CTGGAA	CGCGCCCC--	AGGCGCACCC	AG-----AAA
P.juniperivora	----TTGGAA	CGCGCCCC--	AGGGGCACCC	A------AAA
P.longicolla	----CTGGAA	CGCG---CTT	CGGCGCACCC	AG-----AAA
P.sojae	----CTGGAA	CGCG---CTT	CGGCGCACCC	AG-----AAA
P.sclerotioides	----CTGGA-	CGCG---CTT	CGGCGCACCC	AG-----AAA
P.columnaris	ATTGCTGGAA	CGCG---CTT	CGGCGCACCC	AG-----AAA
Cytospora eucalypticola	----TTGGAA	CGCG---CT-	C-GCGCACCC	AG-----AAA
Mycosphaerella latebrosa	----------	TGAGGGCCCT	CGG----GCC	CGACCTCCCA
M.berberidis	----------	TGAGGGCCTT	CGG----GCT	CGACCTCCAA
M.nubilosa	----C-CGAG	TGAGGG----	CGGC--AGCC	CGACCTCCTA
M.cryptica	----C-CGAG	TGAGGG----	CGCC--CGCC	CGACCTCCAA
Lophodermium conigenum	-AAGAAAAAA	C-ATG-CCTT	CGG----GCT	CTGTTCTTCT
Pseudocercospora eriodendri	----CT-GAG	TGAGGGCTCA	CG------CC	CGACCTCCAA
Guignardia endophyllicola	--CGCCGAAA	TGA---CCTT	C---------	-----TCA--
Phyllosticta pyrolae	-CCGGGGAA-	---GGTCC-T	C---------	-----TCACA

	50	60	70	80
Chaenothecopsis pusilla	CCCACT-GTT	TA------CC	TGT-CCT---	-TGTTGCTTC
ARE1	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
Fungal endophyte MUT 2715	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
Diaporthe phaseolorum	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
D.meridionalis	CCCTTT-GTG	AA------CT	CATACCTT-A	CTGTTGCCTC
D.caulivora	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
D.helianthi	CCCTTT-GTG	AA------CT	TATACCTAT-	CTGTTGCCTC
D.vaccinii	CCCTTT-GTG	AA------CT	TATACCTT-A	TCGTTGCCTC
Phomopsis vaccinii	CCCTTT-GTG	GA------CT	TATACCTT-A	CTGTTGCCTC
P.oryzae	CCCTTT-GTG	AA------CT	TATACCTT--	TTGTTGCCTC
P.amygdali	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
P.quercina	CCCTTT-GTG	AA------CT	TATACCTT-A	CTGTTGCCTC
P.juniperivora	CCCTTT-GTG	AA------CT	GATACCTT-A	CTGTTGCCTC
P.longicolla	CCCTTT-GTG	AA------CT	TATACCT--A	CTGTTGCCTC
P.sojae	CCCTTT-GTG	AA------CT	TATACCTAT-	TTGTTGCCTC
P.sclerotioides	CCCTTT-GTG	AA------CT	TATACCT-TA	CTGTTGCCTC
P.columnaris	CCCTTT-GTG	AA------CT	TATACCT-TA	CTGTTGCCTC
Cytospora eucalypticola	CCCTTT-GTG	AA------CT	TATACCTATA	CTGTTGCCTC
Mycosphaerella latebrosa	CCCTTTTGTG	AACACA-ACT	----------	-TGTTGCTTC
M.berberidis	CCCTTT-GTG	AACACA-ACT	----------	-TGTTGCTTC
M.nubilosa	CCCCAT-GTT	TTCCC--ACC	---------A	C-GTTGCCTC
M.cryptica	CCCCAT-GTT	TTC-CA-ACC	A---------	-TGTTGCCTC
Lophodermium conigenum	CCCTTT-GTT	TAC-CACACT	TA--------	--GTTGCCTT
Pseudocercospora eriodendri	CCCTTT-GTG	AACCAAT-CT	----------	-TGTTGCTTC
Guignardia endophyllicola	CCCTT--GTG	TAC-T-CACT	A---------	-TGTTGCTTT
Phyllosticta pyrolae	CCCTT--GTG	TACCT-TACC	A---------	-TGTTGCTTT

Figure 5 Alignment data of complete ITS1-5.8S-ITS2 sequences of isolate ARE-1 and 25 reference taxa.

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                               ....|....| ....|....| ....|....| ....|....|
                                       90        100        110        120
Chaenothecopsis pusilla        GGCGGGGTCA --CCGTTCTC CCTCC-GG-- -GGGG-GATG
ARE1                           GGCG---TAC -GCTGGCCCC -TA------- --GGG-GTCC
Fungal endophyte MUT 2715      GGCG---CAT -GCCGGCCCC C-A------- --GGG-GCCC
Diaporthe phaseolorum          GGCG---CAG -GCCGGCCCC CTA------- -CGGG-GCCC
D.meridionalis                 GGCG---CAG -GCCGGCCCC CCC---AG-- --GGG-GCCC
D.caulivora                    GGCG---CAG -GCCGGCCCC -CTT------ -GGGG-GCCC
D.helianthi                    GGCG---CAG -GCCGGCCCC CC--CT-G-- --GGG-GCCC
D.vaccinii                     GGCG---CTA -GCTGGCCC- -CT-C----- --GGG-GCCC
Phomopsis vaccinii             GGCG---CTA -GCTGGCCC- -CT-C----- --GGG-GCCC
P.oryzae                       GGCG---CT- -GCTGGT--- -CT-CTAGTA ---GG--CCC
P.amygdali                     GGCG---CAG -GCCGGCCCC -CTTCT---- -GGGG-GCCC
P.quercina                     GGCG---CTA -GCTGGTCC- --TTC----- --GGG-GCCC
P.juniperivora                 GGCG---CTA -GCTGGTCC- --TTC----- --GGG-GCCC
P.longicolla                   GGCG---CAG -GCCGGCCT- -TTT-GTG-A CAAAG-GCCC
P.sojae                        GGCC---TAG -GCCGGCCT- -CTTC----A CTGAG-GCCC
P.sclerotioides                GGCG---CAG -GCCGGCC-- ---TC----A CCGAG-GCCC
P.columnaris                   GGCG---CAG -GCCGGCC-- ---TC----A CTGAG-GCCC
Cytospora eucalypticola        GGCG---TCG -GCTGCCCCC C--TCG---- -GGGG-GTCC
Mycosphaerella latebrosa       GGGG--GCGA CCCTGCCG-- -TTTCG--CG GCGAGCGCCC
M.berberidis                   GGGG--GCGA CCCTGCCG-- -TTTCGA-CG GCGAGCGCCC
M.nubilosa                     GGGG--GCGA CCC-GGCCCC C--GCGC-CG G--GG-CCCT
M.cryptica                     GGGG--GCGA CCC-GGCCGC C--GTGC-CG G--GG-CCCC
Lophodermium conigenum         GGCG---CA- --CCG--CGC C-AGTGG--A TCGAAACCCT
Pseudocercospora eriodendri    GGGG--GCGA CCCTGCCGGC ACTTCG---- CTGGGCGCCC
Guignardia endophyllicola      GGCG--GCGA -CCTGG---- -TTCCGG-CG GCCGGCGCCC
Phyllosticta pyrolae           GGCG--GCGA -CCCGG---- -TTTCGG-CG GCCGGCGCCC

                               ....|....| ....|....| ....|....| ....|....|
                                      130        140        150        160
Chaenothecopsis pusilla        GCCC------ ---------- ---------- GCCGGAGGAT
ARE1                           CTCTG--TCT ----ACAGAG GAGCAGGCGC GCCGGCGGCC
Fungal endophyte MUT 2715      CTCGG-A--- ----GACGAG GAGCAGGCAC GCCGGCGGCC
Diaporthe phaseolorum          CTTGGC---- ----GACAAG GAGCAGGCCC GCCGGCGGCC
D.meridionalis                 CTCGG-A--- ----AACGAG GAGCAGGCCC GCCGGCGGCC
D.caulivora                    CCCGG-A--- ----GACGGG GAGCAG-CCC GCCGGCGGCC
D.helianthi                    CCTGGGA--- -----ACAGG GAGCAG-CCC GCCGGCGGCC
D.vaccinii                     CTCACCCTC- GGG-----TT GAGACGGCCC GCCGGCGGCC
Phomopsis vaccinii             CTCACCCTC- GGGTG---TT GAGACGGCCC GCCGGCGGCC
P.oryzae                       CTCAC--TC- CGGTGAGGAA AA---GGCAC GCCGGCGGCC
P.amygdali                     CTCGT--TC- C--TGACGAG GAGCAGGCTC GCCGGCGGCC
P.quercina                     CTCACCCTC- GGGTG---TT GAGATAGCCC GCCGGCGGCC
P.juniperivora                 CTCACCCTC- GGGTG---TT GAGACAGCCC GCCGGCGGCC
P.longicolla                   CCT------- -GGAGACAGG GAGCAG-CCC GCCGGCGGCC
P.sojae                        CCT------- -GGAGACAGG GAGCAG-CCC GCCGGCGGCC
P.sclerotioides                CTC------- -GGAAACGAG GAGCAG-CCC GCCGGCGGCC
P.columnaris                   CTC------- -GGAAACGAG GAGCAG-CCC GCCGGCGGCC
Cytospora eucalypticola        CTCACCATCT CGGT---GAG GAGCAGGCCC GCCGGCGGCC
Mycosphaerella latebrosa       CC-------- -GGAGGCCTT ---------- ---------C
M.berberidis                   CC-------- -GGAGGCCTT ---------- ---------C
M.nubilosa                     C--------- -GCAGAA--- -------CCC C-------TC
M.cryptica                     C--------- -GGCGGA--- -------CCC C-------TC
Lophodermium conigenum         T--------- ----GAA--- ---------- --------TC
Pseudocercospora eriodendri    CC-------- -GGAGGTCTT ---------- ---------C
Guignardia endophyllicola      C-CAGCCT-T AACTGGCCAG GAC---GCCC ---GGC--T-
Phyllosticta pyrolae           C-CAGCC-CT CACCGGCCAG GAC---GTCA ---GGC--T-
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Figure 5. (continued)

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                               ....|....| ....|....| ....|....| ....|....|
                                      170        180        190        200
Chaenothecopsis pusilla        ACCTTCAAAC TCGTTTGTAA ATGTCGTCTG AGCGA-TGAG
ARE1                           AAGTTAACTC TTGTTTTTAC ACTGAAACTC TGAGAA--AA
Fungal endophyte MUT 2715      AAGTTAACTC TTGTTTTTAC ACTGAAACTC TGAGAA-AAA
Diaporthe phaseolorum          AAGTTAACTC T-GTTTTTAC ACTGAAACTC TGAGCA--CA
D.meridionalis                 AAGCCAACTC TTGTTTTTAC ACCGAAACTC TGAGCA--AA
D.caulivora                    AAGCTAACTC TTGTTTTTAC ACTGAAACTC TGAGAA--AT
D.helianthi                    GACCAAACTC TTGTTTCTAC AGTGGATCTC TGAGT-T-AA
D.vaccinii                     AACC-AACTC TTGTTTTTAC ACTGAAACTC TGAGAAT--A
Phomopsis vaccinii             AACCCAACTC TTGTTTTTAC ACTGAAACTC TGAGAAT--A
P.oryzae                       AAATCAACTC T-GTTTTTAC ACTGAAACTC TGAGAA---A
P.amygdali                     AAGTTAACTC TTGTTTTTAT TGTGAAACTC TGAGAAT--A
P.quercina                     AACCCAACTC TTGTTTTTAC ACTGAAACTC TGAGAAT--A
P.juniperivora                 AACCCAACTC TTGTTTTTAC ACTGAAACTC TGAGAAT--A
P.longicolla                   AACCAAACTC TTGTTTCTAC AGTGAATCTC TGAGTA--CA
P.sojae                        AACTAAACTC TTGTTTCTAT AGTGAATCTC TGAGTA--AA
P.sclerotioides                GACCAAACTC TTGTTTCT-C AGTGGATCTC TGAGTA--AA
P.columnaris                   AACCAGACTC TTGTTTCT-T AGTGGATCTC TGAGTA--AA
Cytospora eucalypticola        AAGTTAACTC TTGTTTTTAC ACTGAAACTC TGAGAAT--A
Mycosphaerella latebrosa       CAACCC---- -TGCATCT-T TGCG----TC GGAGTTTAA-
M.berberidis                   AAACAC---- -TGCATCT-C TGCG----TC GGAGTTTAA-
M.nubilosa                     AACGGC---- -TGGATCT-G TGCG----T- GGAGTAATA-
M.cryptica                     AAC-----TC -TGCATCT-T TGCG----TC TGAGT--GA-
Lophodermium conigenum         A--------- TTG------- -CCG----TC TGAGTACTA-
Pseudocercospora eriodendri    AAACAC---- -TGCATCT-T TGCG----TC GGAGTT----
Guignardia endophyllicola      AAG------- -TGCCC---- -GCCA----- GTA-TACAAA
Phyllosticta pyrolae           AAG------- -CGCCC---- -GCCA----- GTA-TACAAA

                               ....|....| ....|....| ....|....| ....|....|
                                      210        220        230        240
Chaenothecopsis pusilla        AAA-AAAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
ARE1                           AAACAAAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
Fungal endophyte MUT 2715      AAAC-AAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
Diaporthe phaseolorum          AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
D.meridionalis                 AAACACAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
D.caulivora                    AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
D.helianthi                    AAACACAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
D.vaccinii                     AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
Phomopsis vaccinii             AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.oryzae                       AAACACAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.amygdali                     AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.quercina                     AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.juniperivora                 AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.longicolla                   AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.sojae                        AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.sclerotioides                AAAAA-AAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
P.columnaris                   AAACATAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
Cytospora eucalypticola        AAACAAAAAT GAATCAAAAC TTTCAACAAC GGATCTCTTG
Mycosphaerella latebrosa       ----GTAAAT TAAACAAAAC TTTCAACAAC GGATCTCTTG
M.berberidis                   ----GTAAAT TAAACAAAAC TTTCAACAAC GGATCTCTTG
M.nubilosa                     ---------- CAATTAAAAC TTTCAACAAC GGATCTCTTG
M.cryptica                     TAACGAAAAT CAATCAAAAC TTTCAACAAC GGATCTCTTG
Lophodermium conigenum         -----TATAA TAGTTAAAAC TTTCAACAAC GGATCTCTTG
Pseudocercospora eriodendri    -----TAAAT CAAACAAAAC TTTCAACAAC GGATCTCTTG
Guignardia endophyllicola      ACTCATATAT CGATTAAAAC TTTCAACAAC GGATCTCTTG
Phyllosticta pyrolae           ACTCATAAAT TAATTAAAAC TTTCAACAAC GGATCTCTTG
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Figure 5. (continued)

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                              ....|....| ....|....| ....|....| ....|....|
                                     250        260        270        280
Chaenothecopsis pusilla       GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
ARE1                          GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Fungal endophyte MUT 2715     GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Diaporthe phaseolorum         GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
D.meridionalis                GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
D.caulivora                   GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
D.helianthi                   GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
D.vaccinii                    GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Phomopsis vaccinii            GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.oryzae                      GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.amygdali                    GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.quercina                    GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.juniperivora                GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.longicolla                  GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.sojae                       GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.sclerotioides               GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
P.columnaris                  GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Cytospora eucalypticola       GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Mycosphaerella latebrosa      GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
M.berberidis                  GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
M.nubilosa                    GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
M.cryptica                    GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Lophodermium conigenum        GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Pseudocercospora eriodendri   GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Guignardia endophyllicola     GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
Phyllosticta pyrolae          GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
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                              ....|....| ....|....| ....|....| ....|....|
                                     290        300        310        320
Chaenothecopsis pusilla       AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
ARE1                          AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Fungal endophyte MUT 2715     AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Diaporthe phaseolorum         AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
D.meridionalis                AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
D.caulivora                   AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
D.helianthi                   AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
D.vaccinii                    AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Phomopsis vaccinii            AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.oryzae                      AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.amygdali                    AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.quercina                    AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.juniperivora                AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.longicolla                  AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.sojae                       AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.sclerotioides               AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
P.columnaris                  AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Cytospora eucalypticola       AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Mycosphaerella latebrosa      AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
M.berberidis                  AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
M.nubilosa                    AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
M.cryptica                    AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Lophodermium conigenum        AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Pseudocercospora eriodendri   AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Guignardia endophyllicola     AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
Phyllosticta pyrolae          AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA
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Figure 5. (continued)

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                              ....|....| ....|....| ....|....| ....|....|
                                     330        340        350        360
Chaenothecopsis pusilla       ACGCACATTG CGCCCCCTGG TATTCCGGGG GGCATGCCTG
ARE1                          ACGCACATTG CGCCCTCCGG TATTCCGGAG GGCATGCCTG
Fungal endophyte MUT 2715     ACGCACATTG CGCCCTCCGG TATTCCGGAG GGCATGCCTG
Diaporthe phaseolorum         ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
D.meridionalis                ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
D.caulivora                   ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
D.helianthi                   ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
D.vaccinii                    ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
Phomopsis vaccinii            ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.oryzae                      ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.amygdali                    ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.quercina                    ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.juniperivora                ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.longicolla                  ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.sojae                       ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.sclerotioides               ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
P.columnaris                  ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
Cytospora eucalypticola       ACGCACATTG CGCCCTCTGG TATTCCCGAA GGCATGCCTG
Mycosphaerella latebrosa      ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
M.berberidis                  ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
M.nubilosa                    ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
M.cryptica                    ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
Lophodermium conigenum        ACGCACATTG CGCCCTCCGG TATTCCGGAG GGCATGCCTG
Pseudocercospora eriodendri   ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
Guignardia endophyllicola     ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
Phyllosticta pyrolae          ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG


                              ....|....| ....|....| ....|....| ....|....|
                                     370        380        390        400
Chaenothecopsis pusilla       TTCGAGCGTC ATTATCAACC CTCAAGCTCA GCTTGTTGTT
ARE1                          TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGTT
Fungal endophyte MUT 2715     TTCGAGCGTC ATT-TCAACC CTCAAGCACT GCTTGGTGTT
Diaporthe phaseolorum         TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGTT
D.meridionalis                TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGTT
D.caulivora                   TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGTT
D.helianthi                   TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
D.vaccinii                    TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
Phomopsis vaccinii            TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.oryzae                      TTCGAGCGTC ATT-TCAACC CTCAAGCATT GCTTGGTGTT
P.amygdali                    TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.quercina                    TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.juniperivora                TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.longicolla                  TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.sojae                       TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGAT
P.sclerotioides               TTCGAGCGTC ATT-TCAACC CTCAAGCACT GCTTGGTGTT
P.columnaris                  TTCGAGCGTC ATT-TCAACC CTCAAGCACT GCTTGGTGTT
Cytospora eucalypticola       TTCGAGCGTC ATT-TCAACC CTCAAGCCTG GCTTGGTGTT
Mycosphaerella latebrosa      TTCGAGCGTC ATT-TCACCA CTCAAGCCTG GCTTGGTATT
M.berberidis                  TTCGAGCGTC ATT-TCACCA CTCAAGCCTG GCTTGGTATT
M.nubilosa                    TTCGAGCGTC ATT-TCACCA CTCCAGCCCC GCTTGGTATT
M.cryptica                    TTCGAGCGTC ATTA-CACCC CTCCAGCCTC GCTGGGTGTT
Lophodermium conigenum        TTCGAGCGTC ATTA-CAACC CTCAAGCTCT GCTTGGTGTT
Pseudocercospora eriodendri   TTCGAGCGTC ATT-TCACCA CTCAAGCCTG GCTTGGTATT
Guignardia endophyllicola     TTCGAGCGTC ATT-TCAACC CTCAAGCTCT GCTTGGTATT
Phyllosticta pyrolae          TTCGAGCGTC ATT-TCAACC CTCAAGCTCT GCTTGGTATT
```

Figure 5. (continued)

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                                 ....|....| ....|....| ....|....| ....|....|
                                         410        420        430        440
Chaenothecopsis pusilla          GGGTCCGGGT CCCCCC-CGG ---GG-ACCG GCCCGCAAGT
ARE1                             GGGGCACTGC CT-GT--AAA AG-GGCAG-G CCCTCAAA-T
Fungal endophyte MUT 2715        GGGGCACTGC CTTTTC-CGG AA-GGCAG-G CCCTGAAA-T
Diaporthe phaseolorum            GGGGCACTGC CTCCTC-GCG GG-GGCAG-G CCCTGAAA-T
D.meridionalis                   GGGGCACTGC CT-GT--AAA AG-GGCAG-G CCCTGAAA-T
D.caulivora                      GGGGCACTGC CT-GT--AAA AG-GGCAG-G CCCTGAAA-T
D.helianthi                      GGGGCACTGC CT-GTG--AC AG-GGCAG-G CCCTGAAA-T
D.vaccinii                       GGGGCACTGC CTTTACAGAA AG-GGCAG-G CCCTGAAA-T
Phomopsis vaccinii               GGGGCACTGC CTTTACCCAA AG--GCAG-G CCCTGAAA-T
P.oryzae                         GGGGCACTGC -TTTTCAC-- -GAAGCAG-G CCCTGAAA-T
P.amygdali                       GGGGCACTGC CTTGTGTAAA CGAGGCAG-G CCCTGAAA-T
P.quercina                       GGGGCACTGC -TTTTACCCA AG-AGCAG-G CCCTGAAA-T
P.juniperivora                   GGGGCACTGC -TTTTACCCA AG-AGCAG-G CCCTGAAA-T
P.longicolla                     GGGGCACTGC TCTCTGA-CG GGA-GCAG-G CCCTGAAA-T
P.sojae                          GGGGCACTGC CTTCT-AGCG AG-GGCAG-G CCCTGAAA-T
P.sclerotioides                  GGGGCACCGC CT-GT--AAA AG-GGCGG-G CCCTGAAA-T
P.columnaris                     GGGGCACCGC CT-GT--AAA AG-GGCGG-G CCCTGAAA-T
Cytospora eucalypticola          GGGGCATTGC CTTTCGGTAA GAAGGCAG-G CCCTGAAA-T
Mycosphaerella latebrosa         GGG-CGCCGC GG--TGTTCC ----GC-GCG -CCTCAAAGT
M.berberidis                     GGG-CGCCGC GG--TGTTCC ----GC-GCG -CCTCAAAGT
M.nubilosa                       GGG-CGCCGC GGCCT---CC ----GC-GCG -CCTCAATGT
M.cryptica                       GGG-CATCGC GGCCT---CC ----GC-GCG -CCTCAATGT
Lophodermium conigenum           GAG-C-CCGC CCCGCTTACC CGGGGCT-CG -CTTCAAAAT
Pseudocercospora eriodendri      GGG-CGTCGC GGTCTG--CC ----GC-GCG -CCTTAAAGT
Guignardia endophyllicola        GGG-CAACGT CCGCTG--CC -GA--C-GTG -CCTTGAAGA
Phyllosticta pyrolae             GGG-CGACGT CCGCTG--CC GGA--C-GCG -CCTCGAAGA


                                 ....|....| ....|....| ....|....| ....|....|
                                         450        460        470        480
Chaenothecopsis pusilla          C-AGTGAC-G --GCCCGGAC CGTTCTCCAG CGTAGTACAG
ARE1                             CTAGTGGC-G A-GCTCGCCA GGACCCCGAG CGTAGTAG-T
Fungal endophyte MUT 2715        TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
Diaporthe phaseolorum            ACAGTGGC-G A-GCTCGCCA GGACTCCGAG CGCAGTAG-T
D.meridionalis                   CTAGTGGC-G G-GCTCGCCA GGACCCCGAG CGCAGTAG-T
D.caulivora                      TCATTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
D.helianthi                      CCAGCGGC-G A-GCCCGCCG GGACCCCGAG CGTAGTAG-T
D.vaccinii                       TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
Phomopsis vaccinii               TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
P.oryzae                         CTAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
P.amygdali                       TCAGTGGC-G A-GCTCGCCA GGACTCCGAG CGCAGTAG-T
P.quercina                       TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
P.juniperivora                   TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
P.longicolla                     CTAGTGGC-G A-GCTCGCTA GGACCCCGAG CGTAGTAG-T
P.sojae                          CTAGTGGC-G A-GCTCGCTA GGACCCCGAG CGTAGTAG-T
P.sclerotioides                  CTAGTGGC-G A-GCTCGCCG GGACCCCGAG CGTAGTAAAT
P.columnaris                     CTAGTGGC-G A-GCTCGCCA GGACCCCGAG CGTAGTAA-T
Cytospora eucalypticola          TCAGTGGC-G A-GCTCGCCA GGACCCCGAG CGCAGTAG-T
Mycosphaerella latebrosa         CT-CCGGCTG A-GCT-GTCC GT-CTCCAAG CGTCGT-G-A
M.berberidis                     CT-CCGGCTG A-GCT-GTCC GT-CTCCAAG CGTTGT-G-A
M.nubilosa                       CT-CCGGCCG A-GC-CGACC GT-CTCTCAG CGTTGTGGCA
M.cryptica                       CT-CCGGCCG A-GC-CGACC GT-CTCTAAG CGTTGTGGCA
Lophodermium conigenum           C-AGTGGCGG CCGC-CGTCC G-ACCTTCAG CGCAGTAA-T
Pseudocercospora eriodendri      CTTCCGGCTG A-GCT-GTCC GT-CTCTAAG CGTTGTGGAA
Guignardia endophyllicola        CCT-CGGC-G ACGG-CGTCC TAGCCTCGAG CGTAGTAG-T
Phyllosticta pyrolae             CCT-CGGC-G ACGG-CGTCT TAGCCTCGAG CGTAGTAG-T
```

Figure 5. (continued)

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                               ....|....| ....|....| ....|....| ....|....|
                                     490        500        510        520
Chaenothecopsis pusilla        ACTTCATTGA TCTCCCGCTG ATGGGAACGG CCCGGTGTGC
ARE1                           TAAACCC--- TCGCTTT--- GGAAGGCC-- -CTGGCGG-T
Fungal endophyte MUT 2715      TAAACCC--- TCGCTTT--- GGAAGGCC-- -CTGGCGG-T
Diaporthe phaseolorum          TAAACCC--- TCGTTCT--- GGAAGGCC-- --TGGCGG-T
D.meridionalis                 TAAACCC--- TCGCTC---- GGGAGGCC-- -CTGGCGG-T
D.caulivora                    TAAACCC--- TCGCTTT--- GGAAGGCC-- -CTGGCGG-T
D.helianthi                    AACTTC---- TCGCTCC--- GGAAGGCC-- -CTGGCGG-C
D.vaccinii                     TAAACCC--- TCGCTTT--- GGAAGGCC-- -CTGGCGC-G
Phomopsis vaccinii             TAAACCC--- TCGCTTT--- GGAAGGCC-- -CTGGCGG-T
P.oryzae                       TAAACCC--- TCGCTCT--- GGAAGGCC-- -CTGGCGG-T
P.amygdali                     TAAACCC--- TCGCTTT--- GGAAGGA--- -CTGGCGG-T
P.quercina                     TAAACCC--- TCGCTCT--- GGAAGGCC-- -CTGGCGG-T
P.juniperivora                 TAAACCC--- TCGCTCT--- GGAAGGCC-- -CTGGCGG-T
P.longicolla                   TATATC---- TCGTTCT--- GGAAGGCC-- -CTGGCGG-T
P.sojae                        TATATC---- TCGTTCT--- GGAAGGCC-- -CTGGCGG-T
P.sclerotioides                TATATT---- TCGTTCT--- GGAAGGCC-- -CCGGCGG-T
P.columnaris                   TATATT---- TCGTTCT--- GGAAGGCC-- -CTGGCGG-T
Cytospora eucalypticola        TAAACCC--- TCGCTCT--- GGACTGTA-- -CTGGTGCGG
Mycosphaerella latebrosa       TTTCATTA-A TCGCTTC--- GGG-GGC-GG -GCGGCCG-C
M.berberidis                   TTTCATTA-A TCGCTTC--- GAAGTGC-GG -GCGGCCG-C
M.nubilosa                     CTACTGT--T TCGCTGAC-- GGGGA-CCGG TCTGGCG--C
M.cryptica                     CAACTGT--T TCGCTTCC-- GG-GA-CCGG TCTGGCGT-C
Lophodermium conigenum         GC--TCG--- TCGCTGTTAG GGAAGGGTGG -CAAGCGC-C
Pseudocercospora eriodendri    TTTAACTA-T TCGCTTC--- GGAGTGC-GG -GTGGCCG-C
Guignardia endophyllicola      AAAATATC-- TCGCTTT--- GGAGTG---- -CTGGGCGAC
Phyllosticta pyrolae           AA--CATC-- TCGCTTT--- GGAGTG---- -CTAGCCGTT


                               ....|....| ....|....| ....|....| ...
                                     530        540        550
Chaenothecopsis pusilla        CGGCAGCCC- ----CCCCCA AATCTTCGTG AAT
ARE1                           GCCCTGCCGT TAAACCCCCA ACTCTTTGAA AAT
Fungal endophyte MUT 2715      GCCCTGCCGT TAAACCCCCA ACTCTT-GAA AAT
Diaporthe phaseolorum          GCCCTGCCGT TAAACCCCCA ACTCCT-GAA AAT
D.meridionalis                 GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
D.caulivora                    GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
D.helianthi                    GCCCTGCCGT TAAACCCCCA ACTCCT-GAA AAT
D.vaccinii                     GTGCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
Phomopsis vaccinii             GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
P.oryzae                       GCCCTGCCGT TAAACCCCCA ACTCTT-GAA AAT
P.amygdali                     GCCCTGCCGT TAAACCCCCA ACTCTT-GAA AAT
P.quercina                     GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
P.juniperivora                 GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
P.longicolla                   GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
P.sojae                        GCACTGCCGT TAAACCCCCA ACTTCT-GAA AAT
P.sclerotioides                GCCCTGCCGT TAAACCCCCA ACTCCT-GAA AAT
P.columnaris                   GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
Cytospora eucalypticola        GCCCTGCCGT TAAACCCCCA ACTTCT-GAA AAT
Mycosphaerella latebrosa       G----GCCGT TAAA------ -TCTTTCACC AGT
M.berberidis                   G----CCCGT TAAA------ -TATTTCACA AGT
M.nubilosa                     G---CGCCGT TAAAC----- -CCTTTCACC AAT
M.cryptica                     G---CGCCGT CAA-CCCCC- -TCTCTCAC- --T
Lophodermium conigenum         GTCATAC--- --AACCCCCA ------CA-C AA-
Pseudocercospora eriodendri    G----GCCGT TAAA------ -TCTTTATTC AAT
Guignardia endophyllicola      G----GCCGC CGGACAATCG ACCTTCGGTC TAT
Phyllosticta pyrolae           G----GCCGC CGGACAATCG ACCTTTGGTC TAT
```

Figure 5. (continued)

2.2.3 Phylogenetic analysis

Searching for similar sequences to 5.8S rDNA region of isolate ARE-1 in GenBank resulted in 5 taxa with 100 % identity. They were sterile fungal endophytes isolated from Mediterranean plant roots, *Pinus halepensis* and *Rosmarinus officinalis*. The ITS1-5.8S-ITS2 sequences of these isolates were similar with 98-100 % identity. Therefore, one isolate, fungal endophyte MUT 2715, was selected as reference taxon. A total of 21 species with 98-99 % identity and one species with lower percentage identity that have complete data of ITS1-5.8S-ITS2 sequences were also selected. In order to get complete reference taxa, the ITS1-5.8S-ITS2 sequences of isolate ARE-1 was used as the query sequence. Two more similar sequences from *Phomopsis quercina* and *Cytospora eucalypticola* were hit. They were added in the reference taxa.

The alignment data of complete ITS1-5.8S-ITS2 sequences of isolate ARE-1 and 25 reference taxa is shown in Figure 5. The phylogenetic analysis of all taxa with *C. pusilla* as an outgroup taxon yield concensus maximum parsimoneous tree of 710 tree length, with consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC) of 0.6113, 0.3887, 0.6634 and 0.4055, respectively, as shown in Figure 6. In this tree, isolate ARE-1 formed a monophyletic clade with all reference taxa of the family Valsaceae with a 96 % bootstrap support. Based on branch length, as shown in Figure 6, evolution of isolate ARE-1 was found to be most closely related to *Diaporthe caulivora*.

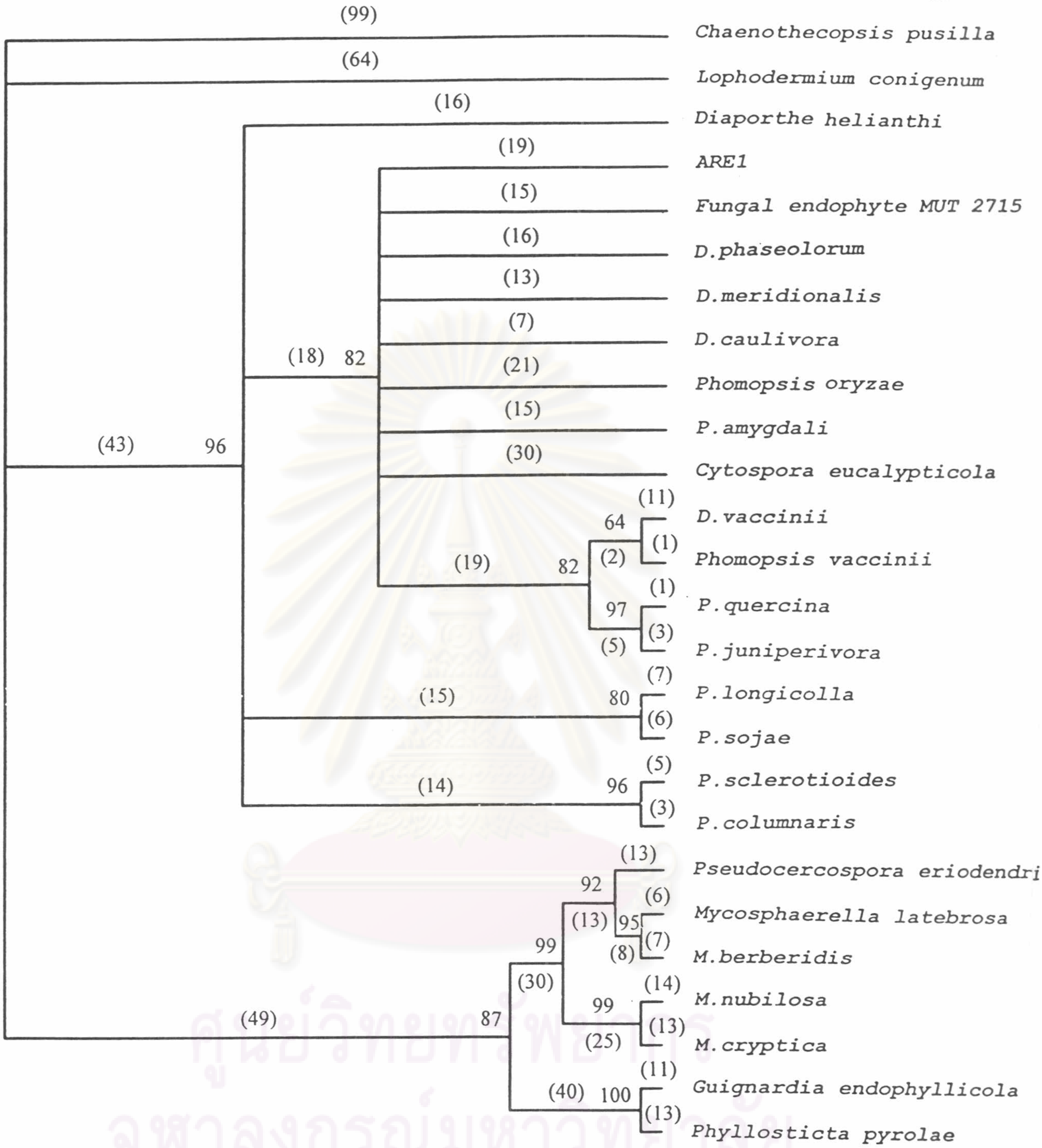


Figure 6 Maximum-parsimony tree generated from the ITS1-5.8S-ITS2 sequences of 26 taxa showing the relationships of ARE1 sequence with reference taxa. The numbers at internal node indicate the percentages of trees from 1,000 bootstrap replications. The numbers in bracket at branches indicate the branch length. *Chaenothecopsis pusilla* was used as an outgroup.

3. Structure elucidation of the isolated compounds.

Five metabolites were isolated from YES culture of the isolate ARE-1. The ethyl acetate extract (AREB 0.9 g) of YES fermentation broth (12L) of the isolate ARE-1 gave four known secondary metabolites, succinic acid monoethyl ester (AREB 3575 HP22, 8.7 mg, 0.96 % of ethyl acetate extract), phenylacetic acid (AREB 485 HP4, 5.1 mg, 0.56 % of ethyl acetate extract), 2-(4′ hydroxy-phenyl)-ethyl acetate (AREB 485HP5, 2.2 mg, 0.24 % of ethyl acetate extract) and 4-hydroxyphenethyl alcohol or tyrosol (AREB 485 HP2+3/4, 2.1 mg, 0.23 % of ethyl acetate extract). While a primary metabolite, ergosterol (ARHM(H) 76, 31.9 mg, 2.28 % of hexane extract), was obtained from hexane extract (ARHM(H) 1.4 g) of ARE1 mycelia. Ergosterol is a major component of cell membrane in most fungi (Weete, 1973).

3.1 Structure elucidation of succinic acid monoethyl ester (AREB3575 HP22)

Compound AREB 3575 HP22 was obtained as yellow oil. The IR absorption spectrum (Figure 7 in Appendix B) exhibited characteristic bands at 3420 cm^{-1} (O-H stretching), 2926 cm^{-1} and 1457 cm^{-1} (C-H stretching), 1732 cm^{-1} and 1653 cm^{-1} (C=O stretching) and 1272 cm^{-1} (C-C stretching). The UV spectrum in MeOH (Figure 8 in Appendix B) of compound AREB 3575 HP22 showed λ_{max} ($\in$) at 203 (3957) nm. The ESI-TOF MS of compound (Figure 9 in Appendix B) displayed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 169.0471 (calculated for $C_6H_{10}O_4Na$ at *m/z* 169.0477).

The 500 MHz ^{1}H-NMR spectrum of compound AREB 3575 HP22 in $CDCl_3$ (Figure 10 in Appendix B) revealed a methyl proton signal at δ 1.28 ppm; two methylene proton signals at δ 2.65 and 2.70 ppm; and an oxygen-bearing methylene proton signal at δ 4.15 ppm.

The 125 MHz ^{13}C-NMR spectrum (Figure 11 in Appendix B) gave six carbon signals. The carbon signals were classified by DEPT 135 spectrum (Figure 12 in Appendix B) and HMQC spectrum (Figure 13 in Appendix B) as one methyl carbon signal at δ 14.1 ppm; two methylene carbon signals at δ 28.8 and 29.0 ppm; one oxygen-bearing methylene carbon signal at δ 60.8 ppm and two carbonyl quaternary carbon signals at δ 172.1 and 176.9 ppm.

The ^{1}H-^{1}H COSY spectra of compound AREB 3575 HP22 (Figure 14 in Appendix B) established the proton connection from H-5 to H-6.

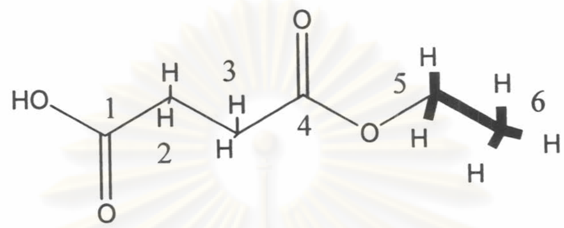


Figure 15 The ^{1}H-^{1}H correlation (bold line) in ^{1}H-^{1}H COSY spectrum of AREB 3575 HP22

The complete ^{13}C assignments of AREB 3575 HP22 were obtained from the HMBC spectra ($^{n}J_{HC}$ = 8 Hz and 4 Hz) (Figures 16 and 17 in Appendix B) showing the following long-range correlations; H-6 (δ 1.28) to oxygen-bearing methylene C-5 (δ 60.8); H-5 (δ 4.15) to C-6 (δ 14.1) and carbonyl carbon C-4 (δ 172.1); and H-3 (δ 2.70) to C-2 (δ 29.0), carbonyl carbon C-1 (δ 176.9) and C-4 (δ 172.1).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of AREB 3575 HP22 in $CDCl_3$ are shown in Figure 18 in Appendix B and summarized in Table 6.

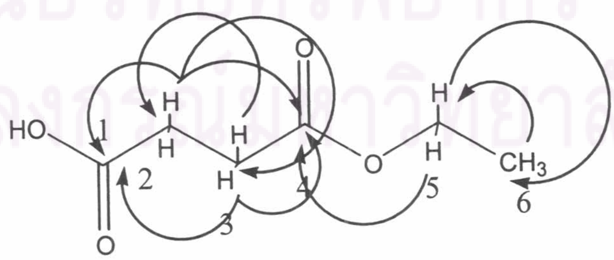


Figure 18 Long-range correlations from HMBC ($^{n}J_{HC}$ = 8 Hz and 4 Hz) spectral data of AREB 3575 HP22 in $CDCl_3$.

Table 6 The [1]H, [13]C-NMR and HMBC spectral data of AREB 3575 HP22 in $CDCl_3$.

Position	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC	
			$^{n}J_{HC}$ = 8 Hz	$^{n}J_{HC}$ = 4 Hz
1	-	176.9	-	-
2	2.65, *t* , (12)	29.0	C-1,C-3,C-4	C-3
3	2.70, *t*, (12)	28.8	C-1,C-2,C-4	C-2
4	-	172.1	-	-
5	4.15, *q*, (3)	60.8	C-4,C-6	C-4,C-6
6	1.28, *t*, (7)	14.1	C-5	C-5

3.2 Structure elucidation of phenylacetic acid (AREB 485 HP4)

Compound AREB 485 HP4 was obtained as yellow oil. The IR absorption spectrum (Figure 19 in Appendix B) showed characteristic bands at 3420 cm^{-1} (O-H stretching), 2915 cm^{-1} (C-H stretching), 1715 cm^{-1} (C=O stretching), 1517 cm^{-1} and 700 cm^{-1} (C=C stretching) and 1236 cm^{-1} (C-O stretching carboxylic group). The UV spectrum in MeOH (Figure 20 in Appendix B) of compound AREB 485 HP4 exhibited λ_{max} (∈) at 205 (8850) nm and 258 (578) nm. The ESI-TOF MS (Figure 21 in Appendix B) demonstrated the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 159.0429 (calculated for $C_8H_8O_2Na$ at *m/z* 159.0422).

The 500 MHz [1]H-NMR spectrum of AREB 485 HP4 in $CDCl_3$ (Figure 22 in Appendix B) presented one methylene proton signal at δ 3.68 ppm and five methine proton signals at δ 7.29-7.39 ppm. The 125 MHz [13]C-NMR spectrum in $CDCl_3$ (Figure 23 in Appendix B) showed eight carbon signals which were classified by DEPT 135 (Figure 24 in Appendix B) and HMQC spectrum (Figure 25 in Appendix B) as one methylene carbon signal at δ 40.9 ppm (C-2); one methine carbon signal at δ 127.3 ppm (C-6); two equivalent methine carbon signals at δ 129.3 ppm (C-4 and C-8); two equivalent methine carbon signals at δ 128.6 ppm (C-5 and C-7); one quaternary carbon signal at δ 133.3 ppm (C-3) and one quaternary carbonyl carbon signal at δ 176.6 ppm (C-1).

The HMBC spectrum ($^nJ_{HC}$ = 8 Hz) of AREB 485 HP4 (Figure 26 in Appendix B) exhibited long-rang correlations of H-2 (δ 3.68) to C-3 (δ 133.3), C-1 (δ 176.6), C-4 (129.3) and C-8 (δ 129.3).

The ^{1}H-^{13}C long-range correlations from HMBC spectrum of AREB 485 HP4 are shown in Figure 27 in Appendix B and summarized in Table 7.

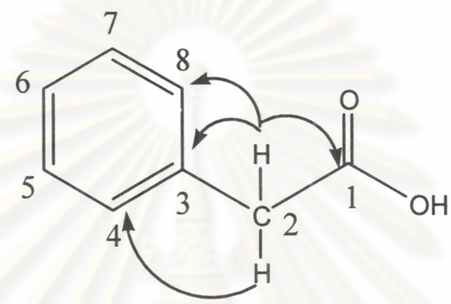


Figure 27 Important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of AREB 485 HP4 in $CDCl_3$.

The downfield of H-2 (singlet at δ 3.68 ppm) was due to a connection to the aromatic moiety. The NOESY experiment (Figure 28 in Appendix B) of AREB 485 HP4 confirmed the correlations of aromatic methine protons (H-4 and H-8) to methylene proton (H-2).

Comparison the spectral data of compound AREB 485 HP4 to reported values in the Aldrich Library of ^{13}C and ^{1}H NMR spectra indicated that AREB 485 HP4 is a known compound, phenylacetic acid (Table 8). This compound was also a metabolite of *Aspergillus niger* as reported by Nair and Burke (1988).

Table 7 The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485 HP4 in $CDCl_3$.

position	AREB 485 HP4		
	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC ($^{n}J_{HC}$ = 8 Hz)
1	-	176.6	-
2	3.68, *s*	40.9	C-1,C-3,(C-4,C-8)
3	-	133.3	-
4	7.29-7.39,*m*	129.3	C-2,C-3,C-5,C-6,C-8
5	7.29-7.39,*m*	128.6	C-3,C-4,C-6,C-7
6	7.29-7.39,*m*	127.3	C-4,C-5,C-7,C-8
7	7.29-7.39,*m*	128.6	C-3,C-5,C-6,C-8
8	7.29-7.39,*m*	129.3	C-2,C-3,C-4,C-6,C-7

Table 8 ^{1}H-NMR (500 MHz in $CDCl_3$) and ^{13}C-NMR (125 MHz in $CDCl_3$) spectral data of AREB 485 HP4 and ^{1}H-NMR (300 MHz in $CDCl_3$) and ^{13}C-NMR (60 MHz in $CDCl_3$) spectral data of phenylacetic acid (Pouchert and Behnke 1993).

position	AREB 485 HP4		Phenylacetic acid (Pouchert and Behnke 1993)	
	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)
1	-	176.6	-	178.1
2	3.68, *s*	40.9	3.60, *s*	41.0
3	-	133.3	-	133.1
4	7.29-7.39, *m*	129.3	7.25-7.35, *m*	129.3
5	7.29-7.39, *m*	128.6	7.25-7.35, *m*	128.5
6	7.29-7.39, *m*	127.3	7.25-7.35, *m*	127.2
7	7.29-7.39, *m*	128.6	7.25-7.35, *m*	128.5
8	7.29-7.39, *m*	129.3	7.25-7.35, *m*	129.3

3.3 Structure elucidation of 2(4′-hydroxy-phenyl) ethyl acetate (AREB485 HP5)

Compound AREB 485 HP5 was obtained as yellow oil. The IR absorption spectrum (Figure 29 in Appendix B) showed characteristic bands at 3420 cm^{-1} (O-H stretching), 2924 cm^{-1}, 1238 cm^{-1} (C-H stretching), 1558 cm^{-1} (C=C stretching) and 1717 cm^{-1} (C=O stretching). The UV spectrum in MeOH (Figure 30 in Appendix B) of compound AREB 485 HP5 exhibited λ_{max} ($\in$) at 202 (8462) nm , 222 (6126) nm , 277 (1799) nm and 284 (1608) nm. The ESI-TOF MS of AREB 485 HP5 (Figure 31 in Appendix B) presented the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 203.0684 (calculated for $C_{10}H_{12}O_3Na$ at *m/z* 203.0684), establishing the molecular formula of this compound as $C_{10}H_{12}O_3$.

The 500 MHz ^{1}H-NMR spectrum of AREB 485 HP5 in $CDCl_3$ (Figure 32 in Appendix B) showed one methyl proton signal at δ 2.05 ppm; two methylene proton signals at δ 2.9 and δ 4.25 ppm; two equivalent methine proton signals at δ 6.8 ppm (H-3′ and H-5′) and two equivalent methine proton signals at δ 7.1 ppm (H-2′ and H-6′). The 125 MHz ^{13}C-NMR (Figure 33 in Appendix B) and HMQC spectral data (Figures 35-37 in Appendix B) revealed ten carbons with were classified by the DEPT 135 (Figure 34 in Appendix B) as one methyl carbon signal at δ 20.9 ppm; two methylene carbon signals at δ 65.1 ppm and δ 34.1 ppm; two equivalent carbons at δ 115.3 ppm; two equivalent carbons at δ 130.0 ppm, and three quaternary carbons at δ 129.9, 154.2 and 171.1 ppm.

The ^{1}H-^{1}H COSY spectrum of AREB 485 HP5 (Figure 38 in Appendix B) revealed the correlations between H-1 and H-2; and H-2′ (or H-6′) and H-3′ (or H-5′).

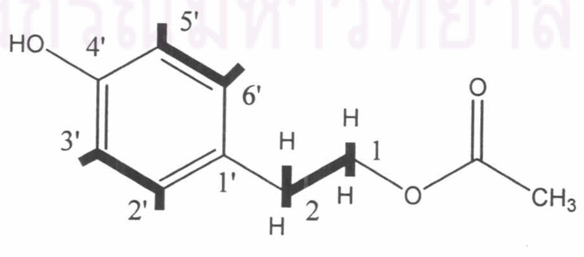


Figure 39 The correlations (bold line) in the ^{1}H-^{1}H COSY spectra of AREB 485 HP5 in $CDCl_3$.

The HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of AREB 485 HP5 (Figures 40-42 in Appendix B) exhibited long-range correlations of H-1 (δ 4.25) to C-2 (δ 34.1), C-1′ (δ 129.9) and carbonyl carbon (δ 171.1); H-2 (δ 2.9) to C-1 (δ 65.1), C-2′ or C-6′ (δ 130.0) and C-1′ (δ 129.9); methyl proton (δ 2.05) to carbonyl carbon (δ 171.1); H-2′ or H-6′ (δ 7.1) to C-2 (δ 34.1), C-1′ (δ 129.9) and C-4′ (δ 154.2); and H-3′ or H-5′ (δ 6.8) to C-1′ (δ 129.9), C-2′ (δ 130.0) and C-4′ (δ 154.2).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of AREB 485 HP5 are shown in Figure 43 in Appendix B and summarized in Table 9.

Table 9 The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485 HP5 in $CDCl_3$.

position	AREB 485 HP5		
	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC ($^{n}J_{HC}$ = 8 Hz)
1	4.25, *t*, (7)	65.1	C-1′, C-2, C=O
2	2.90, *t*, (7)	34.2	C-1, C-1′, C-2′, C-6′
1′	-	129.9	-
2′	7.10, *d*, (8)	130.0	C-2, C-1′, C-4′, C-6′
3′	6.80, *d*, (8)	115.3	C-1′, C-2′, C-4′, C-5′
4′	-	154.2	-
5′	6.80, *d*, (8)	115.3	C-1′, C-3′, C-4′, C-6′
6′	7.10, *d*, (8)	130.0	C-2, C-1′, C-2′, C-4′
C=O	-	171.1	-
CH_3	2.05, *s*	20.9	C=O

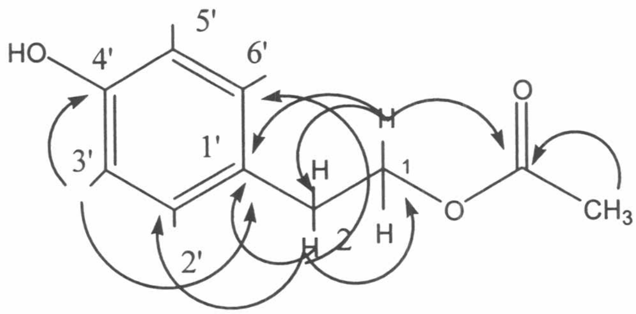


Figure 43 Important ^1H-^{13}C long-range correlations in the HMBC spectrum of AREB 485 HP5 in $CDCl_3$.

Based upon these spectral data, compound AREB 485 HP5 is identified as known compound, 2(4′-hydroxyphenyl) ethyl acetate.

3.4 Structure elucidation of 4-hydroxyphenethyl alcohol (AREB 485 HP2+3/4)

Compound AREB 485 HP2+3/4 was obtained as white yellow oil. The IR absorption spectrum (Figure 44 in Appendix B) showed characteristic bands at 3387 cm^{-1} (O-H stretching), 2926 cm^{-1} (C-H stretching), 1515 cm^{-1} (C=C stretching), 1242 cm^{-1} (C-C stretching), 1050 cm^{-1} (C-O stretching) and 817 cm^{-1} (=C-H bending). The UV spectrum in MeOH (Figure 45 in Appendix B) of compound AREB 485 HP2+3/4 presented λ_{max} ($\in$) at 202 (7545) nm, 222 (6643) nm and 279 (1702) nm. The ESI-TOF MS of compound AREB 485 HP2+3/4 (Figure 46 in Appendix B) showed the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 161.0588 (calculated for $C_8H_{10}O_2Na$ at *m/z* 161.0578) consistent with the molecular formula $C_8H_{10}O_2$.

The ^1H-NMR spectrum of AREB 485 HP2+3/4 in acetone *d*-6 and $CDCl_3$ (9:1) (Figure 47 in Appendix B) exhibited two methylene proton signals at δ 3.7 ppm and δ 2.7 ppm; two equivalent methine proton signals at δ 7.05 ppm (H-2′ and H-6′) and two equivalent methine proton signals at δ 6.72 ppm (H-3′ and H-5′). The ^{13}C-NMR (Figure 48 in Appendix B), DEPT 135 (Figure 49 in Appendix B) and HMQC spectral data (Figures 50 and 51 in Appendix B) showed eight carbons in AREB 485 HP2+3/4 two methylene carbon signals at δ 39.1 ppm and δ 63.8 ppm, two equivalent methine carbon signals at δ 115.4 ppm (C-3′ and C-5′), two equivalent methine carbon signals at δ 130.3 ppm (C-2′ and C-6′), and two quaternary carbon signals at δ 130.6 ppm (C-1′) and δ 156.1 ppm (C-4′).

The ^{1}H-^{1}H COSY spectrum of AREB 485 HP2+3/4 (Figure 52 in Appendix B) presented the proton connections between H-1 and H-2, and between H-2′ (or H-6′) and H-3′ (or H-5′).

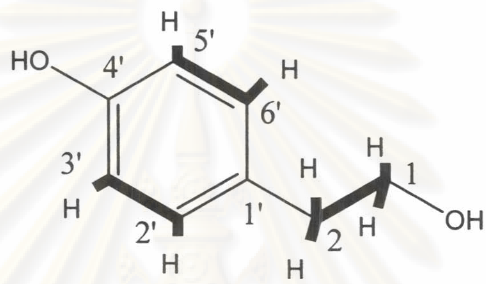


Figure 53 The correlations (bold line) from the ^{1}H-^{1}H COSY spectrum of AREB 485 HP2+3/4 in acetone *d*-6 and $CDCl_3$ (9:1).

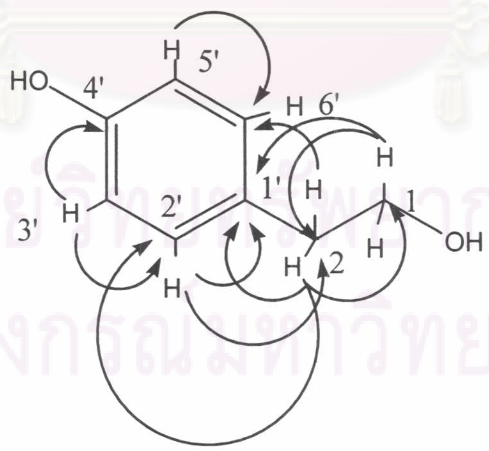
The HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) of AREB 485 HP2+3/4 (Figure 54 and 55 in Appendix B) established long-range correlations of H-1 (δ 3.7) to C-2 (δ 39.1), C-1′ (δ 130.6); H-2 (δ 2.7) to C-1 (δ 63.8), C-1′ (δ 130.6), C-2′ (δ 130.3) and C-6′ (δ 130.3); H-2′ or H-6′ (δ 7.05) to C-2 (δ 39.1), C-3′ (δ 115.4), C-4′ (δ 156.1), and C-1′ (δ 130.6); and H-3′ or H-5′ (δ 6.72) to C-2′ (δ 130.3), C-4′ (δ 156.1), and C-1′ (δ 130.6).

The ^{1}H-^{13}C long-range correlations from the HMBC spectrum of AREB 485 HP2+3/4 are shown in Figure 56 in Appendix B and summarized in Table 10.

Table 10 The ^{1}H, ^{13}C-NMR and HMBC spectral data of AREB 485 HP2+3/4 in acetone *d*-6 and $CDCl_3$ (9:1)

position	AREB 485 HP2+3/4		
	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)	Long-range correlation in HMBC ($^{n}J_{HC}$ = 8 Hz)
1	3.70, *t* , (7)	63.8	C-2, C-1
2	2.70, *t* , (7)	39.1	C-1, C-1′, C-2′, C-6′
1′	-	130.6	-
2′	7.05, *d* , (9)	130.3	C-2, C-1′, C-3′, C-4′, C-6′
3′	6.72, *d* , (9)	115.4	C-1′, C-2′, C-4′, C-5′
4′	-	156.1	-
5′	6.72, *d* , (9)	115.4	C-1′, C-3′, C-4′, C-6′
6′	7.05, *d* , (9)	130.3	C-2, C-1′, C-2′, C-4′, C-5′

Figure 56 Important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of AREB 485 HP2+3/4 in acetone *d*-6 and $CDCl_3$ (9:1).



Comparison the spectral data of compound AREB 485 HP2+3/4 to previously reported values in the Aldrich Library of ^{13}C and ^{1}H NMR spectra, compound AREB 485 HP2+3/4 was identified as 4-hydroxyphenethyl alcohol or Tyrosol (Table 11).

4-Hydroxyphenethyl alcohol has been reported as fungitoxic phenolic compound produced by the endophytic fungus *Epichloe typhina* of *Phleum pratense* (Timothy plant) (Koshino *et al.*, 1988).

Table 11 ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectral data of AREB 485 HP2+3/4 and ^{1}H-NMR (300 MHz) and ^{13}C-NMR (60 MHz) spectral data (in $CDCl_3$ and DMSO-*d*) of 4-hydroxyphenethyl alcohol (Pouchert and Behnke 1993).

position	AREB 485 HP2+3/4		4-Hydroxyphenethyl alcohol (Pouchert and Behnke 1993)	
	δH(ppm), *mult*, (*J* in Hz)	δC (ppm)	δH (ppm), *mult*, (*J* in Hz)	δC (ppm)
1	3.70, *t* , (7)	63.8	3.60	62.8
2	2.70, *t* , (7)	39.1	2.69	38.3
1′	-	130.6	-	129.4
2′	7.05, *d* , (9)	130.3	7.00	129.3
3′	6.72, *d* , (9)	115.4	6.70	114.9
4′	-	156.1	-	155.3
5′	6.72, *d* , (9)	115.4	6.70	114.9
6′	7.05, *d* , (9)	130.3	7.00	129.3

3.5 Structure elucidation of ergosterol (ARHM(H) 76)

Compound ARHM(H)76 was obtained as yellow white wax. The IR absorption spectrum (Figure 57 in Appendix B) established characteristic bands at 3424 cm^{-1} (O-H stretching), 2954 cm^{-1}, 1458 cm^{-1} (C-H stretching), 1658 cm^{-1}(C=C stretching), 1368 cm^{-1} (C-C stretching) and 1037 cm^{-1} (C-OH stretching). The UV spectrum in MeOH (Figure 58 in Appendix B) of ARHM(H)76 showed λ_{max} (∈) at 204 (19115) nm, 260 (11257) nm, 271 (15655) nm, 281 (16231) nm and 292 (10031) nm. Due to degradation was occurred, the peaks at 260, 271, 281 and 292 nm were examined. In the present investigation, ARHM(H)76 was identified as ergosterol by comparison of its ^{1}H and ^{13}C-NMR spectral data with reported values (Adler *et al.*, 1977 and Chobot *et al.*, 1997).

In the ^{1}H-NMR spectrum (Figure 59 in Appendix B), the signals at δ 5.38 ppm (1H, *dd*, J = 2.8, 5.7 Hz) and δ 5.57 ppm (1H, *dd*, J = 2.8, 5.7 Hz) were assigned to H-6 and H-7 of ergosterol. The signals at δ 5.15 - δ 5.31 ppm (2H, *m*) were assigned to H-22 and H-23 of ergosterol. The signals at δ 0.96 and δ 1.00 ppm (2 x Me, *s*) were assigned to Me-18 and Me-19. The signals at δ 1.04, δ 0.84, δ 0.82 and δ 0.92 ppm (4 x Me, *d*, J = 6.6 Hz) were assigned to Me-21, 26, 27 and 28, respectively.

The ^{13}C-NMR spectrum (Figure 60 in Appendix B) of ARHM(H) 76 displayed twenty eight carbon signals. Comparison of these data with reported ^{13}C-NMR values of ergosterol (Chobot *et al.*, 1997) is shown in Table 12.

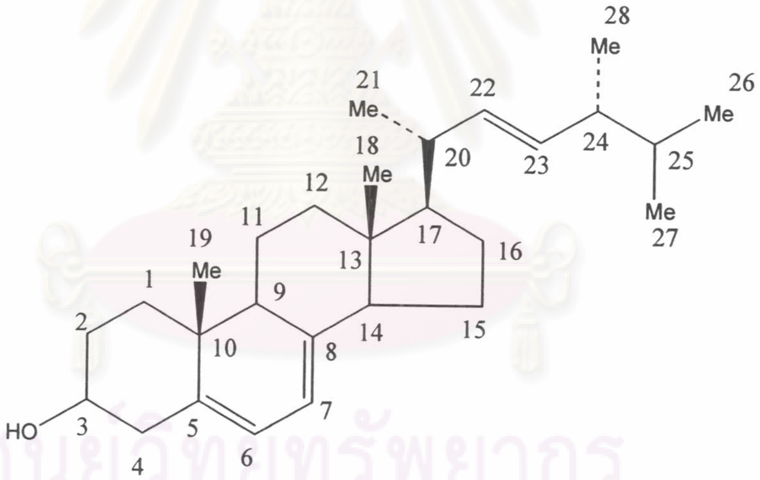


Figure 61 Chemical structure of compound ARHM(H)76.

Ergosterol was previously obtained from the endophytic fungus *Colletotrichum* sp. of *Artemisia annua* (Compositae) (Lu *et al.*, 2000).

Table 12 ^{13}C-NMR spectral data ($CDCl_3$) of ARHM(H)76 and ergosterol in $CDCl_3$ (Chobot *et al*., 1997).

position	Chemical shift (ppm)	
	ARHM(H)76	Ergosterol
1	38.3	38.4
2	31.9	32.0
3	70.4	70.4
4	40.7	40.8
5	141.3	141.4
6	119.5	119.6
7	116.2	116.3
8	139.7	139.8
9	46.2	46.3
10	36.9	37.0
11	21.0	21.1
12	39.0	39.1
13	42.7	42.8
14	54.5	54.5
15	22.9	23.0
16	28.2	28.3
17	55.6	55.7
18	12.0	12.1
19	16.2	16.3
20	40.3	40.4
21	19.6	19.7
22	135.5	135.6
23	131.9	132.0
24	42.7	42.8
25	33.0	33.1
26	21.0	21.1
27	19.9	20.0
28	17.5	17.6

4. Biological activities

Ergosterol (ARHM(H)76) and succinic acid monoethyl ester (AREB 3575 HP22) were tested for biological activities, while other isolated compounds were not biologically evaluated due to limited amount of substances. Only ergosterol showed antituberculosis activity against *Mycobacterium tuberculosis* at MIC of 12.5 μg/ml. Recently, ergosterol has been evaluated against *Mycobacterium tuberculosis* H37Rv for the rational design of new antituberculosis agents (Rugutt 2002). Both ergosterol and succinic acid monoethyl ester were inactive against the malarial parasite *Plasmodium falciparum* K1 strain.

Succinic acid monoethyl ester has been previously reported as volatile oil from custard apple (*Annona reticulata*) (Bartley, 1987). This implies that both the fungal endophyte, ARE-1, and its host plant produce the same metabolite as in taxol producing organisms (Stierle and Strobel, 1995).

The other three metabolites of ARE-1, 4-hydroxyphenethyl alcohol (tyrosol), 2 (4′-hydroxyphenyl) ethyl acetate (tyrosyl acetate) and phenylacetic acid, were reported to have antifungal activity. Tyrosol and tyrosyl acetate could be isolated from various plants such as *Olea europaea* L. (Briante *et al*., 2002), *Croton lechleri* L. (Chen *et al*., 1994) and *Rhodiola rosea* L. (Rohloff, 2002). Additionally, literature data indicated that tyrosol also isolated from fungi for example, *Coniothyrium* sp. (Holler *et al*., 1999), *Ophiostoma crassivaginata* (Ayer and Trifonov, 1995), *Gloeophyllum* sp. (Rasser *et al*., 2000), *Oospora astringenes* (Yamamoto and Nitta, 1962) and the endophytic fungus *Epichloe typhina* (Koshino *et al*., 1988). This supports the idea that endophytic fungi should be a good source for isolation of active compounds without destruction of the forests (Strobel and Long, 1998).