

Horticulture becomes 'high tech'

Multiplex detection and identification of plant pathogens by DNA arrays

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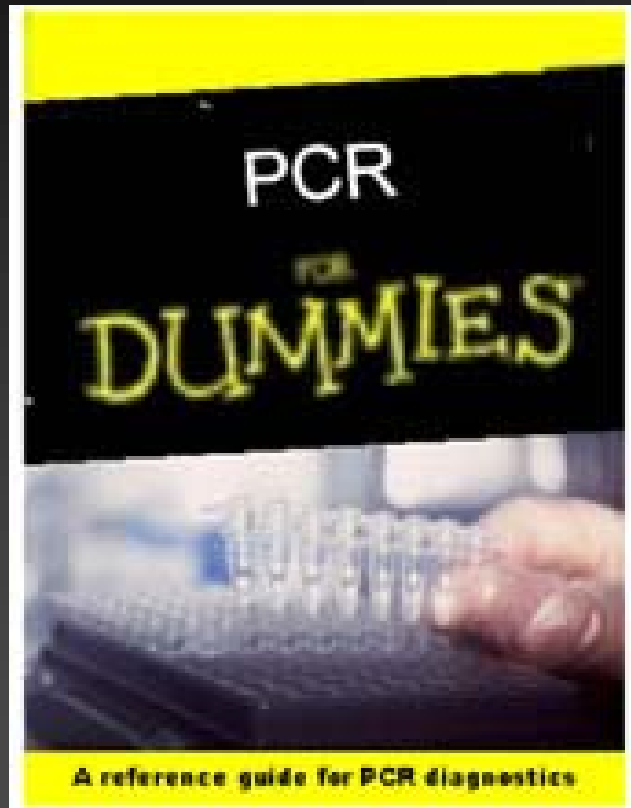
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Main limitations in disease management

Ease of plant pathogen identification



- Lack of a rapid, simple, accurate, and reliable method for qualitative and quantitative (multiplex) detection and identification
- Standard vs molecular procedures
- Limitation of most molecular detection technologies: only single (or a few) species detected per assay

The solution

Multiplex assays – DNA array technology

- Newest generation of DNA diagnostics to detect a comprehensive set of pathogens in a single assay
- Combines *nucleic acid amplification* with the unlimited screening capability of *DNA arrays*: high degrees of sensitivity, specificity, and throughput capacity
- Reduce testing time and costs

Universal primer – PCR based multiplex detection

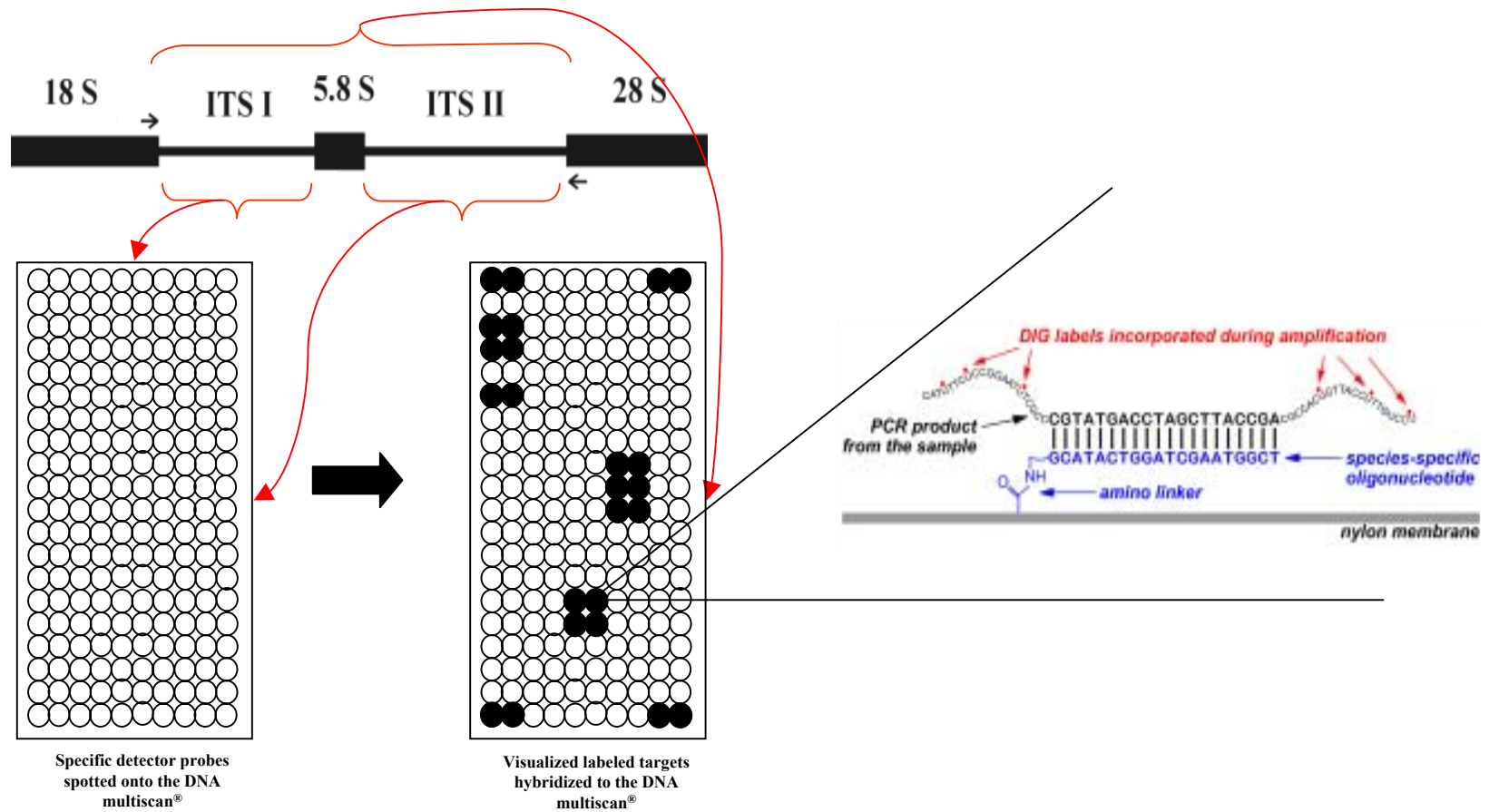
- Hybridization of labeled target amplicons (generated by universal primers) from unknown sample DNA to specific oligonucleotide detector probes bound to a solid support
- Originally developed to screen for human genetic disorders
- Successfully applied to detect and identify human, animal, and plant pathogens

ITS based multiplex detection

- Relatively high gene copy number
- Flanked by highly conserved sequences
- Vary among species
- Abundant ITS sequence information available

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V_dahliae_CBS179.66      CGAGCCCGCCGGTCC-ATCAGTCTCTCTGTTTATAACC-AACGATACTTCT
V_alboatrum_CBS321.91   CGAGCCCGCCGGTAC-ATCAGTCTCTTTATTCATAACC-AACGATACTTCT
V_tricorpus_L28679      CGAGCCCGCCGGTAC-ATCAGTCTCTTTATTTTACC-AACGATACATCT
V_nubilum_AJ292463     CGAGCCCGCCGGTAC-ATCAGTCTCTTTATTTATAACC-AACGATACTTCT
V_nigrescens_AJ292440  CGTGCCCGCCGGCATTATCAGAATCTCTGTTTCGAACCCGACGATACATCT
** *****          *****  *** * **      ***  *****  ***
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Oligonucleotide macro array



Probe specificity

<u>Code</u>	<u>Sequence (5'-3')</u>	<u>Code</u>	<u>Sequence (5'-3')</u>
Uni	TCC TCC GCT TAT TGA TAT GC	Oli13	TGC TGA CTC GCG TT G CAG TG
Dig	GTC CAG ACA GGA TCA GGA TTG	Oli14	TGC TGA CTC CCG TT G CAG TC
Con	GTC CAG ACA GGA TCA GGA TTG	Oli15	AGC TGA CTC CCG TTC CAG TC
Pul1	<u>TGC TGA CTC CCG TTC CAG TG</u>	Oli16	TGC TCA CTC CCG TT G CAG TG
Oli1	AGC TGA CTC CCG TTC CAG TG	Oli17	ACG TGA CTC CCG TTC CAG TG
Oli2	TGC TCA CTC CCG TTC CAG TG	Oli18	TGC TCT GTC CCG TTC CAG TG
Oli3	TGC TGA CTC GCG TTC CAG TG	Oli19	TGC TGA CTC GGC TTC CAG TG
Oli4	TGC TGA CTC CCG TT G CAG TG	Oli20	TGC TGA CTC CCG TT G GTG TG
Oli5	TGC TGA CTC CCG TTC CAG TC	Oli21	TGC TGA CTC CCG TTC CAC AC
Oli6	ACC TGA CTC CCG TTC CAG TG	Oli22	AGC TCA CTC GCG TTC CAG TG
Oli7	TGC TCT CTC CCG TTC CAG TG	Oli23	TGC TCA CTC GCG TT G CAG TG
Oli8	TGC TGA CTC GGG TTC CAG TG	Oli24	TGC TGA CTC GCG TT G CAG TC
Oli9	TGC TGA CTC CCG TT G GAG TG	Oli25	ACC TGA CTC CCG TTC CAG TC
Oli10	TGC TGA CTC CCG TTC CAG AC	Oli26	ACG TGA CTC CCG TTC CAC AC
Oli11	AGC TCA CTC CCG TTC CAG TG	Oli27	AGC TCA CTC GCG TTC GAG TC
Oli12	TGC TCA CTC GCG TTC CAG TG		

Probe specificity

<u>Code</u>	<u>Sequence (5'-3')</u>	<u>Signal</u>	<u>Code</u>	<u>Sequence (5'-3')</u>	<u>Signal</u>
Uni	TCC TCC GCT TAT TGA TAT GC	■	Oli13	TGC TGA CTC GCG TT G CAG TG	
Dig	GTC CAG ACA GGA TCA GGA TTG	■	Oli14	TGC TGA CTC CCG TT G CAG TC	
Con	GTC CAG ACA GGA TCA GGA TTG		Oli15	AGC TGA CTC CCG TTC CAG TC	■
Pul1	<u>TGC TGA CTC CCG TTC CAG TG</u>	■	Oli16	TGC TCA CTC CCG TT G CAG TG	
Oli1	AGC TGA CTC CCG TTC CAG TG	■	Oli17	ACG TGA CTC CCG TTC CAG TG	
Oli2	TGC TCA CTC CCG TTC CAG TG		Oli18	TGC TCT GTC CCG TTC CAG TG	
Oli3	TGC TGA CTC GCG TTC CAG TG	○	Oli19	TGC TGA CTC GGC TTC CAG TG	
Oli4	TGC TGA CTC CCG TT G CAG TG	○	Oli20	TGC TGA CTC CCG TT G GTG TG	
Oli5	TGC TGA CTC CCG TTC CAG TC	■	Oli21	TGC TGA CTC CCG TTC CAC AC	
Oli6	ACC TGA CTC CCG TTC CAG TG	■	Oli22	AGC TCA CTC GCG TTC CAG TG	
Oli7	TGC TCT CTC CCG TTC CAG TG		Oli23	TGC TCA CTC GCG TT G CAG TG	
Oli8	TGC TGA CTC GGG TTC CAG TG		Oli24	TGC TGA CTC GCG TT G CAG TC	
Oli9	TGC TGA CTC CCG TT G GAG TG		Oli25	ACC TGA CTC CCG TTC CAG TC	
Oli10	TGC TGA CTC CCG TTC CAG AC	■	Oli26	ACG TGA CTC CCG TTC CAC AC	
Oli11	AGC TCA CTC CCG TTC CAG TG		Oli27	AGC TCA CTC GCG TTC GAG TC	
Oli12	TGC TCA CTC GCG TTC CAG TG				

Blank = no signal; ○ = weak signal; ■ = strong signal

Fungal isolate	Fgn1	Fgn2	Fox1	Fox2	Vgn1	Vgn2	Vall	Val2	Vdal	Vda2	Con1	Unil	Dig1
<i>Fusarium graminearum</i> HCK PH1	■	■										■	■
<i>Fusarium oxysporum</i> f.sp. <i>conglutinans</i> HCK 81-4	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> CBS 645.78	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> CBS 412.90	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> CBS 646.78	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> CBS 413.90	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> HCK FOL1	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> HCK FOL2	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> MUCL 39789	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> CBS 873.95	■	■	■	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> CBS 101587	■	■	○	■								■	■
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> HCK 0-1090/B	■	■	■	■								■	■
<i>Fusarium solani</i> CBS 165.87		■										■	■
<i>Fusarium solani</i> CABI 17960		■										■	■
<i>Fusarium solani</i> HCK S-66		■										■	■
<i>Nectria haematococca</i> MUCL 20259		■										■	■
<i>Verticillium albo-atrum</i> CBS 451.88					○	■	■	■				■	■
<i>Verticillium albo-atrum</i> CBS 321.91					■	■	■	■				■	■
<i>Verticillium albo-atrum</i> CBS 385.91					○	○	○	○				■	■
<i>Verticillium dahliae</i> CBS 386.49					■	■			■	○		■	■
<i>Verticillium dahliae</i> CBS 179.66					○	■			■	■		■	■
<i>Verticillium dahliae</i> CBS 381.66					○	■			■	■		■	■
<i>Verticillium dahliae</i> RCR V44					○	■			■	■		■	■
<i>Verticillium dahliae</i> RCR PH					○	■			■	■		■	■
<i>Verticillium dahliae</i> RCR 115					■	■			■	■		■	■
<i>Verticillium dahliae</i> RCR 70-21					○	■			■	■		■	■
<i>Verticillium dahliae</i> RCR BB					○	■			■	○		■	■
<i>Verticillium dahliae</i> RCR S39					○	■			■	■		■	■
<i>Verticillium nubilum</i> MUCL 8266												■	■
<i>Verticillium tricorpus</i> MUCL 9792					○	■	■					■	■
Autoclaved ultrapure water													■

Blank = no signal; ○ = weak signal; ■ = strong signal

Multiple probe concept



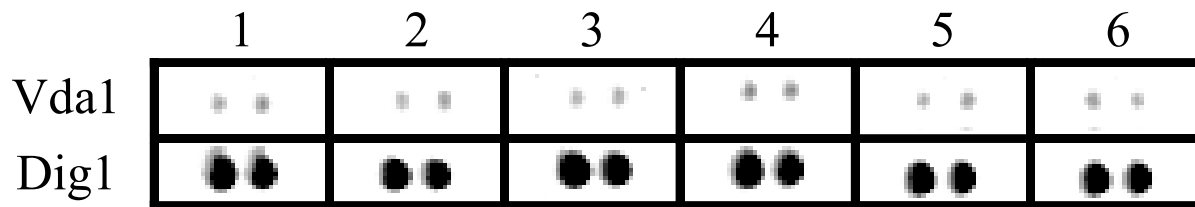
One single probe = no probe

Discriminatory potential

Fungal isolate mixture	Fgn2	Fox2	Vgn2	Val2	Vdal	Con1	Unil	Dig1
<i>F. oxysporum</i> f.sp. <i>lycopersici</i> CBS 645; <i>V. albo-atrum</i> CBS 451.88; <i>V. dahliae</i> CBS 386.49	■	■	■	■	■		■	■
<i>F. solani</i> CBS 165.87; <i>V. albo-atrum</i> CBS 451.88; <i>V. dahliae</i> CBS 386.49	■		■	■	■		■	■
<i>F. oxysporum</i> f.sp. <i>lycopersici</i> CBS 645.78; <i>V. albo-atrum</i> CBS 451.88	■	■	■	■			■	■
<i>F. oxysporum</i> f.sp. <i>lycopersici</i> CBS 645.78; <i>V. dahliae</i> CBS 386.49	■	■	■		■		■	■
<i>V. albo-atrum</i> CBS 451.88; <i>V. dahliae</i> CBS 386.49			■	■	■		■	■
Autoclaved ultrapure water								■

Blank = no signal; ○ = weak signal; ■ = strong signal

Influence of non-target DNA



Signals after amplification of 0.25 pg *V. dahliae* and 250 pg non-target DNA, including DNA from a bacterial (1), oomycete (2), or fungal culture (3) or from tomato plant (4), or sandy soil (5); (6) = no non-target DNA added

Influence of non-target fungal DNA

<i>F. solani</i> template	Hybridization signals for different target DNA amounts of					
	0.25 pg <i>V. albo-atrum</i>		2.5 pg <i>V. albo-atrum</i>		25 pg <i>V. albo-atrum</i>	
	Ratio	Val2	Ratio	Val2	Ratio	Val2
Con	1:0	○	1:0	■	1:0	■
25 pg	1:100	○	1:10	■	1:1	■
250 pg	1:1000	○	1:100	■	1:10	■
2.5 ng	1:10000		1:1000	○	1:100	■
<i>F. solani</i> template	Hybridization signals for different target DNA amounts of					
	0.25 pg <i>V. dahliae</i>		2.5 pg <i>V. dahliae</i>		25 pg <i>V. dahliae</i>	
	Ratio	Vdal	Ratio	Vdal	Ratio	Vdal
Con	1:0	○	1:0	■	1:0	■
25 pg	1:100	○	1:10	■	1:1	■
250 pg	1:1000	○	1:100	■	1:10	■
2.5 ng	1:10000		1:1000	○	1:100	■

Blank = no signal; ○ = weak signal; ■ = strong signal

Diagnosis of greenhouse samples

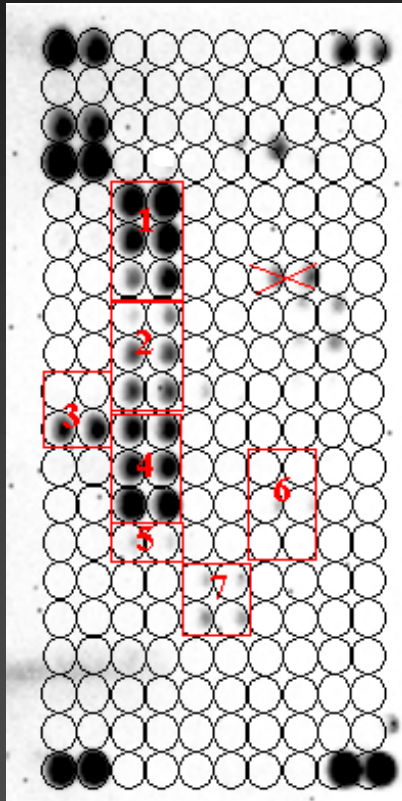
Example: lettuce



- Rotting of the lower leaves, bottom rot
- Small roots

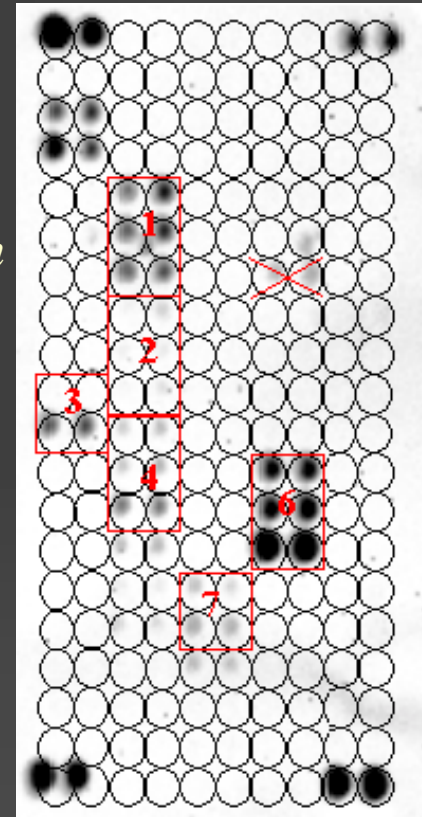
DNA array

Soil



1. *Fusarium* sp.
2. *Fusarium solani*
3. *Botrytis cinerea*
4. *Fusarium oxysporum*
5. *Penicillium* sp.
6. *Rhizoctonia solani*
7. *Pythium* sp.

Roots/lower leaves



Conclusions

- Robust, specific, sensitive, rapid, accurate, simple, cost-effective
- Results can be read as a checklist
- Can be used for routine analysis of samples from different biological sources (plants, seeds, soils, composts, potting mixes, rockwool, water, nutrient solution,...)
 - Additional efforts needed for soil diagnosis
 - Developing sampling strategies to account for spatial variability of inoculum
 - Calculating correlation between inoculum density, disease development, and hybridization signals to aid integrated disease management
- Broad area of application
- Possibility to expand to an unlimited set of microorganisms

DNA Multiscan[®]: Currently detectable organisms

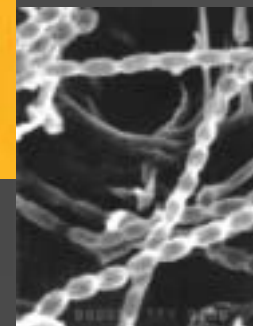
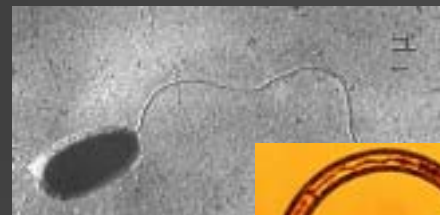
FUNGI & OOMYCETES:

1. Oomycetes
2. *Athelia (Sclerotium) rolfsii*
3. *Botrytis cinerea*
4. *Colletotrichum* spp.
5. *Colletotrichum coccodes*
6. *Colletotrichum gloeosporioides*
7. *Cylindrocladium* spp.
8. *Didymella* spp.
9. *Fusarium* spp.
10. *Fusarium oxysporum*
11. *Fusarium solani*
12. *Penicillium* spp.
13. *Phytophthora* spp.
14. *Phytophthora cactorum*
15. *Phytophthora capsici*
16. *Phytophthora cinnamomi*
17. *Phytophthora cryptogea*
18. *Phytophthora drechsleri*
19. *Phytophthora fragariae*
20. *Phytophthora infestans*
21. *Phytophthora nicotianae*
22. *Phytophthora ramorum*
23. *Plectosphaerella cucumerina*

24. *Pyrenochaeta lycopersici*
25. *Pythium* spp.
26. *Pythium aphanidermatum*
27. *Pythium dissotocum*
28. *Pythium irregulare*
29. *Pythium polymastum*
30. *Pythium sylvaticum*
31. *Pythium ultimum*
32. *Rhizoctonia solani*
33. *Sclerotinia* spp.
34. *Sclerotinia minor*
35. *Sclerotinia sclerotiorum*
36. *Sclerotinia trifoliorum*
37. *Trichoderma* spp.
38. *Trichoderma asperellum*
39. *Trichoderma harzianum*
40. *Trichoderma hamatum*
41. *Verticillium* spp.
42. *Verticillium albo-atrum*
43. *Verticillium dahliae*

BACTERIA:

1. *Pseudomonas cichorii*
2. *Pseudomonas marginalis*
3. *Pseudomonas viridiflava*
4. *Pseudomonas syringae* pv. *porri*
5. *Rhizobium radiobacter* (synonym *Agrobacterium tumefaciens*)
6. *Xanthomonas fragariae*
7. *Ralstonia solanacearum*



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