Development of DNA Arrays for Diagnosis and Prediction of Almond Diseases

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Objectives:

To develop diagnostic DNA arrays for detection of:

1. Known soilborne pests and pathogens of almond.
2. Members of soil-borne microbial communities mediating almond replant disease.

Interpretive Summary:

Of the many methods available for detection of plant pathogens, only array-based approaches afford specific detection and quantification of many microbes simultaneously. Array technology uses DNA “probes” (i.e., either short, DNA fragments--typically 20 to 100s of base pairs long, or whole genomic [total] DNA from the target organisms) to detect DNA in samples. The probes are arrayed in a known pattern on a solid surface (i.e., a nylon membrane, glass slide, or plastic chip). Each position in the array is allocated to a spot of an individual DNA probe that is specific for (i.e., will bind

only to) complementary DNA from a single class or species of organism (the specificity of DNA probes varies depending on their nature). Such arrays may be used as follows: DNA extracted from a sample of interest (i.e., from a root, soil, or microbial culture) is either used directly for testing or first subjected to PCR to amplify target DNA (i.e., the DNA that the array's DNA probes are designed to detect). In either case, the target (sample) DNA is labeled with a dye that will fluoresce so that it can be detected after it has hybridized to the probes. The labeled DNA is allowed to hybridize with the probes on the array. The array is then rinsed, so that the labeled target DNA will be retained (and fluoresce) only over the spots of array probes that complement it (i.e., bind it due to complementary DNA sequence). The binding specificity is relied upon to distinguish between individual genera, or in some cases, species and thereby replace the need for costly sequencing.

It was hoped that an inexpensive macro array format (i.e., one that uses nylon membranes instead of more expensive glass slides or plastic chips for the hybridization platform) could benefit the almond industry as an economical tool for: 1) detecting and quantifying known aggressive pathogens such as species of *Phytophthora* and plant parasitic nematodes and 2) repeated monitoring of microbial community shifts associated with incidence of replant disease (RD) and treatments to control it. Similar macro arrays have been used to examine bacterial community shifts in sheep rumen.

We constructed and tested a prototype macroarray using genomic (i.e., total) DNA purified from the following organisms: *Cylindrocarpon* sp. (two isolates), *Trichoderma* sp., *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora cactorum*, *P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. drechsleri, P. gonapodyides, P. megasperma, P. persica, Pythium helicoides*, and *Pythium* sp. The organisms include fungi associated with RD-affected roots (*Cylindrocarpon destructans*, *Pythium helicoides*) and healthy roots (*Trichoderma* sp.) as well as known pathogens affecting root systems and other parts of almond trees and other crops (e.g., the species of *Phytophthora*, *Colletotrichum*).

The prototype genomic array was tested to optimize it and determine its specificity and sensitivity. Samples containing various concentrations of DNA from individual organisms listed above were prepared. These samples were "probed" in turn using the macroarray at hybridization temperatures of 60, 65 and 70^0 C. It was determined that using an optimal hybridization of temperature 65 ^{0} C, the probing could detect as little as 5 to 10 ng of pathogen DNA in a sample. This level of sensitivity may be sufficient for root and soil diagnostics. However, the specificity of the array was not sufficient for its intended purpose; the array distinguished at a genus level at best. For example, members of the genus *Phytophthora* were distinguished from *Pythium* and the fungal genera (*Cylindrocarpon*, *Verticillium*, *Trichoderma*, and *Colletotrichum*), and from peach, but the array did not distinguish among the fungal genera.

It was concluded that this macroarray format will not fulfill its intended purpose, and alternative approaches are being considered.

Approach, Results, and Conclusion:

DNA was extracted from 18 sources of *Cylindrocarpon* sp. (two isolates), *Trichoderma* sp., *Colletotrichum acutatum*, *Verticillium dahliae*, *Phytophthora cactorum*, *P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. drechsleri, P. gonapodyides, P. megasperma, Pythium helicoides*, a *Pythium* sp. and Nemaguard and Lovell peach rootstocks using a modified CTAB method. The purity of the genomic DNA was good, with an average ratio of 1.90 for A_{260}/A_{280} and 1.55 for A_{260}/A_{230}.

In each of three experiments, multiple sets of positively charged Hybond-N^+ nylon membranes (115 mm x 75 mm) were spotted with known concentrations of probe DNA (i.e., DNA fixed to the membranes in spots and used to probe samples for their target organisms). The probe DNAs were spotted on nylon membranes in amounts ranging from 10 pg to 500 ng DNA, depending on experiment and treatment. The specificity and sensitivity of the probes were tested using samples of DNA from the target organisms.

In the first experiment, the probe DNAs were fixed on the array at 200 ng per spot, and 50 ng of test DNA was present in each sample. The probes distinguished at a genus level between samples of *Phytophthora* and peach DNA, but the probes did not distinguish among sample DNA from different species of *Phytophthora* or between sample DNA from Nemaguard and Lovell peach. Probe DNAs for the *Phytophthora* spp. and for the peach rootstocks did not cross hybridize with sample DNA from *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* and vice versa. However, probe DNAs for *Cylindrocarpon* sp., *C. acutatum*, *Trichoderma* sp., or *V. dahliae* cross reacted (i.e., hybridized indiscriminately) among sample DNA preparations of these same species. The specificity of probes was similar at hybridization temperatures 60^0, 65^0 and 70^0 C. Decreasing the exposure time of X-ray films used to capture fluorescence from 30 to 5 min reduced fluorescence intensities but did not affect probe specificity. None of the labeled whole genomic DNA preparations hybridized with water control spots on the membrane.

In the second experiment, probe DNAs were fixed on the array at 50 ng, 25 ng, 10 ng, 5 ng, 2 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg, 25 pg, or 10 pg per spot and the amount of DNA in samples for an array was held constant at 50 ng. Hybridization temperatures were 60^0, 65^0 and 70^0 C. Specificity and sensitivity generally were not improved over the levels obtained in the first experiments (**Table 1**). That is, regardless of probe spot DNA amount and hybridization temperature, the array distinguished between DNAs from *Phytophthora*, peach, and the fungi in the experimental samples, but it did not distinguish among DNAs from different species of *Phytophthora*, different genera of the fungi, or the two selections of peach (**Table 1**). The intensity of hybridization was proportional to the amount of DNA per spot and the exposure time. Based on results obtained in experiments 1 and 2, it was concluded that the optimum amount of DNA per spot for 5-min exposure to X-ray films was 200 ng.

In the third experiment, probe DNA amount was held constant at 500 ng per spot and sample DNA amount was varied (i.e., 50, 40, 30, 20, 10, 5, or 1 ng). In addition to

samples containing DNA from only one organism (*P. cactorum*, *P. citricola*, or *Pythium helicoides*), a mixed sample containing both Nemaguard and *P. cactorum* DNA was tested. Among the organisms included in this test, probing specificity generally was as observed in the first and second experiments and was considered insufficient for the intended array purposes. It was determined that the macroarray could detect as little as 10 ng and 5 ng of target DNA in a sample for film exposure periods of 5 min and 30 min, respectively (**Figures 1A and 1B**). This level of sensitivity would probably be sufficient to detect plant pathogens in field soils and diseased almond roots and bark tissues.

Overall, it was concluded that the macro array format tested will not fulfill its intended purpose. The macro array exhibited adequate sensitivity, but it had inadequate specificity. Alternative approaches for the diagnostic needs are being considered.

Table 1. Specificity and sensitivity of a prototype nylon membrane-based macro array, experiment 2

Genomic probe DNA on Hybond+ Nylon membrane	Annealing temp. (^{0} C)	Minimum amount of genomic DNA (ng) in probe spot required to hybridize with 50 ng of target sample DNA[b]											
		P. cactorum		*P. citricola*		*P. cinnamomi*		*P. megasperma*		Nemaguard		Lovell	
		5 min[a]	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min	5 min	30 min
P. cactorum	60	25	25	-	25	-	50	-	-	-	-	-	-
	65	25	10	-	-	-	-	-	-	-	-	-	-
	70	25	25	-	25	-	-	-	-	25	25	25	25
P. citricola	60	25	25	25	10	50	25	-	-	-	-	-	-
	65	50	25	25	5	-	25	-	50	-	-	-	-
	70	5	2	5	2	25	5	-	25	50	50	50	50
P. cinnamomi	60	-	25	-	10	50	5	-	-	-	-	-	-
	65	25	10	-	25	25	5	-	-	-	-	-	-
	70	25	10	10	5	5	2	-	25	-	-	-	-
P. megasperma	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	50	25	-	50	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
C. acutatum	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Trichoderma sp.	60	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	-	-	-	-	-	-	-	-	-	-	-
	70	-	-	-	-	-	-	-	-	-	-	-	-
Nemaguard	60	-	-	-	-	-	-	-	-	-	25	-	-
	65	-	-	-	-	-	-	-	-	-	25	-	25
	70	-	-	-	-	-	-	-	-	25	2	25	5
Lovell	60	-	-	-	-	-	-	-	-	25	25	-	25
	65	-	-	-	-	-	-	-	-	25	0.01	25	10
	70	-	-	-	-	-	-	-	-	-	25	-	25

[a] Exposure time of X-ray films to antibody-bound membranes that were covered with CDP-*Star* substrate solution.
[b] "-" indicates that no hybridization was detected under any of the conditions tested.

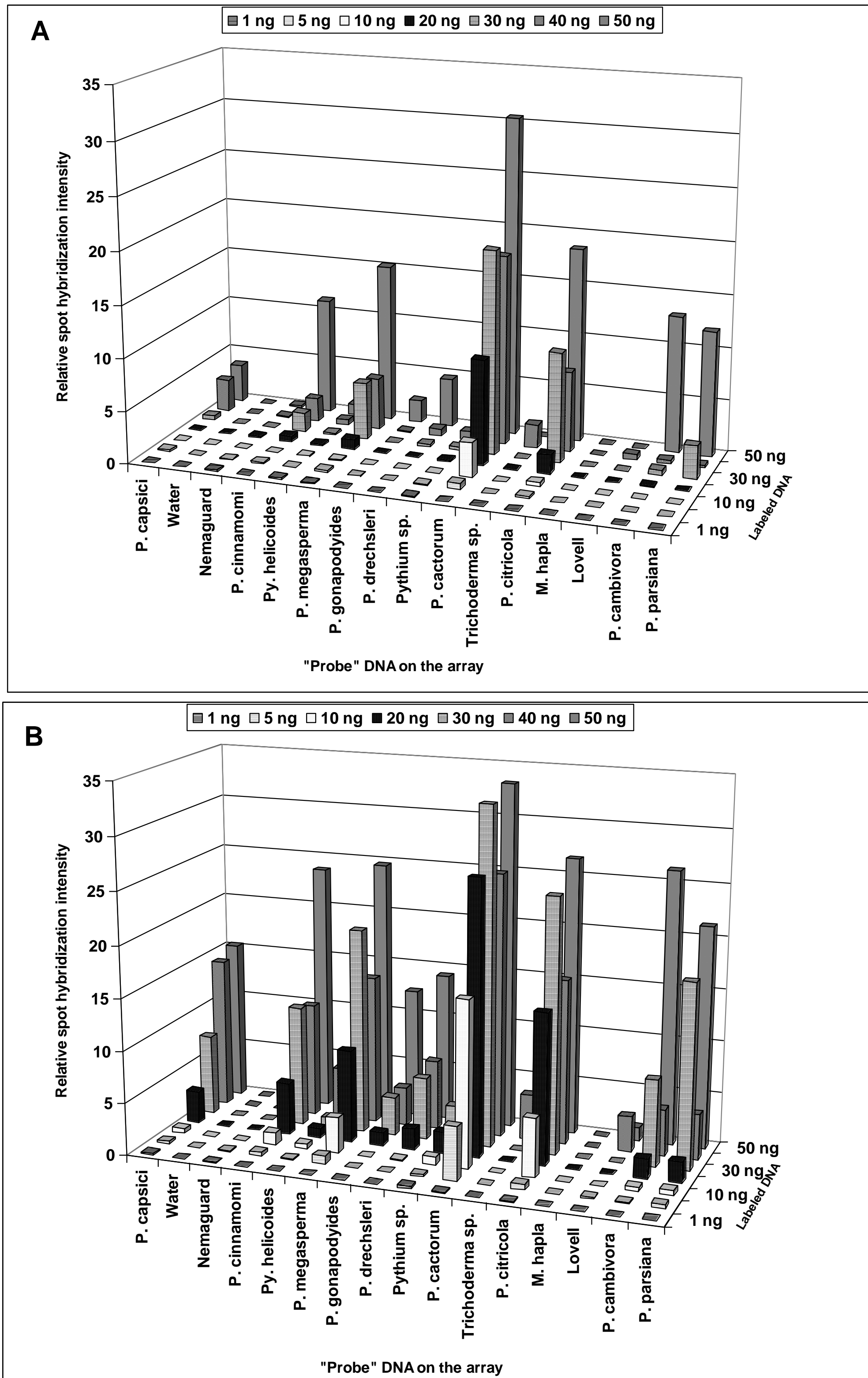

Figure 1. Relative spot intensity values of whole genomic macroarrays hybridized with different amounts of labeled *Phytophthora cactorum* (sample) DNA after exposing hybridized-arrays to films for 5 min (**A**) and 30 min (**B**), experiment 3.