GOBIERNO DE CHILE
FUNDACIÓN PARA LA INNOVACIÓN AGRARIA

PROGRAMA DE FORMA...
Recepcionado 23/10/02
Nº Ingreso 278

CONTENIDO DEL INFORME TÉCNICO

PROGRAMA DE FORMACIÓN PARA LA INNOVACIÓN AGRARIA

1. Antecedentes Generales de la Propuesta

Nombre: **Actualización técnica y generación de redes de colaboración para el desarrollo de proyectos comunes en fitopatología**.

Código : FP-V-2002-1- A - 028

Entidad Responsable Postulante Individual: Instituto de Investigaciones Agropecuarias (INIA)

Coordinador: Inés Marlene Rosales Villavicencio

Lugar de Formación (País, Región, Ciudad, Localidad): Estados Unidos, Milwaukee (Wisconsin), Gainesville, Bradenton (Florida)

Tipo o modalidad de Formación:
- Asistencia al Congreso Anual de la Sociedad de Fitopatología Americana (APS).
- Visita Técnica a los Centros de Investigación de la Universidad de Florida- Departamento de Fitopatología.
- Desarrollo de una incubadora común para proyectos en fitopatología.

Fecha de realización: Inicio: 26 de Julio 2002; Termino: 6 Agosto 2002

Participantes: presentación de acuerdo al siguiente cuadro:

Nombre	Institución/Empresa	Cargo/Actividad	Tipo Productor (si corresponde)
Marlene Rosales	INIA-La Platina	Investigador, Unidad de Biotecnología	

Problema a Resolver: detallar brevemente el problema que se pretendía resolver con la participación en la actividad de formación, a nivel local, regional y/o nacional.

Esta actividad de formación tuvo dos objetivos centrales, el perfeccionamiento técnico del investigador participante y la interacción con investigadores extranjeros de forma de establecer las bases para futuros proyectos comunes en el área de la fitopatología. La asistencia al

Congreso de la Sociedad Fitopatológica Americana nos permitió conocer y actualizarnos en los avances y tendencias de la investigación en el area fitopatológica y otras áreas afines. Este encuentro congregó este año a más de 1500 fitopatólogos y otros expertos, provenientes desde más de 30 países. La asistencia a este evento se conviertió así en una oportunidad para el desarrollo profesional y la maximimización de las interrelaciones con los grupos de investigadores líderes en el mundo, ya que este congreso promueve y entrega posibilidades ilimitadas para el intercambio de ideas e información.

Objetivos de la Propuesta:

Objetivo General: Asistencia a un Congreso Científico Internacional con el objetivo de actualizarce en las avances y tendencias de la investigación en el área de la fitopatología, y promover la interrelación y/o cooperación con grupos de investigación líderes en el mundo.
Se propuso además complementar esta actividad con una visita técnica a los Centros de Investigación de la Universidad de Florida (UF)- Departamento de Fitopatología.

Objetivos Específicos:

1. Asistir al Congreso Anual de la Sociedad Fitopatológica Americana con el ánimo de conocer los últimos avances en el área de la biotecnología vegetal y la fitopatología en particular.
2. Fortalecer el conocimiento en áreas claves que actualmente se estan desarrollando e investigando en nuestro laboratorio, y fomentar la interrelación y/o cooperación con grupos de investigación extranjeros.
3. Resforzar los lazos de interacción con los grupos de investigación en biotecnología vegetal y virología de la Universidad de Florida, con especial énfasis en áreas de transformación genética, biología molecular y caracterización de mosca blanca y los virus transmitidos por este insecto.
4. Establecimiento de cooperación para futuros proyectos de investigación que se desarrollen en las áreas mencionadas en el objetivo específico 3.

2. Antecedentes Generales: describir si se lograron adquirir los conocimientos y/o experiencias en la actividad en la cual se participó (no más de 2 páginas).

La primera actividad programada para esta unidad de capacitación consistió en la asistencia al Congreso Anual de la Sociedad Fitopatológica Americana, en Milwaukee, Wisconsin, con el objetivo de conocer y actualizarnos de los avances en ésta área. Este evento congregó en esta oportunidad a más de 1500 investigadores provenientes desde más 30 países distintos. Este gran evento científico contó con un número importante de sesiones, muchos de éstas vinculadas directamente o indirectamente con los proyectos que hoy en día se ejecutan en nuestro laboratorio o, con aquellos que esperamos desarrollar en un futuro cercano. Debido a la simultaneidad de las sesiones, se debió priorizar la asistencia a aquellas ponencias que fueran de particular interés para la investigadora asistente o a aquellas que se enmarcan dentro de las lineas prioritarias de nuestro laboratorio. Entre éstas se destacan las sesiones de bioseguridad de cultivos, prioridades en la secuenciación de genomas microbianos, la interacción entre bacterias endosimbiónte y la transmisión circulativa de virus, genómica funcional de la interacción planta-patógeno, muerte celular programada en patologías y desarrollo y vectores de expresión viral. Sin duda, los objetivos planteados se

pudieron cumplir satisfactoriamente ya que se presenció una gran cantidad de exposiciones, en diferentes áreas de la patología vegetal. Esto también nos entregó una visión clara de las tendencias de la investigación en esta rama de la ciencia, la que sin duda pasa por conocer las interacciones entre las plantas y sus patógenos. Las genómica funcional toma una posición relevante en este contexto, ya que permitirá dilucidar los mecanismos que hasta ahora son desconocidos en este tipo de interacciones. También se destaca el uso de técnicas de alta sensibilidad y exactitud en el diagnóstico de fitopatógenos,

Sin duda que la gran cantidad de asistentes a este Congreso ofrecen una gran oportunidad para la interacción, el desarrollo profesional y la maximimización de las interrelaciones con los grupos de investigadores líderes en el mundo. En nuestro caso, pudimos establecer contactos con los grupos Norteamericanos que trabajan en Plum Pox Virus, expertos que trabajan en el tema Bemisia tabaci y geminivirus, y Crinivirus (detallados en el punto 5).

La segunda etapa de este proyecto contemplaba una visita técnica a algunos laboratorios del Departamento de Fitopatología de la Universidad de Florida. Allí pudimos visitar el "Gulf Coast Research and Education Center" en Bradenton, donde pudimos visitar el laboratorio de la Dra. Jane Polston. Esta visita nos permitió conocer las facilidades y requerimientos necesarios para el trabajo con mosquita blanca, y se nos dió una introducción al tema del diagnóstico y control de enfermedades causadas por geminivirus, así como también una rápida inmersión en la biología y desarrollo de *B.tabaci*. Aunque ya habíamos establecidos contactos previos para colaboraciones en este tema, esta visita sirvió para formalizar nuestro interés en futuras interacciones.

Posteriormente visitamos el "Citrus Research and Education Center" en Lake Alfred, donde pudimos conversar con los virólogos Dr. Richard Lee y Dr. Ron Brlansky, quienes trabajan en la relación virus-vector y en el diagnóstico y control del virus de la tristeza de los cítricos.

3. Itinerario Realizado: presentación de acuerdo al siguiente cuadro:

Fecha	Actividad	Objetivo	Lugar
26- 07-2002	Salida desde Santiago-Milwaukee (WI)	Llegada a WI el día 27 de Julio, para registrarse en la conferencia y asistir a Orientación inicial	Midest Expres Center, Milwaukee, Wisconsin (WI)
27- 07-2002 al 31- 07-2002	Asistencia a las Sesiones y Conferencias plenarias de Congreso	Actualizar nuestro conocimiento en áreas de interés particular. Resforzar las posibilidades de interacción con grupos internacionales.	Midest Expres Center, Milwaukee, Wisconsin (WI)
31- 07-2002	Traslado a Florida	Continuación de la actividad con la visita técnica a la Universidad de Florida.	Florida

1- 08-2002	Visita Laboratorio Dr. Jane Polston, Gulf Coast Research and Education Center , Bradenton, Florida.	Entrevista con personal del laboratorio. Discutir antecedentes de la situacion chilena v/s FL con respecto a *B.tabaci*, su control, manejo integrado y caracterización molecular de biotipos . Discutir probabilidad de interacción y/o cooperación bilateral. Visita a las instalaciones de invernaderos donde se estudia la biología y comportamiento de *B.tabaci*. Discusión de protocolos y técnicas de detección de geminivirus. Esta visita será dirigida por la estudiante de Doctorado Alba Nava, quien es supervisada por la Dra. Jane Polston.	Bradenton, Florida
2-08-2002	Visita Laboratorio Dr. R.F.Lee, CREC-Lake Alfred.	Entrevista con virologos vegetales del Centro quienes ejecutan proyectos relacionados con transmisión de virus por insectos y transformación genética.	Lake Alfred, Florida
3 - 08 - 2002	Traslado a Gainesville, campus principal de UF	Continuar con la Visita Técnica a UF	Gainesville
5- 08-2002	Campus Principal UF, Departamento Fitopatología	Visita al, Departamento de Fitopatología, Visita Laboratorio Dr. E.Hiebert y reunión con Estudiantes Graduados .	Gainesville
6- 08 – 2002	Traslado a Miami, Viaje Miami-Santiago.		

La entrevista personal con la Chair del Departamento de Fitopatología de la Universidad de Florida, programada para el día 5 de Agosto, debió suspenderse por motivos personales que afectaron a la Dra. Wisler, lo que la llevó a ausentarse de las actividades programadas para esa semana. Similarmente, la Dra. Polston recibió una invitación para asistir al "XII International Congress of Virology-Paris" (27 de Julio- 1 de Agosto 2002) por lo que la entrevista planificada no se llevó a efecto. En su reemplazo, los colaboradores de su grupo se encargaron de nuestra visita, lo que permitió que de todas formas se cumpliera con el objetivo planificado, particularmente aquellos relacionados con las técnicas de detección de geminivirus y el estudio de su vector *Bemisia tabaci*.

5. **Resultados Obtenidos**: descripción detallada de los conocimientos adquiridos. Explicar el grado de cumplimiento de los objetivos propuestos, de acuerdo a los resultados obtenidos. Incorporar en este punto fotografías relevantes que contribuyan a describir las actividades realizadas.

Esta actividad de formación planteó como objetivo general el asistir a un Congreso Internacional para actualizar nuestro conocimientos y promover la interrelación con grupos

internacionales que trabajan en el área de la fitopatología. Sin duda que estos objetivos se cumplieron satisfactoriamente, ya que debido a la variedad y gran número de presentaciones realizadas durante este encuentro fué posible conocer los últimos avances de la investigación en variados temas:

- Estrategias y posiciones frente a la secuenciación de genomas microbianos
- Mecanismos involucrados en la transmisión de virus vegetales
- Novedades y tendencias en el diagnóstico de fitopatógenos
- Bioseguridad de Cultivos

El avance de la tecnología y por sobre todo de los estudios de genómica funcional han cambiado el rumbo de la investigación en ésta y otras áreas. En el caso de la fitopatología, es cada vez más importante conocer las relaciones que ocurren entre las plantas y sus patógenos. De esta forma se espera diseñar estrategias de control más efectivas, mejorar su diagnóstico y detección , así como estudiar la diversidad genética de los patógenos.

Por otra parte, la gran cantidad de asistentes a este encuentro nos permitió establecer contactos con importantes grupos dedicados a investigar algunos virus de importancia económica. Entre ellos podemos destacar a los investigadores que trabajan en el virus de Sharka: Vern Damsteegt y Dr. W.L.Schneider del USDA-ARS Foreign Disease Weed Science Research Unit; Dr. Laurene Levy del USDA-APHIS). quienes manifestaron interés en futuras interacciones con nuestro grupo. Además, en torno al tema *Bemisia tabaci* y geminivirus, pudimos establecer los primeros contactos con las expertas Dra. Judith Brown (University of Arizona) y la Dra. Pamela Anderson (Centro Internacional de Agricultura Tropical, CIAT) quienes nos entregaron su opinión hacerca de la situación de riesgo que enfrenta Chile con la llegada de la mosquita blanca, *B.tabaci*, al norte de nuestro país. En otras áreas, también contactamos al Dr. William M. Wintermantel, Research Plant Pathologist del USDA-ARS (Salinas, California) quien es un especialista en virus transmitidos por mosquita blanca, en especial Crinivirus, tema que esperamos desarrollar proximamente en nuestra unidad.

La segunda etapa de este proyecto contemplaba una visita técnica a algunos laboratorios del Departamento de Fitopatología de la Universidad de Florida. Allí se visitó el laboratorio de la Dra. Jane Polston en el "Gulf Coast Research and Education Center" (Bradenton). Esta visita nos permitió conocer las facilidades y requerimientos necesarios para el trabajo con mosquita blanca, y se nos dió una introducción al tema del diagnóstico y control de enfermedades causadas por geminivirus, así como también una rápida inmersión en la biología y desarrollo de B.tabaci. Aunque ya habíamos establecidos contactos previos para colaboraciones en este tema, esta visita sirvió para formalizar nuestro interés en futuras interacciones. Se anexan algunas fotografías de los hitos más importantes de esta visita.
Posteriormente se visitó el "Citrus Research and Education Center" en Lake Alfred, donde conversamos con los virólogos Dr. Richard Lee y Dr. Ron Brlansky, quienes trabajan en la relación virus-vector y en el diagnóstico y control del virus de la tristeza de los cítricos. Finalmente, en Gainesville contactamos al Dr. Manjunath Keremane, quien está desarrollando un proyecto de producción de anticuerpos de cadena simple en bacteriófagos, los que reconocen específicamente diferentes razas de virus vegetales.

Por lo tanto, desde nuestra perspectiva creemos que los objetivos planteados al presentar esta propuesta se han cumplido en su totalidad y nos deja planteadas excelentes perspectivas de interacción con grupos especializados del area de la Virología Vegetal.

Algunos momentos relevantes de la visita efectuada al Laboratorio de la Dra Jane Polston, Universidad de Florida

A. Visita a las cámaras de crecimiento donde se mantienen las colonias de *B.tabaci*
B. Síntomas del geminivirus TYLCV (Tomato Yellow Leaf Curl Virus) en tomate
C. Colonias de mosquita blanca mantenidas en repollo
D. Explicación demonstrativa de la captura de mosquitas blancas utilizando un aspirador artesanal
E. Actividad demonstrativa de diferenciación de mosquitas machos y hembras
F. Síntomas de

Visita al Citrus Research and Education Center (Universidad de Florida). En la fotografía de izquierda a derecha: Dr. Ron Brlansky, Dra Marlene Rosales y Dr. Richard Lee.

5. Aplicabilidad: explicar la situación actual del rubro en Chile (región), compararla con la tendencias y perspectivas en el país (región) visitado y explicar la posible incorporación de los conocimientos adquiridos, en el corto, mediano o largo plazo, los procesos de adaptación necesarios, las zonas potenciales y los apoyos tanto técnicos como financieros necesarios para hacer posible su incorporación en nuestro país (región).

Chile se ha plateado el gran desafío del desarrollo de la biotecnología en distintas áreas o disciplinas. La biotecnología vegetal en particular se verá beneficiada de los resultados y conocimientos que se generen por parte de los Proyectos de Genómica Funcional Vegetal recientemente seleccionados para su ejecución por grupos de investigación nacionales (PLATAFORMA CIENTIFICA-TECNOLOGICA PARA EL DESARROLLO DE LA GENOMICA EN CHILE). En este contexto, esta actividad de formación permitió conocer la experiencia de grupos norteamericanos que están desarrollando el análisis genómico de microorganismos asociados a plantas y delineando las políticas y prioridades en este tema. También fué interesante conocer cómo los resultados de estas investigaciones han permitido realizar avances en el diagnóstico de fitopatógenos y en el mejoramiento vegetal. Todos estos temas son también un desafío para nuestro país, por lo que conocer la experiencia de otros grupos nos permitirá utilizar más eficientemente los conocimientos que se generen en nuestro país.
La investigación en el área de la Virología Vegetal se está desarrollando rápidamente en nuestro país. Sin duda uno de los principales problemas a abordar es la presencia del Virus Plum Pox que afecta a frutales de carozo en distintas regiones de Chile. El conocer cómo EEUU y Canadá han enfrentado su erradicación y el estudio de sus posibles vectores nos plantea desafíos importantes, tanto a los investigadores del área como a las agencias a cargo del control fitosanitario. Esperamos en el futuro poder iniciar estudios en este tema, apoyados por fondos concursables nacionales, por lo que la interacción con los grupos de investigación que fueron conocidos y contactados durante el desarrollo de esta actividad de formación será clave en el desarrollo de éstos proyectos de investigación..

6. Contactos Establecidos: presentación de acuerdo al siguiente cuadro:

Institución/Empresa	Persona de Contacto	Cargo/Actividad	Fono/Fax	Dirección	E-mail
USDA-ARS Foreign Disease Weed Science Research Unit	Dr. Vern Damsteegt	Investigador			damsteeg@ncifcrf.gov; verndd@hotmail.com
USDA-ARS Foreign Disease Weed Science Research Unit	Dr. W.L.Schneider	Investigador			wschneider@fdwsr.ars.usda.gov
USDA-APHIS	Dra. Laurene Levy	Investigador			laurene.e.levy@aphis.usda.gov

University of Arizona	Dra. Judith Brown	Profesor/ Investigador			jbrown @ag.arizona.edu
Centro Internacional de Agricultura Tropical, CIAT	Dra. Pamela Anderson	Directora Programa Intenacional de Manejo Integrado de Mosca Blanca			cip-ddg-research@cgiar.org
USDA-ARS (Salinas, California)	Dr. William M. Wintermantel	Investigador			wwintermantel @pw.ars.usda.gov
University of Florida	Dr Ronald Brlansky	Profesor/ Investigador			Rhby@lal.ufl.edu
Univerity of Florida	Dr. R.F.Lee	Profesor/ Investigador			rfl@lal.ufl.edu
University of Florida	Dr. Jane Polston	Profesor/Investigador			jep@mail.ifas.ufl.edu

7. Detección de nuevas oportunidades y aspectos que quedan por abordar: señalar aquellas iniciativas detectadas en la actividad de formación, que significan un aporte para el rubro en el marco de los objetivos de la propuesta, como por ejemplo la posibilidad de realizar nuevos cursos, participar en ferias y establecer posibles contactos o convenios. Indicar además, en función de los resultados obtenidos, los aspectos y vacíos tecnológicos que aún quedan por abordar para la modernización del rubro.

Uno de los objetivos principales de esta actividad de formación fue el maximimizar las interrelaciones con los grupos de investigadores líderes en el mundo. Se pudo establecer comunicación con investigadores que trabajan en áreas que actualmente se desarrollan en nuestra unidad de biotecnología, contactos que esperamos mantener en el tiempo, de forma de poder establecer redes de interacción y posibles trabajos en conjunto. Este tipo de inquietud fue también mencionada por los investigadores extranjeros, por lo que vemos posibilidades ciertas de interacción en el futuro cercano. A continuación se detallan algunos aspectos relevantes del trabajo que ellos realizan:

- Dr. Vern Damsteegt y Dr. W.L.Schneider (wschneider@fdwsr.ars.usda.gov) del USDA-ARS Foreign Disease Weed Science Research Unit. Estos investigadores trabajan activamente en caracterización molecular del virus plum pox presente en Pensylvannia, EEUU y los posibles vectores involucrados en su distribución. Ellos manifestaron interés en obtener los aislamientos de PPV chilenos (secuencias, estacas de material infectado, etc) con el objetivo de realizar estudios de variabilidad genética del virus.
- Dra. Laurene Levy (laurene.e.levy@aphis.usda.gov)del USDA-APHIS, quien también centra su investigación en PPV. Ella manifestó interés en futuras cooperaciones con nuestro grupo.

Se conversaron distintas posibilidades de interacción en torno a este tema, en cuanto a áreas de colaboración y posibilidad de acceso a fondos internacionales de investigación.

- En torno al tema Bemisia tabaci y geminivirus, pudimos establecer -os primeros contactos con las expertas Dra. Judith Brown (jbrown@ag.arizona.edu) académica e investigadora de la University of Arizona y la Dra. Pamela Anderson (cip-ddg-research@cgiar.org) (Centro Internacional de Agricultura Tropical, CIAT) quienes nos entregaron su opinión de la situación de riesgo que enfrenta Chile con la llegada de la mosquita blanca, B.tabaci, al norte de nuestro país. La Dra Brown nos señaló su disposición para colaborar en un proyecto de caracterización molecular de biotipos chilenos de mosca blanca.
- Dr. William M. Wintermantel (wwintermantel@pw.ars.usda.gov), Research Plant Pathologist del USDA-ARS (Salinas, California) quien es un especialista en virus transmitidos por mosquita blanca, en especial aquellos miembros de la familia Closteroviridae, áreaque esperamos desarrollar proximamente en nuestra unidad.
- Dr Ronald Brlansky (rhby@lal.ufl.edu), Profesor del Dpto de Fitopatología de UF, virólogo del Citrus Research and Education Center. Dr Brlansky es un experto en microscopía electrónica de transmisión y de barrido. En el Laboratorio de Biotecnología del INIA-CRI La Platina se ejecuta el proyecto FONSAG (Chanchito Blanco) que tiene comprometido estudios de microscopia electrónica de barrido. Se habló la posibilidad de cooperar en estas actividades, para los cual se estableció un en contacto entre el Bioquímico Carlos Aguirre, y el Dr. Brlansky.

8. Resultados adicionales: capacidades adquiridas por el grupo o entidad responsable, como por ejemplo, formación de una organización, incorporación (compra) de alguna maquinaria, desarrollo de un proyecto, firma de un convenio, etc.

9. Material Recopilado: junto con el informe técnico se debe entregar un set de todo el material recopilado durante la actividad de formación (escrito y audiovisual) ordenado de acuerdo al cuadro que se presenta a continuación (deben señalarse aquí las fotografías incorporadas en el punto 4):

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Posters APS	1, 2, 3, 4	Posters varios
Artículo Real Time PCR	5	On-site one tourPCR diagnosis of bacterial diseases
Guía de manejo de moscas blancas	6	Whitefly management guide

Specific detection and quantification of plum pox potyvirus by real-time fluorescent RT-PCR

D.J. Sherman, W.L. SCHNEIDER, A.L. Stone, V.D. Damsteegt, and R.D. Frederick
USDA, Agriculture Research Service, Foreign Disease-Weed Science Research Unit, Fort Detrick, MD 21702

PLUM POX POTYVIRUS (PPV)

PPV Virion

- Causal agent of Sharka, economically devastating disease of *Prunus* species
- 9.7 kb positive sense RNA genome
- 764 X 20 nm particle
- Genome expressed as 350kDa polyprotein precursor proteolytically processed into 9 proteins

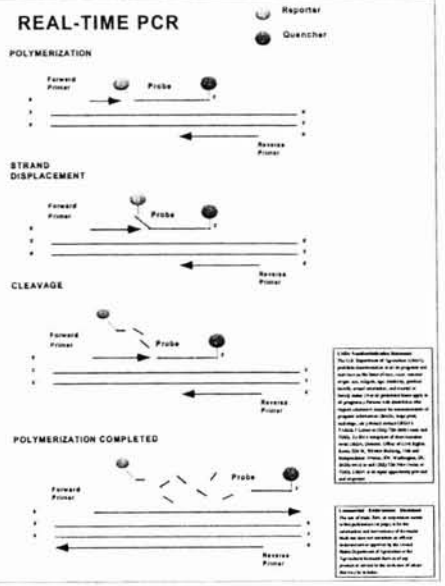
HISTORY

- First identified in Bulgaria in 1915, PPV has since spread across Europe.
- 4 strains found in Europe:
  - D, M, El Amar (EA) and C strains
- PPV (D strain) found in Chile – 1992
- Pennsylvania – 1999
- Canada – 2000

PPV DETECTION METHODS

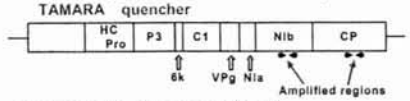
- Enzyme-linked immunosorbent assays (ELISA) are time consuming, crudely quantitative at best, and not always sensitive enough.
- Reverse transcription-polymerase chain reaction (RT-PCR) assays are less labor intensive and more sensitive, but not quantitative.
- Real-time RT-PCR is as sensitive as conventional RT-CPR, with higher specificity and very reproducible instant results.

REAL-TIME PCR



PRIMERS AND PROBES

- Primers and probes designed from conserved regions of the NIB and coat protein (CP) genes
- Probes synthesized with 5' FAM reporter and 3' TAMARA quencher



MATERIALS AND METHODS

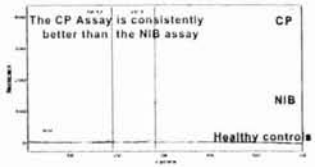
- RNA extraction
  - Conventional, RNA aqueous (Ambion), FTA card (Whatman)
- One step RT-PCR (Invitrogen)

Platinum Taq	
1X Reaction Buffer	
6.0 mM $MgSO_4$	52°C for 15 minutes
200 nM Fwd and Rev. Primer	95°C for 5 minutes
100 nM 3'CP-Fam Probe	40-60 cycles of:
0.5 µL RT/Taq mix per reaction	95°C for 15 seconds
1X Smart Cycler Additive Reagent	60°C for 30 seconds

- Real-time RT-PCR reactions performed in Smart Cycler (Cepheid)

CP AND NIB ASSAY COMPARISON

- Both CP and NIB Primer/Probe sets detect PPV transcript



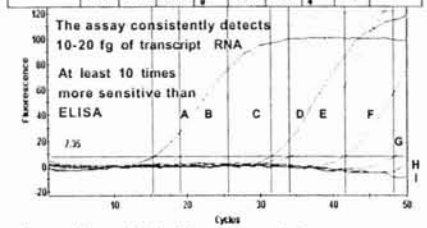
Assay	CT value
CP	19.12
NIB	28.30
CP neg.	0
NIB neg.	0

- The CP Primer/Probe set was chosen for further use

PPV CP ASSAY SENSITIVITY

- The CP Primer/Probe sensitivity was determined using an *in vitro* PPV transcript with the target region.

	A	B	C	D	E	F	G	H	I
Concentration	15 ng	1.5 ng	150 pg	15 pg	1.5 pg	150 fg	15 fg	1.5 fg	negative
Ct value	15.30	18.95	25.67	31.4 9	33.96	41.63	48.2 4	-	-



- Regression analysis of four independent assays determined that the CT values were highly consistent.
- The assay can generate a quantitative standard curve.

PPV CP ASSAY SPECIFICITY

- The assay was tested on total RNA extracted from infected plants and an aphid vector

	Virus and Strain	Host	Results (+/-)
Prunus viruses, PPV Strains	Plum pox virus - D (PPV-D)	***Prunus persica***	+
	Plum pox virus - El Amar (PPV-EA)	***Prunus persica***	+
	Plum pox virus - Cherry (PPV-C)	***Prunus tomentosa***	+
	Plum pox virus - M (PPV-M)	***Prunus persica***	+
	Plum pox virus - PA (Pennsylvania)	***Prunus persica***	+
	Apple chlorotic leaf virus (ACLV)	***Prunus persica***	-
	Tomato ringspot virus (TomRSV)	***Prunus persica***	-
	Myrobalan latent ringspot virus (MLRV)	***Prunus persica***	-
	Peach rosette mosaic virus (PRMV)	***Prunus persica***	-
	Prunus necrotic ringspot (PNRV)	***Prunus persica***	-
Potyviruses	Potato virus Y (PVY)	***Nicotiana tabacum***	-
	Datura potyvirus D437 *	***Nicotiana tabacum***	-
	Maize dwarf mosaic virus (MDMV)	***Sorghum halepense***	-
	Clover yellow vein virus (CYVV)	***Nicotiana benthamiana***	-
Other PPV hosts	Plum pox virus - PA	*Melilotus officianalis*	+
	Plum pox virus - PA	*Nicotiana benthamiana*	+
	Plum pox virus - PA	*Nicotiana edwardsonii*	+
	Plum pox virus - PA	*Nicotiana tabacum*	+
	Plum pox virus - PA	*Nicotiana occidentalis 37-B*	+
	Plum pox virus - PA	*Prunus americana*	+
	Plum pox virus - PA	*Prunus serotina*	+
	Plum pox virus - PA	*Prunus tomentosa*	+
	Plum pox virus - PA	*Prunus persica* leaves	+
	Plum pox virus - PA	" " flower	+
	Plum pox virus - PA	" " fruit	+
	Plum pox virus - PA	" " green stem	+
	Plum pox virus - PA	" " leaf	+
	Plum pox virus - PA	" " leaf bud	+
	Plum pox virus - PA	" " root	+
	Plum pox virus - PA	" " woody stem	-
	Plum pox virus - PA		+

*D-437 is a previously undescribed potyvirus found in *Datura* that cross reacts with PPV antibody (V. Damsteegt, personal communication).

CONCLUSIONS

The PPV CP Real-time RT-PCR assay is:

1. Sensitive
   - Consistently detects 10 fg of PPV RNA
   - At least 10 times more sensitive than ELISA
2. Quantitative
   - When used with a standard curve the assay can be used to accurately estimate viral RNA titer
3. Specific

Detects	Does not detect
All European strains of PPV	Other common *Prunus* viruses
All Pennsylvania strains of PPV	Other potyviruses
PPV in numerous hosts	Plant host mRNAs
PPV in most *Prunus* tissue types	
PPV in aphids	

A LOW TITER RNA ENTITY IN A *CITRUS TRISTEZA VIRUS*-INFECTED MEXICAN LIME PLANT WITH SIMILARITIES TO *OAT BLUE DWARF VIRUS* AND GRAPEVINE FLECK VIRUS

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ABSTRACT

A cDNA with high similarity to part of the *Oat blue dwarf virus* (OBDV; genus *Marafivirus*) genome was obtained from dsRNA extracts of a *Citrus tristeza virus* - infected plant. Northern analyses using the source plant total RNA and RNA extracts from virus purification fractions revealed a genomic RNA of at least 7.5 kb. OBDV coat protein (CP) antibodies react weakly with protein extracts from the source plant and produce a specific band of approximately 28-kDa. Grapevine fleck virus (GFkV; unassigned virus) has many features common to marafiviruses and a 7.5 kb genomic RNA. GFkV CP antibodies do not react with protein extracts from the source plant by ELISA. The entity appears non-graft transmissible to citrus, and a host range study to herbaceous plants concluded the entity could not be mechanically transmitted.

INTRODUCTION

During a sequencing project using dsRNA extracted from one plant infected with *Citrus tristeza virus*, sequences were found which were similar to tymoviruses and marafiviruses, with greatest similarity to part of the *Oat blue dwarf virus* (OBDV) genome between nucleotides 2401-4668 (BLASTN; Altschul *et al.*, 1997). One of these sequences, OBDV-like clone-1 (OL-1) containing a *ca.* 1113 bp insert was used in further investigations. Since the source plant was being assessed for CTV severity and no known marafivirus had been identified as infecting citrus, further tests were performed to identify if the sequences represented a possible new virus or new host range for an already characterized virus. Fig. 1. is the ethidium bromide stained 6% polyacrylamide gel used to separate the dsRNA extracted from the source plant for the sequencing project.

MATERIALS & METHODS

Virus purification: Freshly collected stem bark (18.0g) and leaf midribs (13.2g) were collected from a Mexican lime (*Citrus aurantifolia*) plant infected with *Citrus tristeza virus*. An OBDV purification method was used (Edwards *et al.*, 1997) and this was carried out as described (D'Arcy *et al.*, 1983). Following the final centrifugation on a 10-40% sucrose gradient, 22 x 500 uL fractions were drawn off with a syringe.

RNA analyses: Total RNA was extracted from the virus purification fractions (using 100 uL for slot blot and 50 uL for the size estimations) by a double phenol:chloroform extraction followed precipitation of the aqueous layers in 95% ethanol. Total citrus leaf RNA was extracted in a similar manner with the addition of a lithium chloride treatment. Final pellets from all sources were washed with 70% ethanol, then resuspended in nuclease-free ultra pure water, UV spectrophotometrically quantified, electrophoretically qualified and stored at -80°C until use. For virus citrus transmission studies and size analyses of the RNA from grafted citrus plants, denatured RNA (10 uL) was separated electrophoretically in formaldehyde-1.6% agarose. A RNA ladder was included as the size marker. For RNA extracted from the viral purification fractions and used for size analyses, the final resuspension volume (13 uL) was treated in a similar manner. After electrophoresis the RNA ladder was removed, stained with ethidium bromide, visualized under UV light, and photographed for the size estimation on the blots. The rest of the gel containing control and test RNA samples was incubated with 2X SSC for 30 min. prior to downward capillary transfer of nucleic acids to Hybond-N+ nylon membranes using 20X SSC. Hybridization was performed at 65°C according to Church & Gilbert, (1984), with a 32P-labeled dCTP DNA probe complementary to OL-1, made using a random primers DNA labeling kit and the supplier instructions (Gibco BRL Life Technologies). For slot blot analyses of the RNA from the viral purification fractions, all of the sample was denatured and then vacuum blotted directly onto a Hybond-N+ nylon membrane, with hybridization using similar conditions to the aforementioned.

Serology: Immunoblots of virus purification fractions and grafted plants: SDS-PAGE (16 or 15% acrylamide) was performed on the sucrose gradient fractions 2, 8, 12, 14, 15, 18 and 22. The fractions were heated to 99°C with an equal volume of cracking buffer (63 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) for 5 minutes. A volume of 10 uL /sample/lane were used. Control samples included on each gel were proteins extracted from OBDV oat tissue, from the source plant and a virus-free Mexican lime plant. Biorad broad spectrum protein size markers were used on each gel. One gel was stained with Coomassie Blue to visualize the proteins present while the other was used to transfer proteins to a nitrocellulose membrane then developed with an OBDV antibody.
ELISA: A GFkV indirect double antibody sandwich ELISA was used on extracts from all the citrus plants which were grafted from the source tree with the unknown entity (Boscia *et al.*, 1995). Antibodies were supplied in a kit form (Agritest s. r. l., Tecnopolis, 70010 Valenzano, Italy) with positive and negative grapevine controls. Uninoculated and source tree graft inoculated St George grapevine tissues were also included as samples on the micro plates, as well as buffer only controls. Kit instructions were followed. The hydrolyzed enzyme substrate extinction values were read at 405 nm.

Mechanical inoculation: Inoculum was citrus source leaf tissue titurated in a pre-chilled mortar with a pestle using cold 0.05 M potassium phosphate buffer, pH 7. A tissue:buffer ratio of 1:10 (w/v) was used. The resultant sap was then strained through two layers of sterile muslin. Primary leaves near to full expansion were inoculated with a gloved finger dipped into the inoculum. Test plants were then rinsed gently with tap water and shaded for 12 hr. Receptors of Cotton, *Nicotiana benthamiana*, *N. tabacum*, *Chenopodium quinoa*, *C. amaranticolor*, Maize, Cowpea, Melon, and Spinach were raised from seed in a cooled greenhouse (ambient temperature not greater than 30°C). Plants were kept pest and disease free. Four seedlings per species were used, with three plants being inoculated and one being inoculated with buffer only. Plants were visually assessed for foliar symptoms daily for up to 4 weeks after inoculation.

Graft inoculation: Lateral meristems from the source tree were grafted to two virus-free St. George grapevine (*Vitis rupestris*) seedlings. This cultivar is a used as a susceptible indicator for GFkV. One St. George grapevine seedling was left uninoculated as a comparison. Plants were kept under cooled greenhouse conditions (not exceeding 30°C). The indicator plant foliage growth and stem after grafting was observed for symptoms for 6 weeks after inoculation.

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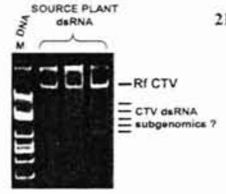

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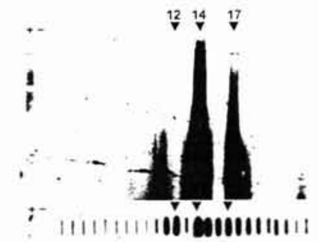

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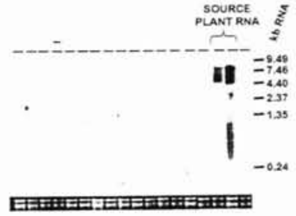

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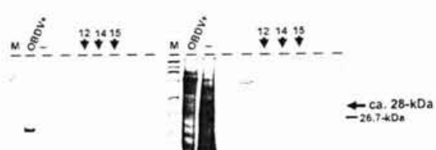

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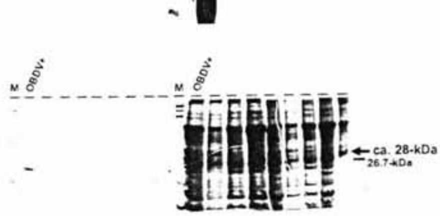

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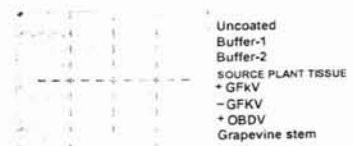

Fig.1: Ethidium bromide stained 6% PAGE of dsRNA extracted from the source Mexican lime plant. Rf: replicative form of CTV. **Fig. 2:** Northern analyses using a probe made from the sequence with similarity to OBDV (OL-1). **A:** Viral purification fractions, upper blot, denaturing gel electrophoresis, lower blot, slot blot analyses. Arrows and numbers indicate the three fractions which hybridize strongly to OL-1 (note fraction 12 was not loaded in the upper gel), the higher the fraction number the lower the fraction in the gradient tube. **B:** Sizing of the genomic RNA of the entity hybridizing to OL-1 from plant and the virus purification fractions, for example, this sample is the positive control from 2A. **Fig. 3:** Northern analyses of total RNA from citrus plants grafted from the source plant using OL-1 as a probe. **Fig. 4:** Immunoblot of proteins extracted from the sucrose gradient fractions using OBDV antibodies (LHS), with corresponding Coomassie stained gel on the RHS. **Fig. 5:** Immunoblot of proteins extracted from plants grafted from the source plant using OBDV antibodies (LHS), with corresponding Coomassie stained gel on the RHS. **Fig. 6:** ELISA of extracts from citrus and grapevine plants grafted from the source plant using GFkV antibodies. This diagram is a cartoon representing the data, the yellow wells represent an interaction, all other wells represent no interaction. Legend to the RHS of the micro plate indicates the duplicated last lane sample controls. Throughout the figures, - represents virus-free tissue or sample, + represents a virus infected sample, indicated as appropriate, and M represents protein marker.

RESULTS & DISCUSSION

The RNA slot blot showed positive reactions with the OL-1 probe for all the RNA extracted from the sucrose gradient fractions (Fig. 2A Lower blot). The reactions for the upper ten sucrose gradient fractions were weak and the strongest reactions were observed to be for the sucrose gradient fractions 11, 12, 14, 15 and 17. The wide spread positive reaction with the probe suggests that the viral particles did not remain intact during the extraction and purification process and that the viral RNA was degraded so that, rather settling in a narrow band in the sucrose gradient, it was dispersed throughout.

The RNA denaturing gel with RNA extracted from the 22 sucrose gradient fractions (Fig. 2A Upper blot) showed strong positive reactions in the lanes corresponding to sucrose gradient fractions 14 and 17. Fraction 12 was not loaded in Fig. 2A, however Northerns were repeated twice more with samples from 12, 14, 16 and 17 (data not shown). Data confirmed fractions 12, 14, and 17 provided the best signals during hybridization. The lower fraction, 17, gave a very distinct band at *ca.* 7.5 kb (putative viral genomic RNA) in Fig. 2A, below which a long dark blur was observed, indicating the presence of degraded genomic and possibly sub-genomic RNA of varying sizes. The genomic RNA band was the same size as the upper band from the source plant RNA when hybridized in a similar manner (Fig 2B). GFkV was at this time the only marafivirus-like virus to have a similar genome size and etiology (prior to Sabanadzovic *et al.*, 2001). The possibility that the unknown entity was GFkV was therefore further investigated. The Northern blot of RNA extracted from citrus plants which had been grafted from the source plant over a period of 5 years (Fig. 3) revealed that none of these plants harbored the OBDV-like entity, therefore the conclusion is that the entity is not graft transmitted to citrus.

The immunoblots using proteins extracted from the sucrose gradient fractions with OBDV antibodies showed a very weak cross reaction with a band of *ca.* 28-kDa (Fig. 4), which is dissimilar in size to that of OBDV coat proteins. This suggests possibly a weak similarity between the coat protein of this unidentified virus and that of OBDV. The immunoblot of source plant graft inoculated plant protein extracts (Fig. 5) with OBDV antibodies also had a very weak association at *ca.* 28-kDa. The ELISA tests (Fig 6 is representative of an example) revealed no cross reaction to GFkV antibodies with any of the sap extracted from the citrus plants grafted from the source plant, the source plant inoculated or uninoculated indicator St. George grapevine plants, or the virus-free grapevine control supplied with the kit. The GFkV positive grapevine control did give a good reaction, indicating the tests were valid.

None of the plant species mechanically inoculated with sap from the source plant were observed with any foliar symptoms different from the mock inoculated controls. Graft inoculation of source tissue to St. George grapevine did not reveal any foliar symptoms characteristic of Grapevine fleck disease; that is, peripheral leaf vein clearing and/or wrinkling of the leaves at least six weeks after infection. Essentially the inoculated plants looked the same as the uninoculated plant.

The cross reaction with the OBDV antibody and the sequence similarity between the known part of the unidentified entity and parts of the OBDV sequence strongly suggest that the entity is a virus similar to OBDV and as such is most likely to be in the genus *Marafivirus*. The OBDV genome is approximately 6.5 kb in length and three capsid proteins have been identified – the most abundant being a putative protein of 22.2-kDa and two other putative proteins of 25.5-kDa and 26.6-kDa, respectively (Edwards *et al*, 1997). OBDV is transmitted only by the aster leafhopper (*Macrosteles quadrilineatus* Forbes) within which the virus replicates. This insect is indigenous to Texas and feeds on many different plant species. The OBDV capsids are similar in size to those reported for the putative capsid proteins for two other marafiviruses: *Maize rayado fino virus* (MRFV; type species of the genus *Marafivirus*) and *Bermuda etched-line virus* (BELV; Izadpanah *et al.*, 2002). GFkV has a single-stranded, positive sense RNA genome *ca.* 7.4 kb size, and coat protein consisting of a single capsid with an estimated molecular mass of *ca.* 28 -kDa, and although GFkV is closely related to marafiviruses and tymoviruses, it has distinct sequence dissimilarity and properties, and may delimit a new viral genus, proposed as the genus *Maculovirus* (Sabanadzovic *et al.*, 2001; Martelli, pers. comm.). GFkV is reported to be graft transmissible although not mechanically transmissible and has no known insect vectors. The ELISA tests with GFkV antibodies infer that the entity is unlikely to be GFkV.

From these studies an unknown RNA entity has been found in a citrus plant with a genome of ca. 7.5 kb. The entity is neither mechanically transmissible to a range of herbaceous and woody plants nor is it graft transmissible to grapevine or citrus. This leads to the conclusion that the entity may be transmitted in another fashion, for instance, by a leaf hopper or by other means. A 1113 nt sequence from the entity has strong similarity to OBDV. BELV is the only other likely marafivirus candidate as this virus has a genome size of the same order of magnitude as the unknown entity. OBDV antibodies very weakly cross react with proteins extracted from the source plant and viral purification fractions at approximately 28-kDa. GFkV antibodies do not react with proteins extracted from the source plant. This entity has marafivirus characteristics, however, further studies to gather more sequence data and electron micrograph visualization of virus particles are needed for confirmation.

ACKNOWLEDGEMENTS

We thank Dr. M. Edwards, USDA-ARS Northern Crop Science Laboratory, Minnesota for the gift of the OBDV antibodies and positive OBDV control. Gratitude goes also to Professor G. V. Martelli, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy, and Agritest s. r. l. for the gift of the GFkV antibody kit. Many thanks to Drs. W. Qiu and L. G. Kovalo from Southwest Missouri State University-Mountain Grove for the advice on GFkV and for supplying the virus-free grapevine seedlings.

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New diagnostic assay based on DNA array for identification and detection of five bacterial pathogens of potato

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The Gram-positive bacterium, *Clavibacter michiganensis* subsp. *sepedonicus* and the Gram-negative bacteria, *Ralstonia solanacearum* and pectolytic erwinias including *Erwinia carotovora* subspp. *atroseptica* and *carotovora* and *Erwinia chrysanthemi*, are important bacterial pathogens of potato. DNA array technology, essentially a reverse dot blot technique, is an emerging methodology useful for identification of DNA fragments and may be applicable for rapid identification and detection of plant pathogens associated with plants. By using conserved primers to amplify common bacterial genome fragments from extracts of potato tubers that might contain the bacterial pathogens, the presence of DNA sequences indicative of pathogenic species can be revealed by hybridization to species-specific oligonucleotide within the array. In this study we explored the feasibility of using DNA array technology for identification and detection of plant pathogenic bacteria by targeting five bacterial pathogens of potato. Both pure cultures of bacteria and potato extracts were evaluated on arrays established with the specific and heterologous oligonucleotide probes to determine the discriminatory potential of this technology and application for identification and detection of bacterial pathogens.

MATERIALS AND METHODS

- **Sequences** of 16S rDNAs and the intergenic spacer (IGS) regions between the 16S-23S rDNA of bacterial strains from the genus *Erwinia* were determined (Fessehaie et al. 2002).
- **Oligonucleotides selected** from the 3' end of the 16S and IGS regions from the pectolytic erwinias and from known sequences of the other bacteria pathogenic on potato, namely, *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* (Pastrik et al. unpublished), were spotted as an array onto nylon membranes.
- **DNA arrays** were used in this non-radioactive hybridization assay for detection of bacterial pathogens provided by mixed genomic DNA's or in inoculated potato samples. **Specificity of oligonucleotides** for their target DNA sequence was evaluated following published methods.

RESULTS

- Phylogenetic analysis of **small IGS sequence** data discerned a consistent relationship among the test strains, which was in agreement with the **16S data** that reflected the accepted species and subspecies structure of the taxon (Fessehaie et al. 2002).
- Sequence data derived from the **large IGS** resolved the strains into coherent groups, however, the sequence information would not allow any phylogenetic conclusion because it failed to reflect the accepted species structure of the test strains.
- Hybridization of **amplicons to the array** and subsequent serological detection of digoxigenin label revealed different hybridization patterns that were distinct for each species and subspecies tested. Furthermore, the assay was used successfully with infected potato samples as illustrated in Figures 1-3.

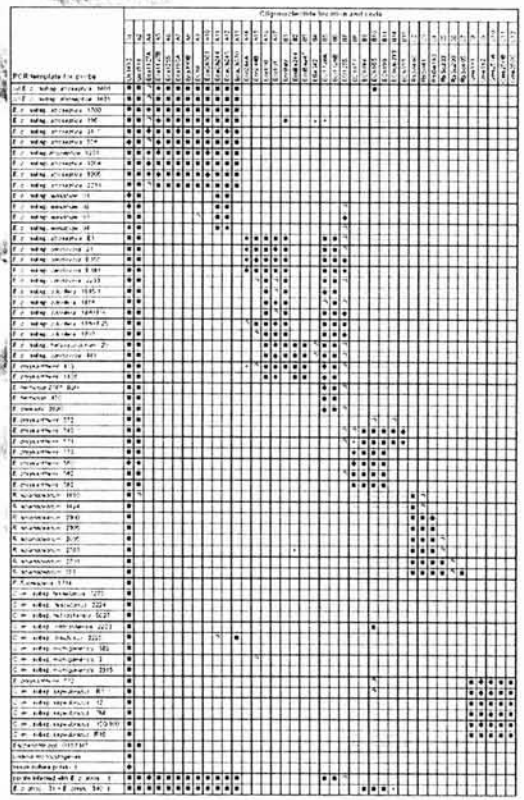

Fig. 1. Hybridization of digoxigenin-labeled PCR amplicons from selected bacterial species and samples to oligonucleotides immobilized on nylon membrane in an array. Hybridization signal strength of each amplicon to an oligonucleotide in the array is expressed in 16 bit gray scale values indicated by the following symbols: ■ = 30,000 - 65, 000; ◆ = 20,000 - 30,000; ● = 10,000 - 20,000; ○ = 1,000 - 10,000; • = 500 - 1,000; <500 = Blank; and – = Not tested. Some membranes are being shown as examples in Fig. 2 (*) and Fig. 3 (†). Oligonucleotide locations for the selected membranes are listed at the top.

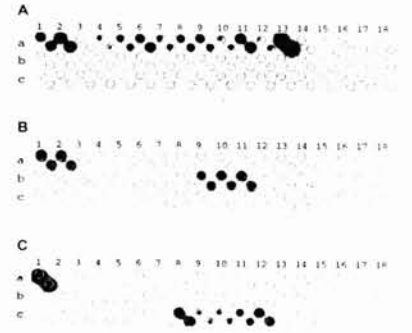

Fig. 2. Hybridization pattern of digoxigenin-labeled PCR amplicons from **(A)** *E. c.* subsp. *atroseptica* strain 31; **(B)** *E. chrysanthemi* strain 340; **(C)** and *C. m.* subsp. *sepedonicus* strain R3 on the oligonucleotide array. Oligonucleotides were spotted, with duplicates on the diagonal, in three rows (a, b and c) of 18 spots. A template to identify spot locations was overlain on the membrane after completion of hybridization and development of the digoxigenin detection procedure. Positive hybridization signals are visualized as dark spots within template circles and computer generated results from these blots summary can be seen in Fig. 1 (*).

Fig. 3. Hybridization pattern of digoxigenin-labeled PCR amplicons obtained from **(A)** a mixture of *E. c.* subsp. *atroseptica* strain 31 and *E. chrysanthemi* strain 340 cells; **(B)** DNA from *E. c.* subsp. *atroseptica*-inoculated potato tuber; and **(C)** DNA from potato tissue culture. Oligonucleotides were spotted, with duplicates on the diagonal, in three rows (a, b and c) of 18 spots (Fig. 3C, A18 is digoxigenin dot). A template to identify spot locations was overlain on the membrane after completion of hybridization and development of the digoxigenin detection procedure. Positive hybridization signals are visualized as dark spots within template circles and computer generated results from these blots can be seen in Fig. 1 (†).

SUMMARY AND CONCLUSION

- Specific oligonucleotide positions discriminated among the *E. carotovora* subsp. *atroseptica, carotovora, odorifera, wasabiae* and *betavasculorum* and between *E. carotovora* and *E. chrysanthemi*. These discriminatory molecular signatures are useful targets from which to derive oligonucleotides for diagnostic purposes.
- DNA arrays were used in this non-radioactive hybridization assay for multiplex detection of bacterial pathogens provided by mixed genomic DNA's or in inoculated potato samples. Hybridization of amplicons was generally restricted to the appropriate homologous oligonucleotides and cross-hybridization with heterologous oligonucleotides was rare (Figures 1-3).
- The new diagnostic assay based on DNA array should prove useful for identification and detection of five bacterial pathogens of potato.

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Canada

A New Member of the Clover Proliferation Phytoplasma Group (16SrVI) Associated with an Elm Yellows in Illinois.

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ABSTRACT

An outbreak of a disease appearing similar to elm yellows (EY) began in the early 1990s in the Chicago suburb of Arlington Heights, Illinois, U.S.A. Over 1000 mature American elm (*Ulmus americana*) trees have since died from the disease. Nested PCR using universal primer pairs previously designed for phytoplasma detection revealed that sixteen of the seventeen trees growing in the outbreak region were positive for phytoplasmas, regardless of the magnitude of symptom expression. All control elms tested negative for phytoplasma. Restriction fragment length polymorphisms (RFLP) and DNA sequence analyses of 16S rDNA of representative samples indicated that the Illinois phytoplasma was not related to the phytoplasma causing EY disease (group 16SrV-A) elsewhere. It is most closely related to clover proliferation phytoplasma (group 16SrVI). We have identified the phytoplasma as representative of a new subgroup (16SrVI-C) of clover proliferation (CP) phytoplasma group. Neither the traditional EY, nor the newly designated Illinois elm yellows (ILEY), phytoplasmas were detected in local leafhopper populations trapped daily between May and September 2000. This is the first report of a phytoplasma related to CP phytoplasma causing elm yellows disease.

INTRODUCTION

More than 1,000 mature American elms have died during the last ten years in the Arlington Heights suburb west of Chicago, IL. The outbreak was first noted in 1991 when ten trees died along residential parkways where there are nearly 10,000 mature elms. The cause of death was unknown, but symptoms clearly differed from those of Dutch elm disease (DED). By 1998, annual losses due to the unknown malady totaled more than 150 trees, roughly equivalent to DED losses in this region. The mortality rate has yet to decline, and during the past two years, symptomatic elms have been detected in adjacent communities giving the appearance that the problem continues to spread. Elm yellows (EY), caused by a leafhopper-transmitted EY phytoplasma (belongs to 16SrV-A) was suspected. However, EY is historically absent from the northern third of Illinois, presumably because winter temperatures limit the reported vector, *Scaphoides luteolus* Van D. and one or more symptoms considered diagnostic of EY, i.e. premature yellowing and epinasty of leaves throughout the canopy, butterscotch discoloration of inner bark and the accompanying odor of wintergreen oil, were absent or erratic in some trees. Our objectives were to determine if the causal agent of the disease was EY phytoplasma, and to assay local leafhopper populations for its presence.

MATERIALS AND METHODS

Sampling: Samples were taken between 1998 and 2000 from seventeen mature parkway elms growing in the vicinity of the outbreak in Arlington Heights. Ten additional trees located thirty miles from the outbreak at The Morton Arboretum, Lisle, IL, were sampled in Fall 2001 to serve as controls. Disease severity ratings (DSR) were based on appearance of the canopy (Fig. 1). DSR values were assigned between 1 and 5 where: 1=healthy or asymptomatic canopy; 2 = up to 25% of the canopy showing sparse and clumpy symptoms; and sometimes discolored; 3 = 25-50% of the canopy showing symptoms; 4 = 50-90% of canopy; 5 = nearly 100% devoid of leaves and tree near death if not dead. Three types of samples, inner bark/phloem, leaves, and wood shavings, were taken when possible. Ten samples of inner bark and phloem were collected from asymptomatic trees (DSR = 1) to serve as controls.

DNA extraction and nested PCR: For leaf tissue, total nucleic acid was extracted as described by Lee et al. (1993). Phloem tissues and wood shavings were extracted by a modified procedure using DNeasy Plant Mini Kit (Qiagen, Valencia, CA) as described by Green et al. (1999). A nested PCR was performed using primer pair P1/P7 followed by R16F2n/R16R2 primer pair. Comparative nested PCR assays using primer pair P1/P7, followed by either a universal primer pair R16F2n/R16R2 or an EY phytoplasma specific primer pair R16(V)F1/R16(V)R1 (Lee et al. 1994) were performed to determine whether EY phytoplasma was also present in five inner bark/phloem samples in which a CP-like phytoplasma was detected.

RFLP and sequence analyses of 16S rDNA: RFLP analyses of the nested PCR products (1.2 kb) were done using up to 12 restriction enzymes: *Mse*I, *Alu*I, *Rsa*I, *Hha*I, *Hae*III, *Hpa*II, *Taq*I, *Hinf*I, *Sau*3AI, *Kpn*I, *Tha*I, and *Bfa*I. Cloning and sequencing of the P1/P7-PCR amplified products from three Arlington Heights trees (926S-E, 746S-V, 825S-H) were performed.

Survey of potential vectors: During the summer of 2000, leafhoppers were collected in the vicinity of the outbreak and separated into 17 morphological groups. Total nucleic acid was extracted from at least 10 individual leafhoppers representing each of the 17 morpho-types and processed via the modified method described above. Nested PCR using primer pair P1/P7 followed by R16F2n/R16R2 was performed.

Fig.1

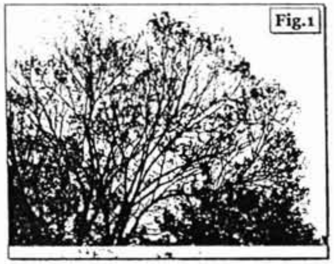


Table 1

[illegible]	[illegible]	[illegible]	Phytoplasma Identification
926S-E	3	pos	ILEY
814S-E	2	pos	na
746S-D	1	pos	ILEY
826S-H	4	pos	ILEY
802S-V	1	pos	ILEY
807S-V	2	pos	na
820S-V	5	pos	na
844S-V	3	pos	na
201W-O	3	pos	na
913S-H	1	pos	na
826S-H	5	neg	na
825S-H	4	pos	ILEY
746-V	4	pos	ILEY
925S-H	4	pos	ILEY
518	4	pos	ILEY
720S-V	5	pos	ILEY
843S-V	4	pos	ILEY

Fig. 2

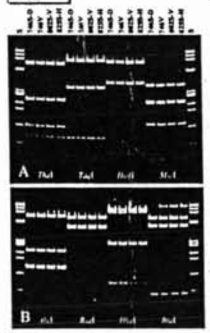
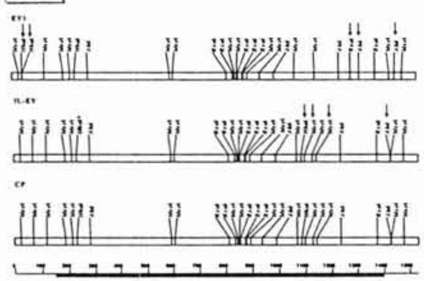


Fig. 3

Fig. 4



RESULTS

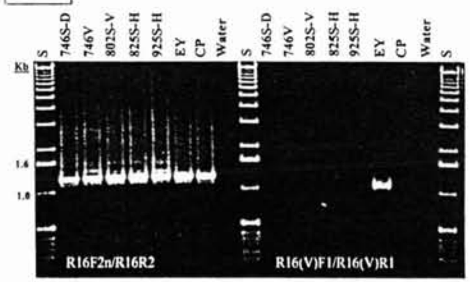
Phytoplasma was detected with universal phytoplasma primers in 16 out of the 17 Arlington Heights trees (Table 1). None of the ten healthy control trees from the Morton Arboretum tested positive for phytoplasma. Collective RFLP patterns based on eight restriction enzymes from eight samples indicate that the phytoplasma present in elms from Arlington Heights were identical to one another (four samples shown in Fig. 2). However, it was distinct from EY phytoplasma and most closely related to the CP phytoplasma (16SrVI-A).

Restriction site analysis derived from sequence data supported RFLP pattern analysis. The henceforth referred to as Illinois elm yellows (ILEY) phytoplasma can be differentiated from EY phytoplasma by four restriction enzymes, *Alu*I, *Hha*I, *Mse*I, and *Rsa*I, and from CP phytoplasma by one enzyme, *Hha*I (Figs 3, 4).

Comparative nested PCR assays using the nested primer pair R16F2n/R16R2 (universal) yielded PCR products for all five ILEY infected elms as well as the two reference phytoplasma strains, EY and CP. However, nested PCR using the EY phytoplasma specific primer pair R16(V)F1/R16(V)R1 gave a positive result only for the reference EY phytoplasma (Fig. 5).

BLAST comparison of the ILEY phytoplasma 16S rDNA sequence indicates about 99% homology to CP phytoplasma and 98.5% homology to multicipita phytoplasma (subgroup 16SrVI-B). Unlike CP phytoplasma, sequences of cloned 16S rDNA indicated ILEY phytoplasma contains two heterogeneous operons (Fig. 4) and has, therefore, been designated as a new subgroup (16SrVI-C). None of the insects assayed were infected with EY, CP or ILEY phytoplasmas.

Fig. 5



DISCUSSION

Until now, EY disease in North America was thought to be caused only by strains of the EY phytoplasma belonging to 16SrV. A nested PCR using the EY phytoplasma group specific primer pair R16(V)F1/R16(V) R1 indicated that EY phytoplasma was absent in the Arlington Heights elms samples. Sequencing and RFLP analyses of PCR products (16S rDNA) confirmed that the ILEY phytoplasma was distinct from EY phytoplasma and is a new subgroup (designated 16SrVI-C) of the clover proliferation (CP) phytoplasma group (16SrVI). It is surprising that a member of the CP phytoplasma group infects elms, as CP phytoplasma is generally considered a pathogen of herbaceous plants.

The presence of ILEY phytoplasma appears well correlated with the eventual development of an EY-like syndrome in Arlington Heights elms, as 16 out of 17 symptomatic trees tested positive for phytoplasma, while all 10 healthy controls were negative. Ten of the 16 positive samples were further analyzed by RFLP and determined to be ILEY phytoplasma. Other symptoms reported to typify the traditional elm yellows disease were, in our experience, less reliable. For example, some trees developed uniformly yellow and epinastic leaves throughout the canopy in mid summer while others remained green. Butterscotch colored phloem accompanied by an aroma of wintergreen oil was readily found in some trees that were exhibiting canopy symptoms, but not in others. Apart from necrosis of the phloem and the rapid death of an infected tree, the sparse and clumped appearance of foliage in the canopy was more reliable in diagnosing this disease. Sampling method and tissue type also appeared to influence detection results. The phloem/bark tissues were found to be the most reliable of those tested for detecting phytoplasma. An improved understanding of seasonal and spatial variation in phytoplasma distribution and concentration would clearly assist in designing more accurate detection methods.

CP phytoplasma vectors, principally the aster leafhopper *Macrosteles quadrilineatus* Forbes, but also the beet leafhopper (*Circulifer tenullus* Baker), are widely distributed in the U.S. and have both migrating and overwintering populations in the Midwest. Attempts to identify the potential vector(s) in this study failed; none of the leafhoppers assayed were found to be carriers of ILEY, CP or EY phytoplasma. It is likely that the vectors of CP phytoplasma, which could carry ILEY, were only transient feeders of elm. An interesting coincidence is that the aster leafhopper typically breeds in the south central United States but during the middle to late 1980s, overwintering populations were present in Wisconsin. Likewise, a severe outbreak of the beet leafhopper transmitted disease, horseradish brittle root disease (caused by *Spiroplasma citri*) occurred in Illinois during the 1980s. Both outbreaks were associated with abnormally high vector populations and appear to coincide with what was probably the initial infection years (middle 1980s) of the elm yellows disease in Arlington Heights, Illinois.

Lee, I.-M.; Gundersen, D. E.; Hammond; R. W.; Davis, R. E. 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* 84: 559-566.

Lee, I.-M.; Hammond, R. W.; Davis, R. E.; Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* 83: 834-842.

Green, M. J.; Thompson, D. A.; MacKenzie, D. J. 1999. Easy and efficient DNA extraction from woody plants for detection of phytoplasmas by polymerase chain reaction. *Plant Disease* 83: 482-485.

Classification of phytoplasmas in the expanded elm yellows group (16SrV) based on 16S rRNA and ribosomal protein gene sequences

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INTRODUCTION

The elm yellows (EY) phytoplasma group (16SrV) represents the third most diverse phytoplasma cluster, next to aster yellows and X-disease phytoplasma groups. The elm yellows phytoplasma strains cause decline of American elms in North America and Eurasian elm species and hybrids in several European countries. Other EY group phytoplasmas associated with diseases in diverse plant genera in various geographical regions are: flavescence dorée (FD) and grapevine yellows phytoplasmas (PGY) grapevine in Europe; rubus stunt (RuS) phytoplasmas in wild and cultivated blackberry (*Rubus* spp) in Europe; cherry lethal yellows phytoplasma (CLY) in cherry in China; peach lethal yellows phytoplasma (PE-A, PE-D) in peach in India; jujube witches'-broom phytoplasma (JWB) in *Ziziphus* spp. in China and India; alder yellows phytoplasmas (ALY) in alders in Germany and Italy; spartium witches'-broom-EY phytoplasma in *Spartium* sp. in Italy; eucalyptus little leaf phytoplasma (ELL) in *Eucalyptus* sp. in Italy; and hemp dogbane decline phytoplasma (HD1) in *Apocynum cannabinum* in New York State. Recently, a new member of the elm yellows group, Virginia Creeper phytoplasma (VC), was reported to infect Virginia creeper (*Parthenocissus quinquefolia*) plants in southern Florida. EY group phytoplasmas have been classified into five subgroups based on 16Sr DNA sequences. This approach was found to be insufficient in differentiating many members of this group, of which several strains have been shown to be distinct based on biological or pathological properties. The aim of this present study was to investigate phylogenetic relationships among members of the expanded EY phytoplasma group based on combined analyses of 16S rRNA and ribosomal protein gene sequences, and to develop and evaluate a new means for a finer strain differentiation.

MATERIALS AND METHODS

Phytoplasma strains. Twenty-two representative members of the EY phytoplasma group were used for this work (Table1).

RFLP analysis. Nested PCR products of 16S rDNA (amplicons using primer pair P1/P7 followed by primer pair R16F2n/R16R2) and ribosomal protein (rp) gene operons (amplicons using primer pair rp(V)F1/rpR1 followed by primer pair rp(V)F1A/rpR1A) were digested with a numbers of restriction enzymes. Putative restriction site maps of representative phytoplasma strains were generated by using the DNASTAR program MapDraw option (DNASTAR, Madison, WI, USA).

Phylogenetic analysis. PCR products amplified by P1A/P7A (1.8 kb, containing 16S rDNA) and by rp(V)F1A/rpR1A (about 1.1 kb) were purified, cloned and sequenced. Partial sequences of 16S rDNA (1.5 kb) and rp gene operon (1.1 kb) from 22 members of EY phytoplasma group and representative phytoplasma strains available in GenBank were aligned and cladistic analyses were performed with PAUP (phylogenetic analysis using parsimony), version 4.0 written by D.L. Swofford (University of Illinois), on a Power Mac G4. Uninformative characters were excluded from analyses. *Acholeplasma laidlawii* was selected as the out-group to root the tree.

RESULTS

RFLP analyses: The *Mse*I and *Alu*I RFLP profiles of the 1.2 kb 16S rDNA fragment were conserved among all 22 representative members of the EY phytoplasma group are unique to this group (Lee et al. 1998) (**Fig. 1, Table 1**). Collective profiles obtained from digests of products with *Rsa*I, *Hpa*II, and *Bfa*I differentiated the EY group into five distinct subgroups. Collective profiles obtained from digests of rp gene sequences with these restriction enzymes differentiated the EY group into 12 distinct rp subgroups (**Fig. 2, Table 1**).

Putative restriction maps: Restriction maps of a 1.1 kb sequence of rp operon (contains *s3* and *l22* genes) cloned from the 12 rp subgroups delineated by RFLP analysis were shown in **Fig. 3**. Eighteen restriction sites were found variable among members of the EY phytoplasma group.

Phylogenetic relationships: One of the most parsimonious trees was deduced by analysis of near full length of 16S rDNA sequences of 26 representative phytoplasmas of distinct phytoplasma groups or subgroups, 20 EY group phytoplasmas and *Acholeplasma laidlawii* (as an out group) (**Fig. 4A**). The EY group phytoplasmas represented a distinct stain cluster (or subclade) in the phytoplasma clade.

Only one phylogenetic tree was deduced by analysis of partial rp gene operon (covering *s3* and *l22*) sequences. Phylogenetic relationships established among members of the EY group were generally in agreement with those deduced based on 16S rRNA gene sequence (**Fig. 4B**). Phylogeny based on rp gene reveled more phylogenetic divergence in the EY phytoplasma group. Twelve distinct phylogenetic lineages were resolved, which were consistent with designated rp subgroups based on RFLP analysis (**Table 1**)

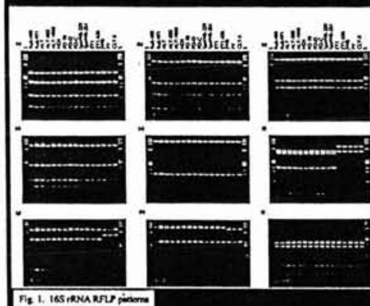


Fig. 1. 16S rRNA RFLP patterns

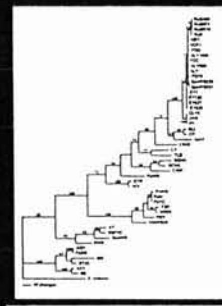


Fig. 4A. Phylogenetic tree based on 16S rRNA sequences

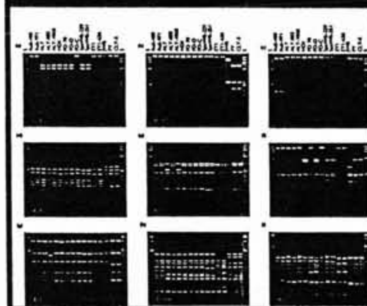


Fig. 2. Rp RFLP patterns

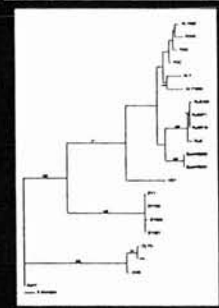


Fig. 4B. Phylogenetic tree based on rp and 16S sequences

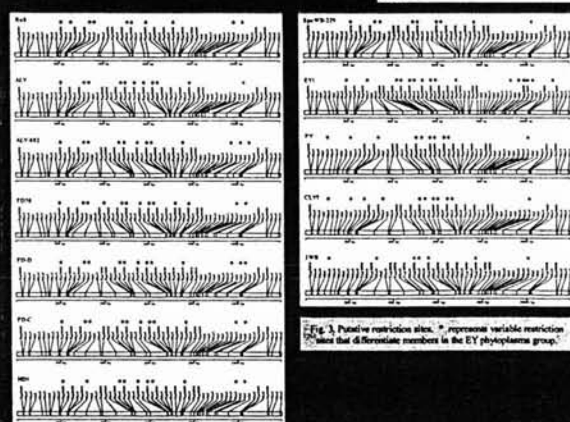


Fig. 3. Putative restriction sites. * represent variable restriction sites that differentiate members in the EY phytoplasma group.

Strain	Disease, host	Origin	RFLP classification subgroup		Reference
			16SrV	rpV	
[illegible]	Rubus stunt, *Rubus* [illegible]	Germany	E	[illegible]	Marcone et al. 1997
[illegible]	Rubus stunt, *Rubus* [illegible]	Italy	E	[illegible]	Marcone et al. 1997
[illegible]	Rubus stunt, *Rubus* [illegible]	Switzerland	E	[illegible]	Marcone et al. 1997
RUS	Rubus stunt, [illegible]	Italy	E	[illegible]	Marcone et al. 1997
ALY	Alder yellows, [illegible]	Italy	C	[illegible]	Marcone et al. 1997
[illegible]	Alder yellows, *Alnus glutinosa*	Germany	C	[illegible]	Marcone et al. 1997
[illegible]	Alder yellows, *Alnus glutinosa*	Italy	C	[illegible]	Marcone et al. 1997
HD1	Hemp dogbane yellows, *Apocynum cannabinum*	NY, U.S.A.	C	[illegible]	Griffiths et al. 1999
FD-[illegible]	Flavescence dorée, *Vitis vinifera*	France	C	[illegible]	[illegible] et al. 1997
FD-D	Flavescence dorée, *Vitis vinifera*	Italy	D	[illegible]	Martini et al. 1999
FD-C	Flavescence dorée, *Vitis vinifera*	Italy	C	[illegible]	Martini et al. 1999
[illegible]	Spartium witches'-broom, *Spartium junceum*	Italy	C	[illegible]	Marcone et al. 1997
[illegible]	Spartium witches'-broom, *Spartium junceum*	Italy	C	[illegible]	Marcone et al. 1997
EY1	Elm yellows, *Ulmus americana*	NY, USA	A	A	Lee et al. 1993
[illegible]	Elm yellows, *Ulmus* [illegible]	Italy	A	A	Marcone et al. 1997
[illegible]	Elm yellows, *Ulmus* [illegible]	Italy	A	A	Marcone et al. 1997
[illegible]	Elm yellows, *Ulmus* [illegible]	Italy	A	A	Marcone et al. 1997
JWB	Jujube witches'-broom, *Ziziphus jujuba*	China	B	[illegible]	[illegible] et al. 1996
PY	Peach yellows, *Prunus persica*	India	B	[illegible]	This paper
CLY-5	Cherry lethal yellows, *Prunus avium*	China	B	[illegible]	[illegible] et al. 1996

Table 1. Subgroups affiliation of representative strains in the EY phytoplasma group

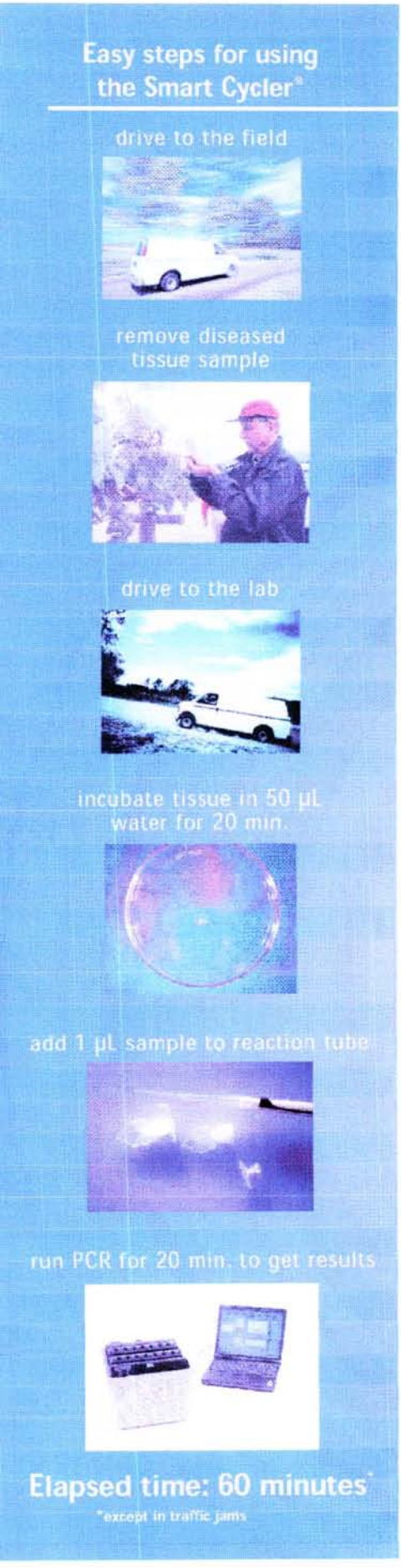
DISCUSSION

16S rRNA gene sequence was probably the most widely used for deducing phylogenetic relationships and molecular classification of microorganisms of interest. This approach has been very useful for classification of microorganisms at genus level or higher taxonomic ranks. Because of its highly conserved nature, however, 16S rRNA gene sequence is not readily useful for differentiation at species level. In the past decade, phytoplasmas have been classified primarily based on 16S rRNA gene sequence. More than twenty major phytoplasma groups and more than 40 subgroups have been identified. The differences in the 16S rDNA sequence homology between two groups are 2.5% or greater. There is a consensus that each group should represent at least one separate species.

According to the revised scheme, the elm yellows phytoplasma group (=16SrV group) was subdivided into five subgroups based on RFLP analysis of 16S rDNA sequences. The variations of 16S rDNA sequences between two subgroups range from 1.0 to 1.8%. The relatively small variations make it uncertain to justify whether the EY phytoplasma group should represent more than one species. But biological and ecological differences among these five subgroups strongly suggest that the EY group consists of highly heterogeneous phytoplasma strains.

In the present study, we have analyzed both 16Sr RNA gene and a rp gene operon sequences for strain differentiation in the EY phytoplasma group. Based on RFLP analysis of 16S rDNA sequences from representative phytoplasma strains, we have identified five 16SrV subgroups, confirming the previous reports based on RFLP or putative restriction site analysis of 16S rDNA. RFLP analysis of rp gene sequences (*l22* and *s3*) resolved 12 distinct subgroups in the EY phytoplasma group. A greater sequence variation (ranging from 1.1 to 6.9%) in rp gene operon was observed between two rp subgroups. The phylogenetic tree deduced by analysis of rp gene sequences resolved 12 distinct lineages consistent with rp subgroups defined by RFLP patterns. Eight rp subgroups identified in the present study were consistent with those identified by Martini et al. (in press).

In contrast to subgroups defined by 16S rDNA sequences, subgroups differentiated based on rp gene sequences appeared to be consistent with their specific ecological niches (specific host plants, vector, or geographical regions). Cross-infection of two or more plant species by members of a given rp subgroup seems rare. Phylogenetic analysis based on both 16S rRNA and rp gene sequences reveled that members of subgroup 16SrV-A represented a rather homogenous strain cluster, whereas, members of subgroup 16SrV-C were highly diverse. Members of Subgroup 16SrV-B that was subdivided into three rp subgroups represented a strain cluster most distantly related to the other members of the EY phytoplasma group. The EY group may represent more than one species.



5

On-site one hour PCR diagnosis of bacterial diseases

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Classical diagnosis of a bacterial disease normally requires 3–4 days to isolate and clone the suspect pathogen and another 5–7 days to confirm the organism's identity by pathogenicity tests. More rapid serological tests are available, but they often lack the sensitivity or result in false positives. Classical PCR tests are very sensitive but usually require 2–3 days. We describe a real-time TaqMan®-based PCR protocol adapted to a field-portable Smart Cycler® (Cepheid, Sunnyvale, CA) for rapid same-day diagnosis of watermelon fruit blotch (WFB), caused by *Acidovorax avenae* subsp. *citrulli*. Major advantages of the Smart Cycler® include portability and rapid cycling times. In direct comparisons with Applied Biosystems Prism™ 7700 Sequence Detection System, we found no differences in specificity and little in sensitivity. Both systems were able to detect 100 fg DNA. Using a Smart Cycler®, WFB can be diagnosed by direct PCR in less than 1 h after sampling using 1 µL of washings of diseased tissue soaked for 20 min in water in a microfuge tube.

Introduction

Plant disease diagnosis has improved greatly with the introduction of PCR (7). Classical PCR assays are available for several plant pathogens (3) and primers are available for the identification and detection of most plant pathogenic bacteria (9). Because classical PCR techniques require time consuming Southern blot hybridization for confirmation, field diagnosticians have shown little interest in using PCR for routine diagnosis. The introduction of real-time PCR, which is based on hybridization to a fluorescent probe sequence, has led to the development of protocols for detection of several plant pathogens (2, 6,10). Such techniques are more sensitive then classical PCR and greatly reduce the time needed for diagnosis since there is no need for agarose gels and Southerns. However, real-time PCR generally requires expensive lab-based equipment. In response to a disease quarantine issue involving shipment of perishable goods or the deliberate release of a pathogen, time becomes an issue of critical importance. To demonstrate the feasibility of rapid on-site diagnosis of a plant disease in one hour or less, we developed a real-time PCR protocol using the portable Smart Cycler® TD [Cepheid, Sunnyvale, CA (3)] for the destructive watermelon fruit blotch disease caused by the bacterium, *Acidovorax avenae* subsp. *citrulli*. Watermelon fruit blotch causes severe losses in watermelon production worldwide. Symptoms of the disease in seedlings grown under greenhouse or field conditions often consist of large black lesions without any water-soaking. This makes presumptive diagnosis based solely upon symptom etiology very difficult. Isolation of bacteria and pathogenicity tests are generally required for diagnosis but take 10–14 days.

Materials and Methods

PCR protocol. The *A. avenae* subsp. *citrulli* specific PCR primers, AacF3 and AacR2 and FAM-labeled probe AacP2 (8), were used in real-time PCR with the following cycling conditions with the Smart Cycler®: 95 °C for 30 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The assays were performed in a total volume of 25 µL containing the following reagents and volumes (µL): 10x PCR buffer (2.50), 25 mM $MgCl_2$ (5.00), 1.25 mM dNTPs (4.00), 25 µM primer AacF3 (1.00), 25 µM primer AacR2 (1.00), 10 µM probe AacP2 (1.00), 5U/µL AmpliTaq® DNA polymerase (0.25), 5x additive reagent containing BSA at 1 mg/mL, Trehalose at 750 nM, and Tween-20 at 1% v/v (Cepheid, 5.00); sterile MBG water (4.25), sample (1.00). The cycle threshold (Ct) values were determined and samples with a Ct value of 38 or less were considered positive. Ct value is defined as the PCR cycle number at which time the signal (fluorescence) of the probe rises above background. The earlier the Ct value the better the PCR performance.

Sensitivity of detection of pure cultures and extracted DNA. For pure cultures, an overnight suspension of *A. avenae* subsp. *citrulli* was adjusted to 0.1 at 600 nm and diluted to $1x10^{-8}$. One microliter aliquots of the 10^{-5} to 10^{-8} dilutions were run for 40 cycles in duplicate using an ABI 7700 and a Smart Cycler®. For viable cell counts in tissue samples, 100 µL of each dilution were plated onto each of five plates of YDC agar and incubated at 36 °C for 2 days. DNA was extracted from cells using standard phenol/chloroform methods (1) and quantitated using a SmartSpec® 3000 (BioRad, Oakland, CA).

Preparation of plant samples.

Watermelon seedlings at the 1st true leaf-stage were injected with a suspension containing 1 x 10^6 cfu/mL of *A. avenae* subsp. *citrulli* into the cotyledon and stem. After 7–10 days incubation in a lighted dew chamber, plants with brown-black lesions on the cotyledon (Fig. 1A) or brown linear lesions on the stem (Fig. 1B) were collected. Discs of tissue were removed from the edges of the cotyledon lesions and 2–5 mm tissue sections were cut from the upper and lower margins of the stem lesions (see arrow) with a scalpel. The tissue was soaked in 50–100 µL sterile water in a microfuge tube for 20 min at room temperature, and 1 µL was immediately used for PCR without DNA extraction.

Results

Sensitivity.

The sensitivity of the Cepheid Smart Cycler® was comparable to the ABI 7700®; both instruments were able to detect at least 100 fg and as few as 10,000 cells of *A. avenae* subsp. *citrulli* per mL (10 cells/µL) of tissue sample extract. Tissue samples containing a mean of 1.1 x 10^4 cfu/mL resulted in Ct values of 32.4 and 34.5 for the Smart Cycler® and 7700®, respectively. Viable cell counts of *A. avenae* subsp. *citrulli* in stem soakates ranged from 10^4 to 10^6cfu/mL.

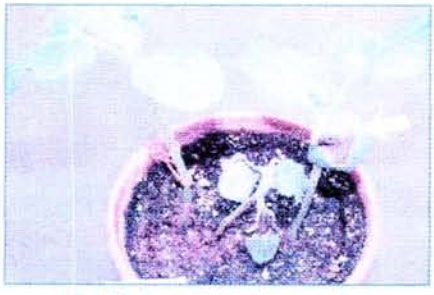

Fig 1A: lesions on the cotyledon

Fig 1B: lesions on the stem

Sampling and detection.

PCR tests were completed in less than 1 h after receiving infected samples. All stem and leaf samples tested were positive whereas all samples from non-inoculated control plants were negative. Typical Ct values ranged from 24.16 to 26.25 (Fig 2).

Fig. 2: Real-time diagnosis of WFB

Discussion

By using the fast cycling Smart Cycler® and sampling without extracting DNA, we were able to detect WFB in seedlings in less than one hour after obtaining samples. Such rapid diagnosis is critical to the successful development of a pest management control program aimed at early detection to prevent spread of the pathogen. The extremely fast cycling of the Smart Cycler® is due in part to the unique design of the PCR reaction tube and optical mechanism. No evidence of PCR inhibition was observed as a result of our sampling protocol. Unlike the protocol developed for rapid detection of *Neisseria gonorrhoeae* (5), our protocol for detection of the watermelon fruit blotch organism does not require extraction of DNA and is therefore much faster.

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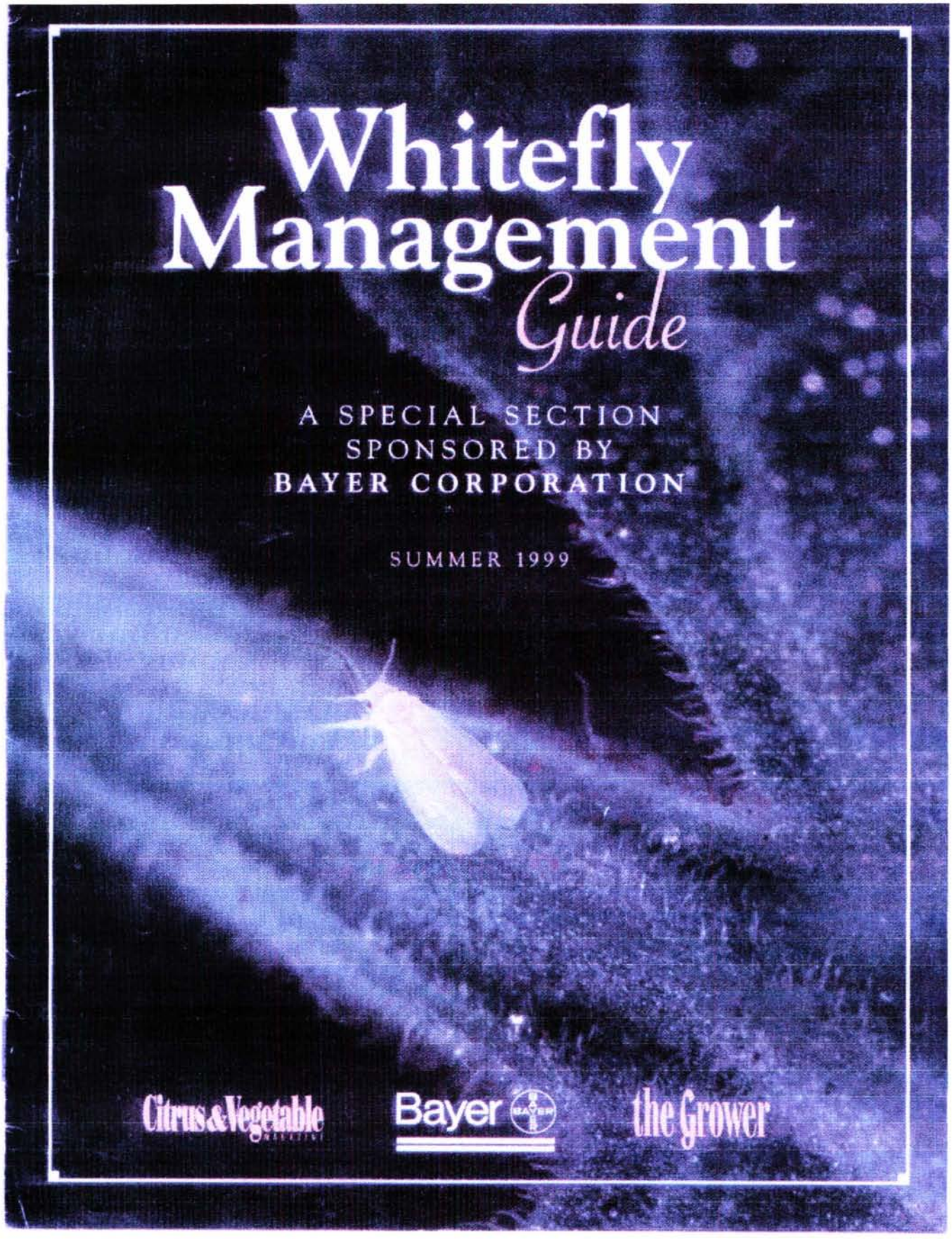
Summary

- Demonstrated for the first time diagnosis of a plant disease by PCR in less than 1 hour.
- Developed simple sampling procedure by soaking tissue 20 minutes in 50 µL water.
- Conducted PCR directly from diseased samples — no DNA extraction needed.
- High sensitivity observed: 100 fg DNA or 10 viable cells/µL were detected.
- The real-time PCR assay was completed in 20 minutes using the Smart Cycler®.
- Using a portable Smart Cycler®, PCR is performed easily in a greenhouse or field.


Whitefly
Management
Guide
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6.

CALIFORNIA

The following are management strategies recommended by the University of California for controlling whiteflies in California:

■ Promptly harvest all host crops and destroy residue immediately thereafter. Prevent regrowth after disking;

■ Control weeds in non-crop areas, including head-rows and fallow fields;

■ Monitor adjacent crops and weeds for increasing whitefly populations;

■ Growers should take an area-wide approach to controlling silverleaf whitefly. Growers should organize with their neighbors so they can have a combined management approach;

■ Get rid of alternative hosts and volunteers; and,

■ Be aware of a control program in your area. Work with your neighbors to create a cultural management program.

FLORIDA

The following are management strategies recommended by the University of Florida for controlling whiteflies in Florida:

■ Plant whitefly-free transplants;

■ Delay planting new crops as long as possible;

■ Do not plant new crops near or adjacent to infested weeds or crops (including tomato, cucurbits, cabbage and potato), abandoned fields awaiting destruction or areas with volunteer plants;

■ Use UV-reflective (aluminum) plastic soil mulch;

■ Control weeds on field edges if scouting indicates whiteflies are present and natural enemies are absent;

■ Manage weeds within crops to minimize interference with spraying;

■ Destroy old crops immediately after harvest and continue destruction of volunteer plants through the fallow period; and,

■ Avoid u-pick or pin-hooking operations unless effective control measures are continued.

Table of Contents

BENEFITS OF ADMIRE IN

Bayer Corporation introduced Admire® Insecticide to the vegetable industry in 1993 in an effort to control silverleaf whitefly, which had invaded Florida in the late 1980s and California in the early 1990s. The whitefly devastated vegetable production in Florida and southern California. Fortunately, treatments of Admire brought the pest under control, restoring crop production schedules and providing growers with higher marketable yields and better marketing opportunities.

To document those results, Bayer commissioned the University of California-Davis to conduct a two-year research study of the economic impact of the whitefly on the California vegetable market and determine how Admire usage had improved vegetable quality, marketability and yield since its introduction.

For the study, the researchers looked primarily at four vegetable crops: broccoli, cauliflower, head lettuce and fresh tomatoes. The following is a summary of the results of the UC-Davis research study showing the effect of the whitefly on each vegetable crop and the results of Admire usage.

BROCCOLI

Whitefly infestations reduce broccoli yields by decreasing the number of marketable crowns and crown size. The pest also delays plant maturity, which causes growers to miss targeted harvest dates. The UC-Davis study determined that treatments with Admire significantly reduced adult whitefly densities on broccoli throughout the season. In addition, as a result of whitefly control, Admire increased marketable yields by 2.11 percent per acre (Table 1) and increased the value of annual returns by 5.63 percent over the traditional foliar application regime (Table 2).

TABLE 1: ADMIRE VS. FOLIAR APPLICATIONS SUMMARY OF CHANGES (% per acre)

Crop	Yield (Y)	Timing (T)	Cost (C)	Y+T-C
Broccoli	2.11	5.63	-0.13	7.87
Cauliflower	1.92	6.78	0.08	8.62
Head Lettuce	3.88	3.62	0.07	7.43
Fresh Tomatoes	0.00	6.46	-0.65	7.11

Yield information is based on the amount of marketable yield improvement of crops treated with Admire compared to crops treated with traditional foliar insect management programs.

TABLE 2: CHANGES IN ANNUAL REVENUES RECEIVED DUE TO SHIFTS IN PLANTING DATES

Crop	Weighted Average Annual Price w/ Admire ($/cwt)	Weighted Average Annual Price w/out Admire ($/cwt)	Change (%)	Difference in Revenue Per Acre
Broccoli	26.18	24.70	5.63	+200.68
Cauliflower	30.45	28.38	6.78	+272.41
Head Lettuce	13.49	13.00	3.62	+119.56
Tomatoes (fresh)	29.13	27.24	6.46	+638.82

Producers averaged $308 more per acre after Admire was used on their broccoli, cauliflower, head lettuce and fresh tomato crops. Increased dollars were based on 1997 average yield statistics provided by the California Agricultural Statistics Service, Sacramento, as seen in Table 4.

TABLE 3: GROSS ANNUAL BENEFITS OF USING ADMIRE

Crop	y-c+t (%)	Gross Value of Production GVP ($)	Fraction Adopted (FA)	Total ($)
Broccoli	7.87	$32,675,850	0.687	1,766,344
Cauliflower	8.62	$18,251,775	0.557	876,049
Head Lettuce	8.48	$89,711,475	0.569	4,325,917
Tomatoes	7.11	$46,587,663	0.338	1,118,528
Total Benefits				8,086,838

CAULIFLOWER

The whitefly decreases the size and weight qualifications in cauliflower if no control is undertaken. The pest also can cause slow crop maturity and delay harvest, which causes growers to miss optimum market prices. By controlling whitefly populations, applications of Admire to cauliflower increased the number of marketable heads by 1.92 percent (Table 1), which resulted in a 6.8 percent change in the value of annual returns versus traditional insect controls (Table 2).

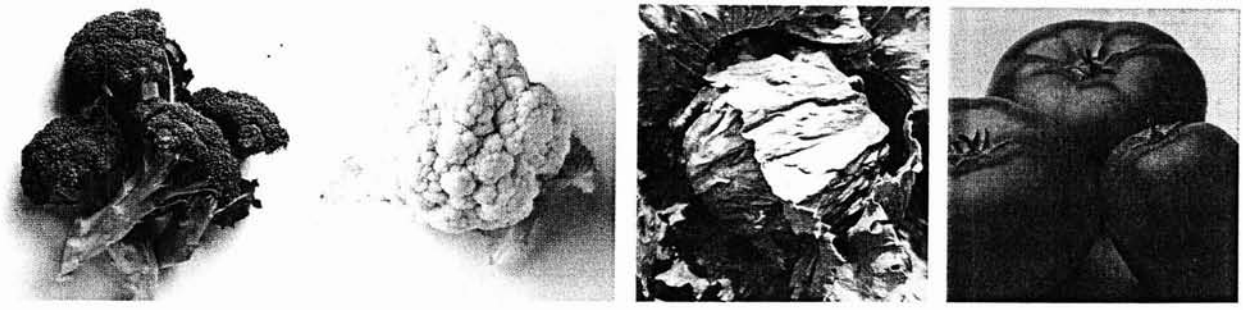
HEAD LETTUCE

In both leaf and head lettuce, whitefly causes color changes and severe weight reduction, which can significantly decrease marketable yields. In addition, feeding by whitefly nymphs also can increase the chance of chlorosis in lettuce. However, with the help of Admire, the head lettuce yield increased by 3.88 percent (Table 1) and the value of annual returns changed by 3.62 percent (Table 2).

FRESH TOMATOES

The whitefly affects tomato quality more than yield because the tomato plant is not a primary host of the whitefly. However, even low infestations can cause significant damage because when the tomatoes are gassed to induce ripening, the immature fruit becomes

VEGETABLE PRODUCTION



apparent. The whitefly also can transmit disease into tomato crops (as we have seen recently with the Tomato Yellow Leaf Curl Virus in Florida). With the treatment of Admire, the amount of damaged culls decreased and the tomato size, color and other marketable qualities increased, which allowed a 6.46 percent increase in the value of annual returns over traditional insect controls (Table 2).

A SHIFT IN PLANTING

Imperial County, Calif., the focal point of the study, is a semi-desert region. Growers in the region have capitalized on the climate, allowing them to produce and sell crops at times when competing produce is less available. These benefits have made this region one of high economic value and the largest center of vegetable production in the Southern California region.

Unfortunately, the silverleaf whitefly also was drawn to the hot climate and chose the county for its breeding ground. Growers were forced to shift their planting times to cooler parts of the year, missing key market periods. However, the study found in cases of crops treated with Admire that growers were able to partially shift their planting times back to the period when they could take advantage of seasonally higher market prices. This shift allowed their average annual returns to increase.

Admire usage also resulted in lower cultivation costs and reduced the need for foliar applications, which decreased overall production costs compared to alternative control programs. Because Admire is taken up by the plant's roots, it works systemically to reduce the need for foliar sprays. This reduction allowed growers to continue with their existing integrated pest management programs.

The increase of yields, timing and a decrease in application costs provided growers in the Imperial County with combined gross annual benefits of $8,086,838 in these four crops (Table 3).

WHAT ADMIRE MEANS FOR YOUR VEGETABLES

The introduction of Admire in 1993 into the vegetable market solved the immediate problem of whitefly infestation and provided a wealth of future benefits to growers and consumers. Growers once again were able to produce a high-yield crop and enjoy an increase in profit. Although this study was based on California crops, the evidence also can be applied to other U.S. vegetable-growing regions and pest-management programs. Admire provides powerful control of pests while maintaining beneficial levels. This season-long control also offers low worker exposures and low-mammalian toxicity rates, which results in high estimated margins of safety. The low application rates also result in reduced insecticide load on the environment. ■

TABLE 4: CHANGE IN VEGETABLE PRODUCTION 1992-1997

Imperial County

Crop	1992			1997			Increase 1992-1997	
	Ave. Yield Per Acre (Tons)	Ave. Price Per Unit ($)	Ave. Income Per Acre ($)	Ave. Yield Per Acre (Tons)	Ave. Price Per Unit ($)	Ave. Income Per Acre ($)	Ave. Yield Per Acre (%)	Ave. Income Per Acre ($)
Broccoli	5.01	378	1893.78	6.78	694	4705.32	26	2811.54
Cauliflower	4.37	512	2237.44	6.58	628	4132.24	34	1894.80
Head Lettuce	9.94	241	2395.54	12.2	306	3733.20	19	1337.66
Fresh Tomatoes	8.89	311	2764.79	16.9	459	7757.10	47	4992.31

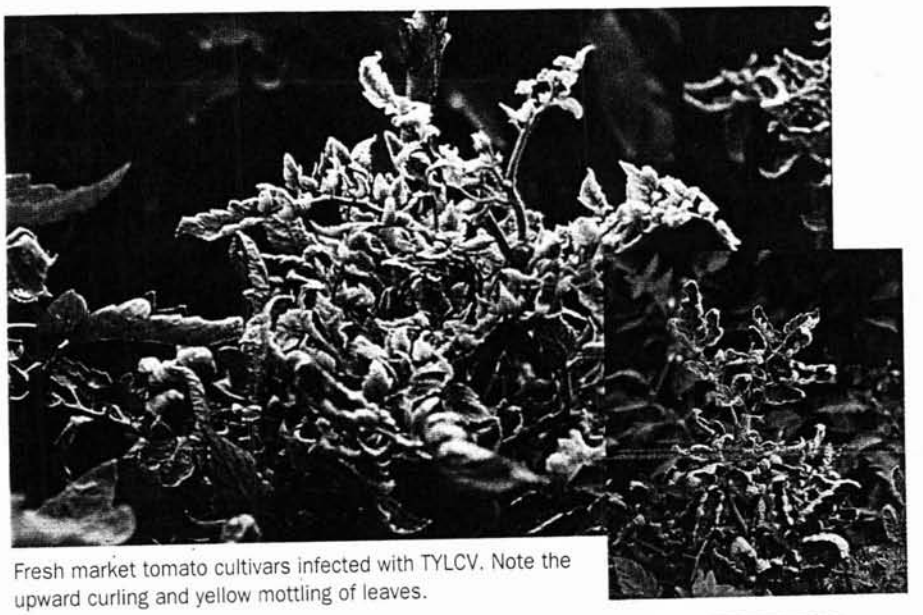
Source: California Agricultural Statistics Service, Sacramento

The chart indicates that since the silverleaf whitefly infestation of 1992, producers have increased their vegetable yields and profits substantially over the five-year period of 1992-1997. Growers experienced an average yield increase of 32 percent and an average income per acre increase of $2,759.08.

WHITEFLY MANAGEMENT GUIDE: TOMATO YELLOW LEAF CURL VIRUS

By David Schuster and Jane Polston

Tomato yellow leaf curl virus (TYLCV) is a whitefly-transmitted geminivirus which is native to the eastern Mediterranean. It found its way to the eastern Caribbean in the early 1990s and was identified in Florida for the first time in July 1997. TYLCV has been found in all tomato production regions of Florida but is of greatest concern in southern Florida. The virus is difficult to contain, and management of whitefly populations at both the beginning and end of a season is critical to maintaining low incidences of infected plants. TYLCV can infect other species of crop plants and weeds, but at this time, it is of concern in tomato production.



Fresh market tomato cultivars infected with TYLCV. Note the upward curling and yellow mottling of leaves.

RECOGNITION OF TYLCV

Two to three weeks after inoculation, mottling and leaf distortion can be seen on the newest leaves. At this stage, TYLCV symptoms resemble those of tomato mottle virus. However, each leaf produced after that will show increasingly severe symptoms which may include markedly reduced size, upward cupping, mottling and chlorotic margins. There is some variation among different cultivars. TYLCV-infected plants will be much shorter than non-infected plants. Infections, which begin when plants are young, will show the most severe symptoms. TYLCV causes flowers to drop prematurely, resulting in fewer fruit. Fruit present on the plant at the time of infection are not noticeably affected, but the number of fruit produced after infection can be reduced by as much as 90 percent. Infections early in the season have a greater impact on yields than later infections.

TRANSMISSION OF TYLCV

TYLCV is transmitted by the whitefly, *Bemisia tabaci* (aka *B. argentifolii*, the silverleaf whitefly), and is not transmitted through seed or mechanical transmission. TYLCV can be acquired by immature whiteflies, but only adults can transmit the virus. The minimum time required for the adult whitefly to acquire the virus is 15 to 30 minutes, and longer feeding periods result in higher transmission rates. Similar time periods are required to transmit the virus. The adults can transmit the virus for most of their life, which can be as long as several weeks. The life cycle from egg to adult is about two to four weeks long, depending on temperatures. Adults can live days or weeks longer under mild winter conditions than they can in the summer.

The movement of TYLCV into a field from a source outside the field is known as primary spread, while movement of virus from plant to plant within a field is known as secondary spread. Interference with these two types of spread often requires different strategies.

MANAGEMENT OF PRIMARY SPREAD OF TYLCV

1) REMOVE SOURCES OF WHITEFLIES AND TYLCV. At this time, infected tomato plants are the primary source of infection. Therefore, new crops

Adult *Bemisia argentifolii*, the silverleaf whitefly

should not be planted near or adjacent to previously infested crops, abandoned fields awaiting destruction or areas with volunteer plants. Crops should be destroyed as soon as harvest is completed and destruction continued through the fallow period. Spraying old fields with a desiccant herbicide combined with sufficient oil to rapidly kill plants and whiteflies, followed as soon as possible with burning of the plants, will prevent whiteflies from migrating from old plants to new fields, weeds and nearby backyards. U-pick operations should be avoided unless effective control measures are maintained. Spring crops should be destroyed as early as possible and fall crops planted as late as possible to create a tomato-free summer period.

2) USE VIRUS-FREE TRANSPLANTS. Where feasible, transplant production facilities should be located away from field production areas and facilities should be covered with fine mesh screening to exclude whitefly adults. Admire should be applied in the greenhouse at 0.5 fl oz per 5,000 plants at least 7 days before shipping.

3) REFLECTIVE MULCHES. The use of aluminum (UV-reflective) polyethylene film as a soil mulch will disorient whitefly adults, reducing the number alighting on plants and thereby reduce the number of infected plants. This plastic is available from numerous sources, but may require a special order.

4) INSECTICIDAL CONTROL. Admire should be applied in the setting water at transplanting. Combinations of pyrethroids with organophosphates, carbamates and Thiodan; Agri-Mek combined with oil; and soap combined with pyrethroids, Lorsban or Thiodan may be applied if the number of immigrating whitefly adults is high. Do not use Provado if plants have been treated with Admire.

5) REPELLENTS. Growers may want to consider the use of low rates of crop oil (0.25 - 0.50 percent) that are repellent and may reduce whitefly feeding and virus transmission.

MANAGEMENT OF SECONDARY SPREAD OF TYLCV

1) ROGUING. Remove infected plants from the field at the first sign of TYLCV-like symptoms. After first tie, infected plants can be removed as sources of virus by killing them (for example, a mixture of diquat (2 qts/100 gals) plus 2 percent oil). Once the population of infected plants reaches 3 percent, roguing may no longer be cost effective.

2) INSECTICIDAL CONTROL. When the efficacy of the Admire begins to decline, the same insecticides for managing primary spread may be used. In addition, the insect growth regulators (IGRs), Knack and Applaud, may be applied when nymphal densities exceed 5 per 10 leaflets (terminal leaflet of the 7th - 8th leaf from the top of 10 plants/2 acres). These products interfere with normal development of immature whiteflies, including eggs, thereby restricting population growth and reducing secondary spread. Because of the effect of IGR's on insect growth and development, do not expect immediate mortality of immature whiteflies. Sprays of Provado should not be used after Admire.

3) REPELLENTS. The use of crop oil also can be used to interfere with secondary virus spread.

The goal is to keep TYLCV incidence and whitefly numbers low, without resorting to the old calendar method of insect control, as this will only encourage the development of other problems such as leafminers and tomato pinworms. This is a time when an IPM scout will be more beneficial than ever in keeping track of whitefly numbers and optimizing the efficacy of your control measures. ■

David Schuster, professor, entomology and Jane Polston, associate professor, virology and epidemiology, University of Florida's Gulf Coast Research and Education Center, Bradenton.

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10. Aspectos Administrativos

10.1. Organización previa a la actividad de formación

a. Conformación del grupo

____ muy dificultosa ____ sin problemas _X___ algunas dificultades

(Indicar los motivos en caso de dificultades)
Inicialmente esta actividad contemplaba la participación de dos miembros del Laboratorio de Biotecnología Vegetal del INIA-CRI La Platina. Problemas personales marginaron a uno de éstos, lo que se tradujo en alteraciones de los planes originales.

b. Apoyo de la Entidad Responsable

__X__ bueno ____ regular ____ malo

(Justificar)
INIA se manifestó favorablemente a la participación de sus investigadores en esta actividad, lo que se tradujo en apoyo económico para financiar aquellos items no cubiertos por FIA.

c. Información recibida durante la actividad de formación

__X__ amplia y detallada ____ aceptable ____ deficiente

d. Trámites de viaje (visa, pasajes, otros)

____ bueno ___X_ regular ____ malo

Los trámites de visa y recibo de pasajes se efectuaron sin contratiempo. El problema surgió a la llegada al evento, ya que inicialmente se acordó compartir una habitación con una estudiante de la Universidad de Texas A.M, razón por la cual se estimaron los gastos de alojamiento en base habitación doble. Desafortunadamente, esta persona desistió de asistir a este congreso dos días antes del inicio del mismo, debido a problemas personales. Ante la imposibilidad de contactar a alguien para compartir los gastos, se utilizó la reservación del hotel de todas formas. Afortunadamente, se recibió una invitación por parte del Dpto de Fitopatología de la UF para utilizar una de las habitaciones reservadas para las estudiantes de la Universidad de Florida, lugar donde la asistente a esta actividad recientemente egresó, hecho que permitió mantener los gastos de la actividad dentro del rango planificado.

e. Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados)
Las recomendaciones son bastante obvias y simples. Organizar las actividades con tiempo y ser realistas en los objetivos planteados (así como los lugares que se planifica visitar). La participación de varias personas en la actividad facilita la organización de

actividades de difusión e informes que siguen a la ejecución de estas actividades, pero puede dificultar la organización previa.
Finalmente creo que el mayor stress de la actividad estuvo en la pérdida de la posibilidad de compartir el hotel en base habitación doble, lo que aumentaría considerablemente el costo de la actividad, hecho que ocurrió lamentablemente casi al momento de la partida. Entonces, mi sugerencia es realizar los cálculos de gastos sólo en función de los participantes locales, y no considerar situaciones como las de compartir gastos con terceros, quienes no aseguran su llagada al evento.

10.2. Organización durante la actividad (indicar con cruces)

Ítem	Bueno	Regular	Malo
Recepción en país o región de destino	X		
Transporte aeropuerto/hotel y viceversa		X	
Reserva en hoteles		X	
Cumplimiento del programa y horarios	X		

En caso de existir un ítem Malo o Regular, señalar los problemas enfrentados durante el desarrollo de la actividad de formación, la forma como fueron abordados y las sugerencias que puedan aportar a mejorar los aspectos organizacionales de las actividades de formación a futuro.

A pesar del alto costo de inscripción que tuvo este Congreso la organización no contempló traslados desde el Aeropuerto al Centro de Convenciones u hoteles, lo que es usualmente cubierto en las reuniones regionales de esta Sociedad (por ejemplo, el año 2001 ante la participación en la Sociedad Fitopatológica del Caribe, todos los gastos de traslado estuvieron incluidos en la inscripción). Esto encareció el costo total de la actividad, ya que se debió recurrir al traslado en taxis desde y hacia el aeropuertos. Afortunadamente, la proximidad del hotel al Centro de Convenciones nos evitó requerir traslados diarios.
En cuanto al hotel, ya se detallaron las dificultades que se tuvieron en el punto 10.1.d.

11. Conclusiones Finales.

Esta actividad de formación permitió a la investigadora participante actualizarse de las tendencias de la investigación en el área de la fitopatología y biotecnología. También se pudo conocer y reencontrarse con investigadores de gran prestigio, algunos de los cuales posiblemente mantendrán ciertos niveles de cooperación con el grupo de Biotecnología del INIA en el futuro cercano. Estos resultados nos dejan plenamente conformes y optimistas de las posibilidades que se puedan desarrollar producto de esta actividad de formación, y esperamos prontamente explorar estas posibilidades.

Además creemos que es extremadamente importante el conocer el estado del arte en la investigación que cada grupo desarrolla. Ciertamente la lejanía de Chile se presenta como un factor adverso al momento de asistir a un Congreso Internacional, ya que viajar a Europa o Norteamérica usualmente representa un gasto difícil de abordar sólo con los dineros aportados

por las instituciones o proyectos de investigación. Es por esto que se agradece la colaboración, compromiso y entusiasmo que La Fundación para la Innovación Agraria presta a este tipo de actividades, lo que sin duda está reflejándose en la calidad y cantidad de proyectos de investigación y desarrollo que se ejecutan en el área silvoagropecuaria.

11. **Conclusiones Individuales**: anexar las conclusiones individuales de cada uno de los participantes de la actividad de formación, incluyendo el nivel de satisfacción de los objetivos personales (no más de 1 página y media por participante).

M. Rosales

Como única participante de esta actividad, quisiera expresar mi satisfacción por el desarrollo de la actividad. Los objetivos planteados en términos de capacitación e interacción se cumplieron a cabalidad, y posiblemente existan posibilidades claras de desarrollar nuevos proyectos producto de los contactos establecidos. Además, creo que la elección de este Congreso fue muy acertada, ya que la calidad de los trabajos presentados fue sobresaliente y existió un gran número de investigadores asistentes. Esto ilustra la diversidad de temas que fueron abordados, y de las grandes posibilidades de interacción que allí se pudieron desarrollar.

También creo importante resaltar el interés mostrado por el público a las actividades de difusión que se comprometieron en la propuesta, en especial estudiantes de pregrado, colegas investigadores fitopatólogos y algunos profesionales que se desempeñan en empresas privadas.

Existen pocas posibilidades de financiamiento a actividades de capacitación y formación como las planteadas en le presente proyecto. Agradezco el apoyo del FIA e INIA en esta actividad, y no dudo que los conocimientos adquiridos, y las interacciones establecidas en el marco de este proyecto se verán reflejados en los trabajos de investigación que emprenda en el futuro.

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Fecha: _____lunes, 21 de octubre de 2002_____

Nombre y Firma coordinador de la ejecución: _____

CENTRO REGIONAL DE INVESTIGACIONES
CRI
LA PLATINA
INIA

AÑO 2002