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Prueba temprana en líneas S_1 de maíz

Early testing of S_1 lines of maize

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Resumen. Se evaluaron 80 líneas de maíz (*Zea mays* L.) S1 derivadas de familias de medios hermanos de poblaciones nativas con buen potencial de rendimiento. El propósito fue seleccionar líneas sobresalientes; las poblaciones nativas son fuente de germoplasma para el mejoramiento genético por selección e hibridación. Se usó un diseño látice 9x9 con tres repeticiones en el campo experimental del Instituto Tecnológico de Roque en el ciclo agrícola P-V 2011. Los resultados del análisis de varianza mostraron diferencias altamente significativas entre las líneas S1 para las variables floración masculina (FM), floración femenina (FF), altura de planta (Apl), altura de mazorca (Amz), número de hojas por encima de mazorca (NHEmz), longitud de mazorca (Lmz), número de hileras (Nhil), granos por hilera (Ghil), total de granos por mazorca (TGmz) y rendimiento (Rend). Todas las variables mostraron gran variabilidad, debido al origen diverso del material evaluado, y la prueba de las líneas S1, que genera mucha variación entre ellas. La comparación de medias detectó siete grupos estadísticos para FF, con intervalo de 77 a 87 días; en FM se obtuvieron 14 grupos estadísticos, con un intervalo de 70 a 86 días. En la variable AMz se obtuvo una variación de 91,4 a 154,6 cm. En la variable longitud de espiga (Lesp), se obtuvo un intervalo de 29,4 a 50,6 cm. Para la variable Rend se identificó al genotipo siete con una producción de 14,65 t/ha, que fue estadísticamente superior al genotipo 81, el testigo H-85, con 12,38 t/ha. La prueba temprana permitió identificar líneas con buenas características agronómicas y alto potencial de rendimiento. Se le considera un método adecuado en un programa de mejoramiento en maíz para eliminar líneas con bajo potencial de rendimiento en una etapa temprana del desarrollo de líneas endogámicas.

Palabras clave: Características agronómicas; Selección; Endogamia; Rendimiento.

Abstract. Eighty S1 maize (*Zea mays* L.) lines derived from half-sib families with good yield potential (greater than landraces in the region) were evaluated, with the purpose of selecting outstanding lines. Native populations are a source of germplasm for genetic improvement through selection and hybridization. Analysis of variance showed highly significant differences between the lines S1 in the variables days to anthesis (FM), silking date (FF), plant height (Apl), ear height (Amz), leaves above ear (NHEmz), ear length (Lmz), row number (Nhil), kernels per row (Ghil), total kernels per ear (TGmz) and grain yield (Rend). All variables showed high variability, an expected result given the origin of the evaluated genotypes. Mean comparisons showed seven statistical groups for FF, with an interval of 77-87 days and 14 statistical groups for FM, with an interval of 70-86 days. For the variable AMz a variation of 91.4 to 154.6 cm was observed. In LESP, the length range was 29.4 to 50.6 cm. For the variable Rend, the genotype seven yielded of 14.65 t/ha, which was statistically superior to genotype 81, the control check, with 12.38 t/ha. Early testing identified inbred lines with good agronomic characteristics and high yield potential. It is considered as an appropriate method in a corn breeding program for eliminating lines with low yield potential at an early stage of development of inbred lines.

Keywords: Agronomic traits; Selection; Inbreeding; Yield.

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INTRODUCCIÓN

El cultivo del maíz en México es de gran importancia por ser un cereal básico en la dieta de la mayoría de la población. Se estima que aproximadamente el 55% de la producción se dedica al autoconsumo. El bajo rendimiento promedio a nivel nacional hace imperativo producir variedades de mayor producción. Uno de los objetivos en un programa de mejoramiento genético es la identificación de líneas superiores de alta aptitud combinatoria general para la formación de buenos híbridos o sintéticos. La evaluación de líneas mediante la prueba temprana permite identificar progenitores potenciales de alto rendimiento, descartando líneas de bajo potencial, ya que es costoso el llevarlas a todas hasta generaciones avanzadas de endogamia. Al respecto, para la formación de híbridos competitivos a nivel comercial, Mendes et al. (2008) señalan la necesidad de identificar líneas progenitoras sobresalientes en etapas tempranas. Esto es en base a sus efectos de aptitud combinatoria general y específica, su comportamiento *per se*, adaptación y producción de semilla (Bekavac et al., 2008).

La identificación y selección de líneas sobresalientes *per se* es una prueba que se podría usar en el mejoramiento genético para formar las posibles combinaciones a fin de aprovechar la heterosis en algunos cruzamientos. Por otra parte, la posibilidad de contar con genes que determinan caracteres cuantitativos deseables permite que estos se puedan concentrar mediante selección paulatina y recombinación para generar poblaciones superiores, con alto potencial de rendimiento (Herrera-Cabreraet al*.,* 2004). Con respecto al uso de la prueba temprana de líneas endogámicas, algunos autores consideran que éstas se pueden seleccionar por su rendimiento *per se*, puesto que éste está positivamente correlacionado con su aptitud combinatoria general (ACG), con valores frecuentemente superiores a 0,5 (González et al., 1990). En niveles avanzados de endogamia, la depresión genética puede dar bajas correlaciones para rendimiento, pero la ACG inicial de las líneas es heredable (Bekavac et al., 2008).

Existen otras pruebas, como la evaluación tardía, que consiste en seleccionar por caracteres agronómicos deseables durante la tercera o cuarta generación de autofecundación; posteriormente, las líneas seleccionadas son cruzadas con un probador para evaluar su capacidad de combinación (González et al*.,* 1990). Esta prueba es un método efectivo de selección. Sin embargo, implica gastos importantes de recursos económicos debido a que durante varios años de trabajo muchas líneas avanzadas son descartadas. El objetivo de este trabajo fue evaluar el potencial de rendimiento de líneas S_1 de maíz mediante la prueba temprana. Este método permite la identificación temprana de líneas de un mayor potencial productivo y la selección de genotipos sobresalientes, antes de producir generaciones avanzadas en cuanto a características agronómicas.

MATERIALES Y MÉTODOS

La investigación se desarrolló en el campo experimental del Instituto Tecnológico de Roque (20° 34´ 54,24" N, 100° 49´ 35,34" O, 1767 msnm, INEGI, 2012).

La población original provino de un compuesto de materiales colectados en los estados de Morelos, Michoacán y Puebla. De ellos se derivaron familias de medios hermanos y se eligieron 169 como las más sobresalientes; de éstas, se seleccionaron 80 líneas S_1 que tenían la suficiente semilla para la evaluación La siembra se realizó en forma manual durante el ciclo agrícola P-V 2011, se depositaron 2 semillas por golpe a una profundidad de 7-10 cm; 15 días después se realizó un raleo para dejar una planta por mata. Se aplicaron cuatro riegos; la precipitación pluvial fue de 333,2 mm en el periodo de junio-agosto. La fertilización utilizada fue 180-60-00, recomendada por el Instituto Nacional de Investigaciones Agrícolas y Pecuarias (INIFAP); se aplicó la mitad del nitrógeno y todo el fósforo a la siembra; la segunda aplicación del nitrógeno se dio a los 45 días. El control de malezas se realizó en forma manual 15-45 días después de la siembra; también se hizo una aplicación con herbicida selectivo Sansón® (Nicosulfuron:2-4,6-dimetoxipirimidin-2-ilcarbomoil-sulfamoil-N,N-dimetilnicotinamida) en dosis única de 1,5 L/ha. Las plagas que se presentaron fueron: trips (*Frankliniella occidentalis*), que se controlaron con Cipermetrína® [(±) alfa ciano-3-fenoxifenil (±) (Cis/trans 3-2,2-dicloroetenil) – 2,2-dimetil ciclopropano carboxilato] en dosis única de 0,5 L/ha; y gusano cogollero (*Spodoptera frugiperda*), controlado mediante una aplicación de Carbofuran en dosis de 20 kg/ha en forma granulada cuando se notaron daños iniciales en el ápice de la planta.

Se utilizó un diseño experimental en látice triple 9 x 9 con 80 líneas endogámicas S_1 más un testigo (H-85); la parcela estuvo constituida por un surco de 5 m de longitud; se sembraron 30 matas por surco, con dos semillas por golpe; la distancia entre plantas fue de 16 cm y la distancia entre surcos fue de 80 cm; la densidad de población fue de aproximadamente 78000 plantas/ha. La cosecha se realizó en forma manual una vez que el grano alcanzó una humedad del 15-16%. Por cada unidad experimental se eligieron seis mazorcas en forma aleatoria, de plantas con competencia completa. Se realizó análisis de varianza para determinar diferencias estadísticas entre los tratamientos, con el programa SAS 9.0; para la comparación de medias se utilizó la prueba de DMS (diferencia mínima significativa) con $P<0,05$.

Variables agronómicas evaluadas. Floración masculina (FM), cuando el 50% más uno de las plantas estaba en antesis. Floración femenina (FF), cuando el 50% más uno de las plantas había emitido estigmas. Altura de planta (Apl), se tomó desde la base de la planta hasta el inicio de la base de la espiga en seis plantas, tomadas al azar, con competencia completa. Altura de

mazorca (Amz), se evaluó en las mismas seis plantas de la variable anterior, desde la base de la planta hasta el nudo donde se insertó la mazorca principal. Número de hojas por arriba de la mazorca (NHEMz): se tomó el promedio de hojas de las mismas seis plantas de la muestra anterior. La evaluación de estas variables se determinó tres semanas después de la floración. Número total de hojas (NH) se consideraron seis plantas por unidad experimental, desde el inicio del tallo hasta la hoja bandera. Longitud de hoja (LH), se midió desde la base de la hoja hasta el ápice de la misma. Ancho de hoja (AH), se tomó la primer hoja arriba de la mazorca de cada planta seleccionada al azar; se midió la parte de mayor anchura en cada hoja evaluada. Longitud de espiga (Lesp), se tomó desde el nudo de inserción hasta el ápice; se midió en las mismas seis plantas de la muestra anterior. Número de ramas de la espiga (Nesp), se tomaron datos de seis plantas y se contaron las ramas de las espigas, excepto la espiga central. Número de mazorcas por planta (NMz) de las seis plantas muestreadas de cada parcela útil. Longitud de mazorca (LgMz), se midió desde la base de la mazorca hasta el ápice de la misma. Número de hileras por mazorca (Nhil), se contó el número de hileras de seis mazorcas elegidas al azar, provenientes de plantas con competencia completa. Granos por hilera (NhilM), se evaluaron las seis mazorcas de cada parcela útil, contando el número de granos de dos hileras por mazorca; se tomó el promedio de ellas. Total de granos por mazorca (TGM), se contó el total de granos de cada mazorca y multiplicando el número de hileras por el número de granos por hilera. Se reportó el promedio. Humedad de grano (HG), se cuantificó tomando una muestra de 250g de las seis mazorcas evaluadas; se utilizó un determinador de humedad portátil marca Dickey-John®. Peso de mil granos en gramos (Pmg), se tomó el peso de seis repeticiones. Rendimiento (Rend) fue el rendimiento obtenido de la parcela útil y se ajustó al 14% de humedad; se realizó el cálculo para transformarlo a rendimiento en t/ha.

RESULTADOS

Los análisis de varianza para las variablesdías a antesis (FM), días a floración femenina (FF), altura de planta (Apl), longitud de mazorca (LgMz), número de hileras por mazorca (Nhil), número de granos por hilera (Nghil), total de granos por mazorca (TGM) y rendimiento (Rend) mostraron diferencias altamente significativas entre líneas. Esto indica que los genotipos evaluados presentaron mucha variación, lo que sugiere que al menos una línea fue diferente al resto en cada una de las variables citadas arriba (Tablas 1-4).

Longitud de hoja (LH), longitud de espiga (Lesp) y número de espiguillas por espiga (Nesp): el análisis de varianza mostró diferencias significativas entre los genotipos evaluados (Tabla 2). El coeficiente de variación para ancho de hoja (AH) fue alto y no se detectaron diferencias significativas entre líneas. Lo mismo se observó para número de espiguillas por espiga; son variables muy afectadas por el ambiente (Tabla 2).

Número de mazorcas por planta (NMz): el análisis de varianza para esta variable no mostró diferencias significativas entre líneas (Tabla 3).

Comparación de medias. La prueba de comparación de medias se realizó para las variables que mostraron significancia estadística entre genotipos en los análisis de varianza. En la prueba de comparación de medias (DMS) para la variable rendimiento, se detectaron cinco grupos de significancia estadística. Esto pone de manifiesto la gran variabilidad en la respuesta de estas líneas. Como se esperaba, en esta primera generación de autofecundación se expresa mucha heterogeneidad, la cual se reduce con el avance en el proceso endogámico y la reducción del grado de heterocigosis. En el primer grupo se observó mucha variación (11,77-14, 18 t/ha) pero fueron estadísticamente iguales al testigo, que tuvo buena productividad (Tabla 5).

Tabla 1. Grados de libertad y cuadrados medios del análisis de varianza de variables agronómicas evaluadas en la prueba temprana en líneas S_1 de maíz.
Table 1. Degree of freedom and mean squares of the analysis of variance of agronomic variables evaluated at early stages on lines $S_1$of corn.

	Cuadrados medios					
FV	**Gl**	**FM**	**FF**	**Apl**	**Amz**	**NHEMz**
Repeticiones	2	44,26	19,13	3124,73	135,2	0,25
Bloques/Rep	24	5,28	7,45	2383,76	225,23	0,46
Líneas	80	25,17**	20,80**	1244,89**	488,10**	0,92**
Error-Intrab	136	7,14	5,32	509,56	124,37	0,36
CV (%)		3,31	2,8	9,4	9,1	10,7

** indica significancia estadística al nivel 0,01 de probabilidad. FM: días a la floración masculina, FF: días a la floración femenina, Apl: altura de planta, Amz: altura de mazorca, NHEMz: número de hojas por arriba de la mazorca.
** statistical significance of the 0.01 level of probability. FM: days to male flowering; FF: days to female flowering; Apl: plant height; Amz: ear height; NHEMz: number of leaves above ear.

Tabla 2. Grados de libertad y cuadrados medios del análisis de varianza de variables agronómicas evaluadas en la prueba temprana en líneas S_1 de maíz.
Table 2. Degree of freedom and mean squares of the analysis of variance of agronomic variables evaluated at early stages on lines S_1 of corn.

	Cuadrados medios				
FV	**Gl**	**LH**	**AH**	**Lesp**	**Nesp**
Repetición	2	114,85	18,88	152,88	54,47
Bloque/Rep	24	160,69	7,58	18,40	90,20
Líneas	80	159,33*	6,99ns	61,02**	98,50*
Error-Intrab	136	90,32	6,26	23,98	51,52
CV (%)		9,5	25,8	12	36

*, ** indica significancia estadística al nivel 0,05 y 0,01 de probabilidad; respectivamente; ns: no significativo. LH: longitud de hoja, AH: ancho de hoja, Lesp: longitud de espiga, Nesp: número de espiguillas.
*, ** statistical significance at the 0.05 and 0.01 levels of probability, respectively; ns: not significant. LH: leaf length; AH: leaf with; Lesp: spike length; Nesp: number of spikelets.

Tabla 3. Cuadrados medios y grados de libertad del análisis de varianza de componentes de rendimiento en evaluación de la prueba temprana en líneas S_1 de maíz.
Table 3. Degree of freedom and mean squares of the analysis of variance for yield components when evaluating early stages on S_1 lines of corn.

	Cuadrados medios				
FV	**Gl**	**NMz**	**LgMz**	**DMz**	**Nhil**
Repetición	2	0,481	6,70	0,66	1,86
Bloques/Rep	24	0,240	1,99	0,42	1,86
Líneas	80	0,391ns	10,80**	0,71ns	5,47**
Error-Intrab	136	0,205	2,27	0,57	1,92
Total	242	0,273	5,09	0,60	3,09
CV (%)		29,1	9,0	16,1	9,9

** indica significancia estadística al nivel 0,01 de probabilidad; ns: no significativo. NMZ: número de mazorcas, LgMz: longitud de mazorca, DMz: diámetro de mazorca, Nhil: número de hileras por mazorca.
** statistical significance of the 0.01 level of probability; ns: not significant; NMZ: number of ears; LgMz: ear length; DMz: ear diameter; Nhil: number of rows per ear.

Tabla 4. Cuadrados medios y grados de libertad del análisis de varianza de componentes de rendimientos mediante la evaluación de la prueba temprana en líneas S_1 de maíz.
Table 4. Degree of freedom and mean squares of the analysis of variance for yield components when evaluating early stages on S_1 lines of corn.

	Cuadrados medios			
FV	**GL**	**Nghil**	**TGM**	**Rend.**
Repetición	2	73,67	23094	11,34
Bloques/Rep	24	23,96	6986,38	6,95
Líneas	80	47,75**	11589**	12,09**
Error-Intrab	136	13,46	2698,16	3,07
CV (%)		11,7	12	16,8

** indica significancia estadística al nivel 0,01 de probabilidad; ns: no significativo. Nghil: granos por hilera, TGM: total de granos por mazorca, Rend: Rendimiento.
** statistical significance at the 0.01 level of probability; ns: not significant. Nghil: number of grains per row; TGM: total number of grains per ear; Rend: yield.

Tabla 5. Comparación de medias mediante la prueba de DMS de variables agronómicas evaluadas mediante la prueba temprana en líneas S_1 de maíz.
Table 5. Mean comparisons using the LSD test for agronomic variables evaluated at early stages on S_1 lines of corn. See tables from 1 to 4 for abbreviations.

Línea	Rend (t/ha)		FF (d)		FM (d)		Amz (cm)		Apl (cm)	Trat	Nmz		Lh		Lesp		Nesp		NHEmz	
7	14,65	a	79	f-i	77	g-k	129,0	c-m	226,5 g-z	7	2,0	ab	101,1	b-y	41,6	c-z	22,0	c-m	5,6	b-e
24	14,18	ab	80	e-h	79	e-i	128,0	c-n	249,5 a-v	24	1,6	a-c	94,8	d-z	44,8	a-m	19,6	c-m	6,3	a-c
72	14,13	abc	78	g-i	77	g-k	110,8	l-y	234,6 c-z	72	1,0	c	101,5	a-y	47,4	a-d	22,0	c-m	6,0	a-d
79	14,09	a-d	80	e-h	80	d-h	119,9	e-u	237,9 c-z	79	1,6	a-c	107,5	a-k	40,2	d-z	23,6	c-j	6,3	a-c
8	13,77	a-e	81	d-g	81	c-g	141,6	ac	281,1 a	8	2,0	ab	100,5	a-y	42,0	c-y	19,0	c-m	6,3	a-c
13	13,55	a-f	80	e-h	79	d-i	134,2	b-h	263,6 a-i	13	1,6	a-c	101,3	a-y	41,3	c-z	22,3	c-m	6,0	a-d
9	13,38	a-g	82	c-f	83	b-e	154,6	a	266,3 a-e	9	1,6	a-c	113,5	a	50,1	ab	27,6	b-e	6,0	a-d
46	13,26	a-g	79	f-i	79	e-i	122,5	c-s	262,7 a-i	46	1,0	c	109,9	a-d	43,5	a-p	16,3	e-m	5,6	b-e
52	13,21	a-g	80	e-h	78	f-j	121,5	d-t	263,7 a-e	52	1,6	a-c	111,0	a-c	41,6	c-z	20,3	c-m	5,0	d-g
34	13,16	a-g	79	f-i	79	e-i	129,9	c-l	259,4 a-n	34	2,0	ab	96,5	c-z	49,1	a-c	16,3	e-m	6,0	a-d
6	12,94	a-g	82	c-f	80	d-h	120,5	d-u	258,7 a-ñ	6	1,6	a-c	109,0	a-h	41,7	c-z	20,6	c-m	6,0	a-d
57	12,52	a-g	80	e-h	79	e-i	124,6	c-p	241,3 c-z	57	1,3	bc	107,5	a-l	40,8	d-z	30,0	bc	6,0	a-d
29	12,42	a-g	79	f-i	77	g-k	126,6	c-ñ	227,7 e-z	29	1,3	bc	99,7	a-y	41,3	c-z	19,6	c-m	5,6	b-e
21	12,40	a-g	80	e-h	80	d-h	126,9	c-ñ	264,3 a-g	21	1,6	a-c	108,6	a-j	44,6	a-ñ	19,0	c-m	5,6	b-e
81	12,38	a-g	77	h-j	70	n	91,4	z	206,2 z	81	1,0	c	104,0	a-t	39,9	d-z	23,6	c-j	6,3	a-c
54	12,34	a-g	79	f-i	79	e-i	128,0	c-n	251,6 a-r	54	1,6	a-c	106,2	a-n	43,1	a-s	24,3	c-i	6,3	a-c
73	12,26	a-g	79	f-i	79	e-i	128,3	c-n	218,3 p-z	73	2,0	ab	98,0	b-z	30,1	z	22,3	c-m	6,0	a-d
71	12,24	a-g	81	d-g	81	c-g	131,2	c-k	234,2 c-z	71	1,3	bc	101,8	a-y	37,9	k-z	26,0	c-g	5,6	b-e
30	11,97	a-g	79	f-i	79	e-i	128,5	c-n	243,6 a-z	30	1,6	a-c	103,1	a-y	44,6	a-n	22,3	c-m	6,3	a-c
20	11,93	a-g	79	f-i	82	b-f	131,5	c-j	220,2 n-z	20	1,0	c	107,0	a-m	41,6	c-z	26,6	c-f	5,6	b-e
12	11,90	a-g	80	e-h	79	d-i	152,0	ab	267,2 a-d	12	1,6	a-c	105,7	a-o	46,4	a-h	21,3	c-m	5,3	c-f
38	11,82	a-g	79	f-i	77	g-k	118,1	f-w	238,8 c-z	38	1,3	bc	100,2	a-y	40,4	d-z	19,3	c-m	6,3	a-c
16	11,77	a-g	81	d-g	80	c-h	125,4	c-p	212,6 s-z	16	53	c	97,1	c-z	29,4	z	19,3	c-m	4,0	g

Medias o valores con la misma letra en cada variable son estadísticamente iguales (DMS, P<0.05).
Means followed by the same letter on each variable are statistically similar (DMS, P<0.05)

Para floración femenina se obtuvieron siete grupos estadísticos, y varió de 77 días en las más precoces a 87 días en las más tardías; para floración masculina se obtuvieron 14 grupos diferentes. En floración masculina se presentó mayor variabilidad, con un valor promedio de 70 días en las precoces y 86 días en las más tardías; esta mayor variabilidad en la floración también se debe a la naturaleza alógama de la especie para asegurar la polinización.

Las variables altura de mazorca y de planta mostraron mucha variabilidad entre líneas, por lo que se puede seleccionar para menor altura de planta y de mazorca (Tabla 5).

En la prueba de comparación de medias (DMS) para la variable número de mazorcas (NMz) se obtuvieron tres grupos de significancia estadística, de los cuales sobresalieron aquellos genotipos que tuvieron dos mazorcas por planta, como los genotipos 7, 8 y 34, que corresponden a los más rendidores. Se obtuvieron 23 genotipos con mayor número de mazorcas por planta comparados con el testigo. Para la variable longitud de hoja se obtuvieron 25 genotipos con mayor longitud de hoja, comparada con el testigo; para las variables longitud de espiga y número de espiguillas, se presentó una amplia variabilidad genética y se observó una gran cantidad de grupos estadísticos (Tabla 5 continuación); nuevamente se manifiesta la gran variabilidad entre líneas S_1. Esto permite eliminar gran cantidad de líneas en esta fase, que no tienen buenas características agronómicas. La longitud de mazorca, número de hileras y de granos por hilera presentaron amplia variabilidad; se obtuvieron 15 líneas con longitud de mazorca similar al testigo; en número de hileras se cuantificaron 11 grupos de significancia estadística. Para el número de granos por hilera se formaron 12 grupos estadísticos, que comprendió desde 28.3 hasta 40 granos por hilera (Tabla 6).

En granos por mazorca se presentó también amplia variabilidad, con valores de 317,2 hasta 572,2. El análisis de estas variables indica la gran variabilidad entre líneas S_1. Este resultado esperado indica que la selección puede continuarse positivamente (Bekavac et al., 2008), debido a la alta variabilidad y heredabilidad en estas variables.

Tabla 6. Comparación de medias mediante la prueba de DMS de los componentes de rendimiento evaluados mediante la prueba temprana en líneas S_1 de maíz.
Table 6. Mean comparisons by the LSD test for the yield components evaluated at early stages on S_1 lines of corn

Trat	Lgmz (cm)		Nhil (No)		Ghil (No)		Tgm (No)	
7	17,6	b-h	14,3	c-h	37,3	ab	519,1	a-i
24	19,0	a-d	14,6	c-g	33,0	b-g	488,5	a-m
72	19,6	ab	14,3	c-h	31,6	b-h	460,6	c-y
79	18,0	a-g	14,6	c-g	37,6	ab	572,6	a
8	19,3	a-c	14,3	c-h	40,0	a	542,1	a-c
13	17,6	b-h	14,6	c-g	38,0	ab	550,1	ab
9	19,3	a-c	14,6	cg	36,3	ad	525,1	a-h
46	17,6	b-h	14,3	ch	32,6	bg	461,5	c-x
52	19,3	a-c	12,3	hk	35,3	ae	436,6	i-y
34	19,0	a-d	12,6	gk	36,3	ad	462,3	c-w
6	17,6	b-h	12,6	gk	36,0	ad	450,9	e-y
57	17,0	c-j	13,6	di	39,6	a	528,4	a-e
29	17,0	c-j	15,6	ad	34,6	ae	527,2	a-f
21	19,0	a-d	18,6	a	34,0	bf	450,0	e-y
81	19,6	ab	13,6	di	33,0	bf	453,3	d-y
54	18,6	a-e	12,6	gk	34,0	bf	456,9	c-y
73	17,6	b-h	15	cf	32,3	bh	476,0	b-q
71	18,3	a-f	13	fk	35,3	ae	480,7	b-o
30	17,3	b-i	14,3	ch	32,3	bh	465,2	b-u
20	17,6	b-h	14,6	cg	36,3	ad	538,8	a-d
12	19,3	a-c	13,3	ej	37,0	ac	481,4	b-ñ
38	16,0	f-m	13,6	di	32,0	bh	448,5	e-y
16	16,3	e-l	13,6	di	32,6	bg	428,0	j-y

Medias o valores con la misma letra en cada variable son estadísticamente iguales (DMS, P<0,05).
Means followed by the same letter on each variable are statistically similar (LSD, P<0.05)

DISCUSIÓN

Los resultados de Días a antesis (FM) y Días a floración femenina (FF) concuerdan con los obtenidos por Reyes et al. (2004). Estos autores mencionan que en la raza Tuxpeño las diferencias en días a floración son atribuidas a la diversidad genética de los materiales. Chávez (1993) también confirmó que la selección aprovecha esta variabilidad presente en las poblaciones. En las poblaciones estudiadas por Bekavac et al. (2008), esta característica presentó la variabilidad genética más alta; los resultados obtenidos en esta investigación en ambas variables de días a floración muestra que las líneas presentan diversidad genética; esto permite considerar que el empleo de la prueba temprana en líneas S_1 es adecuada para identificar y seleccionar genotipos superiores. Las grandes diferencias en el tiempo de floración entre líneas endogámicas son efectos acumulativos de muchos loci (Kliebenstein, 2010).

Los resultados de altura de planta coinciden con Vega et al. (1998), quienes encontraron resultados prometedores en su evaluación de líneas S_1 en prueba temprana, permitiéndoles identificar líneas con buena altura de planta en un tiempo corto. Resultados similares también fueron obtenidos por De la Cruz-Lázaro et al. (2009) y Arellano et al. (2003), que al evaluar genotipos, lograron identificar a los sobresalientes en la variable altura de planta. Además, Antuna et al. (2003) señalan que el mejoramiento a partir de familias de medios hermanos o hermanos completos sirve para mantener la altura de mazorca deseable. De acuerdo con Bekavac et al. (2008), ésta es también una manera de avanzar más rápido en el proceso de selección. Los resultados de esta investigación mostraron que los genotipos evaluados presentaron mucha variabilidad, tanto en altura de planta como en altura de mazorca (Tabla 1). Los coeficientes de variación fueron buenos, lo que indica que los resultados son confiables y que la conducción del experimento fue adecuada.

El resultado de la longitud de mazorca concuerda con Mendes et al. (2008), quienes realizaron trabajos de selección recurrente en maíz en progenies S_2 y encontraron que la longitud media de la mazorca fue mayor que en la población original. No se encontraron diferencias significativas para la variable diámetro de mazorca (Tabla 3), debido posiblemente a que esta variable presenta poca variabilidad en la población original. Nuestro resultado en el número de granos por hilera fueron resultados similares a los obtenidos por Mendes et al. (2008). Estos investigadores encontraron que el número de granos por hilera en progenies S_2 fue mayor al de la población original.

Se sabe que a medida que aumenta el grado de endogamia, las líneas van siendo más homogéneas pero la variabilidad entre ellas aumenta. La gran variabilidad observada en el total de granos por mazorca es importante para el proceso de selección de líneas superiores; ésta es una variable de gran utilidad para el mejoramiento pues es una componente importante del rendimiento (Alvi et al., 2003) (Tabla 4). Las líneas S_1 mostraron amplia variabilidad en rendimiento de grano, resultado esperado como en las demás variables. Al respecto, Mendes et al. (2008) señalan que en los primeros ciclos de selección es posible obtener una ganancia en el rendimiento pero a medida que avanza el proceso endogámico, este tiende a disminuir. Tanner y Smith (1987) obtuvieron resultados similares al evaluar ciclos de selección temprana en familias de medios hermanos. Moll y Smith (1981) evaluaron dos métodos de selección, el de medios hermanos y el de progenies S_1. Después de cinco ciclos de selección, estos autores determinaron que el rendimiento (1) de medios hermanos fue mayor a las poblaciones

adaptadas localmente, y (2) de las progenies S_1 fue 50% mayor al método de medios hermanos, aunque la producción de las líneas S_1 presentó amplia variabilidad. Sierra et al. (2000) obtuvieron líneas tropicales de maíz con buena aptitud combinatoria general y específica mediante un probador, y una buena expectativa de uso por las ventajas que ofrecen desde el punto de vista de la producción de semilla. San Vicente y Hallauer (1993) evaluaron la tasa de depresión endogámica en dos grupos S_1, tanto en líneas generadas antes de 1960 como en otras de después de 1970; en ambas encontraron que en las primeras generaciones la depresión por endogamia es baja.

Las líneas S_1 evaluadas en esta investigación provinieron de diferentes fuentes de familias de medios hermanos. Esquivel et al. (2009) indican que la evaluación de una gran diversidad de germoplasma, aunque es un trabajo arduo, puede redituar importantes resultados. Estos incluyen (1) la identificación de genotipos sobresalientes, (2) la elección de combinaciones que permitan explotar la heterosis, y (3) la posibilidad de contar con genes que determinan caracteres cuantitativos deseables, y que mediante recombinación y selección paulatina pueden concentrarse para generar poblaciones superiores.

CONCLUSIONES

La evaluación de la prueba temprana permitió identificar líneas S_1 prometedoras con buenos rendimientos en comparación con el testigo.

Las características agronómicas de algunas líneas S_1 mostraron potencial para seleccionar genotipos apropiados como progenitores en híbridos.

La prueba temprana es un método apropiado para eliminar líneas con bajo potencial de rendimiento y características agronómicas no deseables, a fin de reducir el número de líneas que se llevan a generaciones avanzadas en un programa de mejoramiento.

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Bacterial diversity in the rhizosphere of a transgenic *versus* a conventional maize (*Zea mays*)

Diversidad bacteriana en la rizosfera de un maíz (*Zea mays*) transgénico, versus otro convencional

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Abstract. Genetically modified crops could cause negative effects on bacterial communities. In this study, we compared the bacterial community structure of two maize cultivars to determine whether the transgenic cultivar exerts a negative effect on bacterial communities inhabiting the rhizosphere. Cultivars included the genetically modified maize (*Zea mays*), with the *pat*-gene conferring resistance to the herbicide glufosinate (synonym: L-phosphinothricin), and the hybrid, conventional maize. Metagenomic DNA was extracted from the rhizosphere of plants grown in a greenhouse. Single-strand conformation polymorphism, based on polymerase chain reaction amplifying a partial subunit rRNA gene was used to characterize and generate genetic profiles that corresponded to the bacterial communities of the amplified products from the rhizosphere of the two maize cultivars. Genetic profiles of the rhizospheres consisted of distinguishable profiles, based on the chosen primer pairs. Similarity analyses of patterns found by binary matrix analyses showed no differences in the bacterial communities of the two cultivars. This analysis showed that the microbial population's structures of the conventional and genetically modified maize were very homogeneous. Genetic modification did not adversely affect the structural bacterial community in the rhizosphere of the transgenic maize cultivar.

Keywords: *Zea mays*; Glufosinate; Metagenomic DNA; Rhizosphere; Single-strand conformation polymorphism.

Resumen. Los cultivos genéticamente modificados pueden causar efectos negativos en las comunidades bacterianas. En este estudio, comparamos las estructuras de comunidades bacterianas de dos tipos de maíz: maíz genéticamente modificado (*Zea Mays*), transformado con el gen *pat* que le confiere resistencia al herbicida glufosinato, y un maíz híbrido convencional. El objetivo fue determinar si el cultivo transgénico ejerce un efecto en las comunidades bacterianas que habitan en la rizosfera. El ADN metagenómico fue extraído de la rizosfera de las plantas crecidas bajo condiciones de invernadero, utilizando suelo de regiones donde anualmente se cultiva el maíz. Se utilizó la técnica de Polimorfismo de Conformación de Cadena Sencilla (SSCP), basada en la reacción de la cadena de la polimerasa amplificando el gen 16S rRNA para caracterizar y generar los perfiles genéticos que correspondieran a las comunidades bacterianas de los productos amplificados de la rizosfera de los dos cultivos de maíz. Los perfiles genéticos de las rizosferas consistieron de perfiles distinguibles, basado en pares de primers seleccionados. El análisis de similitud de patrones encontrados por el análisis de matriz binaria demostró que no existen diferencias significativas en las comunidades bacterianas de ambos tipos de maíz. Este análisis indicó que las estructuras de las poblaciones microbianas del maíz convencional y genéticamente modificado son muy homogéneas. La modificación genética no afectó adversamente a la estructura de la comunidad bacteriana en la rizosfera del cultivo de maíz transgénico.

Palabras clave: *Zea mays*; Glufosinato; ADN Metagenómico; Rizosfera; SSCP.

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INTRODUCTION

Genetically modified or transgenic crops may improve agricultural productivity (Clive, 2013). The global transgenic crop surface area is growing continuously yearly despite the ongoing public debate around the use and commercialization of genetically modified crops. Approximately 175.2 million hectares of genetically modified crops were grown worldwide in 2013 (Clive, 2013). The maize has the greatest number of approved events or traits (introduced genes) followed by cotton, potato, canola and soybean (Clive, 2013). In Mexico, transgenic cotton and soybean were planted in about 0.1 million hectares. Also, experimental plots are being planted in Mexico with transgenic maize (Clive, 2013). However, an important concern is the introduction of transgenic crops into agricultural ecosystems because of their potential ecological affects on soil microbial communities, in particular rhizosphere microbes due to their intimate proximity (Fang et al., 2005; Mulder et al., 2006; Xue et al., 2011).

Abiotic (e.g., soil physicochemical properties), and biotic factors (e.g., animals and grazers, plant phonology and species composition) are assumed to influence the structural and functional diversity of microbial communities in the rhizosphere (Gomes et al., 2001; Mansouri et al., 2002; Berg & Smalla, 2009). Furthermore, soil type has also been indicated as a major factor in determining the composition of rhizosphere microbial communities (Schmalenberger & Tebbe, 2002; Dohrmann & Tebbe, 2005; Fang et al., 2005; Berg & Smalla, 2009). Many studies have postulated that the composition of root exudates varies from plant to plant, and it affects the relative abundance and growth of microorganisms in the bulk soil and rhizosphere (Baudoin et al., 2003; Somers et al., 2004; Aira et al., 2010). Plant roots may exert strong effects on microbial communities on the rhizosphere through rhizodeposition of a specific root exudation and its composition (Bais et al., 2006; Nihorimbere et al., 2011). As a result, transgenic plants might change the soil environment and bacterial consortia qualitatively and quantitatively in the rhizosphere due to release of an altered composition of root exudates (Singh & Mukerji, 2006). In consequence, an altered composition of engineering roots leads to distinct microbial communities in the rhizosphere, and influence their functions (Dunfield & Germida, 2003; Hartmann et al., 2008). The release of proteins or an altered composition of root exudates from transgenic plants has been studied as a model system to evaluate the impact or effect of transgenic properties (Schmalenberger & Tebbe, 2003; Baumgarte & Tebbe, 2005; R. Miethling-Graff et al., 2010; Lottmann et al., 2010).

Moreover, it has been shown that each plants either species or cultivar can select their own specific bacterial community (Schmalenberger & Tebbe, 2002; Dohrmann & Tebbe, 2005; Buée et al., 2009). Therefore, plant genotypes might be more important than other factors in the selection of rhizobacterial communities, (e.g., soil origin, agricultural treatments: Miethling et al., 2000; Wieland et al., 2001, Schmalenberger & Tebbe, 2002; Miethling et al., 2003). However, glufosinate-resistant maize had no effect on the bacterial community composition in a field study (Schmalenberger & Tebbe, 2002). Hart et al. (2009) also showed that crop type (transgenic-glyphosate resistant corn or conventional corn) did not affect the denitrifying bacteria or fungal communities in the rhizosphere. Moreover, other studies in corn showed that no deleterious effects were caused on soil microbial communities after the release of *Bacillus thuringiensis* (Bt) (Cry endotoxin) into the soil. Other environmental factors such as plant genotype, age of plants and field heterogeneity were relatively more important (Saxena & Stotzky, 2001; Saxena et al., 2002; Blackwood & Buyer, 2004; Fang et al., 2005; Xue et al., 2011). Furthermore, Griffiths et al. (2006) concluded that although there are effects of the Bt trait or insecticides on soil microbial and faunal communities, they are relatively small compared with the main effect of soil type (field site) on all measured parameters, which may confound the effects of the natural variation between different maize lines.

Devare et al. (2004) employed a polyphasic approach, in which microbial biomass, activity, and T-RFLP analyses were combined to assess soil microbial ecology they cultivated the non-transgenic isoline CRW Bt corn, and the non-transgenic isoline treated with the pesticide tefluthrin. These authors concluded that CRW Bt corn and tefluthrin did not adversely affect neither the microbial biomass, and activity, nor the bacterial diversity or relative abundance. Moreover, Griffiths et al. (2007) conducted a greenhouse study to determine whether the variation in soil parameters under different, conventional maize cultivars exceeded differences between Bt (*Bacillus thuringiensis* protein, Cry1Ab) and non-Bt maize cultivars. Their results indicated that soil microbial community structure was affected by the plant growth stage but not by the Bt trait, and there was no measurable effect on soil microbial community structure by the Cry1Ab protein. Liu et al. (2008) compared seasonal effects of transgenic rice (express Cry1Ab protein, against lepidopteron pest) and the pesticide triazophos [3-(o,o-diethyl)-1-phenyl thiophosphoryl-1,2,4-triazol] on soil enzyme activities under field conditions. They found seasonal changes in rhizosphere soil microbial community composition throughout rice growth, indicating that the impact of the crop growth stage overweighed the application of triazophos and the *cry*1Ab gene transformation. Bt rice did not affect the rhizosphere soil microbial community composition over 2 years of rice cropping. Also, Kapur et al. (2010) performed a field experiment to determine the ecological consequences of cultivation of Bt cotton. They assessed the culturable and non-culturable microbial species in Bt cotton and non-Bt cotton soils. Their results indicated that cropping of Bt cotton did not adversely affect either the culturable or the non-culturable diversity of the microbial communities.

Few studies have indicated an influence in the composition and diversity of rhizosphere bacterial communities in agricultural soils during cropping of genetically modified crops (Dunfield & Germida, 2001; Castaldini et al., 2005; Lottmann et al., 2010). The effects of transgenic plants on the rhizosphere community have been observed, as in the case of transgenic canola (*Brassica napus*): the composition of rhizosphere bacteria of a transgenic cultivar could be distinguished from that on non-transgenic cultivars (Dunfield & Germida, 2001; Gyamfi et al., 2002; Dunfield & Germida, 2003). Dunfield & Germida (2003) conducted a field experiment to identify differences between the soil microbial community associated with growing genetically modified *versus* conventional canola. They concluded that the changes in the microbial community structure associated with genetically modified plants were temporary, and did not persist into the next field season. Also, Brusetti et al. (2005) found differences between the rhizosphere and bulk soil communities at different plant ages, as well as between transgenic Bt 176 and non-transgenic maize. These authors concluded that root exudates could determine the selection of different bacterial communities. Collectively, these studies seem to indicate that, generally, unintended modifications of rhizosphere-inhabiting communities are possible, but that the degree of variation will be influenced by the plant species and type of modification. However, effects detected to date have been minor in comparison with environmental factors such as agricultural practices, sampling date, soil type, field site, season and plant genotype (Schmalenberger & Tebbe, 2002; Dunfield & Germida, 2003; Griffiths et al., 2007; Lottmann et al., 2010). Also, maize plants (conventional and transgenic) collected 35 days after sowing established different rhizobacterial communities than those collected after 70 days grown in the same field (Schmalenberger & Tebbe, 2002).

In spite of the number of studies already done, further research is needed to clearly differentiate whether the genetic modification (e.g., transgenic maize, plant genotype) could affect the rhizobacterial communities. Moreover, it has been shown that plant age is more selective than field sites (Baumgarte & Tebbe, 2005). However, the effects of the transgenic property at a same growth stage are not clear. The objective of this study was to determine the effects of transgenic (herbicide resistant) *versus* non-engineered or conventional maize on their soil microbial community structures. Soil was recovered from the rhizosphere and analyzed by SSCP (Single Strand Polymorphism Conformation) of PCR-amplified 16S rRNA genes from the community DNA.

MATERIALS AND METHODS

Maize cultivars, soil sampling, and rhizosphere sampling. The transgenic maize (experimental line), an isogenic cultivar from the conventional maize, had the modified bacteria *pat*-gene for encoding for phosphinothricin-acetyltransferase which confers resistance to the herbicide glufosinate. The conventional maize variety used in this study was the hybrid 30P49. The agricultural soil was sandy clay, pH=8; electrical conductivity: 1.38 dS/m; organic matter: 6.75 g/kg; extractable potassium: 894 mg/kg; $N\text{-}NO_3$: 27.15 mg/kg; phosphorus: 10.2 mg/kg. The agricultural field selected to collect the soil samples was a site where conventional hybrid maize is cultivated (Tamaulipas, Mexico). Soil was collected from several sampling points in the field within 15 cm clearance strips and a 10 cm wide perimeter. Individual soil samples were pooled together to make 5 composite samples, followed by sieving (2 mm mesh) (Kapur et al. 2010).

The glufosinate-resistant and conventional maize plants were grown in 2.8 L pots filled with the sampling soil, and all planted pots were kept in a greenhouse (daylight approximately 12 h, average daily temperature ranged from 30 to 35 °C) (Brusetti et al., 2005). Pots were watered regularly with tapwater without fertilizer (Assigbetse et al., 2005). They were arranged in a randomized complete block design with 15 replicates per treatment. Sampling of the maize plants was performed after 30 days of plant growth (V6, vegetative stage). They were seedlings of uniform development. V6 is a phenological stage where nutrients are released by younger roots in the root hair zones, and consequently microbial activity is higher than at later stages (Semenov et al., 1999; Schmalenberger & Tebbe, 2002). Ten plants were randomly chosen from each treatment (Conventional or Transgenic maize plants); they were carefully uprooted to prevent root damage. Whole plants were placed in plastic bags and transported to the laboratory. At the laboratory, five composite samples (replicates) were obtained for each maize type by mixing two of any of the 10 sampled plants. Thereafter, a total of five samples was analysed for each treatment.

Metagenomic DNA extraction. Surrounding soil adhered two millimeters or less in diameter to roots (Hartmann et al., 2008) was separated from the bulk soil by gently shaking the root system (Barriuso et al., 2011). The term "rhizosphere" describes the narrow zone of soil that surrounds the roots (Philippot et al., 2012). Total genomic DNA was extracted from 0.25 g of rhizosphere soil samples using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to their protocol. Extracted DNA was stored at 4 °C. This kit was used because it requires less time per sample, produces less toxic waste, and recovers DNA of higher purity (Dohrmann & Tebbe, 2004).

Amplification of 16S rRNA gene from directly extracted gDNA. For bacterial community analysis, the 16S ribosomal RNA genes were amplified from the extracted community gDNA, at 50 ng/mL according to Baudoin et al. (2003) using primers Com1 (5'- CAG CAG CCG CGG TAA TAC-3') and Com2Ph (5'- CCG TCA ATT CCT

TTG AGT TT-3') (Schwieger & Tebbe, 1998), with one of the primers phosphorylated at the 5′ end, resulting in PCR products corresponding to positions 519–926 of the 16S rRNA gene of *Escherichia coli* (Brosius et al., 1981). Specific primers were chosen to amplify part of the 16S rRNA gene; primers hybridizing to highly conserve regions within this gene were selected for estimates of structural diversity of the most dominant bacteria (Schmalenberger & Tebbe, 2003).

Consequently, selected phylogenetic groups of the bacterial community were amplified with taxon-specific primers followed by a second PCR (nested PCR) with the universal Com-primers (Com1 and Com2Ph). Primers used to amplify members of the α-proteobacteria were F203-α (5'-CCG CAT ACG CCC TAC GGG GGA AAG ATT TAT -3') and R1492-Ph (5'-TAC GG (G/T) TAC CTT GTT ACG ACT T -3') which was phosphorylated at the 5' end (Weisburg et al., 1991; Gomes et al., 2001). For the Actinobacteria, primers used were F243HGC-F (5'- GGA TGA GCC CGC GGC CTA-3') and R1387-Ph (5'-CGG TGT GTA CAA GG CCG GGA ACG-3') which was phosphorylated at the 5' end (Heuer et al., 1997). Annealing conditions in the PCR were 60 s at 63 °C for the Actinobacteria, and 60 s at 56 °C for the α-proteobacteria primers. The thermocycling conditions were those described by Dohrmann and Tebbe (2004). The amplification of the products was confirmed by 3% agarose gel electrophoresis. Briefly, an aliquot of the PCR products (1 μL of the PCR solution was diluted 100-fold) was added as template DNA for the second PCR for each specific group, which was conducted with Com primers, as described above, except that only 25 cycles were run. The PCR reaction mixture of 25 μL contained 0.5 μM of each primer (Alpha DNA, Montreal, QC). Each nucleotide consisted of a triphosphate at a concentration of 0.2 mM (Promega®), and 1.25 U of Taq polymerase (Go Taq, Promega, Madison, WI) with the corresponding 1× PCR buffer containing 1.25 mM $MgCl_2$. All reagents, including the Taq polymerase, were prepared as a master solution that was inserted using a pipette into the PCR tubes. Template DNA (50 ng) was added to a final volume of 25 μL for each PCR. The DNA was quantified by 1.5% agarose gel electrophoresis, using a marker of molecular weight DNA lambda (Promega-Markers® Lambda Ladders) and the Kodak MI Application, Molecular Imaging Software v.5.0.1.27 (Carestream Health, Rochester, NY). The thermocycling was conducted with 200 μL PCR tubes (Axigen®) in a Mastercycler, Eppendorf, Hamburg, Germany. All primers used in this study were synthesized by Alpha-DNA Montreal, QC.

Single strand conformation polymorphism (SSCP) analysis. The SSCP was generated for each sample to assess whether the structural diversity of the bacterial communities associated with transgenic maize were different from those associated with non-transgenic maize cultivars. The PCR products were re-suspended in 8 mL of loading buffer (formamide, EDTA, bromophenol blue, and xylene cyanole); the samples of DNA were denatured for 5 min at 95 °C, then immediately cooled on ice and loaded into the pockets of a non-denaturing, temperature controlled, vertical polyacrylamide gel for SSCP electrophoresis for 7 h at 10 °C and 50 V in a Mini-Protean 3-cell apparatus (Bio Rad). The gel was composed of 0.5 × MDE solutions (Lonza Rockland, Rockland, ME) in 0.5 × TBE buffer, 7.3 cm long, 8 cm wide, and 0.75 mm thick. After electrophoresis, the DNA was visualized with a silver staining kit (Bio-Rad Laboratories, Hercules, CA). Statistical analyses of the SSCP profiles were conducted from gels that had been loaded with the respective rhizosphere samples in a randomized order (Schwieger & Tebbe, 1998; Dohrmann & Tebbe, 2004). The polyacrylamide gels were run under the same conditions, each gel carrying samples of all replicate rhizospheres of the two types of maize.

Digital image analysis of SSCP profiles. SSCP profiles were analyzed by R software (R Foundation for Statistical Computing, Vienna, Austria). Calculation of the similarity matrix was based on a binary matrix. The clustering method used the UPGMA procedure for clustering profiles, based on their similarity (Baumgarte & Tebbe, 2005) to compare the similarity of 16S rRNA gene from SSCP profiles. We analyzed 4 replicates for each treatment for cluster analysis, except sample GM5 (see Figures 2 and 3).

RESULTS AND DISCUSSION

The plant rhizosphere is a dynamic environment in which many factors may affect the structure and species composition of the microbial communities that colonize the roots (Berg & Smalla, 2009). The yield of PCR-amplified DNA obtained from rhizosphere samples was from 65 to 380 ng of DNA/g of rhizospheric soil. It has been previously shown that rhizosphere communities vary between plant species and even between cultivars (Germida & Siciliano, 2001). SSCP profiles looking for dominant bacteria were composed of 5 main bands (Fig. 1).

SSCP targeting *Alpha-proteobacteria* was composed by a similar number of bands, but profiles for the *Actinobacteria* group showed only a few bands (Figs. 2 and 3). These results indicated a low species richness in this type of soil (semi-arid and alkaline). It has been shown that different soil textures affected more the microbial populations than transgenic varieties (Baumgarte & Tebbe, 2005; Fang et al., 2005; Barriuso & Mellado, 2012). Because of this, our study was conducted in the same type of soil. This prevented differences on soil characteristics that may be reflected in the bacterial communities or abundance.

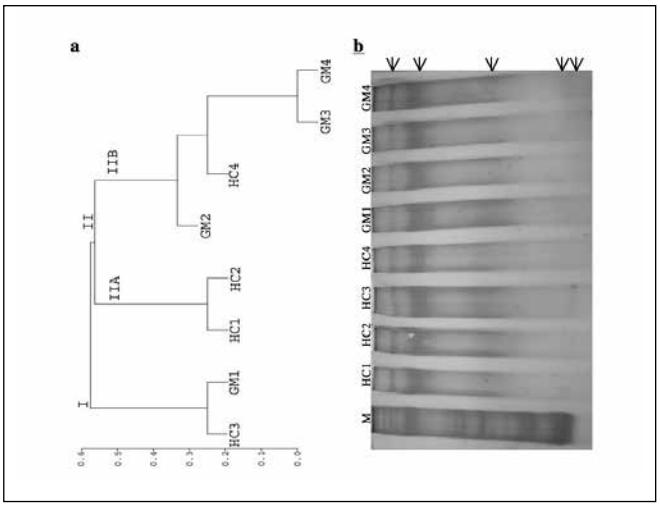

Fig. 1. (a) SSCP genetic profile from bacteria domain, from conventional hybrid (HC1-4) and genetically modified (GM5-8) maize using Marker *Xanthomonas* sp. strain. (b) UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis.
Fig. 1. (a) Perfil SSCP genético del dominio bacteria, las muestras de la rizosfera de maíz HC (carriles 1-4) y del GM (carriles 5-8) (b) Análisis de Clúster UPGMA (del inglés "Unweighted Pair Group Method using Arithmetic averages").

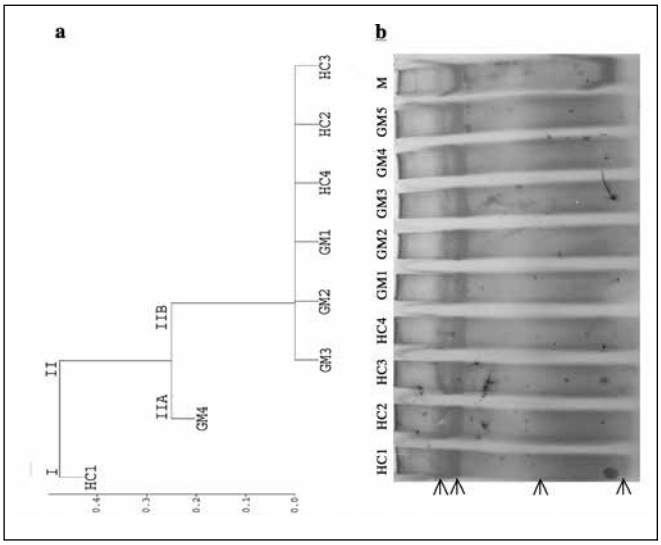

Fig. 2. (a) SSCP genetic profile from group *Alpha-proteobacteria,* from conventional hybrid (HC1-4) and genetically modified (GM5-9) maize using Marker *Xanthomonas* sp. strain. (b) UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis. Exceeding number of samples could not be analyzed on the same gel.
Fig. 2. (a) Perfil SSCP genético del grupo bacteriano específico *Alfaproteobacteria*, las muestras de la rizosfera de maíz híbrido convencional (carriles 1-4) y del GM (carriles 5-9) y utilizando como Marcador (M) la cepa *Xanthomonas* sp. (b) Análisis de Clúster UPGMA (del inglés "Unweighted Pair Group Method using Arithmetic averages"). El número de muestras exceden y no se pudieron analizar en el mismo gel.

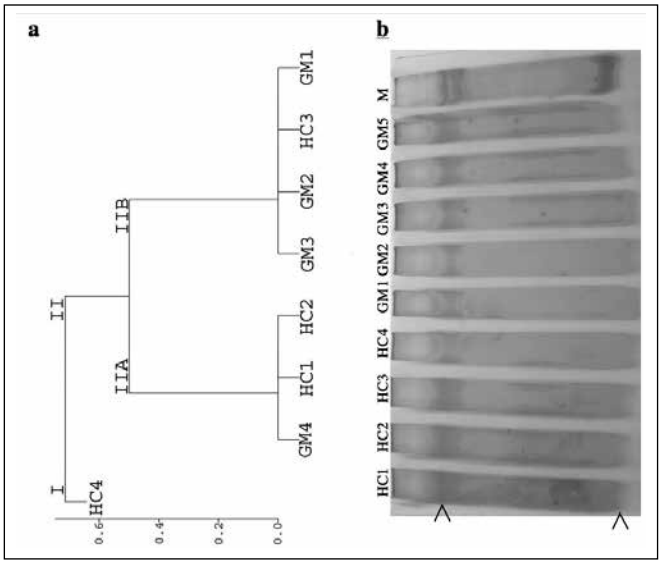

Fig. 3. (a) SSCP genetic profile from group *Actinobacteria*, from conventional hybrid (HC1-4) and genetically modified (GM5-9) maize using Marker *Arthrobacter* sp. strain. (b) UPGMA (Unweighted Pair Group Method with Arithmetic averages) cluster analysis. Exceeding number of samples could not be analyzed on the same gel.
Fig. 3. (a) Perfil SSCP genético del grupo bacteriano específico *Actinobacteria*, las muestras de la rizosfera de maíz híbrido convencional (carriles 1-4) y del GM (carriles 5-9) utilizando como marcador (M) a la cepa *Arthrobacter* sp. (b) Análisis de Clúster UPGMA (del inglés "Unweighted Pair Group Method using Arithmetic averages"). El número de muestras exceden y no se pudieron analizar en el mismo gel.

Cluster analysis of bacterial communities formed two groups (I and II). Group I presented a 42.5% similarity to Group II. Group II was further divided in two subgroups (IIA and IIB), where Group IIA had a similarity of 46% to Group IIB. These subclusters contained most of the bacterial communities from the rhizosphere of both transgenic and conventional maize. Group I contained profiles of conventional and transgenic maize. Using the universal bacterial domain, there were no differences in the structure of the bacterial communities (Fig. 1a). Previous studies have suggested that microbial community composition in maize is independent of the study cultivar (Schmalenberger & Tebbe, 2002; Dohrmann et al., 2013), on soil properties (Baumgarte & Tebbe, 2005), genotypes (Aira et al., 2010), and growth stages (Gomes et al., 2001; Li et al., 2014). Therefore, these results differ from those reported by Aira et al. (2010) who mention that plant genotype modifies the structure of maize rhizosphere microbial communities.

Bacteria domain cluster patterns shared five bands, and some of them were dominant bands in the acrylamide gel (Fig. 1b). This was attributed to members of specific groups, such as profiles of α-*Proteobacteria* (Fig. 2) and *Actinobacteria* (Fig. 3).

The profiles obtained by using the specific primers for α-*Proteobacteria* and *Actinobacteria* were present in lower

numbers than the nonspecific profiles (universal bacterial community primers). This result suggests that the two groups are less represented or less diverse. Further, our results suggest that the dominant members of the bacteria communities were similar and ubiquitous in both types of maize, and there were no important changes at this level in the bacterial structure in either type of maize, as postulated by Baumgarte & Tebbe (2005). From the structural diversity of the α-*Proteobacteria* SSCP profiles, we found four bands that were shared (Fig. 2). Cluster analysis showed that Groups I and II had about 50% similarity. Subgroups IIA and IIB were similar (75%) in the transgenic and conventional maize rhizospheres. Subgroup IIB contained most of the replicates of the α-*Proteobacteria* community in the transgenic and conventional maize rhizospheres. The remaining replicates were contained in Group I (in conventional maize), and only in one replicate of the transgenic maize in Subgroup IIA (Fig. 2). This was most likely due to artifact technical manipulation.

The *Actinobacteria* profiles contained one strong band that was shared by all replicates. These bands could be due to a specific Actinobacteria group highly represented at the rhizosphere of both types of maize. The similarities of the SSCP patterns were low for Groups I and II (28%). Group II contained almost all bacteria communities, and when separated into Subgroups IIA and IIB, similarity between the subgroups was even 50%. Thus, these results indicated that these groups were different, and that the plant can select for species-specific richness (Hartmann et al., 2004). However, both groups shared similar species richness derived from conventional and genetically modified maize. Also, our results indicate that it does not exist a drastic shift in the bacterial populations that inhabited the rhizosphere from both types of maize. Most replicates in Group II from these profiles were similar in transgenic and conventional maize, as found for the α-*Proteobacteria* cluster analysis. The remaining samples of conventional maize rhizospheres were found in Group I (Fig. 3). As it was mentioned before, even for *Actinobacteria,* differences between transgenic and non-transgenic maize were not present.

Differences in the specific profiles of the cultivars, particularly those of the transgenic maize, could not be detected. No differences in bacteria community profiles were found between transgenic and conventional maize rhizospheres, confirming our greenhouse results.

Earlier reports have indicated changes on microbial communities with the use of genetically modified crops (Dunfield & Germida, 2003; Brusetti et al., 2005). However, the SSCP analysis showed that the conformation of the rhizosphere microbial structure did not significantly differ between the conventional and genetically engineered maize with the *pat* gene. Our results are consistent with those reported by Schmalenberger & Tebbe (2002) and Dohrmann et al. (2013) based on the rRNA gene profiling technique and pyrosequencing under the same field and climate conditions. Schmalenberger & Tebbe (2002) found patterns of dominant bacteria in maize rhizosphere. Dohrmann et al. (2013) indicated that the rhizobacterial community of a GM maize did not respond drastically to the presence of proteins in the root tissue. The individual genetic profiles were very similar and reduced, because few bands were observed even if we used the domain bacteria α-*Proteobacteria* and *Actinobacteria*. A few minor variations on microbial community structures could have occurred because of the similar environmental factors. The results presented in this study contribute to the idea that the extent to which the plant influences community composition and structure in the rhizosphere may be different depending not only on the plant species but also on small modifications in their genotype (transgenic plants). This has been shown by other reports (Dunfield & Germida, 2003).

CONCLUSIONS

The present study of bacterial richness in the rhizosphere indicated that herbicide-resistant, transgenic maize did not cause adverse effects or changes on the structure of the microbial community. The structure of the bacterial communities was stable in the *Bacteria* domain, *Alpha-Proteobacteria* and *Actinobacteria* groups from transgenic maize with respect to conventional maize using SSCP analysis. Only a few variations were observed but no drastic changes. This study contributed to resolve some questions about the safe option of using transgenic crops, in this case, herbicide-resistant transgenic maize.

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Antibacterial activity of avocado extracts (*Persea americana* Mill.) against *Streptococcus agalactiae*

Actividad antibacteriana de extractos de aguacate (*Persea americana* Mill.) sobre *Streptococcus agalactiae*

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Abstract. Plants contain numerous constituents and are valuable sources of new biologically active molecules. Avocado (*Persea americana* Mill.) is cultivated and used as food in most tropical and subtropical countries. Its high nutritional value and biological activities, as antioxidant, antimicrobial and analgesic properties, have been thoroughly investigated. Interest in plant extracts with antimicrobial properties has increased as a result of the indiscriminate use of antibiotics, leading to the emergence of resistant bacterial strains. Among bacterial species with clinical importance to multiple hosts, *Streptococcus agalactiae* is outstanding, as it can cause infections especially in humans, fish and cattle. The current study aimed to evaluate the antimicrobial activity of two extracts (ethanol and dichloromethane) from avocado seeds, 'Margarida' variety, against isolates of *S. agalactiae*. Extracts were diluted in ethanol / water (1:1) at a concentration of 100 mg/mL. Antimicrobial activity was tested by the disk diffusion method (antibiogram) against isolates of *S. agalactiae* of human and fish origin.The ethanol extract showed antimicrobial activity only for some isolates of *S. agalactiae* of human origin. The dichloromethane extract showed activity against all isolates of S. agalactiae of both origins. A comparison of the results obtained with dichloromethane extract from isolates of *S. agalactiae* of human or fish origin demonstrated the existence of phenotypic variability among isolates from the same host. However, when comparing measurements obtained in each of the groups, they were statistically similar, showing a lack of interpopulation variability. Thus, it can be verified that the resistance profile of isolates of *S. agalactiae* was independent of host origin and typical of the species.

Keywords: Plant extracts; Disk diffusion method.

Resumen. Las plantas contienen numerosos constituyentes y son fuentes ricas de nuevas moléculas biológicamente activas. El aguacate (*Persea americana* Mill.) es cultivado y utilizado como alimento en la mayoría de los países tropicales y subtropicales, ya que tiene alto valor nutricional, y sus actividades biológicas han sido muy investigadas, entre las cuales la actividad antioxidante, analgésica o antimicrobiana. El interés en extractos vegetales con propiedades antimicrobianas se ha intensificado como consecuencia de la utilización indiscriminada de antibióticos, que llevó a la selección de cepas bacterianas resistentes. Entre las especies bacterianas de relevancia clínica para variados hospederos, se puede destacar la bacteria *Streptococcus agalactiae*, que puede causar infecciones, principalmente en humanos, peces y ganado. El objetivo de ese trabajo fue evaluar la actividad antimicrobiana de dos extractos (etanólico y diclorometanico) de hueso de aguacate variedad margarita en relación a aislados de *S. agalactiae*. Los extractos fueron resuspendidos en etanol/agua en la concentración de 100 mg/mL. La actividad antibacteriana de los extractos fue comprobada usando el método de difusión en discos frente a aislados de *S. agalactiae* de origen humano y peces. El extracto etanólico presentó actividad antimicrobiana solamente para algunos aislados de *S. agalactiae* de origen humano. El extracto diclorometanico presentó actividad antimicrobiana para todos los aislados de *S. agalactiae* de ambos orígenes. La comparación de los resultados obtenidos con el extracto diclorometanico enfrente a los aislados de *S. agalactiae* de origen humano y peces mostró la existencia de variabilidad fenotípica entre aislados del mismo hospedero. Sin embargo, la comparación de las medias obtenidas en cada uno de los grupos fue estadísticamente semejante, demostrando la ausencia de variabilidad interpoblacional. De esta manera, se pudo observar que el perfil de resistencia de aislados de *S. agalactiae* fue independiente del hospedero de origen y característico de la especie.

Palabras clave: Extractos vegetales; Método de difusión en disco.

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INTRODUCTION

The control of bacterial infections is mostly carried out with antibiotics. However, the emergence of resistant bacterial strains has become more frequent, leading to the need of new sources of molecules with antimicrobial activity, which have been found mainly in microorganisms and plants (Cowan, 1999; Mlynarczyk et al., 2010). Natural plant products have been used since ancient times for medicinal purposes as they comprise numerous components and valuable sources of new biologically active molecules (Cowan, 1999; Gupta et al., 2004).

Many plants synthesize antimicrobial secondary metabolites as part of their normal growth and development, often keeping them in tissues that need protection against microbial attack (Gupta et al., 2004). The antimicrobial activity of plant extracts may reside in a variety of different phytochemical constituents, namely terpenoids, essential oils, alkaloids, lectins, polypeptides and polyphenolics and phenolic substances (simple phenols, phenolic acids, quinones, flavones, flavonols and flavonoids, tannins and coumarins) (Gonçalves et al., 2005). The antibacterial activity of these extracts may be ascribable to the combined effects of the polyphenols adsorption on bacterial membrane, leading to its rupture and subsequent leakage of cellular content, and the generation of hydroperoxides (Negi, 2012).

Among plants, avocado (*Persea americana* Mill), originated from Central America, presents a high nutritional value and is cultivated and used as food in most tropical and subtropical countries. Its peel, fruit and leaves are commonly used in America, Antilles and Africa for the treatment of various diseases such as menorrhagia, hypertension, stomach pain, bronchitis, diarrhea and diabetes (Adeyemi et al., 2002). However, avocado seeds are usually discarded during consumption or industrial processes generating residues that could be an economical alternative for treatment of some diseases.

The avocado leaf, stem, fruit and peel have biological activities scientifically proven (Miranda et al., 1997; Adeyemi et al., 2002; Quing-Yi et al., 2005; Gomez-Flores et al., 2008; Castro et al., 2010; Rodríguez-Carpena et al., 2011). Studies with seed demonstrated antioxidant activity and antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas* spp. and *Yarrowia lipolytica*. The Gram-positive bacteria are more sensitive than Gram-negative bacteria (Rodríguez-Carpena et al., 2011). Other seed properties already studied are larvicidal (in *Aedesaegypti*), antifungal (*Candida* spp., *Cryptococcus neoformans* and *Malassezia pachydermatis*) (Leite et al., 2009) and antimicrobial activities against several species including *S. aureus, Enterococcus faecalis, Salmonella Enteritidis, Citrobacter freundii, Pseudomonas aeruginosa, Salmonella Typhimurium, Enterobacter aerogenes* and *Zygosaccharomyces bailii* (Chia & Dykes, 2010).

Phytochemical studies of the avocado seed allowed the identification of several classes of active compounds such as flavonoids, anthocyanins, condensed tannins, alkaloids and triterpenoids in methanolic extracts, while sterols and triterpenes were detected in the hexane extract (Leite et al., 2009).

Several bacterial species are considered of clinical importance because they cause a number of diseases in various hosts. Among these, the species *Streptococcus agalactiae,* a Gram positive, catalase negative and facultatively anaerobic bacteria is remarkable. This species can cause infections in cattle, humans and fish. Furthermore, it can occasionally infect mice, cats, dogs, camels and frogs (Elliot et al., 1990; Figueiredo, et al., 2006; Pereira et al., 2010).

Streptococcus agalactiae is one of the most common causes of perinatal bacterial infections in humans. It is also an opportunistic pathogen of the elderly and immunocompromised people, and may cause pneumonia, meningitis, bacteremia and skin or soft tissue infections (Gibbs et al., 2004; Nakamura et al., 2011). Penicillin is the treatment of choice. However, for patients allergic to β-lactam, erythromycin or clindamycin are prescribed. Mammal isolates are preferably β-hemolytic, but some nonhemolytic have been isolated, and are usually cultivated at 37 °C (Evans et al., 2002; Gibbs et al., 2004).

Besides humans, *S. agalactiae* can infect freshwater and marine fish, either in fish farming or free in the environment (Figueiredo et al., 2006). It is considered the main pathogenic bacteria of different species of fish with high mortality. Naturally or experimentally infected fish exhibit symptoms, such as unilateral or bilateral exophthalmia, corneal opacity, erratic swimming, changes in skin color, skin lesions and ascites (Figueiredo et al., 2006; Pretto-Giordano et al., 2010a). Fish isolates of *S. agalactiae* are usually not hemolytic and are cultivated at 30°C, which may indicate phenotypic adaptations to host (Elliot et al., 1990; Evans et al., 2002; Castro et al., 2008).

S. agalactiae isolates of human source present resistance to tetracycline, clindamycin, erythromycin, chloramphenicol, rifampicin, norfloxacin, levofloxacin, ciprofloxacin, moxifloxacin (Borger et al., 2005; Correa et al., 2011; Nakamura et al., 2011; Ki et al, 2012; Usein et al., 2012). Fish isolates may be resistant to nalidixic acid, gentamicin, neomycin, norfloxacin and streptomycin (Evans et al., 2002; Figueiredo et al., 2006).

The susceptibility of *S. agalactiae* to natural extracts was analyzed in different works. According to Cueva et al. (2012), *S. agalactiae* presented sensitivity to phenolic compounds isolated from wine, epicatechin and gallic acid, and was not sensitive to oenological extracts. It was also sensitive to extracts of wild mushrooms (Alves et al., 2012) and to the essential oil from eight eucalyptus species (Elassi et al., 2012). Leaf extracts of *Calyptranthes clusiifolia*, *Croton floribundus*, *Heisteria silvianii*, *Merremia tomentosa* and *Zanthoxylum riedelianum* also inhibited *S. agalactiae* growth (Castro et al., 2008).

Thus, the aim of this study was to investigate the antibacterial activity of avocado (*P. americana* Mill) seed extracts against

S. agalactiae isolates of human and fish origin. Therefore, comparison of intra- and inter-population variability of resistance profiles was evaluated and indicated the potential therapeutic use of the avocado seed against this bacterial species.

MATERIALS AND METHODS

Plant extracts. In order to obtain seed extracts of avocado (*P. americana* Mill., 'Margarida' variety), the seed was initially separated from the pulp, fragmented, dried and ground into powder. The seed powder was then exposed to a maceration process for a period of seven days, either using ethyl alcohol as solvent, resulting in an extract termed "ethanolic extract", or using dichloromethane as solvent, yielding an extract termed "dichloromethane extract". Subsequently, the extracts were filtered and concentrated in a rotary evaporator. Procedures of maceration, filtration and concentration were repeated once more with both extracts. In order to measure the efficiency of extraction, the obtained extracts were weighed and the ratio between 500g of the initial seed powder and the final weight calculated. Extracts were dissolved in ethanol / water (1:1), stored at room temperature and protected from light until use.

Bacterial strains and culture conditions. The evaluation of 29 *S. agalactiae* isolates recovered from vaginal-rectal swabs and urine of female patients at the University Hospital of Universidade Estadual de Londrina (originally used by Otaguiri et al., 2013) was performed. These isolates had already been characterized for bacterial species confirmation by phenotypic tests (CAMP, KEA, NaCl, hippurate, bacitracin, trimethoprim-sulfamethoxazole, Gram staining and catalase). These isolates were incubated for 24 hours at 37 °C in Muller Hinton blood agar plates (supplemented with 5% sheep blood).

The assessment of 26 isolates of *S. agalactiae* obtained from the Nile tilapia (*Oreochromis niloticus*) with bacterial infection symptoms was conducted. The isolates were collected from different organs, including eyes, brain, liver, heart, blood, visceral fluid and kidney fish collected at fish farming properties located in the northern region of Paraná state and northwest region of São Paulo state, Brazil.The strains had been previously identified as *S. agalactiae* by Gram stain and biochemical assays, and confirmed by more accurate tests, such as API 20 Strep Microtest (BioMerieux) and SlidexStrepto-kit (BioMerieux) (Pretto-Giordano et al., 2010b). These isolates were incubated for 48 hours at 30 °C in Muller Hinton blood agar plates.

Antibiograms. The antibacterial activity of avocado seed extracts against *S. agalactiae* was evaluated by the Disc diffusion method on Muller Hinton blood agar plates, as recommended by CLSI (Clinical Laboratory Standard Institute, 2010). For this purpose, bacteria concentration followed the 0.5 MacFarland scale, yielding an inoculum density of approximately 10^8 CFU/mL (Ostrosky et al., 2008) which was homogeneously distributed over the plates using sterile swabs.

Discs of 6 mm diameter (Laborclin, Brazil) received the application of 10 µL of 100 mg/mL ethanol or dichloromethane extracts. Additionally, other discs received 10 µL of solvents and were used as a negative control. All discs were kept for an hour under a laminar flow for solvent evaporation (Ostrosky et al., 2008).

Biplates were used, forming a duplicate of each isolate per plate. Three discs were placed on each plate side: control, ethanol and dichloromethane extract. In other words, two disks were tested for each extract per strain. Samples of human source were incubated at 37 °C for 24 hours. Strains of fish origin were maintained at 30 °C for 48 hours. At the end of this time, the inhibition zone diameter was measured.

Statistical analysis. The susceptibility test results were analyzed using the Analysis of Variance (ANOVA) followed by the Tukey test or the Mann-Whitney test for interpopulation analysis, at 95% confidence level. Tests were performed with the GraphPad InStat program, version 3.05.

RESULTS

After the extraction procedures, the final weight of extracts was 12.76 g for the ethanolic and 7.48g for the dichloromethane extract. Bacterial inhibition by extracts was evaluated visually by measuring the inhibition zone diameters around disks (disk diameter included) recorded in millimeters. The antimicrobial activity was classified into three levels: low activity (inhibition zone ≤12 mm), moderate activity (inhibition zone between 12 and 20 mm) and strong activity (inhibition zone ≥20 mm), following the criteria adopted in other studies with plant extracts (Rota et al., 2008; Fei et al, 2011).

Antibiogram results of *S. agalactiae* isolates are shown in Table 1 and exemplified in Figures 1 and 2. Both human and fish isolates showed statistical variability in intra-group analysis, exhibiting an inhibition zone between 7 mm e 13 mm for human isolates, and between 9 mm and 12 mm for fish isolates.

For the intergroup analysis, the average of inhibition zones obtained for each group (human and fish origin) was compared. Statistical analysis for ethanolic extract could not be performed, since inhibition zones on plates with fish isolates were not observed. However, differences in susceptibility between strains of human and fish could be observed, given that the first show some susceptible isolates, while in the latter, no susceptible isolates were found (Table 1). The antimicrobial activity of the ethanolic extract, when present, was considered weak, with an inhibition zone between 7 mm and 9.5 mm.

The mean ± standard deviation of the *inhibition zone* diameter for the isolates of human origin observed for the dichlo-

Table 1. Antimicrobial activity of avocado seed extracts against *S. agalactiae* strains.
Tabla 1. Actividad antimicrobiana de extractos de semilla de aguacate contra cepas de *S. agalactiae*.

Isolate (human source)	Inhibition zone diameter (mm)		Isolate (fish source)	Inhibition zone diameter (mm)	
	Ethanolic extract	Dichloromethane extract		Ethanolic extract	Dichloromethane extract
	Mean ± Standard Deviation	Mean ± Standard Deviation		Mean ± Standard Deviation	Mean ± Standard Deviation
6	7.75 ± 0.35	10.75 ± 0.35	**15**	0.00 ± 0.00	10.75 ± 0.35
9	7.75 ± 0.35	11.75 ± 0.35	**16**	0.00 ± 0.00	10.50 ± 0.00
10	4.00 ± 5.66	11.00 ± 0.00	**18**	0.00 ± 0.00	10.75 ± 0.35
11	8.75 ± 1.06	11.00 ± 0.71	**19**	0.00 ± 0.00	11.00 ± 0.00c
12	0.00 ± 0.00	11.00 ± 0.00	**23**	0.00 ± 0.00	11.00 ± 0.00c
13	0.00 ± 0.00	11.25 ± 0.35	**25**	0.00 ± 0.00	10.75 ± 0.35
14	0.00 ± 0.00	12.75 ± 0.35	**26**	0.00 ± 0.00	11.25 ± 0.35 c.e
21	0.00 ± 0.00	11.25 ± 1.06	**29**	0.00 ± 0.00	10.50 ± 0.00
24	0.00 ± 0.00	10.75 ± 0.35	**30**	0.00 ± 0.00	10.75 ± 0.35
25	0.00 ± 0.00	11.25 ± 0.35	**34**	0.00 ± 0.00	10.50 ± 0.00
26	0.00 ± 0.00	10.25 ± 0.35 a	**35**	0.00 ± 0.00	9.75 ± 0.35 d
27	0.00 ± 0.00	10.50 ± 0.00 a	**37**	0.00 ± 0.00	9.75 ± 0.35 d
28	0.00 ± 0.00	10.25 ± 0.35 a	**38**	0.00 ± 0.00	11.00 ± 0.00 c
29	0.00 ± 0.00	10.50 ± 0.00 a	**39**	0.00 ± 0.00	10.25 ± 0.35
33	4.25 ± 6.01	10.50 ± 0.71 a	**40**	0.00 ± 0.00	9.25 ± 0.35d
37	7.25 ± 0.35	11.00 ± 0.00	**42**	0.00 ± 0.00	11.75 ± 0.35 c.e
42	3.50 ± 4.95	11.25 ± 0.35	**44**	0.00 ± 0.00	10.75 ± 0.35
43	0.00 ± 0.00	11.75 ± 1.06	**45**	0.00 ± 0.00	11.25 ± 0.35 c.e
49	0.00 ± 0.00	10.50 ± 0.00 a	**46**	0.00 ± 0.00	10.75 ± 0.35
50	0.00 ± 0.00	11.00 ± 0.71	**47**	0.00 ± 0.00	10.50 ± 0.00
52	0.00 ± 0.00	12.00 ± 0.00	**48**	0.00 ± 0.00	9.50 ± 0.00 d
54	0.00 ± 0.00	10.25 ± 0.35 a	**50**	0.00 ± 0.00	10.50 ± 0.71
56	0.00 ± 0.00	9.75 ± 0.35 a.b	**52**	0.00 ± 0.00	11.00 ± 0.71 c
59	0.00 ± 0.00	11.00 ± 0.71	**53**	0.00 ± 0.00	10.25 ± 0.35
60	0.00 ± 0.00	10.25 ± 0.35 a	**55**	0.00 ± 0.00	10.75 ± 1.06
61	3.50 ± 4.95	10.25 ± 0.35	**56**	0.00 ± 0.00	11.00 ± 0.00 c
62	0.00 ± 0.00	11.00 ± 0.00			
70	0.00 ± 0.00	11.00 ± 0.00			
96	0.00 ± 0.00	11.25 ± 1.06			

a: statistically differs from strain 14; b: statistically differs from strain 52; c: statistically differs from strain 40; d: statistically differs from strain 42; e: statistically differs from strain 48 (P<0,05).
a: difiere estadísticamente de la cepa 14; b: difiere estadísticamente de la cepa 52; c: difiere estadísticamente de la cepa 40; d: difiere estadísticamente de la cepa 42; e: difiere estadísticamente de la cepa 48 (P<0,05).

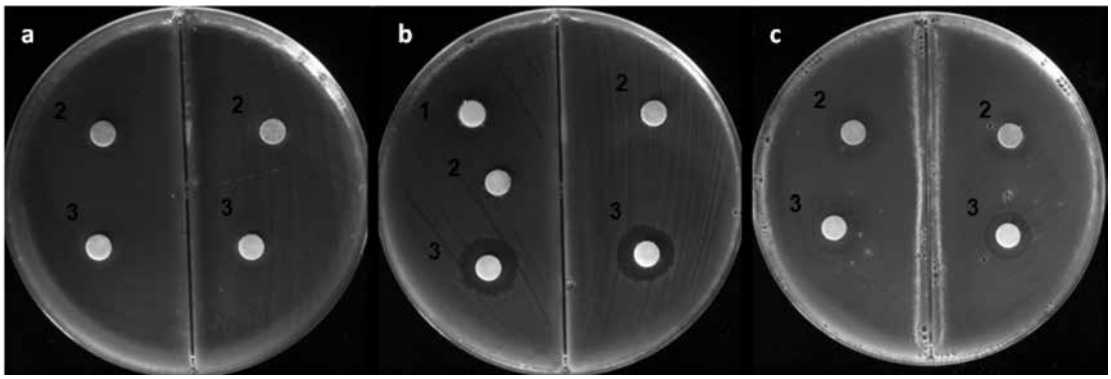

Fig. 1. Antibiogram of *S. agalactiae* isolates of human source: a) strain 6, b) strain 14 and c) strain 25. Disc 1: ethanol/water control; Disc 2: ethanolic extract (100 mg/mL); Disc 3: dichloromethane extract (100 mg/mL).
Fig. 1. Antibiograma de aislados humanos de *S. agalactiae*: a) cepa 6, b) cepa 14 y c) cepa 25. Disco 1: control etanol/agua; Disco 2: extracto etanólico (100 mg/mL); Disco 3: extracto diclorometanico (100 mg/mL).

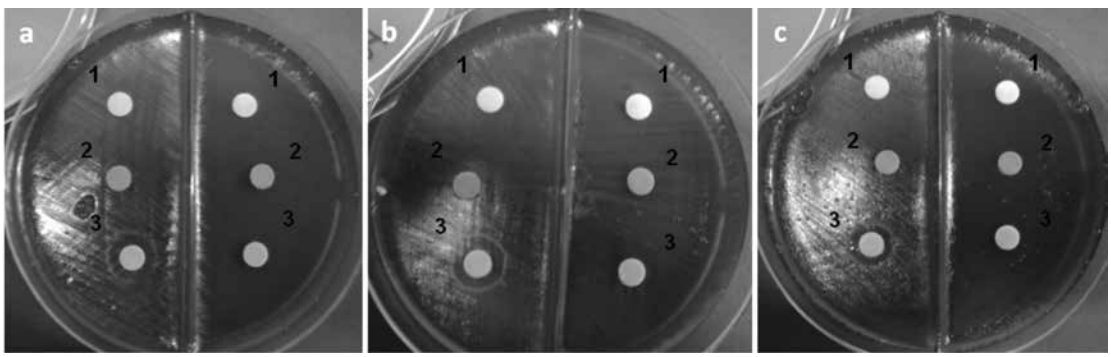

Fig. 2. Antibiogram of *S. agalactiae* isolates of fish source: (a) strain 26, (b) strain 34 and (c) strain 55. Disc 1: ethanol/water control; Disc 2: ethanolic extract (100 mg/mL); Disc 3: dichloromethane extract (100 mg/mL).
Fig. 2. Antibiograma de aislados de peces de *S. agalactiae*: (a) cepa 26, (b) cepa 34 y (c) cepa 55. Disco 1: control etanol/agua; Disco 2: extracto etanólico (100 mg/mL); Disco 3: extracto diclorometanico (100 mg/mL).

romethane extract was 10.93 ± 0.62 mm. On the other hand, fish isolates presented a mean ± standard deviation of 10.61 ± 0.56 mm. The comparison between means was not statistically significant, with p = 0.0897. The dichloromethane extract antibacterial activity was considered weak.

DISCUSSION

Plant extracts are sources of a variety of biotechnology products. Therefore, countless studies have been conducted in order to evaluate characteristics of these extracts, which can be used for the treatment of diseases, due to their antimicrobial, antifungal, analgesic, anti-inflammatory and antitumor activities (Miranda et al., 1997; Adeyemi et al., 2002; Qing-Yi et al., 2005; Leite et al., 2009; Rodríguez-Carpena et al., 2011). Among the commonly evaluated properties, the antimicrobial activity has received special attention, and numerous studies have been conducted, including different avocado extracts (Gomez-Flores et al., 2008; Castro et al., 2010; Chia & Dykes, 2010; Rodríguez-Carpena et al., 2011).

However, although widely used, there are not yet any standardization methods to analyze the antimicrobial activity of extracts of natural products (Ostrosky et al., 2008). The Disk diffusion test is indicated by the FDA (Food and Drug Administration / USA) and established as standard by the

CLSI (Clinical Laboratory Standard Institute / USA, 2010), and, therefore, was the method chosen to conduct this study.

Several *S. agalactiae* isolates of human and fish origin were used in this work, aiming to comprise different phenotypic variations found in isolates from each of the two sources, as well as verify what kind of host presents isolates more susceptible to the evaluated extracts.

Human source isolates used in this study have already been analyzed for capsular type, genotyping by MLVA, antibiotics susceptibility and genetic virulence determinants. The results suggest that even commensal *S. agalactiae* isolates have high potential for virulence and are susceptible to most antimicrobial agents tested (penicillin, ampicillin, vancomycin, etc.) (Otaguiri et al., 2013). However, they presented moderate resistance to erythromycin (19%) and clindamycin (13%) which demands the search for new treatment alternatives, especially for patients allergic to β-lactam antibiotics.

The difference in efficiency of the two extracts can be explained by the difference in polarity of solvents. During the extraction process, polarity influences solubility of the main active substance, leading to difference in their chemical composition and consequently, in their biological activity (Idris et al., 2009). The yield of extraction and concentration of the extract solution can also intervene in the results.

The antimicrobial activity of avocado extracts may be ascribable to its chemical composition. Phytochemical screening highlighted the presence of phenolic compounds in avocado tissues, whose antimicrobial activity is well documented (Idris et al., 2009; Rodriguez-Carpena et al., 2011).

Avocado seed extracts showed low antimicrobial activity against of *S. agalactiae* isolates. This can probably be overcome by increasing extract concentration. The results indicate that the avocado seed is a potential source of antimicrobial substances and arouses considerable interest in new research with more purified extracts for the identification of compounds responsible for the antimicrobial activity.

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Bacteria with capacities of production of biosurfactants isolated from native plants of Baja California, México

Bacterias con capacidad de producción de biosurfantantes aisladas de plantas nativas de Baja California, México

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Abstract. The aim of the present study was to isolate autochthonous microorganisms with biosurfactants capacities from the rhizospere of the cotton (*Gossypium* spp.), cachanilla (*Pluchea sericea*) and salicornia (*Salicornia bigelovii*) in the Mexicali valley. The biosurfactant activity and biosurfactant productions by the strains isolated from the rhizosphere of the cotton (Bs-Alg), cachanilla (Bs-Cach) and salicornia (Bs-Cach01) were determined using oil spreading technique and emulsification activity with corn, olive, soybean and diesel oils, respectively. The analysis of the 16S rRNA showed that strains Bs-Alg, Bs-Cach and Bs-Cach01 are closely related among them and with respect to all *Bacillus subtilis* with more than 99% similarity values. Results showed that all strains had biosurfactant activity. However, Bs-Cach was the only strain that showed a significant biosurfactant activity with all the vegetable oils and diesel. Finally, the isolation of a biosurfactant producing *Bacillus subtilis* strain from native plants of Mexicali valley displayed a substantial potential for production of biosurfactants that can be applied to food industry.

Keywords: Mexicali valley; Biosurfactants; *Bacillus subtilis*; Native plants.

Resumen. El objetivo en el presente estudio fue aislar microrganismos nativos con capacidad biotensioactiva de la rizosfera de algodón (*Gossypium* spp.), cachanilla (*Pluchea sericea*) y salicornia (*Salicornia bigelovii*) en el valle de Mexicali. La actividad del biotensioactivo y producción del biosurfactante por las cepas aisladas de la rizosfera de algodón (Bs-Alg), cachanilla (Bs-Cach) y salicornia (Bs-Cach01) fueron determinados con la técnica de dispersión en aceite y actividad de emulsificante con aceites de maíz, olivo, soya y diésel, respectivamente. El análisis de 16SrRNA mostró que las cepas Bs-Alg, Bs-Cach y Bs-Cach01 están estrechamente relacionados entre sí y con una similitud del 90% con respecto a *Bacillus subtilis*. Los resultados mostraron que todas las cepas tenían actividad biotensioactiva. Sin embargo, B-Cach fue la única cepa que mostró una actividad biosurfactante significativamente con todos los aceites vegetales y diesel. Finalmente, el aislamiento de una cepa de *Bacillus subtilis* procedente de plantas nativas del valle de Mexicali con la capacidad de producir biotensoactivo mostró un potencial para la producción de biotensoactivos que pueden ser aplicados a la industria alimentaria.

Palabras clave: Valle de Mexicali; Biotensoactivo; *Bacillus subtilis*; Plantas nativas.

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INTRODUCTION

Biosurfactants are molecules with a polar and a non-polar region, and hence considered amphipathic. They can be produced by extracellular or intracellular microorganisms, and can reduce surface tension at the air-water interface between two immiscible liquids or between the solid-water interface (Yu et al., 2011). These compounds can be grouped as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds (Biermann et al., 1987). Biosurfactans have been used for (1) serving various environmental problems such as bioremediation of hydrocarbon contaminated soils, (2) enhanced oil recovery and (3) transportation of crude oils (Mulligan, 2005). On the other hand, biosurfactants also have potential applications in agriculture, cosmetics, pharmacy, detergents, personal care products, food processing, textile manufacturing, laundry supplies, metal treatment and processing, pulp and paper processing and paint industries (Banat et al., 2000). Biosurfactants are not yet used in food processing on a large scale due to the numerous protocols set by governmental agencies for alternative ingredients. However, the increase in consumer awareness of adverse allergic effects caused by artificial products has stimulated the development of alternative ingredients such as biosurfactants (Salihu et al., 2009; Banat et al., 2010). Additionally, the biosurfactants show several characteristics such as emulsifying, antiadhesive and antimicrobial activities which suggests their potential application as multipurpose ingredients or additives in diverse areas of the food industry (Nitschke et al., 2010; Malavenda et al., 2010).

In Mexico, the study of microorganisms capable of producing biosurfactants has focused on isolating mesophilic microorganisms from an oil-polluted coastal in Todos Santos Bay (Ensenada BC, Mexico), and a hydrological region RH-27 Tuxpan Nautla in Veracruz Mexico (Martinez-Toledo & Rodriguez-Vazques, 2013; Morales & Paniagua-Michel, 2013). However, studies on the diversity of *autochthonous* microorganisms in the Mexicali valley, which is characterized by the presence of one of the most extreme climates in Mexico, are scarce. The Mexicali valley is located in northeastern Baja California, Mexico (32.55° N, 115.47° W), has a mean annual rain of less than 75 mm, mean July (high) temperature of 43 °C, and mean January highs of 21.1 °C (García-Cueto et al., 2013). Therefore, the objective of the present study was to isolate indigenous microorganisms with biosurfactant capacities from the rhizosphere of native plants of the Mexicali valley.

MATERIALS AND METHODS

Field collection of rhizosphere soil. Soil samples were collected from the rhizosphere of cotton (*Gossypium* spp.), cachanilla (*Pluchea sericea*) and salicornia (*Salicornia bigelovii*) native plants of Mexicali valley, BC, México. Approximately, 100 g soil adhering to the roots of these native plants was collected in sterilized plastic bags and transported to the laboratory at room temperature for isolation of bacteria.

Isolation of biosurfactant-producing bacteria. One gram of rhizosperic soil of each native's plant species has added to 9.0 mL sterile, distilled water in a 10 mL centrifuge tube, which was shaken for 10 sec. Next, 1 mL soil suspension was transferred to another centrifuge tube containing 9 mL sterilized, distilled water using a sterilized pipette to prepare a 1:100 dilution, and 1 mL suspension was again transferred to another tube containing 9 mL sterilized, distilled water (1:1000 dilution). From the dilutions (10^{-3} to 10^{-5}), 1 mL was transferred to a Petri-dish containing nutritive agar, pH 7.2 ± 0.2 at 28 °C, by the spreading plate technique. The plates were then inverted and incubated at 28 °C for 48 hours. Control and replica plates were maintained for each rhizospheric soil of native plants. After incubation, 81.66 ± 3.05, 33.66 ± 3.21, and 63 ± 1.0 colonies-containing plates were selected from the rhizospheres of cotton, cachanilla and salicornia, respectively. These colonies were stored in nutritive agar slants and kept under refrigeration (4 °C) for further experimentation.

Screening for biosurfactant producers. The preliminary screening assays for biosurfactant production were performed using three methods (blood hemolysis test, oil spreading technique and emulsifying activity test). Twenty four-hour-old cultures of the isolates grown in Tryptic Soy Broth (TSB, Merck) were taken to perform screening tests. All screening tests were performed in triplicate. For the isolation of biosurfactant-producing bacteria, one colony from rhizosphere of cotton, cachanilla and salicornia, respectively, were selected from those colonies that had similar morphologies and cultured on blood agar plates for blood hemolysis test (Carrillo et al., 1996). The Petri dishes were incubated at 30 °C for 2 days, and one strain from cachanilla (Bs-Cach), cotton (Bs-Alg) and salicornia (Bs-Cach01) with a clear halo were selected and marked as positive for the presence of biosurfactant-producing bacteria according to Rodrigues et al. (2006). Finally, these strains were inoculated into liquid 5% Tryptic Soy Broth (TSB, Merck) medium supplemented with glycerol (15% final concentration), and stored at -80 °C until the biosurfactant production assay and molecular identification.

Biosurfactant production assay. The biosurfactant production was carried out using Mineral Salt Media (MSM) with glucose (1%) as the sole carbon source at pH 7 according to the proposal of Yesurethinam et al. (2014). Two hundred and fifty milliliters of MSM were inoculated with 3% inoculum of Bs-Cach, Bs-Alg and Bs-Cach01 separately, and incubated at 30 °C for 24 hours with 150 rpm agitation. After the incubation period, the cells were collected by centrifugation at 9000 rpm for 20 min at 4 °C, and the cell free supernatant was used as crude biosurfactant.

Oil spreading assay to determine surfactant activity. The oil spreading assay was utilized to study the surface activities of crude Bs-Cach, Bs-Alg and Bs-Cach01, respectively. This assay was developed by Morikawa et al. (2000) and can be applied when the activity and quantity of biosurfactant is low. For the oil spreading assay, 30 mL of distilled water were added to a petri dish, and 100 μL of corn, olive and soybean oils and diesel were added to the surface of the water, respectively. Twenty microliters of crude biosurfactant from Bs-Cach, Bs-Alg and Bs-Cach01 was then added to the surface of the oil. After 3 min of contact, the diameters of clear zones of triplicate assays from the same sample were determined. MSM broth without cell free supernatant served as the control.

Determination of emulsification activity. The emulsifying activity (EA) of the three strains (BsCach, BsAlg and BsCach01) was assessed against hydrocarbons (diesel), and vegetable oils (corn, olive and soybean). The EA of culture supernatant of the three strains was carried out using the modified emulsification assay described by Cameotra and Bollag (2003). An aliquot of culture supernatant (0.5 mL) was added to 7.5 mL of a 20 mM TM buffer [20 mM Tris-HCl (pH 7) and 10 mM $MgSO_4$] followed by addition of 0.1 mL of each edible vegetable oil and diesel, respectively. After a vigorous vortex for 1 min, the tubes were allowed to stand for 1 h at 30 °C. Finally, the absorbance of triplicate assays from each sample (diesel and vegetable oils) was measured at 540 nm and EA was calculated. One unit of EA was defined as the amount of emulsifier that yielded an absorbance (540 nm) of 0.1 in the assay. MSM broth without cell free supernatant was used as the negative control.

Molecular identification of bacterial isolates. The BsCach (cachanilla), BsAL (cotton) and BsCach01 (salicornia) strains were selected from the colonies based on their ability to form a clear halo on blood agar. Total DNA from Bs-Cach, Bs-Alg and Bs-Cach01 was extracted based on the method of Mendez-Trujillo et al. (2013). The DNA from these strains was amplified by polymerase chain reaction (PCR) with Taq DNA polymerase according to the manufacturer instructions (Invitrogen, Carlsbad, CA, USA). PCR analysis was performed using 25 ng DNA as the template. PCR reactions included the 16S rRNA gene with universal primers 27 forward (AGA GTT TGA TCC TGG CTC AG) and 27 reverse (AAG GAGGTG ATC CAG CCG CA). The PCR reactions were carried out using the following protocol: 94 °C for 5 min (1 cycle), 54 °C for 40 sec, and 72 °C for 1 min (30 cycles). The quality of the PCR reactions analyzed on a 1% Tris acetate EDTA agarose gel, and bands were visualized by staining with ethidium bromide. Images were acquired and stored using the Multidoc-It Digital Imaging system (UVP, Upland, CA, USA). The PCR products were purified using "Purelink® PCR Purification" kit (Invitrogen) and sequencing was carried out using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence data were analyzed using software provided by the Basic Local Alignment Search Tool (BLAST) of GenBank.

Phylogenetic analysis of bacteria isolates. The three sequences obtained, were compared with other DNA sequences using BLAST program of the National Center for Biotechnology Information (Altschul et al., 1997). The sequences with high similarity to those were removed from the GenBank, and a phylogenetic neighbor-joining tree including the obtained isolates and their closest relatives was constructed using MEGA 4.0.

Statistical analysis. Values were expressed as means and standard deviations of triplicate experiments. Statistical analysis was carried out using the Statgraphics Centurion Software (15.2.06 version).

RESULTS

In the present study, phylogenetic analysis of 16S rRNA genes revealed that the Bs-Cach, Bs-Alg and Bs-Cach01 strains formed a stable clade with the members of the genus *Bacillus* spp., showing a sequence similarity of 99% for the 16S rRNA gene. The neighbor-joining method was employed to construct a phylogenetic tree to illustrate the relationships between the 16S rRNA strain sequences and those of other *Bacillus* species (Fig. 1). Thus, the three strains were designated as *Bacillus subtilis* and their sequences were deposited in the GenBank with accession numbers KF669896 (Bs-Alg), KM212950 (Bs-Cach01), and KC256786 (Bs-Cach). In the present study, the results of the oil spreading assay showed that Bs-Cach strain presented the best results with a zone superior to Bs-Alg and Bs-Cach01, respectively (Table 1). The emulsification activity (EA) of the three biosurfactants produced for bacteria cultures from Bs-Alg, Bs-Cach01 and Bs-Cach was tested by measuring the optical density at 540 nm (Table 2). The results showed that only Bs-Cach showed high EA values when it was exposed to different substrate sources used in the present study (corn, olive, soy and diesel). In contrast Bs-Alg and Bs-Cach01 strains showed an unexpected activity close to zero mostly in diesel and oil olive.

DISCUSSION

In the present study the hemolytic, oil displacement and emulsification activity measurements were used to isolate *biosurfactant-producing microorganisms* from the rhizosphere of native plants of Mexicali valley. Our results confirmed that cell free supernatant from Bs-Cach01 (salicornia) and Bs-Cach (cachanilla) strains showed the presence of the surface active compound (biosurfactant), and a high surface activity

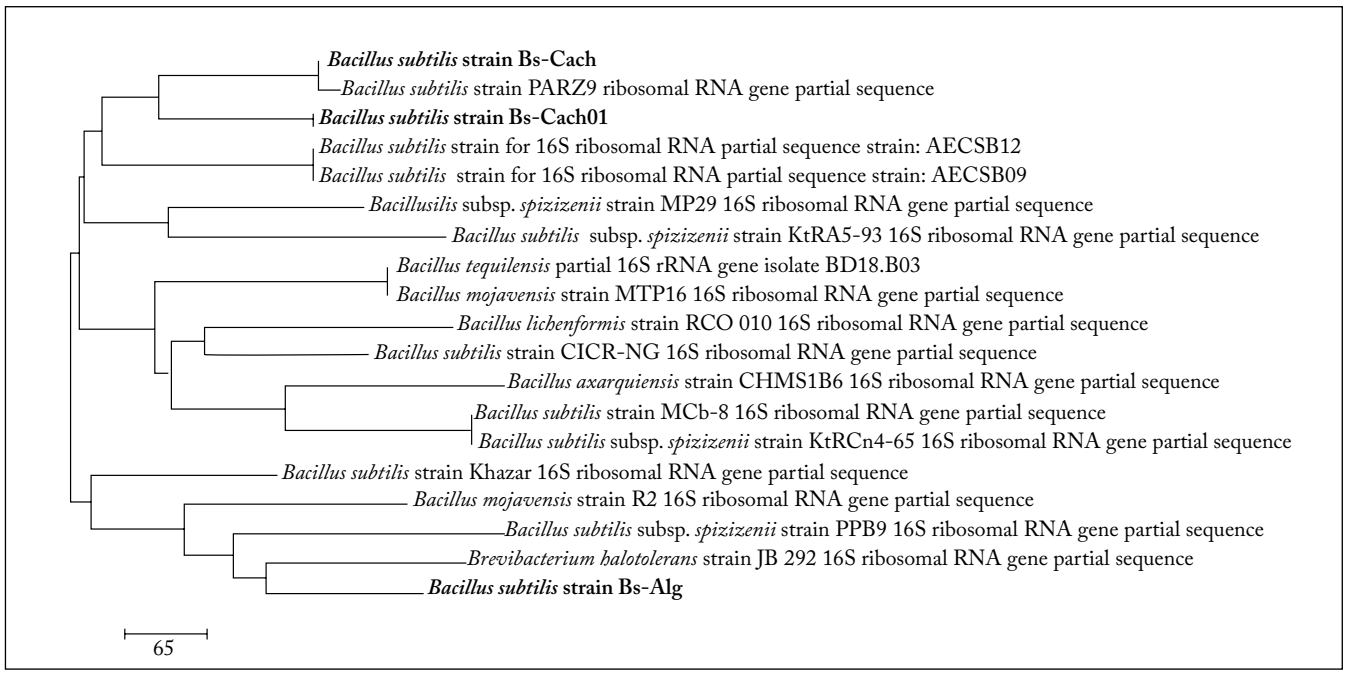

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences constructed using the neighbor-joining method. It shows a close relationship between the 3 strains and the nearest relatives of the genus *Bacillus*. Only values greater than 90% are shown.
Fig. 1. Árbol filogenético basado en las secuencias del gen 16S rRNA construido usando el método de unión al vecino. Muestra una estrecha relación entre las 3 cepas y los parientes más cercanos del género *Bacillus*. Solo se muestran valores mayores a 90%.

Table 1. Comparison of oil spreading efficiency of crude biosurfactant produced by Bs-Cach, Bs-Alg and Bs-Cach01 of natives plants of the Mexicali Valley in different types of oils.
Tabla 1. Comparación de la eficiencia de desparramar el aceite de biosurfactantes crudos producidos por Bs-Cach, Bs-Alg y Bs-Cach01 de plantas nativas del Valle Mexicali en diferentes tipos de aceites.

Source of biosurfactants	Diameter of the clearing zone (mm) in different oils			
	Diesel	Corn	Soybean	Olive
Control (MSM broth)	0	0	0	0
Bs-Cach (Cachanilla)	17.97 ± 0.22	20.6 ± 0.56	9.72 ± 0.56	26.27 ± 1.70
Bs-Cach-Alg (cotton)	0.76 ± 0.67	7.03 ± 0.50	6.25 ± 0.42	7.80 ± 0.54
Bs-Cach01(salicornia)	7.15 ± 0.54	9.24 ± 0.55	6.58 ± 0.38	10.80 ± 0.70

Results are expressed as mean ± standard deviation of values from triplicate experiments.

Table 2. Emulsification activity of crude biosurfactants produced by Bs-Cach, Bs-Alg and Bs-Cach01 of natives plants of the Mexicali Valley in different types of oils.
Tabla 2. Actividad emulsificante de biosurfactantes crudos producidos por Bs-Cach, Bs-Alg y Bs-Cach01 de plantas nativas del Valle de Mexicali en diferentes tipos de aceites.

Source of biosurfactants	Emulsification activity absorbance to 540 nm of different oils			
	Diesel	Corn	Soybean	Olive
Control (MSM broth)	0.39 ± 0.009	0.71 ± 0.001	0.38 ± 0.004	0.50 ± 0.008
Bs-Cach (Cachanilla)	0.007 ± 0.001	0.04 ± 0.003	0.075 ± 0.005	0.0002 ± 0.0004
Bs-Cach-Alg (cotton)	0.044 ± 0.003	0.16 ± 0.002	0.19 ± 0.003	0.037 ± 0.003
Bs-Cach01(salicornia)	7.15 ± 0.54	9.24 ± 0.55	6.58 ± 0.38	10.80 ± 0.70

Results are expressed as mean ± standard deviation of values from triplicate experiments.

in all oils used in this study. However, in this study was not evaluated the surfactant concentration versus the oil spreading activity. Morikawa et al. (2000) reported that the area of oil displacement in oil spreading assays is directly proportional to the concentration of the biosurfactant in the solution.

Similar results were observed by Tomar et al. (2014) and Youssef et al. (2004) in different isolates of microorganisms. Therefore, future studies are necessary to evaluate this possibility using crude biosurfactant from Bs-Cach01 (salicornia) and Bs-Cach (cachanilla) strains. Finally, although the mechanism of oil displacement by biosurfactant has not been fully understood at a molecular level, this assay can be considered as a sensitive and simple method for the measurement of the surface active nature of the biosurfactant (Madhu & Prapulla, 2013). On the other hand, Bs-Cach was only a strain that showed high emulsification activity (EA) on corn, olive, soybean and diesel. These results observed in Bs-Cach were superior to those reported by Olteanu et al. (2011) in new strains of *Bacillus* spp. isolated from different sources. However, the EA from the culture supernatant of Bs-Cach strain was inferior to those of *B. subtilis* B6 and *B. licheniformis* B5 when using corn and sunflower oil, but not olive oil (Sifour et al., 2005). These results indicate that the Bs-Cach strain has both surfactant and emulsifying properties with respect to other strains evaluated in the present study. It is important to consider, however, that even the oil displacement, and use of the emulsification activity are important in the detection of biological surfactants in culture media. These assays are insufficient for the differentiation of bioemulsifiers from biosurfactants. This is due to the fact that bioemulsifiers are best known for emulsification of liquids without significant changes in surface/interfacial tension of their growth medium (Satpute et al., 2008). This could explain the results observed in Bs-Cach01 and Bs-Alg with respect to their emulsifying activity and oil spreading assay.

On the other hand, diverse studies on the evaluation of the emulsifying ability (EA) of biosurfactants are in general related to environmental biotechnology, because many properties of this compound are widely used in remediation technologies of both organic and metal contaminants (Pacwa-Plociniczak et al., 2011). Nevertheless in the present investigation we detected that the biosurfactant and bioemulsifier activity of *Bacillus subtilis* (Bs-Cach) isolated from roots of cachanilla plants form a stable emulsion with oils used in the food industry. This is interesting because the isolation of biosurfactant-producing bacteria from native plants from Mexicali valley displayed a substantial potential for production of biosurfactants and bioemulsifiers that can be applied to the food industry.

CONCLUSION

This study represents the first report about the production of biosurfactant/ bioemulsifiers by *Bacillus subtilis* strains isolated of cachanilla (*Pluchea sericea*), a native plant species of the Mexicali valley. Bs-Cach strain showed the formation of a stable emulsion with oils, suggesting its potential as an emulsifying agent in the food industry. Finally, to our knowledge, use of oil spreading and emulsifying activity represent two simple assays that can be used in the detection of biosurfactant/bioemulsifiers production by indigenous microorganisms.

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Turnip mosaic virus infecting kale plants in Ordu, Turkey

El virus del mosaico del nabo infecta plantas de *Brassica oleracea* var. *acephala* en Ordu, Turquía

Sevik MA

Abstract. *Brassica oleracea* var. *acephala* L. (kale) is widely grown in the Black Sea Region of Turkey. Kale growing has not been common in the other regions of Turkey. A number of diseases can seriously affect *Brassica* crop production. Field surveys were done to determine the occurrence of viruses in kale-growing areas in Ordu in 2013-2014. Leaf samples were collected from kale plants and tested for the presence of *Turnip mosaic virus* (TuMV), *Cauliflower mosaic virus* (CaMV), *Turnip yellow mosaic virus* (TYMV), and *Cucumber mosaic virus* (CMV) by DAS-ELISA and bioassays. Result of serological and biological tests showed that 7.7% of these samples were infected with TuMV. However, CaMV, CMV, and TYMV were not detected in any of the tested kale plants. The occurrence of TuMV suggested that the virus might be an important threat for the kale crops in the province. This is the first report of occurrence of the virus on kale in Ordu, Turkey.

Keywords: *Brassica*; Kale; Survey; Disease; Virus.

Resumen. *Brassica oleracea* var. *acephala* L. (kale) crece ampliamente en la región del Mar Negro de Turquía. El crecimiento de kale no ha sido común en las otras regiones de Turquía. Varias enfermedades pueden afectar seriamente la producción de cosecha de *Brassica*. Se hicieron estudios de campo para determinar la ocurrencia de virus en áreas de crecimiento de kale en Ordu en 2013-2014. Se recolectaron muestras foliares de plantas de kale y se estudió en las mismas la presencia del virus del mosaico del nabo (TuMV), el virus del mosaico del coliflor (CaMV), el virus del mosaico amarillo del nabo (TYMV), y el virus del mosaico del pepino (CMV) por DAS-ELISA y bioensayos. Los resultados de pruebas serológicas y biológicas mostraron que el 7.7% de estas muestras estaban infectadas con TuMV. Sin embargo, CaMV, CMV, y TYMV no fueron detectados en ninguna de las plantas muestreadas de kale. La ocurrencia de TuMV sugirió que el virus podría ser una importante amenaza para las cosechas de kale en la provincia. Este es el primer informe sobre la ocurrencia del virus en kale en Ordu, Turquía.

Palabras clave: *Brassica*; Kale; Estudio; Enfermedad; Virus.

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INTRODUCTION

Kale (*Brassica oleracea* var. *acephala* L.) is a leafy herbaceous, biennial or perennial, plant in the family Brassicaeae. It is widely grown as a leafy green vegetable in the Black Sea Region of Turkey (Balkaya & Karaagac, 2005; Okumus & Balkaya, 2007). Annual kale production in Turkey is 85000 t according to 2013 recofards (TurkStat, 2013). Commercial growing for fresh production is mainly concentrated in the Black Sea region of Turkey (Balkaya & Yanmaz, 2005).

Cabbage vegetables, like the *Brassica* group, are perceived as very valuable food products. They have a very good nutritive value, high antioxidant activity and pro-healthy potential. Especially, kale is characterized by good nutritional and pro-healthy properties (Sikora & Bodziarczyk, 2012). Diseases are important factors limiting the production of leafy greens. Severe disease development can reduce quality to the point where the crop is unmarketable. Pathogens that cause the most common diseases of leafy greens are fungi, bacteria and viruses. A number of viral pathogens such as the *Turnip mosaic virus* (TuMV), *Cauliflower mosaic virus* (CaMV), *Turnip yellow mosaic virus* (TYMV) and *Cucumber mosaic virus* (CMV) can seriously affect *Brassica* crop production (Raybould et al., 1999; Moreno et al., 2004; Spence et al., 2007).

To date, there is no data on the incidence and distribution of these viruses in kale plants in the Ordu province, which has one of the largest kale production capacities in Turkey (TurkStat, 2013). This paper reports the results of a survey of kale plantings in Ordu, Turkey, during late summer 2013 and early spring 2014 to determine the incidence of common Brassica viruses (TuMV, CaMV, TYMV and CMV).

MATERIALS AND METHODS

Surveys and sample collection. Kale, grown on an area of approximately 5532 ha, is one of the most important crops in Ordu province. Five districts were selected from the major kale-producing districts, based on the intensity of kale production in Ordu province. Surveys were conducted in Camas, Catalpinar, Fatsa, Gurgentepe, and Unye districts of Ordu province. Forty-seven sub-locations were selected from these 5 districts based on the size of the kale production. Samples were collected in a proportional and representative way in accordance with quantities cultivated of the region. A total of 234 samples of kale plants were randomly collected from five kale cultivation locations in Ordu province from late November 2013 to early March 2014 (Fig. 1). Leaf samples collected randomly from non-symptomatic and symptomatic plants (Fig. 2) were placed in plastic bags and brought to the laboratory. All collected samples were tested by DAS-ELISA in duplicate and bioassays.

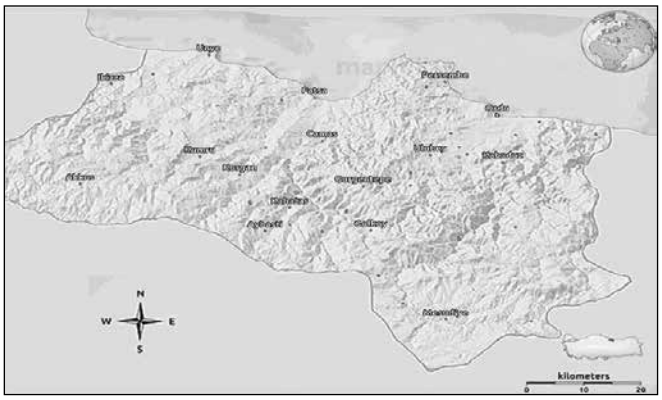

Fig. 1. Map of Ordu province of Turkey, showing surveyed locations.
Fig. 1. Mapa de la Provincia de Ordu en Turquía, mostrando los lugares muestreados.

Fig. 2. Symptoms associated with natural infection by TuMV on kale plant.
Fig. 2. Síntomas asociados con la infección natural por TuMV en plantas de *Brassica oleracea* var. *acephala*.

Serological analysis. Polystyrene 96-well plates (TPP) and polyclonal antiserum kits for TuMV, CaMV, TYMV, and *CMV* (Bioreba) were used in the study. Related chemicals of ELISA were provided from Laboratory of Virology, Department of Plant Protection, University of Ondokuz Mayis. Serological testing, the double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA), was used to detect viruses in kale samples. In the DAS-ELISA test, the leaves of kale plants were grounded (1 g leaf/5 mL buffer) in the extraction buffer (Phosphate Buffered Saline; pH: 7.4) containing 0.05% Tween-20, and 1% skimmed milk powder. ELISA Absorbance values were read at 405 nm using a microplate reader (Tecan Spectra II, Grodig/Salzburg, Austria). Tests were conducted using four negative controls on each plate for each virus. While negative controls were included healthy samples, a reaction was considered positive if ELISA readings were more than twice the average absorbance value of healthy controls (Spence et al., 2007).

Bioassays. Biological studies were done using TuMV isolates that gave the highest ELISA readings from different geographical locations (Camas, Catalpinar, and Unye). The saps obtained by grinding TuMV-infected kale leaves in 0.01 M phosphate buffer (pH: 7.0) (Korkmaz et al., 2008) were mechanically inoculated to indicator plants (i.e., *B. oleracea* var. *capitata, B. oleracea* var. *acephala, B. campestris* subsp. *rapa, Raphanus sativus* and *Chenopodium quinoa)* using carborundum powder as abrasive. The inoculated plants were maintained in a plant growth room at 24 ˚C. The occurrence and type of symptoms were investigated on those inoculated leaves. Inoculated plants were tested for the presence of viruses by DAS-ELISA.

RESULTS

Field survey. Eighteen out of 234 samples (7.7%) reacted positively in DAS-ELISA to TuMV from the viruses examined. The results of serological and biological tests showed that kale plants were only infected with TuMV in Ordu, but none of the samples were infected with CaMV, TYMV, and CMV in this region. Occurrence of the virus in kale plants was found in most *Brassica*-growing areas of Ordu province, including Camas, Catalpinar, and Unye districts. The highest infection rate was found in kale fields in Camas (24%), followed by fields in Unye (8.7%), and Catalpinar (3.3%) (Table 1).

Table 1. Occurrence of viruses in kale samples collected in Ordu in 2013-2014.
Tabla 1. Ocurrencia de virus en muestras de *Brassica oleracea* var. *Acephala* colectadas en Ordu en 2013-2014.

		Viruses*			
Regions	**Samples tested**	**TuMV**	**CaMV**	**TYMV**	**CMV**
Camas	50	12 (24.0%)	0	0	0
Catalpinar	60	2 (3.3%)	0	0	0
Fatsa	30	0 (0.0%)	0	0	0
Gurgentepe	48	0 (0.0%)	0	0	0
Unye	46	4 (8.7%)	0	0	0
Total	234	18 (7.7%)	0	0	0

*: TuMV (Turnip mosaic virus), CaMV (Cauliflower mosaic virus), TYMV (Turnip yellow mosaic virus), and CMV (Cucumber mosaic virus).

In the current study, TuMV was detected in kale plants showing symptoms of leaf mosaic, leaf distortion, mid rib narrowing, and reduction in overall plant growth. However, a few leaf samples that showed symptoms did not react with four antisera used in serological tests. The leaf samples were possibly infected with other disease-causing agents or the symptoms may have been caused by nutrient deficiencies.

Mechanical inoculation tests. Inoculated plants showed different symptoms (Table 2). Necrotic local lesions and mosaic in *B. oleracea* var. *capitata* subvar. *alba*, severe mosaic and leaf malformation in *B. oleracea* var. *acephala* and *B. campestris* subsp. *rapa,* mosaic in *R. sativus,* and necrotic local lesions in *C. quinoa* were observed.

Table 2. Symptoms caused by TuMV in test plants inoculated, and DAS-ELISA positive samples.
Tabla 2. Síntomas causados por TuMV en las plantas inoculadas, y muestras positivas a DAS-ELISA.

Plants	**Symptom*/ DAS-ELISA****
B. oleracea var. *capitata* var. *alba*	NLL, M / +
B. oleracea var. *acephala*	SM, LM / +
B. campestris subsp. *rapa*	SM, LM / +
R. sativus	M / +
C. quinoa	NLL / +

*: M: mosaic, SM: severe mosaic, LM: leaf malformation, NLL: necrotic local lesions
**: + = virus detected in DAS-ELISA.
*: M: mosaico, SM: mosaico severo, LM: hoja malformada, NLL: lesiones necróticas locales.
**: + = virus detectado en DAS-ELISA.

DISCUSSION

The kale is one of the most important vegetable crops in the Black Sea Region of Turkey (Balkaya & Yanmaz, 2005). A survey of kale plants for the presence of selected viruses in Ordu, Turkey was carried out in 2013-2014. All collected samples were tested by DAS-ELISA in duplicate and bioassays. Result of serological and biological tests showed that the kale plants were infected with TuMV in Ordu, located in the Middle Black Sea Region, Turkey. Occurrence of TuMV was recorded in Camas (24.0%), followed by kale fields in Unye (8.7%), and Catalpinar (3.3%). These results revealed that kale grown in commercial fields were infected quite commonly with TuMV. Likewise, a total of 142 samples collected from fields planted to Brassicaceae crops in the southwest Marmara region of Turkey during the 2004-06 growing seasons were tested by DAS-ELISA. Of those tested, 19 plants (13.4%) including white cabbage (7 plants), Brussels sprouts (1 plant), radish (7 plants), wild radish (3 plants) and wild mustard (1 plant) were infected with TuMV (Korkmaz et al., 2008). A total of 532 Brassicaceae reservoir weed samples were collected from plants with virus-like symptoms in Iran. The samples were tested for the presence of TuMV by DAS-ELISA using specific antibodies. Among those tested, 340 samples (64%) were in-

fected with TuMV (Farzadfar et al., 2009). Similarly, many reports have showed that one of the most widespread virus of *Brassica* crops was TuMV worldwide (Walsh & Jenner, 2002; Eiras et al., 20007; Gładysz & Hanus-Fajerska, 2009).

Raybould et al. (1999) reported four viruses (CaMV, TuMV, BWYV, and TYMV) in five natural populations of *B. oleracea* in Dorset (UK). All four viruses were common; 60% of plants were infected with CaMV, 43% with TuMV and BWYV, and 18% with TYMV. Alan (2012) tested winter vegetable species in the Eastern Mediterranean region of Turkey for the presence of a number of viruses, including TuMV and CaMV. Ten cabbage and one cauliflower plants were found to be infected with TuMV and CaMV, respectively. Contrarily to the reports of Raybould et al. (1999), and Alan (2012), CaMV, TYMV, and CMV were not detected in kale samples collected from Ordu, Turkey in the current study.

Positive findings for TuMV were observed in the biological tests to confirm the findings in DAS-ELISA. TuMV isolates were inoculated to test plants. Symptoms of isolates showed that TuMV caused necrotic local lesions and mosaic in cabbage, severe mosaic and leaf malformation in kale and turnip, mosaic in radish, and necrotic local lesions in quinoa. These symptoms were similar to those previously described for TuMV (Farzadfar et al., 2009).

Turnip mosaic virus belongs to the genus *Potyvirus* within the family Potyviridae (Fauquet et al., 2005), and is probably the most common and important virus in Brassicaceae crops throughout the world (Farzadfar et al., 2009). Aphids and weed hosts play a major role in the epidemiology of the virus. TuMV is transmitted in a non-persistent manner by at least 89 aphid species (Walsh & Jenner, 2002). Non-persistent transmission by aphids is the most difficult to avoid because once the aphids carrying virus have attacked the host plant, infection occurs immediately. However, the number of plants that can be infected is very low. Winter crops such as kale provide growers with an early season product because they grow well in cool weather. Harvesting goes from the end of November to the end of February each year in the surveyed regions (Balkaya & Yanmaz, 2005). Fortunately, the cold and wet conditions during winter have kept aphid populations low in the Black Sea Region of Turkey. In the present study, TuMV was detected in limited areas of the Ordu province and showed low incidence, most probably due to the reduced vector populations and activity (Sastry & Zitter, 2014).

TuMV is not seed-borne. It survives in weeds or volunteer host plants outside the growing season. Weed and wild plants also play a major role in the high incidence of the virus. Occurrence of the virus in weeds was found in most crop and ornamental *Brassica*-growing areas of Iran by Farzadfar et al. (2009). Samples collected from Brassicaceae weeds during 2003-2008 were tested by DAS-ELISA. The highest infection rate of TuMV was found for *Rapistrum rugosum* (82/82, 100%), followed by *Sisymbrium loeselii* (73%), *S. irio* (28.9%) and *Hirschfeldia incana* (20%) (Farzadfar et al., 2009). However, the role of weeds and wild plants in the epidemiology of TuMV was not studied in the present work.

In conclusion, virus diseases continue to be a problem in the production of *Brassica* crops in Turkey. This is particularly true of the diseases caused by aphid-borne viruses like TuMV, CaMV and CMV. However, TuMV causes the most serious damage to cabbage crops (Spence et al., 2007). This is the first report which used serological and biological methods to identify the viruses of kale crops in Ordu. We showed that TuMV was the major virus in kale crops at the Black Sea Region of Turkey during 2013 to 2014. Vectors and weeds may have a significant effect on virus epidemiology, and knowledge of weed reservoirs and vectors of viruses would be essential to understand the epidemiology. Therefore, further studies are needed to determine the role of weed hosts and the major vector species of TuMV in the region. To our knowledge, this is the first report of natural occurrence of TuMV on kale plants in Ordu, Turkey.

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Distribución espacial y fluctuación poblacional de *Tetranychus urticae* (Koch) y *Phytoseiulus persimilis* (Athias-Henriot) en cultivos de rosal

Spatial distribution and population fluctuation of *Tetranychus urticae* (Koch) and *Phytoseiulus persimilis* (Athias-Henriot) in rose crops

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Resumen. Se determinó la distribución espacial y vertical, y la fluctuación poblacional de *Tetranychus urticae*, y su depredador *Phytoseiulus persimilis*, en cuatro variedades de rosal (Royal, Samuray, Red Baiser y Keiro) bajo condiciones de invernadero. Se realizaron 9 muestreos para la plaga y 8 para el depredador. Del 20 de Septiembre al 18 de noviembre de 2013, *T. urticae* y *P. persimilis* presentaron una distribución agregada en la mayoría de las fechas de muestreo. Al mismo tiempo, *P. persimilis* no modificó la distribución vertical de *T. urticae*. También se observó un comportamiento sincrónico entre el depredador y su presa en las cuatro variedades estudiadas, aunque solo los cultivares Royal y Samuray presentaron una correlación significativa (r= -0,708, P<0,05; r= 0,702, P<0,05, respectivamente).

Palabras clave: Rosal; Distribución espacial y vertical; *Phytoseiulus persimilis*; *Tetranychus urticae*; Fluctuación poblacional.

Abstract. Spatial and vertical distributions, and population fluctuations of *Tetranychus urticae* and its predator *Phytoseiulus persimilis* were determined in four varieties of rose (Royal, Samuray, Red Baiser and Keiro) under greenhouse conditions. Nine samples for the pest and 8 samples for the predator were obtained. From 20 September to 18 November 2013, *T. urticae* and *P. persimilis* presented an aggregated distribution pattern during most of the sampling dates. At the same time, *P. persimilis* did not change the vertical distribution of *T. urticae*. A synchronous behavior between both predator and prey was observed in the four study varieties, although only the cultivars Royal and Samuray presented a significant correlation (r= -0.708, P<0.05; r= 0.702, P<0.05, respectively).

Keywords: Rosal; Spatial and vertical distribution; *Phytoseiulus persimilis*; *Tetranychus urticae*; Population fluctuation.

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INTRODUCCIÓN

Las plantas ornamentales del género *Rosa* L. (Rosales: Rosaceae) se cultivan en todo el mundo y representan uno de los cultivo florícolas más populares (Chow et al., 2009). El ácaro de dos manchas, *Tetranychus urticae* Koch (Acari: Tetranychidae), es una plaga importante de los cultivos ornamentales bajo invernadero en todo el mundo (Van de Vrie 1985), y la principal plaga en rosas de corte (Casey et al., 2007). Esto es debido a que alteran los procesos fisiológicos de las plantas como la fotosíntesis y la respiración, y afectan el crecimiento, la floración y la fructificación (Hall y Ferree, 1975).

Uno de los depredadores más efectivos de *T. urticae* es *Phytoseiulus persimilis Athias-Henriot* (Acari: Phyotoseiidae) (Gerson y Smiley, 1990). Al respecto, se han desarrollado varios estudios que intentan comprender la relación depredador-presa en ambas especies con respecto a su distribución espacial y temporal (Nachman, 1981; Cross, 1984; García-Mari et al., 1991; Ryoo, 1996; Nachman, 2006; Gómez-Moya y Ferragut, 2009; Alatawi et al., 2011). Croft et al. (2004) señalan que *Phytoseiulus persimilis* es un depredador especialista que sobrevive consumiendo arañas rojas.

En cada estrato de la planta existen factores bióticos y abióticos que influyen sobre el ácaro y su biología. Entre los factores abióticos, la temperatura y la humedad son estímulos de importancia en el movimiento de los ácaros sobre la planta (Jeppson et al., 1975). El entendimiento del cambio estacional y de la dispersión vertical de las plagas y sus depredadores en las plantas es fundamental para el desarrollo de una estrategia de liberación óptima (Lilley et al., 1999). Los objetivos de esta investigación fueron determinar (1) el patrón de distribución espacial y vertical, y la fluctuación poblacional de *T. urticae* y su depredador *P. persimilis* para un control biológico eficiente en cuatro variedades de rosal bajo condiciones de invernadero, (2) cuáles son las variedades de rosal más susceptible o resistente al ataque de *T. urticae*, y (3) en qué variedad se desempeña mejor *P. persimilis.*

MATERIALES Y MÉTODOS

El presente trabajo se realizó en las instalaciones del Departamento de Parasitología de la Universidad Autónoma Agraria Antonio Narro (UAAAN) en Buenavista, Saltillo, Coahuila, México. Para el estudio se utilizaron las especies *T. urticae, P. persimilis* y cuatro variedades de rosal (*Rosa*sp.). La colonia de *Tetranychus urticae* se inició con material biológico recolectado en huertas de manzano en la localidad de Huachichil, Municipio de Arteaga, Coahuila. Los ácaros recolectados en el campo (previamente identificados) fueron colocados en plantas de frijol para incrementar su población, bajo condiciones de invernadero a una temperatura de 27 ± 2 °C. La obtención de *Phytoseiulus persimilis* fue a través de la empresa KOPPERT Biological Systems S.A., México.

El estudio se llevó a cabo en una cama de siembra de 60 cm x 9 m; se utilizaron cuatro variedades de rosal: 1.- Red Baiser, 2.- Samuray, 3.- Royal, 4.-Keiro. Se colocaron 10 plantas por variedad a una distancia de 10 cm, bajo condiciones de invernadero. Un año con siete meses después de la siembra, se realizó una infestación inducida en forma aleatoria con 100 hembras adultas recién apareadas por planta. Una semana después se realizó un muestreo de la población de la araña roja, e inmediatamente se liberaron 12 hembras adultas de *P. persimilis* por planta en forma aleatoria. Después de una semana, se iniciaron una serie de muestreos semanales del depredador y la presa en los estratos superior, medio e inferior de tres foliolos por estrato, y en tres plantas de cada variedad. El conteo se realizó con ayuda de un microscopio portátil de 30X.

Análisis estadístico. Se realizó la prueba de Kruskal-Wallis para determinar diferencias entre las variedades con respecto a la abundancia de la arañuela roja sobre las cuatro variedades de rosal (Zar 2010). Los modelos utilizados incluyeron:

(1) el índice de Morisita (1959) para determinar el tipo de agregación:

$$[\Sigma(X(X-1))/((\Sigma X)(\Sigma X-1))]N$$

donde X es el número de individuos en la i-ésima unidad muestreal, ΣX es el número de individuos en todas las unidades muestreales y N número de unidades muestreales;

(2) Taylor (1961):

$$(v=am^b)$$

que describe una relación potencial entre la media y la varianza, donde *a* corresponde al antilogaritmo de la intersección con la ordenada, *b* a la pendiente de una línea de regresión que determina el tipo de distribución espacial, y m a la densidad media de la muestra, y

(3) Iwao (1968):

$$(\overline{X}^*=\alpha+\beta\overline{X})$$

donde β es la pendiente de la línea de regresión que determina el tipo de dispersión espacial. Valores positivos y negativos del parámetro α del modelo de Iwao (1968) son indicadores de la atracción y repelencia (competencia), respectivamente, entre los organismos (Badii y Castillo, 2009). Se realizó la prueba de significancia t (α=0,05) para los parámetros que determinan el tipo de disposición espacial, teniendo como hipótesis que los valores para los parámetros del modelo de Taylor "b" y de Iwao "β"son iguales, menores o mayores a la unidad. Por último, se usaron los métodos de correlación Pearson o Spearman (Restrepo y González, 2007) para medir el grado de relación o asociación existente entre la abundancia del ácaro fitófago *T. urticae* y la de su depredador *P. persimilis*, utilizando el programa SAS/STAT (SAS 2001).

RESULTADOS

En análisis para la población inicial, la prueba no paramétrica (Kruskal-Wallis) arrojó diferencias significancias (χ^2=7,9191; gl=3, P<0,05) entre la abundancia del número de ácaros sobre las variedad. La variedad Royal presentó el mayor número de ácaros (i.e., 629), seguida por Keiro, Reb Baiser y Samuray con 460, 363 y 120 ácaros/planta, respectivamente.

Patrón de distribución espacial de *Tetranychus urticae*. En todos los muestreos el modelo de Morisita indicó una distribución agregada, mientras que los modelos de Taylor e Iwao indicaron una distribución uniforme en una fecha en las variedades Red Baiser y Samuray (Tabla 1). El parámetro α, indicó que *T. urticae* presentó repelencia en seis de nueve fechas en la variedad Red Baiser, seis de ocho en la variedad Samuray, siete de nueve en la variedad Royal y tres de seis en la variedad Keiro (Tabla 1). Las pruebas de significancias de t (α=0,05; gl=7) confirmaron el tipo de disposición espacial que indicaron los modelos de Taylor e Iwao. Se registró una mayor abundancia sobre las variedades Royal y Red Baiser con 5650 y 3789 ácaros/planta, respectivamente, y una menor abundancia en Samuray y Keiro con 1268 y 1263 ácaros/planta, respectivamente. (Tabla 2).

Patrón de distribución espacial de *Phytoseiulus persimilis*. En la mayoría de las fechas el modelo Morisita mostró que *P. persimilis* presentó un patrón de distribución agregada en las variedades Red Baiser y Royal, Samuray y Keiro, mientras que el modelo de Tyalor presentó una distribución agregada en cuatro fechas en las variedades Keiro y Royal, en dos fechas en la variedad Reb Baiser, y una fecha en la variedad Samuray. La distribución fue al azar en tres fechas en la variedad Red Baiser, y uniforme en dos fechas en las variedades Samuray y Red Baiser. A su vez, el modelo de Iwao presentó una distribución agregada en la variedad Keiro en todas las fechas en que se pudo llevar a cabo el método, y una distribución uniforme en dos fechas en las variedades Samuray y Royal (Tabla 3). El parámetro α indicó que *P. persimilis* presentó competencia intraespecífica por alimento en todas las fechas en la variedad Keiro, en cinco fechas en la variedad Red Baiser, en tres fechas en la variedad Royal y en una en la variedad Samuray (Tabla 3). Hubo una mayor abundancia sobre las variedades Red Baiser y Keiro con 91 y 83 ácaros/planta, respectivamente. En las variedades Royal y Samuray se observaron 66 y 58 ácaros/planta, respectivamente.

Distribución vertical de *T. urticae* y *P. persimilis*. El mayor porcentaje de la población inicial de *T. urticae* se observó en el estrato inferior de las plantas con el 45, 54, 56 y 53% en las variedades Red Baiser, Samuray, Royal y Keiro, seguido por el estrato superior con 30, 27 y 43%, respectivamente. Después de la liberación del depredador, la arañuela de dos manchas permaneció en el estrato inferior. La población de *P. persimilis* en las variedades Royal y Keiro se observó mayormente en el estrato medio con 47 y 48%, respectivamente, en la variedad Samuray en el estrato superior con 66%, y en la variedad Red Baiser en el estrato inferior (40%) (Fig. 1).

Fluctuación poblacional. En relación a la fluctuación poblacional registrada de *T. urticae* y su depredador *P. persimilis*, se observó en general que la población de la plaga disminuyó a medida que se fue incrementando la población del depredador. La correlación de estas dos especies mostró significancia de acuerdo a Pearson en la variedad Royal, y por Spearman en la variedad Samuray (Fig. 2). *Phytoseiulus persimilis* fue más eficiente sobre la variedad Keiro, donde el fitófago alcanzó una población total de un ácaro/planta a la sexta semana de la liberación, mientras que en la variedad Red Baiser mostró menos eficiencia con un total de 67 ácaros/planta (Fig. 2, Tabla 2).

DISCUSIÓN

Se encontraron diferencias significativas en la abundancia de la arañuela roja sobre las variedades de rosal. Las diferencias observadas en este estudio entre variedades son probablemente debidas a la existencia de mecanismos de defensa de la planta (Peralta y Tello, 2011). En este sentido, las cuatro variedades de rosal ofrecen condiciones diferentes que afectan la biología de *T. urticae*. Estas condiciones involucran aspectos químicos, nutritivos, aspectos físicos como la pubescencia, la resistencia de los tejidos, la estructura superficial de las hojas, la superficie disponible para cada individuo y los componentes secundarios de las plantas (Agrawal, 2000; Balkema-Boomstra et al., 2003; Biswas et al., 2004). Estos factores pueden reducir la calidad alimenticia, reduciendo la digestibilidad y afectando a la fertilidad, el crecimiento, la densidad poblacional, la supervivencia, el desarrollo y la mortalidad de los estados juveniles de las arañuelas rojas (Peralta y Tello, 2011).

Se ha registrado un patrón de distribución agregado de *T. urticae* en rosas (Hilarión et al., 2008) y en conjunto con *P. persimilis* (Nachman, 2006). Este patrón de distribución es común en insectos y ácaros (Badii, 1994). Ambas especies de ácaros tuvieron distintas distribuciones. El hecho de encontrar algunas fechas en las que el ácaro depredador muestra diferentes tipos de distribución en varios de los índices quizás se deba a la sensibilidad del modelo. Como lo sugiere Taylor (1984), también es posible que en algunos casos las especies se reproducen muy rápido de modo que cambia su densidad poblacional, y por consecuencia su disposición espacial. Otros estudios realizados con *P. persimilis* (Nachman, 1981; Ryoo, 1996) indican que la dispersión de la presa dentro de la planta, y la coincidencia espacial entre el depredador y la misma, determinan la respuesta funcional del depredador.

En el primer muestro (sin la presencia del depredador), la arañuela de dos manchas fue hallada en el estrato inferior, lo

Tabla 1. Indices de agregación de *Tetranychus urticae* en cuatro variedades de rosal.
Table. 1. Indexes of aggregation of *Tetranychus urticae* on four varieties of roses.

Fecha	m	σ^2	Mor	Tay		r		Iwao		r		m	σ^2	Mor	Tay		r		Iwao		r	
				RED BAISER											SAMURAY							
20 sep 2013	40,33	1392,25	1,74	1,97	ns	0,911	***	1,47	*	0,88	**	13,33	148,50	1,68	0,61	ns	0,533	ns	0,91	ns	0,91	***
30 sep 2013	33,22	767,44	1,59	0,81	ns	0,583	ns	0,76	*	0,73	*	12,56	79,28	1,38	1,96	ns	0,777	*	2,83	ns	0,83	**
07 oct 2013	78,33	1719,00	1,24	2,16	ns	0,773	*	1,40	*	0,78	*	22,56	353,53	1,58	1,62	ns	0,880	**	1,68	ns	0,94	***
14 oct 2013	59,33	1806,50	1,44	2,36	ns	0,978	***	3,61	*	0,98	***	7,33	85,25	2,31	2,07	ns	0,957	***	2,09	ns	0,97	***
21 oct 2013	61,56	2009,28	1,46	2,00	ns	0,960	***	2,80	*	0,97	***	62,67	1074,00	1,23	2,17	ns	0,870	**	2,72	ns	0,89	***
28 oct 2013	51,78	832,44	1,26	2,07	ns	0,880	**	2,64	*	0,85	***	15,89	69,61	1,19	2,26	ns	0,920	***	3,14	ns	0,88	**
04 nov 2013	63,67	3316,50	1,71	2,03	ns	0,986	***	2,77	*	0,95	***	6,00	41,50	1,89	1,66	ns	0,816	**	2,49	ns	0,94	***
11 nov 2013	25,33	517,00	1,68	2,19	ns	0,988	***	3,56	*	0,98	***	0,56	1,03	2,70	1,14	ns	0,839	**	2,78	ns	0,96	***
18 nov 2013	7,44	33,53	1,42	2,22	ns	0,956	***	3,88	*	0,98	***	0	0	0	0	-	0		0	ns	--	--
				ROYAL											KEIRO							
20 sep 2013	69,89	5256,86	1,95	2,57	ns	0,842	**	1,80	*	0,93	***	51,11	3515,61	2,18	1,32	*	0,852	**	1,08	ns	0,83	**
30 sep 2013	118,33	12556,75	1,79	1,78	ns	0,956	***	1,94	*	0,87	**	4,00	48,25	3,53	1,10	*	0,92	***	1,14	ns	0,97	***
07 oct 2013	208,33	9140,75	1,18	3,54	ns	0,831	**	2,66	*	0,91	***	19,89	172,11	1,34	1,62	*	0,828	**	1,90	ns	0,82	**
14 oct 2013	68,78	1140,19	1,20	2,35	ns	0,932	***	1,73	*	0,90	***	43,22	1074,94	1,49	1,92	*	0,946	***	3,16	ns	0,96	***
21 oct 2013	76,67	2345,75	1,34	1,68	ns	0,885	**	1,51	*	0,96	***	15,67	212,50	1,72	1,64	*	0,945	***	1,64	ns	0,97	***
28 oct 2013	48,89	788,11	1,28	2,03	ns	0,968	***	1,99	*	0,95	***	6,00	36,00	1,75	1,86	*	0,898	***	2,60	ns	0,86	**
04 nov 2013	26,44	287,28	1,33	2,60	ns	0,923	***	2,42	*	0,94	***	0,11	0,11	φ	φ		--	--	φ	--	--	--
11 nov 2013	9,00	32,50	1,26	1,13	ns	0,644	ns	1,37	*	0,74	*	0,11	0,11	φ	φ		--	--	φ	--	--	--
18 nov 2013	1,44	1,53	1,04	1,42	ns	0,67	*	2,73	*	0,88	**	0,22	0,44	φ	φ		--	--	φ	--	--	--

* P<0,05; ** P<0,001; ***P<0,0001; ns: no significativo; φ: Ácaros observados en un folíolo; 0: No se observaron ácaros; Mor: Índice de Morisita; Tay: Índice de Taylor; Iwao: Índice de Iwao; r: coeficiente de correlación; m: media muestral;σ^2: varianza muestral.

Tabla 2. Población de *Tetranychus urticae* en cuatro variedades de rosal..
Table 2. Population of *Tetranychus urticae* on four varieties of roses.

Fecha	RED BAISER													SAMURAY											
	T	Estrato Superior				Estrato Medio				Estrato Inferior				T	Estrato Superior				Estrato Medio				Estrato Inferior			
		N	M	Po	EE	N	m	Po	EE	N	M	Po	EE		N	m	Po	EE	N	M	Po	EE	N	m	Po	EE
20/09/2013	363	110	36,67	0	48,18	90	30,00	0	27,62	163	54,33	0	44,84	120	32	10,7	0	7,767	23	7,67	0	6,807	65	21,7	0	18
30/09/2013	299	121	40,33	0	44,97	118	39,33	0	24,85	60	20,00	0	6,00	113	44	14,7	0	12,58	23	7,67	0	6,658	46	15,3	0	7,77
07/10/2013	705	142	47,33	0	30,09	241	80,33	0	56,22	322	107,33	0	10,07	203	32	10,7	0	4,041	53	17,7	0	20,43	118	39,3	0	17,6
14/10/2013	534	129	43,00	0	31,58	105	35,00	0	23,43	300	100,00	0	43,71	66	11	3,67	0	3,512	14	4,67	0	5,033	41	13,7	0	14,6
21/10/2013	554	189	63,00	0	45,71	87	29,00	0	20,81	278	92,67	0	49,70	564	148	49,3	0	43,43	159	53	0	15,72	257	85,7	0	31
28/10/2013	466	120	40,00	0	28,79	120	40,00	0	22,61	226	75,33	0	27,23	143	47	15,7	0	9,504	36	12	0	5,568	60	20	0	10,4
04/11/2013	573	147	49,00	0	61,02	113	37,67	0	37,74	313	104,33	0	65,58	54	1	0,33	1	0,577	22	7,33	0	3,215	31	10,3	0	8,74
11/11/2013	228	15	5,00	1	4,58	56	18,67	0	6,43	157	52,33	0	15,01	5	1	0,33	2	0,577	1	0,33	2	0,577	3	1	1	1,73
18/11/2013	67	16	5,33	2	1,53	18	6,00	0	6,00	33	11,00	1	8,19	0	0	0	3	0	0	0	3	0	0	0	3	0
		ROYAL													KEIRO											
20/09/2013	629	121	40,33	0	40,53	159	53,00	0	19,67	349	116,33	0	118,42	460	196	65,33	0	63,13	18	6,00	0	3,61	246	82,00	0	72,64
30/09/2013	1065	198	66,00	0	52,12	405	135,00	0	174,81	462	154,00	0	102,56	36	7	2,33	0	2,52	5	1,67	2	1,53	24	8,00	2	12,17
07/10/2013	1875	497	165,67	0	91,49	533	177,67	0	114,02	845	281,67	0	54,63	179	55	18,33	0	16,04	57	19,00	0	17,35	67	22,33	0	10,79
14/10/2013	619	159	53,00	0	17,44	136	45,33	0	9,50	324	108,00	0	25,71	389	88	29,33	0	9,29	100	33,33	0	32,33	201	67,00	0	43,41
21/10/2013	690	97	32,33	0	12,22	205	68,33	0	30,60	388	129,33	0	32,93	141	13	4,33	0	3,51	34	11,33	0	14,57	94	31,33	0	6,03
28/10/2013	440	127	42,33	0	24,83	86	28,67	0	14,19	227	75,67	0	24,11	54	18	6,00	0	7,94	21	7,00	2	6,24	15	5,00	2	6,24
04/11/2013	238	79	26,33	0	11,93	37	12,33	0	7,23	122	40,67	0	18,77	1	0	0	2	0	0	0	3	0	1	0,33	2	0,58
11/11/2013	81	11	3,67	0	3,786	29	9,67	0	5,51	41	13,67	0	3,06	1	0	0	2	0	1	0,33	2	0,577	0	0	3	0
18/11/2013	13	4	1,33	2	1,155	5	1,67	1	1,53	4	1,33	2	1,53	2	2	0,67	2	1,15	0	0	3	0	0	0	3	0

T = Total de ácaros; N = Número de individuos por estrato; m = promedio de ácaros por foliolo; EE = Error estándar; Po = Número de unidades con cero individuos.

Tabla 3. Indices de agregación de *Phytoseiulus persimilis* en cuatro variedades de rosal.
Table 3. Indexes of aggregation of *Phytoseiulus persimilis* on four varieties of roses.

	Taylor								Iwao				Taylor									Iwao				
Fecha	m	σ^2	Mor	b		r		b		A		R		m	σ^2	Mor	b		r		b		α		r	
	RED BAISER													SAMURAY												
30 sep 2013	1	4	4,00	0,82	ns	0,796	*	0,72	ns	-0,01736	ns	0,97	***	0,11	0,11	0	0	--	--	--	0	--			--	--
07 oct 2013	0	0	0	0				0						0,33	1,00	0	0	--	--	--	0	--			--	--
14 oct 2013	0,67	0,75	1,20	1,00	ns	0,802	**	1,33	ns	-0,019	ns	0,71	*	0,33	0,50	φ	φ	--	--	--	φ	--			--	--
21 oct 2013	0,56	0,53	0,90	1,00	ns	0,917	***	2,03	ns	-0,079	ns	0,91	***	1,33	5,00	3,00	1,61	ns	0,90	***	3,60	ns	-0,378	ns	0,99	***
28 oct 2013	1,56	3,28	1,68	1,22	ns	0,824	**	1,73	ns	-0,125	ns	0,74	*	3,67	13,25	1,65	0,97	ns	0,88	**	0,95	ns	0,261	ns	0,91	***
04 nov 2013	3,56	4,78	1,09	1,17	ns	0,836	**	1,38	ns	-0,130	ns	0,830	**	0,67	0,50	0,60	0,96	ns	0,92	***	0,50	ns	0,056	ns	0,71	*
11 nov 2013	1,22	2,69	1,96	1,00	ns	1,00	***	1,00	ns	-5,05E19	ns	1,00	***	0	0	0	0		0		0				0	
18 nov 2013	1,56	2,53	1,38	0,66	ns	0,536	ns	0,76	ns	0,096	ns	0,66	*	0	0	0	0		0		0				0	
	ROYAL													KEIRO												
30 sep 2013	0,11	0,11	0	0		0		0				0		0	0	0	0		0		0	0			0	
07 oct 2013	0	0	0	0		0		0				0		0	0	0	0		0		0	0			0	
14 oct 2013	0,89	1,36	1,61	1,44	ns	0,741	*	1,86	ns	-0,143	ns	0,74	*	0,89	0,86	0,96	1,25	ns	0,91	***	2,84	ns	-0,323	ns	0,930	***
21 oct 2013	0,22	0,19	φ	φ				φ				--		3,11	29,4	3,50	1,47	ns	0,97	***	1,77	ns	-0,157	ns	0,99	***
28 oct 2013	1,11	1,86	1,60	1,39	ns	0,445	ns	1,85	ns	-0,037	ns	0,79	**	4	9,25	1,30	1,49	ns	0,82	**	2,21	ns	-0,460	ns	0,88	**
04 nov 2013	2,33	7,25	1,84	1,38	ns	0,881	**	1,40	ns	-0,005	ns	0,92	***	1,22	3,69	2,62	1,55	ns	0,89	**	3,50	ns	-0,353	ns	0,98	***
11 nov 2013	1,89	3,86	1,52	0,10	ns	0,05	ns	0,49	ns	0,080	ns	0,58	ns	0	0	0	0	0	0		0	0	0		0	
18 nov 2013	0,78	1,94	3,00	1,15	ns	0,899	***	0,05	ns	0,04167	ns	0,21	ns	0	0	0	0	0	0		0	0	0		0	

* P<0,05; ** P<0,001; ***P<0,0001; ns: no significativo; φ: Ácaros observados en un foliolo; 0: No se observaron ácaros; Mor: Índice de Morisita; Tay: Indice de Taylor; Iwao: Indice de Iwao; r: coeficiente de correlación; m: media muestral; σ^2: varianza muestral.

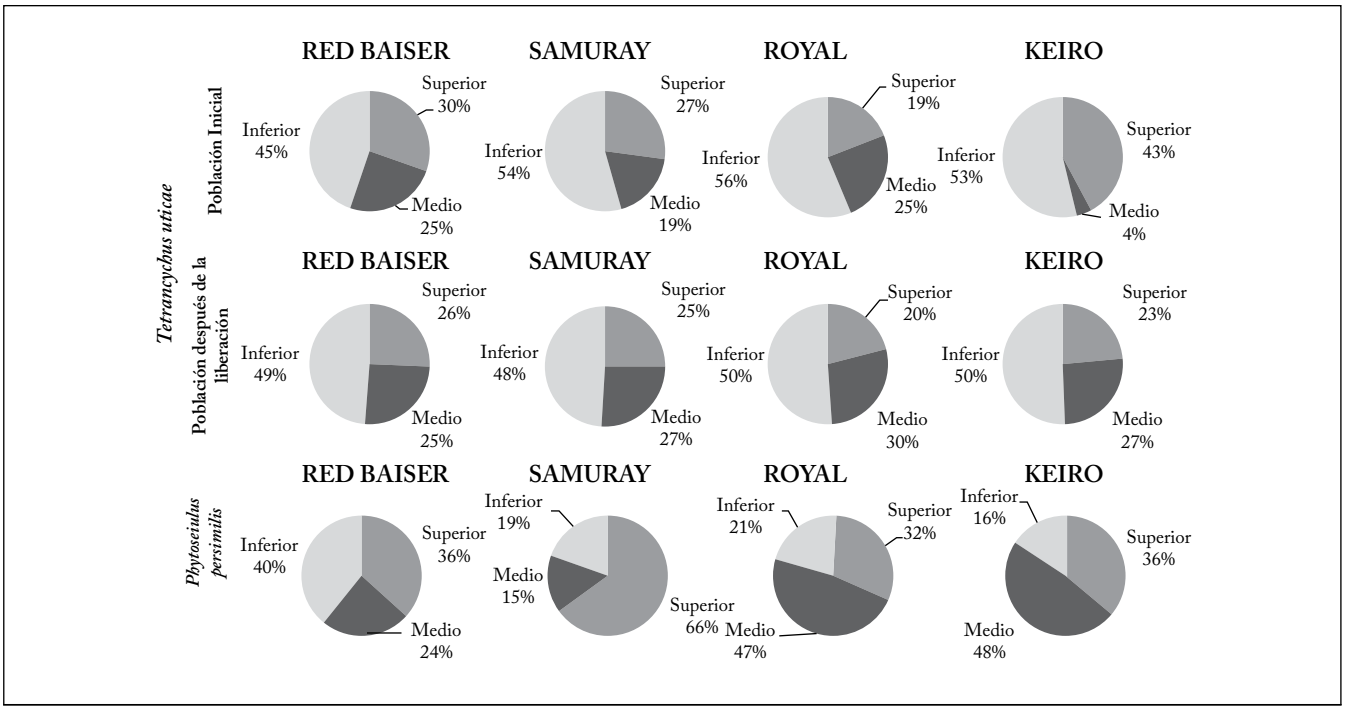

Fig. 1. Distribución vertical de ácaros móviles de *Tetranychus urticae* y *Phytoseiulus persimilis* en cuatro variedades de Rosal.
Fig. 1. Vertical distribution of the mobile mites *Tetranychus urticae* and *Phytoseiulus persimilis* on four varieties of roses.

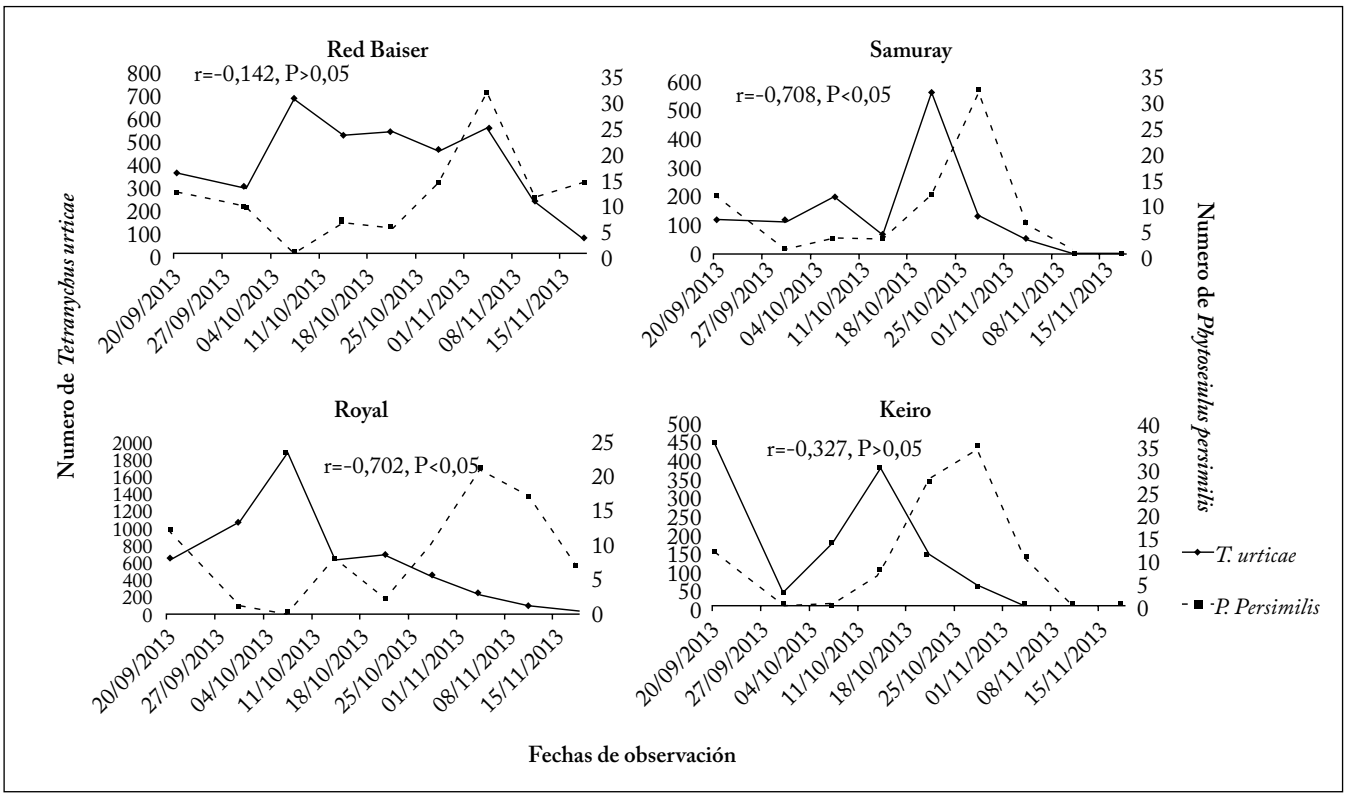

Fig. 2. Fluctuación poblacional de *Tetranychus urticae* y *Phytoseiulus persimilis* en cuatro variedades de Rosal.
Fig. 2. Population fluctuation of *Tetranychus urticae* and *Phytoseiulus persimilis* on four varieties of roses.

Tabla 4. Población de *Phytoseiulus persimilis* en cuatro variedades de rosal..
Table 4. Population of *Phytoseiulus persimilis* on four varieties of roses.

Fecha	RED BAISER													SAMURAY										
	T	Estrato Superior				Estrato Medio				Estrato Inferior				T	Estrato Superior				Estrato Medio				Estrato Inferior			
		N	M	Po	EE	N	m	Po	EE	N	m	Po	EE		N	m	Po	EE	N	m	Po	EE	N	m	Po	EE
30/09/2013	9	6	2,00		3,46	2	0,67		1,15	1	0,33		0,58	1	0	0		0	1	0,33		0,577	0	0		0
07/10/2013	0	0	0,00		0,00	0	0,00		0,00	0	0,00		0,00	3	0	0		0	0	0		0	3	1,00		1,73
14/10/2013	6	4	1,33		1,15	2	0,67		0,58	0	0,00		0,00	3	2	0,67		1,15	1	0,33		0,58	0	0		0
21/10/2013	5	1	0,33		0,58	3	1,00		1,00	1	0,33		0,58	12	10	3,33		3,21	1	0,33		0,58	1	0,33		0,58
28/10/2013	14	3	1,00		1,00	1	0,33		0,58	10	3,33		2,08	33	25	8,33		1,53	5	1,67		0,58	3	1,00		1,00
04/11/2013	32	13	4,33		2,52	7	2,33		2,31	12	4,00		2,00	6	1	0,33		0,58	1	0,33		0,58	4	1,33		0,58
11/11/2013	11	5	1,67		2,08	1	0,33		0,58	5	1,67		2,08	0	0	0		0	0	0		0	0	0		0
18/11/2013	14	1	0,33		0,58	6	2,00		2,00	7	2,33		1,53	0	0	0		0	0	0		0	0	0		0
	ROYAL																		KEIRO							
30/09/2013	1	0	0		0	0	0		0	1	0,33		0,577	0	0	0		0	0	0		0	0	0		0
07/10/2013	0	0	0		0	0	0		0	0	0		0	0	0	0		0	0	0		0	0	0		0
14/10/2013	8	2	0,67		1,15	3	1		1,73	3	1,00		1	8	2	0,67		0,58	4	1,33		1,53	2	0,67		0,58
21/10/2013	2	2	0,67		0,58	0	0		0	0	0		0	28	6	2		2,65	18	6		9,54	4	1,33		0,58
28/10/2013	10	5	1,67		1,53	5	1,67		1,53	0	0		0	36	14	4,67		1,15	15	5		5,20	7	2,33		1,53
04/11/2013	21	2	0,67		1,15	11	3,67		3,79	8	2,67		2,517	11	8	2,67		2,89	3	1		1	0	0		0
11/11/2013	17	8	2,67		2,52	7	2,33		2,08	2	0,67		1,155	0	0	0		0	0	0		0	0	0		0
18/11/2013	7	2	0,67		1,15	5	1,67		2,08	0	0		0	0	0	0		0	0	0		0	0	0		0

N = Número de individuos por estrato; m= promedio de ácaros por hoja; EE = Error estándar; Po = Número de unidades con cero individuos.

cual concuerda con los resultados encontrados por Hilarión et al. (2008). Estos autores observaron la mayor población de esta arañuela en la hoja del tercio inferior en plantas de rosa variedad Versilla (alrededor del 72%). El resto de dicha población se halló en hojas del tercio medio (22%) y superior (6%). Después de la liberación del depredador, no se modificó la preferencia espacial del fitófago, que continuó con un mayor porcentaje poblacional en el estrato inferior. Gómez-Moya y Ferragut (2009) observaron que el depredador *P. persimilis* alteró la distribución vertical de la arañuela de dos manchas, aumentando el porcentaje de fitófagos encontrados en las hojas inferiores de 24 a 49%, y reduciéndolo en las hojas superiores de 76 a 51%.

La distribución vertical de *P. persimilis* fue diferente en los cuatro cultivos de rosal. En las variedades Royal y Keiro se estableció principalmente en el estrato medio, mientras que en las variedades Red Baiser y Samuray lo hizo en los estratos inferior y superior, respectivamente (Fig. 2). Moya y Ferragut (2009) y Nachman (1981) reportaron que *P. persimilis* presentó una mayor tendencia a desplazarse hacia las hojas superiores. La eficacia de los depredadores en el consumo de sus presas influye en la distribución de éstas en la planta. Cuanto más eficaz es el depredador consumiendo a la presa con mayor rapidez, ésta se mueve hacia la parte superior de la planta lo que contribuye a su al escape del depredador (Moya y Ferragut, 2009).

La fluctuación poblacional de *P. persimilis* fue similar a la de *T. urticae* sobre los tres estratos de las variedades Samuray y Keiro, en donde el depredador controló en menor tiempo al fitófago (Tablas 2, 4). Gómez-Moya y Ferragut (2009) registraron que la eficacia del depredador dependió de su habilidad para distribuirse espacialmente de acuerdo con su presa, agrupándose en las hojas o partes de la planta donde la población de la arañuela de dos manchas fue mayor. Dichos autores mencionaron que la agregación de los fitoseidos fue debido a que pasan más tiempo en las zonas donde se concentra la presa y el potencial biótico es mayor, lo que se manifiesta en una mayor tasa de supervivencia y fecundidad.

Los métodos de Pearson y Spearman indicaron una asociación significativa entre *T. urticae* y *P. persimilis* en las variedades Royal y Samuray lo que significa una adecuada relación presa-depredador. Al mismo tiempo, no se encontró significancia en las variedades Red Baiser y Samuray. Probablemente esto se debió a que el grado de alimentación no fue suficiente, lo que se reflejó en los resultados obtenidos por los métodos estadísticos.

En las cuatro variedades, las poblaciones de *T. urticae* y *P. persimilis* presentaron un crecimiento sincrónico durante el periodo de estudio; es decir, un incremento en la población de la plaga fue seguido por un incremento en la población del depredador (Fig. 1). Los incrementos en la población del depredador se registraron a partir del décimo día después de incrementarse la población de la plaga. Al respecto, Badii y Flores (1993) mencionan que la selección natural favorece a aquellos depredadores que están bien sincronizados con sus presas, y al mismo tiempo favorece a aquellas presas que pueden evitar esta sincronización.

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