TESIS DOCTORAL

"Las enmiendas orgánicas del suelo en el control de las Fusariosis del espárrago y del tomate"

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Córdoba, Noviembre de 2014

TITULO: *Las enmiendas orgánicas del suelo en el control de las Fusariosis del espárrago y del tomate*

AUTOR: *Ana Isabel Borrego Benjumea*

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Córdoba, Noviembre de 2014

TÍTULO DE LA TESIS:

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DOCTORANDO/A: Ana Isabel Borrego Benjumea

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo **"Las enmiendas orgánicas del suelo en el control de las Fusariosis del espárrago y del tomate"**, del que es autora la Ingeniera Agrónoma Dña. Ana Isabel Borrego Benjumea, se ha realizado en el Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible del CSIC (IAS-CSIC) de Córdoba. El trabajo tiene carácter de investigación y se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en la Universidad de Córdoba.

Los resultados que se han ido generando durante la realización de la Tesis Doctoral se han expuesto y discutido en diversos foros y congresos científicos nacionales e internacionales, dando lugar a comunicaciones y publicaciones.

Comunicaciones y publicaciones:

- **Borrego-Benjumea, A.,** Melero-Vara, J.M., Basallote-Ureba, M.J., Abbasi, P.A. 2012. Characterizing *Fusarium* isolates from asparagus fields and managing *Fusarium* crown and root rot with soil organic amendments. Southwestern Ontario Regional Association of the Canadian Pythopathological Society (SORA-CPS) *Annual Meeting,* London, Ontario (Canada), (Nov 3).

- **Borrego-Benjumea, A.**, Basallote-Ureba, M.J., Melero-Vara, J.M., Abbasi, P.A. 2012. Caracterización de aislados de *Fusarium* patógenos de espárrago y manejo de la Podredumbre de raíces y cuello con enmiendas orgánicas de suelo. *XVI Congreso de la Sociedad Española de Fitopatología*, Málaga (Spain), (17-21 Septiembre).

- **Borrego-Benjumea, A.,** Melero-Vara, J.M., Basallote-Ureba, M.J., Abbasi, P.A. 2012. Characterizing *Fusarium* isolates from asparagus fields and managing *Fusarium* crown and root rot with soil organic amendments. *2012 Canadian Phytopathological Society Meeting*, Niagara Falls, Ontario (Canada), (24-27 June).

- **Borrego-Benjumea, A.**, Lazarovits, G. 2011. Efficacy of organic amendments in combination with temperature in the control of Fusarium wilt of tomato. *6th IOBC Working Group Meeting on Multitrophic Interactions in Soil,* Cordoba (Spain), (4-7 April).

- **Borrego-Benjumea, A.**, Basallote-Ureba, M.J., Melero-Vara, J.M. 2010. Eficacia de enmiendas orgánicas, temperatura del suelo y cultivares en el control de la podredumbre de raíces y cuello de espárrago. *XV Congreso de la Sociedad Española de Fitopatología*, Vitoria (Spain), (27 Septiembre-1 Octubre).

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Artículos publicados en revistas científicas indexadas (SCI, Science Citation Index):

- **Borrego-Benjumea, A.**, Melero-Vara, J.M., Basallote-Ureba, M.J. 2014. Effect of substrate organic amendments and incubation temperature for different periods on the control of Fusarium crown and root rot caused by *F. oxysporum*, *F. proliferatum*, and *F. solani***. Manuscrito en preparación para su envío a la revista *Spanish Journal of Agricultural Research*.

- **Borrego-Benjumea, A.**, Basallote-Ureba, M.J., Abbasi, P.A., Lazarovits, G. and Melero-Vara, J.M. 2014. Effects of incubation temperature on the organic amendment-mediated control of *Fusarium* wilt of tomato. Annals of Applied Biology, 164: 453-463.

- **Borrego-Benjumea A.,** Basallote-Ureba M.J., Melero-Vara J.M., Abbasi P.A. 2014. Characterization of *Fusarium* isolates from asparagus fields and influence of soil organic amendments on Fusarium crown and root rot. Phytopathology, 104: 403-415.

Artículos publicados en revistas científicas no indexadas y de divulgación científica:

- **Borrego-Benjumea, A.,** Melero-Vara, J.M., Basallote-Ureba, M.J., Abbasi, P.A. 2013. Characterizing *Fusarium* isolates from asparagus fields and managing *Fusarium* crown and root rot with soil organic amendments. Annual Meeting, 2012 The Canadian Phytopathological Society, in: Canadian Journal of Plant Pathology, 35: 103.

- **Borrego-Benjumea, A.**, Melero-Vara, J. M., Basallote-Ureba, M. J. 2012. Efectividad del aporte de compost de alperujo en el control de la Fusariosis del tomate. Iniciativas del grupo de trabajo, en: Boletín de Compostaje para la Producción Ecológica. 1er Trimestre de 2012. Consejería de Agricultura y Pesca. Junta de Andalucía.

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- **Borrego-Benjumea, A.**, Melero-Vara, J.M., Basallote-Ureba, M.J. 2010. Effects of soil organic amendments and incubation temperature on the control of *Fusarium oxysporum* f. sp. *asparagi*. Acta Horticulturae, 883:369-375.

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- **Borrego-Benjumea, A.**, Melero-Vara, J. M., Basallote-Ureba, M. J. 2010. Efecto del compost de alperujo en combinación con la solarización del suelo en el control de *Fusarium oxysporum* f. sp. *asparagi*. Separata Técnica nº 3. en: Boletín de Compostaje para la Producción Ecológica. 2º Trimestre de 2010. Consejería de Agricultura y Pesca. Junta de Andalucía.
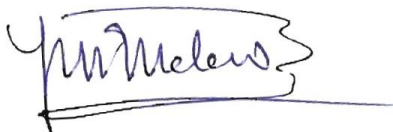
Capítulos de libro:

- **Borrego-Benjumea, A.**, Basallote-Ureba, M.J., Melero-Vara, J.M. 2012. Etiology and Management of Asparagus Crown and Root Rot. 16 pages. Book Chapter in: *Fusarium*: Epidemiology, Environmental Sources and Prevention. Nova Science Publishers, Inc., New York, USA. ISBN: 978-1-61942-562-0.

Por todo ello, se autoriza la presentación de la Tesis doctoral.

Córdoba, 10 de Noviembre de 2014

Firma de los directores

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TÍTULO DE LA TESIS:
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DOCTORANDO/A: Ana Isabel Borrego Benjumea

ESCRITO RAZONADO DEL RESPONSABLE DE LA LÍNEA DE INVESTIGACIÓN

(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca a la Universidad de Córdoba).

El trabajo **"Las enmiendas orgánicas del suelo en el control de las Fusariosis del espárrago y del tomate"**, realizado por la Ingeniera Agrónoma por la Universidad de Córdoba Dña. Ana Borrego Benjumea bajo la dirección del Dr. José María Melero Vara, Profesor de Investigación del Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible, CSIC, y la Dra. María José Basallote Ureba, Investigadora Titular del Instituto de Investigación y Formación Agraria y Pesquera "Las Torres-Tomejil", y que ha sido realizada bajo mi tutoría puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Agronomía de la Universidad de Córdoba.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de Noviembre de 2014

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"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa."

Mahatma Gandhi

RESUMEN

La Podredumbre de raíces y corona (PRC) del espárrago (*Asparagus officinalis* L.), de etiología compleja, incluyendo varias especies de *Fusarium,* y la Fusariosis vascular (FV) del tomate (*Solanum lycopersicum* L.) ocasionada por *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), están presentes en la mayoría de las zonas de cultivo del mundo y causan graves pérdidas económicas. Su control es difícil debido a la prolongada supervivencia de los patógenos en el suelo y su fácil diseminación con el material de propagación. Además, los cultivares de espárrago suelen tener escasa resistencia, que es difícil de implementar por la amplia diversidad genética dentro de las especies de *Fusarium* patogénicas de espárrago y, aunque los cultivares de tomate resistentes a la Fusariosis han estado disponibles desde hace décadas y proporcionan un cierto grado de control, la aparición y desarrollo de nuevas razas patogénicas supone un problema constante. De hecho, no existen variedades de tomate comercialmente aceptables con una resistencia suficiente. Para el manejo de estas enfermedades hay pocas opciones de control químico disponibles. Una alternativa sostenible y ecológica es el uso de enmiendas orgánicas de suelo, que pueden reducir el impacto de hongos de suelo fitopatógenos (Lazarovits, 2001; Noble y Coventry, 2005; Bonanomi *et al.*, 2007; Melero-Vara *et al.*, 2011). Este trabajo se ha realizado en el marco de una agricultura ecológica, utilizándose residuos orgánicos de la industria agrícola y pesquera de la zona (gallinaza, pellet comercial de gallinaza, compost de alpeorujo y emulsión de pescado) como enmiendas orgánicas de suelo en ambos patosistemas *Fusarium* spp./espárrago y *Fusarium oxysporum* f. sp. *lycopersici* / tomate.

El primer objetivo de la presente Tesis Doctoral fue evaluar la eficacia de la temperatura y periodo de incubación del sustrato en combinación con las enmiendas orgánicas gallinaza, pellet de gallinaza y compost de alpeorujo, en el control de la PRC del espárrago causada, en España, por *Fusarium oxysporum* (*Fo*)*, F. proliferatum* (*Fp*) *y F. solani* (*Fs*)*.* Para ello se determinó la viabilidad de los propágulos de los aislados de *Fusarium* spp. en un sustrato artificialmente infestado y enmendado que se incubó a 30 ó 35ºC durante diferentes periodos (15, 30 y 45 días). Por lo general, la viabilidad de los inóculos estuvo considerablemente afectada por la temperatura y el periodo de incubación, produciéndose una mayor reducción de aquélla cuando se combinó con la aplicación de enmiendas orgánicas. La mayor reducción de la viabilidad se produjo con los periodos de incubación más largos (45 días) y a 35ºC se alcanzaron mayores pérdidas de viabilidad que a 30ºC, siendo el pellet de gallinaza la enmienda orgánica más efectiva en reducir la viabilidad de *Fo* y *Fs*. Sin embargo, la viabilidad de los propágulos de *Fp* fue generalmente similar a las del testigo infestado sin aporte de enmienda, sobre todo cuando la incubación fue a 30ºC. Tras la incubación, fueron trasplantadas plántulas de espárrago del cv. Grande a macetas con los sustratos infestados por las distintas especies de *Fusarium*, y a los tres meses se determinó la severidad de las lesiones en el sistema radical y el peso fresco de las plantas. Los resultados mostraron disminuciones muy significativas de la severidad de síntomas radicales. El inóculo de *Fp* causó una menor severidad de síntomas de raíz que los aislados *Fo* y *Fs*. En el tratamiento con pellet de gallinaza, la disminución de la severidad de síntomas fue mayor, especialmente a 30ºC (a los 30 y 45 días en el caso de *Fo*, y a los 15 días en el caso de *Fs*), así como con gallinaza al 2% (con *Fo* y *Fs* a las dos temperaturas de incubación y, generalmente, durante periodos de 30 y 45 días), y con compost de alpeorujo (por lo general con 15-30 días de incubación). Los incrementos del peso fresco de las plantas de espárrago fueron, generalmente,

significativamente elevados para infestaciones del sustrato con *Fo* y *Fs*, con cualesquiera de las enmiendas orgánicas evaluadas.

El segundo objetivo consistió en la identificación y caracterización de aislados de *Fusarium* de plantaciones comerciales de espárrago asociados con la PRC en Canadá, y la evaluación de la eficacia de enmiendas orgánicas en el control de la PRC del espárrago evaluando su impacto en las poblaciones de los patógenos más importantes allí y de bacterias del suelo, así como en el desarrollo de la enfermedad. En este estudio se identificaron 93 aislados de *Fusarium* spp., obtenidos de 11 plantaciones comerciales de espárrago en el Suroeste de Ontario (Canadá), como *F. oxysporum* (65.5%)*, F. proliferatum* (18.3%)*, F. solani* (6.4%)*, F. acuminatum* (6.4%) y *F. redolens* (3.2%) en base a caracteres morfológicos y análisis de PCR con cebadores especie-específicos. Las caracterizaciones posteriores de estos aislados de campo mediante análisis de PCR con inter-secuencias simples repetidas (ISSR) revelaron una considerable variabilidad entre aislados pertenecientes a las diferentes especies de *Fusarium*. Los resultados de pruebas de patogenicidad con aislados de *Fusarium* recuperados de dichos campos, mostraron un amplio rango de agresividad entre ellos. El 50% de los aislados fueron patogénicos para espárrago, y el 22% causaron los síntomas más severos. *F. oxysporum*, *F. solani y F. acuminatum* fueron muy patogénicos, mientras que *F. proliferatum* y *F. redolens* resultaron ser débilmente agresivos. *F. oxysporum* y *F. proliferatum* fueron las especies predominantes, pero la agresividad determinada en pruebas de patogenicidad mostró también la importancia de *F. solani* (45% de frecuencia de aislamiento). Por lo tanto, estas tres especies pueden considerase como las más importantes de *Fusarium* asociadas a la PRC en cultivo de espárrago en el Suroeste de Ontario.

Se evaluó en un invernadero el control de la PRC en suelos infestados con los dos aislados más patogénicos (*Fo*-1.5 de *F. oxysporum* y *Fs*-1.12 de *F. solani*) a los que se añadieron distintas dosis de las enmiendas orgánicas pellet de gallinaza, compost de alpeorujo y emulsión de pescado, utilizando tres cultivares de espárrago de diferente susceptibilidad. Los resultados mostraron que estas tres enmiendas redujeron la severidad de los síntomas de la PRC del espárrago en los sistemas aéreo y radicular, y que la reducción de la enfermedad, en la mayoría de los casos, fue más evidente en los cultivares Jersey Giant y Grande que en el cultivar Mary Washington. Además, los tres subproductos orgánicos aumentaron los pesos fresco y seco de las plantas, y el incremento de la biomasa estuvo asociado con la reducción de la severidad de síntomas. El pellet de gallinaza al 1% fue la enmienda más eficaz, ya que reducía consistentemente la severidad de la enfermedad (de 42 a 96%) y aumentaba el peso de las plantas (de 77 a 152%) en los tres cultivares de espárrago, respecto al tratamiento testigo infestado sin aporte de enmienda. Se cuantificaron las poblaciones de *Fusarium* y bacterias totales tras 1, 3, 7 y 14 días de haber realizado las aplicaciones de enmiendas al suelo. Se observó una disminución gradual de las poblaciones de *Fusarium* y, en contraste, un aumento constante de las densidades de población de bacterias cultivables totales en suelos enmendados con cualquiera de las enmiendas orgánicas citadas. Esto fue más frecuente a las dosis más altas de las enmiendas orgánicas utilizadas. Además, esas dosis fueron también más eficaces en la reducción de la severidad de la PRC y en el aumento del peso de las plantas. Se concluye pues, que las enmiendas orgánicas del suelo, en especial el pellet de gallinaza, logran disminuir la severidad de la enfermedad y promover el crecimiento de plantas, posiblemente por la disminución de la población de patógenos y la mejora de la actividad bacteriana en el suelo.

En el último objetivo se determinó la eficacia de enmiendas orgánicas en combinación con la temperatura de incubación del suelo, en la FV del tomate, evaluándose el papel de las concentraciones de NH_3 y HNO_2 en el desarrollo de la enfermedad. Además se desarrolló un protocolo de PCR cuantitativa en tiempo real para lograr la detección y cuantificación precisas de *Fol* en el suelo. El uso generalizado de esta técnica permitirá cuantificaciones mucho más fiables que las convencionales de dilución seriada de suelo en placas para llegar a establecer estrategias óptimas de control. Primeramente se infestó artificialmente una mezcla de sustrato con un aislado patogénico de *Fol* y se añadieron las enmiendas orgánicas comerciales gallinaza, pellet de gallinaza y compost de alpeorujo, a varias dosis. Los sustratos infestados y enmendados se incubaron a 30 ó 35ºC durante dos periodos (15 y 30 días), tras los cuales se determinó la viabilidad del inóculo de *Fol* por el método de dilución en placas en medio de agar semi-selectivo para *Fusarium*. Se observó que la incubación del sustrato reducía la viabilidad del inóculo, siendo dicha reducción mayor a la temperatura de incubación más alta (35ºC) y cuando el período de incubación fue más largo (30 días). Tras la incubación, fueron trasplantadas plántulas de tomate cv. Monfavet a macetas conteniendo el sustrato enmendado e infestado por *Fol*, y a las cuatro semanas se determinó la severidad de las lesiones en el sistema radical. Se observó que a medida que aumentaba la dosis de la enmienda añadida, se reducía proporcionalmente la severidad de la enfermedad. Además, la enfermedad alcanzó niveles significativamente menores en los sustratos incubados durante 30 días a ambas temperaturas, en comparación con los sustratos incubados durante sólo 15 días.

Posteriormente se infestó artificialmente un suelo natural con el mismo aislado de *Fol* y, seguidamente, se añadieron las enmiendas orgánicas comerciales pellet de gallinaza y compost de alpeorujo a diferentes dosis. Los suelos infestados y enmendados se incubaron a temperatura ambiente (23 ± 2°C) ó a 35ºC durante varios periodos (7, 14 y 28 días), en los que se midieron el pH de los suelos y la concentración de los compuestos tóxicos NH_3 y HNO_2. La cuantificación de los propágulos de *Fol* se realizó al final de la incubación del suelo (28 días) mediante PCR cuantitativa en tiempo real, con sondas TaqMan. El efecto de las enmiendas sobre las poblaciones de *Fol* fue significativo. Se obtuvo la mayor reducción en la cantidad de ADN de *Fol* cuando el suelo enmendado con pellet de gallinaza se incubó a 35°C. La incubación del suelo también tuvo un papel importante en los procesos de liberación de NH_3 y HNO_2, ya que la temperatura más alta (35ºC) aceleró el proceso de mineralización. Las reducciones en las concentraciones de ADN parecían relacionarse con la acumulación de altas concentraciones de NH_3 (27,3 mM) en suelos enmendados con pellet de gallinaza al 2% e incubados a temperatura ambiente o a 35°C. Tras la incubación, fueron trasplantadas a macetas con el suelo arenoso infestado por *Fol*, plántulas de tomate cv. Bellestar, y a las cuatro semanas se determinó la severidad de las lesiones en el sistema radical y el peso seco de las plantas. La severidad de las plantas cultivadas en suelos incubados a temperatura ambiente se redujo en más del 40%, y en más del 73% cuando se incubó a 35°C, independientemente de la dosis de pellet de gallinaza. Este fue el tratamiento más eficaz en la reducción de la viabilidad de *Fol* y, consiguientemente, de la severidad de la enfermedad. En cambio, la CA redujo la viabilidad de los propágulos de *Fol* a cualquier dosis, pero la severidad se redujo significativamente sólo a la dosis más alta (3%), tanto a TA como a 35°C. Además, tanto el pellet de gallinaza como el compost de alpeorujo difirieron en sus efectos sobre los bioensayos de tomate, incrementando el primero hasta 3,5 veces el peso de la planta, como resultado de la reducción de la severidad de los síntomas. Se concluyó que el efecto combinado de las enmiendas orgánicas del suelo y la incubación a temperaturas moderadas,

como aproximación a la solarización sub-óptima del suelo, lograban reducir los propágulos de *Fol* y, en consecuencia, la severidad de la enfermedad en las plantas de tomate. El manejo con pellet de gallinaza, cuando se combina con la incubación del suelo, es una medida de control eficaz contra la FV del tomate.

De la información científico-técnica generada en esta Tesis, se pueden realizar recomendaciones de cómo emplear algunas de las enmiendas orgánicas en combinación con su incubación en suelos infestados por los distintos *Fusarium* spp. implicados como agentes causales de las enfermedades referidas, a determinados regímenes térmicos y por periodos relativamente breves (inferiores a cuatro semanas), subóptimos para procesos exitosos de solarización del suelo. Se requiere una expansión de estos estudios para determinar qué condiciones óptimas puedan garantizar la eficacia de estos métodos de control en situaciones de invernadero o de campos donde ocurren estas problemáticas fitopatológicas, al tiempo que permitan la implementación de técnicas por parte de los agricultores.

Aparte de estos beneficios, cabe valorar los efectos favorables sobre el medio ambiente, derivados del reciclaje de subproductos de industrias agrícolas y pesquera, como la gallinaza, pellet de gallinaza, compost alpeorujo y emulsión de pescado, que pueden ser muy contaminantes. Estas enmiendas de suelo podrían reducir considerablemente la dependencia de pesticidas químicos y fertilizantes inorgánicos de síntesis para lograr sistemas agrícolas sostenibles. Concomitantemente, podría deducirse una mejora de los rendimientos de estos cultivos.

SUMMARY

Fusarium crown and root rot (FCRR) of asparagus (*Asparagus officinalis* L.), with a complex etiology including several *Fusarium* spp., and Fusarium wilt (FW) of tomato (*Solanum lycopersicum* L.), caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), occur in most growing areas of the world and can cause serious economic losses. Its control is difficult due to the long survival of pathogens in the soil and their easy spreading by propagation material. In addition, asparagus cultivars available often have low resistance levels and, due to the wide genetic diversity within species of *Fusarium* pathogenic of asparagus, implementation of resistance is difficult to develop. On the other hand, tomato cultivars resistant to *Fusarium* have been available for decades and provide a certain degree of control, but the emergence and development of new pathogenic races is a constant problem. In fact, commercially acceptable varieties of tomato with complete resistance are not frequent. Therefore, there are few chemical control options available for the management of these diseases. A sustainable and environmentally friendly alternative is the use of organic soil amendments that can greatly reduce the impact of soilborne plant pathogens (Lazarovits, 2001; Noble y Coventry, 2005; Bonanomi *et al.*, 2007; Melero-Vara *et al.*, 2011). This work has been conducted in the framework of organic farming since organic by-products from agricultural and fishing industries in the area (poultry manure, commercial pellet of poultry manure, olive residue compost and fish emulsion) have been tested as organic soil amendments aiming disease control for the pathosystems *Fusarium* spp./asparagus and *Fusarium oxysporum* f. sp. *lycopersici* / tomato.

The first objective of this Thesis was to evaluate the effectiveness of temperature and incubation period in combination with several organic amendments (poultry manure, commercial pellet of poultry manure, olive residue compost) in substrate mixture, on the management of FCRR of asparagus caused by *Fusarium oxysporum* (*Fo*)*, F. proliferatum* (*Fp*) *and F. solani* (*Fs*), the most important in Spain*. The propagule viability of *Fusarium* spp. isolates was determined on a substrate artificially infested and amended which was incubated at 30 or 35ºC for periods of 15, 30 and 45 days. Overall, the viability of the inocula was significantly affected by temperature and incubation period, resulting in a further reduction of the former when combined with the application of organic amendments. The greatest reduction in pathogens viability occurred with the largest incubation period (45 days) and larger loss of viability was achieved at 35ºC than at 30ºC, with pellet of poultry manure as the most effective organic amendment in reducing the viability of *Fo* and *Fs*. However, the viability of propagules of *Fp* was usually similar to that in infested and un-amended control, especially when incubation was at 30ºC. After incubation, seedlings of asparagus cv. Grande were transplanted into pots containing substrates infested by the different species of *Fusarium*. After three months, the severity of symptoms in the root system and fresh weight of the plants were assessed. Results showed highly significant decreases in the root symptom severity. *Fp* inoculum caused a lower severity than *Fo* and *Fs* isolates. After substrate treatment with pellet of poultry manure, reduction of symptoms severity was higher particularly at 30ºC (by 30 and 45 days incubation of *Fo*-infested substrate, and by 15 days incubation of *Fs*-infested substrate). Similarly, reductions in severity were observed with the higher poultry manure doses (in *Fo* and *Fs*-infested substrate incubated at both temperatures and, usually, for 30 and 45 days), and olive residue compost (usually by 15-30 days incubation). Fresh weight increase of asparagus plants were overall, significantly high in *Fo* and *Fs* substrate infestations with any of the three organic amendments tested.

Summary

The second objective was the identification and characterization of *Fusarium* species associated with FCRR of commercial asparagus fields in Southwestern Ontario, Canada, and evaluation of the effects of organic amendments in the management of FCRR of asparagus assessing their impact on populations of pathogens most important in SW Ontario and soil bacteria, and on the disease development, in order to assess its management. Ninety-three *Fusarium* isolates were identified as *F. oxysporum* (65.5%), *F. proliferatum* (18.3%), *F. solani* (6.4%), *F. acuminatum* (6.4%), and *F. redolens* (3.2%) based on morphological or cultural characteristics and PCR analysis with species-specific primers. The inter-simple-sequence repeat (ISSR) PCR analysis of the field isolates revealed considerable variability among the isolates belonging to different *Fusarium* species. The results of pathogenicity assessment showed a wide range in virulence among the *Fusarium* field isolates. Fifty percent of the field isolates were pathogenic to asparagus, and 22% caused the most severe symptoms on asparagus. *F. oxysporum*, *F. solani*, and *F. acuminatum* were highly pathogenic, whereas *F. proliferatum* and *F. redolens* were found to be weakly aggressive. *F. oxysporum* and *F. proliferatum* were the predominant species, but the virulence determined by pathogenicity tests showed also the importance of *F. solani* (45% isolation frequency), thus these three species can be considered as the most important species of *Fusarium* associated with the FCRR of asparagus fields in Southwestern Ontario.

The management of FCRR in soils infested with the two most pathogenic isolates (*F. oxysporum* Fo-1.5 and *F. solani* Fs-1.12) and amended with soil organic amendments of pelleted poultry manure, olive residue compost, and fish emulsion was evaluated in a greenhouse using three asparagus cultivars of different susceptibility. The results of the study showed that all the three organic amendments reduced stem and root system severity of FCRR on asparagus, and disease reduction in most cases was more conspicuous in the moderately susceptible cvs. Jersey Giant and Grande than in the highly susceptible cv. Mary Washington. Moreover, all three organic byproducts also increased fresh and dry plant weights, and the biomass increase was associated with the reductions in symptoms severity. Application of pellet of poultry manure at 1% was the most efficient since it consistently reduced FCRR severity by 42-96% and increased plant weights by 77-152% on all three cultivars, as compared to the *Fusarium* control treatment. Populations of *Fusarium* and total bacteria were enumerated after 1, 3, 7 and 14 days of soil amendment. A gradual decline in *Fusarium* populations was observed in contrast with a steady increase in the population densities of total culturable bacteria in soils amended with each of the three organic byproducts. This was more frequent at the highest rates of organic amendments used. Furthermore, these higher amendment rates used were also most effective in reducing FCRR severity and increasing plant weights. In conclusion, soil organic amendments, especially pellet of poultry manure, may achieve decrease of disease severity and promote plant growth possibly by decreasing pathogen population and enhancing bacterial activity in the soil.

The last objective of this study was to determine the effects of organic amendments in combination with soil incubation temperature on Fusarium wilt of tomato evaluating the role of the concentrations of NH_3 and HNO_2 in the disease development. Further, a real-time PCR protocol was developed to achieve detection and precise quantification of *Fol* on soil. The generalized use of this technique will allow inoculum quantifications much more reliable than the conventional serial soil dilutions on dishes to establish optimal control strategies. First, a substrate mixture was artificially infested with a pathogenic isolate of *Fol* and amended with organic amendments (poultry manure, pellet of poultry manure and olive residue compost) at several rates. Then it was incubated at 30 or 35ºC for

two periods (15 and 30 days), after which the viability of *Fol* inoculum was determined by dilution plating on *Fusarium*-selective agar medium. It was observed that the substrate incubation reduced the viability of the inoculum, and that reduction was higher at the highest incubation temperature (35ºC) and when the incubation period was longer (30 days). After incubation, tomato seedlings cv. Monfavet were transplanted to pots containing *Fol*-infested and amended substrate, and four weeks later root symptom severity was determined. Disease severity was proportionally reduced as the rate of amendment added increased. Furthermore, disease levels were significantly lower in substrates incubated for 30 days at both temperatures, as compared to substrates incubated for only 15 days.

Subsequently, a natural soil was artificially infested with the same *Fol* isolate and then organic amendments (pellet of poultry manure and olive compost residue) at different rates were added. Soil was incubated at room temperature (23 $\pm$ 2°C) or 35ºC for periods of 7, 14 and 28 days, at which soil pH and concentration of toxic compounds NH_3 and HNO_2 were measured. Quantification of *Fol* propagules was performed at the end of soil incubation (28 days) by real-time PCR with TaqMan probes. The effects of amendments on *Fol* populations were significant. The highest decreases in *Fol* DNA resulted when soil amended with pellet of poultry manure was incubated at 35ºC. Soil incubation temperature also had an important role in the processes of NH_3 and HNO_2 release, as the highest temperature (35ºC) accelerated the process of mineralization. The reductions in DNA concentrations were most likely related to the accumulations of high concentrations of NH_3 (27.3 mM) in soils treated with 2% pellet of poultry manure and incubated at room temperature or at 35ºC. After incubation, seedlings of tomate cv. Bellestar were transplanted into pots containing the *Fol*-infested sandy soil, and after four weeks the root symptom severity and the plant dry weight were assessed. Severity of plants grown in soils incubated at room temperature decreased by over 40%, and more than 73% when incubated at 35ºC, regardless of the rate of pellet of poultry manure. That was the most effective amendment in reducing the viability of *Fol*, and consequently the disease severity. Instead, ORC reduced the viability of *Fol* at any dose, but the severity was significantly reduced only at the highest dose (3%), both at RT and at 35ºC. Furthermore, both PPM and ORC differed in their effects on tomato bioassays, the former enhanced up to 3.5-fold plants dry weight, as a result of reducing tomato symptoms severity.

It was concluded that the combined effect of organic soil amendments and incubation at moderate temperatures, as an approach to sub-optimal soil solarization, reduced *Fol* propagules and therefore the disease severity on tomato plants. Managing manure with pellet, when combined with the incubation of soil is a method of effective control against FV tomato. The management with pellet of poultry manure, when combined with soil incubation, is an effective control measure against FW of tomato.

The use as soil amendments of agricultural and fish-processing industrial by-products such as poultry manure, pelleted poultry manure, olive residue compost, and fish emulsion, is found as an effective method of managements of FCRR of asparagus and FW of tomato, while significantly reducing the dependency on chemical pesticides and fertilizers for achieving sustainable farming systems.

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INTRODUCCIÓN GENERAL Y OBJETIVOS

INTRODUCCIÓN GENERAL

LA PODREDUMBRE DE RAÍCES Y CORONA DEL ESPÁRRAGO

El espárrago (*Asparagus officinalis* L.) es una planta herbácea perenne de la familia *Liliaceae* cuyo cultivo puede dar rendimientos económicamente rentables durante 10-15 años, después de tres años de establecimiento del cultivo. Esta planta fue domesticada en Grecia hace más de 2000 años (Bailey y Bailey, 1976), posiblemente desde formas silvestres originarias de Asia, pero fueron los romanos quienes introdujeron este cultivo en Europa septentrional. La planta de espárrago está formada por tallos aéreos ramificados y una parte subterránea, denominada "garra", constituida por un tallo subterráneo denominado corona o rizoma, plataforma a partir de la cual se producen las yemas que darán lugar a los turiones o espárragos, y las raíces principales o de almacenamiento.

La producción mundial de espárragos ha tenido recientemente un creciente auge, aumentando el 40% de la superficie en los últimos 20 años (FAO, 2011), por ser un producto preferencial en el mercado internacional que permite obtener elevados beneficios, dado el incremento del consumo y la variedad de preparaciones. España es el sexto país en importancia de producción de espárrago a nivel mundial, y segundo de la UE con 11.047 ha y producciones de 58.400 toneladas anuales (FAO, 2011), siendo uno de los principales cultivos hortícolas en el país, cuya superficie se ha expandido mucho en el sur durante las dos últimas décadas, principalmente porque pueden exportarse espárragos frescos a otros países de la UE a principios de temporada (Serrano, 2003). Sin embargo, en el resto de España la superficie ha disminuido un 40% en los 10 últimos años, concentrándose más del 70% de la producción en Andalucía, principalmente en Huétor-Tájar (Granada) (www.magrama.gob.es).

La Podredumbre de raíces y corona (PRC) causada por *Fusarium* spp. es una de las principales enfermedades del espárrago, con importancia económica en todo el mundo (Elmer, 2001). Esta enfermedad fue descrita primero en Massachusetts a principios del siglo XX (Stone y Chapman, 1908) y desde entonces se ha convertido en un factor limitante en las principales zonas productoras de espárrago (Elena y Kranias, 1996; Elmer *et al.*, 1996). En España esta enfermedad fue detectada por primera vez a finales de los 70 del siglo pasado, resultando afectados por la enfermedad el 60% de los campos inspeccionados en el norte del país 10 años después (Tello *et al.*, 1985). La PRC está asociada con el decaimiento prematuro de las plantaciones y con problemas de replantación. Afecta a plantas adultas, pero también devasta plántulas y plantaciones jóvenes (Elmer, 2001).

Sintomatología

Los síntomas de la enfermedad incluyen podredumbres y coloración vascular de raíces, rizomas y tallos; lesiones necróticas sobre las superficies de la raíz y del tallo; reducción del tamaño de la planta; amarilleo y senescencia de los tallos y turiones y, en casos graves, la muerte de la planta (Cohen y Heald, 1941; Johnston *et al.*, 1979; Schreuder *et al.*, 1995; Elmer, 2001; Corpas-Hervias *et al.*, 2006). Consecuentemente, el rendimiento del cultivo decae lentamente en cultivos de 5-10 años de edad, tanto por una menor producción individual de las plantas enfermas como por la disminución en la densidad de plantas debida a la muerte de las más afectadas (Grogan y Kimble, 1959) (Fig. 1).

Etiología

Agentes causales

La PRC es una enfermedad de etiología compleja, con varios *Fusarium* spp. patógenos de suelo como agentes causales asociados a las raíces de espárragos (Elmer, 2001). Las principales especies

implicadas son *Fusarium oxysporum* Schlecht. emend. Snyder y Hansen f. sp. *asparagi* y *F. proliferatum* (Matsush.) Nirenberg (Damicone y Manning, 1985; LaMondia y Elmer, 1989; Elmer, 2001; Schreuder *et al.*, 1995; Corpas-Hervias *et al.*, 2006). *F. redolens* Wollenw. f. sp. *asparagi* Baayen, morfológicamente similar a *F. oxysporum*, parece ser también un patógeno importante en el complejo de esta enfermedad (Baayen *et al.*, 2000b). *F. verticillioides* (Saccardo) Nirenberg (syn = *F. moniliforme* Sheld.) (Seifert *et al.*, 2003) y *F. solani* (Martius) Apple y Wollenw. emend. Snyder y Hansen, también han sido asociados con la PRC (Endo y Burkholder, 1971; Johnston *et al.*, 1979; Tu, 1980; LaMondia y Elmer, 1989; Schreuder *et al.*, 1995; Corpas-Hervias *et al.*, 2006). Además, otras especies de *Fusarium* spp., como *F. subglutinans* (Wollenw. y Reink.) Nelson, Tousson y Marasas, *F. culmorum* (W.G. Smith) Saccardo, *F. equiseti* (Corda) Saccardo y *F. lateritium* Nees, pueden ser componentes etiológicos de menor importancia del complejo de la enfermedad (Gindrat *et al.*, 1984; Tello *et al.*, 1985; Blok y Bollen, 1995; Durán, 2001; Elmer, 2001; Corpas-Hervias *et al.*, 2006). En el sur de España las especies más importantes asociadas con el decaimiento lento del espárrago son *F. oxysporum* f. sp. *asparagi*, *F. proliferatum*, *F. solani* y *F. verticillioides* (Corpas-Hervias *et al.*, 2006; Wong y Jeffries, 2006).

Figura 1. Plantación de espárrago afectada por la PRC (a). Necrosis en base del tallo y atrofia radical (b). Coloración vascular del rizoma (c). Necrosis en raíz principal y raíces secundarias (d).

Aunque los cultivos de *F. proliferatum* y *F. oxysporum* en Agar Patata Dextrosa (APD) suelen asemejarse, estas especies se distinguen fácilmente por la presencia de microconidias en cadena en *F. proliferatum* y la presencia de clamidosporas y microconidias en falsas cabezas en *F. oxysporum*. *F. solani* puede distinguirse de *F. oxysporum* al examinar la longitud de las fiálidas portadoras de microconidias (muy largas en *F. solani* y cortas en *F. oxysporum*) y por las propias microconidias: las de *F. solani* tienden a ser más anchas, de forma más oval y con pared más gruesa que las de *F. oxysporum* (Leslie y Summerell, 2006).

Distribución

La distribución de *Fusarium* spp. patógenos de espárrago varía entre cultivos y áreas, dependiendo de las condiciones ambientales que pueden favorecer diferencialmente una o varias de las especies (Blok y Bollen, 1996a; Schreuder *et al.*, 1995; Elmer *et al.*, 1997; Corpas-Hervias *et al.*, 2006; Vujanovic *et al.*, 2006; Wong y Jeffries, 2006).

F. oxysporum f. sp. *asparagi* y *F. proliferatum* son las especies predominantes en los cultivos de espárrago en EE.UU. (Elmer, 2001), y una o ambas de estas especies han sido aisladas de rizomas con síntomas en América, Australia, Europa, Japón, Nueva Zelanda, Sudáfrica y Taiwán (Tu, 1980; Schreuder *et al.*, 1995; Blok y Bollen, 1996a; Elmer *et al.*, 1997; Elmer 2001; Nahiyan *et al.*, 2011).

La tasa de aislamiento de *F. oxysporum* y *F. proliferatum* encontrados en cultivos de Sudáfrica y España fue similar (Schreuder *et al.*, 1995; Wong y Jeffries, 2006), pero *F. oxysporum* fue más frecuente en rizomas de viveros de espárragos en España (Corpas-Hervias *et al.*, 2006).

En muchas zonas frías de Europa, *F. oxysporum* f. sp. *asparagi* y F. *redolens* f. sp. *asparagi* están asociados a la podredumbre de rizomas y raíces del espárrago (Van Bakel y Kerstens, 1970; Baayen *et al.*, 2000b) y *F. culmorum* se asocia con la podredumbre del pie y el tallo (Baayen *et al.*, 2000a). En cambio, *F. proliferatum,* ocasiona la misma sintomatología que las dos primeras especies, más frecuentemente en climas más cálidos (Moretti *et al.*, 1997; Elmer, 2001), teniendo una presencia baja o nula en países del norte de Europa (Blok y Bollen, 1996a; Wong y Jeffries, 2006). Sin embargo, en estudios realizados en el este de Canadá, con condiciones de temperaturas muy bajas, se encontró frecuentemente *F. proliferatum* (Vujanovic *et al.*, 2006), contrastando con la falta de recuperación de aislados de *F. proliferatum* y *F. verticillioides* en los Países Bajos (Van Bakel y Kerstens, 1970; Blok y Bollen, 1996a), y la baja presencia de *F. proliferatum* en Reino Unido, donde *F. oxysporum* apareció como predominante (Wong y Jeffries, 2006).

Otras especies de *Fusarium*, como *F. acuminatum*, *F. avenaceum*, *F. chlamydosporum*, *F. equiseti*, *F. verticillioides*, *F. solani* y *F. subglutinans* han sido aisladas de campos de espárrago en Australia (Sangalang *et al.*, 1995), Europa (Blok y Bollen, 1997; Corpas-Hervias *et al.*, 2006; Wong y Jeffries, 2006), Sudáfrica (Schreuder *et al.*, 1995) y EE.UU. (Elmer, 2001).

Patogenicidad y rango de huéspedes

Muchas formas patógenas de *Fusarium* han sido reconocidas por su patogenicidad selectiva y designadas como *formae speciales*, una categoría intraespecífica para taxones caracterizados más patológica que morfológicamente (Ainsworth *et al.*, 1971).

Fusarium oxysporum f. sp. *asparagi* es un patógeno cortical relativamente específico del espárrago (Blok y Bollen, 1997) y, aunque fue descrito originalmente para los aislados de *F. oxysporum* patógenos de espárrago, otros estudios encontraron que esta patogenicidad también se produjo en aislados de *F. oxysporum* obtenidos a partir de un amplio número de huéspedes y con una gran diversidad genética (Elmer y Stephens, 1989; Elmer, 1991; Yergeau *et al.*, 2005; Vujanovic *et al.*, 2006;

Wong y Jeffries, 2006). Sin embargo, *F. proliferatum* y *F. solani* producen lesiones en un amplio rango de especies (Leslie y Summerell, 2006).

En un estudio llevado a cabo en los Países Bajos se concluyó que *F. oxysporum* f. sp. *asparagi* tiene un estrecho rango de huéspedes como patógeno restringido al espárrago, pero una amplia gama de huéspedes como parásito, colonizando tejidos radicales de otras 11 especies de plantas, como leguminosas (mostrando el guisante y el altramuz síntomas leves), zanahoria, maíz, remolacha azucarera, trigo y pastos, mientras que otros cultivos, como cebolla y puerro, estuvieron libres de la colonización de la raíz (Blok y Bollen, 1997). En contraste con estos resultados, en un trabajo reciente con aislados de *F. oxysporum*, *F. proliferatum* y *F. solani* recuperados a partir de plantas de espárrago sintomáticas en España, la colonización de las raíces de plantas de cebolla inoculadas con un aislado de *F. oxysporum* obtenido de espárrago, alcanzó un nivel similar a la del espárrago. Por otra parte, se observaron síntomas en raíces de plantas de ajo inoculadas con las tres especies de *Fusarium* estudiadas, y *F. solani* también fue patogénica para la cebolla. *F. oxysporum* y *F. solani* mostraron un cierto grado de especialización patogénica en la familia *Liliaceae* que contrasta con la falta de especificidad de *F. proliferatum* (Molinero-Ruiz *et al.*, 2011). En este sentido, análisis filogenéticos utilizando secuencias de traslación del factor de elongación 1 alfa (EF1α) indicaron que los aislados de *F. oxysporum* patógenos de espárrago están repartidos en varios subtipos dentro de las especies del complejo, lo que avala la hipótesis de que, en esta especie, la patogenicidad al espárrago es un rasgo relativamente no especializado (Wong y Jeffries, 2006).

Tanto *F. oxysporum* f. sp. *asparagi* como *F. proliferatum* son las especies predominantes y más patogénicas en la mayoría de los cultivos de espárrago. Sin embargo, la patogenicidad de *F. solani* en espárrago depende de los aislados: mientras que en EE.UU. y Sudáfrica los aislados recuperados eran no patogénicos o ligeramente virulentos (LaMondia y Elmer, 1989; Schreuder *et al.*, 1995), en Taiwán, España y Japón se encontraron aislados muy virulentos (Tu, 1980; Corpas-Hervias *et al.*, 2006; Nahiyan *et al.*, 2011), y en el Reino Unido se observó una gran diversidad patogénica (Wong y Jeffries, 2006).

Un método fácil y rápido para diferenciar subespecíficamente aislados de *Fusarium* spp. es su clasificación en grupos de compatibilidad vegetativa (VCG), es decir, aislados fúngicos cuyas hifas pueden establecer contacto, fusionarse y producir un heterocarión estable. *F. oxysporum* f. sp. *asparagi* es una *forma specialis* heterogénea compuesta por varias poblaciones distintas, ya que han sido identificados al menos 43 VCGs. Análogamente, la virulencia de *F. verticillioides* está presente en al menos 13 VCGs (Elmer *et al.*, 1996).

Epidemiología: supervivencia y factores que favorecen la infección

Por tratarse de un cultivo perenne, los patógenos de espárrago pueden permanecer todo el año en su huésped. *F. oxysporum* es más común en rizomas y raíces que *F. proliferatum*, debido a que la primera sobrevive muy bien en el suelo como saprófito y forma clamidosporas adaptadas a sobrevivir en el suelo durante muchos años, al igual que *F.solani*. Sin embargo, *F. proliferatum*, más frecuente en tallos, forma esporodoquios en los que produce conidias que pueden dispersarse con facilidad y rapidez por el aire o por salpicaduras del agua de lluvia o riego por aspersión, y además pueden sobrevivir en el suelo en forma de restos de micelio con paredes delgadas y endurecidas (Durán, 2001; Elmer, 2001).

Fusarium oxysporum y *F. proliferatum* se transmiten con las semillas y las garras de plantación (Inglis, 1980; Blok y Bollen, 1996; Doan y Carris, 1996; Durán, 2001; Elmer, 2001; Corpas-Hervias *et al.*, 2006). En las semillas pueden estar localizados en las rugosidades de su superficie, de las que pueden eliminarse por los tratamientos usuales de desinfección, o bien internamente, forma en la que pueden transmitirse (Inglis, 1980; Elmer, 2001). Los daños originados por las prácticas de cultivo o por

la incidencia de factores abióticos o bióticos, como la sequía, o por insectos y virus, predisponen a las infecciones por *Fusarium* en la planta (Stephens y Elmer, 1988; Nigh, 1990; Elmer *et al.*, 1996). La edad de la planta también influye en la severidad de los ataques, siendo las garras jóvenes más susceptibles a *F. oxysporum* f. sp. *asparagi* que las viejas (Grogan y Kimble, 1959).

La temperatura óptima para la infección es muy similar a la óptima de crecimiento del patógeno (Graham, 1955). Aunque el óptimo térmico para *F. oxysporum* y *F. verticillioides* en suelo es 25ºC. *F. oxysporum* predomina en suelos con capacidad de retención de agua del 21% y valores de pH neutro, en tanto que *F. verticillioides* ocurre más frecuentemente en suelos ácidos (pH 6) y algo más húmedos (26%) (Tu, 1985). El encharcamiento es muy perjudicial para el espárrago porque disminuye el oxígeno del suelo y favorece el crecimiento de *F. oxysporum* (Nigh, 1990).

El espárrago produce toxinas que limitan el establecimiento de nuevas plantaciones en campos previamente cultivados de espárrago. Al tratarse de un cultivo perenne que puede ser productivo hasta más de 20 años, se acumulan en el suelo constantemente patógenos y sustancias autotóxicas presentes en los tejidos del espárrago (Elmer, 2002). Además, los residuos radicales de espárrago en descomposición del cultivo anterior también liberan toxinas alelopáticas, especialmente compuestos aromáticos tales como los ácidos cumárico, cafeico y ferúlico (Hazelbrook *et al.*, 1989; Hartung y Putnam, 1990). Este factor, junto con la PRC, contribuye al problema del decaimiento prematuro de las plantaciones (Blok y Bollen, 1996a; Elmer *et al.*, 1996) y a la posterior problemática de replantación que se produce cuando se vuelve a plantar espárrago en suelos que anteriormente se han dedicado a dicho cultivo (Grogan y Kimble, 1959). La inhibición del crecimiento del espárrago podría mitigarse mediante la selección apropiada de variedades que reduzcan la persistencia de esas autotoxinas y la acumulación de aleloquímicos de residuos de raíces durante la replantación (Yeasmin *et al.*, 2013). Además, los *Fusarium* spp. no se ven afectados por las sustancias aleloquímicas, lo que les permite proliferar en ausencia de competidores (Blok y Bollen, 1996b). Estas toxinas inhiben a microorganismos beneficiosos, tales como las micorrizas arbusculares (Pedersen *et al.*, 1991; Matsubara *et al.*, 1995), *Trichoderma* spp. y *Gliocladium* spp . (Blok y Bollen, 1996b), causando la reducción del vigor de la planta y una mayor susceptibilidad a la PRC (Elmer, 2002).

Control de la Podredumbre de raíces y corona del espárrago

Para prevenir la PRC del espárrago y asegurar la rentabilidad de los cultivos, es necesario utilizar diferentes medidas de control, incluyendo el empleo de material de plantación y de suelo libres de inóculo (Di Lenna *et al.*, 1988; Blok y Bollen, 1996c). El uso de cultivares resistentes, el control químico, físico o biológico, o la combinación de varias de estas medidas de control se han ido utilizando a lo largo del tiempo para controlar o reducir el efecto de las pérdidas ocasionadas por esta enfermedad en el cultivo del espárrago.

Cultivares resistentes

La estrategia con más éxito para el control de enfermedades causadas por *Fusarium* en muchos cultivos hortícolas es el uso de variedades resistentes, cuando éstas están disponibles (Mace *et al.*, 1981; Fravel *et al.*, 2003). En espárrago no se dispone de cultivares bastante resistentes a *Fusarium* spp., debido a la dificultad de encontrar resistencia a estos agentes patógenos, aunque es bien conocida la existencia de una fuente de resistencia a *Fusarium* en *Asparagus densiflorus* (He *et al.*, 2001). Sin embargo, varios investigadores (Mc Collum, 1988; Marcellan y Camadro, 1996; 1999) han intentado, sin éxito, obtener híbridos de *A. densiflorus* y *A. officinalis* que mostraran la resistencia a *Fusarium* del parental resistente. No obstante, se ha incrementado el banco de germoplasma usado por los mejoradores con híbridos masculinos de espárrago, que suponen un avance significativo de la selección de cultivares resistentes a la PRC en campo (Ellison y Kinelski, 1985; Elmer *et al.*, 1996). La

falta de resistencia del huésped es debida, en parte, a la amplia diversidad genética dentro de las poblaciones de *Fusarium* spp. patógenas y a la falta de uniformidad genética de cultivares de espárrago (Stephens *et al.*, 1989; Blok y Bollen, 1997; Lassaga *et al.*, 1998). Estudiando la reacción de 11 cultivares diferentes de espárrago a las infecciones ocasionadas por aislados españoles de *F. oxysporum, F. proliferatum, F. verticillioides* y *F. solani*, los cvs. Dariana y Verde-Morado mostraron valores más bajos de severidad que los restantes, y mucho menores pérdidas de peso seco en las plantas inoculadas, en comparación con los testigos no inoculadas. Por el contrario el cv. Grande, ampliamente utilizado, fue el más susceptible a los aislados de *Fusarium* spp. evaluados (Corpas-Hervias *et al.*, 2006).

Control químico

Las especies de *Fusarium* que causan la PRC del espárrago, sobreviven en el suelo, lo que hace que su control por métodos químicos, como la aplicación de fungicidas durante el desarrollo de la planta, suela resultar ineficaz o difícil. Además, la escasa especificidad de estos productos afecta a toda la microflora presente en el suelo (Fravel *et al.*, 2003). La fumigación del suelo puede ser un método eficaz en la erradicación del inóculo del suelo pero, aparte de su elevado coste, plantea problemas ambientales y de seguridad (Fravel *et al.*, 2003). Además, ya no se permite el uso del Bromuro de Metilo, el fumigante más eficaz para la eliminación de patógenos de suelo, por su efecto destructivo sobre la capa de ozono de la estratosfera que protege la vida terrestre de las radiaciones nocivas (Gamliel *et al.*, 2000; Katan, 2000; Basallote *et al.*, 2010).

En el control químico de la PRC del espárrago, las estrategias utilizadas incluyen la desinfección de las semillas y de las garras de plantación, así como la fumigación del suelo. Las especies de *Fusarium* que causan la PRC en espárrago pueden ser transmitidas por las semillas; si se limitan a la superficie de estas, se pueden eliminar fácilmente mediante una desinfestación en dos fases, con hipoclorito sódico y con benomilo en acetona (Damicone *et al.*, 1981; Stephens y Elmer, 1988). El tratamiento de las garras con benomilo y captafol reduce los niveles de enfermedad por un período corto e incrementa el tamaño y el vigor de las plantas (Manning y Vardaro, 1977) aunque no siempre es efectivo (Lacy, 1979), y el uso de metil-tiofanato en las garras o su aplicación en los surcos de siembra puede mejorar, en ocasiones, el establecimiento del cultivo (Counts y Hausbeck, 2008). La fumigación del suelo alcanza un control parcial ya que los patógenos suelen volver a colonizar el suelo después del tratamiento (Manning y Vardaro, 1977; Lacy, 1979; Damicone *et al*, 1981). El uso de productos químicos fungicidas en los cultivos ofrece también posibilidades de controlar la PRC (Lacy, 1979; Falloon *et al*, 1989; Reid *et al.*, 2002). Así, en estudios en invernadero, los tratamientos con benomilo y fludioxonil incrementaron el peso de la raíz y redujeron la enfermedad en plantas de espárrago infectadas con *F. oxysporum* f. sp. *asparagi* y *F. proliferatum* cuando en el suelo había un bajo nivel de inóculo, mientras que, con una densidad de inóculo alta, fludioxonil, como fungicida de contacto, solo restringió la muerte de plántulas causada por *Fusarium* spp. (Reid *et al.* 2002).

Control cultural

Rotación de cultivos

La rotación de cultivos a largo plazo puede ser eficaz en la erradicación o reducción de la densidad del inóculo en el suelo (Curl, 1963), pero este método es impracticable en el cultivo del espárrago debido a su condición casi perenne, que restringe además determinadas labores culturales.

Esterilización del suelo

Otras estrategias para el control de las Fusariosis, que actúan directamente sobre el suelo, son la esterilización del mismo con vapor o agua caliente. Pero estos métodos, que pueden suprimir

eficazmente patógenos, malas hierbas, nematodos e insectos (Kita, 2004), son demasiado caros para la aplicación en campo, ya que requieren equipos costosos y un elevado gasto de combustible (Gamliel *et al.*, 2000; Katan, 2000), en tanto que el control por inundación del suelo requiere grandes cantidades de agua (Blok *et al.*, 2000).

Solarización del suelo

La solarización del suelo es un método hidrotérmico que aumenta la temperatura del suelo mediante la retención de la energía de la radiación solar, utilizando para ello láminas de plástico transparente que se depositan sobre el suelo previamente humedecido, reduciendo así los patógenos y la incidencia de enfermedades. En suelos solarizados se producen múltiples mecanismos que, primariamente, afectan a la inactivación térmica del patógeno, debida al incremento de la temperatura del suelo (Katan *et al.*, 1976), o al debilitamiento de los propágulos patógenos que se hacen más susceptibles a la competencia o actividad antagónica de la microflora autóctona del suelo (Stapleton, 2000). Muchos microorganismos saprofíticos, incluyendo varios antagonistas, son más tolerantes al calor que los patógenos de plantas (Stapleton, 2000). Además, al igual que otras técnicas de desinfección del suelo, la solarización suele promover el crecimiento de las plantas por mecanismos tales como la mejora de la estructura del suelo, la liberación de nutrientes minerales (Chen *et al.*, 1991) y la estimulación de rizobacterias promotoras del crecimiento de plantas (Gamliel y Stapleton, 1993). La principal limitación de esta técnica es el uso de materiales plásticos tradicionales, ya que la gestión de residuos plásticos (quema en la finca o en vertederos), tiene costes ambientales y económicos para los agricultores. Una posible solución sería el uso de plásticos biodegradables que se degradan poco a poco tras el laboreo, debido a la acción de los microorganismos del suelo (Al-Kayssi y Al-Karaghouli, 2002). La solarización, ya sea sola o combinada con enmiendas orgánicas, o la inundación del suelo, son alternativas eficaces para el control de fitopatógenos de suelo en áreas o en condiciones específicas (Blok *et al.*, 2000).

Enmiendas orgánicas del suelo

Las industrias agrícola y pesquera generan anualmente una cantidad impresionante de residuos orgánicos reciclables, como residuos de cultivos, estiércol y deyecciones animales, harinas vegetales y animales, etc. Su re-utilización en la agricultura implica que puedan ser evitados los métodos alternativos de eliminación, tales como su combustión o desecación, transformación e incluso su posible vertido al medio ambiente (Fig. 2).

La adición de materiales orgánicos a suelos o sustratos se ha utilizado comúnmente en los sistemas agrícolas para mejorar las condiciones del suelo para el crecimiento vegetal (Hader *et al.*, 1992; Lazarovits, 2001). Afectan a la aireación, la estructura, el drenaje, la capacidad de retención de agua, la disponibilidad de nutrientes y la ecología microbiana del suelo, cambiando drásticamente el ambiente para el crecimiento y la supervivencia de cultivos y microorganismos (Drinkwater *et al.*, 1995; Davey, 1996). Además constituyen una alternativa para proteger los cultivos de enfermedades causadas por patógenos de suelo, ya que suprimen una amplia variedad de estos (Lazarovits, 2001; Litterick *et al.*, 2004; Noble y Coventry, 2005; Bonanomi *et al.*, 2007; Avilés *et al.*, 2011). Sin embargo, los mecanismos de supresión aún no se comprenden completamente. Cualquier información sobre la forma en que las enmiendas orgánicas proporcionan supresión de la enfermedad es esencial para optimizar y mejorar la eficacia en la reducción de las enfermedades que estos materiales orgánicos puedan proporcionar (Lazarovits *et al.*, 2005).

Figura 2. Subproductos de la industria agroindustrial utilizados como enmiendas orgánicas del suelo: gallinaza (a), pellet de gallinaza (b), compost de alpeorujo (c).

Un efecto general, o a largo plazo, de las enmiendas orgánicas es su potencial para mejorar la actividad microbiana, incluidos los agentes de control biológico, conduciendo al establecimiento de condiciones naturales supresivas de la enfermedad (Hoitink y Boehm, 1999; McKellar y Nelson, 2003; Abbasi *et al.*, 2008). Las enmiendas orgánicas aumentan la actividad y la diversidad de las comunidades microbianas residentes por el aumento del contenido de materia orgánica del suelo enmendado y, por lo tanto, pueden proporcionar un ambiente más favorable para el control biológico natural de patógenos de suelo, mediante una mayor competencia por los nutrientes existentes en el suelo, aumentando así el efecto de los microorganismos antagonistas (Tsao y Oster, 1981; Conn y Lazarovits, 1999).

La supresividad de los composts a enfermedades de plantas se ha atribuido a interacciones complejas entre características abióticas y poblaciones de microorganismos y plantas, incluyendo competición microbiana por nutrientes y nichos ecológicos, antibiosis, producción microbiana de enzimas líticas, degradación de ácidos grasos, parasitismo, cambios en la disponibilidad de nutrientes e inducción de resistencia huésped (Hoitink y Boehm, 1999; McKellar y Nelson, 2003; Kavroulakis *et al.*, 2005; Borrero *et al.*, 2006; Pérez-Piqueres *et al.*, 2006; Avilés *et al.*, 2011). Así, el uso de compost mejoró el rendimiento y el número de turiones, así como la biomasa microbiana en plantaciones de espárrago con problemas de replantación (Ngouajio y Counts, 2012).

Los efectos a corto plazo pueden ser más específicos para un determinado tipo de enmienda, que se traducen en una reducción inmediata de inóculo del patógeno (Tsao y Oster, 1981; Tenuta y Lazarovits, 2002a; Mazzola *et al.*, 2007). Así, enmiendas con alto contenido en nitrógeno y ácidos grasos volátiles (AGV) causan casi una inmediata reducción en las poblaciones de patógenos a través de metabolitos tóxicos de corta duración (Tenuta *et al.*, 2002; Conn *et al.*, 2005; Lazarovits *et al.*, 2005). Los productos como estiércol de pollo o gallinaza, harinas animales y otros aplicados al suelo en grandes cantidades, se descomponen por los microrganismos del suelo y liberan grandes cantidades de amoníaco (NH_3) que, a niveles de pH neutro o ácido, se convierte en amonio (NH_4^+), elevando el pH. Si éste llega a 8,0 o más, el NH_4^+ (no tóxico para las plantas o los patógenos) se convierte en el NH_3 (gas volátil muy tóxico) (Tsao y Oster, 1981; Lazarovits, 2001; Tenuta y Lazarovits, 2002a). A través de la nitrificación bacteriana, el NH_4^+ se transforma en nitrito (NO_2^-) y luego en nitrato (NO_3^-). Si el pH baja por debajo de 5,5, el NO_2^- toma la forma HNO_2, que es extremadamente tóxico para los patógenos, pero también para muchos cultivos y semillas de malas hierbas (Lazarovits, 2001; Tenuta y Lazarovits, 2002a).

La presencia de estos productos activos depende en gran medida del contenido de materia orgánica del suelo, el pH del mismo y su capacidad de amortiguación (Tenuta y Lazarovits, 2002a). Los compuestos tóxicos volátiles también se pueden producir mediante la creación de condiciones anaeróbicas y reductoras en el suelo, mediante la incorporación de material orgánico fresco como el brócoli o el pasto (Blok *et al.*, 2000). En el manejo de la PRC del espárrago, el uso de estos abonos verdes combinado con la solarización, que mejora la concentración y la persistencia de las sustancias tóxicas en el perfil del suelo, redujo las poblaciones de *F. oxysporum* f. sp. *asparagi* tras una incubación de 15 semanas, debido a la liberación de compuestos volátiles tóxicos generados por los glucosinolatos presentes en residuos de crucíferas, tras las condiciones anaeróbicas desarrolladas simultáneamente (Blok *et al.*, 2000).

Las deyecciones líquidas ganaderas, como los purines de cerdo, tienen altas concentraciones de AGV tóxicos, como los ácidos acético, butírico, propiónico, valérico y otros (Tenuta *et al.*, 2002; Conn *et al.*, 2005), que mejoran el control de las enfermedades. Se ha encontrado que sólo los purines con cantidades suficientes de estos AGV proporcionaban una reducción significativa de la enfermedad, y sólo en los suelos donde el pH fue menor de 5,0, condición que permite la actividad biológica de los ácidos (Tenuta *et al.*, 2002; Conn *et al.*, 2005). En suelos con niveles de pH> 6,0, los AGV existen como sales que no tienen actividad biológica (Tenuta *et al.*, 2002). Los efectos de las enmiendas orgánicas del suelo sugieren que ambos componentes, químicos y biológicos, de estas pueden contribuir a la supresión de la enfermedad (Abbasi *et al*, 2002; Bulluck y Ristaino, 2002), y es probable que varios mecanismos contribuyan simultáneamente a la actividad con ciertos productos en algunos suelos.

La emulsión de pescado o de nutrientes solubles de pescado son subproductos líquidos de la industria de transformación de pescado y se han utilizado principalmente como fertilizantes (Ceci, 1975; Aung *et al.*, 1984). También se ha demostrado su uso eficaz en el control de enfermedades foliares (Abbasi *et al.*, 2003) y de suelo (Abbasi *et al.*, 2004; 2006). La aplicación de la emulsión de pescado como enmienda del suelo libera ácidos grasos volátiles, siendo el acético el más importante, cuya toxicidad es uno de los mecanismos de reducción de la enfermedad, junto con grandes cantidades de ácidos orgánicos presentes en la emulsión de pescado, que también son tóxicos para los patógenos (Abbasi *et al.*, 2006; 2009).

La enmienda del suelo con biocarbón ha sido estudiada en el control de la PRC del espárrago. El biocarbón es un producto mineral rico en carbono que puede resistir la descomposición de CO_2 durante siglos, lo que ayuda a la mitigación del cambio climático debido a la reducción de CO_2 en la atmósfera por el secuestro de carbono mediante la degradación térmica (pirolisis) de materiales orgánicos en ausencia casi completa de oxígeno (Lehman *et al.*, 2011). El biocarbón redujo la PRC tras su aplicación al suelo infestado por *F. oxysporum* f. sp. *asparagi* y *F. proliferatum*, y estuvo asociado con la colonización de las raíces por micorrizas arbusculares, lo cual resulta beneficioso para reducir la infección por los patógenos y, consiguientemente, el desarrollo posterior de la enfermedad (Elmer y Pignatello, 2011). Por otra parte, el efecto del biocarbón en la alcalinización de suelos ácidos podría contribuir a la supresión de la enfermedad, ya que disminuye la disponibilidad de Fe a los patógenos, reduciendo la enfermedad y promoviendo la actividad de microorganismos antagonistas.

Cloruro de sodio

En el control de la PRC del espárrago, el uso de cloruro de sodio (NaCl) como una enmienda del suelo puede ser una estrategia prometedora del manejo de la enfermedad, por ser el espárrago un cultivo muy tolerante a la salinidad. Su aplicación reduce significativamente las poblaciones de *F. proliferatum* y *F. oxysporum* de la rizosfera (Elmer, 1992). En experimentos en cámara de crecimiento, en invernadero y en pequeñas parcelas de campo, NaCl fue más eficaz que otras formas de sales de cloruro (CaCl₂, NH₄Cl y MnCl₂) en el control de la enfermedad causada por ambos patógenos, aunque esto no tuvo lugar en parcelas comerciales (Reid *et al.*, 2001; Elmer, 2003). El uso de NaCl proporciona además beneficios inmediatos en la supresión de la enfermedad y el aumento de los rendimientos, cuando las circunstancias obligan a restablecer espárragos en campos abandonados (Elmer, 2002). Por otra parte, esta es una práctica con mayor viabilidad económica que la fumigación del suelo (Manning y Vardaro, 1977). Sin embargo estas aplicaciones salinas pueden causar una pérdida de la estructura del suelo, su acidificación, desplazamiento de nutrientes, contaminación de las aguas subterráneas y daños en los cultivos de rotación sensibles (Elmer, 2003).

Control biológico

Otro método alternativo es el control biológico mediante el uso de Agentes de Control Biológico (ACB) a través de mecanismos como antibiosis, competencia, supresión, parasitismo, resistencia inducida, hipovirulencia y depredación. Ello puede implicar la pre-inoculación de las plantas con cepas no patógenas de *F. oxysporum* o el uso de microrganismos antagónicos.

En condiciones experimentales, el uso de cepas no patógenas de *F. oxysporum* ha sido efectivo en el control biológico de enfermedades de marchitez y podredumbre de muchos cultivos diferentes causadas por una diversidad de *formae speciales* de *F. oxysporum*. La capacidad de biocontrol se logra por mecanismos de acción que conforman la competencia por los nutrientes, que pueden privar a las clamidosporas de elementos requeridos para la germinación, la competencia con las cepas patógenas para los sitios de infección en las raíces, y la resistencia sistémica adquirida (SAR) provocada por reacciones de defensa de la planta. La eficacia del biocontrol depende mucho de las condiciones bajo las que se realiza la aplicación en campos comerciales (Chet, 1990; Fravel *et al.*, 2003).

El control biológico con *Trichoderma* spp. es una alternativa potencial al control químico de varios agentes fitopatógenos de suelo (Harman *et al.*, 2004). En particular, las especies de *Trichoderma* son eficaces en el control de enfermedades de podredumbre de raíces y corona en muchos cultivos (Verma *et al.*, 2007). Sin embargo, el nivel de control de enfermedades causadas por *F. oxysporum* ha resultado, en ocasiones, inadecuado o inconsistente (Elad *et al.*, 1980; Hadar *et al.*, 1979; Larkin y Fravel, 1998; Nel *et al.*, 2006). Es sabido que un aislado particular de *Trichoderma* puede ser diferencialmente eficaz contra diversos patógenos (Bell *et al.*, 1982; Harman *et al.*, 1989). En experimentos llevados a cabo en invernadero, con plantas de espárrago infestadas por *F. oxysporum* f. sp. *asparagi* y *F. proliferatum*, el tratamiento con *T. harzianum* T-22 aumentó el peso de la raíz y disminuyó la enfermedad, en comparación con el control infestado, cuando se utilizó un bajo nivel de patógenos, pero no fue eficaz a altas tasas de inóculo (Reid *et al.*, 2002).

Se ha observado en varios cultivos la capacidad de *Trichoderma* spp. para aumentar el crecimiento de plantas, con la mayoría de los aislados colonizando y creciendo en asociación con las raíces de las plantas (Harman, 2000; 2006; Harman *et al.*, 2004). Este mecanismo, designado como "competencia de la rizosfera" (Harman, 2000), parece ser operativo en el caso de los aislados de *T. asperellum* T108 y *T. harzianum* T2 (ambos recuperados de la rizosfera del espárrago), y promovió un mayor desarrollo de las partes aéreas de las plantas de forma simultánea a la colonización profusa de sus raíces. En pruebas de patogenicidad, el efecto antagonista de los dos aislados de *Trichoderma* spp. contra *F. oxysporum* y *F. proliferatum* en espárrago, resultó en el control de la enfermedad. Sin embargo, la eficacia del biocontrol ejercida por ambas cepas de *Trichoderma* dependió de la virulencia del aislado de *Fusarium* utilizado (Rubio-Pérez *et al.*, 2008).

Pseudomonas fluorescens es otro microorganismo beneficioso que está implicado en la supresión de la PRC del espárrago y puede ser un indicador útil de la salud del suelo y de la raíz (Elmer, 1995; 2003).

Los hongos formadores de micorrizas arbusculares también se han mostrado eficaces en el control de enfermedades ocasionadas por patógenos transmitidos por el suelo, mediante la inducción de proteínas de defensa de la planta (Somssich y Hahlbrock, 1998; Pozo *et al.*, 2002) y barreras físicas (Sharma *et al.*, 1992). Estos hongos son biotrofos que sobreviven en el sistema radicular hasta la madurez de los cultivos y, por lo tanto, pueden proporcionar a las raíces, a lo largo de todo el ciclo del cultivo, mayor resistencia contra patógenos de suelo (Sharma *et al.*, 1992). En plántulas de espárrago inoculadas con el hongo formador de micorrizas arbusculares *Glomus fasciculatum* (Thaxt. sensu Gerd.) Gerd. y Trappe y con *F. oxysporum*, en condiciones de campo y de invernadero, los niveles de enfermedad y de poblaciones rizosféricas de *F. oxysporum* fueron más bajos que en las plantas no inoculadas con *G. fasciculatum* (Wacker *et al.*, 1990).

LA FUSARIOSIS VASCULAR DEL TOMATE

El tomate (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) es una planta perenne de la familia *Solanaceae* de porte arbustivo, que se cultiva como anual. Esta planta es originaria de Colombia, Ecuador, Perú, Bolivia y Chile (Nuez, 2001) y se cultiva en todo el mundo para su consumo tanto fresco como procesado de diferentes modos.

El tomate es el segundo cultivo hortícola más importante en el mundo después de la patata ocupando una superficie de 4,7 millones ha, que producen más de 159 millones de toneladas. España es el noveno país en producción de tomates, dedicándose a este cultivo una superficie de 51.200 ha, y produciéndose anualmente unos 3,8 millones de toneladas (FAO, 2011).

La Fusariosis vascular (FV) o Marchitez vascular causada por *Fusarium oxysporum* está presente en la mayoría de las zonas productoras de tomate del mundo. Fue descrita por primera vez en Inglaterra a finales del siglo XIX (Massee, 1895). Esta enfermedad causa grandes pérdidas en las variedades susceptibles, y cuando las temperaturas aéreas y del suelo son muy altas durante gran parte del periodo de cultivo. Las plantas infectadas se atrofian y, rápidamente, se marchitan hasta que

finalmente mueren. Ocasionalmente, campos enteros de tomates mueren o quedan gravemente dañados antes de la recolección (Jones *et al.,* 1991; Agrios, 2005). En las áreas del sur de EE.UU. y Europa, la enfermedad es más destructiva en cultivos al aire libre, pero en las zonas del norte está limitada, por las bajas temperaturas, a cultivos en invernadero (Jones *et al.*, 1991).

Sintomatología

Los primeros síntomas de la enfermedad consisten en un ligero aclaramiento de las nervaduras de los folíolos jóvenes más externos. Las plantas infectadas en la etapa de plántula suelen marchitarse y mueren poco después de la aparición de los primeros síntomas. En las plantas más viejas, se aclaran las nervaduras de sus hojas y se produce epinastia foliar, la planta se achaparra, amarillean sus hojas inferiores, se forman ocasionalmente raíces adventicias, se marchitan sus hojas y tallos jóvenes, observándose defoliación, necrosis marginal de sus hojas persistentes y, finalmente, la muerte de la planta. Con frecuencia, estos síntomas aparecen sólo en un lado del tallo y avanzan hacia la parte superior de la planta hasta que destruyen el follaje y se produce la muerte del tallo. Los frutos que ocasionalmente resultan infectados se pudren y desprenden, sin que aparezcan manchas en ellos. Las raíces también resultan infectadas y, después de un período inicial en el que las plantas quedan achaparradas, se pudren sus raíces laterales más pequeñas. En cortes transversales del tallo, cerca de la base de la planta infectada, se puede observar un anillo marrón en el área de los haces vasculares (Jones *et al.*, 1991; Agrios, 2005) (Fig. 3).

Figura 3. Síntomas de la FV en tomate: amarilleo y marchitez (a-c), necrosis de raíces (d-e), retraso del crecimiento y muerte (f).

Etiología

Agente causal

La FV del tomate está causada por *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W. C. Snyder y H. N. Hans., ascomiceto mitospórico presente en la mayoría de las zonas productoras de tomate. Este patógeno produce tres tipos de esporas asexuales: microconidias, producidas con más frecuencia en el interior de los vasos de la planta infectada; macroconidias, que aparecen con gran frecuencia sobre la superficie de la planta destruida por el patógeno; y clamidosporas, que sobreviven en el suelo durante más tiempo (Agrios, 2005; Leslie y Summerell, 2006).

Patogenicidad

Dentro de una *forma specialis*, suelen distinguirse las razas por su patogenicidad específica para diferentes cultivares. Hasta la fecha hay descritas tres razas de *F. oxysporum* f. sp. *lycopersici*, las razas 1, 2 y 3 (Clayton, 1923; Alexander y Tucker, 1945; Grattidge y O'Brien, 1982) siendo la raza 3 la más devastadora. Las razas 1 y 2 están muy generalizadas en todo el mundo. La raza 3 se encontró en 1966 en Brasil, y posteriormente se ha citado en diversos lugares, pero su actuación más intensa parece ocurrir en Australia (Grattidge y O'Brien, 1982).

Aunque en bastantes *formae especiales* se ha comprobado que los grupos de compatibilidad vegetativa (VCG) están correlacionados con las razas, en *F. oxysporum* f. sp. *lycopersici* los VCG y las razas no están estrechamente relacionados. Algunos VCG de este patógeno contienen múltiples razas, y las razas 1 y 2 de *F. oxysporum* f. sp. *lycopersici* se han encontrado en múltiples VCG (Elias y Schenieder, 1991).

Epidemiología: supervivencia y factores que favorecen la infección

Entre cultivos, el patógeno sobrevive en los restos de plantas infectadas, principalmente en forma de clamidosporas, pero también como micelio. Cuando las plantas sanas se desarrollan en suelo infestado, los tubos germinativos de las esporas y el micelio penetran directamente los ápices radiculares en los puntos de su intersección con las raíces laterales, o a través de heridas. El micelio del hongo se propaga intercelularmente a través del córtex de la raíz y penetra en los vasos del xilema. Se produce la obstrucción de los vasos por el micelio, esporas, geles, gomas y tilosas, al tiempo que se observa déficit hídrico de las plantas infectadas. Cuando el volumen de agua disponible para las hojas es inferior al requerido para su funcionamiento, se cierran los estomas, las hojas se marchitan y, como consecuencia, muere la planta. El hongo invade entonces masivamente los parénquimas de las plantas, llega a la superficie de los tejidos muertos y allí genera esporas profusamente (Jones *et al.*, 1991; Agrios, 2005).

En general, los factores que favorecen el desarrollo de la enfermedad son las temperaturas del suelo y del aire en torno a 28ºC, y una humedad del suelo óptima para el crecimiento vegetal. La diseminación de *F. oxysporum* f. sp. *lycopersici* a larga distancia se produce a través de semillas, material de plantación o suelo. La maquinaria agrícola, las salpicaduras de lluvia o el viento favorecen la dispersión a corta distancia (Jones *et al.*, 1991; Agrios, 2005).

Control de la Fusariosis vascular del tomate

Como se ha comentado anteriormente en el manejo de la PRC del espárrago, es importante el uso de material de plantación y de suelo libres de inóculo para prevenir la enfermedad.

Cultivares resistentes

En el control de la FV del tomate, la utilización de cultivares resistentes ha sido el medio más eficaz para el control de la enfermedad (Beckman, 1987; Amini 2009). Sin embargo, es posible la aparición de nuevas razas del patógeno que superen la resistencia del huésped, por mutación aleatoria espontánea o parasexualidad, en los cultivares actualmente cultivados (Sidhu y Webster, 1979; Beckman, 1987; Tello y Lacasa, 1988; Jones *et al.*, 1991; Cai *et al*, 2003) por lo que se necesitan opciones alternativas para el manejo de *F. oxysporum* f. sp. *lycopersici*.

Control químico

Al igual que las especies de *Fusarium* que causan la PRC del espárrago, *F. oxysporum f. sp. lycopersici* sobrevive en el suelo, dificultando su control por métodos químicos. En varios estudios realizados sobre el control químico de la FV del tomate, tanto *in vitro* como en invernadero, se ha determinado que el uso de algunos fungicidas sistémicos pueden controlar la enfermedad, pero sólo parcialmente (Song *et al.*, 2004; Amini y Sidovich, 2010). En ensayos *in vitro* para controlar *F. oxysporum* f. sp. *lycopersici*, la mezcla de metamidoxime y oxicloruro de cobre tuvo un fuerte efecto sinérgico y podrían ser utilizados como base para formular un nuevo producto que controle las enfermedades de tomate (Nedelcu y Alexandri, 1995). También tiram y topsin-M fueron efectivos reduciendo las poblaciones de *F. oxysporum* f. sp. *lycopersici* en más de un 80% a los 45 días (Dwivedai *et al.* 1995). En experimentos *in vitro* y en invernadero, los fungicidas procochoraz (un imidazol) y bromuconazole (un triazol) fueron eficaces contra el patógeno, seguidos por benomilo, carbendazima y fludioxonil. Sin embargo, fludioxonil y bromuconazole fueron fitotóxicos en las plántulas de tomate (Amini y Sidovich, 2010). Otros compuestos químicos como el cloruro de cobre, el cloruro férrico y el sulfato de manganeso, también resultaron eficaces en el control de *F. oxysporum* f. sp. *lycopersici* mediante inducción de resistencia en plantas de tomate susceptibles (Mandal y Sinha, 1992). En el tratamiento de desinfección de semillas, el uso del fungicida sistémico carboxina, combinado con los fungicidas preventivos de amplio espectro tiram o captan, se mostró efectivo para eliminar *F. oxysporum* f. sp. *lycopersici* (El-Shami *et al.*, 1993).

Control cultural

Algunas de las técnicas de control cultural generales para *Fusarium* comentadas anteriormente para el manejo de la PRC del espárrago, como la rotación de cultivos y la esterilización y solarización del suelo, son aplicables también al control de la FV del tomate. El método de la rotación de cultivos también es impracticable en el cultivo del tomate debido a su carácter intensivo y altamente especializado, además de la práctica cada vez más extendida de cultivo fuera del suelo o en contenedores hidropónicos.

Enmiendas orgánicas del suelo

Para la FV del tomate, el uso de otras estrategias de control como la aplicación de enmiendas orgánicas se está convirtiendo en una alternativa eficaz para el control de esta enfermedad.

El aporte al suelo de abonos verdes, como los residuos de crucíferas, redujo la FV del tomate disminuyendo el número de clamidosporas en el suelo (Smolinska, 2000), y la combinación de residuos de hojas forestales con estiércol animal incrementó el porcentaje de plántulas sanas al menos en un 60%, especialmente con el uso del estiércol (Padmodaya y Reddy, 1999).

Los compost derivados de residuos agro-industriales, como los obtenidos del procesado de los residuos de almazara, usados como sustratos de crecimiento, disminuyeron el efecto de *F. oxysporum* f. sp. *lycopersici* y *Fusarium oxysporum* f. sp. *radicis-lycopersici* en plantas de tomate (Ntougias *et al.*, 2008; Kavroulakis *et al.*, 2010), debido a interacciones entre las características abióticas del suelo, cambios en las poblaciones microbianas y, tal vez, la reducción de la susceptibilidad de las plantas (Borrero *et al.*, 2004; Ntougias *et al.*, 2008; Avilés *et al.*, 2011). Los compost de orujo de vid y de corcho redujeron el efecto de *F. oxysporum* f. sp. *lycopersici* en plantas de tomate, debido a las poblaciones microbianas que intervenían en la supresividad del sustrato (Borrero *et al.*, 2004; 2006). Otros compost, como los derivados de lodos de aguas residuales fueron efectivos en la supresión de la enfermedad en la etapa temprana de crecimiento de las plantas (Cotxarrera *et al.*, 2002). El vermicompost, o compost de lombriz comercial, tiene un efecto supresor de los hongos patógenos e inhibió la infección de plantas de tomate por *F. oxysporum* f. sp. *lycopersici* al incrementar considerablemente el número total de microorganismos y de poblaciones de bacterias y hongos antagonistas (Szczech *et al.*, 1993; Szczech, 1999).

La combinación del aporte de enmiendas orgánicas con solarización del suelo, precedida por su riego, resultó efectiva contra una amplia gama de patógenos de suelo, entre ellos *F. oxysporum* f. sp. *lycopersici* (Momma *et al.*, 2005), ya que se redujeron considerablemente los niveles de propágulos infectivos por las condiciones de agotamiento de oxígeno y la reducción de los valores del potencial redox. Sin embargo, cuando se aplican estos tratamientos por separado, la inactivación de los patógenos es mucho más limitada (Blok *et al.*, 2000; 2008).

Control biológico

Se han utilizado numerosos ACB para la protección de plantas de tomate contra la enfermedad de la FV, que incluyen diversas especies fúngicas, como *Trichoderma spp.*, *Pythium oligandrum* y *Penicillium oxalicum* (De Cal *et al.*, 2000; Floch *et al.*, 2003; Silva y Bettiol, 2005; Mohamed y Haggag,2006) y bacterianas, como *Achromobacter xylosoxydans*, *Brevibacillus, Pseudomonas* y *Streptomyces* (Ramamoorthy *et al.*, 2002; Minuto *et al.*, 2006; Moretti *et al.*, 2008; Chandel *et al.*, 2010).

La cepa T34 de *T. asperellum*, aislada de un compost supresivo para *Fusarium* (Trillas y Cotxarrera, 2003) es un agente de control microbiano eficaz contra la FV del tomate (Cotxarrera *et al.*, 2002; Borrero *et al.*, 2012), permitiendo el uso de fuentes de amonio en la solución nutritiva en cultivos hidropónicos, como la perlita, sin un riesgo excesivo de incrementar la enfermedad (Borrero *et al.*, 2012).

La capacidad de *Brevibacillus brevis* para influir en el desarrollo de la FV del tomate fue investigada en plantas crecidas en placas de Petri y en macetas en invernadero, reduciéndose notablemente el desarrollo de síntomas en ambos sistemas, además de que se potenció el incremento de altura de las plantas y la longitud de las raíces (Chandel *et al.*, 2010).

Streptomyces griseoviridis, comercializado como biofungicida, puede controlar la enfermedad mediante la colonización de la rizosfera antes de ponerla en contacto con los patógenos. Este formulado fue muy eficaz contra *F. oxysporum* f. sp. *lycopersici* en suelos infestados artificialmente (Minuto *et al.*, 2006).

El uso de cepas no patógenas de *F. oxysporum* y *F. solani* ha proporcionado reducciones significativas en la incidencia de la enfermedad, mostrándose éstas como ACB eficaces (Larkin y Fravel, 1998). Numerosos estudios que utilizan *P. oxalicum* y *F. oxysporum* no patógenos (Duijff *et al.*, 1998; Larena *et al.*, 2003; Shishido *et al.*, 2005; Alam *et al.*, 2011), han demostrado una reducción significativa en la incidencia y severidad de esta enfermedad.

Entre los ACB utilizados contra la FV del tomate se encuentran también los hongos formadores de micorrizas arbusculares (Akköprü y Demir 2005; Steinkellner *et al.*, 2012; Hage-Ahmed *et al.*, 2013) y algunas rizobacterias (Alabouvette *et al.*, 1985; Duijff *et al.*, 1999; Shanmugam y Kanoujia, 2011). Ambos tipos de microorganismos, miembros importantes de la rizosfera, son simbiontes eficaces que protegen las plantas contra los patógenos, incrementando el crecimiento de aquéllas y su tolerancia a diversos factores de estrés.

BIOLOGÍA MOLECULAR

La complejidad etiológica de la PRC del espárrago comprende la asociación de varias especies de *Fusarium* como agentes causales, por lo que es indispensable la identificación precisa y rápida de estos patógenos para reducir sus efectos perjudiciales en los cultivos.

El gran desarrollo de las técnicas de biología molecular que ha tenido lugar en las dos últimas décadas ha hecho posible que su aplicación alcance disciplinas como la Patología Vegetal (Bridge *et al.*, 1998). Así, el empleo de modernas técnicas moleculares ha hecho posible la identificación rápida y objetiva de los hongos a nivel interespecífico, e incluso intraespecífico (Nitschke *et al.*, 2009). Se han utilizado herramientas experimentales basadas en técnicas de biología molecular, tales como la reacción en cadena de la polimerasa (PCR), para investigar la diversidad genética entre los aislados de *Fusarium* spp. de muchos cultivos. Para examinar la diversidad de especies de *Fusarium* en el cultivo del espárrago se han utilizado métodos tales como la electroforesis en gel con gradiente desnaturalizante (PCR-DGGE) (Yergeau *et al.*, 2005), el análisis de PCR con cebadores especie-específicos (Mulè *et al.*, 2004) o el análisis de polimorfismos en la longitud de los fragmentos de restricción (RFLP) (von Bargen *et al.*, 2009). El análisis con inter-secuencias simples repetidas (ISSR), un nuevo tipo de marcador molecular de ADN polimórfico mediante un único cebador, se utiliza también

para explorar las diferencias intra e interespecífica de una amplia variedad de *Fusarium* spp. (Bayraktar y Dolar, 2011; Vitale *et al.*, 2011).

Para mejorar eficazmente el manejo de la FV del tomate es fundamental un conocimiento previo y preciso acerca de las razas y de la densidad del patógeno en el suelo. Para ello es necesario el uso de métodos o técnicas de detección y cuantificación precisa y fiable de *F. oxysporum* f. sp. *lycopersici* en suelo. Se han aplicado de manera rutinaria para la cuantificación de hongos, los métodos basados en el cultivo microbiológico, tales como la dilución del suelo, con la siembra en medio selectivo y la enumeración de los propágulos de hongos usando unidades formadoras de colonias (UFC), (Bridge y Spooner, 2001).

Mediante la tecnología de PCR cuantitativa en tiempo real (qPCR) se puede cuantificar con precisión la biomasa del patógeno. Las aplicaciones de esta técnica se basan en el uso de fluorocromos inespecíficos (Holland *et al.*, 1991) o sondas específicas (sondas de hibridación o sondas exonucleasa) (Schaad *et al.*, 2003). Las técnicas basadas en sondas específicas, como las sondas TaqMan, permiten medir la producción de productos de PCR mediante un sistema de sondas marcadas mediante dos fluorocromos. Esta tecnología se está utilizando cada vez más en Fitopatología para la detección y cuantificación precisa de los agentes fitopatógenos, incluso cuando los niveles de infección son muy bajos.

Por otra parte, el uso de subproductos de la industria agrícola y de procesamiento de pescado, como el pellet de gallinaza, el alpeorujo compostado y la emulsión de pescado, como enmiendas orgánicas del suelo es una alternativa para reducir la severidad de estas enfermedades al tiempo que se reducen grandes cantidades de residuos orgánicos de estas industrias, y la dependencia de los fertilizantes químicos para lograr sistemas agrícolas sostenibles. Para ello es necesario estudiar los mecanismos asociados con el uso de estos productos en los patosistemas de espárrago y tomate.

OBJETIVOS

1. Determinar la eficacia de la combinación de temperatura, periodo de incubación del sustrato y diferentes enmiendas orgánicas del sustrato en el control de la Podredumbre de raíces y corona causada por *F. oxysporum, F. proliferatum y F. solani*, evaluando su impacto sobre el desarrollo de la enfermedad en las plantas de espárrago.

2. Identificar y caracterizar aislados de *Fusarium* de plantaciones comerciales de espárrago asociados con la Podredumbre de raíces y corona, y determinar la eficacia de diferentes enmiendas orgánicas en el control de la enfermedad evaluando su impacto en las poblaciones del patógeno y bacterianas del suelo, y en el desarrollo de la enfermedad.

3. Determinar la eficacia de diferentes enmiendas orgánicas en combinación con la temperatura de incubación del suelo en la Fusariosis vascular del tomate evaluando el papel de las concentraciones de NH_3 y HNO_2 en el desarrollo de la enfermedad, y el desarrollo de protocolos de PCR cuantitativa en tiempo real para lograr la detección y cuantificación de *F. oxysporum* f. sp. *lycopersici* en el suelo.

OBJECTIVES

1. To determine the efficacy of temperature and duration of substrate incubation in combination with different organic amendments applied for the control of Fusarium crown and root rot caused by *F. oxysporum, F. proliferatum and F. solani*, and evaluation of their impacts on disease development in asparagus plants.

2. To identify and characterize *Fusarium* isolates from asparagus fields, associated to Fusarium crown and root rot, and to determine the efficacy of different organic amendments in the control of this disease by the evaluation of impacts on soil populations of the pathogen and total bacteria, and on disease development.

3. To determine the efficacy of different organic amendments in combination with temperature of soil incubation on Fusarium wilt of tomato, evaluating the role of NH_3 and HNO_2 concentrations on disease development, and update real time PCR protocols to achieve detection of *F. oxysporum* f. sp. *lycopersici* in the soil.

CAPÍTULO I

Effect of substrate incubation temperature and soil organic amendments on the control of Fusarium crown and root rot of asparagus caused by *F. oxysporum*, *F. proliferatum*, and *F. solani*

Effect of substrate incubation temperature and soil organic amendments on the control of Fusarium crown and root rot of asparagus caused by *F. oxysporum*, *F. proliferatum*, and *F. solani*

ABSTRACT

Fusarium oxysporum (*Fo*), *F. proliferatum* (*Fp*) and *F. solani* (*Fs*) are the causal agents associated with the roots of asparagus causing crown and root rot of asparagus, a disease inflicting serious losses worldwide. Substrate mixtures were artificially infested with either *Fo*5*, Fp*3 or *Fs*2 and thereafter amended with either poultry manure (PM), the pellet of this amendment (PPM), each at 1 and 2% concentrations, or olive compost residue (ORC) at 3 and 6% concentrations. The propagule viability of *Fusarium* spp. isolates was determined on a substrate artificially infested and amended which was incubated at 30 or 35°C for different periods (15, 30 and 45 days). Generally, the viability of the inocula was significantly affected by temperature and incubation period, resulting in a further reduction of the former when combined with the application of organic amendments. The greatest reduction in viability occurred with longer incubation times (45 days) and lower loss of viability was achieved at 30ºC than at 35°C, with PPM as the most effective organic manure amendment reducing the viability of *Fo*5 and *Fs*2. However, the viability of propagules of *Fp*3 was generally similar to the infested and un-amended control, especially when incubation was at 30ºC. After incubation, seedlings of asparagus cv. Grande were transplanted into pots containing substrates infested by the different species of *Fusarium*. After three months of incubation in greenhouse, the severity of symptoms in the root system and the fresh weight of the plants were assessed. Results showed highly significant decreases in the root symptom severity. *Fp*3 inoculum caused a lower severity than *Fo*5 and *Fs*2 isolates. After PPM treatment, reduction of severity was particularly higher at 30ºC (by 30 and 45 days in *Fo*5-infested substrate, and by 15 days in *Fs*2-infested substrate) as well as the higher PM doses (in *Fo*5 and *Fs*2-infested substrate incubated at both temperatures and, usually, for 30 and 45 days), and ORC (usually by 15-30 days of incubation). Fresh weight increases of asparagus plants were, generally, significantly high in *Fo*5 and Fs2 substrate infestations with any of the three organic amendments tested.

INTRODUCTION

Fusarium crown and root rot (FCRR), probably one of the most important diseases of asparagus in the world (Schreuder *et al.*, 1995; Elmer *et al*., 1996; Blok and Bollen, 1997), has a complex etiology, with several *Fusarium* spp. as causal agents associated with the roots of asparagus (Elmer, 2001). In Spain the most important species are: *Fusarium oxysporum* f. sp. *asparagi*, *F. proliferatum*, *F. verticillioides* (syn. *F. moniliforme*) and *F. solani* (Seifert *et al*., 2003; Corpas-Hervias *et al*., 2006; Wong & Jeffries, 2006). The FCRR acts on adult plants, but also devastates seedlings and young plantations. The *Fusarium* species involved in the FCRR depend on the geographic area, although its symptoms are similar. These include vascular staining and rotting of roots, rhizomes and stems; necrotic lesions on the surface of the root and stem; reduced plant size; yellowing and senescence of stems and crowns and, in severe cases, death of the plant (Johnston *et al*., 1979; Elmer, 2001). Consequently, crop yield declines slowly, because of the lower individual production of diseased plants and by the decrease in plant density due to the death of the most affected.

This disease is difficult to control due to, among other factors, the long-term survival of the pathogen in the soil and its easy propagation by planting material (Elmer, 2001; Corpas-Hervias *et al*, 2006). Furthermore, chemical treatments are ineffective or provide short term protection (Elmer, 2001). Moreover, the use of methyl bromide, the most effective for the suppression of soil pathogens, is no longer allowed, for its destructive effect on the ozone layer in the stratosphere that protects life on Earth from harmful radiation (Gamliel *et al*, 2000; Katan, 2000; Basallote *et al*., 2010).

Soil solarization is a non-chemical method of soil disinfestation that increases the soil temperature by retaining the energy of solar radiation using transparent polyethylene sheets, under suitable climatic conditions, to reduce pathogens and disease incidence. The multiple mechanisms involved affect the thermal inactivation of the disease, due to increased soil temperature (Katan et al., 1976), or the weakening of pathogen propagules that become more susceptible to competition or antagonistic activity of the indigenous soil microflora (Stapleton, 2000). Soil solarization, either alone or combined with organic amendments, or soil flooding, is effective in controlling many soilborne plant pathogens (Katan, 1981; Blok *et al*., 2000; Klein *et al.*, 2011; Melero-Vara *et al*., 2011).

Soil organic amendments (OAs) including animal manures, composts and green manures, may suppress or reduce many soilborne pathogens (Noble & Coventry, 2005; Bonanomi *et al.*, 2007; Avilés *et al.,* 2011). Organic amendments affect aeration and soil structure, drainage, water holding capacity, nutrient availability and microbial ecology of soil (Davey, 1996). The suppression of pathogens by incorporation of OAs in the soil depends on several mechanisms. A factor involved in the survival of the pathogen is the production of toxic compounds, such as volatile fatty acids (acetic, propionic and isobutyric acids) during the microbial degradation of manure. The mechanism of suppression of microsclerotia and other fungal propagules for nitrogen-rich amendments, such as poultry manure or its pelletized form, is due to the production of ammonia and nitrous acid following degradation of the amendments by microorganisms (Tenuta & Lazarovits, 2002). Another possible mechanism of action of the OAs, such as composts, is the decrease in the disease severity by significantly increases of microbial population present in the amendment (Tsao & Oster, 1981; Conn & Lazarovits, 1999). This leads to greater competition for nutrients in the soil and ecological niches, increasing the effect of antagonistic microorganisms, antibiosis, microbial production of lytic enzymes, fatty acid degradation, parasitism, changes in nutrient availability and induction of host resistance (Tsao & Oster, 1981; Hoitink & Boehm, 1999; Borrero *et al.,* 2006; Avilés *et al*., 2011).

More recently, a new method of soil disinfestation, biological disinfection was proposed to control soilborne pathogens, which combine the addition of OAs to irrigation and solarization creating soil anaerobiosis. This method was effective against a wide range of soilborne pathogens, including *F. oxysporum* f. sp. *asparagi* and *F. oxysporum* f. sp. *lycopersici* (Blok *et al*., 2000; Shinmura, 2000).

The objectives of this study were to determine the efficacy of heat-treatments at sublethal temperatures at several incubation periods combined with different organic amendments with by-products from the agricultural industry, such as poultry manure, pelleted poultry manure and olive residue compost, on pathogen population and the impact of these organic amendments on the root symptom development and on the fresh weight of asparagus plants.

MATERIALS AND METHODS

Fungal isolates

Three monoconidial isolates of *Fusarium* from our collection and maintained in sterile soil at 4ºC, i.e. *F. oxysporum* (*Fo*5), *F. proliferatum* (*Fp*3) and *F. solani* (*Fs*2), which had proven pathogenic to asparagus (Corpas-Hervias et al., 2006) were selected. Inocula of these isolates were obtained by incubation of flasks with sterile potato-dextrose broth (PDB) to which four actively growing mycelium disks on potato dextrose agar (PDA) plates per flask were added. Incubation of PDB cultures was for 7 days on an orbital shaker at 150 rpm continuously, at 25ºC and a 12 h photoperiod. Four-layer sterile gauze was used to filter the conidial suspensions recovered from the flasks, and then their concentrations were estimated using a hemocitometer.

Infestation of substrate

Sand:silt mixture (2:1, v/v) was used as substrate in pot experiments conducted in greenhouse. This substrate was autoclaved at 121ºC for 60 min, twice in consecutive days. Afterwards, a volume of each conidial suspension, calculated according to the concentration, was poured in each bag of sterile substrate in order to reach 10^5 microconidia·g^{-1}, and vigorously shaken under aseptic conditions trying to homogenize the distribution in the substrate. Sterile distilled water was added instead in the un-infested controls. Substrate bags were then incubated at 25ºC for 30 days in the dark, with aseptic aeration every other day in a laminar air flow chamber at the time that the content was homogenized again. Chlamydospore formation was confirmed in all infested bags after the incubation period.

Organic amendments

Three organic amendments (OA) obtained as byproducts of different agricultural activities, i.e., poultry manure (PM), pellet of PM (PPM) and olive residue compost (ORC) were tested in this study. Several physicochemical features of those are given in Table 1.

Effect of organic amendments and incubation period and temperature on the viability of propagules of *Fusarium* spp. pathogenic to asparagus

The three isolates of *Fusarium* spp. with two incubation temperatures (30 and 35ºC) during three periods (15, 30 and 45 days), and the three OA above mentioned, were included in this experiment. After the batches of substrate separately infested with the three isolates were incubated during 30 days, 45 g-aliquots were placed in sterile polypropylene 50-ml test tubes (25 mm diameter) and PM or PPM at rates 1 and 2% (w/w) and ORC at 3 and 6% rates were added separately and homogeneously mixed with the differently infested substrate, then closed with plastic plugs and incubated in the dark at 30 or 35ºC for 15, 30 and 45 days. There were nine replications for every combination of treatments.

Table 1. Nitrogen, phosphorous and potassium contents, pH and electric conductivity of the organic amendments used in this study

Organic amendments	Nitrogen (%)	Phosphorous (%)	Potassium (%)	pH	CE (dS/cm)
Poultry manure	2.4	3.4	1.9	7.5	2.9
Pellet of poultry manure	4.0	1.0	2.0	6.3	11
Olive residue compost	1.5	0.7	2.0	8.7	3.2-3.5

For determination of the inocula viability in the substrate, samples (1 g) were taken just after the application of organic amendments (time 0) as well as after incubation periods of 15, 30 and 45 days, then kept at 4-6ºC until lab processing. Plate dilution method was used with a semi-selective agar medium for *Fusarium* in

order to determine inoculum viability (Bouhot & Rouxel, 1971). Original suspensions were obtained by magnetic stirring for 1 min of the substrate samples in flasks with 150 ml sterile water-agar (0.1%), then serial dilutions 10^{-1} and 10^{-2} were made, and three aliquots of 1ml of each of them were transferred onto Petri plates with that medium (as replications). After incubation at 25ºC for 2 days in the dark followed by three additional days with a 12-h photoperiod, the colony-forming units (CFU) were enumerated in all plates. Inoculum viability was then expressed as log-transformed data of the CFU.g^{-1} substrate, and percentage reductions of viability were calculated by the quotients between the log-values of CFU for treated and un-treated samples.

Effect of organic amendments and incubation period and temperature on disease development

One bioassay was conducted in a greenhouse using the infested and amended substrate with seedling of asparagus cv. Grande, moderately susceptible to *Fusarium* spp. (Corpas-Hervias et al., 2006).

Asparagus seeds were surface-disinfested by dipping them for 3 min in aqueous solution of Na-hypochlorate at 20% (50 g/l active chlorine) supplemented with 0,05% Tween 20, then three times washed with sterile distilled water at 5 min interval. Thereafter, disinfested seeds were aseptically placed on Petri dishes (eight seeds per dish) with water-agar 0.6%, and incubated at 28ºC in the dark for 7-8 days. The germinated seeds were planted in flats with autoclaved sand (60 min at121ºC, twice in two consecutive days), and incubated for 2-3 weeks in a growth chamber (16 h fluorescent light at 23ºC and 8 h dark at 18ºC).

Infested substrate in each tube was added to each of three containers (6 cm x 6 cm x 18 cm) filled with 150 g of infested sand-peat mixture (1:1, v/v). As negative and positive controls, *Fusarium* un-infested and infested substrates lacking organic amendment, were used respectively. One asparagus seedling was transplanted to each container. The flats holding these containers with the plants were incubated in a greenhouse (photoperiod of 16 h and mean temperature ranging 20-24ºC) for 12 weeks, and watered as needed. There were three replications (containers) for each combination of treatments. Independent experiments were performed for every *Fusarium* spp. isolate with a three-factorial design, i.e., treatment OAs (PM, PPM and ORC), temperatures (30 and 35ºC) and period of incubation (15, 30 y 45 days). Disease symptoms (chlorosis, wilt and necrosis) development was observed weekly until the end of each experiment. Then, asparagus plants were pulled out the containers, their root systems were rinsed under tap water to allow the evaluation of severity of lesions affecting both the crowns, roots and aboveground plant parts (stems) using a percentage scale 1-100% of tissues showing chlorosis, necrosis or wilt (Molinero-Ruiz et al., 2011). Furthermore, fresh weight of plants from each treatment was recorded. Finally, isolation on PDA dishes was tried with samples of affected tissues, to confirm the infections due to *Fusarium*. Each experiment was repeated.

Data analyses

Homogeneity of variance for the experimental error between replications was shown for the separate analyses of every experiment. The percentages of severity of symptoms were angle-transformed prior to conducting the analysis of variance (ANOVA) and averages were compared by LSD test ($P \leq 0.01$). The ANOVA for the viability of *Fusarium* were performed after the log-transformation of CFU. For the experiments with different organic amendments of the substrate, average severities were compared by LSD tests ($P \leq 0.05$). Statistix 9.0 (Analytical Software, Tallahassee, FL, USA) was the program used for all the ANOVAs.

RESULTS

Effect of organic amendments and incubation period and temperature on the viability of propagules of *Fusarium* spp. pathogenic to asparagus

Fusarium oxysporum

After 15 days incubation at 30ºC there was a significant reduction of CFU of *Fo5* following the application of PPM-2% (Fig. 1A). When incubation duration was 30 or 45 days the reductions of viability of this pathogen

were of 22 and 54% or 26 and 76%, respectively for the amendments with PPM-1 and 2%; in addition to 25% reduction of *Fo*5 viability when substrate amended with PM-2% was incubated for 45 days, but not with PM-1% nor with un-amended control (Fig. 1A).

However, the incubation at 35ºC for 15 d determined reductions in the CFU of *Fo*5 with all organic amendments tested, which were significantly larger for longer periods of incubation, especially for PPM-1 and 2% amendments. Thus, for the latter rate viability reduction increased from 57 to 93% as the incubation increased from 15 to 45 days, but PM-1 and 2% amendments incubated at 35ºC achieved much lower reductions in *Fo*5 viability (7-27%). The amendment with ORC-6% incubated at 35ºC for only 15 days achieved CFU reductions of 31%, whereas the un-amended control determined 27% reductions for incubation periods of at least 30 days (Fig. 1B).
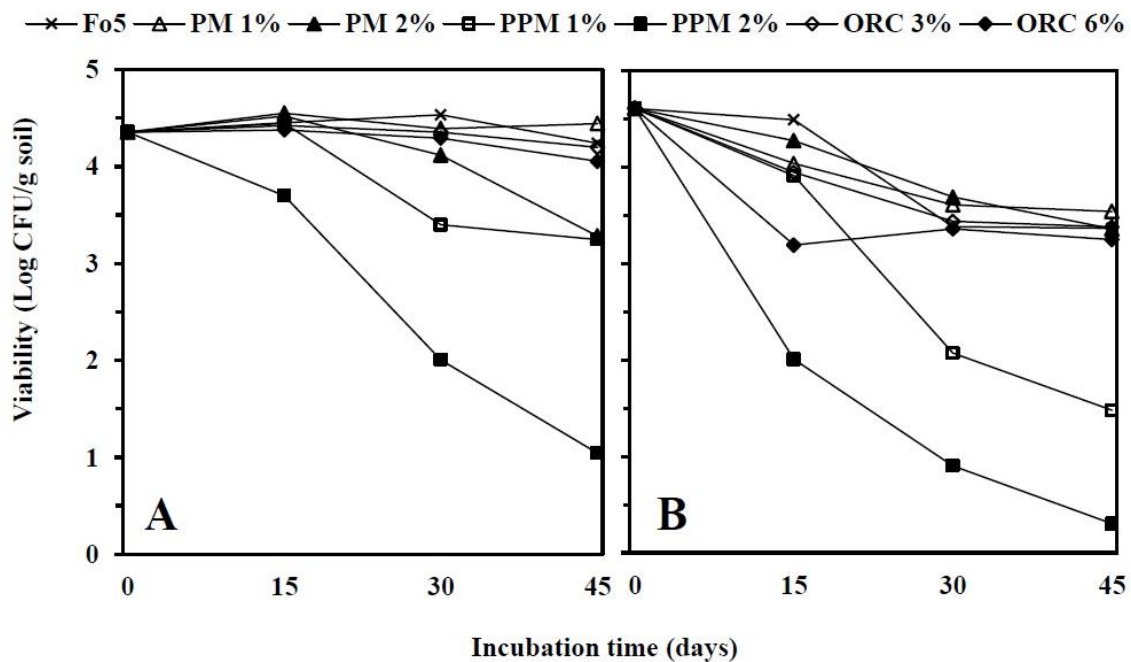
Figure 1. Viability of *Fo*5 isolate of *F. oxysporum* in a substrate incubated at 30ºC **(A)** and 35ºC **(B)**. After 30 days incubation with this pathogen, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)·g^{-1} soil, and are the average of results from two experiments, each with three replications for every treatment

Fusarium proliferatum

Viability of *Fp*3 propagules in all amended substrates was significantly reduced at both incubation temperatures regardless of the period of incubation, except for the PM-1% amendment (Fig. 2). When incubated at 30ºC amended substrates that viability decreased gradually with period of incubation, as compared to the un-amended control (Fig. 2). Amendments with PM-1 and 2% determined significant decrease in *Fp*3 viability, respectively reaching 17 and 10%, after 45 days incubation, whereas pathogen populations significantly decreased in 27 and 22%, respectively, after 45 days incubation with ORC-3 and 6% (Fig. 2).However, larger reductions were found in PPM-1 and 2%, respectively reaching 11 and 19%, and 28 and 54%, after 15 and 45 days incubation. In contrast, *Fp*3 populations increased slightly over initial inoculum after 15 and 30 days incubation periods (Fig. 2A*)*.

Viability of *Fp*3 propagules in all treatments incubated at 35ºC decreased progressively with time of incubation. The reduction in viability was over 44% after 15 days incubation for all the amendments except for PM-1 and 2% (only 19 and 27%) as well as for the un-amended control. Following incubation for 30 and 45 days, viability was reduced over 35 and 46%, regardless of the treatments (Fig. 2B). Maximal reductions were reached with PPM-1 and 2% amendments, for all incubation periods, although the lowest *Fp3* viability (93%) corresponded to incubation with PG-2% for 45 days (Fig. 2B).
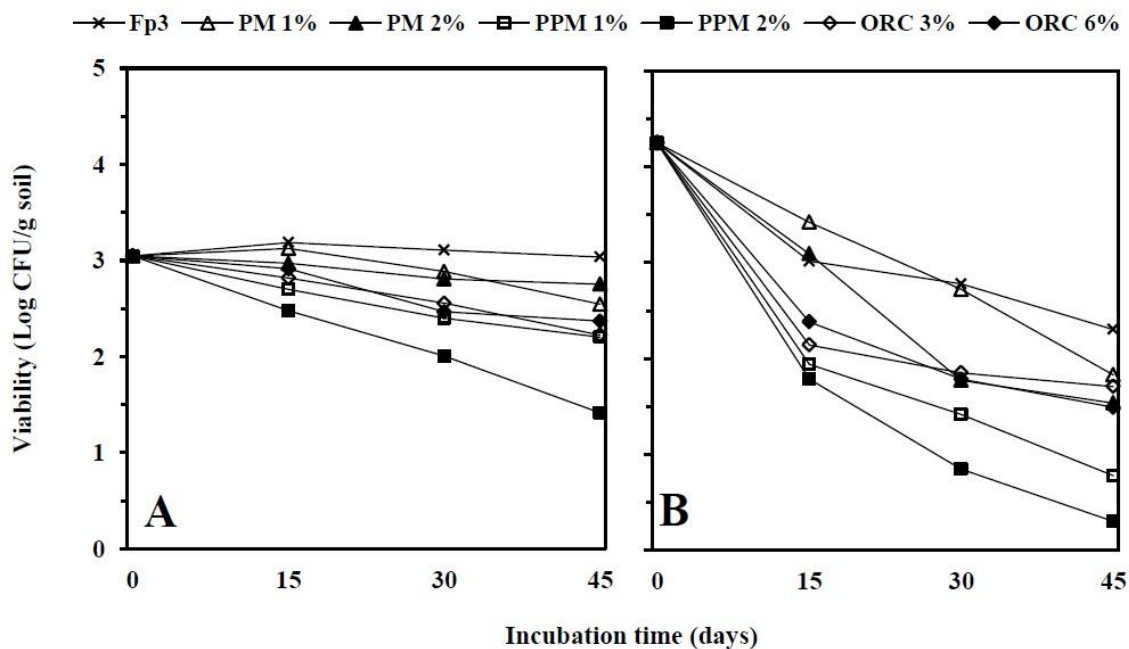
Figure 2. Viability of *Fp*3 isolate of *F. proliferatum* in a substrate incubated at 30ºC **(A)** and 35ºC **(B)**. After 30 days incubation with this pathogen, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)·g^{-1} soil, and are the average of results from two experiments, each with three replications for every treatment.

Fusarium solani

Viability of inoculum of *Fs*2 was significantly reduced both in all amended substrates and their un-amended controls, irrespective of temperature and period of incubation (Fig. 3).

PPM-amended substrates incubated at 30ºC for 15 days significantly reduced *Fs*2 viability (17 and 21%, for rates 1 and 2%, respectively). Amendment with PM at both application rates reduced *Fs*2 viability in 5-6%. After incubating for 30 days, following amendment with PPM-1 and 2%, viability was further decreases (31 and 51%, respectively) whereas ORC amendments achieved 18 and 26% reductions, according to rates. All amendments evaluated reduced inoculum viability by 23-67%, after 45 days incubation, in contrast with a decrease of only 13% for the un-amended control (Fig. 3A).

Viability of *Fs*2 was reduced by 21 and 27% in substrates amended with PPM-1 and 2%, respectively, which were incubated at 35ºC for 15 days. Similar conditions of incubation for ORC-amended substrate at 3 and 6% rates, determined 19% reduction of inoculum, and only in 15 and 12% for PM-1 and 2% amendments. The latter viability reductions were similar to that achieved in un-amended control (10%). Incubation for 45 days at

35ºC reached viability reductions over 65% for all amendments, which were maximal (83 and 100%) for PPM-1 and 2%, in contrast with the un-amended control (Fig. 3B).
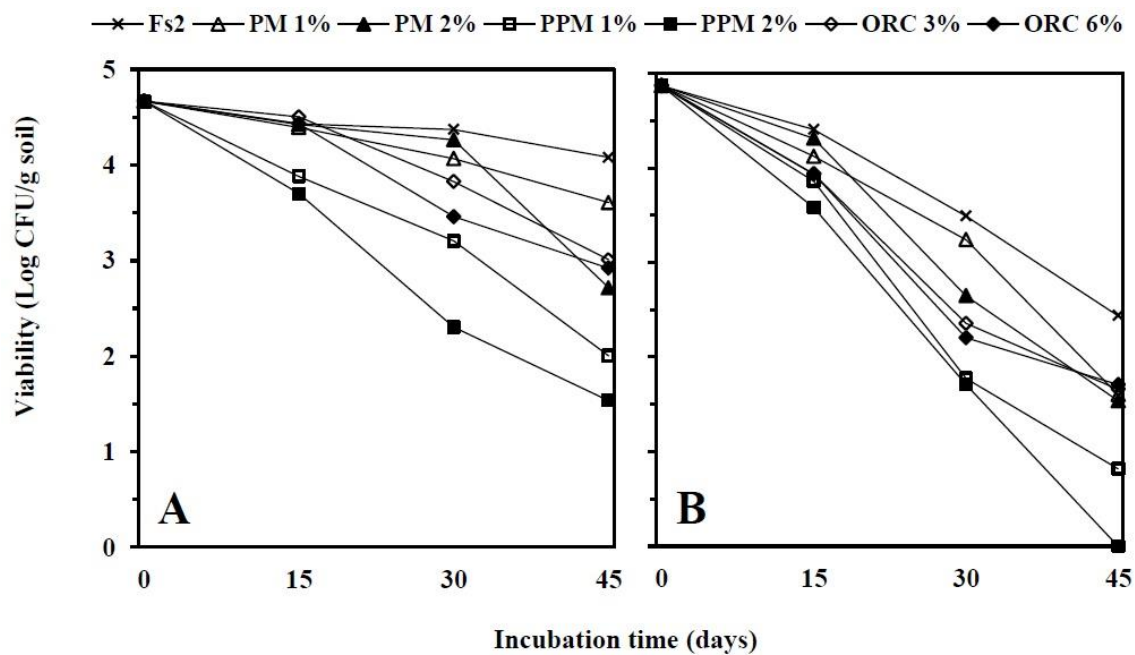
Figure 3. Viability of *Fs*2 isolate of *F. solani* in a substrate incubated at 30ºC **(A)** and 35ºC **(B)**. After 30 days incubation with this pathogen, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)·g^{-1} soil, and are the average of results from two experiments, each with three replications for every treatment.

Effect on the severity of root symptoms

Three months after incubation all the asparagus plants infected by *Fo*5, *Fs*2 and *Fp*3 showed symptoms typically associated with FCRR, such as brown or reddish-brown lesions of roots, necrosis in the insertion of feeding rootlets with storage roots, necrotic flecks, more or less extensive necrosis of the stem base and both types of roots, as well as wet rots of roots. Restricted growth of radical system is eventually observed in the very severe reactions.

Fusarium oxysporum

Addition of OAs to the substrate infested with *Fo*5 showed a significant ($P \leq 0.05$) effect on the reduction of symptoms severity due to FCRR, for most of the treatments evaluated (Fig. 4). After 15 days of substrate incubation at 30ºC, PM-1% and ORC-6% determined severity reductions much larger (65 and 76%) than the un-amended control (Fig. 4A). When incubated for 30 days at this temperature, symptoms severity was reduced by 39% for PM-1% and 2% treatments, by 27 and 88% for PPM-1 and 2%, and by 22 and 51% for ORC-3 y 6%, respectively. Severity reductions after 45 days incubation were maximal, when compared to untreated control of *Fo*5-infested substrate, the most effective amendments being PM-2% and PPM-1 y 2%, with reductions of 86, 77 and 89%, respectively (Fig. 4A).
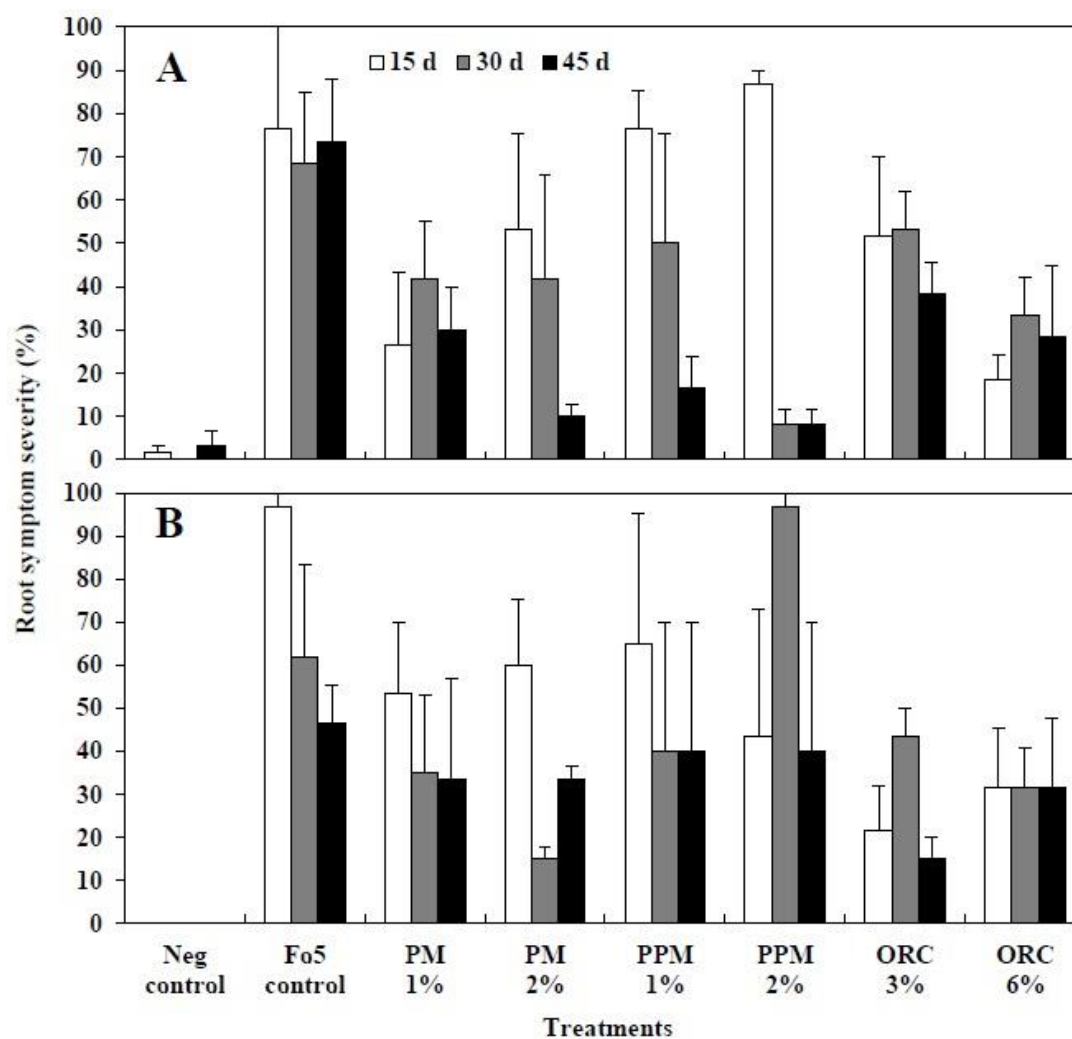
Figure 4. Severity of symptoms in root of asparagus 'Grande'. Seedlings were grown in *F. oxysporum* (*Fo*5) infested soils amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

Incubation at 35ºC for 15 days resulted in maximal severity reductions (78 and 67%, respectively for ORC-3 and 6% amendments), whereas largest reduction (76%) after incubation for 30 days, corresponded to PM- 2% treatment. For substrate incubated for 45 days, reductions in severity were lower than for shorter periods, being 68 and 32% for ORC-3 and 6% respectively, and 29 and 14% for amendments with PM-1 and 2%, and PPM-1 and 2%, respectively (Fig. 4B).

Fusarium proliferatum

Substrate incubation at 30ºC for 15 days, determined 92% reduction of symptoms severity when amended with ORC-3%. The maximal reductions of severity after incubation for 30 days were 89 and 83% respectively for PM-1 and 2%, and 78% for PPM-1%. The period of incubation of 45 days determined 78% reduction in severity for the amendment with PM-2%. All these reductions contrasted with the very high severity of symptoms for PPM2%, overcoming the values in the untreated, infested substrate (Fig. 5A).

After 15 days substrate incubation at 35ºC, all the amendments evaluated, with the exception of PPM-2%, significantly reduced symptoms severity at both concentrations, those reductions being 80 and 60% for PM-1 and

2%, 67 and 73% for ORC-3% and 6%, respectively, whereas for PPM-1 y 2% they were 53 and 13%. When the incubation period was 30 days, only ORC amendments, at 3 and 6%, reduced severity by 67% (Fig. 5B). After 45 days incubation, the most effective amendments were PPM-1%, PM-2% and ORC-3%, with severity reductions of 82, 72 and 72%, respectively.
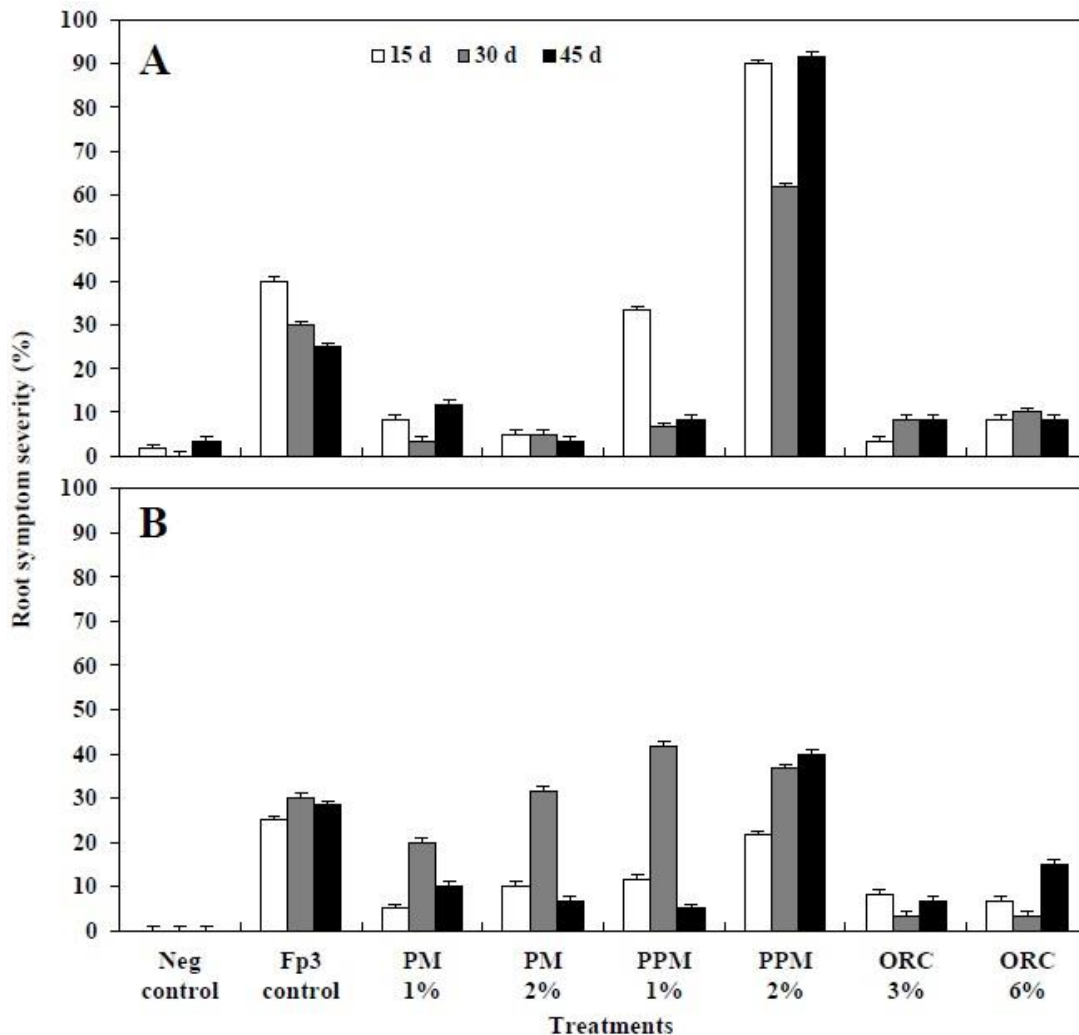
Figure 5. Severity of symptoms in root of asparagus 'Grande'. Seedlings were grown in *F. proliferatum* (*Fp*3) infested soils amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium solani

After 15 and 30 days incubation of the substrate infested with isolate *Fs*2 and amended with PPM-1%, reductions in severity of symptoms on asparagus cv. Grande were *ca.* 79 and 81%. Amendments with PM-1 and 2% respectively reduced severity by 17 and 41% after 15 days incubation and by 56 and 88% after 30 days incubation. Severity reductions of 59 and 72%, and 75 and 88% corresponded, respectively, to incubations for 15 and 30 days following amendments with ORC-3 and 6%. After 45 d incubation, PM-1 and 2% determined reductions by 50 and 83%, whereas 92% was found for ORC-6% amendment (Fig. 6A).

Severity reductions for *Fs*2 infested substrate, as compared to the un-amended control, were 60-92%, irrespective of the amendment, when incubation was at 35ºC for 15 days. However, after incubations for 30 days at this temperature, severity reductions were as high as high as 83 and 78% for amendments with PM-2% and ORC-6%, whereas it reached 83% for PM-2% after 45 days incubation (Fig. 6B). Surprisingly incubation for 30-45 days with PPM-2% increased severity over that of the un-amended infested control, at both temperatures, which was also true for the 30 days incubation at 35ºC when amended with PPM-1%.
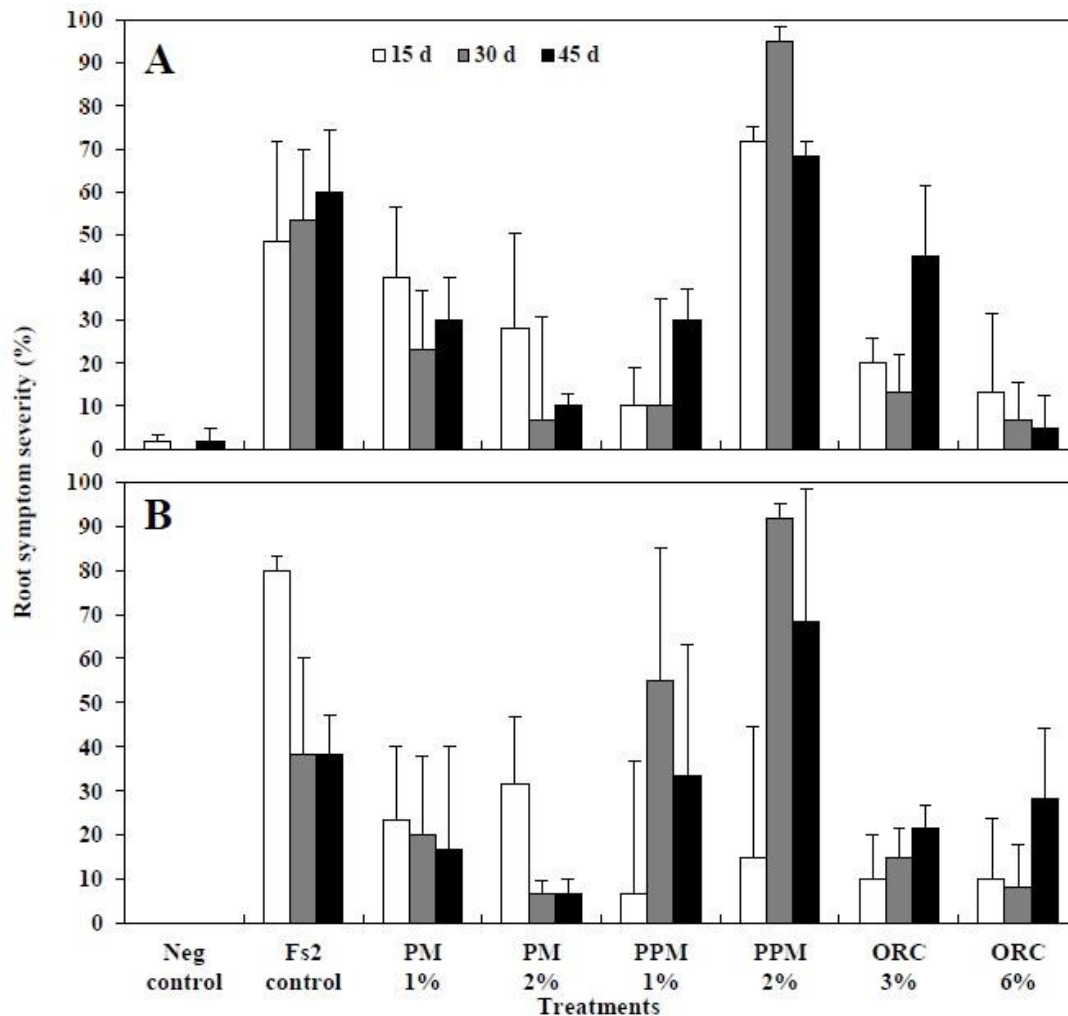
Figure 6. Severity of symptoms in root of asparagus 'Grande'. Seedlings were grown in *F. solani* (*Fs*2) infested soils amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

Effect on fresh weight of plants

Fusarium oxysporum

Fresh weight of asparagus plants grown on *Fo*5-infested substrate and incubated at 30ºC for 15 days, increased by 390 and 216% when amended with PM-1 and 2%, and by 173 and 193% for ORC-3 and 6%, respectively. Furthermore, incubation for longer determined weight increases over the infested, un-amended control, of 338 and 314% when PPM-2% was applied. However, when substrate was amended with PPM-1%, significant fresh weight increase (by 140%) only occurred after 45 days incubation (Fig. 7A).

When incubated at 35ºC for 15 days, all organic amendments provided significant increases of the fresh weight of asparagus plants. Incubation at 35ºC for 30 days after PM-2% amendment determined increase by 243% of the fresh weight, but PPM-2% required 45 days incubation to achieve fresh weight increase of only 57%, but maximal fresh weight of plants was achieved after only 15 days incubation, similarly to the amendment of the substrate with ORC-6% (Fig. 7B).
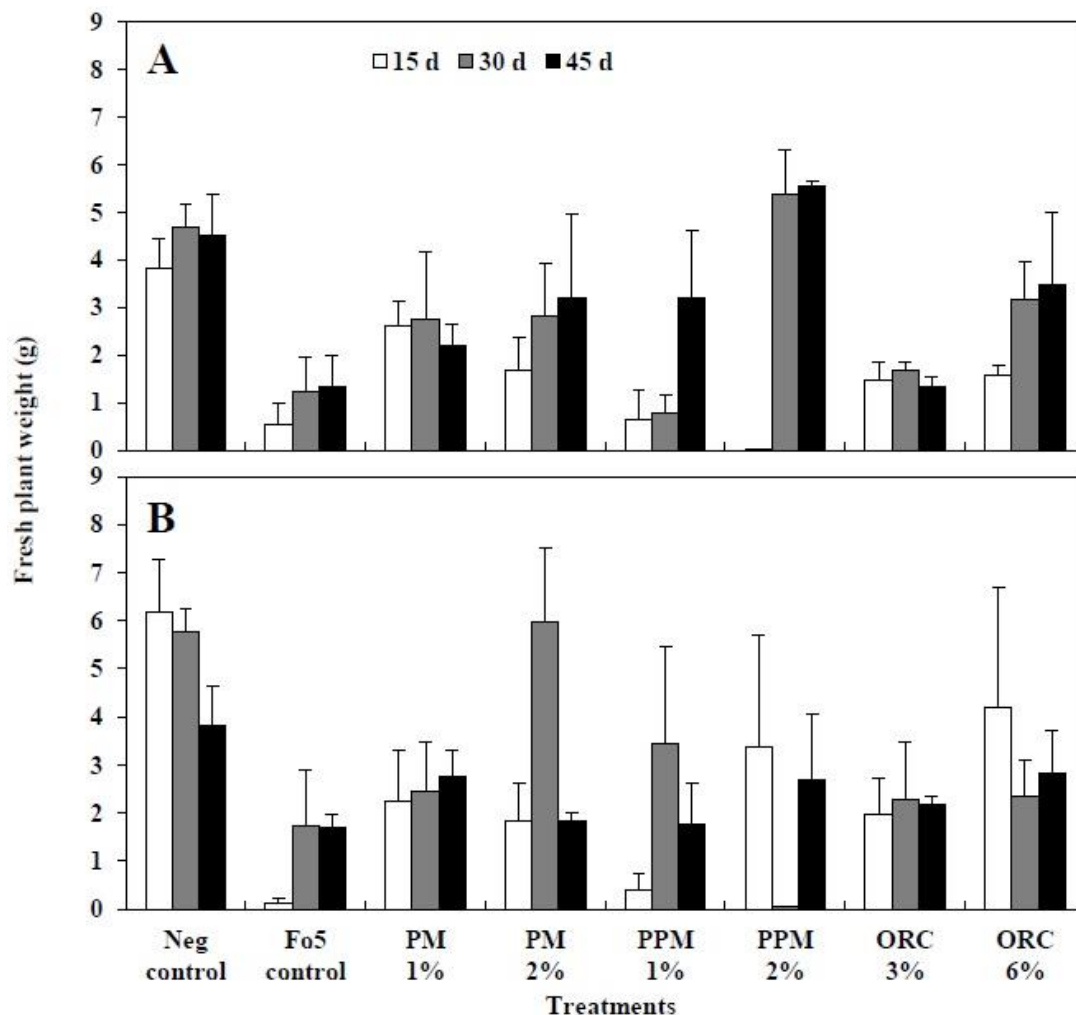
Figure 7. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in soils infested with *F. oxysporum* (*Fo*5) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium proliferatum

Fresh weight of plants grown on *Fp*3-infested substrate and incubated at 30ºC for 15 days, increased by 78% with PM-2% amendment, and by 80-85% for ORC-3 and 6%, respectively. As incubation period increased to 30 and 45 days, except for substrate amended with PPM-2%, fresh weight of asparagus plants increased, irrespective of the amendments tested and their rates, reach maximal weight increases to 172, 158% and 144%, respectively for amendments with PM-1%, PPM-1% and ORC-3% after 45 days incubation (Fig. 8A).

All the organic amendments increased fresh plant weight (by 64-238%) when incubated for 15 days at 35ºC. However, extending this incubation to 30 days determined only slight weight increases for PM-1% and ORC-6% (6 and 9%, respectively), whereas all the other treatments, as well as incubation for 45 days resulted in decreased fresh weight of plants (Fig. 8B).
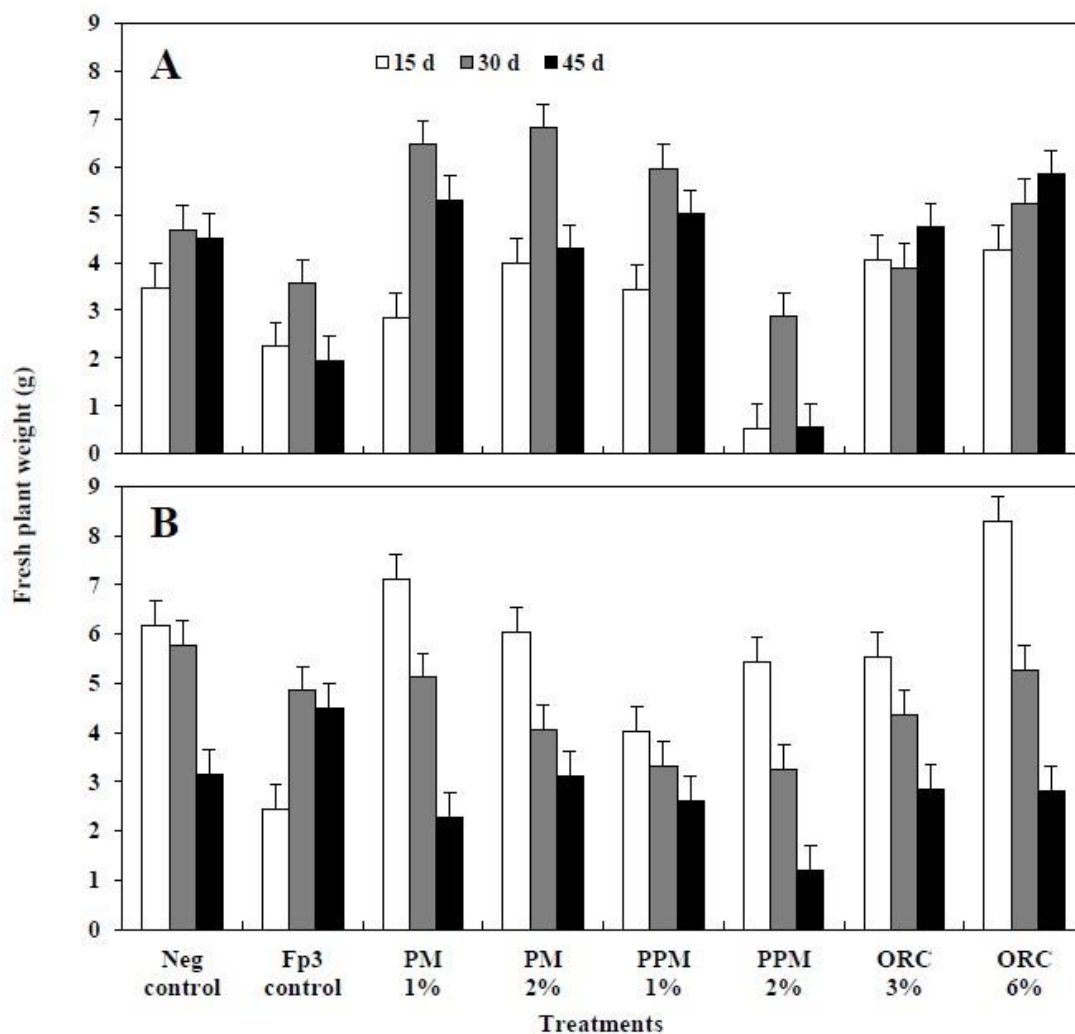
Figure 8. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in soils infested with *F. proliferatum* (*Fp*3) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium solani

Asparagus plants 'Grande' grown on *Fs*2-infested substrate incubated at 30ºC for 15 days following the amendment with PPM-1% achieved maximal increase of fresh weight (93%) whereas, after 30 days incubation, increases were slightly higher (104%) for PM-2% and PPM-1%, but still higher (118 and 162%) in substrates amended with ORC-3 and 6%, respectively. After 45 days incubation, all amendments evaluated increased the fresh weight of plants, with maximal increases (449%) for ORC-6%, and *ca*. 344% for PM-1 and 2% (Fig. 9A).

When substrate incubation was at 35ºC for 15 days, all the amendments determined asparagus fresh plant weight increases in the range 596-797%. After incubation for 30 days, only ORC-6% achieved significant increase of plant weight (163%). However, incubation for 45 days determined very low weight increases of plants grown in all amended substrates, except for PPM-2% and ORC-3% amendments, which decreased weight (Fig. 9B).
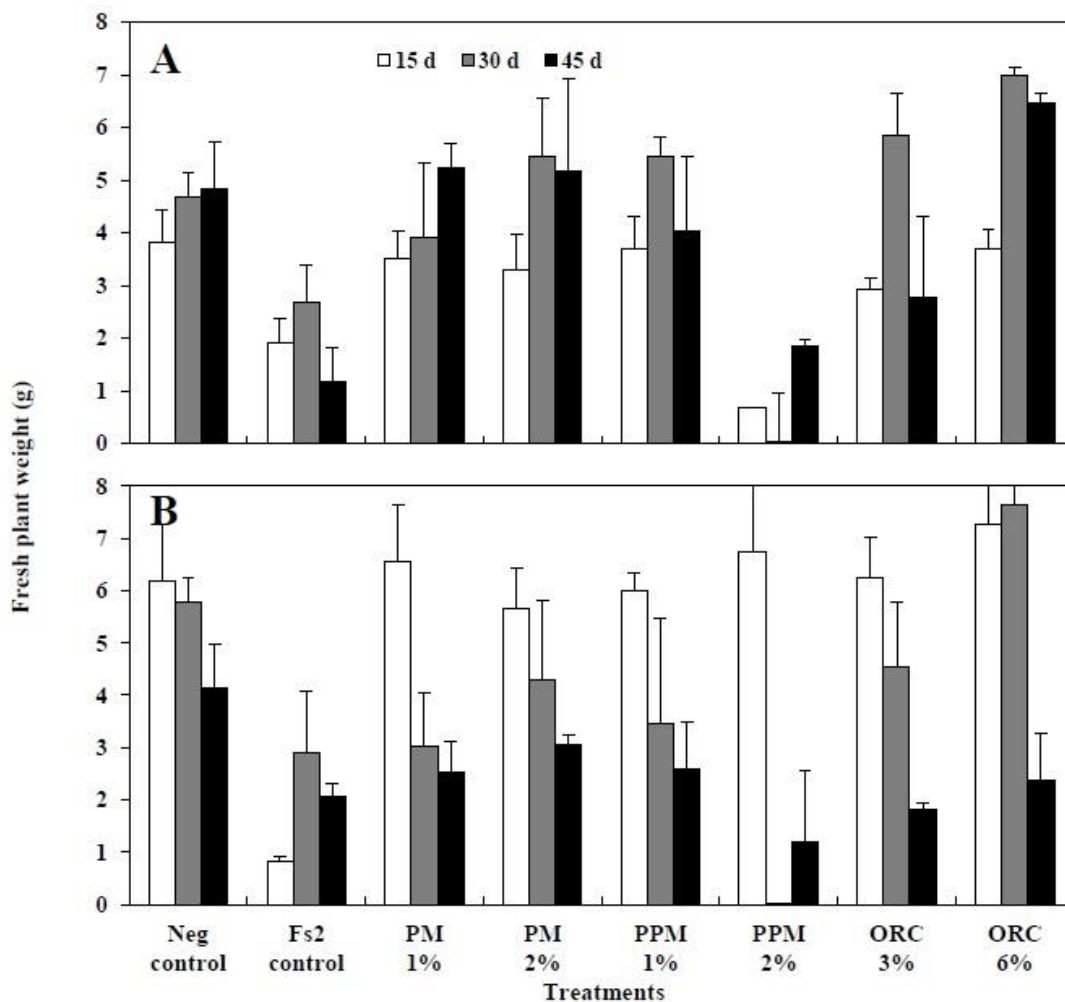
Figure 9. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in soils infested with *F. solani* (*Fs*2) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC **(A)** and 35ºC **(B)**. Values are the average of results from two experiments, each with three replications for every treatment.

DISCUSSION

Our results showed that temperature and period of incubation considerably affect the viability of the three isolates of *Fusarium* (*Fo*5, *Fp*3 y *Fs*2) studied, and combining this with the application of organic amendments to the infested substrates further increased the reduction of fungal propagules viability. This agrees with the loss of viability of several soil-borne plant pathogenic fungi, including *F. oxysporum* f. sp. *asparagi*, when amendment of soils with plant residues combined with soil solarization was performed (Blok *et al.* 2000).

Viability of inocula of *Fo*5 and *Fs*2 followed analogous patterns, since both started with densities in the substrate around 4.5-4.9 (log values) and *Fo*5 decreased to 3.5-4 for most of the treatments after incubation for 45 d, depending on temperature, whereas *Fs*2 viability decreased much faster (to 1.5-2.5). However, the most effective treatments were PPM-1 and 2%, with a more pronounced effect of the higher rate of amendment, reaching elimination of *Fs*2 after 45 day incubation at 35ºC, whereas decreases to 2.5 were also found for the un-amended substrate for similar conditions. This suggests a larger effect of temperature for *Fs*2 than in the case of *Fo*5.

We observed a progressive decline in pathogen viability as incubation period increased, which was more pronounced at 35 than at 30ºC, the most effective amendment being pellet of poultry manure for *Fo*5 y *Fs*2 isolates, and this effect was slightly larger with 2% than with 1% rate. This seems to relate to the higher N content in PPM (4%) as compared to the other amendments tested (2.4 and 1.5%). As a matter of fact, the N-richest compounds liberate and accumulate higher amounts of volatile toxic compounds such as NH_4 and HNO_2 during organic matter decomposition, which are able to eliminate many soil-borne fungi (Tsao & Oster, 1981; Tenuta & Lazarovits, 2002a). In contrast, isolate *Fp*3 usually kept inoculum viability much similar to those in the un-amended infested control, mainly when incubated at 30ºC, and even for PPM-1% treatment.

The symptoms we observed are coincident with those reported in asparagus crops affected by FCRR elsewhere (Blok & Bollen, 1995; Corpas-Hervias *et al.*, 2006; Elmer, 2001). Three months after asparagus seedlings cv. Grande were transplanted to pots with substrates infested with the different *Fusarium* spp. isolates, the effects of the substrate treatments were highly significant reductions of root severity symptoms. However, this severity decrease was much more pronounced for PPM-2 and 1% (mainly after 30-45 days incubation with PPM-1% at 30ºC in the case of *Fo*5, whereas 15 days incubation favored decrease of symptoms severity in the case of *Fs*2), as well as for PM-2% (when *Fo*5 and *Fs*2 were incubated, usually for 30-45 days, at both temperatures), and for ORC-3 and 6% amendments (mainly for *Fs*2 infestation, after 15-30 days incubation). Disease reduction after different organic amendments (including poultry manure) of soils infested with *F. oxysporum, F. solani* and *F. equiseti, F. oxysporum* f sp. *cumini* and *F. oxysporum* f sp. *spinaciae* were also reported by other research groups (Escuadra & Amemiya, 2008; Israel *et al.*, 2011; Martínez *et al.*, 2011).

The lower severity of root symptoms caused by isolate *Fp*3, as compared to *Fo*5 and *Fs*2, except for incubation at 30ºC of substrate amended with PPM-2%, is likely due to poor survival of *F. proliferatum* in bare soils, as this species does not produce chlamydospores; therefore the starting inoculum density was lower for this than for the two other species tested (Elmer *et al.*, 1996; Elmer, 2001). Furthermore, using *F. proliferatum* at 1.3 x 10^4 CFU·g^{-1} (similar to that in our experiments) Reid *et al.* (2002) showed a low root severity (20%), in agreement with our results for *Fp*3. For the other treatments, however, we found much lower root severity values than the un-amended control, mainly for ORC-3 and 6%, but also for PM-1 and 2% and PPM-1% treatments.

For substrate infested with *Fp3*, the lack of effectiveness of PPM-2%, regardless of temperature and period of incubation, together with the significant increase of root severity following substrate incubation at 30ºC, suggest that phytotoxic symptoms occur, but those seem to be reduced when incubation at 35ºC was conducted. The reason is likely that dissipation of the volatile compounds generated by PPM-2%, mainly ammonia, develops quickly at 35ºC (Lazarovits, 2001; Tenuta & Lazarovits, 2002a). To this regard it is interesting the comparison of N content and electrical conductivity between PPM and PM: those for PPM are, respectively, about double and 4-times that of PM (Table 1). A similar effect was also observed when high concentrations of PPM were applied to pots with substrate infested with *Fusarium oxysporum* f. sp*. dianthi* and incubated at high temperature before transplanting rooted cuttings of carnation (Nava Juárez, 2013), but these phytotoxic symptoms were absent from plants growing in greenhouse on soil to which equivalent dosages of PPM were applied (Melero-Vara *et al.,* 2011). This is likely due to a stronger confinement of toxic compounds in the reduced volume of pots.

Concerning the increases of fresh weight of asparagus plants, substrates infested with *Fo5* were significantly higher than the un-amended control for several treatments, depending on the particular combinations of amendment and period and temperature of incubation, except significant decreases in the case of PPM-1 and 2% incubated for 15 or 30 days and ORC-3% (particularly when incubated at 30ºC). In the case of *Fp3* decrease of fresh weight of plants was significant for PPM-2% amendment, mainly when incubation at 30ºC, but also with longer periods of incubation at 35ºC, thus confirming the phytotoxicity hypothesis suggested above. Nevertheless, plants fresh weight increases were more common in *Fs*2-infested substrate, although it is worthy to mention that most pronounced effects occurred after 15 days incubation, but again significant decreases were noticed for PPM-2% amendment, except for incubation at 35ºC during only 15 days. A similar effect of effectiveness optimization by the incubation at 35ºC was noticed for substrates incubated only for 15 days, whereas periods of 30-45 days incubation at 30ºC for amendments with PM-1 and 2%, and PPM-1% seemed to be optimal, in contrast with the dreadful results for PPM-2%. This seems to correlate with the severity of the root system observed after 3 months development of asparagus plants in pots with infested substrate organically amended.

Summarizing, asparagus disease levels decreased when the integration of temperature, period of incubation an organic amendment used resulted in reducing inoculums viability, which corresponded in most cases with longest periods of incubation. Therefore, transferring our results to the application of organic amendments to the soil in nurseries that produce planting material and to commercial asparagus fields requires experimental implementation before general advices are offered to the growers. Thus, it is convenient to determine the impact of such organic amendments on the pathogen and bacterial soil populations as well as on disease development, and to evaluate the role of NH_3 and HNO_2 on the latter. From these studies we should be able to conclude which organic amendments (mainly for PPM-2%) do not cause phytotoxicity problems for asparagus plants growing in nurseries and fields, such as it was considered for the control of Fusarium wilt of carnation (Melero-Vara et al., 2011).

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LITERATURE CITED

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CAPÍTULO II

Characterization of *Fusarium* isolates from asparagus fields in southwestern Ontario and influence of soil organic amendments on Fusarium crown and root rot

Characterization of *Fusarium* Isolates from Asparagus Fields in Southwestern Ontario and Influence of Soil Organic Amendments on Fusarium Crown and Root Rot

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ABSTRACT

Borrego-Benjumea, A., Basallote-Ureba, M. J., Melero-Vara, J. M., and Abbasi, P. A. 2014. Characterization of *Fusarium* isolates from asparagus fields in southwestern Ontario and influence of soil organic amendments on Fusarium crown and root rot. Phytopathology 104:403-415.

Fusarium crown and root rot (FCRR) of asparagus has a complex etiology with several soilborne *Fusarium* spp. as causal agents. Ninety-three *Fusarium* isolates, obtained from plant and soil samples collected from commercial asparagus fields in southwestern Ontario with a history of FCRR, were identified as *Fusarium oxysporum* (65.5%), *F. proliferatum* (18.3%), *F. solani* (6.4%), *F. acuminatum* (6.4%), and *F. redolens* (3.2%) based on morphological or cultural characteristics and polymerase chain reaction (PCR) analysis with species-specific primers. The inter-simple-sequence repeat PCR analysis of the field isolates revealed considerable variability among the isolates belonging to different *Fusarium* spp. In the in vitro pathogenicity screening tests, 50% of the field isolates were pathogenic to asparagus, and 22% of the isolates caused the most severe symptoms on asparagus. The management of FCRR with soil

organic amendments of pelleted poultry manure (PPM), olive residue compost, and fish emulsion was evaluated in a greenhouse using three asparagus cultivars of different susceptibility in soils infested with two of the pathogenic isolates (*F. oxysporum Fo*-1.5 and *F. solani Fs*-1.12). Lower FCRR symptom severity and higher plant weights were observed for most treatments on 'Jersey Giant' and 'Grande' but not on 'Mary Washington'. On all three cultivars, 1% PPM consistently reduced FCRR severity by 42 to 96% and increased plant weights by 77 to 152% compared with the *Fusarium* control treatment. Populations of *Fusarium* and total bacteria were enumerated after 1, 3, 7, and 14 days of soil amendment. In amended soils, the population of *Fusarium* spp. gradually decreased while the population of total culturable bacteria increased. These results indicate that soil organic amendments, especially PPM, can decrease disease severity and promote plant growth, possibly by decreasing pathogen population and enhancing bacterial activity in the soil.

Additional keywords: molecular analysis.

Asparagus (*Asparagus officinalis* L.) is a low-input and high-value perennial crop able to give profitable yields for 10 to 15 years after 3 years of crop establishment. Canada has a cultivated area of 1,559 ha and a production of 6,599 t/year (26) and >70% of asparagus is produced in Ontario. Fusarium crown and root rot (FCRR) is probably one of the most important diseases of asparagus worldwide (8,20,47). FCRR has been associated with asparagus decline, accounting for the reduction in quantity and size of the spears produced, and the death of plants (38). Furthermore, early decline or replant disease are denominations of the damage caused to asparagus crops when old asparagus fields are replanted with asparagus; yields are slowly reduced in 5 to 10 years on the old crop, and plants are unable to establish productive plantings (28).

The FCRR has a complex etiology, with the occurrence of several soilborne *Fusarium* spp. as causal agents associated with asparagus roots (17). *Fusarium oxysporum* Schltdl., *F. proliferatum* (Matsush.) Nirenberg, and, to a lesser extent, *F. culmorum* (W. G. Sm.) Sacc. are the main species involved in asparagus plantations in North America and recognized as causal agents of several symptoms, such as dwarf asparagus, wilt and root rot,

seedling blight, foot rot, and stem and crown rot (17). In Canada, asparagus crops also suffer from severe a FCRR problem (29,30) that frequently compromises the production profitability but there is little information available regarding the distribution and prevalence of *Fusarium* spp. associated with FCRR. The diversity of *Fusarium* populations in asparagus fields has been reported within the Quebec growing areas in eastern Canada (54,59,60).

With the development of modern molecular techniques, rapid and objective identification of fungi at species or even at intraspecific level is now possible (42). Polymerase chain reaction (PCR)-based techniques have been used to investigate the genetic diversity among isolates of *Fusarium* spp. of many crops. Methods such as PCR–denaturing gradient gel electrophoresis (58), PCR analysis with species-specific primers (39), or restriction fragment length polymorphism analysis (52) have been used to examine *Fusarium* spp. diversity in asparagus crop. Analysis with inter-simple sequence repeats (ISSR), a new kind of molecular marker of polymorphic DNA by a single primer, is also utilized to explore the intra- and interspecific differences of a wide variety of *Fusarium* spp. (6,51).

Although attempts have been made to control FCRR through cultural, biological, and fungicidal approaches (9,18,19,24,32,45), the management of FCRR can be challenging, particularly in the fields where disease has been established due to the long-term nature of this crop. The choice of land with no prior history of FCRR is usually the first measure to avoid an FCRR problem.

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Fungicide drench application of benomyl and fludioxonil to asparagus seedlings in the greenhouse were reported to increase asparagus root weight and decrease the disease under low levels of *F. oxysporum* and *F. proliferatum* (45). In contrast, application of these fungicides via drip irrigation in fields with a history of asparagus production resulted in a lack of significant differences among treated and untreated plots (16). Thus, the chemical control of this disease is not consistent and, additionally, it may have risks to humans and negative effects on the environment (27), therefore being incompatible for sustainable agriculture.

Alternatively, organic amendments can provide control of several soilborne plant diseases (4,11,33,35,43). Fusarium diseases have been managed with soil organic amendments in many horticultural crops, including asparagus, tomato, carnation, cucumber, strawberry, and spinach (9,13,21,23,25,31,37). In asparagus, soil amendments with fresh broccoli or grass subsequently covered with air-tight plastic significantly reduced the populations of *F. oxysporum* f. sp. *asparagi* after 15 weeks of incubation (9). Similarly, animal manures, such as raw or pelleted poultry manure, and composts derived from agroindustrial wastes, combined with the incubation of soil at different temperatures and periods, showed a reduction in the density of viable inocula of *F. oxysporum* f. sp. *asparagi*, *F. proliferatum*, and *F. solani* (12; A. Borrego-Benjumea, *unpublished data*). However, despite the importance of FCRR in North America, there is little information available regarding the management of this asparagus disease in Canada with soil organic amendments.

The objectives of this study were to identify and characterize the *Fusarium* spp. associated with FCRR of asparagus in southwestern Ontario using DNA-based PCR methods and ISSR-PCR analysis, and to investigate the effects of organic amendments such as animal manures, composts, and fish emulsions from the agricultural and fish-processing industry on populations of pathogens and soil bacteria, and on disease development, in order to assess its management.

MATERIALS AND METHODS

Isolation of *Fusarium* spp. from asparagus ecosystems. Eleven commercial asparagus fields in southwestern Ontario, Canada, were surveyed in 2011 and 2012 to collect plant and soil samples for this study. From each field, 5 to 10 plants were arbitrarily taken. The asparagus plants were dug from the soil with a spade. Plant roots were shaken to collect soil from the rhizosphere for isolation purposes. Plants from each field were pooled and placed in plastic bags and brought to the laboratory. They were air dried, placed in paper bags, stored at 5°C, and used within a week for making isolations. The rhizosphere soil samples from the plants of each field were pooled and mixed to make one soil sample per field. The air-dried rhizosphere soil was placed in plastic bags, stored at 5°C, and used within a week for making isolations.

Fusarium isolations from asparagus plant tissues were made from stems (at the base near the intersection), crowns (or rhizomes), and roots (including storage and secondary roots) as described previously (15,22,54), with some modifications. Because the root surface is greater than the crown surface in the root system of asparagus plants, the percentages of the different parts assayed were 16% basal stem pieces, 34% crown pieces, and 50% root pieces. The plant tissues were thoroughly washed under running tap water and cut into 4- to 5-cm pieces, surface disinfested by dipping in 1% sodium hypochlorite solution (20% household bleach) for 1 min, and air dried for 10 min under sterile conditions. Then, each piece was cut into small, 0.5-cm pieces with a sterile scalpel and placed on the surface of potato-dextrose-agar (PDA) medium, supplemented with antibiotics (streptomycin sulfate at 100 mg/liter and neomycin sulfate at 12 mg/liter) (Sigma-Aldrich, St. Louis, MO).

Isolations of *Fusarium* from rhizosphere soil were performed by the plate-culturing method as described previously (22,54), with some modifications. The soil sample from each field was thoroughly mixed and a 10-g subsample was taken for isolation on *Fusarium*-selective agar mediums (14,53) containing agar, peptone, streptomycin sulfate, neomycin sulfate, and pentachloronitrobenzene (PPA) or myclobutanil (MBA). Each subsample was diluted in 0.1% water agar and dilutions (10^{-2} to 10^{-5}) were spread on the surface of PPA and MBA plates. The plates were incubated in the dark at 24°C for 7 to 10 days.

Culturing and identifying *Fusarium* spp. The individual *Fusarium*-like colonies from the plates were transferred to PDA plates and incubated in the dark at 24°C for 10 days for growth and sporulation. Microscopic observation of fungal structures was also performed to characterize the isolates to *Fusarium* genus. In total, 93 *Fusarium* cultures with different colony morphologies were recovered from PDA plates and further purified. The monosporic cultures were subcultured on PDA and used for further characterization. A first approach for the identification of the isolates was based on morphological characteristics on PDA medium. Cultural characteristics such as colony color and morphology were assessed by visual examination, and morphology of macro- and microconidia was recorded from cultures grown on PDA by microscopic examination (5,34). Later, this primary identification was confirmed or corrected by molecular characterization with PCR analysis with species-specific primers and ISSR-PCR analysis.

Molecular assays. In order to extract DNA for PCR analysis, *Fusarium* isolates were grown on PDA plates for 7 days and three to four 8-mm-diameter plugs with fungal mycelium were aseptically transferred to 50-ml aliquots of sterilized liquid potato dextrose broth in 150-ml Erlenmeyer flasks. Cultures were grown at room temperature (22°C) for 6 days under low agitation (110 rpm). *Fusarium* mycelial mats were freeze dried and ground in liquid nitrogen with a mortar and pestle. Total genomic DNA of fungal isolates was isolated from dried mycelium (≈100 mg) using the E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-Tek, Norcross, GA), according to the manufacturer's protocol. DNA was recovered and dissolved in elution buffer and stored at –20°C until PCR amplification. DNA quality was assessed by gel electrophoresis. Concentrations of DNA were determined by spectrophotometer NanoDrop, ND-1000 (NanoDrop Technologies, Wilmington, DE).

The colonies of *Fusarium* spp. obtained above were identified based on PCR analysis with species-specific primer pairs developed by Yergeau et al. (58) for all *Fusarium* spp., Williams et al. (56) for *F. acuminatum*, Mulè et al. (39) for *F. oxysporum* and *F. proliferatum*, and Bogale et al. (10) for detection of *F. redolens*. The *F. solani*-specific primer pair FSOL1/2 were designed on the basis of the sequence of *Fusarium* field isolate *Fs*-1.12 with Primer-BLAST (46). Previously, the nuclear ribosomal internal transcribed spacer (ITS) region of the isolate *Fs*-1.12 was amplified with universal primers ITS4 and ITS5 (55), using as cycling parameters 10 min at 95°C; followed by 40 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C; and a final extension for 10 min at 72°C. The resulting products were purified and analyzed on the 3730 Genetic Analyzer (Applied Biosystems, Forest City, CA) at the London Regional Genomics Centre DNA Sequencing Facility. The sequences of the species-specific primers are given in Table 1. Three known asparagus-pathogenic isolates—*F. oxysporum* f. sp. *asparagi* (*Foa*4), *F. proliferatum*, (*Fp*9), and *F. solani* (*Fs*2) (15)—were used as positive controls, and sterile, double-distilled H$_2$O instead of DNA was included as negative control in each experiment. The reaction mixtures were prepared in a total volume of 25 µl with a final concentration of 2.5 µl of 10× PCR buffer, 0.8 mg/ml of bovine serum albumen (BSA), 2.5 mM MgCl$_2$, and 0.2 mM each dNTP. For each reaction, 1.25 U of Taq polymerase (Invitrogen, Carlsbad, CA),

0.4 µM each primer, and ≈25 ng of fungal template DNA were used. PCR conditions for the FSOL1/2 primer pair were an initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 50 s, and extension at 72°C for 1 min; a final extension at 72°C for 5 min; followed by cooling at 4°C until recovery of the samples. For the other primer pairs, PCR conditions were same as described by the authors mentioned above. Amplified products were separated by 1.5% agarose gel electrophoresis in 1× Tris-acetate-EDTA buffer and visualized after staining with GelRed (10 µg/ml).

All 93 *Fusarium* isolates from asparagus fields were further characterized with ISSR-PCR to investigate the degree of genetic variability among and within different *Fusarium* spp. Three known asparagus isolates of *Fusarium* (*Foa*4, *Fp*9, and *Fs*2) were also included for comparisons and as positive controls. DNA from these isolates was extracted as described above. Eighteen individual primers, based on the production of distinct and reproducible polymorphic banding patterns (51), were used for PCR assays: $(AAG)_6$, $(AC)_8T$, $(ACA)_5$, $(AG)_8TA$, $(AG)_8TC$, $(CAA)_5$, $(CCA)_5$, $(CTC)_4$, $(GA)_6GG$, $(GA)_8C$, $(GA)_8T$, $(GACA)_4$, $(GAG)_4GC$, $(GT)_6CC$, $(GTC)_6$, $(GTG)_5$, $(AG)_8T$, and M13. PCR was performed in a total volume of 20 µl containing 2 µl of 10× PCR buffer, BSA at 0.6 mg/ml, 2 mM of $MgCl_2$, 0.4 µM primer, 10 mM dNTPs mix, 1 U of Taq polymerase (Invitrogen), and 25 ng of template DNA. Amplification was performed as follows: 5 min at 94°C; 1 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C for 40 cycles; 7 min at 72°C; followed by cooling at 4°C. Amplified products were separated by 0.8% agarose gel electrophoresis in 0.5× Tris-borate-EDTA buffer and visualized after staining with GelRed (10 µg ml^{-1}) under UV light, and images were acquired by Gel Doc 2000 System (Bio-Rad, Hercules, CA). A commercial gel documentation system (Quantity One 4.2.1; Bio-Rad) was used as support for the scoring of bands as presence (=1) or absence (=0).

Pathogenicity tests. In vitro experiments were carried out to assess the pathogenicity of 77 isolates selected from the 93 *Fusarium* isolates obtained above from commercial asparagus fields and identified by molecular analysis. The in vitro pathogenicity of *Fusarium* isolates in asparagus seedlings was determined as described previously (15,48), with some modifications. Asparagus seed of 'Jersey Giant', 'Mary Washington', and 'Grande' (tolerant, susceptible, and moderately tolerant to *Fusarium* spp., respectively) were surface disinfested by immersion for 3 min in 20% household bleach supplemented with 0.05% Tween 20 and rinsed three times with distilled H_2O, air dried under sterile conditions, and placed on the surface of water agar (0.6%) medium. The plates were incubated in the dark at 28°C for 7 to 8 days for emergence. Individual plantlets were transferred aseptically to test tubes containing 15 ml of Murashige and Skoog basal salt mixture media with Minimal Organics (MP Biomedicals Inc., Santa Ana, CA). The tubes were loosely capped to allow air exchange and incubated for 2 weeks in a growth cabinet (16 h of fluorescent light at 23°C and 8 h of darkness at 18°C), until inoculation.

Fusarium isolates were grown on PDA plates for 10 days. The spore suspension used as inoculum was obtained after filtering it through four layers of sterile cheesecloth. The spore concentration was determined using a hemacytometer and adjusted to 1×10^7 conidia/ml. To inoculate the plants, 1 ml of the appropriate spore suspension was deposited next to the roots. Sterile water was added to control plantlets. Plants were incubated for 3 weeks as above. The assay included three replications (plantlets) for each of the 77 isolates tested. At the end of the assay, severity of the symptoms was assessed on a percentage scale of the root system affected on a 1 to 5 scale (15), where 1 = 1 to 20, 2 = 21 to 40, 3 = 41 to 60, 4 = 61 to 80, and 5 = 81 to 100% of the root system affected, including crowns and roots. Those isolates that produced lesions on ≥20% of the root system were considered to be pathogenic on asparagus (22).

Soil amendments and microbial assays. Three separate greenhouse experiments were conducted, each with a different asparagus cultivar, to evaluate the effects of various soil treatments on FCRR development on asparagus, on plant growth, on soil populations of *Fusarium* spp. and culturable bacteria, and on soil pH. These greenhouse experiments were repeated at least twice.

The natural soil used in the three greenhouse experiments was sandy soil (85% sand, 7% silt, 8% clay, and 3.6% organic matter, pH 6.9) from a field location in Thamesville, Ontario, Canada. This field had been previously under asparagus crop 'Guelph Millennium' for several years. Several soil samples (n = 25 to 30) were arbitrarily collected from the top layer of soil to a depth of up to 20 cm. The soil samples (total of 300 kg) were mixed into one large composite sample and air dried by spreading on a bench for 48 h or until soil was in workable condition (≈10% moisture). Soil subsamples were randomly taken from the large sample to

TABLE 1. Oligonucleotide sequences of species-specific primers

Primer name	Species specificity	PCR (bp)^z	Primer sequence (5′–3′)	Reference
Alfie1-GC	*Fusarium* spp.	450	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG GGGTCGTCATCGGCCACGTCGACTC	58
Alfie2			CCTTACCGAGCTCRGCGGCTTC	
FAC F	*Fusarium acuminatum*	600	GGGATATCGGGCCTCA	56
FAC R			GGGATATCGGCAAGATCG	
CLOX1	*F. oxysporum*	534	CAGCAAAGCATCAGACCACTATAACTC	39
CLOX2			CTTGTCAGTAACTGGACGTTGGTACT	
CLPRO1	*F. proliferatum*	526	TGCATCAGACCACTCAAATCCT	39
CLPRO2			GCGAGACCGCCACTAGAT	
Red-F	*F. redolens*	386	ATCGATTTTCCCTTCGACTC	10
Red-R			CAATGATGATTGTGATGAGAC	
FSOL1	*F. solani*	398	GCTCTCCAGTTGCGAGGTGT	This study
FSOL2			TTGCTTCGGCGGGAACAGAC	

^{z} Polymerase chain reaction (PCR) product.

TABLE 2. Chemical analyses of organic material used in this study

Organic material	Nitrogen (%)	Phosphorus (%)	Potassium (%)	pH	Electrical conductivity (mS/cm)
Pelleted poultry manure	4.0	1.0	2.0	6.3	11.0
Olive residue compost	1.5	0.7	2.0	8.7	3.2–3.5
Fish emulsion	5.0	1.5	1.8	2.4–2.8	19.0–20.5

determine the density of *Fusarium* population by the plate culturing method. Each 10-g soil sample was shaken with 90 ml of 0.1% sterile water agar on a rotary shaker at 100 rpm for 10 min, and the suspension was then serially diluted by placing 1 ml in 9 ml of saline water (NaCl at 8.5 g/liter) in tubes, and 1 ml from each of 10^{-2} to 10^{-5} dilutions was plated onto PPA medium in petri dishes. The density of *Fusarium* spp. was expressed as the number of colony-forming units (CFU) per gram of soil. The large soil sample was kept in plastic bags for up to 2 weeks after air drying and stored at 5°C.

Three organic amendments from the agricultural and fish-processing industry used in this study included a pelleted poultry manure (PPM; Envirem Technologies Inc., NB, Canada), an olive residue compost (ORC; Department of Agriculture and Fisheries, Andalucía, Spain), and a fish emulsion (FE) prepared from whole menhaden fish (*Brevoortia patronus* Goode and *B. tyrannus* Latrobe) (Omega Protein, Houston, TX). A nutrient analysis of these amendments is given in Table 2.

Based on the results of in vitro pathogenicity assessment, two pathogenic *Fusarium* isolates, *Fo*-1.5 (*F. oxysporum*) and *Fs*-1.12 (*F. solani*), were selected for the greenhouse experiments. The inocula of these isolates were separately grown on sterilized millet seed in 1-liter flasks containing 50 g of millet seed and 100 ml of H_2O as described previously (18), with some minor modifications. The contents of the flasks were autoclaved for 1 h on two consecutive days. The flasks were then kept at room temperature in a laminar flow hood to cool down before infestation with 10 5-mm-diameter PDA plugs obtained from the outer margins of actively growing colonies of each isolate. Inoculated flasks were incubated at room temperature for 14 days and hand shaken daily for 15 s. The colonized seed from the flasks was air dried for 24 h in a laminar flow hood under sterile conditions. The dried, colonized seed was ground in a mill for 30 s at 25,000 rpm to achieve 1- to 2-mm particle size. *Fusarium* populations of ground inoculum were enumerated by the plate culturing method as described above, and expressed as the number of CFU per gram of inoculum. Soil was bagged according to treatments and infested with the ground millet seed inocula of *Fusarium* isolates *Fo*-1.5 or *Fs*-1.12 separately. The infested soil was incubated at 24°C for 24 h in the dark prior to adding the corresponding soil treatments.

The first greenhouse experiment was conducted with Jersey Giant and *Fusarium* isolates *Fo*-1.5 and *Fs*-1.12. For each treatment, an aliquot of 2.5 kg of soil from the large soil sample was infested with the millet seed inoculum of *Fo*-1.5 (4 g/kg of soil at 9×10^6 CFU/g) or *Fs*-1.12 (6 g/kg of soil at 7×10^7 CFU/g), separately, both by mixing in plastic bags. This was enough soil to fill five replicate 10-cm-diameter plastic pots per treatment. The infested soil from bags was incubated for 24 h at 24°C in the dark before amending with the following treatments: benomyl (0.5 g/liter of soil) as chemical control, PPM (0.5 and 1% *m/m* (weight/weight) soil), ORC (3 and 6% *m/m* soil), and FE (0.5% *m/m* soil). The pathogen-noninfested and -infested soils served as negative and *Fusarium* controls, respectively. The amended soil was incubated for 14 days at 24°C in the dark prior to planting asparagus seedlings. The treated soils were also sampled at 0, 1, 3, 7, and 14 days after amendment for determination of population of *Fusarium* spp. and culturable bacteria, and for soil pH.

In the second greenhouse experiment, Mary Washington asparagus was used. The millet seed inoculum (3 g/kg soil) of either isolate *Fo*-1.5 (6×10^7 CFU/g) or *Fs*-1.12 (1×10^7 CFU/g) was mixed with 3.8 kg of soil (from the large composite soil sample) per treatment by mixing in plastic bags. The soil from each bag was enough to fill five replicate 12.5-cm-diameter plastic pots per treatment. The infested soil from bags was incubated for 24 h at 24°C in the dark before amending with the soil treatments as in the first experiment. The amended soil was

incubated for 14 days at 24°C in the dark prior to planting. The amended soils were also sampled at 0, 1, 3, 7, and 14 days after amendment for determination of populations of *Fusarium* spp. and culturable bacteria, and for soil pH.

The third greenhouse experiment was conducted with Grande and *Fusarium* isolate *Fs*-1.12. For each treatment, 2.5 kg of soil from the large composite soil sample was infested with the millet seed inoculum (16 g/kg of soil at 1×10^7 CFU/g) by mixing in plastic bags. This was enough soil to fill five replicate 10-cm-diameter plastic pots per treatment. The pathogen-noninfested and -infested soils served as negative and *Fusarium* controls, respectively. The infested soil from bags was incubated for 24 h at 24°C in the dark before amending with the soil treatments as indicated for experiments 1 and 2, except that ORC was applied at 1 and 3% *m/m* soil and FE at 0.1 and 0.5% *m/m* soil. The treated soil was incubated for 10 days at 24°C in the dark before planting asparagus seedlings. Differently in this experiment, the amended soils were sampled at 0, 3, 7, and 10 days after amendment for determination of population of *Fusarium* spp. and culturable bacteria, and for soil pH.

Populations of total *Fusarium* spp. (*Fo*-1.5, *Fs*-1.12, and other *Fusarium* spp. present in natural soil) and culturable bacteria were enumerated to determine the effect of the different treatments on soil microflora by the plate-culturing method as described above, using soil dilutions plated onto semiselective media (bacteria: tryptic-soy-broth-based agar medium; *Fusarium* spp.: PPA medium), and expressed as the number of CFU per gram of soil. Plates were incubated at 24°C for 2 days for bacteria and 4 days for *Fusarium* spp. Three replicate plates were used for each dilution. For enumeration of *Fusarium* spp., 1 ml from each of 10^{-2} to 10^{-5} dilutions was plated and, for bacteria, 1 ml from each of 10^{-5} to 10^{-7} dilutions.

To determine the soil pH, soil subsamples (8 g) from each treatment were placed in polyethylene stomacher bags and 40 ml of distilled water was added to each of them. The bags were shaken for 15 min at 200 rpm. The pH of the soil–water mixture was determined as described previously (49).

To evaluate the development of FCRR disease, Jersey Giant, Mary Washington, and Grande asparagus, of different susceptibility to *Fusarium* spp., were assessed. Asparagus seed of these cultivars were surface disinfested as described above, transferred to water agar medium (0.6%), and kept in the dark at 28°C for 7 to 8 days. Once roots began to emerge, seedlings were transferred to trays with sterile sand and placed for 2 weeks in a growth chamber with daily cycles of 16 h of light at 24°C and 8 h of darkness at 19°C. The amended soil from each bag was mixed again and transferred to five pots. One seedling each of Jersey Giant and Grande and two seedlings of Mary Washington were transplanted into each corresponding pot and maintained in a greenhouse for 12 weeks at 22 to 24°C under a combination of daylight and supplemental lighting (photoperiod = 16 h of light; light intensity = 225 μE m^{-2} s^{-1}) in a randomized completed block design. Pots were watered daily as required. At the end of each experiment, asparagus plants were removed from the pots and their root systems were rinsed in tap water. The severity of symptoms was assessed by the percentage scale of the stem system affected on a 1 to 5 scale (15), where 1 = 1 to 20, 2 = 21 to 40, 3 = 41 to 60, 4 = 61 to 80, and 5 = 81 to 100% of the aerial part (stems) showing chlorosis, necrosis, or wilt (15) and of the root system affected, including crowns and roots, as described above. Fresh weights were recorded for each treatment after rating the plants and dry weights were taken after the plants had dried for 48 h in an oven at 70°C.

Statistical analyses. The bands generated by ISSR primers from each gel were scored as present (= 1) versus absent (= 0) and the resulting data matrix was analyzed by Minitab statistical software (version 16.0; Minitab Inc., State College, PA). The genetic similarity matrix was subjected to cluster analysis with

complete linkage and the Euclidean distance option of the software to generate a dendrogram. All experiments were repeated at least twice and yielded similar results. Separate analyses of each experiment showed homogeneous variance of the experimental error between replications. Data of symptom severity were angle transformed and analyzed using analysis of variance (ANOVA) ($P \leq 0.01$). ANOVA of *Fusarium* population and culturable bacteria were performed on previous log CFU transformation of data. The means were compared using the least significant difference test ($P = 0.01$) for pathogenicity experiments and ($P = 0.05$) for the soil organic amendments experiments. All analyses were performed with Statistical analyses of data supported by Statistix 9.0 software (Analytical Software, Tallahassee, FL).

RESULTS

Culturing and identifying *Fusarium* spp. Of the 93 field isolates of *Fusarium* spp. collected from 11 asparagus fields in southwestern Ontario, 77 were obtained from asparagus plants (7% from basal stems, 29% from crowns, and 64% from roots), and 16 from rhizosphere soil. Of those isolates, 61 were identified as *F. oxysporum* (65.5%), 17 as *F. proliferatum* (18.3%), 6 as *F. solani* (6.4%), 6 as *F. acuminatum* (6.4%), and 3 as *F. redolens* (3.2%), based on cultural characteristics and morphological criteria. *F. oxysporum* was present in all fields and was the dominant species, followed by *F. proliferatum* and *F. solani* (73 and 45% isolation frequency, respectively). *F. acuminatum* was isolated from three fields and *F. redolens* was only found in one field (Table 3). Of the 77 isolates obtained from asparagus plants, 66% belong to *F. oxysporum*, 15% to *F. proliferatum*, 8% to *F. acuminatum*, 8% to *F. solani*, and 3% to *F. redolens.* Of the 16 isolates from rhizosphere soil, 63% belong to *F. oxysporum*, 31% to *F. proliferatum*, and 6% to *F. redolens*.

Molecular assays. The species-specific PCR assays confirmed the identity of most of these field isolates of *Fusarium* spp. associated with asparagus by amplifying bands of the expected size for each species (Fig. 1). The primer set Alfie1-GC/2 amplified a single 450-bp fragment from all field isolates, confirming their identity as *Fusarium* spp. (Fig. 1A). The primer sets CLOX1/2 and CLPRO1/2 produced the expected 534- and 526-bp fragments specific for *F. oxysporum* and *F. proliferatum* isolates, respectively (Fig. 1B and 1C), and FAC F/R produced the expected 600-bp fragments specific for *F. acuminatum* (Fig. 1D). The primer set FSOL1/2 produced the 398-bp fragment specific for *F. solani* isolates (Fig. 1E) but a very faint band was also amplified in 10 isolates of *F. oxysporum*, whereas Red-F/R produced a 386-bp fragment specific for *F. redolens* isolates (Fig. 1F).

The ISSR-PCR analysis of 93 *Fusarium* field isolates using 18 ISSR primers revealed a considerable intraspecific and interspecific variability among isolates of *Fusarium* spp. Clear and reproducible DNA fragments were scored consistently in repeated amplifications from all isolates. The amplified fragments ranged in size from 225 to 3,000 bp, and the number of ISSR bands obtained with each primer varied from 18 to 33. A PCR amplification pattern generated by using the ISSR primer (AAG)$_6$ is shown in Figure 2. The presence or absence of these bands was analyzed with complete linkage and Euclidean distance. The analysis of ISSR data clustered the isolates belonging to the same

species into the same groups (Fig. 3). The dendrogram indicated that *F. oxysporum* isolates formed two clusters. The genetic similarity found in each one of these clusters was 71 and 65%. The genetic similarity for *F. solani* and *F. redolens* was 58%. The similarity of *F. proliferatum* and *F. acuminatum* was 51.3 and 50%, respectively (Fig. 3).

Pathogenicity tests. The common symptoms developed on the root system were brown to reddish-brown necrotic lesions of variable length. In all three cultivars, a wide range in virulence was observed between the *Fusarium* field isolates. The average root rot severity of isolates showed a wide range (2 to 94%) with the three asparagus cultivars tested and, depending on the cultivar, 47 to 51% of the isolates resulted in root severity values $\geq 20\%$ of the root system (Fig. 4). Only 22% of the field isolates, including five *F. oxysporum* isolates (*Fo*-1.3, *Fo*-1.4, *Fo*-1.5, *Fo*-6.1, and *Fo*-11.4), four *F. solani* isolates (*Fs*-1.12, *Fs*-4.5, *Fs*-6.3, and *Fs*-7.3), and five *F. acuminatum* isolates (*Fa*-3.6, *Fa*-10.1, *Fa*-10.2, *Fa*-10.4, and *Fa*-10.6), caused severity values $\geq 50\%$ in the three cultivars (Fig. 4A to C).
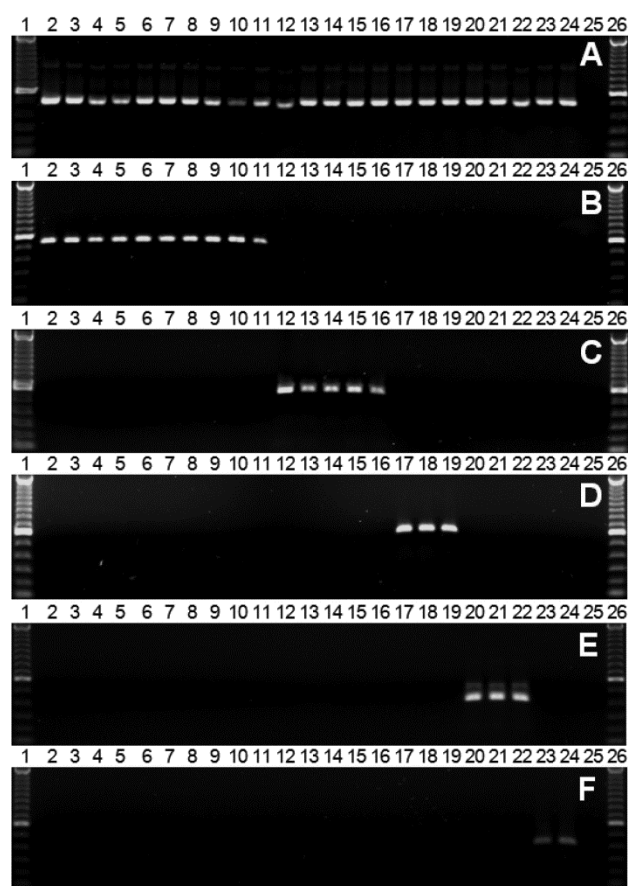
Fig. 1. Polymerase chain reaction detection of different *Fusarium* spp. with species-specific primer sets. **A,** Alfie1-GC/2; **B,** CLOX1/2; **C,** CLPRO1/2; **D,** FAC F/R; **E,** FSOL1/2; and **F,** Red-F/R. Lanes 1 and 26, GeneRuler 100-bp DNA Ladder; lanes 2 to 11, *Fusarium oxysporum*; lanes 12 to 16, *F. proliferatum*; lanes 17 to 19, *F. acuminatum*; lanes 20 to 22, *F. solani*; lanes 23 to 24, *F. redolens*; and lane 25, no DNA (or negative) control.

TABLE 3. Frequency of the *Fusarium* spp. isolated from asparagus of 11 commercial fields in southwestern Ontario

| *Fusarium* spp. | Isolation frequency (%) on the 11 fields | | | | | | | | | | |
	1	2	3	4	5	6	7	8	9	10	11
Fusarium acuminatum	0	0	13	0	0	0	0	0	0	50	17
F. oxysporum	94	57	37	50	50	75	75	62	62	38	83
F. proliferatum	0	0	37	25	50	12.5	12.5	38	38	12	0
F. redolens	0	43	0	0	0	0	0	0	0	0	0
F. solani	6	0	13	25	0	12.5	12.5	0	0	0	0

The most pathogenic isolates of the predominant species, *F. oxysporum, F. proliferatum*, and *F. solani,* were *Fo*-1.5, *Fp*-7.2, and *Fs*-1.12 and their average severity was 76, 37, and 92%, respectively. Based on these results, the isolates *Fo*-1.5 and *Fs*-1.12, with highest pathogenicity, were selected for further experiments.

Soil amendments and microbial assays. The evaluation of the effect of organic soil amendments on FCRR in the first experiment showed that the severity of symptoms on Jersey Giant plants in *Fo*-1.5-infested soils was very low, and there were no significant differences between the soil treatments (Fig. 5A); whereas, in *Fs*-1.12-infested soils, stem and root system severity were significantly ($P < 0.05$) reduced by all treatments (Fig. 5B). The highest reduction (93 and 90% in stem and root severity, respectively) compared with the *Fusarium* control treatment, for which the severity was moderate, corresponded to PPM at 1% (Fig. 5B). With the rest of amendments, the stem and root system disease severities were similar to those of benomyl (by 63% in both stems and roots), the decreases being 71 and 66% for 0.5% PPM; 44 to 53 and 55 to 74% for 3 and 6% ORC, respectively; and 75 and 82% in the case of 0.5% FE (Fig. 5B), respectively. In the second experiment, the severity of symptoms was high in Mary Washington asparagus plants grown in *Fo*-1.5-infested soils. Only the 1% PPM treatment significantly ($P < 0.05$) reduced the symptom severity on the stem (by 42%), and the applications of 0.5 and 1% PPM and 6% ORC significantly ($P < 0.05$) reduced root severity (by 28 and 44%, and 27%, respectively), compared with the *Fusarium* control treatment (Fig. 5C). Mary Washington plants grown in *Fs*-1.12-infested soils had higher symptom severity than the plants grown in *Fo*-1.5-infested soils. Only the benomyl and 0.5% PPM treatments significantly ($P < 0.05$) reduced severity on the stem (by 22 and 18%, respectively) compared with the *Fusarium* control treatment (Fig. 5D); however, there were no significant differences between *Fusarium* control and amended treatments on root severity of plants in *Fs*-1.12-infested soils (Fig. 5D). In the third experiment, where Grande plants were grown in *Fs*-1.12-infested soils, low severity of aerial symptoms was observed in contrast to the *Fusarium* control, regardless of the treatment. However, root severity showed a significant ($P < 0.05$) reduction with the application of any treatment; benomyl as well as the soil amendments with 0.5% FE and 0.5 and 1% PPM were the most effective on disease control (100, 97, 95, and 89% reduction, respectively) compared with the *Fusarium* control (Fig. 6).

The evaluation of the effect on plant growth in the first experiment showed that both fresh and dry weights of Jersey Giant plants were not affected by any amendment treatments in soils infested with isolate *Fo*-1.5 (Fig. 7A). In soils infested with isolate *Fs*-1.12, the addition of benomyl and the treatments with 0.5 and 1% PPM significantly ($P < 0.05$) increased fresh (by 68,

81, and 104%, respectively) and dry (by 64, 55, and 109%, respectively) weights of Jersey Giant plants compared with the *Fusarium* control treatment (Fig. 7B). In the second experiment, the 1% PPM amendment to *Fo*-1.5-infested soil significantly ($P < 0.05$) increased the fresh (by 77%) and dry (by 100%) weights of Mary Washington plants compared with the *Fusarium* control

Fig. 3. Dendrogram derived from cluster analysis of similarities between *Fusarium* isolates from asparagus fields based on inter-simple sequence repeats polymerase chain reaction analysis. Analysis was performed with complete linkage and Euclidean distance options of Minitab statistical software (version 15.1; Minitab Inc., State College, PA).

Fig. 2. Agarose gel showing the inter-simple sequence repeats polymerase chain reaction amplification of DNA from different *Fusarium* spp. with primer (AAG)₆. Lane 1, GeneRuler 100-bp DNA ladder; lanes 2 to 24, *Fusarium* field isolates; lanes 25 to 27, known asparagus-pathogenic isolates (lane 25, *F. oxysporum* f. sp. *asparagi*; lane 26, *F. proliferatum*; and lane 27, *F. solani*); and lane 28, no DNA (or negative) control.

treatment (Fig. 7C), and only the benomyl treatment to *Fs*-1.12-infested soil significantly (*P* < 0.05) increased the fresh weight (by 50%) of Mary Washington plants compared with the *Fusarium* control treatment (Fig. 7D). In the third experiment, fresh weights of Grande asparagus plants were significantly (*P* < 0.05) higher in soil amended with 0.5 and 1% PPM, 0.5% FE, and benomyl, with an increase of 138, 152, 131, and 100% over the *Fusarium* control. A similar effect was observed for dry weight of plants, with the highest values corresponding to the three amendments above mentioned (Fig. 8).

In the study of the effect of soil chemical and organic treatments on soil populations of *Fusarium* (*Fo*-1.5, *Fs*-1.12, and other *Fusarium* spp. present in natural soil), in the first experiment, the *Fusarium* populations in the treated soils gradually decreased compared with the untreated *Fusarium* control soils (Fig. 9A and B). In *Fo*-1.5-infested soils, benomyl, 1% PPM, and 3% ORC decreased *Fusarium* populations after 1 day post treatment; whereas, with 0.5% PPM, 6% ORC, and 0.5% FE treatments, the pathogen population also started to decrease after 7 days compared with the *Fusarium* control treatment (Fig. 9A). After 14 days of treatment, the reduction in pathogen population was most effective with benomyl and 1% PPM. In *Fs*-1.12-infested soils, *Fusarium* populations were higher and all chemical and amendment treatments gradually decreased the *Fusarium* populations starting from day 1 after treatments, and the reduction in pathogen populations was most obvious after 14 days with the 1% PPM, 6% ORC, and 0.5% FE amendments (60, 56, and 62% reduction, respectively), compared with the *Fusarium* control

treatment (Fig. 9B). In the second experiment, all chemical and amendment treatments to *Fo*-1.5- and *Fs*-1.12-infested soil significantly (*P* < 0.05) and gradually decreased the pathogen populations from the day 1 after receiving treatments (Fig. 9C and D). After 14 days, 1% PPM and benomyl were the most effective treatments in reducing the *Fusarium* populations in *Fo*-1.5- and *Fs*-1.12-infested soils, respectively (Fig. 9C and D). In the third experiment, all chemical and amendment treatments significantly (*P* < 0.05) decreased the *Fusarium* populations in the soil infested with *Fs*-1.12 after 10 days (Table 4). The 1% PPM, benomyl, and 0.5% FE were the best treatments in reducing the pathogen populations after 66 days of treatment applications (Table 4).

In the study of the effect on soil populations of total culturable bacteria, in the first experiment, the bacterial populations were significantly (*P* < 0.05) higher in the soils treated with the organic amendments than in the *Fusarium* and chemical control soils (Fig. 10A and B). The highest increase in population of culturable bacteria occurred in *Fs*-1.12-infested soils amended with 0.5 and 1% PPM (19.8 and 15.7% increase) and 6% ORC (16.4% increase) after 14 days of incubation (Fig. 10B). In the second experiment, the populations of culturable bacteria were also significantly higher in soils treated with organic amendments compared with the *Fusarium* and chemical control soils (Fig. 10C and D). All organic amendment treatments were equally effective in increasing the populations of culturable bacteria. After 14 days of incubation, an average increase of 14.6 to 19.4% was observed with organic amendments (Fig. 10C and D). In the third experi-
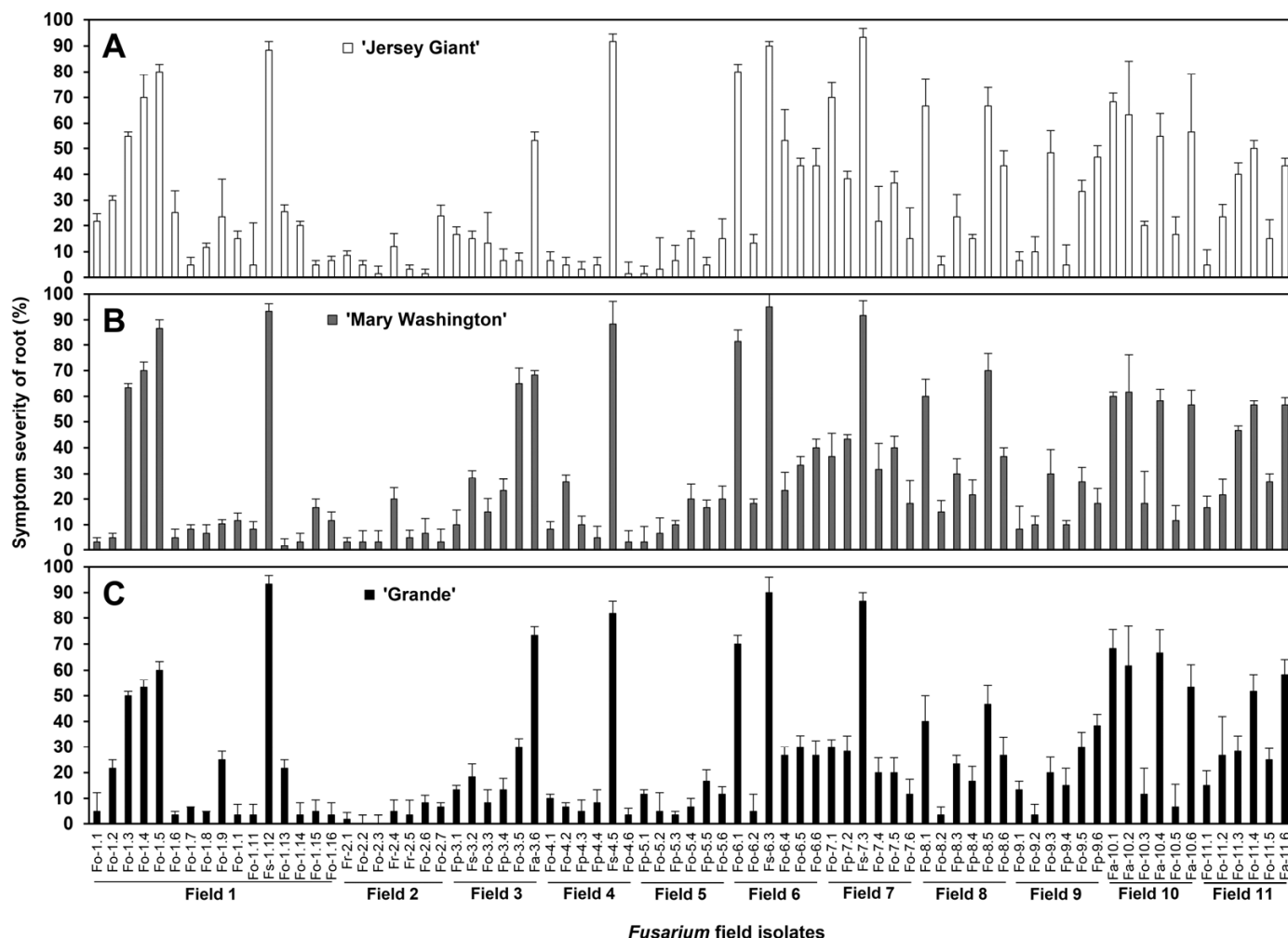
Fig. 4. Severity of root symptoms on the seedlings of the three asparagus cultivars **A,** 'Jersey Giant'; **B,** 'Mary Washington'; and **C,** 'Grande' inoculated with field isolates of *Fusarium* spp. Error bars represent standard error.

ment, the populations of total culturable bacteria were significantly ($P < 0.05$) higher in the soils treated with organic amendments (by 9% at least) than in the *Fusarium* and chemical control soils, where the population decreased rapidly after 10 days of treatment (Table 4).

The determination of the effect on soil pH in the first experiment showed that soil amendments of 3 and 6% ORC increased soil pH after 1 day of treatment and pH stayed above throughout the 14-day incubation period (Fig. 11A). The soil pH also increased immediately after the 0.5% FE treatment but decreased after 3 days (Fig. 11A). Similar trends were also seen with ORC and FE treatments in *Fs*-1.12-infested soils (Fig. 11B). The 1% PPM treatment also increased the pH after 1 day of incubation in

this experiment (Fig. 11B). All other treatments had no significant effect on soil pH during the entire incubation period. In the second experiment in soils infested with isolate *Fo*-1.5, the pH increased significantly after 1 day of incubation in soils treated with 3 and 6% ORC and 1% PPM (Fig. 11C). A sharp decrease in pH of the soil treated with 0.5% FE after 7 days was also noticed in this experiment (Fig. 11C). A similar trend in soil pH was also noticed in *Fs*-1.12-infested soils, except that 1% PPM significantly decreased the pH after 7 days and 0.5% FE decreased pH after 3 days (Fig. 11D). The soil pH was not affected by any other treatments during the entire incubation period. In the third experiment in soils infested with isolate *Fs*-1.12, the 3 and 6% ORC treatments increased soil pH after 3 days of incubation

TABLE 4. Effect on population of isolate *Fs*-1.12 and total culturable bacteria, and on soil pH[y]

| | Log CFU/g of soil | | | | | | Soil pH | | | |
| | Isolate *Fs*-1.12 | | | Total culturable bacteria | | | | | | |
Treatment[z]	0 day	10 days	66 days	0 day	10 days	66 days	0 day	3 days	7 days	10 days
Controls										
Negative	1.09 o	1.16 no	1.27 n	7.53 i	7.61 g	7.68 h	7.11 gh	7.13 fgh	7.09 gh	7.10 gh
Fusarium	6.94 a	6.33 c	6.65 b	8.39 f	8.12 ef	7.93 g	7.15 fg	7.16 efg	7.15 fg	7.13 fgh
Chemical	6.94 a	3.13 j	2.22 m	8.39 f	7.73 cde	7.55 h	7.15 fg	7.13 fgh	7.17 efg	7.12 fgh
PPM 0.5%	6.94 a	4.46 e	2.99 k	8.39 f	9.14 bcd	9.06 ab	7.15 fg	7.16 fgh	7.19 efg	7.04 h
PPM 1%	6.94 a	3.33 i	2.19 m	8.39 f	9.28 de	8.87 abc	7.15 fg	7.32 cd	7.42 bc	7.22 def
ORC 1%	6.94 a	4.82 d	3.53 h	8.39 f	9.26 def	9.10 ab	7.15 fg	7.40 bc	7.48 bc	7.50 b
ORC 3%	6.94 a	3.96 f	3.35 i	8.39 f	9.10 de	8.91 abcd	7.15 fg	7.64 a	7.71 a	7.67 a
FE 0.1%	6.94 a	4.81 d	3.84 g	8.39 f	8.86 ef	8.79 cd	7.15 fg	7.21 ef	7.12 efg	6.90 i
FE 0.5%	6.94 a	3.83 g	2.82 l	8.39 f	9.00 de	9.13 a	7.15 fg	7.27 de	6.92 i	6.78 j

[y] Values are the average ($n = 6$) of two experiments with three replications for each treatment. Values followed by the same letter within each variable are not significantly different according to the least significant difference test ($P \leq 0.01$).

[z] The *Fs*-1.12-infested soil was amended after 24 h of pathogen inoculation with benomyl (0.5 g liter^{-1} of soil), pelleted poultry manure (PPM; 0.5 and 1%), olive residue compost (ORC; 1 and 3%), and fish emulsion (FE; 0.1 and 0.5%).
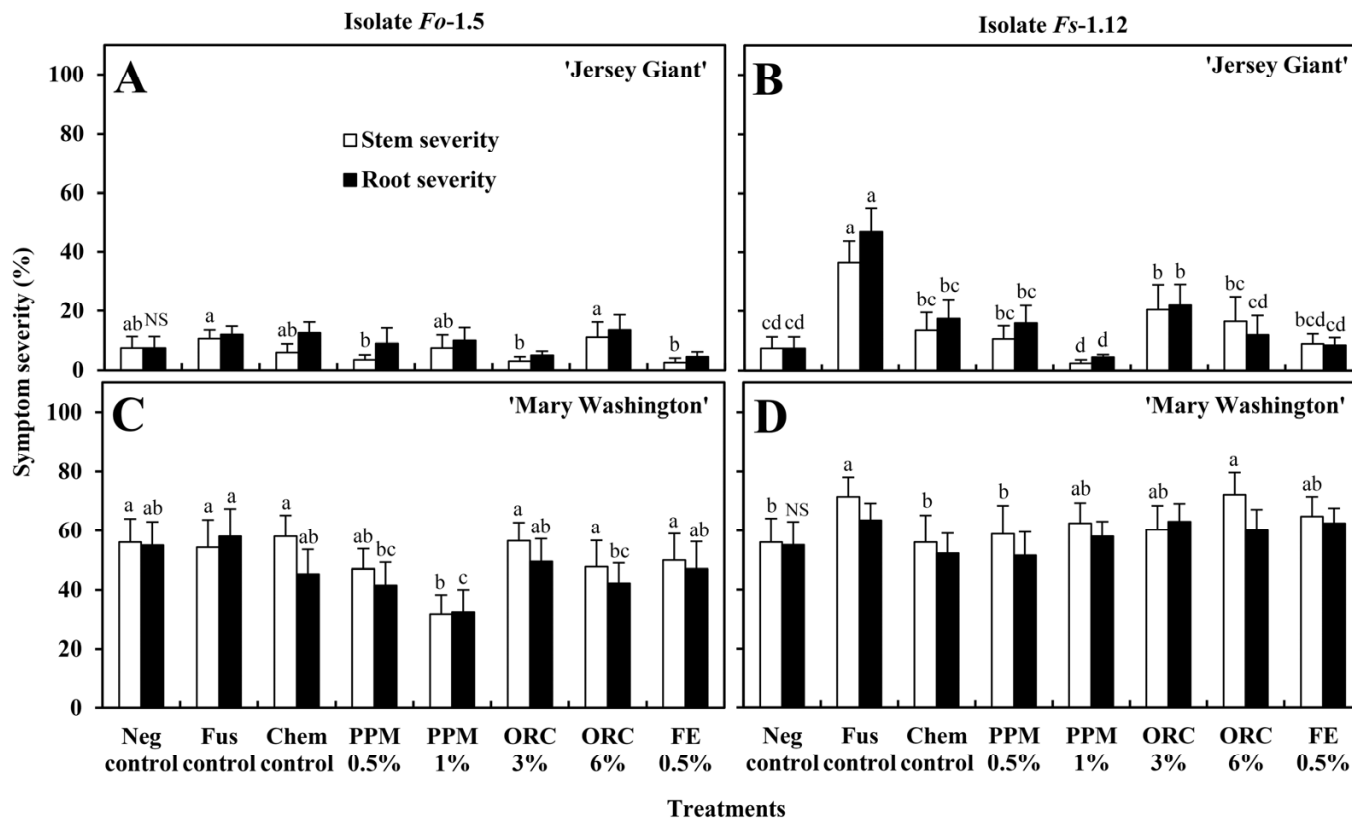
Fig. 5. Severity of symptoms in stem and roots of asparagus **A and B,** 'Jersey Giant' and **C and D,** 'Mary Washington'. Seedlings were grown in soils infested with *Fo*-1.5 and *Fs*-1.12 isolates and treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 0.5 and 1%), olive residue compost (ORC; 3 and 6%), or fish emulsion (FE; 0.5%) after 14 days of incubation. Error bars represent standard error and different letter notation indicates significant difference in stem and root severity according to the least significant difference test ($P = 0.05$). Nonsignificant results are labeled NS ($P > 0.05$). Values are the average ($n = 10$) of two experiments, with five replications for each treatment.

(Table 4). The soil pH was also increased with the 1% PPM treatment but, after 7 days, it decreased. With the addition of 0.5 and 0.1% FE, the pH significantly decreased after 7 and 10 days of incubation, respectively (Table 4).

DISCUSSION

FCRR is an important disease of asparagus with a very complex etiology, because several soilborne *Fusarium* spp. are associated as causal agents and have been frequently isolated from the affected plants. This disease is also known as one of the contributing factors in asparagus decline. In this study, 93 isolates of *Fusarium* spp. collected form 11 established asparagus fields with a history of FCRR in southwestern Ontario were identified based on morphological characteristics and PCR analysis with species-specific primers. Their identity was confirmed as *F. acuminatum*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, and *F. solani*. Only *F. oxysporum* was found in all investigated fields and
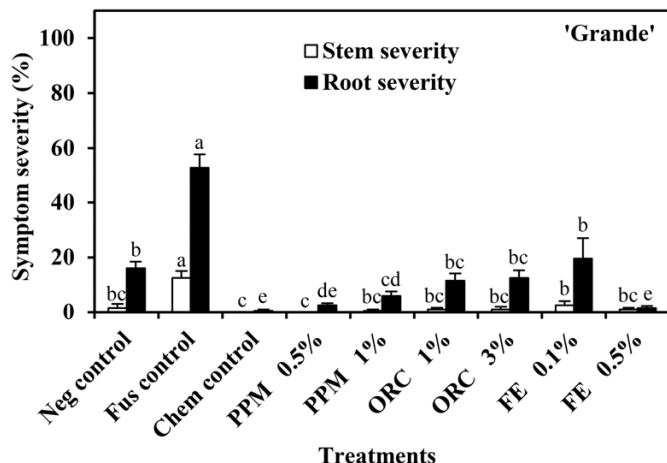
Fig. 6. Severity of symptoms in stem and root of asparagus 'Grande'. Seedlings were grown in *Fs*-1.12-infested soils treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 0.5 and 1%), olive residue compost (ORC; 1 and 3%), or fish emulsion (FE; 0.1 and 0.5%) after 10 days of incubation. Error bars represent standard error and different letter notation indicates significant difference according to the least significant difference test (*P* = 0.05). Values are the average (*n* = 10) of two experiments, with five replications for each treatment.
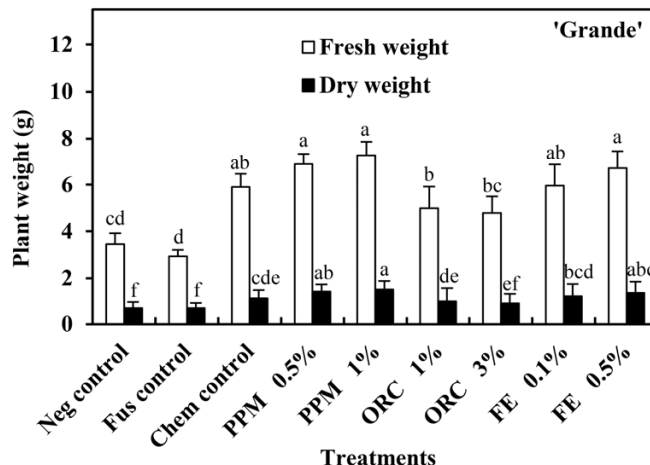
Fig. 8. Plant fresh and dry weights of 'Grande' asparagus. Seedlings were grown in *Fs*-1.12-infested soils treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 0.5 and 1%), olive residue compost (ORC; 1 and 3%), or fish emulsion (FE; 0.1 and 0.5%) after 10 days of incubation. Error bars represent standard error and different letter notation indicates significant difference according to the least significant difference test (*P* = 0.05). Values are the average (*n* = 10) of two experiments, with five replications for each treatment.
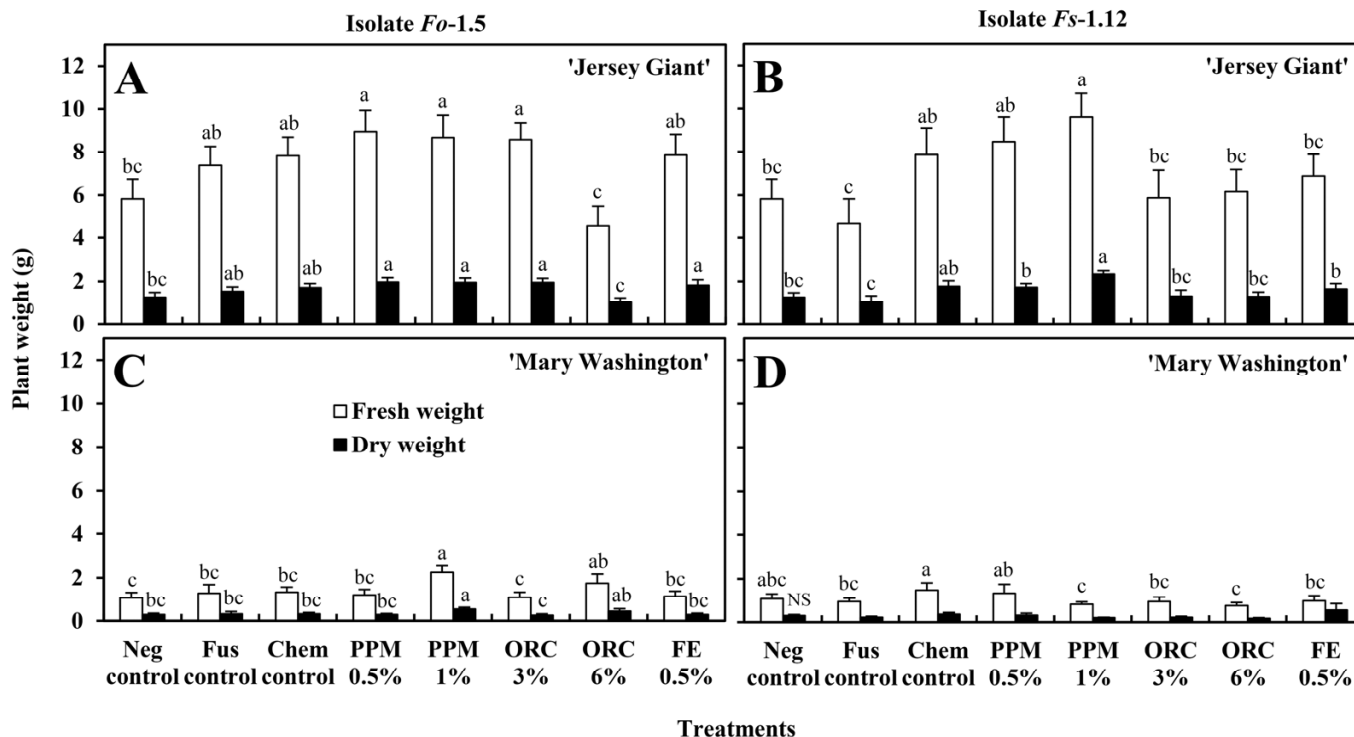
Fig. 7. Plant fresh and dry weights of asparagus **A and B,** 'Jersey Giant' and **C and D,** 'Mary Washington'. Seedlings were grown in soils infested with *Fo*-1.5 and *Fs*-1.12 isolates and treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 0.5 and 1%), olive residue compost (ORC; 3 and 6%), or fish emulsion (FE; 0.5%) after 14 days of incubation. Error bars represent standard error and different letter notation indicates significant difference in stem and root severity according to the least significant difference test (*P* = 0.05). Nonsignificant results are labeled NS (*P* > 0.05). Values are the average (*n* = 10) of two experiments, with five replications for each treatment.

Fig. 9. Population of *Fusarium* isolates *Fo*-1.5 and *Fs*-1.12 in asparagus **A and B,** 'Jersey Giant' and **C and D,** 'Mary Washington' experiments at 1, 3, 7, and 14 days after infestation. Infested soils were treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 1%), olive residue compost (ORC; 6%), or fish emulsion (FE; 0.5%) 24 h after infestation. Values are expressed as log CFU g^{-1} of soil and are the average (*n* = 6) of two experiments, with three replications for each treatment.
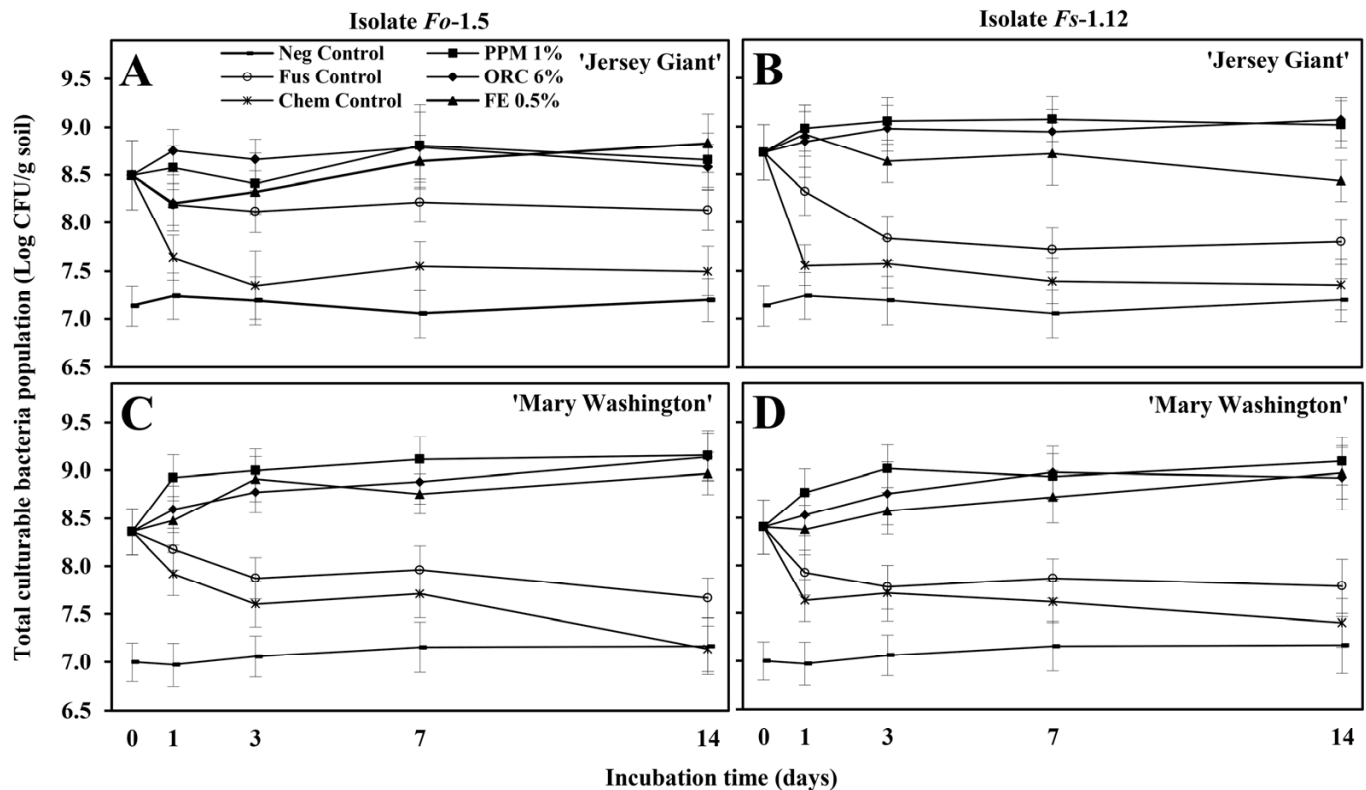
Fig. 10. Population of total culturable bacteria in *Fo*-1.5- and *Fs*-1.12-infested soils in **A and B,** 'Jersey Giant' and **C and D,** 'Mary Washington' experiments at 1, 3, 7, and 14 days after infestation. Infested soils were treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 1%), olive residue compost (ORC; 6%), or fish emulsion (FE; 0.5%) 24 h after infestation. Values are expressed as log CFU g^{-1} of soil and are the average (*n* = 6) of two experiments, with three replications for each treatment.

was the most frequently isolated, representing ≈65.5% of all *Fusarium* isolates. *F. proliferatum* represented 18.3% and the rest of the *Fusarium* spp. were much less frequent (<6.5%). Further characterization of these field isolates by ISSR-PCR analysis revealed considerable variability between the isolates belonging to different *Fusarium* spp.

The diversity of *Fusarium* spp. associated with asparagus has been reported from Canada (54) and other countries (15,40,57). There was a large dissimilarity in the dominant species of *Fusarium* between different regions or continents. In a previous study on the diversity of *Fusarium* spp. from asparagus fields in eastern Canada, Vujanovic et al. (54) found *F. oxysporum* and *F. proliferatum* as predominant and abundant, whereas *F. redolens, F. solani,* and *F. acuminatum* were less frequently found, with *F. redolens* being the third most frequent in the area of Quebec and *F. solani* and *F. acuminatum* more common in Ontario. Similar to our results, the frequency of isolation in asparagus fields in Japan was 68, 28.6, and 2.5% for *F. oxysporum, F. proliferatum,* and *F. solani*, respectively, but *F. redolens* was absent (40). A similar frequency pattern was found in asparagus fields in the United Kingdom and Spain (57). However, isolations from asparagus nurseries in Spain indicated high frequencies of *F. proliferatum, F. oxysporum,* and *F. verticillioides* (syn. *F. moniliforme*) (15).

The recovery of *Fusarium* spp. is strongly influenced by host organ; for example, generally, *F. proliferatum* infects mainly the crown and other *Fusarium* spp., such as *F. oxysporum*, colonize in roots. Therefore, the plant portion affects the isolation rate of *Fusarium* spp. (17,20). In our study, due to the greater surface of roots than surface of crown (or rhizome) existing in the whole root system of asparagus plants, the ratio of root pieces sampled (including storage and secondary roots) was higher than the proportion of crown pieces (ratio of 1.5:1). This could explain the higher percentage of *F. oxysporum* in the 11 fields. Accordingly, most of the 93 *Fusarium* isolates were recovered mainly from roots (53%) and crowns (24%). The diversity and abundance of

Fusarium spp. can also be influenced by available soil nutrients (60) as well as by other abiotic and biotic factors (59).

The results of pathogenicity assessment showed a wide range in virulence among the *Fusarium* field isolates. *F. oxysporum, F. solani,* and *F. acuminatum* were highly pathogenic, whereas *F. proliferatum* and *F. redolens* were found to be weakly virulent. However, other studies reported different results in the virulence of isolates. Blok and Bollen (7) found *F. oxysporum* to be the most important virulent species in asparagus in the Netherlands, whereas extremely virulent isolates of *F. solani* were associated with asparagus in Spain and the percentage of *F. proliferatum* isolates associated with root dry weight loss was high (15). In Japan, the United Kingdom, and Spain, *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* were the species most frequently associated with diseased asparagus plants and are considered important biotic factors for asparagus decline in those countries (40,57). In this study, *F. oxysporum* and *F. proliferatum* were the predominant species but the virulence determined by pathogenicity tests also showed the importance of *F. solani* (45% isolation frequency); thus, these three species can be considered the most important species of *Fusarium* associated with the FCRR of asparagus fields in southwestern Ontario. The results of this study also showed a similar level of susceptibility in the three cultivars to FCRR. However, in the greenhouse bioassays, Jersey Giant and Grande were less susceptible to FCRR than Mary Washington. Both in vitro pathogenicity tests and greenhouse control bioassays confirmed that the *F. solani* field isolate *Fs*-1.12 was consistently more aggressive and caused severe FCRR symptoms on all three asparagus cultivars compared with the *F. oxysporum* isolate *Fo*-1.5.

The greenhouse studies indicated that PPM, ORC, and FE as preplant soil amendments reduced stem and root system severity of FCRR on asparagus, and disease reduction in most cases was more conspicuous in the moderately susceptible Jersey Giant and Grande than in the most susceptible Mary Washington. All three
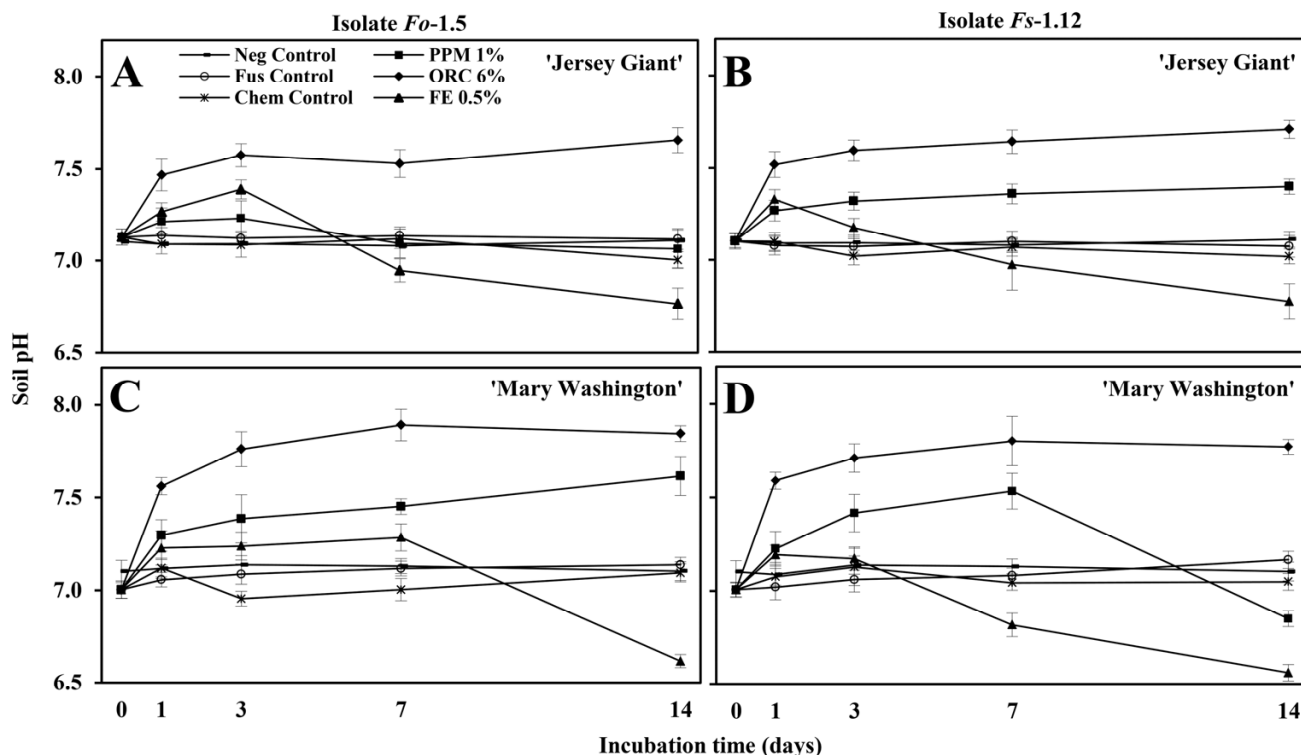
Fig. 11. Soil pH in *Fo*-1.5- and *Fs*-1.12-infested soils in **A and B,** 'Jersey Giant' and **C and D,** 'Mary Washington' experiments at 1, 3, 7, and 14 days after infestation. Infested soils were treated with benomyl (0.5 g liter^{-1} of soil) or amended with pelleted poultry manure (PPM; 1%), olive residue compost (ORC; 6%), or fish emulsion (FE; 0.5%) 24 h after infestation. Values are expressed as log CFU g^{-1} of soil and are the average (*n* = 6) of two experiments, with three replications for each treatment.

organic products also increased fresh and dry plant weights, and the biomass increase was associated with reductions in symptoms severity and was higher when the moderately susceptible cultivars were grown in *F. solani Fs*-1.12-infested soil. The results of the study also showed a gradual decline in *Fusarium* populations in contrast with a steady increase in the population densities of total culturable bacteria in soils amended with each of the three organic products. This was more frequent at the highest rates of organic amendments used. Furthermore, these higher amendment rates were also most effective in reducing FCRR severity and increasing plant weights.

Soil organic amendments can play an important role in disease control and plant growth promotion, possibly by reducing the pathogen inoculum, an argument supported by our current study as well as some previous reports. Borrego et al. (12) found a reduction in the viability of *F. oxysporum* f. sp. *asparagi* inoculum in the soils amended with PPM and ORC and incubated for several weeks at 30 and 35°C prior to planting in greenhouse pot experiments. They also found a reduction in disease severity and an increase in plant growth when asparagus seedlings were planted in such soils after the end of the incubation period. Similarly, a reduction in soil populations of *F. oxysporum*, *F. solani,* and *F. equiseti* in sweet pepper was achieved after solarization of the soils amended with fresh sheep and chicken manure (36). Soil amendment of poultry manure or compost reduced the severity and impact of Fusarium wilt on strawberry (25) and carnation (37), and soil amendment of ORC improved suppressiveness against *F. oxysporum* f. sp. *radicis-lycopersici* and *Phytophthora nicotianae* in tomato (44). Dairy compost enhanced crop yield, spear number, and microbial biomass in the asparagus fields with replant problems (41), and soil amendment of FE improved disease suppressiveness against seedling damping-off and root rots, potato scab, and Verticillium wilt diseases (1–3). In most of these cases, disease control by organic soil amendments was reciprocated with an increased microbial activity in the amended soil.

On the other hand, depending on the rates and types of soil organic amendments used, toxic metabolites released during the bacterial breakdown of some organic products in the soil may also have played a role in disease suppression. Poultry manures are nitrogen-rich amendments that can eliminate many plant pathogens due to the release and accumulation of compounds such as NH_3 and HNO_2 that can be toxic to plant pathogens (49,50). Composts made of olive residues used as plant growth substrates were found to reduce the effects of *F. oxysporum* f. sp. *radicis-lycopersici* (44) but this was related to interactions between soil abiotic characteristics, changes in microbial populations, and, perhaps, the reduced susceptibility of the plants (4,13,44). Organic byproducts used in this study may have provided disease control and plant growth promotion, possibly by decreasing pathogen populations and enhancing total bacterial activity as well as the activity of fungal antagonists (not determined in this study) in the soil. Any role of toxic compounds is less likely but cannot be overruled.

Another possible mechanism for disease suppression is competition between soil microorganisms and *Fusarium* spp. The addition of organic amendments to soil strongly influences the soil microflora (23). In our study, only populations of total culturable bacteria were determined but the densities of beneficial or deleterious fungi were not reflected. Thus, in the *Fusarium* spp.-infested soils incubated for 24 h prior to the treatments, an increase in total culturable bacteria was observed in those infested soils compared with the noninfested soils. After the addition of all organic amendments, the bacterial activity gradually and continuously increased in these amended soils. This result supports previous reports, where the use of compost on asparagus fields enhanced the soil bacterial biomass (41) and the use of FE caused the increment of bacteria in soil or substrate (1). Moreover, these

reports also showed an increase in the fungal antagonists. Fungal and bacterial antagonists are reported to play a role in *Fusarium* population structure, which is strongly influenced by soil nutrient availability such as P fertilization and Mn, Fe, Ca, and dehydrogenase activity (60). However, abiotic factors (soil structure, organic content, NH_4, Na, and Cu) have more influence on *Fusarium* spp. abundance (59). Thus, soil microbial structure could be an important factor in controlling FCRR (29).

In conclusion, the use of agricultural and fish-processing industry byproducts such as PPM, ORC, and FE as soil amendments was found to be an effective method to decrease FCRR severity in asparagus while significantly reducing dependency on chemical pesticides and fertilizers for achieving sustainable production. Our results indicated that organic products such as PPM could be considered in integrated disease management for their effectiveness in decreasing pathogen population and, consequently, disease severity, and promoting plant growth. In addition, we showed the bacterial activity as a response after application of amendments but the role of other microbial groups such as fungi should be also considered. The use of these organic byproducts for management of FCRR needs to be investigated in fields with replant problems. Moreover, the accurate and reliable identification and characterization of *Fusarium* spp. in fields using DNA-based PCR methods can be important for the development of effective control strategies.

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CAPÍTULO III

Effects of incubation temperature on the organic amendment-mediated control of Fusarium wilt of tomato

RESEARCH ARTICLE

Effects of incubation temperature on the organic amendment-mediated control of Fusarium wilt of tomato

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Abstract

Organic soil amendments play important roles in the reduction of plant diseases caused by soil-borne plant pathogens. This study examined the combined effects of concentrations of organic amendments, temperature and period of incubation in soil on the management of Fusarium wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). In an experiment with substrate mixture, *Fol* reduction was higher when the soils were incubated at 35°C than at 30°C. Disease severity was proportionally reduced as the volume of amendment added increased. Furthermore, disease was significantly lower in substrates incubated for 30 days at both temperatures, as compared to substrates incubated for only 15 days. The most effective control was achieved with pelletised poultry manure (PPM). In experiments with natural sandy soil, the effects of amendments on *Fol* populations, measured by real-time quantitative PCR with TaqMan probes, were significant. The highest decreases in *Fol* DNA resulted when the soil was amended with 2% PPM and incubated at 35°C. The reductions in DNA concentrations was most likely related to the accumulations of high concentrations of NH_3 (27.3 mM) in soils treated with 2% PPM and incubated at room temperature (RT; 23 ± 2°C), or at 35°C. Severity of plants grown in soils incubated at RT decreased by over 40%, and more than 73% when incubated at 35°C, regardless of the rate of PPM. The results indicate that the management with PPM, when combined with heating or solarisation, is an effective control measure against Fusarium wilt of tomato.

Introduction

Fusarium wilt of tomato (*Solanum lycopersicum*) is caused by a soil-borne fungal pathogen, *Fusarium oxysporum* f. sp. *lycopersici* W. C. Snyder & H. N. Hans. (*Fol*). The disease occurs throughout most tomato-growing areas of the world and can cause serious economic losses. The pathogen causes vascular wilt by infecting plants through the roots and growing internally through the root cortex to xylem vessels, where it is carried upward to the sap stream. Symptoms include wilting and death at seedling stage, stunting of the plants, yellowing of leaves, wilting of leaves and stems and defoliation. A brown ring appears in the area of the vascular bundles, in cross sections near the base of the infected plant stem (Jones *et al.*, 1991).

Resistant cultivars have been the most effective means of controlling Fusarium wilt of tomato (Beckman, 1987). However, the occurrence and evolution of new pathogenic races are a continuous problem (Jones *et al.*, 1991). The control of soil-borne plant pathogens, including *Fusarium*, using chemical methods is difficult, and few chemical control options are available for growers to effectively manage such pathogens (McDonald & Linde, 2002). Moreover, the use of methyl bromide, the most effective fumigant for soil-borne pests (e.g. Gamliel *et al.*, 2000), is no longer allowed in the EU and developed countries because of the associated environmental hazards (Basallote-Ureba *et al.*, 2010).

Organic soil amendments (OAs) have shown to reduce the effects of several soil-borne plant pathogens

(Lazarovits, 2001; Litterick *et al.*, 2004; Noble & Coventry, 2005; Bonanomi *et al.*, 2007; Avilés *et al.*, 2011; Melero-Vara *et al.*, 2011, 2012; Borrego-Benjumea *et al.*, 2014). The soil amendments more effective for disease control were found to be compost and organic wastes (Bonanomi *et al.*, 2007). Nitrogen-rich amendments, such as poultry manure, have shown to eliminate many plant pathogens as a result of the decomposition of the organic matter leading to the release and accumulation of compounds, such as NH_3 and HNO_2 that are toxic to plant pathogens (Tsao & Oster, 1981; Tenuta & Lazarovits, 2002a). These volatile products killed microsclerotia, the resting structures of *Verticillium dahliae*, which are particularly difficult to eradicate in soil (Tenuta & Lazarovits, 2002a). The release of NH_3 and HNO_2 into the soil because of the degradation of OAs is one of the mechanisms involved in reducing or eliminating pathogen populations in soil, but pH is a crucial factor in determining the relative amounts of toxic NH_3 and HNO_2 versus the non-toxic NH_4^+ or NO_2^- compounds (Tenuta & Lazarovits, 2002b). When soil microorganisms degrade amendments rich in nitrogen, any excess nitrogen is as NH_3, which is rapidly converted to NH_4^+ increasing the soil pH. This brings about NH_4^+ ions to convert back to NH_3, which is very toxic and formed only at pH 7 or above (Ells *et al.*, 1991). When NH_4^+ is converted to NO_2^-, soil pH drops rapidly as H^+ are released and, when pH is below 5.5, NO_2^- starts to be converted to HNO_2 (nitrification), which is highly toxic to many fungal plant pathogens such as *F. oxysporum* and *V. dahliae*, as well as to weeds and other pests (Tsao & Oster, 1981; Tenuta & Lazarovits, 2002a). The production of such compounds also likely reduced Fusarium wilt and root galls of carnation, respectively caused by *F. oxysporum* f. sp. *dianthi* and *Meloidogyne incognita* (Melero-Vara *et al.*, 2011, 2012), as well as crown and root rot of asparagus caused by *Fusarium* spp. (Borrego-Benjumea *et al.*, 2014). Composts made of olive residues derived from agroindustrial wastes used as plant growth substrates were found to reduce the effects of *Fol*, but this was related to interactions between soil abiotic characteristics, changes in microbial populations and perhaps the reduced susceptibility of the plants (Borrero *et al.*, 2004; Ntougias *et al.*, 2008; Avilés *et al.*, 2011).

Soil solarisation has proven feasible for the control of different soil-borne plant pathogens in a variety of crops, primarily by physical modes of action; the effect is mainly because of the high solar radiation and to the temperatures usually reached during the summer (Katan *et al.*, 1976; Katan, 1981; González-Torres *et al.*, 1993; Chellemi, 2002). Combining organic amendments with soil solarisation has been an effective method of controlling fungi, weeds and other pests (Stapleton & Duncan, 1998; Klein *et al.*, 2011b; Melero-Vara *et al.*, 2011, 2012).

The impact of any management strategies on Fusarium wilt of tomato or other soil-borne fungal diseases can be verified by monitoring the population densities of the pathogen in soil. The methods using soil dilutions plated onto semi-selective media are commonly used for enumeration of fungal propagules, which are usually expressed as colony-forming units (CFU) (Bridge & Spooner, 2001). However, molecular methods based on polymerase chain reaction (PCR), such as real-time PCR, are now more popular and have been shown to rapidly and accurately detect and/or quantify the amount of *F. oxysporum* genomic DNA in several crop diseases, such as the Fusarium wilts of daisy (*F. oxysporum* f.sp. *chrysanthemi*) (Pasquali *et al.*, 2004), watermelon (*F. oxysporum* f. sp. *niveum*) (Zhang *et al.*, 2005), cotton (*F. oxysporum* f. sp. *vasinfectum*) (Abd-Elsalam *et al.*, 2006), tomato (*F. oxysporum* f. sp. *lycopersici*) (Inami *et al.*, 2010) and chickpea (*F. oxysporum* f. sp. *ciceris*) (Jiménez-Fernández *et al.*, 2011) and Fusarium wilt and crown rot of basil (*F. oxysporum* f. sp. *basilici*) (Pasquali *et al.*, 2006).

The objectives of this study were to determine the effects of incubation at moderately high temperature on (a) organic amendment-mediated control of Fusarium wilt of tomato, (b) concentrations of NH_3 and HNO_2 for disease development and (c) pathogen populations or *Fol* DNA in substrate mixture and natural sandy soil.

Materials and methods

Fusarium inoculum and infestation of substrate mixture or soil

The tomato wilt pathogen used in this study was a monosporic isolate of *F. oxysporum* f. sp. *lycopersici* race 2 (*Fol*-C) originally isolated from an infected tomato plant in London (ON, Canada). The pathogenicity of this isolate was confirmed in previous assays. This pathogen was grown in potato dextrose broth in flasks in which culture plugs of the fungus obtained from the edge of the colony were added just before incubating on a rotary shaker (150 rpm) at 25°C for 7 days. The spore suspension was filtered through four layers of sterile cheesecloth and the spore concentration was determined using a haemocytometer.

A sand : silt (2:1, v/v) substrate mixture and a natural sandy soil (1.0% organic matter, pH 6.9), collected from an experimental farm located near Delhi (ON, Canada), were used in pot experiments conducted in a growth chamber. The experiment with substrate mixture served to establish conditions prior to the experiments with natural sandy soils where comprehensive studies were performed. The substrate mixture was sterilised by autoclaving at 121°C for 60 min twice for two consecutive days prior to infesting with the pathogen spore suspension

to reach a density of 10^6 microconidia g^{-1} soil, and the contents were mixed vigorously and homogenised. The infested substrate mixture was incubated at 25°C in darkness for 30 days, airing on alternate days in a laminar flow hood. After incubation, the formation of chlamydospores was confirmed, and aliquots of 45 g of the infested substrate were placed into 50-mL plastic tubes. The natural soil was also infested with the pathogen spore suspension as described above.

Organic amendments

The three organic amendments from the agricultural industry evaluated for Fusarium wilt control in growth chamber assays were poultry manure (PM) from local farmers, commercially available pelletised poultry manure (PPM) from Envirem Technologies Inc. (NB, Canada) pasteurised to ensure that the material is devoid of pathogens and weed-propagules and olive residue compost (ORC), resulting from the controlled fermentation of the by-products from the olive oil extraction process along with sheep manure (obtained from the Department of Agriculture and Fisheries, Andalucía, Spain). Results of the NPK analyses as well as pH and electrical conductivity (EC) values of these materials are presented in Table 1.

Incubation temperature and Fusarium wilt of tomato bioassay

Bioassay with substrate mixture

After incubation of the infested substrate mixture at 25°C, aliquots of 45 g were placed into 50-mL plastic Falcon tubes and amended with OAs [PM at 1 and 2%, PPM at 0.5 and 1% or ORC at 3 and 6% (w/w)] and the contents were mixed thoroughly. The tubes were capped with lids that allowed some air exchange and incubated in the dark at 30 or 35°C for 15 and 30 days. A bioassay with the amended substrate mixture described above was carried out in growth chamber with seedlings of tomato cv. Monfavet, proven as susceptible to *Fol*-C in previous assays. Plastic pots (9.5 cm × 9.5 cm × 9.5 cm) were filled with a sand : peat (1:1) potting mixture and three planting holes per pot were made. The infested substrate of each tube was distributed among the three planting holes of each pot. Pots without OAs with un-infested and *Fol*-infested

substrates served as negative and positive *Fol* inoculated controls, respectively. One tomato seedling (2–3 true-leaf stage) grown in vermiculite for 12 days in a growth chamber was transplanted into each of the three holes per pot. Pots were kept in a growth chamber for 4 weeks (15 h of fluorescent light at 22°C and 9 h of darkness at 19°C) and were irrigated as needed. The bioassay included three pots (replications) with three plants per pot for each treatment combination. The experiment was conducted as a factorial design, involving three factors: the duration of the incubation (15 and 30 days), incubation temperature (30 and 35°C) and the nature of OA (PM, PPM, ORC). Development of disease symptoms (chlorosis and wilt) was recorded weekly and the severity in shoots (percentage of chlorotic and wilted leaves and stem) was evaluated at the end of the experiment. Symptomatic shoot tissues of all the plants were sampled and transferred to potato dextrose agar (PDA) medium to confirm the infection by *Fol*.

Bioassays with natural sandy soil

On the basis of the results of the bioassay with substrate mixture, PPM and ORC (organic amendments, respectively with high and low nitrogen contents) were selected to carry out two bioassays with natural sandy soil (one with PPM and another with ORC).

Aliquots of 450 g dry soil were placed in 600-mL plastic containers and infested with 50 mL of the spore suspension (at a density of 10^6 microconidia g^{-1} soil) as described above. Sterile water was added to the negative control soil. Following the infestation of soil with the inoculum, PPM was added at 0.5, 1 and 2% and ORC at 1, 2 and 3% (w/w) and thereafter thoroughly mixed with the soil. The containers were covered with lids that allowed air exchange and were incubated for 28 days in the dark at room temperature (RT; 23 ± 2°C) or in incubators set at 35°C. All the experiments included five replications (containers) per treatment combination. Treatments were arranged in a factorial design involving two factors: two temperature regimes of soil incubation (RT: 23±2°C; and 35°C) and the amendments with the three rates of OAs (PPM or ORC). In order to study the mechanisms by which these organic amendments control Fusarium wilt disease, soils samples were collected at 0, 7, 14 and 28 days of incubation for determination of soil pH and chemical analyses of soil water suspensions for the quantification of NH_3, NH_4^+, HNO_2, NO_2^- and NO_3^- (Tenuta & Lazarovits, 2002*a*) on the same day of collection. Samples collected after 28 days of incubation were also analysed for determination of inoculum viability of *Fol*. These samples were stored at −20°C until laboratory processing.

Table 1 Characteristics of the organic amendments tested

Organic Amendments	N (%)	P (%)	K (%)	pH	EC (mS cm^{-1})
Poultry manure	2.4	3.4	1.9	7.5	2.9
Pelletised poultry manure	4.0	1.0	2.0	6.3	–
Olive residue compost	1.5	0.7	2.0	8.7	3.5

Two bioassays with amended soil (one with PPM and another with ORC), as described above, were carried out in growth chamber using the tomato cv. Bellestar, proven as susceptible to *Fol*-C in previous assays. The soil samples (400 g) of each container were poured into plastic pots (9.5 cm × 9.5 cm × 9.5 cm). As negative and positive *Fol* inoculated controls, pots without OAs with un-infested and *Fol*-infested soils were used, respectively. Tomato seedlings (2–3 true-leaf stage) were transplanted into each pot. Plants were irrigated as needed and grown for 4 weeks in the growth chamber under the same conditions as above. The bioassays included five pots (replications) with five plants per pot for each treatment combination. The experiment followed a factorial design involving two factors: two temperature regimes of soil incubation (RT and 35°C) and the treatments with OAs (three rates of PPM or three rates of ORC). Disease symptom development (chlorosis and wilt) was observed once every week. At the end of each experiment, the plants were removed from the pots. Disease severity on shoots (percentage of chlorotic and wilted leaves and stems) was assessed. Then, for each treatment, dry weight of shoots and roots per pot were determined after the plants had dried for 48 h at 70°C. Symptomatic shoot tissues of all the plants were sampled and transferred to PDA medium to confirm the infection by *Fol*.

Effectiveness of soil treatments on reduction of *Fol* densities

Quantification of Fusarium inoculum in substrate mixture by CFU method

In the experiment with the substrate mixture, the viability of *Fol* inoculum was determined by dilution plating on Fusarium-selective agar medium (Bouhot & Rouxel, 1971). Substrate samples (1 g) were taken at 0 (prior to adding the OAs), 15 and 30 days incubation of substrate-amendment mixture. Each sample was shaken with 99 mL of sterile 0.1% water agar on a rotary shaker (100 rpm) for 10 min. The suspension was serially diluted and 1 mL from each of 10^{-2} dilutions was plated onto each of three plates of the selective medium. The plates were incubated at 25°C and the CFU were counted 5 days after incubation. Inoculum density was expressed as log of CFU per gram of soil.

Quantification of Fol *DNA in natural sandy soil by real-time PCR*

In the experiments with sandy soil, the density of *Fol* inoculum in soil was determined by real-time PCR analysis.

DNA extraction. Fungal genomic DNA for standard curves was extracted from fungal mycelium, using the E.Z.N.A. SP Fungal DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. To extract DNA from soil, un-infested and infested soil samples incubated for 28 days were lysed using the FastPrep®-24 instrument running at $6.0\,\mathrm{m\,s^{-1}}$ for 40 s. DNA was extracted from 250 mg sample using the PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) and stored at −20°C until use.

Polymerase chain reaction primers and probe. The real-time PCRs were carried out using a primer pair (5′-CCG AAT TGA GGT GAA GGA CAG-3′ and 5′-CCG AAG TAC CCA TTG AGA GTG-3′), designed previously (Lievens *et al.*, 2009). The primer pair amplifies the SIX1 gene of *F. oxysporum* f. sp. *lycopersici*. This gene is carried by all the current races of *Fol* but not by other *formae speciales*, non-pathogenic isolates in *F. oxysporum* and other fungal species, and can be used for unambiguous identification of *Fol* races (Lievens *et al.*, 2009). The fluorogenic TaqMan® probe (5′-(FAM) CGT AGG TCC CAG GCG ATT TAG GC (BHQ1)-3′) used was designed using Primer3 software (Rozen & Skaletsky, 2000). This probe was labelled at 5′-terminal nucleotide with 6-carboxy-fluorescein (FAM) fluorescent reporter and at 3′-terminal nucleotide with the minor groove binding non-fluorescent Black Hole Quencher (BHQ1). An *in silico* test was performed for the primer-probe combination by running the primer sequences against the NCBI GenBank database to ensure the specificity of the primers and probe prior to synthesis.

Standard curves and real-time polymerase chain reaction amplification. Standard fluorescent amplification curves representing an exponential growth of PCR products based on cycle thresholds (Ct) for 10-fold dilution series of pure fungal culture genomic DNA (13, 13×10^{-1}, 13×10^{-2}, 13×10^{-3}, 13×10^{-4} and $13 \times 10^{-5}\,\mathrm{ng\,\mu L^{-1}}$), prepared in triplicate and used in real-time PCR reactions were constructed for the *Fol* isolate detected by the corresponding specific primer pairs. Concentrations were determined by absorbance at 260 or 280 nm using a spectrophotometer NanoDrop, ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA). Ct values were calculated to indicate significant fluorescence signals rising above background during the early cycles of the exponential amplification phase of the real-time PCR process. Quantitative PCR amplifications were carried out using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), in a total volume of 15 µL consisting of 1.5 µL template DNA, 1.5 µL (2 µM) of primers, 1.5 µL (1 µM) of TaqMan® probe, 7.5 µL of 2×

EXPRESS qPCR Supermix Universal (Invitrogen, Carlsbad, CA, USA), 2.1 μL (10 μg μL^{-1}) of bovine serum albumin (BSA) and 0.9 μL of sterile distilled water. Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 94°C, 20 s at 57°C and 30 s at 72°C, in addition to 2-min pre-incubation at 50°C. There were three replicated samples per treatment and two PCR assays per sample. The experimental Ct values were transformed into amounts (expressed as nanograms) of pathogen DNA.

Statistical analyses

All experiments were repeated at least twice, and yielded similar results. Separate analyses of each experiment showed homogeneous variance of the experimental error between replications. Data were analysed using analysis of variance (ANOVA; $P \leq 0.01$) and the means were separated using the LSD test ($P \leq 0.01$), except the data on CFU assay which were performed at $P \leq 0.05$. All data analyses were performed using Statistix 9.0 software (Analytical Software, Tallahassee, FL, USA).

Results

Soil chemical analyses in sandy soil experiments

In the experiment with PPM, the soil pH was significantly ($P \leq 0.01$) affected by the amendment rates, soil incubation temperature and incubation period. For all treatments pH remained below 8.5 during the entire period of incubation except for soil treated with 2% PPM and incubated at 35°C. The pH of this treatment reached over 9.0 from 7 to 28 days of incubation at 35°C (Fig. 1A and 1B). Soil incubation at RT with 2% PPM spiked pH to 8.5 after 7 days but had fallen back to pH 7.5 by day 14. Soil pH decreased below 6.5 when the other rates of PPM had been applied and incubated at RT, when compared to pH 6.9 in un-amended soils. Similarly, in soils treated with the lowest rate of PPM and incubated at 35°C for 28 days, pH decreased to 6.0 (Fig. 1A and 1B).

Accumulation of NH$_3$ and NH$_4^+$ occurred only in amended soils (Fig. 1C to 1F). The concentration of NH$_3$ by day 7 was significantly higher in soils amended with 2% PPM (Fig. 1C and 1D) than in other treatments. The concentration of NH$_4^+$ was also highest in soils amended with 2% PPM, that is 3.4 and 4.0 mM at RT and 35°C, respectively, and lowest at the lower rates of PPM (Fig. 1E and 1F). With 2% PPM, the increase of NH$_3$ concentration was more pronounced in soils incubated at 35°C (70.1 mM) than in those incubated at RT (27.3 mM, Fig. 1C and 1D), and coincided with the rise in pH above 8.5. However, the increases in both NH$_3$ and NH$_4^+$ concentrations sharply declined after incubation
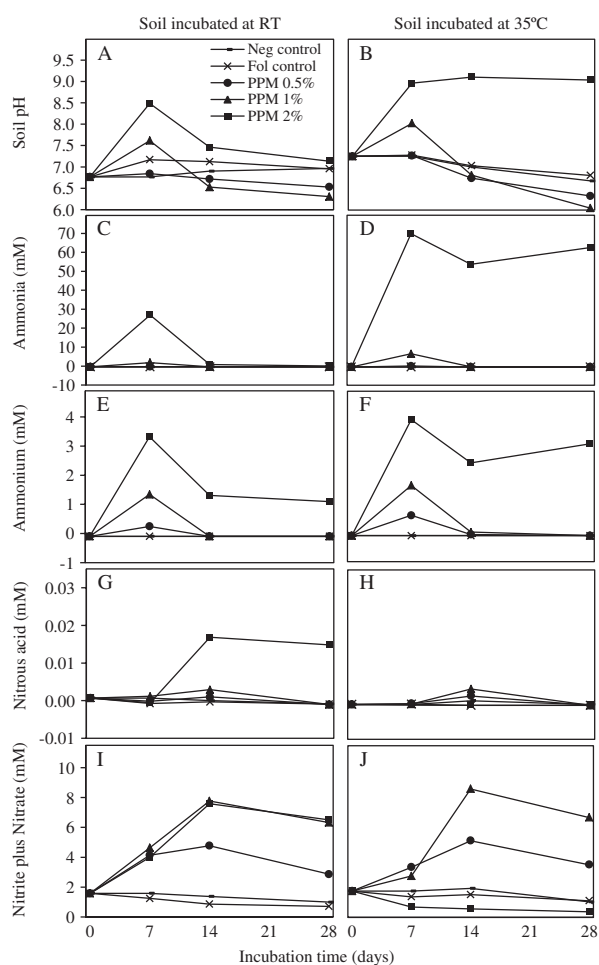
Figure 1 Soil pH (A, B), and concentrations of ammonium (C, D), ammonia (E, F), nitrous acid (G, H) and nitrite plus nitrate (I, J), in a sandy soil artificially infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*-C) and amended with pelletised poultry manure (PPM) following the incubation at RT and 35°C in the dark, for 7, 14 and 28 days. Values are the average of the results from two trials, each having five replications per treatment.

for 14 days at RT, but remained high until 28 days of incubation in 2% PPM samples at 35°C (Fig. 1D and 1F).

There was a significant ($P \leq 0.01$) increase in the concentration of HNO$_2$ (0.018 and 0.016 mM, respectively) after 14 and 28 days of incubation at RT in soils amended with 2% PPM (Fig. 1G). Under these conditions, soil pH decreased following the initial increase to pH 8.5 at 7 days incubation (Fig. 1A). In contrast, at the same rate of PPM, soil pH rose over 8.5 during the entire incubation period at 35°C (Fig. 1B) at which HNO$_2$ is not formed. The concentration of HNO$_2$ among the different treatments did not change significantly during incubation at 35°C (Fig. 1H).

The concentration of NO$_2^-$ plus NO$_3^-$ in un-amended soils did not vary significantly during incubation at any of the temperature regimes tested. In amended soils, the

total of these compounds accumulated to high levels regardless of incubation temperatures and periods, with the only exception of 2% PPM incubated at 35°C, where very little NO_2^- plus NO_3^- was found ($P \leq 0.01$). The maximum concentrations of NO_2^- plus NO_3^- were recorded with PPM, irrespectively of the rate, after 14 days incubation, with a moderate decrease from then onwards (Fig. 1I to 1J).

In the experiment with ORC amendment there were no significant differences in soil pH between the un-amended soil incubated at RT or 35°C, but soil pH increased significantly when the dosage of compost added to soils was increased (data not shown). Neither NH_4^+ nor NH_3 was detected in any of the soils amended with ORC, and concentrations of HNO_2 were almost negligible (data not shown).

Fusarium wilt of tomato bioassay

Bioassay with substrate mixture

Disease symptoms consisted of yellowing, wilting and defoliation of leaves, death and stunting of plants and vascular colouration at the base of stems. A significant ($P \leq 0.01$) interaction was found in the relationships between temperature/incubation period and soil amendments. More than 70% of the tomato plants in the *Fol* inoculated control were diseased, with an average stem disease severity rating of 41 and 53%, by 15 and 30 days of incubation at 30°C, respectively, and of 53 and 31%, by 15 and 30 days of incubation at 35°C, respectively (Table 2). Thus, the highest temperature (35°C) and the longest incubation period (30 days) showed the greatest reduction of the disease in the *Fol* inoculated control plants. This response of incubation period was consistent across the various amendments tested.

The addition to the substrate of all OA rates, except PM 1%, significantly ($P \leq 0.01$) reduced severity of symptoms by an average of the OAs rating of 25–68% by 15 days and 41–91% by 30 days of soil incubation (Table 2). On the whole, all the treatments effectively reduced stem severity when OAs were applied at the highest rates (PM-2% by 40–81%; PPM-1% by 60–91%; ORC-6% by 48–68%; Table 2). The largest relative reduction of severity (91%) resulted when substrate mixture amended with PPM 1% was incubated at 35°C for 30 days. Also, under these conditions ORC-amended treatment (66% severity reduction) appeared to achieve good disease control (Table 2).

Bioassays with natural sandy soil

Symptomatic plants showed symptoms similar to those above mentioned. In bioassays with PPM-amended soils

Table 2 Effect of incubation temperature of a substrate mixture artificially infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*-C) and addition of different organic amendments[a] on mean severity[b] of symptoms in tomato cv. Monfavet

| | Disease Severity (%)[c] | | | |
| | 30°C | | 35°C | |
Treatments	15 days	30 days	15 days	30 days
Negative control	6.5 ± 1.5 e	2.9 ± 1.1 f	5.3 ± 1.3 h	2.6 ± 1.2 d
Fol control	41.1 ± 4.3 a	53.2 ± 1.8 a	52.5 ± 3.9 a	30.6 ± 2.7 a
PM 1%	17.5 ± 1.6 c	22.3 ± 3.2 c	27.5 ± 1.0 e	28.8 ± 3.0 a
PM 2%	24.8 ± 2.5 b	10.1 ± 2.5 e	30.2 ± 1.3 d	16.1 ± 2.6 b
PPM 0.5%	27.9 ± 3.6 b	21.4 ± 4.6 c	32.5 ± 3.2 c	15.8 ± 1.6 b
PPM 1%	14.7 ± 4.1 d	18.1 ± 3.2 d	21.1 ± 2.2 f	2.7 ± 0.6 d
ORC 3%	26.3 ± 1.8 b	22.7 ± 3.8 b	39.5 ± 3.0 b	12.1 ± 0.9 c
ORC 6%	21.2 ± 3.2 c	18.6 ± 6.7 d	16.8 ± 1.2 g	10.4 ± 2.8 c

[a]Poultry manure (PM), pelletised poultry manure (PPM) and olive residue compost (ORC) were incorporated into the infested substrate and incubated in the dark.
[b]After incubation, tomato seedlings were planted and incubated in a growth chamber. Plants were rated after 4 weeks for Fusarium wilt using a percentage scale of chlorotic and wilted leaves and stems.
[c]Values are the mean ± SE of the results from two trials, each having three replications per treatment. Values followed by the same letter within each column are not significantly different according to LSD's test ($P \leq 0.01$).

the severity of Fusarium wilt of tomato plants was reduced as compared to *Fol* inoculated control soils (Fig. 2A). Moreover, the reduction of disease severity caused by *Fol* differed significantly ($P \leq 0.01$) in soils amended with increasing rates of PPM, mostly with incubation at high temperature. The greatest activity of PPM was found at the 1% rate as the severity was reduced by 40 and 95% at RT and 35°C, respectively (Fig. 2A). Plants grown in soils with 2% PPM frequently showed phytotoxic symptoms when incubated at 35°C. Plant growth was significantly increased in soils amended with PPM rates 0.5 and 1%, with greater plant dry weights at 1% rate than at 0.5% (Fig. 2B). These increases were consistent with reductions in the severity of symptoms in plants grown in soil incubated at 35°(Fig. 2A). The interaction of soil incubation temperatures with the rates of organic amendments was also significant ($P \leq 0.01$), as combining 1% amendment with incubation at RT and 35°C increased plant growth to 2.4 and 3.5-fold, respectively, over *Fol* inoculated control (Fig. 2B).

In bioassays with ORC amendment, severity in *Fol* inoculated control treatment was around 15%. As compared to the *Fol* inoculated control, only the reduction in severity attributable to the addition of ORC at 3% was significantly ($P \leq 0.01$) greater, reaching 100 and 95%, at RT and 35°C, respectively (data not shown). Significantly ($P \leq 0.01$) greater plant dry weights were only observed in the un-amended soil (data not shown).
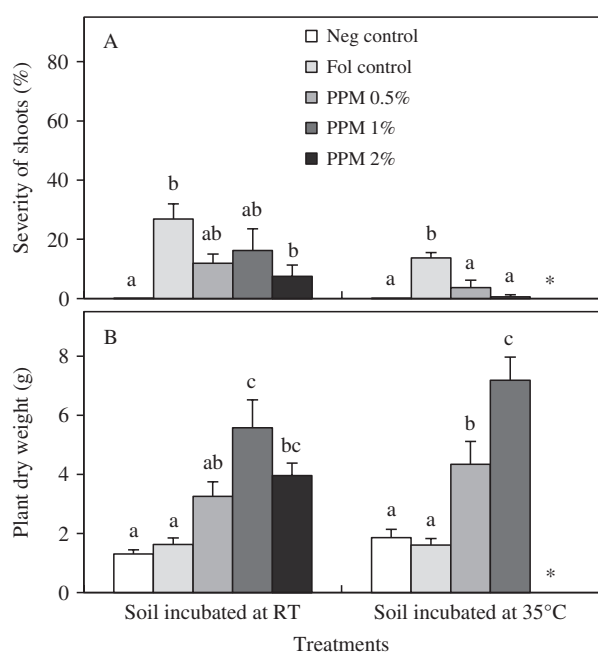
Figure 2 Effect of the amendment with pelletised poultry manure (PPM) of a sandy soil artificially infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*-C), and incubation in the dark at RT and 35°C, for 28 days, on severity of tomato plants (A) cv. Bellestar and on their dry weights (B). After incubation, tomato seedlings were planted and kept in a growth chamber for 4 weeks. Values are the average of the results from two trials, each having five replications per treatment. Error bars represent standard errors of the mean. Bars headed by the same letter were not significantly different according to LSD's test (P≤0.01). Asterisk (*) indicates the plants that showed phytotoxic symptoms.

Effectiveness of soil treatments on reduction of *Fol* densities

Quantification of Fusarium inoculum in substrate mixture by CFU method

The incubation temperature of infested substrate influenced the viability of *Fol*, which was reduced significantly ($P \leq 0.05$), especially at 35°C (Table 3). The incubation period also significantly ($P \leq 0.05$) decreased inoculum viability with incubation for 30 days being more effective than 15 days. Thus, the reduction in inoculum viability was greater when incubations lasted longer and at a higher temperature. The addition of all OA rates to the substrate significantly ($P \leq 0.05$) reduced the viability of *Fol* inoculum as compared with the control treatment (Table 3). The interaction of temperature/incubation period with OA was also significant. The two rates of PPM significantly reduced *Fol* viability over 74–86% and 92–94% after 15 or 30 days of incubation at 30 and 35°C, respectively, over the control (Table 3). Poultry manure added at 1 and 2% significantly decreased

Table 3 Effect of incubation temperature and different organic amendments[a] on the viability of *F. oxysporum* f. sp. *lycopersici* (*Fol*-C) in a substrate mixture

|	*Fol* Viability (Log of CFU g^{-1} soil)[b]			
	30°C		35°C	
Treatments	15 days	30 days	15 days	30 days
Fol control	6.43 ± 0.87 a	6.14 ± 0.86 a	6.29 ± 0.06 a	5.94 ± 0.07 a
PM 1%	6.21 ± 0.89 ab	5.77 ± 0.58 b	5.96 ± 0.07 b	5.58 ± 0.08 b
PM 2%	5.95 ± 0.88 bc	5.75 ± 0.46 b	5.87 ± 0.06 bc	5.59 ± 0.09 b
PPM 0.5%	6.10 ± 0.89 b	5.51 ± 0.79 d	5.61 ± 0.07 ef	5.15 ± 0.07 c
PPM 1%	5.84 ± 0.89 c	5.58 ± 0.69 cd	5.45 ± 0.06 f	4.97 ± 0.08 d
ORC 3%	6.18 ± 0.87 ab	5.70 ± 0.34 bc	5.80 ± 0.06 cd	5.56 ± 0.09 b
ORC 6%	6.08 ± 0.86 bc	5.68 ± 0.23 bc	5.67 ± 0.06 de	5.44 ± 0.09 b

[a]Poultry manure (PM), pelletised poultry manure (PPM) and olive residue compost (ORC) were incorporated into the infested substrate and incubated in the dark.
[b]Values are the mean ± SE of the results from two trials, each having three replications per treatment. Values followed by the same letter within each column are not significantly different according to LSD's test ($P \leq 0.05$).

($P \leq 0.05$) inoculum viability over 67 and 82% after 15 or 30 days of incubation at 30 and 35°C, respectively, as compared to the un-amended control (Table 3). Similarly, the treatment with ORC at 3 and 6% significantly reduced *Fol* viability over 69 and 87% after 15 or 30 days of incubation at 30 and 35°C, respectively (Table 3).

Quantification of Fol *DNA in natural sandy soil by real-time PCR*

The specificity of the primer and probe pair was examined by performing PCR assays with DNA from *Fol*-C isolate. The primers and TaqMan® probe amplified a 138-bp single amplicon of the SIX1 gene from pure cultures of *Fol*-C isolate. The precision of the real-time PCR assay was validated using serial dilutions of *Fol*-C isolate DNA. The qPCR assay detected DNA of *Fol*-C isolate in all soil samples infested but not in un-infested controls. The fluorescent signal was proportional to the log concentration of DNA. There was a linear relationship ($R^2 = 0.978$ to 0.988) between *Fol*-C genomic DNA concentration and real-time quantification cycles using the primers and TaqMan® probe.

In the experiment with PPM amendment, the quantities of DNA, from the standard curve constructed with known concentrations of DNA from pure cultures of the isolate *Fol*-C, ranged from 0.043 to 6.939 pg g^{-1} of soil in infested soil samples collected following 28 days of incubation. The temperature of incubation of infested soil had an effect on fungal survival, reducing significantly ($P \leq 0.01$) *Fol*-C DNA concentrations from 6.9 to 4.3 pg DNA g^{-1} soil incubated at RT and at 35°C,
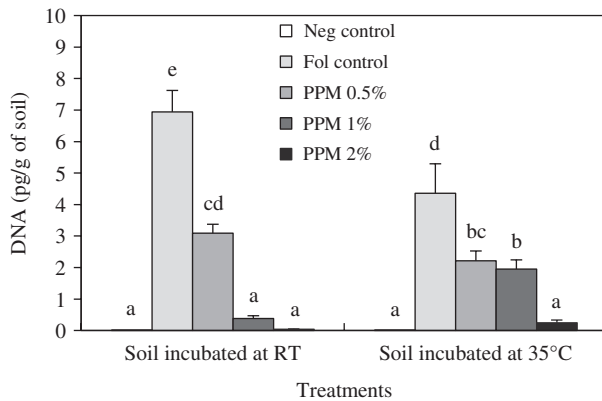
Figure 3 Estimation of absolute DNA quantities of a sandy soil artificially infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*-C) and amended with pelletised poultry manure (PPM), based on values shown in the standard curve from the real-time quantitative PCR analysis. Values are the average of the results from two trials, each having five replications per treatment. Error bars represent standard errors of the mean. Bars headed by the same letter indicate no significant difference according to LSD's test ($P \leq 0.01$).

Figure 4 Estimation of absolute DNA quantities of a sandy soil artificially infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*-C) and amended with olive residue compost (ORC), based on values shown in the standard curve from the real-time quantitative PCR analysis. Values are the average of the results from two trials, each having five replications per treatment. Error bars represent standard errors of the mean. Bars headed by the same letter indicate no significant difference according to LSD's test ($P \leq 0.01$).

respectively, in the *Fol* inoculated control soils (Fig. 3). In PPM-amended soils incubated at RT, detection of *Fol*-C DNA gradually decreased with increasing rates of PPM, such that significantly lower values ($P \leq 0.01$) than *Fol* inoculated control were found in the treatments with 0.5, 1 and 2% PPM (Fig. 3). When incubation was at 35°C, *Fol*-C DNA recovery in PPM-treated soil was similar to that found in soil incubated at RT for the comparative rates of PPM treatment, except for 1% rate, which showed significantly lower DNA recovery when incubated at RT than at 35°C (Fig. 3).

In experiment with ORC, from the standard curve constructed, the quantities of *Fol*-C DNA ranged from 0.119 to 0.543 pg g^{-1} of soil in infested soil samples collected following 28 days of incubation. The temperature of incubation of infested soil had an effect on fungal survival, reducing significantly ($P \leq 0.01$) *Fol*-C DNA concentrations from 0.54 pg DNA g^{-1} soil incubated at RT to 0.29 pg DNA g^{-1} soil incubated at 35°C, in the *Fol* inoculated control soils (Fig. 4). During incubation at RT, the amount of inoculum was significantly reduced ($P \leq 0.01$) in soils amended with ORC at 1% and higher rates. In soils incubated at 35°C, there was a significant reduction ($P \leq 0.01$) between soils un-amended and amended at 2 and 3% ORC (Fig. 4).

Discussion

In this work, the combined effect of organic amendments of soil and moderate temperatures of incubation, as an approach to sub-optimal soil solarisation, was found to

be effective in reducing the viability of propagules of *F. oxysporum* f. sp. *lycopersici* (*Fol*) and, consequently, the disease severity in tomato plants.

This is consistent with results reported on the combination of organic amendments and soil solarisation on the survival of soil-borne plant pathogens such as *Rhizoctonia solani*, *V. dahliae* and some *formae speciales* of *F. oxysporum*, for example *Fol*, *F. oxysporum* f. sp. *asparagi*, *F. oxysporum* f. sp. *radicis-cucumerinum* and *F. oxysporum* f. sp. *dianthi* (Blok *et al.*, 2000; Borrego-Benjumea *et al.*, 2010, 2014; Klein *et al.*, 2011*a*; Melero-Vara *et al.*, 2011). Moreover, incorporation of plant residues from field crops in soil and exposure to solarisation for 2 weeks under field conditions in Israel resulted in 95–100% mortality of *F. oxysporum* f. sp. *radicis-lycopersici* (Klein *et al.*, 2011*b*), the control of *F. oxysporum* f.sp. *cumini* was improved by combining prolonged heating with incorporation of on-farm wastes and irrigation (Israel *et al.*, 2011), and reductions over 72% on populations of *F. oxysporum*, *Fusarium solani* and *Fusarium equiseti*, all three pathogens of sweet pepper, were achieved in soils amended with fresh sheep and chicken manure and then solarised (Martínez *et al.*, 2011).

In this work, the highest temperature (35°C) of soil incubation for the longer period (30 days) tested reduced inoculum viability to the greatest extent. Another study (Bennett, 2012) reported an effective reduction of the viability of *F. oxysporum* f. sp. *vasinfectum*, causing Fusarium wilt of cotton, with incubation at 39–41°C for 6–10 days. When we added the highest rates of each amendment in combination with sandy soil incubation at 35°C, the

control of the disease was usually enhanced, but PPM was the most effective treatment in reducing *Fol* viability and, consequently, disease severity. Furthermore, both PPM and ORC differed in their effects on tomato bioassays, the former enhanced up to 3.5-fold plants dry weight, as a result of reducing tomato symptoms severity and the beneficial effect of N as a fertiliser. In contrast, wilt severity reduction in ORC-amended soil was not associated to tomato dry weight, as it was higher in un-amended plots.

The most effective control achieved might be associated with higher nitrogen content with PPM (4.0% N) than with ORC (1.5% N) amendments. Soil incubation temperature had an important role in the processes of NH_3 and HNO_2 release, as the highest temperature (35°C) accelerated the process of mineralisation. In our work, soil HNO_2 concentrations were high only for PPM-2% sample, at 14–28 days of incubation at RT, likely corresponding to increased rates of nitrification and rapid declines in soil pH. Although soils amended with high nitrogen-containing materials can undergo exposure to toxicity from both NH_3 and HNO_2, overall microorganism populations have been shown to increase by 100 to 1000 times, indicating that these products are selectively toxic to them (Conn & Lazarovits, 1999).

Phytotoxicity leading to a high mortality of tomato was observed in sandy soil amended with 2% PPM and incubated at 35°C, likely because of the increased NH_3 release from that amendment. Similarly, Ells *et al.* (1991) reported inhibition of both germination and seedling growth of cucumber in sand–soil mixtures with toxic levels of NH_3 released after amendment with alfalfa hay. Also, NH_3 above 10 ppm was found phytotoxic to cotton (Megie *et al.*, 1967). However, this effect was not observed in carnation plants in a greenhouse, when soils naturally infested by *F. oxysporum* f. sp. *dianthi*, were amended with PPM at 2.5 kg m^{-2} (Melero-Vara *et al.*, 2011).

All these processes did not occur in soil amended with ORC, with much lower N content than PPM. As disease level was also reduced to some extent by ORC amendments, a different mechanism of action is suggested to occur, likely based on shifts in microbial populations that may provide antagonism or else induction of resistance of plants to Fusarium wilt, for which incubation at 35°C for 30 days seems to be important. Thus, the use of ORC on asparagus enhanced the soil bacterial biomass while the *Fusarium* population decreased (Borrego-Benjumea *et al.*, 2014).

Poultry manure, whether in the pelletised form or another, can play an important role in reducing economic losses from tomato diseases, especially for farmers using organic production systems, as disease reduction and increased biomass were demonstrated. However, further works are necessary to test the effectiveness of this strategy under field conditions. The effectiveness of PPM and ORC was conclusively shown to reduce the populations of *Fol*, by the generation of bioactive metabolites. When organic amendments are combined with soil solarisation, the rates of application can be reduced to economically feasible levels and the length of the solarisation could be much reduced from the 6–8 weeks usually required for conventional treatments.

Moreover, disposal of considerable amounts of organic wastes generated in some agricultural industries such as broiler and olive oil production can be used as soil amendments for disease suppression, while significantly reducing the dependency on chemical fertilisers for achieving sustainable farming systems.

The commonly used CFU quantification of fungal pathogens in soil measures only the viable cells *in vitro*, but microorganisms with similar colony morphologies to the target agent, whether pathogenic or not, will also be counted. As many types of *Fusaria* reside in most soils, we cannot assume that we only counted *Fol* in this study. In contrast, our determinations of the amount of genomic *Fol* DNA in artificially infested soil indicate that DNA quantification from this particular organism can be accurately measured. Specific primer and probe development is an important step to quantify only the pathogen DNA in infested soils. The accurate and reliable detection and quantification of *Fol* populations in soil achieved in this study improve the management of Fusarium wilt of tomato.

Acknowledgements

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DISCUSIÓN GENERAL

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Esta Tesis se ha centrado en el estudio del control de la Podredumbre de raíces y corona (PRC) del espárrago y de la Fusariosis vascular (FV) del tomate, que ocasionan graves pérdidas económicas en la mayoría de las zonas de cultivo del mundo. La prolongada supervivencia en el suelo de las *Fusarium* spp. implicadas en la PRC y de *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), respectivos agentes causales de estas enfermedades, y su fácil diseminación con el material de propagación dificultan la lucha contra ellas.

Los estudios de diversidad de *Fusarium* spp. aislados de plantas de espárrago de 11 campos del SO de Ontario (Canadá), con 66% de *F. oxysporum* y 15% de *F. proliferatum*, se asemejan a los resultados de Sudáfrica (Schreuder *et al.*, 1995), EE.UU. (Elmer, 2001), España y Reino Unido (Wong y Jeffries, 2006), Este de Canadá (Vujanovic *et al*, 2006), y Japón (Nahiyan *et al.*, 2011), señalándolas como predominantes. *Fusarium acuminatum*, *F. redolens* y *F. solani* se aislaron con frecuencias menores en algunos países, incluyendo Canadá, según los resultados expuestos en esta Tesis y además, *F. verticillioides* se aisló, aunque con baja frecuencia, de plantas de vivero en España (Corpas-Hervias *et al.*, 2006).

En nuestro estudio etiológico de la PRC de espárrago en el SO de Ontario, los aislados de *Fusarium* spp. se recuperaron con mayor frecuencia de las raíces que de las coronas, y esta misma tendencia se observó, más acusadamente, en los re-aislamientos realizados de las plantas inoculadas, al finalizar los experimentos. Las pruebas de patogenicidad realizadas con dichos aislados, mostraron a *F. oxysporum*, *F. solani* y *F. acuminatum* como los más patogénicos, mientras que la reacción a la inoculación con *F. proliferatum* y *F. redolens* fueron menos graves, señalándose diferencias y coincidencias con resultados obtenidos en otros países; en España sería destacable la elevada agresividad de algunos aislados de *F. solani*, que puede considerarse la segunda especie a este respecto, tras *F. oxysporum*, en sintonía con lo descrito en Taiwán y Japón (Tu, 1980; Nahiyan *et al.*, 2011).

El desarrollo de estrategias, basadas en resultados consistentes, para el manejo de la PRC de espárrago y la FV de tomate requiere identificar y caracterizar objetiva y fiablemente los *Fusarium* spp. patógenos, lo que es posible gracias a técnicas de biología molecular como el uso de métodos de PCR basados en ADN, así como detectar y cuantificar con rapidez y precisión las poblaciones de dichos patógenos en el suelo. En el Capítulo 3 se ha cuantificado el ADN genómico de *Fol* en el suelo utilizado métodos moleculares basados en PCR en tiempo real. Los resultados obtenidos han demostrado que la cuantificación de ADN a partir de este organismo, en particular, se puede lograr con precisión (Borrego-Benjumea *et al.*, 2014b).

Para el manejo de enfermedades es fundamental la búsqueda de alternativas que integren diversas medidas de control compatibles con una agricultura sostenible, que reduzcan la dependencia de agroquímicos (fertilizantes y pesticidas de síntesis) y las grandes cantidades de residuos orgánicos generados en industrias agrícolas y agroalimentarias. Este trabajo se ha enfocado principalmente a la

utilización de subproductos orgánicos como enmiendas orgánicas del suelo para luchar contra las enfermedades ya mencionadas, en el contexto de sistemas agrícolas sostenibles, e incluso ecológicos.

Los aportes de varias enmiendas orgánicas del suelo han demostrado reducir los impactos de diversos patógenos de suelo (Lazarovits, 2001; Noble y Coventry, 2005; Bonanomi *et al.*, 2007; Melero-Vara *et al.*, 2011; 2012). La mayoría de las enmiendas orgánicas supresivas de enfermedades se encuentran en los compost y los residuos orgánicos (Bonanomi *et al.*, 2007). En nuestras investigaciones, la eficacia de las enmiendas estuvo supeditada a la propia enmienda, así como a la temperatura y a la duración de la incubación de los sustratos enmendados. Las condiciones óptimas, para *F. oxysporum* y *F. solani* estuvieron asociadas con el empleo de pellet de gallinaza (PG) y periodos de 15 días de incubación a 35º C y de 30-45 días a 30ºC. No obstante, también se lograron resultados satisfactorios con gallinaza (GA), compost de alpeorujo (CA) y emulsión de pescado (EP), a diversas concentraciones y temperaturas de incubación. El control de *F. proliferatum*, patógeno de espárrago, parece más fácil, ya que incubando a 30ºC con la mayoría de las enmiendas orgánicas evaluadas, se consiguieron buenos resultados. En coincidencia con *F. oxysporum* f. sp. *asparagi* y *F. solani*, el inóculo de *Fol* se redujo significativamente incubándolo a 35ºC con PG al 2%, durante 4 semanas, y a temperatura ambiente con dosis menores de PG (Borrego *et al.*, 2014a).

La mayor eficacia del PG en la reducción del inóculo en el sustrato puede deberse a su mayor contenido en N, comparado con el de GA y el del CA lo que, unido a la incubación a 35ºC, facilitó la liberación de NH_3 y HNO_2, gases de probada toxicidad para otros muchos hongos fitopatógenos (Tsao y Oster, 1981; Tenuta y Lazarovits, 2002a). No obstante, GA y CA, aunque con menor contenido en N, fueron bastante efectivas frente a *F. solani*, uno de los patógenos del complejo etiológico de la PRC del espárrago.

A la semana de aportarse PG, e independientemente de la dosis utilizada, se cuantificaron los valores máximos de NH_3 y pH en el suelo enmendado. Dichos valores se mantuvieron altos hasta las 4 semanas de incubación a 35ºC cuando se utilizaba la dosis del 2%. Así se consiguió un efecto fungitóxico más prolongado, pero también se detectaron síntomas de fitotoxicidad que llegaron a causar la muerte de algunas plantas, repercutiendo en el peso al finalizar los experimentos, lo que hizo no recomendable su utilización.

Estos mismos efectos se observaron en experimentos llevado a cabo en macetas, para controlar la Fusariosis vascular del clavel (Nava Juárez, 2013). Sin embargo, no se detectaron en experimentos realizados repetidamente en invernaderos con suelos infestados con *F. oxysporum* f. sp. *dianthi* (Melero-Vara *et al.*, 2011), probablemente por la disipación más rápida de los compuestos tóxicos volátiles y su lixiviación a perfiles más profundos del suelo que en el caso de quedar confinados por más tiempo en el reducido volumen de sustrato (0,5 L) de las macetas utilizadas.

A pesar de que el NH_3 y el HNO_2 liberados resultaron tóxicos para *Fusarium* spp., su toxicidad parece selectiva ya que las poblaciones bacterianas presentes en los sustratos enmendados con PG y CA se incrementaron en relación con el testigo sin enmienda orgánica, lo que confirma lo observado previamente por Conn y Lazarovitz (1999) en cultivos de patata.

Además de controlar la enfermedad, los tratamientos con enmiendas orgánicas tuvieron efectos beneficiosos en la biomasa producida por el cultivo, en parte debido al control de los patógenos, pero

también por sus propiedades como fertilizantes y mejoradores de la estructura de los suelos, sugiriendo la posibilidad de reducir las dosis de abonados minerales. Los incrementos de peso de las plantas de espárrago cv. Grande fueron más consistentes cuando se había infestado en suelo con *F. solani* (efectos similares en casi todas las enmiendas orgánicas evaluadas) que cuando se trataba de *F. oxysporum*.

En otros cultivares de espárrago menos susceptibles a la enfermedad, como Mary Washington y Jersey Giant, los efectos beneficiosos se limitaron a determinadas enmiendas, dosis de aplicación y periodos de incubación, dependiendo de la especie de *Fusarium* (Borrego-Benjumea *et al.*, 2014a; b). De acuerdo con nuestros resultados, la combinación del aporte de las enmiendas orgánicas con la solarización del suelo ha demostrado mejorar la eficacia de la desinfección con dichas enmiendas *per se*, frente a una amplia gama de *formae speciales* de *F. oxysporum* patógenos de diferentes cultivos (Blok *et al.*, 2000; Melero Vara *et al.*, 2011; Klein *et al*, 2011; Avilés *et al.*, 2011).

Cabe esperar que los progresos que se deriven de nuestros resultados, en avances de experimentación agraria de campo, permitan un control integrado de enfermedades de interés relevante, al tiempo que se haga un uso creciente de las enmiendas orgánicas ensayadas, de bajo costo y fácil acceso para muchos agricultores. Esto supondría un beneficio al Medio Ambiente, por los menores insumos requeridos por los cultivos en cuanto a fertilizantes y pesticidas y, no menos importante, por el reciclaje de los residuos agrarios mencionados.

CONCLUSIONES

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1. *Fusarium oxysporum, F. proliferatum* y *F. solani* pueden ser consideradas como las especies más importantes de *Fusarium* asociadas con la Podredumbre de raíces y corona (PRC) en el cultivo del espárrago en el Suroeste de Ontario.

2. *F. oxysporum* y *F. solani* de la colección española de aislados se mostraron significativamente más agresivos al cv. Grande de espárrago que *F. proliferatum*.

3. El pellet de gallinaza al 1% fue la enmienda más efectiva reduciendo la viabilidad de *Fusarium* spp. en sustratos infestados y la severidad de la PRC en espárrago fue menor a 30ºC, e incrementaba el peso de las plantas.

4. Las mayores dosis de gallinaza y compost de alpeorujo redujeron también la severidad de las infecciones por *F. oxysporum*-5 y *F. solani*-2, cuando se incubaron a 30 y 35ºC, usualmente durante 30 y 45 días con gallinaza y 15-30 días con compost de alpeorujo.

5. Las enmiendas orgánicas del suelo, en especial el pellet de gallinaza, pueden disminuir la severidad de la Fusariosis vascular del tomate y promover el crecimiento de plantas, posiblemente por la disminución de la población de patógenos y la mejora de la actividad bacteriana en el suelo, pero el uso del pellet de gallinaza al 2% puede ocasionar fitotoxicidad.

6. Los niveles de Fusariosis vascular del tomate disminuyeron cuando la combinación temperatura-enmienda orgánica resultó en la reducción de la viabilidad del inóculo de *F. oxysporum* f. sp. *lycopersici*.

7. El ADN de *F. oxysporum* f. sp. *lycopersici* puede cuantificarse con precisión en suelos artificialmente infestados con cebadores específicos y sondas TaqMan.

8. El aporte de enmiendas en combinación con la temperatura reduce la cantidad de ADN en el suelo de *F. oxysporum* f. sp. *lycopersici*.

9. Cuando se aplica pellet de gallinaza al suelo se observa una relación entre la reducción en cantidad de ADN de *F. oxysporum* f. sp. *lycopersici* en el suelo, la reducción de la severidad de síntomas y el incremento del peso seco de las plantas.

10. La temperatura de incubación y el pH del suelo tuvieron un papel importante en el proceso de generación de NH_3 y HNO_2, a partir de la descomposición de la materia orgánica rica en N, siendo el pellet de gallinaza la enmienda que determinaba los valores máximos de NH_3 y pH en el de suelo.

11. Los tratamientos con enmiendas orgánicas tuvieron efectos beneficiosos en la biomasa producida por el cultivo, en parte debido al control de los patógenos, pero también por sus propiedades como fertilizantes y mejoradores de la estructura de los suelos.

CONCLUSIONS

1. *Fusarium oxysporum, F. proliferatum* and *F. solani* can be considered as the most important species of *Fusarium* associated with the Fusarium crown and root rot (FCRR) in asparagus crop in Southwest Ontario.

2. *F. oxysporum* and *F. solani* from the Spanish collection of isolates showed significantly more aggressive to asparagus cv. Grande than *F. proliferatum.*

3. Pellet of poultry manure at 1% was the most effective amendment reducing the viability of *Fusarium* spp. in infested substrate and the severity of FCRR of asparagus was lower at 30ºC, as well as increasing the weight of the plants.

4. The higher rates of poultry manure and olive residue compost also reduced severity of infections by *F. oxysporum*-5 and *F. solani*-2 when incubated at 30 and 35ºC, usually for 30 and 45 days with poultry manure and 15-30 days with olive residue compost.

5. Soil organic amendments, especially pelletized poultry manure, can reduce the severity of Fusarium wilt of tomato and promote plant growth, possibly by decreasing the population of pathogens and enhancing bacterial activity in the soil, but use of 2% pellet of poultry manure may cause phytotoxicity.

6. Fusarium wilt of tomato decreased when the combination temperature-organic amendment resulted in reduced viability of *F. oxysporum* f. sp. *lycopersici* inoculum.

7. *F. oxysporum* f. sp. *lycopersici* DNA can be precisely quantified in artificially infested soil with specific primers and TaqMan probes.

8. Addition of amendments in combination with temperature reduces the amount of DNA in soil of *F. oxysporum* f. sp. *lycopersici.*

9. When pellet of poultry manure is applied to the soil, a relationship appeared between the reduction in the amount of DNA from *F. oxysporum* f. sp. *lycopersici* in the soil, reduction of severity of symptoms and increase of dry weight of plants.

10. Incubation temperature and soil pH played an important role in the process of generation of NH_3 and HNO_2, from decomposition of organic material rich in N, with the amendment pellet of poultry manure determined maximum values of NH_3 and in soil pH.

11. Organic amendment treatments had beneficial effects on the crop biomass, in part due to the control of pathogens, but also for their properties as fertilizers and enhancers of soil structure.

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