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PCR-SSCP analysis of *Fusarium* diversity in asparagus decline in Japan

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Abstract The diversity of *Fusarium* populations in asparagus (*Asparagus officinalis* L.) decline fields in Japan was estimated by PCR-SSCP (single-stranded conformational polymorphism) analysis of the ITS2 regions of the nuclear rRNA genes. This method was used to rapidly and objectively identify pathogens associated with roots of plants showing symptoms of asparagus decline collected from fields in five regions across Japan. Over 651 fusarial isolates were obtained, and were easily differentiated into three principal species. *Fusarium oxysporum* f. sp. *asparagi* was most frequently isolated from the domestic five regions (68%), whereas *Fusarium proliferatum* (28.6%) was less frequent. *Fusarium solani* was found much rarely (2.5%). The frequency of isolation of *Fusarium proliferatum* increased gradually from the north to the south of Japan, though considerable differences were found between fields in each region, as well as regional differences among the *Fusarium* populations. Most of the fusarial isolates were highly pathogenic in vitro. These results reveal that *Fusarium oxysporum* f. sp. *asparagi* and *Fusarium proliferatum* are important biotic factors which lead to asparagus decline in Japan.

Keywords *Asparagus officinalis* L. · *Fusarium oxysporum* f. sp. *asparagi* · *Fusarium proliferatum* · *Fusarium solani* · Pathogenicity

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Introduction

Asparagus (*Asparagus officinalis* L.) is a high market-value and long-term perennial vegetable crop with a production cycle of up to 15 years or more (Hamel et al. 2005). Peak production occurs after 5–8 years (Elmer 2001), but then marketable yields decrease as a result of yellowing ferns and growth suppression. Spears become smaller in the middle to end period of their cultivation. This phenomenon is known as asparagus decline (Pontaroli and Camadro 2001). Diseased plants often appear stunted and chlorotic (Elmer 2001) and are characterized by vascular discoloration in the crown and base of the infected stem, with reddish-brown root lesions. These eventually turn to wilts and crown rots (Schreuder and Lamprecht 1995; Pontaroli and Camadro 2001). Replanting asparagus in former asparagus fields is a common

practice in asparagus-producing regions of the world, but this often results in plants suffering from early decline and other replant symptoms such as initial growth suppression and death of replants. In such cases, the economic life of replanted fields is decreased relative to newly planted fields (Blok and Bollen 1995). Asparagus decline is associated with both abiotic and biotic factors (Yang 1982; Elmer et al. 1996; Yergeau et al. 2006). Abiotic factors are mainly related to the release of allelopathic compounds (Yang 1982; Hartung and Stephens 1983; Hazebroek et al. 1989; Blok and Bollen 1993), but also nutrient imbalance, deterioration of soil physiochemical conditions, cultural factors, and excessive harvesting pressure (Hamel et al. 2005; Yergeau et al. 2006; González and del Pozo 2008). Biotic factors are principally infestations by soil-borne pathogens that cause crown and root rot, mainly *Fusarium oxysporum* f. sp. *asparagi* (Foa), *Fusarium proliferatum* (Fp) (Schreuder and Lamprecht 1995; Yergeau et al. 2004; Wong and Jeffries 2006), and *Fusarium redolens* (Fr) (Baayen et al. 2000).

Traditionally, the diversity of *Fusarium* species in asparagus fields is assessed on the basis of isolation and enumeration of species growing on selective media (Vujanovic et al. 2002). However, this approach is problematic, because isolation and identification of numerous isolates of this organism is a difficult and highly time-consuming task (Yergeau et al. 2006). Modern molecular techniques permit more effective and rapid solutions for the large number of samples required in large-scale ecological studies. For example, polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP) analysis has been used to identify minor sequence differences in target *Fusarium* DNA (Wong and Jeffries 2006). This approach enables large numbers of isolates to be prescreened for sequence similarities thus drastically reducing the samples needed for subsequent sequencing (Brandão et al. 2002). Furthermore, SSCP profiles can often be diagnostic at the species level, thus enabling rapid analysis of populations. Thus, SSCP offers an inexpensive yet sensitive, rapid and convenient method for determining DNA sequence variation in a large number of samples.

Diseases which affect asparagus, such as asparagus decline, have become more important as the production of asparagus has increased world-wide. Losses through disease decrease the farmer's motivation to grow this crop, causing a problem in Japan and abroad. The development of both biological and chemical control measures for asparagus decline has become an important topic of research (Blok et al. 1997; Elmer et al. 1996; Elmer 2003). However, these methods are not necessarily appropriate to control asparagus decline in all asparagus-producing regions in the world. Furthermore, the biotic agents responsible for asparagus decline may differ world-wide. *Fusarium oxysporum* f. sp. *asparagi* and *Fusarium proliferatum* are the commonest causes of asparagus decline in asparagus-producing regions in the United States of America, Canada and South Africa (Schreuder and Lamprecht 1995; Elmer et al. 1996; Vujanovic et al. 2006), whereas *Fusarium oxysporum* f. sp. *asparagi*, *Fusarium proliferatum* and *Fusarium redolens* are responsible for asparagus decline in Europe (Blok and Bollen 1995, Baayen et al. 2000; Wong and Jeffries 2006). It is not clear what the distribution and diversity of the main *Fusarium* species are within asparagus decline fields in Japan. In this study, we have used PCR-SSCP to study the diversity of *Fusarium* species isolated from asparagus plants showing decline symptoms across a range of regions of Japan.

Materials and methods

Isolation of Fusarium from symptomatic asparagus plant roots

Using an intensive sampling regime, 651 fusarial isolates were obtained from root samples from twenty asparagus fields in five regions (Hokkaido, Fukushima, Nagano, Kagawa and Saga) from the North to the South of Japan (Fig. 1). The cultivars sampled in the fields were 'Welcome' (18 fields), 'Harukitaru' (1 field in Fukushima) and 'Sanukinomezame' (1 field in Kagawa). Plantation age ranged from 1 to 13 years. Root samples were taken from 5 to10 plants showing decline symptoms in each field on a randomized basis. Ten root segments (0.5 cm) from each plant, with or without lesions, were surface-sterilized in 70% (v/v) ethanol for 10 s and 10% (v/v) NaClO for 15 min. These were subsequently rinsed three times with sterilized distilled water before being transferred to Komada's medium (Komada 1975) in a standard Petri dish. The Petri dishes were incubated for 7–10 days

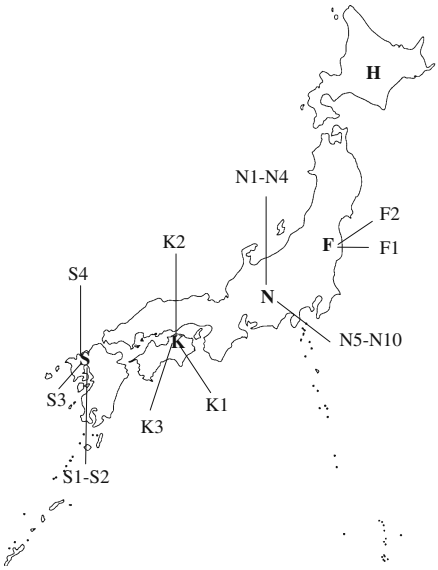

Fig. 1 Regions in Japan where asparagus samples were collected. *H* Hokkaido; *F* Fukushima; *N* Nagano; *K* Kagawa; *S* Saga. Number shows field ID

until fungal colonies emerging from the root segments. Fungal colonies were transferred at least three times onto potato dextrose agar (PDA) medium, via hyphal transfer, to obtain pure cultures.

Recovery of high-molecular weight of DNA

Fungal mycelium (0.1 g fresh weight) was taken from actively growing colonies and triturated in a 2.0 ml eppendorf tube containing 300 μl Puregene cell lysis solution (Gentra Systems), then kept on ice. The mycelium was crushed in the tube with a mini-homogenizer (Pellet Mixer, Sansyo) and 150 μl of 3 M sodium acetate was added. The contents were mixed and incubated at −20°C for 10 min. The precipitated slurry was clarified by centrifugation at 14,000 rpm for 5 min. Finally, the clear supernatant was transferred to a clean microcentrifuge tube and the volume of supernatant recorded. An equal volume of isopropanol was added, mixed gently, and the tube incubated for 5 min at room temperature. Precipitated DNA was collected by centrifugation at 14,000 rpm for 5 min. The isopropanol was carefully poured off and the sample was washed in 200 μl of 70% filter-sterilized ethanol for 15 min, and centrifuged at 14,000 rpm for 5 min. The DNA pellet was air-dried prior to resuspension in 70 μl TE (10 mM Tris-HCl and 1 mM EDTA at pH 8) buffer, and stored at 4°C.

PCR-SSCP analysis

PCR (PTC-200 DNA engine, MJ Research) targeted the ITS2 regions of nuclear rRNA genes. The 20 μl reaction mixture was comprised of *Taq* DNA polymerase 10X reaction buffer (Promega, Madison, WI), 4 μl; 25 mM Mg2+ solution, 2.25 μl; dNTP mix (4 mM each), 1.75 μl; 25 mM 5.8S ITS3 forward primer (5′ GCATCGATGAAGAACGCAGC 3′, Sigma-Genosys) and ITS4 reverse primer (5′ TCCTCCGCTTATTGATATGC 3′, Sigma-Genosys), 1 μl each; *Taq* DNA polymerase, 0.1 μl; extracted DNA, 1 μl; and sterile MilliQ water to 20 μl. The PCR program consisted of 35 cycles with a denaturing step of 95°C for 45 s, an annealing step of 50°C for 1 min and a polymerization step of 72°C for 3 min. PCR products were checked by agarose gel electrophoresis and visualized on a UV transluminator. Finally, SSCP analysis was conducted (Wong and Jeffries 2006) and the ITS2 regions of the rRNA gene cluster of *Fusarium* species from Japan were identified by comparing with the SSCP-ITS2 profiles (Fig. 2) of known isolates. Isolates of *F.oxysporum*, *F.proliferatum*, *F.solani*, *F.redolens* of Wong and Jeffries (2006) were used as co-migrating reference points on every SSCP gel.

Axenic assay of pathogenicity

Two hundred and twenty-three *Fusarium* isolates were selected for pathogenicity tests. Seeds of asparagus (*Asparagus officinalis* L., cv. Welcome) were surface-sterilized in 70% (v/v) ethanol for 10 s and immersed in 10% (v/v) NaClO for 15 min. The seeds were rinsed thrice in sterilized distilled water, sown onto water agar (0.6%) medium, and then incubated in the dark for 7–10 days at 25°C in a growth chamber. On germination, individual seedlings were transferred (421 cc glass bottle closed by plastic cap) to 50 ml Knop's agar medium (Blok and Bollen 1995) and grown for at least 14 days in a growth chamber (25°C with 16-h photoperiod). Seedlings that were more than 4 cm tall were inoculated at the base of their shoots with 3 mm cubes of fusarial mycelium cut from cultures grown on PDA. Inoculated plants were grown for a further 5 weeks under similar conditions. Five replicates were used per isolate. The pathogenicity of an isolate was scored according to the number of seedlings with root lesions. However, since an ability to cause a lesion in at least one plant demonstrates

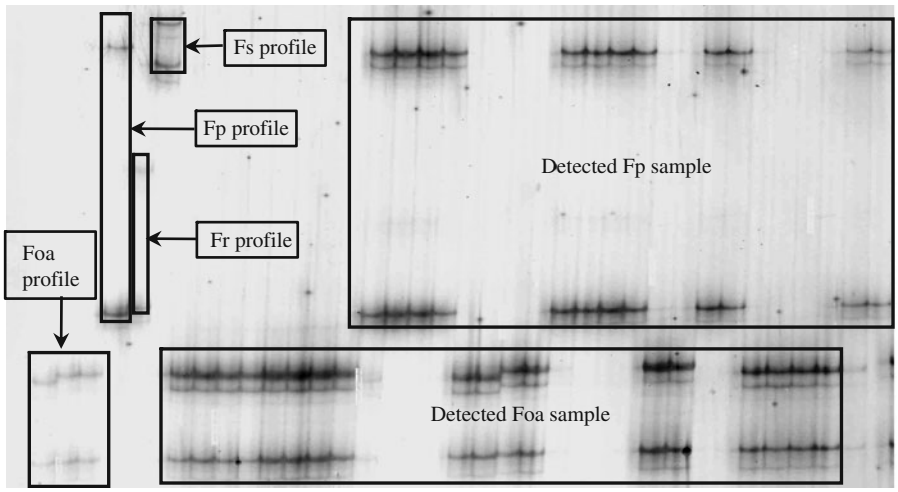

Fig. 2 Representative SSCP (single-stranded conformational polymorphism) gel showing the typical results from ITS2-SSCP profiling of a selection of *Fusarium* isolates in Japanese fields. *Foa Fusarium oxysporum* f. sp. *asparagi*; *Fp Fusarium proliferatum*; *Fs Fusarium solani; Fr Fusarium redolens*

some degree of pathogenicity, isolates are described as 'highly pathogenic' if their pathogenicity scores were 4 or 5.

Results

In total, 651 fusarial isolates were compared from 20 representative fields showing symptoms of asparagus decline from the northern to the southern part of Japan. Most isolates were differentiated easily into the three principal reference species using PCR-SSCP analysis: *Fusarium oxysporum* f. sp. *asparagi* (Foa), *Fusarium proliferatum* (Fp) and *Fusarium solani* (Fs). The SSCP profiles showed a considerable variation in the relative proportions of each species isolated across the fields in each region (Fig. 3). The majority of the *Fusarium* isolates in the Hokkaido region were Foa (82.5%) and Fp (12.5%), with Fs being less abundant (5%). In the Fukushima region, the most dominant species within the isolates from field F1 was Foa (87.5%), with Fp being the next most frequent isolate (12.5%), whereas in F2 Foa and Fp were found in equal proporations (50%). In the Nagano region, 378 fusarial isolates were obtained from 10 representative fields (N1-N10). The SSCP profiles of isolates from the N2-N4 fields showed that the common fusarial pathogen was Foa, and the next most frequent was Fp, with Fs being less abundant in the N3 (13.9%) and N4 (19%) fields. However, in field N1, Fp was dominant (92.9%), with Foa only representing 7.1% of isolates. Interestingly, in the fields N5-N10, Foa was the dominant isolate in the N6 (92.7%) and N9 (100%) fields, whereas Fp was in the majority in the N7 (62.5%) and N10 (58.3%) fields. Foa was most frequently obtained from the K1 (81.8%) and K3 (62.5%) fields, with Fp representing 18.2% (K1) and 37.5% (K3) of isolates respectively. Conversely, Fp (76.5%) was the major isolate and Foa (23.5%) was the minor isolate from field K2. From the SSCP profiles from isolates from the Saga region, Foa was dominant (100%) in fields S1 and S4, but made up a smaller proportion in fields S2 (75%) and S3 (60%). *Fusarium proliferatum* accounted for most of the other isolates from fields S2 (25%) and S3 (22.9%). Interestingly, 17.1% of isolates in field S3 were not identified, but their SSCP patterns suggested

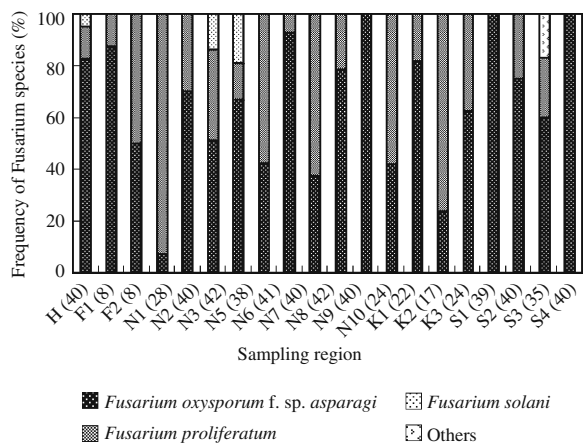

Fig. 3 Relative frequency of isolation of *Fusarium* species in different fields of Hokkaido, Fukushima, Nagano, Kagawa and Saga. *H* Hokkaido; *F* Fukushima; *N* Nagano; *K* Kagawa; *S* Saga. Number in parentheses shows total *Fusarium* isolates in each field

that they were fusaria. In summary, SSCP profiles showed a wide distribution of species variation between locations in the relative proportions of isolates (Fig. 4). Though three fusarial species were isolated from the Nagano region, the most frequently obtained was Foa (61.4%), followed by Fp (34.9%). Samples from Fukushima, Kagawa and Saga all yielded isolates of Foa and Fp. Foa was dominant in Fukushima (68.8%), Kagawa (58.7%) and Saga (84.4%), with, Fp making up 31.2%, 41.3% and 11.7% of the isolates respectively. Overall, Foa was the most frequently isolate from the five regions (68%), with *F. proliferatum* being less frequent (28.6%), whereas *F. solani* was rarely isolated (2.5%). Each region showed considerable intraregional variation.

A sub-set of 223 isolates was selected from the 651 *Fusarium* isolates identified by PCR-SSCP. All isolates were highly pathogenic (Table 1). Eighty percent of the Foa (112/140) had a pathogenicity score of 5 out of 5, whereas 10.7% scored 4. For the other two species, 84% of Fp isolates and 62.5% of Fs isolates scored 5, whilst 8% of Fp isolates and 12.5% of Fs isolates scored 4. Though there were no non-pathogenic fuasarial isolates in this study, one Foa and two Fs isolates had a low score of 1.

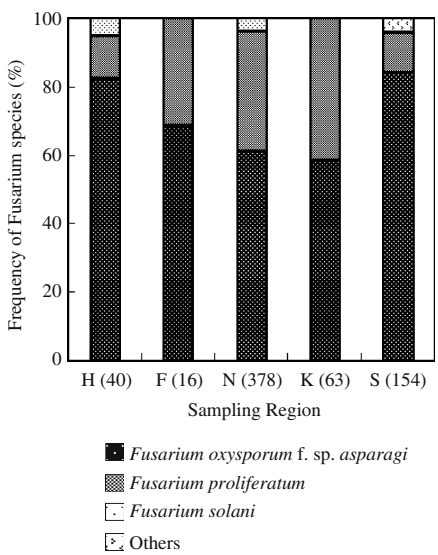

Fig. 4 Relative frequency of isolation of *Fusarium* species in each sampling region. *H* Hokkaido; *F* Fukushima; *N* Nagano; *K* Kagawa; *S* Saga. Number in parentheses shows total *Fusarium* isolates in each region

Table 1 Relative pathogenicity scores of *Fusarium*

Pathogenicity scores[a]	Number of isolates[b]					
	Foa	%	Fp	%	Fs	%
5	112	80.0	63	84.0	5	62.5
4	15	10.7	6	8.0	1	12.5
3	7	5.0	5	6.7	0	0
2	5	3.6	1	1.3	0	0
1	1	0.7	0	0	2	25.0
0	0	0	0	0	0	0
Total	140	100	75	100	8	100

[a] Disease rating scale: pathogenecity of each fusarial isolates was scored according to the number of seedlings with root lesions.

[b] Foa, *Fusarium oxysporum* f. sp. *asparagi*; Fp, *Fusarium proliferatum*; Fs, *Fusarium solani*.

Discussion

The use of SSCP analysis allowed large numbers of samples to be rapidly screened for sequence variation without the need for sequencing. In this study, SSCP analysis of the ITS2 region proved to be a rapid and objective method for identifying fusarial isolates associated with asparagus decline. PCR-SSCP analysis showed that *Fusarium* population diversity within each region of Japan was as varied as that found from the northern to the southern part of Japan. Isolates of Foa and Fp were commonly obtained from asparagus plants showing decline symptoms in each region, but relative proportions varied among fields. Isolates from these two species usually scored highly in pathogenicity tests, with most isolates causing lesions on all the seedlings tested. Our findings that Foa, Fp and Fs comprised 68%, 28.6% and 2.5% respectively, of all the isolates is similar to other field studies of diseased asparagus. Wong and Jeffries (2006) found that Foa, Fp, *Fusarium redolens* (Fr) and Fs represented 69%, 7%, 1% and 15%, respectively, of 209 isolates recovered from asparagus roots from a single field in UK, and 40%, 39%, 12% and 7%, respectively, of 156 isolates recovered from asparagus roots from six asparagus fields in Spain. One major difference between the current study and the latter study is that *Fusarium redolens* was completely absent from the Japanese isolates, despite Fr being recorded as a very important causal agent of asparagus root rot, crown rot and spear rot (Baayen et al. 2000). Climatic

factors, including soil moisture, may be unfavorable for the disease development of Fr in Japan, an issue worthy of further investigation.

The SSCP profiles from our study suggested that Foa is the most important species in Japan causing asparagus decline because of its high frequency of isolation, as was the case in the UK, Netherlands and USA (Blok and Bollen 1997; Elmer 2001; Wong and Jeffries 2006). *Fusarium proliferatum* was also ubiquitous and was the second most abundant *Fusarium* species in this study *Fusarium proliferatum* is also one of the main causal agents of crown and root rot in North and Central America, Australia and South Africa (Damicone and Manning 1985; Elmer et al. 1997; Doan and Carris 1998). The present study also confirms *Fusarium solani* as a minor pathogen causing asparagus decline, found only in the Hokkaido and the Nagano samples. This species is often found colonizing the root and crown tissue of asparagus (Elmer 2000) and was recovered in 2–20% of samples from the USA, South Africa and Australia (Elmer 2001). The Fs isolates were pathogenic in the in vitro tests, supporting the observations of Schreuder and Lamprecht (1995). This species is commonly associated with asparagus, but the relative importance of Fs in *Fusarium* crown and root rot is still unclear (Elmer et al. 1997)

Fusarium proliferatum was a more frequent isolate in the warmer climates of southern Italy (Moretti et al. 1997), North and Central America, Australia and South Africa (Elmer 2000, 2001). In contrast, Blok and Bollen (1995), failed to recover any Fp isolates in Netherlands due to the cooler soil temperatures. The dominance of Foa, and the marginalization of Fp in the Hokkaido region, may reflect this trend as this area has a cooler climate. In contrast, Saga, a warmer region than Hokkaido, Fukushima and Nagano, had a considerably higher percentage of Foa than that of Fp in its asparagus decline fields, suggesting that other factors may also influence the relative proportions of *Fusarium* species. The movement of asparagus seedlings from place to place may be one such factor. This may also imply that colonization of asparagus by Foa is more dependent on the resident soil population of Foa than transmission from seed-borne isolates. Additional studies are required to assess the *Fusarium* species associated with asparagus seed distribution in Japan. However, we found considerable field and regional differences in the *Fusarium* population in asparagus decline fields, similar to observations from Australia, New Zealand and Spain (Elmer et al. 1997; Wong and Jeffries 2006). Diversity among the *Fusarium* isolates from Japan did not correlate with plantation age, but we would expect that populations of pathogenic strains would build up with time. For example, Hamel et al. (2005) observed a positive correlation between the plantation age and Fusarium crown and root rot development in Quebec, Canada. Further studies are also required in this context.

Our results show that *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* are the most frequently species associated with diseased asparagus plants, suggesting they are the main pathogens and important biotic factors leading to asparagus decline in Japan.

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