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Stem blight resistance of *Asparagus kiusianus* and its hybrid with *A. officinalis*

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Abstract Resistance of cultivated asparagus (*Asparagus officinalis*), wild *A. kiusianus* and their interspecific hybrids to stem blight caused by *Phomopsis asparagi* (Sacc.) Bubák was evaluated. *A. kiusianus* had higher resistance than *A. officinalis* although there were individual variations. The resistance of interspecific hybrids between *A. officinalis* and *A. kiusianus* was mostly intermediate, but some individuals had strong resistance equivalent to *A. kiusianus*. The results suggest that it is possible to introduce the resistance of *A. kiusianus* to stem blight into *A. officinalis* through interspecific hybridization for further *Asparagus* breeding.

1. Introduction

Asparagus (*Asparagus officinalis* L.), of the family Asparagaceae, is a perennial crop widely cultivated in the world. The crop is cultivated from northern to southern regions in Japan, and is an important vegetable. It is expected that production of the crop will increase due to its high profits in many places. The southwestern region of Japan has an advantage because spears emerge earlier in this region than in the northern region. Several diseases such as stem blight, leaf spot and brown spot cause serious problems in open field culture in warm regions. Therefore, rain protected culture is necessary (Kobayashi and Shinsu, 1990). Among these diseases, stem blight caused by *Phomopsis asparagi* (Sacc.) is the most serious disease in Japan, Korea, China and Southeast Asia and causes devastating damage to the crop in economically producing fields; the disease is also distributed in Australia, Greece, Italy, New Zealand and USA (Davis, 2001; Elena, 2006; Udayanga *et al*., 2011). Recently disease damage was also reported in northern parts of Japan (Sonoda, personal communication). The causal fungus first forms small lesions or spots on the lower part of the asparagus stem. The lesions continue to expand and produce yellowish-brown spindle shaped spots (Sonoda *et al*., 1997). Pycnidia usually appear at the central part of a spot and become secondary sources of infection (Sakai *et al*., 1992). The symptoms are different with aging of stems, and result in systemic symptom, and finally stems are killed (Fukutomi, 1993). Thus, production of cultivars with strong resistance to stem blight has become an urgent issue. The genus *Asparagus* is composed of about 100-300 (Bailey, 1944; Dahlgren *et al*., 1985) species mainly distributed in arid regions of the Mediterranean, Africa and Asia (Dahlgren *et al*., 1985). The species are widely used not only as food source but also for medicinal or ornamental purposes. Sonoda *et al*. (1997, 2001) reported that there were varietal differences of stem blight resistance but there were no strong resistant cultivars in *A. officinalis*. They also reported that several *Asparagus* species had strong disease resistance, but it was impossible to produce hybrids by interspecific crosses with *A. officinalis*. Kunitake *et al*. (1996) produced somatic hybrids between *A. officinalis* (2n=2x=20) and *A. macowanii* (2n=2x=20) by protoplasts electrofusion for the purpose of introduction of disease resistance characteristics into asparagus. However, the hybrids failed to mature because of their abnormal genome composition. Marcellán and Camadro (1999) also performed interspecific hybridization between *A. officinalis* and *A. densiflorus* (Kunth) Jessop cv. Sprengeri (2n=6x=60), but the

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endosperm failed to develop normally, leading to subsequent embryo abortion.

Asparagus kiusianus (2n=2x=20) is a dioecious species native to the coastal region of the Japan Sea from Yamaguchi to northern Kyushu. It is, therefore, expected that the species is adapted with the climate of these regions with high disease or salt resistance. Ito *et al*. (2011) successfully produced interspecific hybrids between *A. officinalis* and *A. kiusianus* and also obtained backcross progenies.

The objective of this study is to demonstrate the usefulness of *A. kiusianus* for producing stem blight disease resistant asparagus cultivars, making cultivation in open field culture in southwest region of Japan as well as in other warm climate areas in the world possible. Evaluation of disease resistance was performed in *A. kiusianus*, cross compatibility between *A. officinalis* and *A. kiusianus* and disease resistance in the interspecific hybrids was surveyed.

2. Materials and Methods

Plant materials

Asparagus officinalis cv. UC157F$_1$, as the control cultivar (Sonoda *et al*., 1997), and *A. kiusianus* Keya line from Fukuoka, Japan were used for inoculation tests in 2010 to compare the resistance to stem blight in these two species (Table 1). UC157F$_1$ and *A. kiusianus* (Nijinomatsubara line from Saga, Japan) were used for crossing experiments. UC157F$_1$ and its intraspecific F$_1$ hybrids, *A. kiusianus* (Nijinomatsubara line from Saga, Keya line and Uminonakamichi line from Fukuoka, Japan) and interspecific F$_1$ hybrids of *A. officinalis* with *A. kiusianus* (OK) were used for inoculation tests in 2011 and 2012 (Table 1).

Inoculum

Phomopsis asparagi strain P1, preserved in Saga Prefectural Agricultural Experiment Station, was cultured on potato sucrose agar (PSA) medium at 25°C for one month. After sporulation, conidiospores were suspended in sterilized water and adjusted to the inoculum density of 2×10^6 spores/ml for use in the following tests.

Interspecific cross

Interspecific crosses between *A. officinalis* (female) and *A. kiusianus* (male) and intraspecific cross in *A. officinalis* cv. UC157F$_1$ were made by hand pollination in a greenhouse of Kyushu University Farm in 2010. Obtained seeds were germinated at 25°C in plastic petri dishes, and germinated seeds were sown in vermiculite in plastic cell trays in an incubator. They were transplanted in plastic pots six months after seed sowing and transferred. Genomic DNA was extracted from young cladodes of each plant by modified CTAB method (Stajner *et al*., 2002). Hybridity of interspecific progenies was confirmed by three SSR markers (Caruso *et al*., 2008), AG2, AG7 and AG10, followed by Takeuchi *et al*. (2012).

Evaluation of stem blight resistance for inoculation test

Inoculation tests were performed three times from November 2010 to January 2011 with UC157F$_1$ and *A. kiusianus*, Keya line grown in plastic pots. Seven to 10 plants were used for each test. The plants were inoculated with the vinyl tape and cotton method by reference to the protocols previously described by Sonoda *et al*. (1997, 1999, 2001). Absorbent cotton moistened with spore suspension was put around the basal part (2-5 cm below the lowest branching nodes) of the stem, and covered with vinyl tape. The plants were maintained at 25°C with 90% humidity for three days, followed by incubation at 25°C and at 60-70% humidity in a growth chamber. The spears, which emerged two to three weeks after cutting the areal stems of the seedlings, were examined. Disease severity was determined by scoring 0-4 grades for each plant weekly until five weeks after inoculation. The disease severity grade (DSG) was: 0 = no lesion; 1 = small-sized lesion (<1 cm in length); 2 = spread lesion; 3 = large-sized lesion (>half of the aerial stem) or defoliation; 4 = aerial part death. Percentage of infected plants and disease indices were calculated, as follows:

$$\text{Percentage of infected plants} = \frac{\text{Number of infected plants}}{\text{Total number of plants employed}} \times 100$$

$$\text{Disease index (DI)} = \frac{\Sigma(\text{Number of plants classified into each grade} \times \text{DSG number})}{\text{Total number of plants employed} \times 4} \times 100$$

The degree of pycnidia formation was also surveyed. Resistance in UC157F$_1$ and its intraspecific F$_1$ hybrids, *A. kiusianus* (Keya line, Nijinomatsubara line and Uminonakamichi line) and interspecific F$_1$ hybrids between them was evaluated by inoculation test three times from August to September and from November to December in 2011 and from October to November in 2012. Assessments were performed according to the procedure as described above.

Table 1 - Plant materials used for inoculation tests

Species	Cultivar and line	Tested year	No. of individuals investigated
A. officinalis	UC157F$_1$	2010	14
A. officinalis	UC157F$_1$, F$_1$ progenies of ' UC157F$_1$'	2011, 2012	25
A. kiusianus	Keya line	2010	15
A. kiusianus	Keya line, Uminonakamichi line, Nijinomatsubara line	2011, 2012	21
Interspecific hybrid	Hybrids between *A. officinalis* and *A. kiusianus*	2011, 2012	25

3. Results

Evaluation of the resistance to stem blight in A. officinalis *and* A. kiusianus

Most of the plants in both species showed pathogenic symptoms on the stems (Table 2). All the inoculated asparagus cultivars began to show blight by one week after inoculation and the aerial parts of all plants were dead within five weeks. In contrast, *A. kiusianus* showed lower DI than *A. officinalis* and more than half of the plants survived five weeks after inoculation although they exhibited symptoms. There were differences of susceptibility among *A. kiusianus* accessions, whereas all *A. officinalis* accessions showed high susceptibility with severe symptoms. Pycnidia formation was frequently observed in *A. officinalis* from two to four weeks after inoculation (data are not shown). A few accessions showed pycnidia formation in *A. kiusianus*, whereas some other individuals showed no symptoms possessing strong resistance.

Interspecific crosses between A. officinalis *and* A. kiusianus

Fruit set rate and number of seeds in interspecific crosses between *A. officinalis* (female) and *A. kiusianus* (male) were lower than those in intraspecific crosses among *A. officinalis* (Table 3). Germination rate of the interspecific hybrid was high (76.1%) at 25°C. Hybridity of the progenies was confirmed by three SSR markers (Caruso *et al*. 2008), AG2, AG7 and AG10 (data are not shown). The hybrid plants showed intermediate morphology between the parents.

Evaluation of the resistance to stem blight in the interspecific hybrids

Most of the hybrid plants and their parents showed pathogenic symptoms (Fig. 1, Table 4). The aerial stems of all inoculated *A. officinalis* showed blight by one week after inoculation and most of them faded within four weeks (Fig. 2 D). In contrast, the aerial stems of *A. kiusianus* and the interspecific hybrids survived even four weeks after

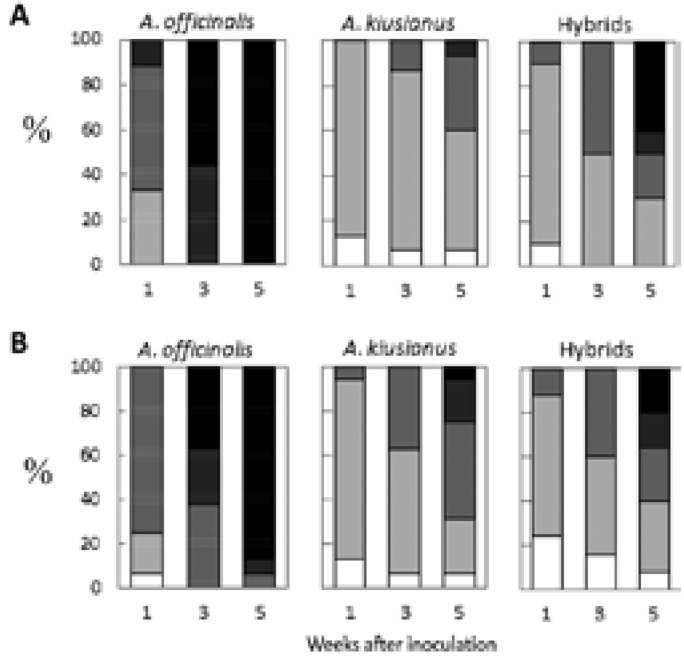

Fig. 1 - Frequency of individuals with their disease severity grades (DSG) in *A. officinalis*, *A. kiusianus* and F_1 hybrids screened for resistance to stem blight. Tests were performed in August (A) and November (B) in 2011. ☐ 0 = no lesion; ■ 1 = small-sized lesion (<1 cm); ■ 2 = spread lesion; ■ 3 = large-sized lesion (>half the size of plant) or defoliation; ■ 4 = aerial part death.

Table 2 - Development of disease symptoms in *A. officinalis* and *A. kiusianus* inoculated with *P. asparagi*

Species	Percentage of infected plants					Disease index				
	1 (z)	2	3	4	5	1	2	3	4	5
A. officinalis	100 a	100 a	100 a	100 a	100 a	45 a	54 a	88 a	98 a	100 a
A. kiusianus	77 a	85 a	93 a	93 a	93 a	25 b	38 b	54 b	63 b	69 b

(z) Weeks after inoculation.
Different letters in a column indicate significant difference according to student's *t*-test (P≦0.05).

Table 3 - Result of interspecific crosses between *A. officinalis* and *A. kiusianus*

Seed parent	Pollen parent	No. of pollinated flowers	No. of obtained fruits (%)(z)	No. of obtained seeds (n) (y)	No. of germinated seeds (%)(x)	No. of obtained seedlings
A. officinalis ×	*A. kiusianus*	301	27 (9.0)	67 (2.5)	51 (76.1)	42
A. officinalis ×	*A. officinalis*	46	25 (54.3)	119 (4.8)	104 (87.4)	45

(z) No. of obtained fruits/No. of pollinated flowers)×100.
(y) No. of obtained seeds/No. of obtained fruits.
(x) (No. of germinated seeds/No. of obtained seeds)×100.

Table 4 - Development of disease symptoms in *A. officinalis*, *A. kiusianus* and their hybrids inoculated with *P. asparagi*

Species	Percentage of infected plants					Disease index				
	1 [z]	2	3	4	5	1	2	3	4	5
A. officinalis	95 a	100 a	100 a	100 a	100 a	30 a	35 a	60 a	90 a	100 a
A. kiusianus	81 a	88 a	88 a	88 a	88 a	27 a	31 a	34 b	37 b	40 b
Hybrids	86 a	92 a	92 a	92 a	95 a	29 a	36 a	40 ab	47 b	59 b

[z] Weeks after inoculation.
Different letters in a column indicate significant difference according to Turkey's multiple range test (P≦0.05).

the inoculation (Fig. 2 E, F). The DI's in *A. kiusianus* and the interspecific hybrids were lower than those in *A. officinalis* although lesion was formed in most of the plants (Table 4). The disease severity grade of *A. officinalis* was high compared with that of *A. kiusianus* and the interspecific hybrids (Fig. 1). Most *A. kiusianus* showed lower grades even five weeks later. The interspecific hybrids showed varied degrees of symptoms and the distribution of DSG was intermediate between the parental species. Diseased lesions were observed in all the stems of *A. officinalis* and numbers of pycnidia were frequently formed four weeks after inoculation (Figs. 3, 4). There was little pycnidia formation in *A. kiusianus* and interspecific hybrids, while lesions were observed in the stems of most plants of *A. kiusianus* and the interspecific hybrids. The hybrids showed intermediate susceptibility between their parent species and their pycnidia formation rates were as low as the degree of *A. kiusianus* (Fig. 4).

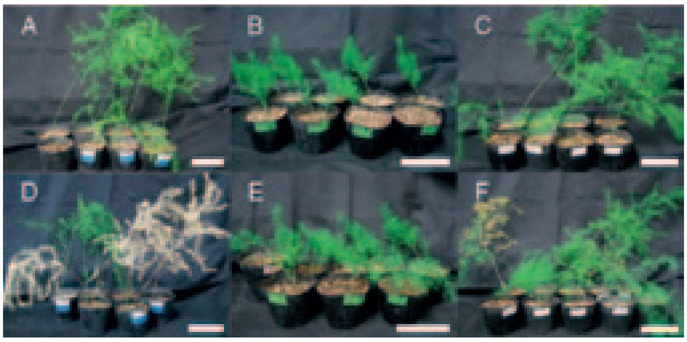

Fig. 2 - Disease symptoms of *A. officinalis* (A, D), *A. kiusianus* (B, E) and F_1 hybrids (C, F) inoculated with *P. asparagi*. A-C: 1 week after inoculation, D-F: 4 weeks after inoculation. *Scale bars* 9 cm.

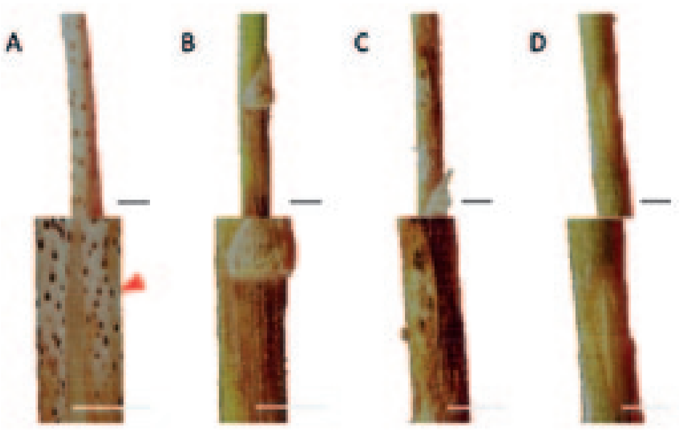

Fig. 3 - Lesion on stems of *A. officinalis* (A), *A. kiusianus* (B) and interspecific hybrids (C, D) inoculated with *P. asparagi*. Arrows indicate pycnidia. *Scale bars* 1 mm.

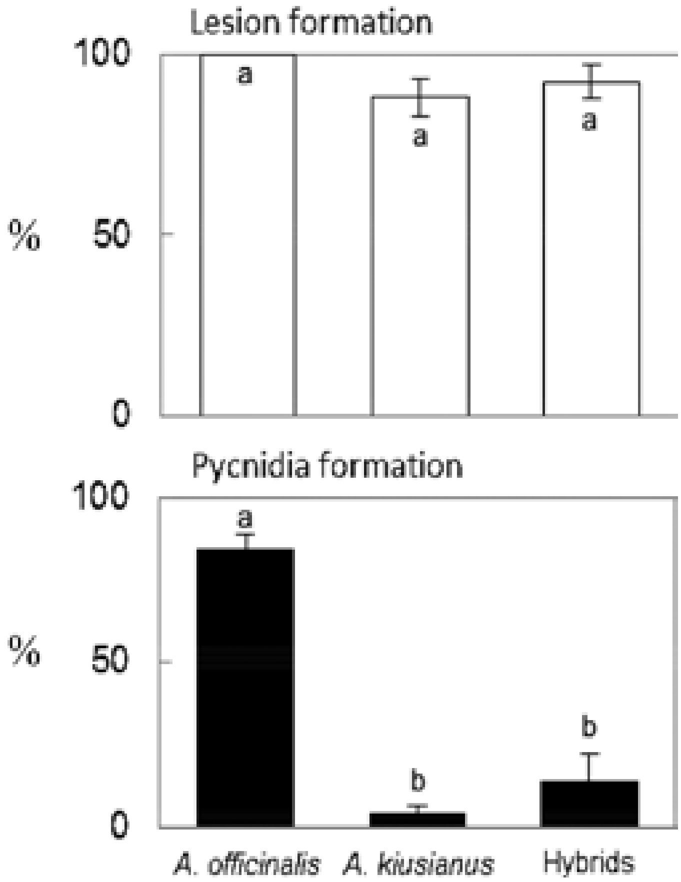

Fig. 4 - Rates of lesion and pycnidia formation of *A. officinalis*, *A. kiusianus* and interspecific hybrids four weeks after inoculation with *P. asparagi*. Bars represent SE (n=3). Different letters indicate significant difference according to Turkey's multiple range test (P≦0.05).

4. Discussion and Conclusions

The symptoms were severer in this study than those reported by Sonoda *et al*. (1997, 1999, 2001). DI was nearly 100 in *A. officinalis* four weeks after inoculation in this study, while it ranged from 42 to 79 in another investigation (Sonoda *et al*., 1997). This might be due to aggressive pathogenicity of the *phomopsis* strain used or differences in environmental conditions from the previous study (Sonoda *et al*., 1997). The results of the present study show that *A. kiusianus* had higher resistance than *A. officinalis* with various degrees of resistance among individuals. Although the lesions were observed in most *A. kiusianus* plants, the resistant accessions displayed reduced disease development and varying levels of resistance. Pycnidia were infrequently formed on the stems of *A. kiusianus*, restricting the spread the pathogen. It is thought of as incomplete (or partial) resistance (Poland *et al*., 2009). The rate-reducing resistance or partial resistance is believed to be effective against a large number of pathogen genotypes and durable resistance since it is non-race specific (Niks and Rubiales, 2002; Poland *et al*., 2009). Therefore, the resistance identified in *A. kiusianus* will be useful for asparagus breeding programs.

There were differences in disease resistance among *Asparagus* species (Sonoda *et al*., 2001). *Asparagus densiflorus*, *A. virugatus*, *A. asparagoides* and *A. macowanii* were highly resistant. They are, however, genetically distant to *A. officinalis* and extremely difficult to obtain viable interspecific hybrids. In contrast, *A. kiusianus* is genetically a closer species to *A. officinalis* than other *Asparagus* species based on the analysis of non-coding cpDNA regions (Fukuda *et al*., 2005, 2011; Kubota *et al*., 2011), although *A. kiusianus* is endemic to Japan, far from of the habitat of *A. officinalis* which is the Mediterranean. Ito *et al*. (2011) obtained interspecific crosses between *A. officinalis* and *A. kiusianus* and their backcross progenies, suggesting the possibility of producing disease resistant asparagus cultivars. It was indicated in this study that the resistance derived from *A. kiusianus* was inherited in the interspecific hybrids, although it was variable among individuals.

Further study with these hybrids and their backcross progenies are needed in order to screen the genotypes with a higher level of resistance to stem blight for breeding disease-resistant asparagus cultivars.

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