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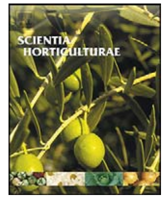

Pathogenicity of dried-shrink disease and evaluation of resistance in a germplasm collection of sea buckthorn (*Hippophae* L.) from China and other countries

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ABSTRACT

Sea buckthorn (*Hippophae* L.) is a woody, dioecious plant that can be grown in dry and poor soil conditions. It has recently received world-wide attention as a new berry crop with a very high nutritional value and unique medicinal properties, as well as a means of combating soil erosion. A major problem is, however, dried-shrink disease (DSD), which destroys sea buckthorn plants and halts commercial production. In this study, we investigated symptoms and pathogenicity of DSD, isolated and identified pathogens using a combination of morphology and ITS sequences, and evaluated the disease index (DI) of different cultivars in the field. DSD infection causes isolated yellowing of plant tissues, and as the disease develops the bark putresces, dries and shrinks, and finally the plant dies. DSD infects older plants (>3 years), often at the plant base. Death of plant parts above the infected area does not influence root vitality, and a new plant can therefore be regenerated. Combining morphological characters and ITS sequences, we isolated and identified four fungal pathogens causing DSD, including *Fusarium acuminatum*, *F. oxysporum*, *Fusarium camptoceras* and *Phomopsis* spp. DI values for 27 cultivars ranged from 0 to 10.4. About half of the cultivars were regarded as resistant (DI < 3.2) to DSD, and half as susceptible (DI > 6.7). Cultivar 'Zhongguoshaji' (DI = 4.2) showed an intermediate level of DSD resistance. Our data provides basic information for further studies of the mechanism of pathogenicity, and for breeding of DSD-resistant cultivars.

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1. Introduction

The sea buckthorn (*Hippophae* L., Elaeagnaceae) genus contains 6 species and 12 subspecies of woody, deciduous shrubs or small trees (Bartish et al., 2002; Sun et al., 2002; Swenson and Bartish, 2002). All species of this genus are restricted to the Qinghai-Xizang plateau and adjacent areas except for *Hippophae rhamnoides* L., which is naturally distributed in cold-temperate regions of over 40 countries like China, the Indian Himalayas and Central Asia, Russia, and many countries in Central and North Europe (Heinz and Barbaza, 1998; Roy et al., 2001). This species has also been introduced into Canada, USA, Bolivia, Chile, Korea and Japan. Sea buckthorn is dioecious, wind pollinated, and obligately outcrossing. It reproduces both vegetatively through root suckers and by seeds that are dispersed by birds and frugivorous animals.

Berries and leaves of sea buckthorn are rich in nutrients and bioactive substances such as vitamins, carotenoids, flavonoids, polyunsaturated fatty acids, etc., which have high medicinal and nutritional value, especially in cancer and cardiovascular disease therapy, treatment of gastrointestinal ulcers and as a liver protective agent (Yao et al., 1992; Wolf and Wegert, 1993; Sadek and Abou-Gabal, 1999; Vikberg and Itamies, 1999; Suleyman et al., 2001; Agrawala and Goel, 2002; Goel et al., 2002; Zadernowski et al., 2002; Cheng et al., 2003; Tsydendambaev and Vereshchagin, 2003; Zeb, 2004; Zhang et al., 2005; Ruan and Zheng, 2006; Ruan et al., 2007; Andersson et al., 2008a,b, 2009). Recently, sea buckthorn has been planted as a new berry crop for obtaining important bioactive compounds. In addition, its good adaptability, rapid growth, ability to act as protection against wind and sand drift, and to assist in soil and water conservation and improvement of soil by efficient nitrogen fixation, allow sea buckthorn to be widely used in vegetation construction and restoration of degenerated ecosystems (Hou et al., 1995; Wei et al., 1998; Ruan et al., 2000; Ruan and Li, 2002; Chen and Chen, 2003; Li, 2004). In China alone, artificial plantations of 1.5 million ha have been established since 1985, with an increase of 0.1 million ha/year.

Dried-shrink disease (DSD) is a dangerous pathogen that destroys sea buckthorn and halts commercial production. In China alone, DSD decreases fruit yield by 30–40% (Zhang et al., 2001) and eliminates 4000 ha of mature plantations annually. Some studies on DSD have been reported, including development and causative agents (Du, 2001, 2002; Zhang et al., 2001), epidemiology (Zhang

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and Xin, 2002), effective treatments for control of DSD (Zhang, 2002), antagonistic *Cladothrix actinomyces* (Li and Liu, 2006; Liu et al., 2006; Liu, 2007), screening and application of antagonistic fungus (*Trichoderma* sp. and *Penicillium* sp.) (Zhang, 2006), and symptoms of infected plants (Ruan et al., 2008). However, there are no reports on systematic surveys of pathogenicity and evaluation of DSD resistance of different cultivars, especially in the identification of pathogens.

In this study, we have (i) investigated symptoms and pathogenicity of DSD by long-term observations and measurements in the field, (ii) isolated and identified pathogens using a combination of morphological characters and ITS sequences, (iii) evaluated the disease index (DI) of different cultivars in the field, and (iv) made a primary selection of cultivars with DSD resistance.

2. Materials and methods

2.1. Symptoms and pathogenicity

More than 80 cultivars (http://www.shajimiaomu.com) from China and other countries were grown in our sea buckthorn orchard located in Fuxing City (E121°01′–l22°56′, N41°41′–42°56′), Liaoning Province, China, with a mean annual rainfall of 539 mm and annual evaporation of l 800 mm. Mean annual temperature is 7.5 °C, and weather falls into the category of north-temperature continental monsoonal climate.

Since 1998, we have been observing symptoms of DSD in our sea buckthorn orchards located in Fuxing and Dalian City of Liaoning Province and Ansai county of Shannxi Province of China, including changes in the color of leaves (e.g. chlorosis, etiolation and paling), leaf and shoot decay (e.g. leaf spot and blight, tip necrosis and abscesses), severe plant destruction (e.g. dry rot, wet decay and soft rot), and wilt caused by destroyed vascular bundles (e.g. sympodial branches, shrinkage, tubercles or galls and leaf rolling).

In 2008 and 2009, we recorded, investigated and analyzed the age when plants were infected and finally died of DSD, tissue samples of infected plants, seasonal variation in DSD infection, and the influence of infected plants on other, vegetatively connected plants.

2.2. Isolation and identification of pathogens

In the field, we collected fruiting plants, 5–8 years old, which were infected by DSD in July 2007. Ten pieces (0.1 cm × 0.05 cm) were cut from the epidermis, phloem and xylem of infected roots and stems and branches, respectively, with a sterilized scalpel. Cut pieces were placed in PDA culture medium (200 g potato starch, 1000 ml water, 10 g glucose and 20 g agar) and incubated for 10 d at 25 °C. Different fungi were isolated and observed using a fluorescence microscope (Olympus IX71) (400×), and their morphological characters were recorded. We deduced their taxonomical classification by morphological characters (color, size and shape of mycelium, structure of pycnidium, etc.). For three fungi whose taxonomical classification could not be precisely determined only by morphological characters, further identification was achieved by analysis of ITS sequences. Genomic DNA was extracted from the fungus using the CTAB method (Saghai-Maroof et al., 1984). Polymerase chain reaction (PCR) was performed using a GeneAmp PCR 9700 system (Applied Biosystems). PCR reactions were performed in 25 μl volumes with the following reaction components: 10–100 ng template DNA, 10× *ExTaq* buffer (TaKaRa), 100 μM of each dNTP, 1.5 mM $MgCl_2$, 0.1 μM of each primer, and 1.25 U of *ExTaq* (TaKaRa). PCR cycling conditions were: 94 °C (10 min), then 25 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), followed by a final 72 °C (10 min) step. Forward sequencing primers were ITS1 (5′–3′: TCCGTAGGTGAACCTGCGG) and reverse ITS4 (5′–3′: TCCTCCGCTTATTGATATGC) (White et al., 1990). Target PCR product fragments (∼531, 535 and 554 bp, respectively) were cleaned using the QIAquick PCR purification Kit (Qiagen) before sequencing. The cleaning process involved the following steps: (i) 5 volumes of Buffer PB were added to 1 volume of the PCR sample then mixed; (ii) a QIAquick spin column was placed in a 2-ml collection tube provided by the manufacturer; (iii) DNA was bound by applying the sample to the QIAquick column and centrifuging for 30–60 s; (iv) flow-through was discarded and the QIAquick column was replaced into the same tube; (v) to wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30–60 s; (vi) flow-through was discarded and the QIAquick column was replaced into the same tube; the column was centrifuged for an additional 1 min at maximum speed; (vii) the QIAquick column was placed in a clean 1.5 ml microcentrifuge tube; and (viii) to elute DNA, 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O was add to the center of the QIAquick membrane and the column was centrifuged for 1 min. Sequences were read on an ABI 377 Prism automated sequencer operated by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Possible sequence misreads were checked by comparison of the forward strand with the reverse strand sequence in all cases. Three new sequences were contrasted with the sequences in Genbank using Blast Contrast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST tree display was created from genetic distances calculated using standard methods from Jukes–Cantor aligned sequences (Jukes and Cantor, 1969) for nucleotide comparisons. The trees themselves were then built from these distance matrices using the Fast Minimum Evolution (FastME) (Desper and Gascuel, 2002) method.

2.3. Evaluation of disease index of different cultivars in the field

During 1998–2008, in our sea buckthorn orchard, 27 sea buckthorn cultivars (Table 1) (200–450 plants per cultivar) were observed and investigated to assess whether they were naturally infected by DSD and to determine the symptoms of DSD after infection under natural conditions. The disease grade (Table 2) of each selected plant was evaluated. The DI of each cultivar was calculated using the following formula: $DI = \{[\sum(NPDG \times VDG)]/[TNIP \times MVDG]\} \times 10$, where NPDG is the number of plants of each disease grade (Table 2), VDG is the value of each disease grade, TNIP is the total number of investigated plants, and MVDG is the maximum value of different disease grades among different investigated plants for this cultivar. Statistically, a normality test was used to determine whether the DI values of different cultivars were well-modeled by a normal distribution or not, using the one-sample Kolmogorov–Smirnov test (SPSS v. 11.0 software). The DI values were then used to classify cultivars as resistant or susceptible, combined with the symptoms of each cultivar infected by DSD. In addition, we tested the difference in DI values between DSD-resistant and -susceptible groups, using the two-sample *t*-test (SPSS v. 11.0 software).

3. Results

3.1. Symptoms and pathogenicity of DSD

Our observations showed that the primary symptoms of DSD can usually be seen in April or May. Leaves of infected plants gradually become yellow at the middle or end of June; yellow leaves then gradually senesce. Fruits of infected plants do not develop properly and retain a pre-mature color. They also show early drop at the end of June, in contrast to fruits of normal plants which mature about 10 d into July. If infected by DSD, the side-branches start to bend down in June, and then dry up and finally die in July. If the plant

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