Pak. J. Bot., 45(5): 1825-1829, 2013.

EFFECTS OF HOST GROWTH STAGE, RE-ISOLATION AND CULTURE MEDIUM ON SCREENING FOR RESISTANCE TO STEM ROT DISEASE CAUSED BY *SCLEROTIUM ROLFSII* SACC. IN JERUSALEM ARTICHOKE

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Abstract

Consistently replicable methods of inoculation are necessary to efficiently screen Jerusalem artichoke (*Helianthus tuberosus* L.) genotypes for resistance to the soilborne fungal pathogen *Sclerotium rolfsii* Sacc. The fact that plant age, type of inoculum and source of inoculum can affect severity and incidence of stem rot disease caused by *S. rolfsii* are factors that must be assessed and controlled in seeking disease-resistant accessions The objective of this work was to investigate the effects of seedling stage, source of *S. rolfsii* inoculum (serial *In vitro* subculture and re-isolation of *S. rolfsii* from infected plants), and culture medium used to prepare inoculum on stem rot disease. Seedlings of Jerusalem artichoke (Kaen Tawan # 2) at 6-, 8- and 10-leaf stages were inoculated with sorghum seed that had been infested with *S. rolfsii* derived from different sources (serial *In vitro* subculture or re-isolated from symptomatic host tissue, and cultured in either potato dextrose agar (PDA) or a sorghum-based medium; the infested seed were placed in wounds made at the base of the stem). In a greenhouse, the experiment was set up as a 3 x 2 x 2 factorial in a randomized complete block design with six replications; the experiment was repeated once. Plants at the 6-leaf stage were more susceptible than those at the 8- or 10-leaf stages. *S. rolfsii* inoculum derived from serial *In vitro* subculture caused more severe stem rot symptoms than inoculum derived by re-isolation from symptomatic host plants, but the difference was not significant for disease incidence. Inoculum culture on the sorghum-based medium resulted in a higher incidence of stem rot than PDA. The highest incidence of disease was observed with inoculum grown on PDA from serial *In vitro* subculture and on the sorghum-based medium that had been re-isolated from symptomatic host tissue. In addition, disease severity was higher when inoculum was grown on PDA from serial *In vitro* subculture than from sorghum-based medium that had been sub-cultured *In vitro*.

Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) has high potential for use as health food. The plant produces large amounts of inulin, a source of dietary fiber that is beneficial to human health (Niness, 1999; Stevens *et al*., 2001; Cherbut 2002). Jerusalem artichoke is used to produce a variety of products such as health food products (Danilčenko *et al*., 2008), animal feed (Zaky, 2009) and bio-ethanol (Yildiz *et al*., 2006).

Jerusalem artichoke can be grown commercially in the tropics (Pimsaen *et al*., 2010; Puangbut *et al*., 2011). However, stem rot disease caused by *Sclerotium rolfsii* poses a threat to the crop (Sennoi *et al*., 2010). Yield losses caused by the fungus have also been reported in the United States (Koike, 2004). The disease is prevalent in warm climates especially under high temperature and high humidity (Kwon *et al*., 2008; Yaqub & Shahzad, 2011), and it affects a wide variety of plants, including most vegetables, flowers, legumes, cereals, forage plants and weeds (Agrios, 2005). The disease also causes serious losses on major food crop such as peanut (Rakh *et al.*, 2011) and cassava (Banito *et al*., 2010).

Although commercial interest in Jerusalem artichoke is increasing, a consensus on optimal methods for screening this plant for resistance to *S. rolfsii* has not emerged. A first step toward this goal would be to clarify host, pathogen, and/or environmental influences on this disease during resistance screening trials. Although young chickpea plants are more susceptible to the disease than older ones (Hussain *et al*., 2006; Sconyers *et al*., 2007), possible impacts of plant age on stem rot caused by *S. rolfsii* in Jerusalem artichoke have not been characterized. Variation in pathogenicity of different *S. rolfsii* isolates was reported (Sarma *et al*., 2002; Sennoi *et al*., 2010), but another challenge in assessing resistance to *S. rolfsii* is to maintain cultures of the pathogen. A primary cause of culture collection loss has been the inability to store and maintain individual isolates in the state in which they were originally collected (Day & Stacey, 2008). It is also unclear whether repeated serial passage through *In vitro* subculturing affects pathogenicity of *S. rolfsii*, but this factor needs to be assessed in order to develop reliable screening techniques.

The type of culture medium often affects mycelial growth rate of fungi (Hubballi *et al*., 2010), and types of media should be compared to determine the most effective medium for inoculation. Screening of Jerusalem artichoke genotypes for resistance to *S. rolfsii* requires reliable and effective inoculation methods. Objectives of this work were investigate the effects of seedling stage, cultural status of *S. rolfsii* inoculum (serial *In vitro* subculture *vs.* re-isolation of *S. rolfsii* from infected plants) and medium used to produce inoculum on incidence and severity of stem rot disease caused by *S. rolfsii* in Jerusalem artichoke.

Materials and Methods

The experiment was conducted in an open-sided greenhouse at Khon Kaen University (KKU) Agronomy Farm, Khon Kaen, Thailand, in July 2011 and repeated in September 2011. Treatments were arranged in 3 × 2 × 2 factorial combinations in a randomized complete block

design with six replications due to the shading effect from sun in each replication because one side of the experiment was shaded by a lager greenhouse. Three developmental stages of seedlings (6-, 8-, and 10-leaf stages) were assigned as factor A, two sources of *S. rolfsii* (serial *In vitro* subculture and re-isolation from symptomatic host) were assigned as factor B, and two types of inoculum (PDA and sterilized sorghum seeds) were assigned as factor C. There were four pots per treatment subplot. During the first and second runs of the experiment, air temperature ranged from 23.9 to 31.9 ^{o}C and 23.9 to 31.6 ^{o}C and relative humidity ranged from 83 to 99 % and 90 to 96 %, respectively. Air temperature on the inoculation dates ranged from 23.0 to 30.5 ^{o}C in experiment 1 (15 July) and 23.5 to 30.2 ^{o}C in experiment 2 (12 September).

A recommended genotype by Jerusalem artichoke Improvement Project, Khon Kaen University (Kaen Tawan # 2) for commercial cultivation was used in the experiment. Its tubers were cut into small pieces with 2 to 3 active buds, and incubated in charred rice husks for one week to facilitate germination. The germinated tuber pieces were then transferred to plug trays for a week until each seedling had two leaves. The three sets of seedling stage treatments were prepared at four-day intervals. They were later transferred to square pots with the size of 8 × 8 × 9 cm containing steamed and charred rice husk (1:1). The soil of the experiment belongs to Roi-et series (Re**;** fine-loamy, mixed, subactive, isohyperthermic Aeric Kandiaquults).

Plants showing typical symptoms of stem rot were collected from KKU farm. Single sclerotia of *S. rolfsii* were surface-sterilized using 70% alcohol for 1 minute and rinsed with sterilized water for 1 minute. The samples were then transferred to potato dextrose agar (PDA) medium in petri dishes and incubated at room temperature (25 ± 2°C) for 3 days. The mycelium was later purified by subculture, and the isolate was stored in test tubes at room temperature (Sennoi *et al*., 2010). The sclerotia from the test tubes were transferred to potato dextrose agar (PDA) medium and incubated at 25 ± 2°C for 10 days until new sclerotia were produced. After incubation, the sclerotia were stored at room temperature for 3 months, and then transferred to new PDA medium. For the serial *In vitro* subculture treatment, subculture was done for four passages, and then sclerotia were again transferred to PDA for use as inoculum. For the host-derived inoculum treatment, mycelia plugs (0.5-cm-diameter) from this PDA medium were used to infect Jerusalem artichoke seedlings; after 7 days, the fungus was re-isolated. For, mycelia plugs from same PDA medium were transferred to new PDA medium for 10 days to obtain sclerotia. Inoculation was performed when sclerotia from two sources were ready.

Sclerotia from the two sources was firstly cultured in PDA medium for 3-5 days, and later it was sub-cultured to PDA medium for 5-7 days and steamed sorghum seeds for 7-10 days. For preparation of PDA inoculum, surface-sterilized sclerotia from serial *In vitro* subculture and re-isolation were transferred to PDA (20 ml/Petri dish) and then cut by 0.5-cm-diameter cork borer for PDA plug. Sorghum inoculum was prepared at least 4 days in advance because the method took at least 4 days longer than preparation of PDA inoculum. The method for preparation of sorghum seed inoculum was described previously (Sennoi *et al*., 2010). Briefly, mycelia plugs were transferred to sorghum medium, which was made by autoclaving sorghum seeds and then weight for 30 g per bottle.

The method of inoculation was the same for both inoculum types. Two wounds of 0.5cm length were made by a sterilized sharp blade at the crown of each stem, and two PDA plugs (0.5-cm diameter) or sorghum seed inoculum were attached closely to the wounds. The wounds were covered with moistened cotton with sprayed water once after inoculation to facilitate infection. The water was supplied to the experiment regularly to avoid stress. The data were recorded daily from 1 day after inoculation to 30 days after inoculation for number of infected plants and then converted to percent infected plants (infected plants/total plants) ×100) and the number of days to permanent wilting was noted. Data for each experiment were analyzed statistically for each parameter and error variances between 2 experiments were tested for homogeneity. Data with homogeneity of variance were subjected to combined analysis of variance for the two experiments according to a factorial in RCBD for factor main effects and interaction (Hoshmand, 2006). Least significant difference (LSD) was used to compare mean differences. All calculations were done using STATISTIX 8 software program (Analytical software, Tallahassee, Florida).

Results and Discussion

The difference between experiments was statistically significant for the number of days to permanent wilting ($p<0.01$) whereas disease incidence was similar between experiments (Table 1). Our previous study indicated that days to permanent wilting could better identify the difference among Jerusalem artichoke genotypes than other traits (Sennoi *et al*., 2012). The average number of days to permanent wilting was less for the first experiment (4 days) than the second experiment (6 days). Furthermore, disease severity was greater in the first experiment. Differences among growth stages of seedling were significant for disease incidence and days to permanent wilting ($p<0.01$).

Inoculum preparation methods (serial subculture *vs.* re-isolation from host) were significantly different for days to permanent wilting ($p<0.01$), but they were not statistically different for disease incidence. In addition, medium (PDA vs. sorghum grain) for inoculum preparation differed significantly for disease incidence ($p<0.01$), but it not for days to permanent wilting. Furthermore, interactions between the inoculum preparation method and medium were found for disease incidence ($p<0.01$) and days to permanent wilting ($p<0.01$). Three-way interaction among stage of plants, inoculum preparation method, and medium was significant for days to permanent wilting ($p<0.01$). Variation in disease incidence at 7 days after inoculation was highest as indicated by high F-ratio and low coefficient of variation (C.V.) value.

Table 1. Mean square error from combined analysis of variance for disease incidence at 7 days after inoculation and days to permanent wilting of Jerusalem artichoke due to infection by *Sclerotium rolfsii* in an open-sided greenhouse.

Sources of variation	Df	Disease incidence	Days to permanent wilting
Experiment (E)	1	351.6	169.0**
Rep within experiment	10	1032.1	4.7
Stage of plant (S)	2	16410.6**	263.5**
Sources of pathogen (P)	1	108.5	103.4**
Media (M)	1	1410.6**	0.1
S×E	2	481.8	33.8**
P×E	1	8789.1**	103.4**
M×E	1	7296.0**	96.7**
S×P	2	30.4	10.1
S×M	2	169.3	6.9
P×M	1	8789.1**	152.1**
S×P×M	2	976.6	86.9**
S×P×M×E	7	1569.3**	18.8**
Pooled error	121	710.3	2.4
C.V. (%)		45.8	28.8

* Significant for $p<0.05$
** Significant for $p<0.01$

Plants inoculated at the 6-leaf stage had much higher disease incidence than those inoculated at 8- and 10-leaf stages (Fig. 1). Disease incidence ranged from 31 to 81%, 11 to 58%, and 5 to 50%, for plants inoculated at 6-, 8- and 10-leaf stages, respectively. Disease incidence increased from 1 day after inoculation until 13 days of inoculation, then showed little change until 30 days after inoculation; therefore, data were presented only until 13 days after inoculation. However, at 9 to 13 days after inoculation, 10-leaf stage (48-50%) was not statistically significant from 8-leaf stage (56-58%).

Plants inoculated at 6-leaf stage took the shortest time (3 days) to permanent wilting followed by the plants inoculated at 8-leaf stage (5 days), whereas the plants inoculated at 10-leaf stage took the longest time (8 days) (Fig. 2). According to previous study on effect of chickpea seedling age to collar rot caused by *S. rolfsii* in Pakistan, younger seedlings were more susceptible than older seedlings (Hussain *et al*., 2006). The phenomenon of increasing resistance to fungal diseases with seedling age has also been demonstrated other pathosystems. For example, in red crown rot of soybean caused by *Calonectria ilicicola*, tap and lateral root colonization levels were reduced approximately 50% when the pathogen was placed in soil 1 week after planting compared to late plantings (Kuruppu *et al*., 2004). Inoculum preparation methods (serial subculture vs. re-isolation from the host) were not significantly different for disease incidence, but the plants inoculated with sub-cultured inoculum required less time (4 days) to cause permanent wilting than those inoculated with the re-isolated fungus (6 days) (Fig. 3). Some pathogens need re-isolation from host pathogenicity in order to maintain pathogenicity, including *Phytophthora erythroseptica*, *Diatrypaceae* species and *Alternaria alternata* (Salas *et al*., 2000; Hubballi *et al*., 2010; Trouillas & Gubler, 2010). However, study on the influence of re-isolation of *S. rolfsii* disease has not been reported previously. The inoculation method in the present study used sclerotia, which are specialized survival structures of *S. rolfsii*; we speculate, based on our results, that loss of pathogenicity in sclerotia is unlikely to be impacted by whether or not it has been repeatedly subcultured since isolation from a symptomatic host.

Inoculum that has been prepared on sorghum seeds generally had higher disease incidence than did when prepared on PDA, especially 5 days after inoculation (Fig. 4). From 5 to 13 days after inoculation, disease incidence ranged from 58 to 75% for sorghum-derived inoculum compared to 42 to 51% for PDA. However, days to permanent wilting were not statistically different. For pink rot disease in potato caused by *Phytophthora erythroseptica*, intact potato tuber plug inoculum resulted in higher disease incidence than crushed tuber plugs and agar-culture plugs (Salas *et al*., 2000). A study screening of peanut for resistance to *S. rolfsii* found that agar-derived inoculum had higher disease incidence than oat seed inoculum in greenhouse test, but in field trials oat seed inoculum resulted in higher disease incidence (Shokes *et al*., 1996). Chickpea seed meal extract agar medium was use for culture method of *S. rolfsii* (Amber *et al*., 2012). However, the use of small agar disks was not better than the use of sorghum seed inoculum because it was difficult to handle, time-consuming and dried quickly.

Interactions of inoculation medium were found for disease incidence and days to permanent wilting (Table 1). For PDA inoculum, serially subcultured inoculum had higher disease incidence than inoculum that had recently been re-isolated from symptomatic host plants (58%) (Fig. 5). For sorghum inoculum, *S. rolfsii* from re-isolation had higher infection than did *S. rolfsii* from in serial subculture (84%). In addition, PDA inoculum with *S. rolfsii* from serial subculture took less time to permanent wilting than did PDA inoculum from re-isolation (3 days) (Fig. 6). It is possibly due to *S. rolfsii* from re-isolation had higher potential to infect on plant part (sorghum seed) than *S. rolfsii* from serial subculture. In previous *Phytophthora* pod rot study, pod of cocoa in the field after removal of the beans are a good source of re-infection (Robin, 1997). However, sorghum inoculum derived from both methods of inoculum preparation did not have statistical differences for days to permanent wilting.

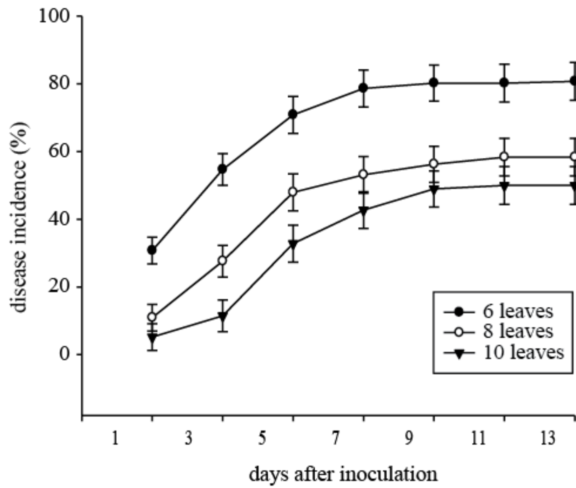

Fig. 1. Effect on disease incidence of seedling stage to *Sclerotium rolfsii* stem rot in Jerusalem artichoke

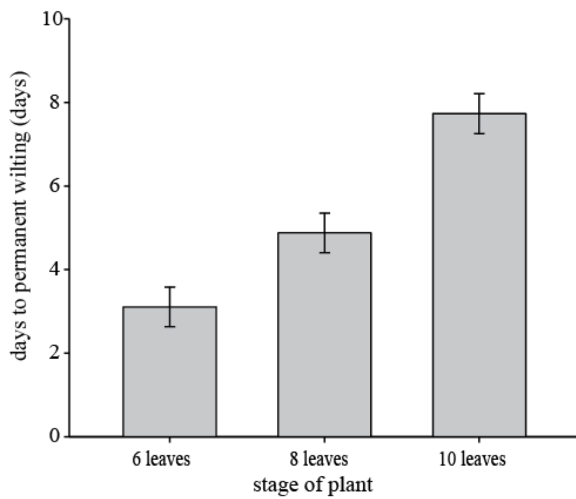

Fig. 2. Effect on permanent wilting of seedling stage to *Sclerotium rolfsii* stem rot in Jerusalem artichoke.

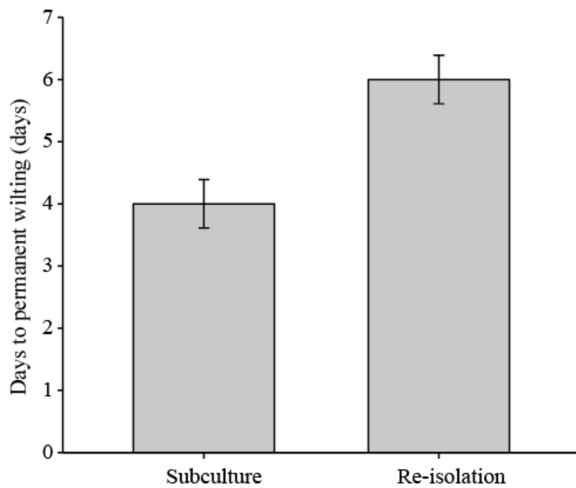

Fig. 3. Effect of *Sclerotium rolfsii* from serial subculture and re-isolation on days to permanent wilt in Jerusalem artichoke.

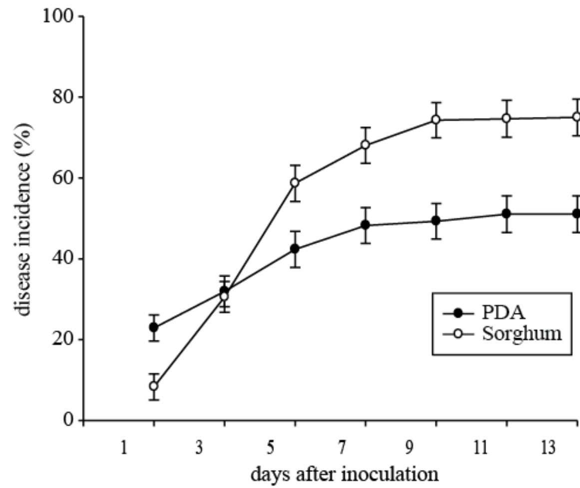

Fig. 4. Effect of media of inoculation on *Sclerotium rolfsii* disease in Jerusalem artichoke.

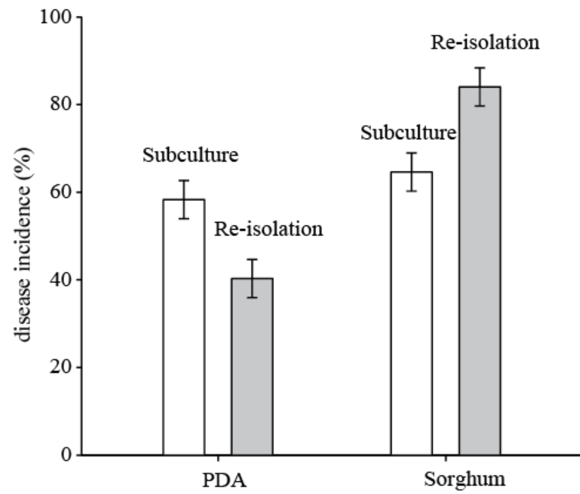

Fig. 5. Effect of media of inoculums and *Sclerotium rolfsii* from serial subculture and re-isolation on disease incidence of Jerusalem artichoke.

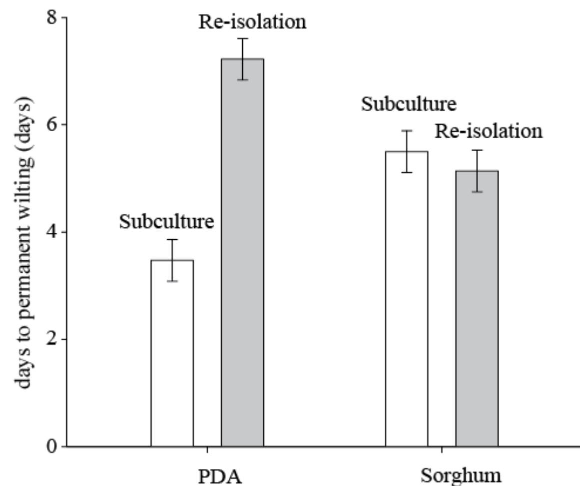

Fig. 6. Effect of media of inoculums and *Sclerotium rolfsii* from serial subculture and re-isolation on days to permanent wilting of Jerusalem artichoke.

Acknowledgements

This research was funded by a grant from the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral Degree from the Office of the Higher Education Commission, Thailand. Grateful acknowledgement are made to the Thailand Research Fund (TRF), the Commission for Higher Education (CHE) and Khon Kaen University (KKU) for providing financial supports to this research through the Distinguished Research Professor Grant of Professor Dr Aran Patanothai and the Peanut and Jerusalem Artichoke Improvement for Functional Food Research Group, KKU and Plant Breeding Research Center for Sustainable Agricultural, Khon Kaen, Thailand. The Plant Gene Resource of Canada and Leibniz Institute of Plant Genetics and Crop Plant Research, Germany, are acknowledged for their donation of Jerusalem artichoke germplasm. Finally, many thanks are extended to Dr. Mark L. Gleason, Dept. of Plant Pathology, Iowa State University, Ames, Iowa, USA for his kind suggestion.

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(Received for publication 8 May 2012)