RESEARCH ARTICLE

SABRAO Journal
of Breeding and Genetics
53 (1) 70-78, 2021

DEVELOPMENT OF AN OXALIC ACID ASSAY TO EVALUATE *SCLEROTIUM ROLFSII* RESISTANCE IN JERUSALEM ARTICHOKE

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SUMMARY

Stem rot caused by *Sclerotium rolfsii* causes major losses on Jerusalem artichoke (*Helianthus tuberosus* L.) in Thailand and other tropical countries, but resistance breeding efforts have been minimal. In this study, an oxalic acid assay was used to evaluate resistance to *S. rolfsii* in stems and tubers of Jerusalem artichoke. Preliminary evaluation with varying concentrations of oxalic acid showed that symptoms consistent with those produced by *S. rolfsii* occurred only on excised stems. Subsequently, excised stems of five Jerusalem artichoke accessions (PI 650103, PI 547238, PI 547230, PI 650095 and PI 65009) were partially immersed in three concentrations of oxalic acid (20, 30 and 40 mM). Treatments were arranged in a randomized complete block design with five replications. Two runs of the experiment were conducted in a dew chamber at 100% relative humidity and 27°C. Lesion length was measured from the excised ends of the stems at 1 to7 days after application of oxalic acid. Differences in lesion length were observed among Jerusalem artichoke accessions at all evaluation times; the highest variation was found at 6 days after treatment. Oxalic acid at a concentration of 40 mM gave the greatest lesion length at all evaluation times, but a concentration of 20 mM resulted in the largest F-ratio among accessions, indicating that it may be the most suitable concentration for screening Jerusalem artichoke accessions for stem rot resistance.

Keywords: Stem rot, susceptible, rapid technique, sunchoke

Key findings: The oxalic acid assay method may be useful for evaluation of *S. rolfsii* resistance in Jerusalem artichoke and can be used a basis to develop effective screening techniques. 20 mM oxalic acid evaluated at 6 DAT showed highest variation in lesion length among five Jerusalem artichoke accessions.

Manuscript received: October 6, 2020; Decision on manuscript: December 10, 2020;
Accepted: January 25, 2021.

Communicating Editor: Dr. Ramakrishnan M. Nair

INTRODUCTION

Jerusalem artichoke (*Helianthus tuberosus* L.) is a perennial tuber crop originating in temperate North America (Kays and Nottingham, 2008). Its tubers are consumed either as fresh vegetables or cooked. The tubers contain inulin, which is valuable as raw material to produce health food products and animal feed additives (Zaky, 2009). Inulin is beneficial to human health as it can reduce the risk of obesity, insulin-dependent diabetes mellitus (type 2), and heart disease (Orafti, 2005). It is also a source of fructose syrup for the food industry and fructans for medical or dietetic purposes (Huang *et al.*, 2012). Jerusalem artichoke has become a new crop with high potential for inulin production in Thailand (Puttha *et al.,* 2012) and as a biomass crop in China (Yang *et al.,* 2015).

Stem rot caused by the soil borne fungus *Sclerotium rolfsii* is a significant problem for Jerusalem artichoke production worldwide and yield losses up to 60% have been reported (McCarter and Kays, 1984). Crowns and tubers of Jerusalem artichoke were infected with white mycelium, and later rounded, tan sclerotia were formed to survive under adverse environment (Koike, 2004). The use of resistant cultivars is potentially a sustainable way to control the disease. The pathogen has an exceptionally wide host range and can cause severe losses in many agricultural crops. Therefore, attempts have been made to find sources of resistance in several crops, including peanut (Shew *et al.,* 1987), cowpea (Fery and Dukes Sr., 2002), and chickpea (Akram *et al.,* 2008). In earlier work in our program at Khon Kaen University, Thailand, several Jerusalem artichoke accessions from different sources were evaluated for resistance to *S. rolfsii* (Sennoi *et al.,* 2013).

Development of a rapid and efficient screening method is important to identify genotypes with superior resistance. Several inoculation methods have been used for screening plants for resistance to pathogenic fungi in greenhouse or laboratory settings, such as agar disk techniques (Shokes *et al.* 1996). However, high levels of variability encountered in these trials can sometimes mask differences in host resistance levels. In addition, the available methods for screening accessions for resistance to *S. rolfsii* are impractical because they are labor-intensive, time-consuming, and difficult to conduct reliably. Consequently, development of simpler and more effective methods would provide an advantage for breeders in developing commercially acceptable cultivars with high levels of resistance.

Oxalic acid is known to play a key role in pathogenicity of *S. rolfsii* by disrupting host defense mechanisms (Bateman and Beer 1965; Kritzman *et al.*, 1977; Punja, 1985). In addition, oxalic acid is very important in pathogenesis of *Sclerotinia sclerotiorum* (Godoy *et al.,* 1990; Ferrar *et al.,* 1993). Oxalic acid assays were developed to evaluate soybean (Wegulo *et al.,* 1998) and canola cultivars for resistance to *S. sclerotiorum* (Bradley *et al.,* 2006). In addition, an oxalic acid assay was developed to assess resistance among hosta (*Hosta kikutii* and *Hosta* spp.) cultivars to petiole rot caused by *S. rolfsii* var. *delphinii* (Xu *et al.,* 2009). A potential advantage of these assays is that screening can be done in the absence of the pathogen, which simplifies their use. However, a rapid technique for screening Jerusalem artichoke for resistance to *S. rolfsii* by oxalic acid assay has not been reported. The objective of this study was to assess the feasibility of developing an oxalic acid method for screening of Jerusalem artichoke for resistance to *S. rolfsii*.

MATERIALS AND METHODS

Effect of oxalic acid on tuber rot of Jerusalem artichoke

In a preliminary trial, tubers of two Jerusalem artichoke genotypes (TUB and JA (VOL GA2) were harvested in June 2012 from the North Central Regional

Plant Introduction Station(NCRPIS), a U.S. Department of Agriculture facility in Ames, Iowa, U.S.A. The tubers were washed in tap water, then air dried at room temperature and stored at 7-10 °C.

The tubers were surface-sterilized in 10% sodium hypochlorite for 60 s, rinsed with sterile distilled water and air dried under ambient laboratory conditions. Tubers were placed in 17.9 cm × 25.4 cm plastic containers labeled with concentrations of 0 (control), 10, 20, 30, 40 and 50 mM oxalic acid. The tip of a cotton swab was placed in contact with the base of a tuber and fastened to the tuber with adhesive tape. Twenty µl of each oxalic acid concentration were dispensed onto the tip of the cotton swab with a pipette. As a control, sterile distilled water (20 µl) was dispensed on each cotton swab tip.

The oxalic acid treatments at concentrations of 0 (control), 10, 20, 30, 40 and 50 mM were arranged in a randomized complete block design with 10 replications (10 containers). The containers were immediately placed in a dew chamber at 100% relative humidity and 27 °C (Xu *et al.,* 2009) under 14h light and 10h dark per day (Wegulo *et al.,* 1998).

Effect of oxalic acid on intact stems of Jerusalem artichoke seedlings

Fifty seeds of a Jerusalem artichoke accession, PI664624, obtained from NCRPIS, were germinated using gibberellic acid (GA) and pre-chilling method in incubators at the Iowa State University Seed Testing Laboratory. The germinated seeds were planted in 4:3:4 peat moss, metro mix and coarse perlite, respectively, for 1 week in plastic flats in a growth chamber (25 °C under 14 h light and 10 h dark). Healthy plants were transplanted to 12.7 cm-diameter pots (1 plant per pot) for 1 week in the same chamber. The plants were used for experiments when they were at the eight-leaf stage. A cotton swab was placed in contact with the stem base of each seedling, after which 20µl of 50 mM oxalic acid was dispensed on to the tip of the cotton swab with a pipette. The treated seedlings were incubated under the same environmental conditions as for inoculated tubers.

Effect of oxalic acid on stem rot of excised stems

Plant materials were prepared from seeds of Jerusalem artichoke as described above. Stems of genotypes PI 650103, PI 547238, PI 547230, PI 650095 and PI 650091 were cut transversely at the soil line using a surface-sterilized scalpel. Mature leaves and petioles were removed from the stem except for two apical, fully-developed leaves. Each plant was placed immediately in a test tube containing oxalic acid solution (5 ml of 20, 30, or 40 mM oxalic acid). The amount and concentrations of OA were applied based on the protocol of Wegulo *et al.* (1998). As a control, an excised stem of each genotypes was placed in a test tube containing sterile distilled water (5 ml) for each replication. Test tubes containing Jerusalem artichoke stems were arranged in test tube racks in a randomized complete block design with five replications. Blocking was done due to slightly difference of the light of each shelf in the dew chamber. For each genotype, there were two test tubes per concentration for each replication. After treatment, the test tubes were placed in a dew chamber at 100% relative humidity and 27 °C (Xu *et al.,* 2009). The dew chamber was set up with 14h light and 10h dark (Wegulo *et al.,* 1998). The experiment was conducted twice.

Lesion length was measured daily 1 to 7 days after treatment (DAT) (Wegulo *et al.,* 1998). Error variances between the two trials of the excised-stem assay were tested for homogeneity; data sets passing the homogeneity of variance criterion were subjected to combined analysis of variance for the two trials. Least significant difference (LSD) was used to

compare mean differences. All calculations were done using STATISTIX 8 software program (Analytical Software, Tallahassee, Florida, USA).

RESULTS

Preliminary trials

In the preliminary trials, inoculation of tubers did not cause symptoms on immature tubers after incubation in a dew chamber at 100% relative humidity at 27 °C for 14 days. In addition, the cotton swab method on intact seedlings of Jerusalem artichoke resulted in localized lesions at the inoculation site after 1 day of inoculation, after which the lesions did not progress beyond the immediate vicinity of the cotton swab (data not shown). In a preliminary trial, OA concentrations less than 50 mM did not result in visible damage to the stem

Stem immersion trials

In the replicated trials in which excised stems were immersed in oxalic acid solutions, discoloration and softening at the bases of the stems was noted on all accessions at all evaluation dates and concentrations in both runs of the experiment. The two runs of the experiment did not differ significantly for lesion length among accessions at 1, 3, 4, 5, 6 or 7 DAT (Table 1). Therefore, the data were pooled for the two experiments. Jerusalem artichoke accessions were significantly different ($P < 0.01$) for lesion length at 3 to 7 DAT. Significant differences ($P < 0.01$) among concentrations of oxalic acid were observed for lesion length at 1 to 7 DAT, and higher concentrations of oxalic acid and longer duration of evaluation time resulted in increased lesion length. Lesions lengths ranging from 4.4 to 15.5 cm were observed across oxalic acid concentrations and evaluation dates. The longest lesions were observed for an oxalic acid concentration of 40 mM at 7 DAT, and the shortest lesions were found for an oxalic acid concentration of 20 mM at 1 DAT.

Jerusalem artichoke accessions responded differently to oxalic acid treatment in this experiment. Accessions PI 650091 and PI 547238 had shorter stem lesions than accessions PI 547230, PI 650095 and PI 650103 for most concentrations and observation dates. Lesion length in all Jerusalem artichoke accessions increased most rapidly between 2 and 5 DAT (Figure 1).

Responses of Jerusalem artichoke accessions to oxalic acid concentrations at 6 DAT are shown in Figure 2. Higher concentration of oxalic acid tended to result in longer lesion length for accessions PI 650103 and PI 650091. Conversely, PI 650095 had shorter lesion length when oxalic acid concentration was higher. Variation in lesion length among accessions was determined by F-test value and coefficient of variation (CV) (Table 2). Treatment with 20 mM oxalic acid evaluated at 6 DAT showed the highest variation for lesion length in five Jerusalem artichoke accessions. Lesion lengths ranging from 9.6 to 18.5 cm were observed in Jerusalem artichoke accessions immersed in 20 mM oxalic acid and evaluated at 6 DAT. The PI 650091 had the shortest lesions, whereas PI 547230 had the longest lesions (Figure 3).

DISCUSSION

Results of this study offer evidence that a simple, relatively rapid assay for resistance to *S. rolfsii* in Jerusalem artichoke may be feasible. This assay – using immersion of excised stems in oxalic acid – required a week or less to reveal differences in resistance among Jerusalem artichoke accessions. The biological rationale for the assay is that *S. rolfsii* utilizes oxalic acid as a primary weapon during pathogenesis. By substituting oxalic acid for the pathogen itself, breeders could substantially streamline screening for resistance in Jerusalem

Table 1. Mean squares for overall ANOVA of stem lesion length in Jerusalem artichoke (*Helianthus tuberosus*) evaluated at 1, 3, 5 and 7 days after treatment (DAT). Excised stems of Jerusalem artichoke were immersed in a range of concentrations of oxalic acid, and length of stem discoloration from the excised end was measured. Means shown represent two runs of the experiment.

Source of Variation	d.f.	Lesion length						
		1 DAT	2 DAT	3 DAT	4DAT	5 DAT	6DAT	7 DAT
Experiment (E)	1	1.94ns	4.16*	1.06ns	0.47ns	0.10ns	0.17ns	0.02ns
Rep within experiment	4	0.61ns	0.74ns	1.04ns	0.28ns	0.33ns	0.89ns	0.64ns
Accessions (A)	4	1.82**	1.77**	19.95**	108.68**	177.94**	199.39**	139.21**
Concentrations (C)	2	10.58**	22.65**	19.72**	89.66**	12.25**	7.83**	13.63**
A × C	8	0.34ns	0.26ns	3.23**	9.45**	7.48**	18.82**	15.36**
Pooled error	126	0.27	0.31	0.38	0.54	0.51	0.46	0.47
CV (%)		10.92	10.1	7.86	6.76	5.16	4.84	4.59

ns, *, **Non-significant, significant at 0.05 and 0.01 probability level

Table 2. F-ratios for lesion length in Jerusalem artichoke accessions treated with three concentrations of oxalic acid evaluated at 1, 2, 3, 4, 5, 6 and 7 days after treatment (DAT). Excised stems were immersed in a range of concentrations of oxalic acid, and length of stem discoloration from the excised end was measured. Values shown represent two runs of the experiment.

Oxalic acid concentrations	1 DAT	2 DAT	3 DAT	4 DAT	5 DAT	6 DAT	7 DAT
20 mM	3.19*	1.95ns	11.62**	47.35**	137.29**	290.24**	267.21**
CV (%)	12.82	11.96	9.05	8.05	5.88	5.01	4.62
30 mM	0.92ns	1.64ns	30.82**	90.09**	93.05**	99.94**	89.88**
CV (%)	10.16	10.22	7.89	6.44	4.73	4.51	4.50
40 mM	4.07**	3.30*	24.94**	78.54**	148.32**	97.44**	96.19**
CV (%)	10.69	9.31	7.41	6.41	4.70	5.26	4.97

ns, *, **Non-significant, significant at 0.05 and 0.01 probability level

artichoke, resulting in significant savings of time and cost. In developing screening assays for resistance to two different pathogens that utilize oxalic acid in pathogenesis, Wegulo *et al.* (1998) and Xu *et al.* (2009) demonstrated that results of oxalic acid-based assays could discriminate among cultivar resistance for soybean and hosta, respectively, and they found that these results were correlated to ratings of assay methods that required presence of both the host and the pathogen. In the case of an oxalic acid assay for resistance in Jerusalem artichoke to *S. rolfsii*, subsequent studies are needed to validate ability of the assay to discriminate resistance levels among cultivars in multiple field trials before it can be utilized reliably by breeders.

Although Jerusalem artichoke accessions used in this research were not pre-selected to represent a wide range of levels of resistance to *S. rolfsii,* the stem-immersion assay revealed significantly different levels of resistance among five arbitrarily selected accessions. The variation noted among these accessions may prove useful for Jerusalem artichoke breeding efforts in the future, once the assay method is further validated. Although the number of accessions in this study was small, the genotypes with the highest and lowest resistance to oxalic acid could be used as check genotypes to assess the levels of resistance in large scale screening of other germplasm sources.

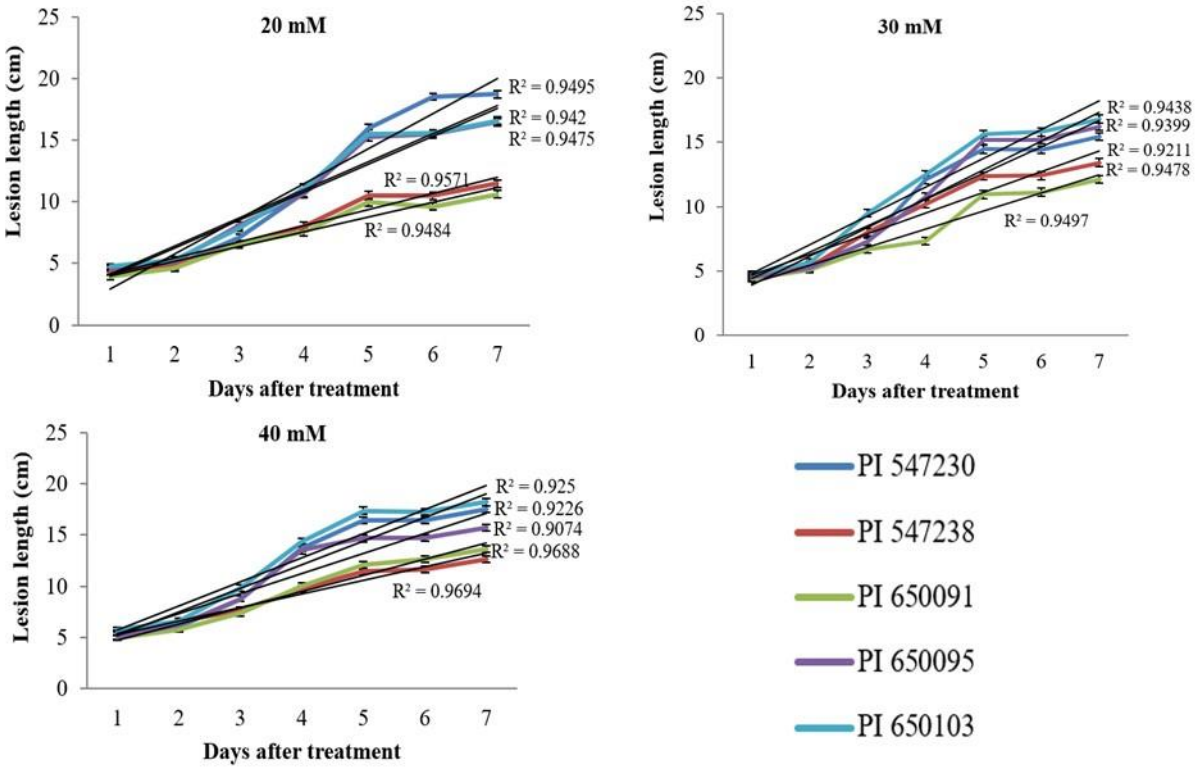

Figure 1. Effect of oxalic acid on lesion length of excised stems of five accessions of Jerusalem artichoke evaluated at 1 to 7 days after treatment in different concentrations of oxalic acid**.**Means shown represent two separate runs of the experiment**.**

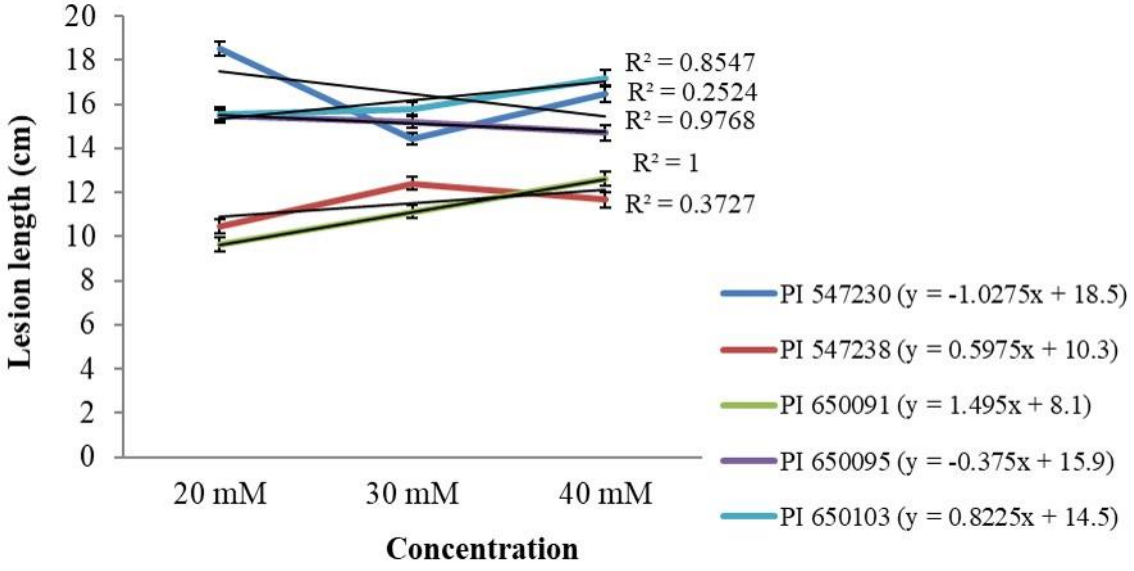

Figure 2. Lesion length of stem rot in five accessions of Jerusalem artichoke at 6days after treatment with different concentrations of oxalic acid.

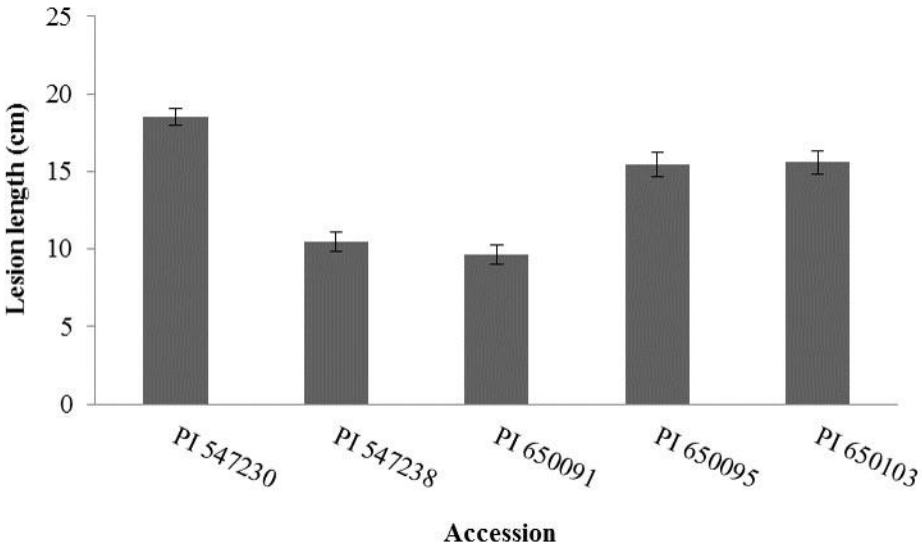

Figure 3. Lesion length of excised stems of five accessions of Jerusalem artichoke at 6 days after treatment with 20 mM oxalic acid. Means shown represent two runs of the experiment.

The stem immersion assay was the most promising approach among several methods that were tried in preliminary experiments. Application of oxalic acid to the surface of tubers of Jerusalem artichoke did not result in lesion development. In a previous investigation in hosta, application of oxalic acid to leaves resulted in symptoms similar to those caused by *S. rolfsii* var. *delphinii* (Xu *et al.,* 2009). Determining suitable plant parts for discrimination among accessions is important for the success of OA-based screening assays.

Oxalic acid plays a key role in plant pathogenicity of *S. rolfsii* (Punja 1985) and *Sclerotinia sclerotiorum* (Godoy *et al.,* 1990; Ferrar *et al.,* 1993; Kabbage *et al.,* 2013). The secreted OA is produced to destroy the host cell wall (Bosamia *et al.* 2020). Stem rot caused by *S. sclerotiorum* and *S. rolfsii* in Jerusalem artichoke had similar symptoms, and both fungi secrete oxalic acid to degrade host defenses (Cassells and Walsh, 1995). In other crops, evaluation of resistance to *S. sclerotiorum* using an oxalic acid assay was effective in soybeans (Wegulo *et al.,* 1998), canola (Bradley *et al.,* 2006), and common bean (Steadman *et al.* 2001). To the best of our knowledge, evaluation of resistance to *S. rolfsii* using this method has not been reported previously for Jerusalem artichoke.

The OA stem immersion assay protocol may require further optimization. Suitable concentration and time for evaluation are important for development of a reliable screening assay. In this study, the optimal concentration was 20 mM, and the optimal evaluation time was 6 DAT. In previous investigations, the optimal concentrations of oxalic acid for soybean and hosta plants were 40 mM and 50 mM, respectively (Wegulo *et al.,* 1998; Xu *et al.,* 2009). Use of data from a single assessment date can be economical and time-saving when the number of cultivars under evaluation is large, and additional savings can be realized by using a single OA concentration rather than multiple concentrations.

The criterion of days to permanent wilting has been used for evaluation of *S. rolfsii* resistance in intact Jerusalem artichoke plants under greenhouse conditions (Sennoi *et al.* 2013). In that study, this trait gave the greatest ability to discriminate resistance levels among accessions. In this study, however, days

to permanent wilting were not useful because it did not result in measurable differences among accessions. Lesion length was more suitable for evaluation because the variation among five Jerusalem artichoke accessions was greatest for that response variable. Regardless of the suitable variable, factors that should be considered in resistance breeding assays include uniformity of the plants (stage), environmental control, and an optimal oxalic acid concentration solution to ensure the consistency of trial outcomes.

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. Assistance was also received from the Peanut and Jerusalem Artichoke Improvement Project for the Functional Food Research Group, and the Thailand Research Fund for providing financial support through the Senior Research Scholar Project of Professor Dr. Sanun Jogloy (Project no. RTA6180002). The North Central Regional Plant Introduction Station, USDA in Ames, IA, USA is acknowledged for their donation of Jerusalem artichoke accessions. Many thanks for the laboratory and greenhouse space provided by the Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA, USA.

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