Susceptibility of yellow starthistle to *Puccinia jaceae* var. *solstitialis* and greenhouse production of inoculum for classical biological control programs

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**A B S T R A C T**

In anticipation of large-scale distribution of a Turkish isolate of *Puccinia jaceae* var. *solstitialis* in California for biological control of yellow starthistle (*Centaurea solstitialis*,YST), susceptibility of YST within the state was determined and a protocol for bulk inoculum production was developed. Inoculation was made of 62 field accessions of YST representative of the range of habitats in California. These were determined to be equally susceptible to infection by the isolate approved for release in the United States in 2003. To support a program to speed establishment by release at many locations statewide, protocols for artificial increase of inoculum were developed. Over 64 g of urediniospores were produced with a mass-production system under greenhouse conditions from 2003 to 2006. Yield of inoculum varied by season, with peak production occurring from early spring through early summer. A large-scale urediniospore harvest also was made from a field plot at Davis, California. Our results show that susceptibility of YST in California is not likely to limit establishment of *P. jaceae* for biological control, and that production of this or other obligate pathogenic fungi (biological control agent) is possible for support of statewide release and research programs.

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1. Introduction

A permit was issued in April, 2003, by the USDA, Animal & Plant Health Inspection Service (APHIS), for introduction and field release of the exotic rust fungus *Puccinia jaceae* Oth var. *solstitialis* (Savile, 1970), herein referred to as *P. jaceae*. *P. jaceae* is an aecious, macrocyclic, obligate pathogen selected as a classical biological control for YST. The objective of this release was to complement existing insect agents for biological control of yellow starthistle (YST, *Centaurea solstitialis* L.) in California, and ultimately in the western United States, where YST is a major pest plant (DiTomaso and Gerlach, 2000; Pitcairn et al., 2006). Only a small amount of inoculum was delivered to the California Department of Food & Agriculture (CDFA) from the USDA, Agricultural Research Service (ARS), Foreign Disease-Weed Science Research Unit (FDWSRU) microbial containment greenhouse facility at Ft. Detrick, MD (Melching et al., 1983). This delivery was made with the approval by APHIS following extensive evaluation of the pathogen for safety and efficacy (Berner and Bruckart, 2005; Bruckart, 1989, 2006).

The APHIS permit was issued with restrictions, including: (1) release of only one isolate of *P. jaceae*, (2) delivery of inoculum from containment that is free of contaminants, and (3) release limited specifically to sites in California. Increase of inoculum in Maryland outside of containment was not allowed, since this would constitute “release” of *P. jaceae*, which had not been requested in the permit proposal. The result was delivery of 100 mg *P. jaceae* urediniospores produced within the containment greenhouse facility at FDWSRU. Inoculum was checked for purity before removal from the containment facility and hand-carried at ambient temperature to the CDFA, Plant Health and Pest Prevention Services, Biological Control Program, in Sacramento. Delivery was made under permit No. 47494, issued by APHIS to CDFA (Woods et al., 2004).

Given the extent of the YST infestation in the western United States (Pitcairn et al., 2006) and the urgency to control the plant in California, we decided to: (1) test YST from various locations and habitats in the state to determine if it was uniformly susceptible to the permitted isolate, and (2) field inoculate YST at several locations in order to speed establishment of *P. jaceae* and damage anticipated from the disease. For the latter, a large-scale production program was established to provide sufficient inoculum for widespread distribution of the pathogen. Additional inoculum was also needed for proposed field research projects. There is

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practically no information about large-scale production of inoculum for an obligate fungal pathogen in the literature, so protocols had to be developed for this purpose. In this paper, we describe both tests for susceptibility of YST accessions from California to *P. jaceae*, and procedures for mass production and collection of urediniospores of *P. jaceae* for field release and study.

2. Materials and methods

2.1. Fungal isolate and inoculum source

Isolate FDWSRU 84-71 was collected originally in Turkey by Dr. Sarah Rosenthal (USDA-ARS, retired) and evaluated as a candidate for biological control of YST at the FDWSRU. Following the issuance of a permit by APHIS for introduction, inoculum was removed from quarantine and hand carried to the CDFA as described in the Introduction.

2.2. Spore production in the greenhouse

2.2.1. Plant production

Field collected YST seeds, stored at room temperature, were germinated on wet blotter paper in plastic Petri plates on a laboratory bench. After 3–5 days, two seedlings were transplanted to each 10-cm (4-in.) plastic pot containing a commercial potting soil mix. Potted plants were moved to a small conventional greenhouse maintained at 26 °C, and supplemental light was used to maintain a 16-h photoperiod. By the seventh week, plants had developed 6–10 true leaves, each 4–8 cm in length.

2.2.2. Inoculum procedure

Inoculum was prepared by suspending 50 mg of urediniospores in 100 ml of water containing three drops of Tween 20 (polyoxyethylene sorbitan monolaurate; Fisher Scientific, Fair Lawn, NJ). Spores were allowed to “wet” for 20 min before filtering the suspension through four layers of cheesecloth. The entire volume of filtered suspension was applied to YST plants using plastic, 250 ml finger pump household spray bottles at a rate of 2.5 mg urediniospores/plant. Leaves were sprayed to runoff. Inoculated plants were incubated overnight in the dark in a dew chamber (Percival Scientific, Boone, IA) and then were moved to the greenhouse.

2.2.3. Spore harvest

Urediniospore harvest began 2 weeks after inoculation. A custom made twin-shell cyclone spore harvester was attached to a 60 L (=16 gallon), wet/dry vacuum cleaner, which provided suction necessary to operate the harvester. Each inoculated plant was inspected for infection before spores were harvested. Harvested spores were collected in glass vials attached to the harvester. After harvest, spores were weighed, transferred to small screw top glass vials, and stored in a refrigerator. Most spores were bulked and transferred to a −70 °C ultra-cold freezer for long-term storage within 1 month of harvest. The remaining spores were used either for urediniospore production or for field releases associated with *P. jaceae* establishment or research programs (Woods et al., 2008). Spores were harvested three times per week, if possible, (usually on Monday, Wednesday, and Friday) until individual plants were no longer productive, i.e., when pustule numbers declined to fewer than five sporulating pustules on each of the three most infected leaves. Each inoculation included 20–30 pots, each with two plants per pot. Inoculations were staggered so that there was an average of 50 pots being harvested at any particular time. Harvests began in August, 2003, and continued into early 2006. Additional dew was not provided for infected plants, and greenhouse conditions were insufficient for natural re-infection of plants.

To determine how long infected YST plants produced inoculum, detailed records of harvest dates were maintained for 206 individual pots inoculated from November 2003 through February 2004. Spore production data were not recorded for individual plants.

In order to examine seasonal variation in spore production, harvest data were averaged by month for 29 of the 35 months between August 2003 and June 2006 (Fig. 2). Data for inoculum production were analyzed using Proc GLM with Statistical Analysis System (SAS) software Version 8 (SAS Institute, Cary, NC). Means were separated on the basis of probabilities of similarity (Proc. PDIFF in SAS); those with *P* < 0.05 were considered statistically significantly different.

2.2.4. Pest control

Artichoke pests, including aphids, whiteflies, spidermites, and mealybugs were a consistent threat to spore production. Spidermites were observed actively consuming spores, and all artichoke pests limited both plant vigor and spore production. Considerable effort was made to suppress infestations. Infested plants were culled on a regular basis and insecticidal sprays were applied routinely to prevent establishment of arthropods. AVID® (Abamectin) was applied every 2 weeks in combination with either Floramite® (Bifenazate) or Endavor® (Pymetrozine).

2.3. Field collection of *P. jaceae* spores

A 2-year graduate thesis study started in 2006 at the Vegetable Crops Field Station in Davis (Yolo County), California provided an opportunity to collect urediniospores from field-inoculated plants (O’Brien, 2008). Three- to four-week-old seedlings were planted at the end of January in 1 m² plots at varying densities of YST and wild oat. After 4–5 weeks, at the beginning of March, rosettes in each plot were inoculated with 100 mg urediniospores in 200 ml water plus 12 drops Tween 20 per liter. Plots that had escaped infection during the first inoculation were re-inoculated at the end of March. A total of 2.4 g of spores were used for inoculation in 2006, and 3 g were used in 2007. Inoculated plots were tented overnight to maintain humidity and improve conditions for infection (Bennett et al., 1991; Fisher et al., 2007; Emge et al., 1981). By April 30, 2006, and March 23, 2007, disease in epidemic proportion had developed in the plots. Spores were harvested as described, with power supplied by a gasoline generator.

Two attempts to collect spores were made in 2006. The first was on June 20, when YST was in full bloom, and the second was on July 12, at conclusion of the study. One collection was made in 2007 on June 26. Spores were harvested from plants in the exterior, or buffer zone, of each plot, leaving the interior 0.5 m² undisturbed (O’Brien, 2008).

2.4. Testing YST accessions from California for susceptibility

YST seed was collected from around the state during a survey of biological control agents (Pitcairn et al., 2008). From this collection, 62 accessions were selected as representative of the geographic range and diversity of YST in California (Fig. 1). Six plants from each accession were inoculated as described and visually evaluated for infection 8, 10, 13 and 17 days after inoculation.

2.5. First releases

On July 8, 2003, following delivery of spores from FDWSRU, 20 eight- to 12-week-old YST plants were inoculated as described with 10 mg of urediniospores at the CDFA greenhouse in Sacramento. This was to initiate the *P. jaceae* inoculum production pro-
gram and constituted the first inoculation of YST outside of containment in the United States.

The first field inoculation was made on July 9, 2003, in Napa County, CA. A 1 m² plot was marked with stakes, irrigated with water to provide a favorable microclimate for infection, and at sunset, spray-inoculated with 50 mg urediniospores suspended in 250 ml water plus three drops of Tween 20 (0.13%). This then became the first plant pathogen to be fully approved and released in the field for classical biological control of a weed in the continental US following the modern permitting process required by APHIS.

3. Results

3.1. Susceptibility of California accessions

All accessions representing the range of geographic distribution of YST in California (Fig. 1) were susceptible to infection by Puccinia jaceae var. solstitialis.
3.2. Spore production

3.2.1. Spore productivity of individual plants

Plants began producing enough spores for harvest 2 weeks after inoculation. Harvesting was continued for several weeks until pustule numbers declined below the non-productive threshold. Loss in plant productivity resulted primarily from damage to the leaves and occasionally to petioles from the vacuum harvester. Damage was greatest on the most severely infected leaves, which could only survive a couple of harvests. Additionally, individual pustules appear to have a limited productive lifespan, declining in size and number over a period of several weeks even on non-damaged leaves. Since no additional dew period treatments were administered to infected plants after harvests, and because greenhouse conditions were insufficient for re-infection, plants gradually became non-productive. Mean productivity, in days from inoculation to discard of individual plants was 90.7 (SE = 1.85, maximum = 145 days). Plants survived for an average of 26 harvests (SE = 0.58, maximum = 49). Plants with moderate infection levels were infected after harvests, and because greenhouse conditions for infection, but we have also had reasonable success with a room cleaner, containing more than 75% spores and only 25% contamination (primarily plant trichomes and insect parts).

3.2.2. Greenhouse production and harvests

Large-scale production was initiated in the fall of 2003 with inoculations of 40 plants each in September and October. Spore yield increased rapidly through mid-December. Many of these original plants then declined in quality, and spore production decreased accordingly. Staggered planting and inoculation of YST was initiated at this time. Yield varied greatly from day-to-day, but it was always highest on Mondays, when plants had 2 days for spore generation. Monthly yield also varied significantly (P = 0.05) on a seasonal basis, with two peak periods of production each year (Fig. 2). Greatest spore production occurred between March and May, and lowest yields occurred during late summer months. A second, smaller peak developed in late fall. A total of 14.6 g of urediniospores were produced in 2003, 16.2 g in 2004, 23.6 g in 2005, and 10.4 g during the first 6 months of 2006. Periodic testing of spore viability indicated that germination rates did not vary during greenhouse production cycles, and there was no significant decline in germination after storage at –70 °C.

3.2.3. Field collection of P. jaceae

Field collection of spores in 2006 resulted in the harvest of 32.2 and 7.2 g of spores on June 20 and July 7, respectively. The first harvest took 2 h, and the second took 1.5 h. The first collection was composed almost entirely of high quality urediniospores (i.e., good germination), but it also contained 50% sand contamination by volume (based on microscopic examination and grid intercept of subsamples). Telia were more prevalent during the second harvest, but only urediniospores were collected by vacuum (teliospores are not friable). urediniospores in the second sample germinated at a lower rate, and there was much more sand contamination (75%) than before. In 2007, 12.3 g urediniospores were harvested, germination was good, and there was 50% sand contamination. Greenhouse spore collections were consistently less contaminated (i.e., cleaner), containing more than 75% spores and only 25% contamination (primarily plant trichomes and insect parts).

4. Discussion

The plan for a program of widespread release of P. jaceae in California was justified on the basis of the uniform susceptibility of YST accessions representing various habitats and locations in the state. Based on limited pre-release evaluations, there was confidence that plants inoculated anywhere in the state would likely be infected under the right environmental conditions. Findings in this study are consistent with a report indicating similar genetic diversity within and between North American YST populations (Sun, 1997), and suggests that all YST in the US would be uniformly susceptible to isolate FDWSRU 84-71. Thus, differences found in infection rate and damage by P. jaceae between field sites would likely be the result of environmental factors.

Uniformity in target plant susceptibility is a legitimate issue that can affect success in biological weed control. Clement (1994) described resistance of certain populations of yellow starthistle to specific thistle-head insects, and a number of target weeds have differed in susceptibility to specific pathogen isolates, including pathogen of rush skeletonweed (Cullen, 1986) and Salsola species (Ryan and Ayres, 2000; Bruckart et al., 2004). This was not an issue for YST and P. jaceae in California.

Production of large amounts of P. jaceae urediniospores was accomplished under greenhouse conditions in this study in a ‘low-tech’ but labor intensive manner. Specialized equipment included a twin-shell cyclone spore harvester and an ultra-cold freezer, the latter used for long-term storage of urediniospores. A commercial dew chamber was used to provide optimal conditions for infection, but we have also had reasonable success with a room humidifier inside a plastic tent. Other equipment included a wet/dry canister-type vacuum unit, collection vials, and a greenhouse.

Extensive personnel time was required to harvest the spores; each harvest taking from 1–4 h. Planting, watering, and pest control also required considerable time. In general, the process of large-scale urediniospore production for biological control was reasonable and successful. Story et al. (1994, 1996), found that labor costs were more than 70% of the total input in production of two insect species for a spotted knapweed management project.
Labor cost for the present project was similarly high, but less than costs of greenhouse construction and energy for maintenance of temperature (heating and air conditioning).

Seasonal fluctuation in urediniospore yield was significant in our study. This was likely due in large part to changes in host quality throughout the year. Plant growth rates, along with spore yield, declined each winter as day length declined below 16 h and when supplemental lighting became the primary light source. Plant growth rates, along with spore yield, again increased starting in February and continued to increase through the spring. Longer photoperiods during the summer months also changed the YST plant quality to that of a less succulent and hairier nature, despite use of air conditioning and application of shading compound on the greenhouse. Seasonal effects were not reversed by supplemental lighting or by mechanical temperature control (heating and air conditioning). For these reasons, it may be more efficient and economical to produce inoculum only during the most favorable times of year as part of an intensive program.

Fisher et al. (2008) demonstrated that viability of urediniospores declined over a period of 10 weeks after storage in the laboratory at ambient temperatures. Although yellow starthistle can be inoculated artificially in the field over a period of several months (Fisher et al., 2007), ideal temperature and moisture conditions in California usually occur in the field during a 2- to 3-month-period in the spring. Field inoculations in California were most successful in the early spring (Woods et al., 2008; Fisher et al., 2007), which is also the time that greenhouse production is highest. Therefore, any large-scale, intensive spore production program would need to be conducted a year before planned field inoculations of P. jaceae, and low temperature storage would then be required to preserve the large quantity of spores needed for such an activity. Ultra-cold storage of spores was a very effective means of preserving inoculum in this study.

Leaf disease severity was not specifically evaluated in this study. However, pustule density varied between individual plants, and plants with the most pustules consistently produced the most spores during harvest; i.e., heavily-infected plants were essential to large-scale production of inoculum. We observed that severely infected leaves had a shorter lifespan and that severely infected plants were no longer productive 2–3 weeks after the start of sporation. Preliminary data from a field and growth chamber study on damage by the rust disease support this finding (Woods and Popescu, 2007a,b, personal observations). Shishkoff and Bruckart (1993) also noted both a large variation in pustule number per leaf under controlled inoculations and that pustule number was correlated with rate of leaf senescence. Plants with moderate infection levels proved to be the most durable and amenable to long-term spore production. These plants also were less prone to vacuum damage or stress abscission.

The interplay of moisture and temperature is of major importance in successful infection of YST. Our greenhouse production system provided nearly ideal conditions for infection and harvest at certain times of the year. In contrast, high yield from field plots was not anticipated, because optimum conditions for infection and spread seldom exist in the field. Inoculation of P. jaceae at several release sites in the state resulted in low infection rates and virtually no secondary spread, so field collection of inoculum was generally not practical at these sites. The 2006 field site at Davis had ideal conditions for an epidemic (i.e., vigorous plant growth along with warm temperatures and sustained leaf wetness), so infection was very good and a large volume of spores was harvested. Because a different field was used each year, annual inoculation was required to initiate new epidemics. Field production of spores was excellent at the site in Davis, but the use of field sites, in general, is not likely to be reliable for inoculum production, unless artificial measures, e.g., a misting system for “dew”, can be added to the process. Only one other field location has been found where the rust disease developed to levels similar to the Davis site (Woods et al., 2008). In contrast, greenhouse production under controlled conditions consistently resulted in excellent yields, particularly in the spring.

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