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Some Effects of Abiotic Stress on Infection of Dyer's Woad (*Isatis tinctoria L.*) by *Puccinia thlaspeos C.* Schub.: Implications for Biological Control

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Abstract: Problem statement: The rust pathogen, *Puccinia thlaspeos*, is being studied as a potential biocontrol agent for the noxious weed dyer's woad. Although its disease etiology is well understood, very little has been learned about the effect of environmental stresses on infection. **Approach:** Dyer's woad plants were exposed to different levels of oxidative stress, salinity stress, osmotic stress, dehydration, and cold stress before being inoculated with the rust pathogen. Rust infections were subsequently detected in asymptomatic tissue using rust-selective primers with the polymerase chain reaction. **Results:** Mild abiotic stress appears to enable dyer's woad plants to develop cross-tolerance to the rust pathogen. Plants exposed to the mildest level of salinity were only 60% infected. Those exposed to the lowest osmotic stress were only 50% infected while plants exposed to the shortest period of dehydration, or cold stress were both only 70% infected. Control plants were 100% infected for all experiments. On the other hand, exposing plants to mild oxidative stress did not lower infection while the highest level of oxidative stress significantly lowered infection to 55%. **Conclusion:** Cross-tolerance to multiple stresses often a desirable trait for plants of economic importance, is a cause for concern in biocontrol of weeds because of its potential to adversely impact the efficacy of mycoherbicides.

Key words: *Puccinia thlaspeos*, abiotic stress, abscisic acid, dehydration stress, dyer's woad, mycoherbicide, rust pathogen, oxidative stress, osmotic stress, pathogens, ethylene, salicylic acid, cross-tolerance, rust fungus, mannitol, sodium chloride, noxious weed, *Isatis tinctoria*

INTRODUCTION

Dyer's woad (*Isatis tinctoria*) is an invasive biennial or short-lived perennial plant that is problematic in the western United States. It was introduced into North America sometime during the colonial period as a source of blue dye, and is believed to have entered the western part of the continent in the early 1900's as a contaminant in alfalfa. Since then, it has spread rapidly and has been reported in most parts of the Western United States, leading to its designation as a noxious weed in a number of states (Flint and Thompson, 2000; Kropp *et al*., 1996; 2002).

In agricultural areas, dyer's woad can be controlled with herbicides. However, many large infestations of dyer's woad occur in inaccessible or environmentally sensitive areas where traditional methods cannot be used. In these circumstances, biological control is an attractive alternative for managing this noxious weed.

The rust fungus *Puccinia thlaspeos* has recently received much attention as a potential biological control agent for dyer's woad (Kropp *et al*., 2002). This rust fungus asymptomatically infects rosettes of dyer's woad in the first year of its life cycle and when the plants bolt during the second year, disease symptoms appear. Symptomatic woad plants become severely stunted, chlorotic and malformed, with very little seed production (Kropp *et al*., 1996; 1997; 1999). Though extensive studies have been conducted on the ecology, disease etiology, inoculation techniques, colonization, establishment and dispersal of *P. thlaspeos* (Flint and Thomson, 2000; Kropp *et al*., 1996; 1997; 1999; 2002), very little is known about the effects of environmental factors on disease initiation.

Plants are subjected to numerous biotic and abiotic stresses during the completion of their life cycles. Abiotic stresses include factors such as drought, salinity, extreme temperatures, oxidative stress and

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flooding while biotic stress is the result of challenges from pathogens and herbivores. An abundance of research exists on the effects of individual stresses on plant growth and productivity (Chen *et al.*, 1983; Downton and Loveys, 1981; Zeevart and Creelman, 1988) and studies have shown that plants respond to them in similar ways. Hence plants that are resistant to one type of stress can develop cross-tolerance to others. This is typically beneficial to crop plants but it could potentially work against the use of plant pathogens to control noxious weeds.

Under natural conditions, it is likely that almost any time an infection is initiated, the host plant could be facing some level of abiotic stress that could lead to cross-tolerance. Thus, the goal of this study was to examine the effect of different levels of selected abiotic stresses (oxidative, salt, osmotic, dehydration and cold stress) on the infection of dyer's woad by *P. thlaspeos.*

MATERIALS AND METHODS

Growth and inoculation of dyer's woad: Silicles of dyer's woad were harvested from field populations and threshed manually to obtain seeds that were stored at 4°C until further use. The seeds were planted in 10 cm pots that contained steam-sterilized potting mixture, consisting of perlite, peat moss, and vermiculite, mixed in a ratio of 1:1.3:1, respectively. Prior to experimentation, the plants were grown for 4 weeks under greenhouse conditions using a light and temperature regime of 16 h days at 25°C and 8 h nights at 18°C and watered to saturation on alternate days. Fully expanded leaves on the 4-week-old seedlings were used for all experimental treatments.

Prior to inoculation, inoculum that had been prepared from diseased dyer's woad plants collected in the field (Kropp *et al*., 1997) was surface-sterilized with 0.6% sodium hypochlorite, soaked in sterile distilled water for 3 min, and rinsed thoroughly. The surface-sterilized inoculum was incubated for 6 h in a dew chamber that was maintained at a temperature of 15°C ±1 to reduce the germination lag period before inoculating the plants. To inoculate the leaves, the inoculum was then positioned over the leaves to ensure that the apical two centimeters of the leaf would be saturated with basidiospores. Inoculations were carried out in the darkness at night. Care was taken to ensure that the leaf tissue was not damaged during inoculation. The inoculated plants were then held in a dew chamber for a period of 12 h before being transferred to the greenhouse. After growing the plants for 3 weeks, they were assayed for rust infections. Inoculations were done following the stress treatment described below.

Abiotic stress treatments: To study the effects of abiotic stress on rust infection in dyer's woad, plants were subjected in turn to: oxidative stress, cold stress, dehydration stress, osmotic stress and salinity stress. Each stress category was tested at four different levels to determine whether there was a dose-dependent effect on infection rates. Control plants were not subjected to any abiotic stress, but were exposed to only the rust pathogen. Each level of abiotic stress treatment and the corresponding control treatments consisted of 8-10 replicate plants depending on the experiment that were arranged in a completely randomized design. Following stress treatments, controls and inoculated plants were watered on a regular basis to ensure that the treatments in each category did not undergo further stress that might affect the outcome of the study. Within group comparisons were conducted using Welch's t-test of unequal variances (Welch, 1947) to explore the significance of the effect of specific stresses on rust infection compared to the control. $P<0.05$ was considered statistically significant.

Oxidative stress: Dyer's woad was exposed to different levels of paraquat (methyl viologen; Sigma, St. Louis, MO) to assess the effect of oxidation stress on infection (Mittler *et al*., 1999; Vranova *et al*., 2002). Dyer's woad was sprayed with 10, 20, 50, or 100 µM paraquat in a 0.1% Tween 20 solution until complete coverage of the plant. Control plants were treated with 0.1% Tween 20 solution. After plants were sprayed with different levels of paraquat, they were incubated for 2 h in the greenhouse before inoculation with the rust pathogen. After inoculation, the plants were maintained in the greenhouse until being assessed for infection rates.

Salinity stress: The effect of salinity stress on rust infections of dyer's woad was examined by watering the plants twice with sodium chloride solutions at different concentrations (50, 100, 200, or 300 mM) to create varying stress levels. To ensure that the plants were under salinity stress from the time of basidiospore germination to intercellular mycelial growth in the host, each treated plant was watered with 100 mL of the appropriate saline solution 24 h prior to inoculation and then again 2 h prior to inoculation with 100 mL of saline solution. After treatment, the plants were allowed to recover by watering 24 h after inoculation and grown under greenhouse conditions until tested for infection.

Osmotic stress: Plants were watered with 25, 50, 100, or 200 mM mannitol to study the effect of osmotic stress on infection. All plants within each treatment

were watered twice with 100 mL of the different mannitol solutions. The first treatment was done 24 h prior to inoculation so that the plants would be stressed at the time of inoculation while the second application was done 2 h prior to inoculation to ensure continued stress at the time of basidiospore germination and fungal growth within the host. All plants were watered 24 h after inoculation and infection rates were determined after three weeks growth in the greenhouse.

Dehydration stress: The effect of dehydration stress on infection rates was examined by withholding water for a period of 3-7 days. Plants that were not watered for more than 7 days did not recover. Control plants were watered on a daily basis. In all treatments involving dehydration stress, inoculation was carried out 24 h before the end of the stress period to ensure that the plants continued to face stress during the infection period. After treatment, all plants were grown under greenhouse conditions and watered regularly until the inoculated leaves were assayed for infection using PCR.

Cold stress: The effect of cold stress on infection rates was studied by subjecting dyer's woad plants to 4°C for 6, 12, 24, or 48 h. After the plants were subjected to cold stress, they were immediately transferred to the dew chamber and inoculated without allowing for recovery. Control plants were left in the greenhouse until they were used for inoculations. All plants were transferred to the greenhouse after inoculation and incubated until infection rates were ascertained by PCR.

Detection of rust infections in asymptomatic plant tissue: Since infected dyer's woad plants are initially asymptomatic, rust infections were determined using PCR to detect fungal DNA. Previous studies (Kropp *et al*., 1996) show that, after inoculation, the rust fungus moves at the rate of 0.25 cm week^{-1} in the dyer's woad tissue. Since the plants in the present study were incubated for three weeks after inoculation before being sampled, the fungus would have moved at least 0.75 cm away from the point of inoculation, during that time. Thus to determine whether infections had occurred, sampling was conducted at 0.75 cm away from the point of inoculation. Infections were considered to be successful only if the fungus could be found in this zone alone; care was taken to avoid collecting tissue from the point of inoculation in order to avoid false positives from basidiospore inoculum remaining on leaf surfaces. As an added precaution, the sampled leaf tissue was rinsed vigorously for 90 sec under running cold water before DNA was extracted to remove any potential remaining inoculum on the plant surface.

Extraction of DNA and PCR was done by using the Extract-N-Amp Plant PCR kit (Sigma, St Louis, MO). Leaf tissue disks (one-half centimeter in diameter) were cut from the leaves using a standard paper punch and DNA was extracted according to kit directions. PCR was carried out using the Extract-N-Amp PCR reaction mix containing either two universal primers, F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and R635 (5'-GGGTCCGTGTTTCAAGACGG-3') to amplify a 620 base pair portion of the large ribosomal subunit (Kropp *et al*., 1995), or a rust-selective primer set, F63 and Rust1 (5-GCTTACTGCCTTCCTCAATC-3'). The universal primers were used as a check to ensure the presence of amplifiable DNA in the leaf samples while the rust-selective primers were used to confirm the presence of rust infections in the leaf samples. The amplification parameters used were those of Kropp *et al*. (1997; 1997).

RESULTS

Effect of oxidative stress on infection: Plants sprayed with 100 μM of paraquat showed 55% infection and were significantly less infected than the untreated control plants that were 100% infected (Fig. 1). No significant differences were found for infection rates for plants exposed to the other levels (10, 20 and 50 μM) of paraquat. Plants sprayed with 10 and 20 μM of paraquat were 100% infected while the rate for plants sprayed with 50 μM of paraquat was 80% (Fig. 1).

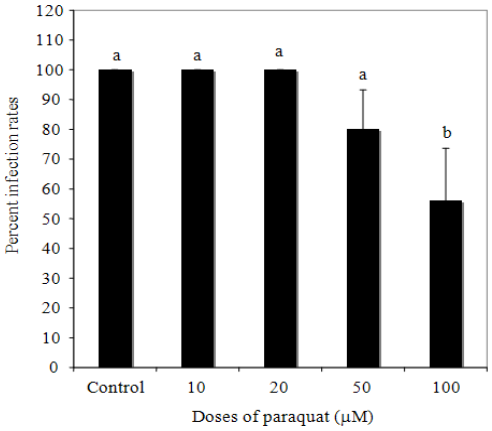

Fig. 1: Effect of oxidative stress on rust infection of dyer's woad. Control plants were sprayed with water and infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means with different letters (a, b) are statistically different using Welch's t test ($p<0.05$)

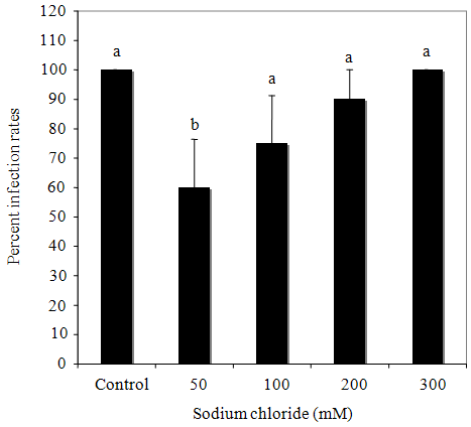

Fig. 2: Effect of salinity stress on rust infection of dyer's woad. Control plants were watered regularly without sodium chloride. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means with different letters (a, b) are statistically different using Welch's t test ($p<0.05$)

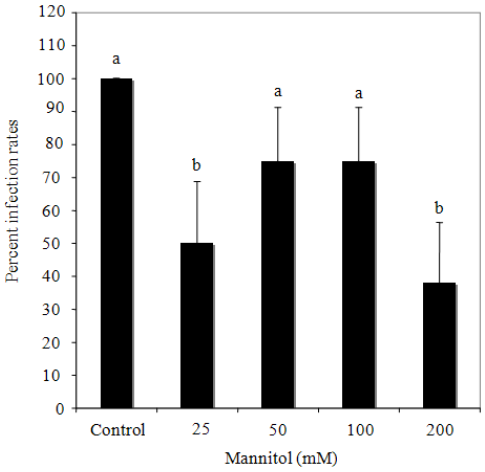

Fig. 3: Effect of osmotic stress on rust infection of dyer's woad. Control plants were watered regularly without mannitol. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means with different letters (a, b) are statistically different using Welch's t test ($p<0.05$)

Effect of salinity stress on infection: Plants watered with 50 mM of sodium chloride had significantly lower infection rates than the control plants (Fig. 2). However, no statistically significant differences in infection were observed between controls and the plants exposed to the other three levels of sodium chloride.

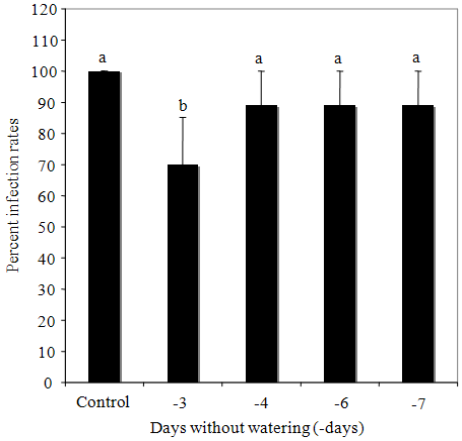

Fig. 4: Effect of dehydration stress on rust infection of dyer's woad. Control plants were watered regularly. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means with different letters (a, b) are statistically different with Welch's t test ($p<0.05$)

Of the plants treated with 100 mM sodium chloride, 75% were infected, while plants watered with 200 and 300 mM were 90 and 100% infected respectively (Fig. 2).

Effect of osmotic stress on infection: Plants watered with 25 or with 200 mM mannitol showed significantly less infection than the control plants (Fig. 3). No statistically significant differences were found between infection levels of control plants and plants exposed to 50 and 100 mM mannitol. The infection level for plants watered with 50 and 100 mM of mannitol was 75% while controls were 100% infected (Fig. 3).

Effect of dehydration stress on infection: The within-group differences between different dehydration stress treatments were examined using Welch's t-tests (Fig. 4). Infection was significantly less for plants that had water withheld for 3 days than for unstressed controls that were all infected. However, no statistically significant differences were found for infection rates of control plants and plants that were not watered for 4-7 days, which were all 89% infected.

Effect of cold stress on infection: Plants exposed to 4°C for 6 h were 70% infected and significantly different from untreated controls (Fig. 5). No statistically significant differences were found between the control plants and plants exposed to cold for the three longer periods of time. Plants exposed for 12, 24 and 48 h were 88% infected while controls were all infected.

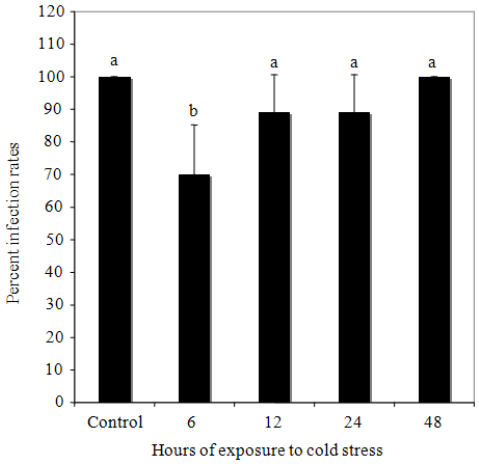

Fig. 5: Effect of cold stress on rust infection of dyer's woad. Control plants maintained in the greenhouse, were not subjected to cold stress of any kind. Infection was detected by PCR using rust specific primers three weeks after rust inoculation. Means with different letters (a, b) are statistically different using Welch's t test ($p<0.05$)

DISCUSSION

In the natural environment, plants are subjected to various biotic and abiotic stresses. The perception of stress and the relay of such information within the plant are brought about by various signal transduction pathways. Increasing evidence suggests that, when subjected to multiple stresses at the same time, there is a considerable amount of crosstalk between stress signaling pathways (Fujita *et al*., 2006; Narusaka *et al*., 2004). Some of the key players involved in the crosstalk between biotic and abiotic stress signaling include phytohormones and Reactive Oxygen Species (ROS).

The production of ROS is vital in key processes such as programmed cell death and hypersensitive responses that curtail pathogen growth (Levine *et al*., 1994). Application of paraquat is known to create oxidative stress leading to the accumulation of ROS in the apoplast (Mittler *et al*., 1999; Vranova *et al*., 2002). In our study, application of low doses of paraquat did not affect infection rates (Fig. 1). However, as the doses of paraquat increased, infection rates decreased accordingly. This is presumably because the accumulation of ROS led to the activation of the plant defense responses that in turn lowered infection rates.

In contrast, we also observed that infection levels were not significantly lower than those for the control after exposing dyer's woad to moderate or severe levels of dehydration stress, salinity stress, osmotic stress and low temperature stress (Fig. 2-5). This is potentially explained by the role of phytohormones in the biotic-abiotic stress interaction. Plants facing salinity stress, dehydration stress, or low temperature stress are known to have elevated levels of abscisic acid (ABA) (Chen *et al*., 1983; Downton and Loveys, 1981; Zeevart and Creelman, 1988). The phytohormone ABA has been shown to play a key role in stress response (Chandler and Robertson, 1994; Fujita *et al*., 2006; Giraudat *et al*., 1994). On the other hand, a different set of phytohormones (salicylic acid, jasmonic acid and ethylene) are involved in the defensive responses to challenge by a pathogen (Glazebrook, 2005). Several studies have shown that ABA and defense response pathways act antagonistically to each other (Anderson *et al*., 2004; Mauch-Mani and Mauch, 2005). For example, a study conducted by Audenaert *et al*. (2002) using ABA-deficient tomato mutant *sitiens*, found that salicylic acid-mediated resistance to the pathogen *Botrytis cinerea* was abolished upon the exogenous application of ABA suggesting that elevated levels of ABA could inhibit SA-mediated responses in certain plants. A different study using the same ABA-deficient mutant found that it was more resistant to *Pseudomonas syringae* than wild-type plants; this mechanism was also SA-mediated (Thaler and Bostock, 2004). Additional studies have demonstrated the antagonistic interactions between ABA and jasmonate/ethylene signaling wherein ABA-deficient mutants displayed greater resistance to the pathogens, however this resistance was abolished on ABA application (Anderson *et al*., 2004; Beaudoin *et al*., 2000; Ghassemian *et al*., 2000).

These studies concluded that when the plants faced abiotic and biotic stresses at the same time, the ABA-mediated stress response took precedence over the SA-mediated defense response. Based on this, it is not unreasonable to postulate that when dyer's woad faces moderate to severe abiotic stress, ABA levels rise and suppress SA-mediated defense responses that would make the plant even more vulnerable to the rust fungus.

Another interesting finding of our study was that exposure of dyer's woad to mild, sub-lethal levels of abiotic stress appeared to initiate cross-tolerance to the rust pathogen. Challenge with mild stress appeared to tilt the balance in favor of the host and this was reflected in lowered infection rates (Fig. 2-5). This has been reported in few other instances. The reasons for the lowered infection rates are not well known, although, this has been observed in a few other

instances and one can develop hypothetical explanations for this based on literature reports. For example, Mittra *et al*. (2004) found that wheat exposed to mild doses of the heavy metal cadmium developed resistance to subsequent infections by *Fusarium oxysporum*. In another study, winter wheat subjected to cold acclimation developed resistance to pink snow mould, caused by *Microdochium nivale* (Kuwabara *et al*., 2002). The data from this study showed that cold acclimation leads to the accumulation of thaumatin-like proteins (i.e., pathogenesis-related proteins) in the apoplast that contributes to resistance to *M. nivale*. Studies have also shown that the exposure of tobacco plants to sub-lethal levels of ultraviolet and ozone stimulated the accumulation of salicylic acid and pathogenesis-related proteins that conferred resistance to the tobacco mosaic virus (Yalpani *et al*., 1994).

CONCLUSION

The phenomenon of cross-tolerance is important in agriculture because it helps crop breeders develop varieties that show tolerance to multiple stresses (Bowler and Fluhr, 2000). However, cross-tolerance is potentially undesirable when mycoherbicides are being developed because much of the study in selecting biocontrol agents is done under carefully controlled conditions. Yet, in the natural environment, the target plants are constantly subjected to abiotic stresses. Thus, the development of cross-tolerance to a potential biocontrol agent could reduce the efficacy of biocontrol. It could also help explain why biological control agents that work well in the greenhouse sometimes do less well in the field. This factor should be taken into consideration during the development and release of potential biocontrol agents for noxious weeds.

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