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*Department of Forest Science, Oregon State University, Corvallis, OR, USA*

# Energy Dissipation and Photoinhibition in Douglas-Fir Needles with a Fungal-Mediated Reduction in Photosynthetic Rates

Daniel K. Manter

Authors' address: USDA Forest Service, PNW Research Station, 3200 Jefferson Way, Corvallis, OR 97331, USA (correspondence to Daniel K. Manter. E-mail: dmanter@fs.fed.us)

With 5 figures

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## Abstract

The dissipation of absorbed light and potential for photooxidative damage was explored in Douglas-fir (*Pseudotsuga menziesii* ) seedlings with and without *Phaeocryptopus gaeumannii* infection. The presence of *P. gaeumannii* significantly reduced net $CO_2$ assimilation rates from ca. 6 $\mu$mol/m$^2$/s to 1.5 $\mu$mol/m$^2$/s, without any significant impact on chloroplast pigments. The partitioning of absorbed light-energy to photochemistry or thermal dissipation was determined from chlorophyll fluorescence measurements. Maximum thermal dissipation for both control and infected needles was ca. 80%, consistent with the similar xanthophyll pool sizes in the two treatments. At high photosynthetic photon flux density (PPFD), when thermal dissipation was maximized, the lower photochemical utilization in infected needles resulted in greater amounts of excess absorbed light (ca. 20 and 10% for the infected and control needles, respectively). A second experiment, monitoring changes in photosystem II (PSII) efficiency ($F_v/F_m$) in response to a 1 h high light treatment (PPFD = 2000 $\mu$mol/m$^2$/s) also suggests that infected needles absorb greater amounts of excess light. In this experiment, declines in $F_v/F_m$ were 1.5 times greater in infected needles, despite the similar xanthophyll pool sizes. Furthermore, increases in minimum fluorescence (178 and 122% of initial values for the infected and control needles, respectively) suggest that the reduction in PSII efficiency is largely attributable to photooxidative damage. Finally, reductions in PSII efficiency under high light conditions provide a plausible explanation for the greater pathogenicity (e.g. premature needle abscission) of *P. gaeumannii* in sun-exposed foliage.

## Introduction

In healthy plants light absorption may exceed photosynthetic utilization resulting in an excess of absorbed light (Demmig-Adams and Adams, 1996; Demmig-Adams et al., 1996), which unless thermally dissipated can lead to photooxidative damage. Thermal dissipation results in a reduced efficiency of PSII reaction centres, i.e. reduction in $F_v/F_m$, of which, a major component is related to the presence of the de-epoxidized xanthophyll pigments (zeaxanthin and antheraxanthin, Z and A). The exact mechanism of Z and A energy dissipation from PSII centers is unclear (cf. Demmig-Adams and Adams, 1992; Horton and Ruban, 1992); however, a close correlation between thermal dissipation and the levels of Z and A has been reported for several plant species (Demmig-Adams and Adams, 1996). Furthermore, the maximum thermal dissipation capacity has an upper limitation based on the size of the xanthophyll pool [i.e. xanthophylls per unit chlorophyll (Chl), Demmig-Adams and Adams, 1994].

At high light, when thermal dissipation may be maximized (Demmig-Adams and Adams, 1996), any reduction in photochemical quenching – without a corresponding decline in total light absorption – will result in greater amounts of excess absorbed light. One situation where this may occur is at low temperatures, as the Calvin cycle is reduced to a greater extent than electron transport (Baker, 1994). Higher antioxidant pools during winter (Logan et al., 1998) suggest the presence of photooxidative conditions (triplet exited Chl, singlet oxygen formation, etc.) at low temperatures; however, because of an increased thermal dissipation capacity, photooxidation is less than expected based on summer measurements. For example, the size of the xanthophyll pool increases during winter due to both an increase in xanthophylls and a decline in chlorophyll (Ottander et al., 1995; Logan et al., 1998).

The alteration of needle physiology by pathogenic fungi may represent another scenario where the total

 

dissipation of absorbed light-energy by thermal dissipation and photochemical quenching is reduced, resulting in greater quantities of excess absorbed light and photooxidative conditions. For example, the biotrophic foliar pathogen of Douglas-fir [*Psuedotsuga menziesii* (Mirb.) Franco], *Phaeocryptopus gaeumannii* (Rhode) Petrak, causes a significant decline in photosynthetic rates by an apparent occlusion of needle stomata (i.e. fungal fruiting bodies emerge from stomatal cavities reducing gas exchange) without any apparent changes in intrinsic PSII efficiency (Manter et al., 2000). Assuming that light absorption and thermal dissipation capacity are unchanged, this decline in photosynthesis (i.e. photochemical quenching) would result in excess absorbed light and potential photooxidative damage in infected needles. The presence of photooxidative damage in *P. gaeumannii*-infected needles is unknown; however, symptom development (i.e. needle abscission) is exacerbated in sun-exposed foliage (Manter, 2001), or the conditions were the balance between light absorption and dissipation may be most critical, suggesting that photooxidation may influence *P. gaeumannii* pathogenicity and symptom development.

The purpose of this study was to determine if photooxidative damage occurs in *P. gaeumannii*-infected Douglas-fir needles. The three primary objectives were to (i) determine the relative rates of photochemical and thermal dissipation, (ii) quantify the chloroplast pigments and (iii) determine changes in PSII efficiency under high light exposures in seedlings with and without *P. gaeumannii* infection.

## Materials and Methods

### Plant material

All plant material consisted of potted 2-year-old Douglas-fir (*P. menziesii* ) seedlings (open-pollinated Oregon north-coast seed source, Starker Forests, Corvallis, OR, USA) with (infected) and without (control) *P. gaeumannii* infection. Infected seedlings were inoculated by placing seedlings under the canopy of a heavily *P. gaeumannii*-infected Douglas-fir plantation (Salal plot, Hansen et al., 2000) for 4 weeks in June 2001. Except during the inoculation period, all seedlings were stored in a cold frame at the Oregon State University Botany Farm (Corvallis, OR, USA), fertilized with Osmocote Pro 18-8-8 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) and irrigated as needed. All subsequent measurements were conducted on current-year needles in February 2002.

### Disease assessments

The colonization of *P. gaeumannii* is reported as the per cent of needle stomata with visible fruiting bodies (pseudothecia density), based on the procedure outlined in Manter et al. (2000). Pseudothecia density was quantified from 10-needle samples from randomly selected seedlings and reported values are the average of six control and six infected seedlings.

### Pigment analysis

Ten needles were removed from randomly selected infected and control seedlings (three per treatment) at predawn, and immediately frozen in 2 ml micro-centrifuge tubes immersed in liquid nitrogen, where they were stored until extraction. Pigments were extracted by homogenizing (2 min at 4200 rpm ) frozen needles with a mini-bead beater (Biospec Products, Bartlesville, OK, USA). After homogenization, samples were suspended in 1 ml 85% acetone, incubated on ice for 15 min, centrifuged at 12000 $\times \boldsymbol{g}$ for 5 min and the supernatant decanted. This procedure was repeated a second time, and the combined supernatants were filtered (0.45 $\mu$m filter) and stored at 40°C until HPLC analysis. Quantification of pigments by HPLC followed the conditions outlined in Thayer and Björkamn (1990). All pigments [chlorophyll (Chl), violaxanthin (V), antheraxanthin (A), zeaxanthin (Z), neoxanthin (Neo), lutein (Lut) and $\beta$-carotene ($\beta$-car)] are reported per unit needle DW using the conversion factors of Thayer and Björkman (1990) and dry weights determined from an adjacent 10-needle sample, dried at 70°C for 72 h.

### Gas exchange and fluorescence measurements

Gas exchange and chlorophyll fluorescence of attached needles were measured using a LiCor 6400 (LiCor Inc., Lincoln, NE, USA) on randomly selected infected and control seedlings (six per treatment). Following an overnight dark-adaptation period, the intrinsic PSII efficiency in the absence of thermal dissipation ($F_v/F_m$ @ PPFD = 0 $\mu$mol/m$^2$/s) was measured for each selected seedling. In addition, gas exchange and fluorescence light response curves (PPFD = 20, 50, 100, 200, 500, 750, 1000, 1500 and 2000 $\mu$mol/m$^2$/s) were measured, with a 15 min equilibration period at each set point.

Chlorophyll fluorescence parameters measured included maximum ($F_m$) and minimum ($F_o$) fluorescence of dark-adapted needles; and steady-state ($F_s$), maximum ($F'_m$) and minimum ($F'_o$) fluorescence of light-exposed needles. The efficiency of open PSII centres for dark ($F_v/F_m$) and light-adapted ($F'_v/F'_m$) needles, and the potential rate of electron transport (ETR or $0.83 \cdot \text{PPFD} \cdot F'_v/F'_m \cdot qP$) were computed based on the equations of Gentry et al. (1989), where $F_v$ is $F_m - F_o$, $F'_v$ is $F'_m - F'_o$, and $qP$ is $(F'_m - F_s)/(F'_m - F'_o)$. The fraction of light absorbed by PSII centers that is thermally dissipated [D or $1 - (F'_v/F'_m)$], utilized by photochemistry (P or $F'_v/F'_m \cdot qP$) or potentially going into singlet oxygen formation [O or $1 - (D + P)$] was based on the equations of Demmig-Adams et al. (1996) and the potential rate of singlet oxygen formation was calculated as $0.83 \cdot \text{PPFD} \cdot O$. The 0.83 coefficient for the ETR and O calculations corrects for light absorbency of a typical $C_3$ plant (as suggested in Harley et al., 1992).

The response of the ratio of variable to maximum fluorescence ($F_v/F_m$) to a high light treatment was also determined on a second random sample of six control and six infected seedlings. Following an overnight

Table 1
Presence of *Phaeocryptopus gaeumannii* (PD or pseudothecia density), intrinsic PSII efficiency in the absence of thermal dissipation ($F_v/F_m$), chlorophyll ($\mu$mol/g$^{-1}$ DW) and xanthophyll pigments (nmol/g$^{-1}$ DW) concentrations in current-year-old needles from seedlings with and without *P. gaeumannii* infection. Reported values are the arithmetic mean and individual standard error (n = 3). Means with different letters are significantly different (ANOVA, $p < 0.05$).

| TRT | PD (%) | $F_v/F_m$ | Chl | V + A + Z | Neo | Lut | $\beta$-Car |
|---|---|---|---|---|---|---|---|
| Control | 0.0 (0.0)[a] | 0.803 (0.007)[a] | 2.86 (0.17)[a] | 485.2 (49.2)[a] | 139.2 (11.9)[a] | 753.2 (65.3)[a] | 691.2 (78.2)[a] |
| Infected | 30.3 (2.7)[b] | 0.796 (0.006)[a] | 2.79 (0.13)[a] | 511.9 (51.2)[a] | 132.3 (12.2)[a] | 722.1 (57.1)[a] | 682.9 (74.8)[a] |

dark-adaptation period, seedlings were exposed to a high light treatment (2000 $\mu$mol/m$^2$/s for 1 h followed by a recovery period of 100 $\mu$mol/m$^2$/s for 3 h). To prevent excessive heat build-up, seedlings were cooled with two conventional box fans. On each seedling, sample branches exposed to the target PPFD levels (±5%) were selected and tagged. At each time point, $F_v/F_m$ was measured by removing three needles from each tagged branch, affixing them to index cards (abaxial side facing-up), dark-adapting for 10 min under a black cloth, and then recording chlorophyll fluorescence.

### Statistical analysis

Data were analysed with the statistical package Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, MD, USA). Treatment differences associated with the parameters shown in Table 1 were tested by ANOVA on untransformed data. Regression analysis was used to estimate the linear relationship shown in Fig. 2. Gas exchange and fluorescence light-response curves were measured using a total of six seedlings (i.e. each exposed to the range of PPFD levels stated above) and graphical points represent the arithmetic mean and individual standard error.

## Results

Inoculated seedlings had significantly higher levels of *P. gaeumannii* colonization than control needles (30.0 ± 2.7 and 0.0%, respectively), but similar levels of predawn PSII efficiency or $F_v/F_m$ and chloroplast pigments (Table 1). Despite the insignificant changes in $F_v/F_m$ and chlorophyll content, the presence of *P. gaeumannii* resulted in approximately a three-fold reduction in net $CO_2$ assimilation ($A_{net}$, Fig. 1). Simultaneous measurements of chlorophyll fluorescence and gas exchange showed a single linear relationship between ETR and $A_{net}$ for both control and infected needles (Fig. 2), which was similar to that observed for other $C_3$ species (e.g. Cheng, 1999).

The fraction of absorbed light going into thermal dissipation (D), photochemistry (P) and potential single oxygen formation (O) is shown in Fig. 3. Because of the lower photosynthetic rates in the diseased seedlings, a smaller fraction of absorbed light was necessary to support photosynthesis in diseased needles. For

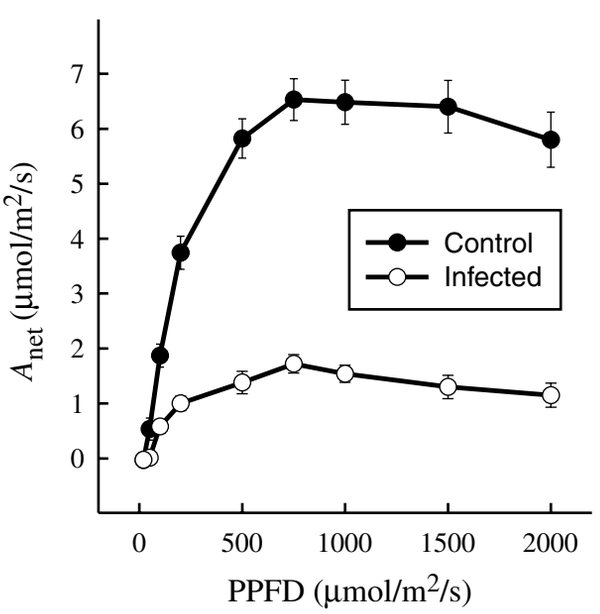


Fig. 1 Response of net $CO_2$ assimilation ($A_{net}$) to increasing irradiance. Each observation is the arithmetic mean and individual standard error for six seedlings

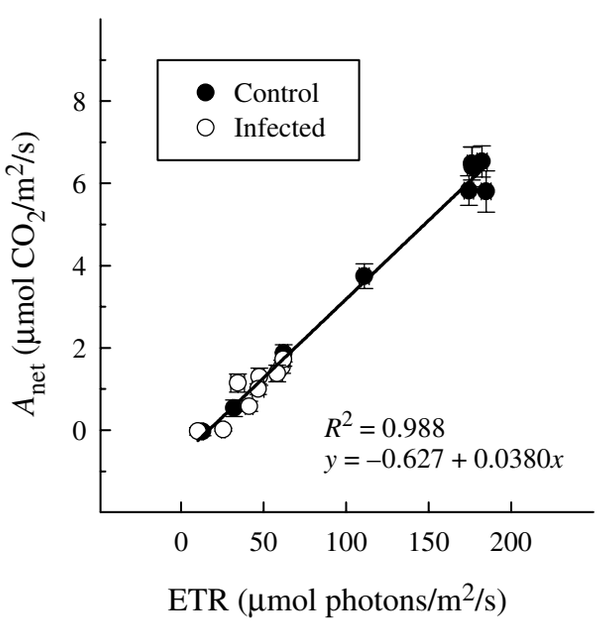


Fig. 2 Relationship between net $CO_2$ assimilation ($A_{net}$) and electron transport rate (ETR). Each observation is the arithmetic mean and individual standard error for six seedlings

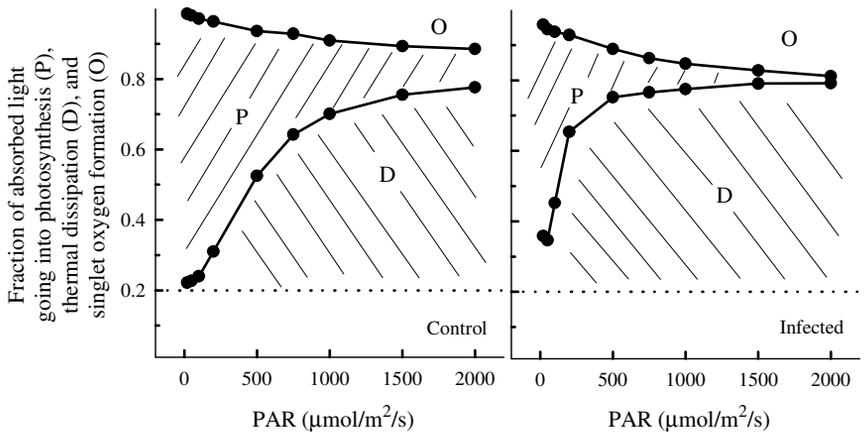


Fig. 3 Fraction of absorbed light going into thermal dissipation (D), photochemistry (P) and potential singlet oxygen formation (O). Each point is the mean and standard error of six seedlings. Each observation is the arithmetic mean and individual standard error for six seedlings, standard errors are smaller than the symbol size

example, at a PPFD value of 500 $\mu$mol/m$^2$/s, ca. 15 and 45% of the absorbed light was utilized for photochemistry for the infected and control needles, respectively. For both infected and control needles, the proportion of light thermally dissipated increased with increasing irradiance, approaching a maximum near 80%; however, the infected needles reached maximum thermal dissipation at a much lower light intensity (ca. 500 vs. 2000 $\mu$mol/m$^2$/s). Because of the apparent limit on thermal dissipation and reduced photochemical quenching, greater amounts of excess light energy were absorbed by infected foliage. Figure 4 shows the potential rate of singlet oxygen formation, and at the highest PPFD level infected foliage had rates approximately 1.5 times that of control foliage.

The recovery of available open PSII centres, upon transfer from a high- to a low-light environment, was assessed for both infected and control needles. For all needles, a logarithmic response in the rate of change in $F_v/F_m$ was observed during both the light and recovery treatments (Fig. 5). After 1 h, the number of open PSII centres declined from 80 to 60 and 50% for the control and infected needles, respectively. The recovery rates were similar for both infected and control needles; however, the control needles had fully recovered by the end of the 3-h period, whereas the infected needles had only recovered to 70%.

## Discussion

Infection by the foliar pathogen of Douglas-fir, *P. gaeumannii*, results in a significant reduction in $A_{net}$. In the current study, when fungal colonization levels reached 30% pseudothecia density, a common level of colonization in Douglas-fir forest plantations in the Oregon Coast Range (Hansen et al., 2000, Manter, 2001) $A_{net}$ was reduced three-fold without any significant impact on chlorophyll content. Although chlorosis (needle yellowing) is a common symptom in

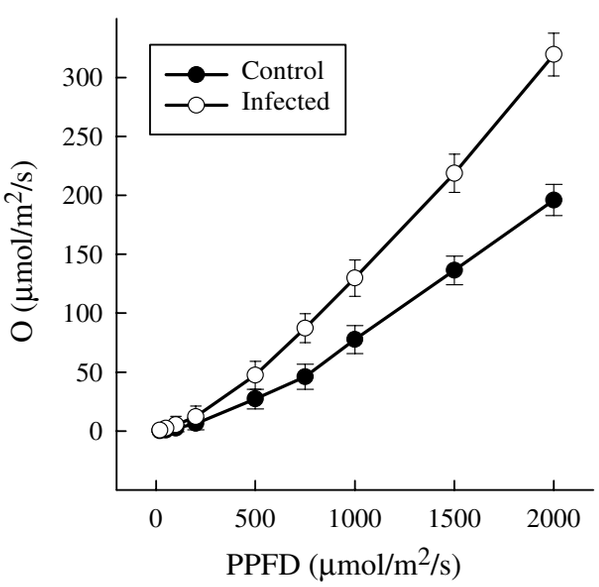


Fig. 4 Response of the potential rate of singlet oxygen formation [O or $0.83 \cdot \text{PPFD} \cdot F'_v/F'_m \cdot (1 - qP)$] to increasing irradiation. Each observation is the arithmetic mean and individual standard error for six seedlings

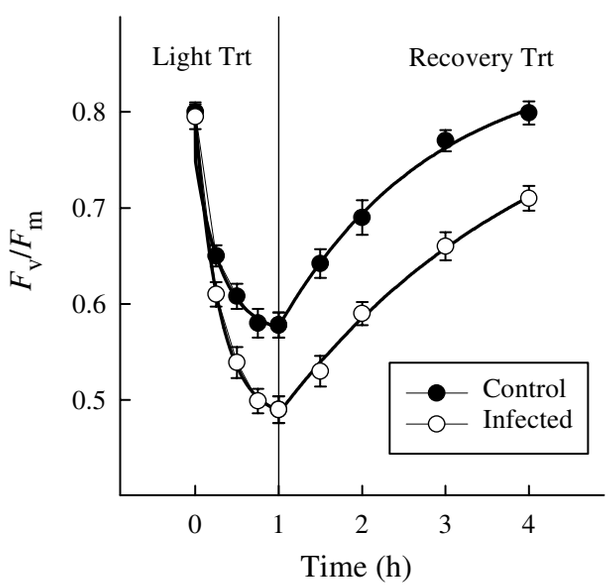


Fig. 5 Change in the intrinsic efficiency of open PSII reaction centers ($F_v/F_m$) during and after a 1-h exposure to a high light (2000 $\mu$mol/m$^2$/s) treatment. Each observation is the mean and standard error of six seedlings

*P. gaeumannii*-infected foliage (Hansen et al., 2000; Manter 2001), it is not a requirement for significant declines in $A_{net}$. Furthermore, the secondary nature of needle chlorosis is supported by a several month time-lag between fungal colonization and the onset of needle chlorosis (Manter, 2001).

The use of chlorophyll fluorescence to estimate rates of light dissipation depends upon light absorption. In healthy $C_3$ plants, foliage typically absorbs ca. 83%, of incident light (as suggested in Harley et al., 1992). However, many foliar diseases reduce light absorption by either a reduction in chlorophyll content or fungal hyphae on needle surfaces blocking light penetration (Wood et al., 1988; Coghlan and Walters, 1992; Scholes and Rolfe, 1996). While light absorption was not directly measured in this study, it appears that light absorption was not influenced by *P. gaeumannii* infection, because similar chlorophyll contents and a linear relationship between calculated ETR and $A_{net}$ were observed for both control and infected foliage.

Chlorophyll fluorescence was used to estimate the proportion of light-energy partitioned to D, P and O. The apparent maximum of D, and lower rates of P, resulted in a greater amount of excess light energy absorbed by infected needles. At the highest light intensity, O was approximately 1.5 times greater in infected needles. A mechanistic basis for reduced photosynthetic rates in *P. gaeumannii*-infected needles has previously been reported (Manter et al., 2000), and appears to be associated with the physical occlusion of stomata and needle gas exchange. D was calculated based on the suggestion of Demmig-Adams et al. (1996), not the parameters *qN* or *NPQ*, as long-term depression of $F_m$ may lead to an underestimation of D. For both infected and control needles, D reached a maximum of approximately 80%, although the earlier induction of D in infected needles resulted in maximal D at a much lower light intensity, 500 and 2000 $\mu mol/m^2/s$ for the infected and control needles, respectively. Previous studies of the role of the xanthophyll cycle in D may explain the faster induction and similar maximum levels of D in control and infected needles.

In previous studies it has been shown that D is strongly correlated with the amount of Z and A present in the xanthophyll pool (e.g. Verhoeven et al., 1996), which is dependent upon a low pH in the thylakoid lumen. The pH optimum of violaxanthin de-epoxidase, the enzyme controlling conversion of V to Z and A, is approximately 5.0 (Yamamoto, 1979). The earlier induction of D in infected needles is consistent with this mechanism, i.e. the lower photosynthetic rates of infected needles should result in a lower thylakoid lumen pH and greater de-epoxidation of V. Similarly, maximum D is related to the size of the xanthophyll pool relative to chlorophyll content (Adams and Demmig-Adams, 1994; Demmig-Adams and Adams, 1994). Thus, the similar maximum rates of D are expected, as no differences in either xanthophyll or chlorophyll contents were detected in *P. gaeumannii*-infected seedlings. In other studies, xanthophyll contents have been reported to increase under photooxidative conditions, e.g. a four-fold increase in the xanthophyll pool has been reported in Douglas-fir during winter (Adams and Demmig-Adams, 1994). Although an increase in xanthophyll pools may be possible under some conditions, the current study suggests that xanthophyll pools were already at their maximum levels in control needles and could not be increased in infected needles.

In order to determine if the excess absorbed photons result in photooxidative damage to open PSII centres, changes in $F_v/F_m$ were measured during acclimation and recovery from a high light treatment. Sustained reductions in $F_v/F_m$ have variously been attributed to photooxidative damage to PSII centres (e.g. Franklin et al., 1992) and/or sustained de-epoxidized V, particularly at low temperatures (e.g. Adams and Demmig-Adams, 1995). In the current study, the decline in $F_v/F_m$ during the high light treatment is best explained by photooxidative damage, as no differences in xanthophyll pool sizes were detected in study 1; the $F_v/F_m$ decline in infected needles was 1.5 times greater than controls, or approximately equal to the estimate of potential singlet oxygen formation; measurements were conducted at temperatures of ca. 22C; and $F_o$ values at the end of the 1 h treatment period were significantly higher in infected needles ($F_o$ values were $178 \pm 14$ and $122 \pm 12\%$ of $F_o$ values at time zero for the infected and control seedlings, respectively), indicating damage to the D1 protein of PSII (e.g. Franklin et al. 1992). Furthermore, if the recovery of $F_v/F_m$ was associated with the pH dependent removal of de-epoxidized V, and not PSII damage, then different rates of recovery would be expected as the *P. gaeumannii*-infected needles had significantly lower photosynthetic rates. Instead the similar rates of recovery are more likely due to similar rates of *de novo* synthesis of D1 protein and repair of damaged PSII reaction centers. Finally, because of the greater damage levels in infected needles, and similar rates of recovery, the number of functional PSII centres at the end of the 3-h recovery period remained significantly reduced in the infected needles.

This study shows that photooxidative damage appears to occur in Douglas-fir needles exposed to high light and is higher in needles infected with the fungal pathogen, *P. gaeumannii*. For example, maximum $A_{net}$ was observed at a moderate light intensity (PPFD = 750 $\mu mol/m^2/s$) but declined by 10 and 34% at a high light intensity (PPFD = 2000 $\mu mol/m^2/s$) for the control and infected needles, respectively. For both treatments, V-associated dissipation increases with increasing light exposure reaching a threshold near 80%; however, because of the lower photosynthetic quenching in infected needles, increasing amounts of photooxidative conditions result.

The previous observation that sun-exposure exacerbates physiological impact of *P. gaeumannii* is

consistent with the results of this study. Direct observation showed that $A_{net}$ was maximized at moderate light intensities and declined as light intensity and photooxidative conditions increased. Upon return to a low light intensity, PSII efficiency in damaged needles can recover and return to predamage levels. The recovery is best explained by the *de novo* synthesis of D1 protein. Therefore, the combined effects of reduced PSII efficiency and the respiratory costs of PSII repair appear to influence *P. gaeumannii* pathogenicity and needle function and longevity.

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