1-12-2015

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Received for publication _____.

This paper is a portion of a M.S. Thesis submitted by T. Maughan.

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Abstract. Freezing temperatures are a major limitation to strawberry production in temperate regions, and protected-cultivation strategies such as the use of tunnels and row covers are used to minimize this limitation. In order to optimize management under protected cultivation, it is necessary to understand the damage thresholds for strawberry plant tissues. The effects of freezing temperatures (-3, -5, and -7 ˚C) on leaf CO_2 assimilation were evaluated on 'Chandler', 'Seascape' and 'Jewel' strawberry (*Fragaria* × *ananassa*). Growth chambers were used to expose plants to freezing temperatures under carefully defined conditions. Net assimilation was then measured on the cold-exposed leaves, after the plants had been returned to 10 ˚C. Exposure to -3 ˚C did not significantly reduce CO_2 assimilation when compared to plants maintained at 10 ˚C d/5 ˚C night. However, leaves exposed to -5 ˚C for one night had a net CO_2 assimilation rate that was 49% of the control. When leaves were first exposed to a conditioning night of -3 ˚C and then exposed to -5 ˚C the net assimilation rate was 62% of the untreated control. Repeated exposure to -5 or -7 ˚C night temperatures resulted in a further decrease in net assimilation after each successive exposure. Leaves exposed to -7 ˚C for one night had a net assimilation rate of 6% of the control. Leaves exposed to -5 ˚C or -7 ˚C did not show any recovery over a 28-d monitoring period. There was no significant difference among cultivars in the sensitivity of leaves to cold temperatures. These results indicate that protected cultivation systems should be managed to maintain strawberry leaf temperatures above -5 ˚C in order to preserve full photosynthetic activity of existing leaves which would extend the growing season of the crop.

Keywords. Cold hardiness, photosynthesis, carbon assimilation, recovery, *Fragaria* × *ananassa*

1. Introduction

Strawberries are produced in areas ranging from mild maritime to severe temperate continental climates. The plants are remarkably adaptable to a wide range of conditions and growing systems (Darrow, 1966). Despite this adaptability, temperature is a major limiting factor in production. Plant growth responds predictably to temperature. For strawberry, baseline temperature for growth is just above freezing (Galletta and Himmelrick, 1990), with growth rates increasing with temperature to an optimum of 20 to 26 ˚C (Darrow, 1966). Growth slows dramatically above the optimum temperature with higher temperatures eventually resulting in tissue necrosis (Carlen et al., 2009; Hancock, 1999).

Strawberry plants acclimate to cold conditions and can survive sub-freezing temperatures by tolerating ice formation in crown tissues. This is accomplished by water moving from within the cell to outside the cell to form extracellular ice (Hancock, 1999; Koehler et al., 2012; Warmund, 1993). Significant work has been done to assess cold temperature damage on crowns and inflorescences. Crowns have been found to be severely injured at -9 ˚C when unprotected (Galletta and Himmelrick, 1990; Nestby and Bjorgum, 1999; Warmund, 1993) and killed at about -12 ˚C when acclimated, with some variation by cultivar (Darrow, 1966). Once inflorescences begin to expand in the spring, floral organs are susceptible to damage at -1 ˚C (Hummel and Moore, 1997; Maas, 1998).

Although somewhat limited, work has also been done to assess cold temperature damage on leaves. Even in relatively cold temperate regions, leaves may remain green throughout the winter months. However, it is not known whether these leaves maintain photosynthetic activity and contribute to continued plant growth once environmental conditions improve. Research on cold temperature damage in strawberry leaves has been conducted on detached leaves or excised leaf disks. Detached leaves sustain significant

damage, as assessed by solute leakage, when exposed to temperatures between -5 and -12 ˚C (O'Neill et al., 1981; Owens et al., 2002).Working with detached leaves does not allow determination of tissue recovery from cold temperature damage. We are unaware of published reports investigating photosynthetic response of attached leaves to freezing temperatures.

The bulk of commercial strawberry production in North America occurs in mild maritime climates where temperatures rarely drop to levels that would damage leaves. However, small-scale production continues throughout North America to target the increasing demand for locally grown food. In regions with cold fall and winter temperatures and frequent spring frost events, growing strawberries under protected cultivation such as high tunnels, low tunnels, or floating row covers is becoming more common (Fernandez, 2001; Himmelrick et al., 2001; Rowley, 2010). Since protected cultivation involves actively managing temperature, understanding the critical temperature thresholds for plant injury is essential to developing optimized management strategies. Knowing the temperature at which the leaves lose photosynthetic capacity will provide guidelines in employing and managing these protected-cultivation strategies. In this study we investigated the effect of cold temperature exposure on leaf injury and subsequent recovery, as determined by photosynthetic activity.

2. Materials and Methods

2.1 *Plant production.* Cold-stored dormant plants were obtained from a commercial nursery (Lassen Canyon Nursery, Redding, CA). The cultivars 'Chandler', 'Seascape' and 'Jewel' were selected to represent adaptation to different production systems in the United States, namely California June-bearing and day neutral production systems, and Northeast production. Plants were established in 2.0 dm^3 containers of soilless media (1:1:1 peat moss, vermiculite, and perlite) and fertilized three to four times a week with a

93 water soluble 20N-10P-20K fertilizer diluted to 100 ppm. Plants were grown under
94 greenhouse conditions of 22 °C d/16 °C night, with a day length of 14 h maintained with
95 supplemental light from metal-halide lamps until five fully expanded trifoliate leaves
96 were present (typically 4 weeks), and then used for the specific experiments.
97 Inflorescences were removed upon emergence.
98 2.2 *Freezing tests.* Prior to the beginning of freeze tests, plants were acclimated for 7 d in
99 a walk-in growth chamber (EGC Plant Growth Chamber; Chagrin, OH) at 10 °C d/5 °C
100 night temperatures, with a light period of 9 h, at a light intensity of 200 to 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$.
101 Once acclimated, individual plants were selected for uniformity and transferred to
102 an environmental test chamber (Tenney Model TUJR, Winona, MN) for exposure to one
103 of the freezing regimes. The test chamber's performance was verified using
104 thermocouples connected to a CR 1000 data logger (Campbell Scientific, Logan, UT).
105 The freezing cycle was programmed to simulate a high tunnel during a cold night in the
106 winter, where temperatures regularly fall below 0 °C (Maughan, 2013). Briefly, as lights
107 turned off in the growth chamber, a selected plant was moved to the environmental test
108 chamber. Pots were placed in an insulated box to prevent freezing of the roots and crown
109 during the freezing cycle. Air temperature was then held at 5 °C for 4.5 h, and then
110 slowly decreased to the target freezing temperature over 3.5 h. Once the target freezing
111 temperature was reached, it was held for 4 h, and then the chamber temperature gradually
112 increased to 5 °C over a 3-h period. The plant was then returned to the growth chamber,
113 where leaf injury was determined based on net CO_2 assimilation rate (*A*), using a portable
114 infrared gas analyzer (LI-6400, Li-Cor; Lincoln, NE) equipped with a LED supplemental
115 light head that supplied 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light. Injury assessment was carried out on the
116 youngest fully expanded leaf and data recorded continuously for 4 h. The controls were

117 untreated plants of the same developmental stage, kept in the growth chamber at a
118 constant 10 °C d/ 5 °C night temperature regime.
119 2.3 *Temperature step-down.* Selected plants from each cultivar (‘Chandler’, ‘Seascape’
120 and ‘Jewel’) were exposed to successively lower temperatures (-3, -5, -7, -9, and -11 °C)
121 in the environmental test chamber on five consecutive nights. Each morning the plant was
122 moved back to the growth chamber and leaf gas exchange was monitored for 4 h to
123 determine *A*. The experiment was repeated on four replicate plants of each cultivar.
124 2.4 *Repeated freeze.* Acclimated ‘Chandler’ plants were subjected to the same target
125 temperature, (-3, -5, or -7 °C) for three consecutive nights, and *A* monitored between
126 freeze cycles for 4 h immediately upon removal from the test chamber. The -5 and -7 °C
127 trials were replicated five times and the -3 °C trial was replicated twice.
128 2.5‘*Conditioned’ repeat freeze.* Acclimated ‘Chandler’ plants were subjected to a
129 conditioning night of -3 °C, followed by three consecutive nights of -5 °C using the
130 methods described above. Gas exchange was monitored for 4 h periods in the morning
131 between each freezing cycle. This trial was replicated three times. In a second trial that
132 was also replicated 3 times, ‘Chandler’ and ‘Seascape’ plants were conditioned for one
133 night of -3 °C, followed by six consecutive nights of -5 °C.
134 2.6 *Recovery.* On four consecutive nights, two acclimated plants were exposed to either -
135 5 or -7 °C as described above, and then returned to the growth chamber. After the fourth
136 night, leaf *A* was measured every 30 s for 15 min on the youngest fully expanded leaf and
137 the second-oldest leaf on each of these plants. Measurements were repeated every 4 d
138 until 28 d after initial exposure. Measured leaves were tagged to ensure repeated
139 measurement on the same leaf. This trial was replicated four times.
140 2.7 *Field-grown comparison.* Fall-planted ‘Chandler’ and ‘Seascape’ plants from the
141 Greenville Research Farm in North Logan, UT (41.735 N latitude and 1455 m elevation)

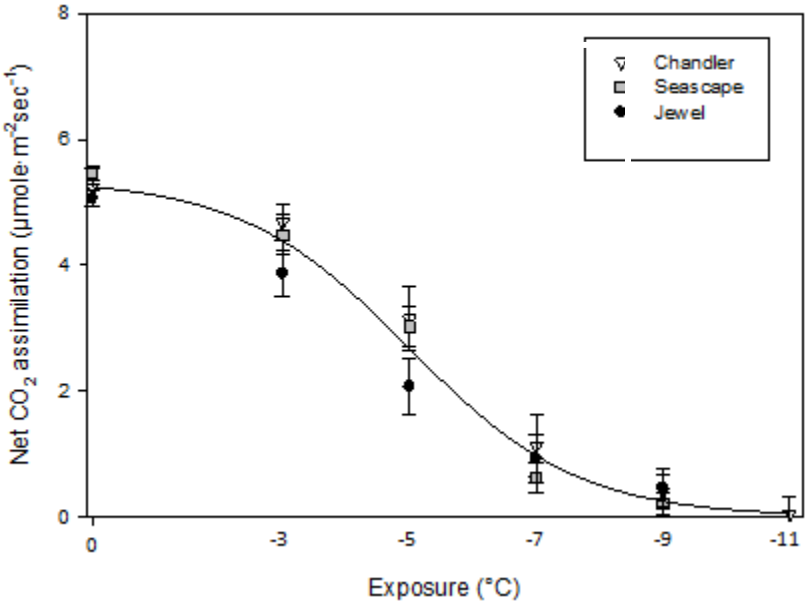
were dug on 1 March, 2013, just as they were breaking winter dormancy. Two replicate plants with overwintering leaves still intact were removed from each treatment and transplanted into 2.0 dm^3 pots. Plants were from another experiment and grown in three different treatments, an unprotected outdoor field, under high tunnel protection, or under a low tunnel within a high tunnel (Maughan, 2013). Potted plants were moved to the 10 °C d/ 5 °C night growth chamber (11 h day/ 13 h night, mimicking spring conditions), and *A* was measured on over-wintering leaves approximately 3 h after being brought into the growth chamber.

2.8 *Statistical analysis.* Data were subjected to analysis of variance (ANOVA) by standard procedures using the PROC REGWQ in SAS (version 9.3, SAS Institute, Cary, NC). Each series of experiments were analyzed as completely randomized designs. The step-down trial was analyzed using a non-linear regression to determine the LT_{50} (temperature resulting in 50% loss of *A* activity). A sigmoid 3 parameter curve was fit to the data (f = a/(1+exp(-(x-x0))/b) where a = max value, b = slope at x0 and x0 = LT_{50}, using Sigma Plot (Version 10.0, Systat Software, San Jose, CA).The assimilation recovery trial was analyzed as a repeated measures design using orthogonal contrast statements in PROC GLM. The cultivar comparison experiments were analyzed as a cultivar by temperature factorial. Means separation was by Tukey-Kramer at the 0.05 level of significance.

3. Results

3.1 *Temperature step-down.* Strawberry leaves were exposed to incrementally lower temperatures for five consecutive nights, with *A* measured the day after each exposure. Plants exposed to -3 °C for 4 h had *A* rates that were not significantly different from control plants. As plants were exposed to colder temperatures, there was a significant reduction in *A* with each successively colder temperature (Fig. 1). There was no

167 difference in *A* response among the three cultivars tested (Chandler, Seascape, and Jewel;
168 $P = 0.11$). Nonlinear regression with data combined from all cultivars predicted an LT_{50}
169 of -5.3 °C. The predicted LT_{50} for 'Chandler', 'Seascape' and 'Jewel' was -5.80 ±0.33
170 °C, -5.45 ±0.35 °C and -5.07 ±0.16 °C, respectively.
171



172

173 Figure 1. Extinction curve showing the effect of exposure to progressively colder
174 temperatures on net CO_2 assimilation. Symbols represent mean for individual
175 cultivars ± standard error (N=4).

176 3.2 *Repeat freeze.* Leaves of the cultivar 'Chandler' exposed to three consecutive cycles
177 of -3 °C had the same leaf *A* as untreated controls. Leaves exposed to consecutive nights
178 of -5 °C had *A* rates of 49%, 26% and 10%, respectively, which was a statistically
179 significant reduction in *A* after each successive night. Plants exposed to -7 °C had *A* rates
180 that were not significantly different from zero after a single night exposure (data not
181 shown).

3.3 '*Conditioned' repeat freeze.* Interestingly, 'Chandler' plants exposed to a single night of -5 °C in the repeat freeze experiment showed lower leaf *A* than plants first exposed to -3 °C, then exposed to -5 °C the following night as seen in the step-down experiment. Expressed as a percent of the untreated control, *A* was 49% after a single night exposure to -5 °C, compared to 89% of control after -3°C and then -5 °C (Fig. 1). Similarly, *A* was reduced more by one night of -7 °C (A; 6% of control) than when exposed to -7 °C (A; 62% of control) in the step-down study. These results suggest that previous exposure to sub-zero temperatures improves subsequent cold temperature tolerance.

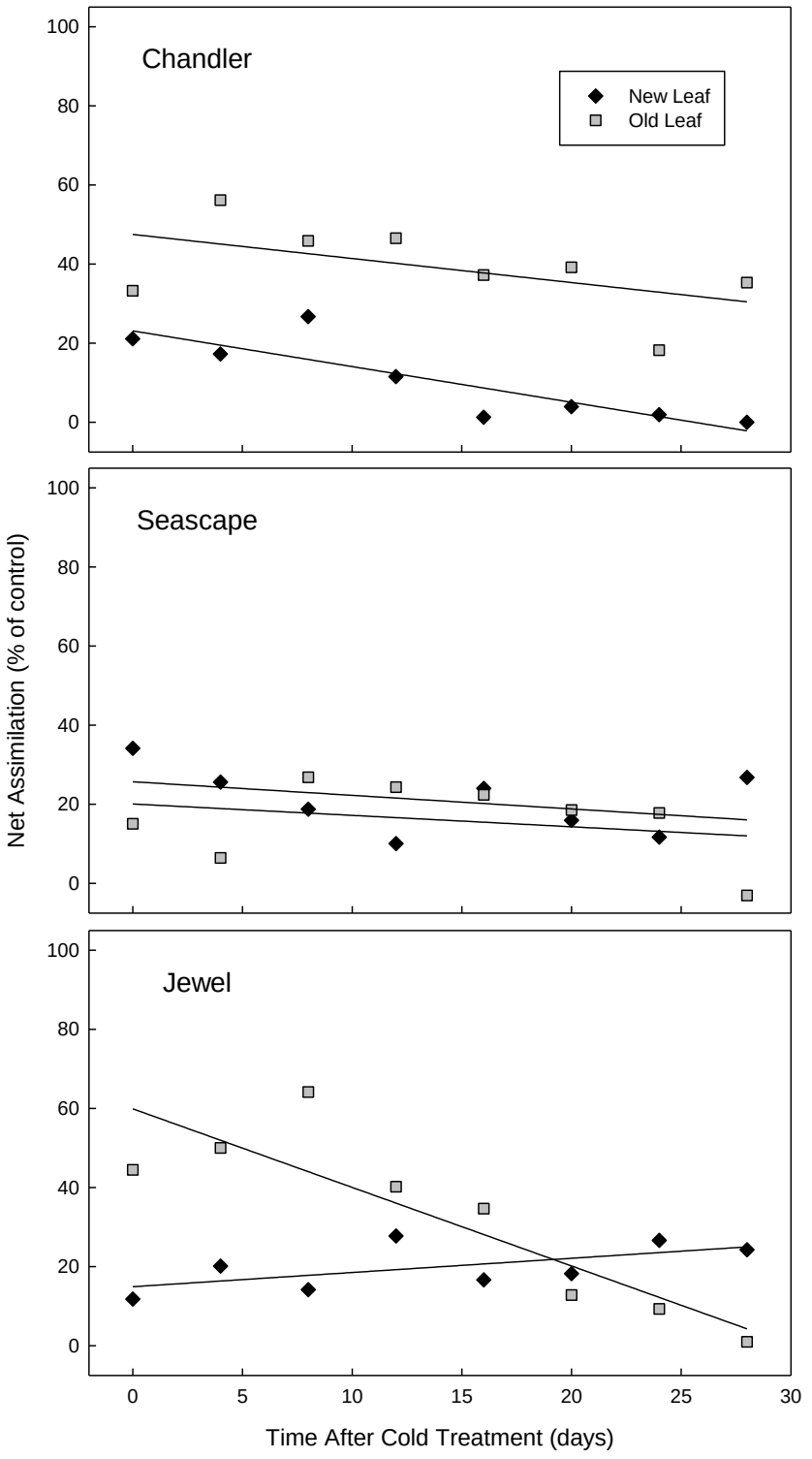
To test this hypothesis, plants were exposed to a single night of -3°C followed by three nights of -5 °C and compared to plants that received three nights of -5 °C without the -3 °C conditioning (Table 1). Conditioning followed by a single night of -5°C resulted in a 38% reduction in *A*, which was significantly different from the 51% reduction in the non-conditioned plants. However, *A* capacity continued to decrease linearly with each successive night of cold exposure in both conditioned and non-conditioned plants. For non-conditioned plants, *A* rate after day 3 was significantly different from *A* rate after day 1 ($P < 0.001$). Although the *A* rates also trended downward for the conditioned plants, differences between the first and third exposure were not statistically significant at $P < 0.05$.

Table 1. The effect of a single conditioning night at -3°C on leaf photosynthetic activity of 'Chandler' strawberry over three nights of -5°C. Values are percent of control plants kept at 10 °C d/ 5 °C night.

Conditioning	Day		
	1	2	3
	(% of control)		
None	49a[a]	27a	10a
-3 °C	62b	46b	42b

[a]Values within a column followed by the same letter are not significant at $P \leq 0.05$.

3.4 *Recovery over time.* Due to the level of damage observed from exposure to -5 and -7 °C, experiments were conducted to determine the ability of leaves to recover from a single exposure to -5 °C. For all three cultivars ('Chandler', 'Seascape' and 'Jewel'), leaves exposed to -5 °C sustained less damage, as measured by *A* capacity, than those exposed to -7 °C. Although leaf *A* was monitored for 28 d, there was no statistically significant increase in *A* for any of the cultivars for young or old leaves over that period (Fig. 2).



230

231 Figure 2. Long-term effect of a single night of -5 °C exposure on net

232 assimilation, expressed as % of untreated control. Data points are the mean of 4

233 replicate plants. None of the slopes were significantly greater than zero,

234 indicating no recovery over 28 d.

3.5 *Field-grown comparison plants.* 'Chandler' and 'Seascape' strawberries grown under unprotected field conditions had significantly lower *A* than plants grown with the protection of a high tunnel ($P = 0.0003$, Table 2). The additional protection provided by a low tunnel within the high tunnel did not improve photosynthetic rate over a high tunnel alone ($P = 0.086$), although high tunnel + low tunnel managed plants had slightly higher *A*, which corresponds to warmer mid-winter temperatures recorded in this treatment (Maughan, 2013). There was no statistically significant difference between the two cultivars evaluated ($P = 0.55$). Unprotected field grown, high tunnel, and high tunnel + low tunnel grown plants had photosynthetic rates that were 88, 66 and 59% lower, respectively, than the greenhouse-grown control plants held in a growth chamber at 10 °C d/ 5 °C night (Table 2).

Table 2. Net CO_2 assimilation rate (*A*) among field-grown, high tunnel (HT) and low tunnel (LT) strawberry plants, and greenhouse-grown plants kept at 10 °C d/ 5 °C night.

Cultivar	Treatment			
	Greenhouse	Field	HT	HT + LT
Chandler	9.23	0.59b	3.08a	3.97a
Seascape	8.76	1.64b	3.07a	3.39a
Analysis of Variance		*P*		
Treatment		<0.001		
Cultivar		0.548		

Numbers within a row followed by the same letter are not significantly different. Greenhouse plant values listed as a reference and are not included in statistical analysis.

4. Discussion

Strawberries have been successfully produced in the Intermountain West using a combination of high tunnels and low tunnels (Maughan, 2013; Rowley et al., 2010). In these high tunnel systems, strawberries are planted in the fall and harvested very early the following spring. Fall growth is important for high yields, as plants need to develop adequate roots, branch crowns and flower buds. Plant development continues in the tunnels during the winter due to adequate growing temperatures, despite low light levels. Early fall and late spring frosts are common throughout the Intermountain West and these conditions may contribute to lower productivity. Therefore, providing minimum temperature thresholds will help growers make better decisions regarding temperature management within the tunnels, including when supplemental heat might be justified (Maughan, 2013).

Work by O'Neill et al. (1981) and Owens et al. (2002) found that significant damage (measured by solute leakage) occurred when excised leaf disks were exposed to temperatures between -5 and -12 °C. Our data with intact leaves attached to the plant supports these findings. We found a significant drop in *A* rate after a single night exposure to -5 °C, with a nearly complete loss of *A* capacity after multiple exposures to -5 °C, or a single night of exposure to -9 °C. Leaves exposed to these cold conditions did not recover and thus would not contribute to subsequent plant growth. Although LT_{50} is traditionally used to describe the temperature at which half of the plants die, in this study LT_{50} was used in reference to the temperature at which there was a 50% reduction of the net CO_2 assimilation.

The effect of a single conditioning night at -3 °C on *A* with subsequent exposure to colder temperatures was particularly interesting. It is generally accepted that strawberry plants acclimate to cold temperatures, typically this acclimation is

accomplished within 7 days (Darrow, 1966). Based on our results from the step-down and repeat freeze experiments, we found some acclimation occurs after only one night exposure to freezing temperatures. However, even with a conditioning night, *A* activity continues to decline with repeated exposure to sub-critical temperatures.

The lack of recovery in photosynthetic capacity after exposure to damaging cold temperatures suggests that plants with freezing damage to the leaves would recover by producing new leaves to support further growth, rather than repairing damaged leaves. Therefore, to gain the most benefit from protected cultivation, canopy temperatures should remain above -5 °C. While high tunnels have been shown to have air temperature significantly warmer than outside air during the day (Wien, 2009), additional heating may be warranted at night when air temperature differences are not as great. The analysis of the field-grown plants further indicates benefits of using protected cultivation since *A* rates of leaves grown in high tunnels were significantly higher than those kept outdoors.

Growth chamber studies may underestimate the potential damage that occurs to leaves in the field, as none of the leaves in the growth chambers were simultaneously exposed to extreme cold and bright light conditions, as would be the case at sunrise when the air temperatures are often the coldest. Theoretically, freezing temperatures in conjunction with high light levels would be more damaging than gradually warming frozen leaves in darkness prior to light exposure, as measured in this study. This is due to an increased susceptibility to light stress at low temperatures as seen by Powles et al. (1983). As this is a common condition of field or tunnel grown strawberries in the Intermountain West, a more complete understanding of leaf damage would require additional investigation of the effect of freezing temperatures coupled with exposure to sunlight. Even with the theoretically increased damage of both light exposure and freezing temperatures, plants under at least high tunnels had an average of a 400%

297 increase in photosynthetic activity over unprotected plants where temperatures dropped
298 below -5 °C on multiple occasions.

299 **5. Conclusion**

300 In conclusion, leaves exposed to -3 °C for 4 h did not experience a significant reduction
301 in net CO_2 assimilation. Regression analysis indicated the LT_{50} was between -5 and -6 °C
302 for all cultivars tested (Fig. 1), with 'Chandler', 'Seascape' and 'Jewel' being -5.80 °C, -
303 5.45 °C and -5.07 °C, respectively. Exposure to -3 °C before exposure to -5 and -7 °C
304 improved cold temperature tolerance of leaves. When leaves were exposed to -5 and -7
305 °C without conditioning exposure to freezing temperatures, more severe damage was
306 observed, as indicated by a significant reduction in photosynthesis. Furthermore, young
307 and old leaves exposed to a single night of -5 °C did not recover lost photosynthetic
308 activity even after 28 d at 10 °C d/5 °C night. Strawberry plants in protected cultivation
309 systems should be kept above -5 °C to minimize leaf damage and promote continued
310 growth.

311 **6. Acknowledgements**

312 The authors thank James Frisby (Utah State University) and Elizabeth Gordon (Li-Cor
313 Biosciences) for technical assistance. Funding was provided by grants from the research
314 foundation of the North American Strawberry Growers Association, the Utah Dept. of
315 Agriculture and Food Specialty Crop Block Grant Program and by the Utah Agricultural
316 Experiment Station, Utah State University, and approved as journal paper number 8675.

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