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Rapid screening methods to identify chilling tolerance in sweet basil (*Ocimum basilicum* L.)

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ABSTRACT

Sweet basil (*Ocimum basilicum* L.) is a chilling sensitive crop and therefore highly susceptible to chilling injury (CI) both in the field and during shipping when temperatures drop below 10°C. CI manifests in leaf browning and wilting, leading to visual damage of the leaves and rendering the produce unmarketable. Such low temperature occurs during unexpected cold events including frost early in the season at the preharvest stage or during shipping, processing or storage at the postharvest stage. Effective screening procedures are critical to identify new sources of chilling tolerance in basil and to evaluate tolerance in breeding populations. This study presents two methods that screen and assess sweet basil for chilling tolerance at the pre- and postharvest stage with visual ratings using five-level indices and high-throughput image phenotyping.

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is a chilling sensitive crop with injury occurring at temperatures ≤10°C and severe injury at ≤5°C (Cantwell and Reid, 1993; Lange and Cameron, 1994). Chilling sensitivity is common in plants of tropical origin including basil and other economically important crops such as rice, maize, soybean, cotton and tomato (Chinnusamy et al., 2007; Ding et al., 2019; Simon, 1998). However, in sweet basil, the final product is the fresh leaves and as a result, any damage due to chilling injury (CI) that leads to visual discoloration and injury to the leaves results in a lower quality or unmarketable product (Cantwell and Reid, 1993; Lange and Cameron, 1994; Lange and Cameron, 1997).

Basil is susceptible to CI during the pre- and postharvest stages of production. Field-grown basil experiences CI with unexpected frosts, seasonal weather fluctuations or any other time the ambient temperature drops below 10°C (Cantwell and Reid, 1993; Dudai et al., 2017; Lange and Cameron, 1994; Lange and Cameron, 1997). Harvested and processed basil is often shipped in refrigerated trucks with other herbs as most herbs are not chilling sensitive and low temperatures minimize disease and decay (Aharoni et al., 2010). Although bulk shipping eliminates the cost of separate shipping arrangements, it may sacrifice the quality and marketability of fresh-cut basil. Growers can at least recognize when CI occurs before harvest or during cleaning, sorting and grading at the farm, but they cannot control for injury that develops after the harvest leaves the farm. Unfavorable shipping or storage conditions result in the delivery of poor quality produce, thus impacting future sales and markets and incurring great economic loss (Vigneault et al., 2009).

The main symptoms of CI in basil manifest as brown discoloration or necrotic lesions in the

interveinal leaf area, wilting and general loss of or changes in the aroma profile (Cantwell and Reid, 1993; Cozzolino et al., 2016). Commercial management strategies including harvesting in the afternoon (Aharoni et al., 2010; Lange and Cameron, 1994), acclimating plants with less severe low temperatures (Lange and Cameron, 1994; Lange and Cameron, 1997), applying plant hormone treatments (Satpute et al., 2019) or supplementing with artificial lighting (Dudai et al., 2017; Jensen et al., 2018) can lessen the severity of the injury. However, growers, processors or distributors may still suffer sizable economic losses even with these added expenses.

Response to cold temperatures is a complex, quantitative trait and therefore difficult to quantify (Cantwell and Reid, 1993; Ding et al., 2019). Many factors influence cold stress physiology, including time of day of harvest (Lange and Cameron, 1994), age of the crop (Cantwell and Reid, 1993), water availability (Hussain et al., 2018; Satpute et al., 2019), hormone or chemical contamination in the postharvest environment (Cantwell and Reid, 1993; Satpute et al., 2019), and artificial light (Dudai et al., 2017; Jensen et al., 2018). Chilling assays must account for these variables otherwise results will be inconsistent and populations unstable.

The Food and Agriculture Organization (FAO) reports that frost and freezing temperatures incur more economic losses in the US than any other weather hazard. For example, one frost event in 1990 alone cost California ~$500 million in fruit loss and 450,000 ha of tree damage (Snyder and de Melo-Abreu, 2005). CI in crops is not only caused by cold weather events but also by refrigerated shipping and storage (Lange and Cameron, 1997; Vigneault et al., 2009). Developing robust and accurate assays to screen for cold tolerance in crops is important to combat pre- and postharvest losses.

Development of chilling tolerant varieties is highly desirable to prevent such major pre- and postharvest crop loss (Dudai et al., 2020). Since CI was recognized and reported in the literature, there has not been a release of commercial sweet basils that are chilling tolerant (Dudai et al., 2020; Ribeiro and Simon, 2007). The current study examines two methods for effectively screening and assessing sweet basil for chilling tolerance at the pre- and postharvest stages with two different methods for visualization and phenotyping using a cold tolerant breeding line and cold sensitive commercial control.

MATERIALS AND METHODS

Preharvest plant material. CB15 and VDFSSP were sourced from Van Drunen Farms Specialty Seeds (VDFSS, Momence, IL, USA). CB15 is a cold tolerant sweet basil breeding line developed at Rutgers University (New Brunswick, NJ, USA). CB15 is among many sister lines being developed for chilling tolerance with the most stable and consistent response to low temperature both at Rutgers and at a commercial greenhouse operation (data not shown). VDFSSP is a sweet basil variety developed by VDFSS with no chilling tolerance.

Postharvest plant material. F_2 individual plants (n=69) derived from a cross between the chilling sensitive cultivar ‘Perrie’ and the chilling tolerant cultivar ‘Cardinal’ (Dudai et al., 2018) were grown in a greenhouse in Newe Ya’ar Research Center during the spring of 2019. Parental lines were developed at the Unit of Aromatic and Medicinal Plants of the Agricultural Research Organization, Newe Ya’ar Research Center (Ramat Yishay, Israel).

Preharvest chilling assay. Seeds were sown in 128 cell plug trays and spaced diagonally to each other, at least one row and one column apart to maximize cool air flow. Six trays were assessed at 0.56°C and 2°C and four trays were assessed at 3°C in a randomized block design. Sixteen individuals of each line were planted per tray and separated by two spacer columns for a total of 96 seedlings for refrigerator settings 0.56°C and 2°C and 64 seedlings for 3°C. Seedlings were grown to the 4-8 true leaf stage (~6 weeks after seeding). Trays were watered between 0700 and 0900 HR and placed in a solid door reach-in refrigerator (Avantco A-49R-HC 54”, 2020, 41.3 ft^3) on the middle and bottom shelves in the dark for 24 h. Refrigerators were set to 0.56°C, 2°C and 3°C for the first, second and third assay, respectively. Govee Wireless Thermometers/Hygrometers (H5074001) were positioned near trays to assess temperature and humidity every 15 minutes once readings stabilized. Assays were conducted at Rutgers New Jersey Agricultural Experiment Station (NJAES), New Brunswick, NJ, USA.

Postharvest chilling assay. Individual leaves (n=4-5) from all mature F_2 plants were harvested and put in a Ziploc bag. When the number of leaves permitted, 2 bags were collected per individual. The bags were stored at 4°C for 5 days, moved to room temperature for 2 days and then analyzed for necrotic

lesions via image-based phenotyping. After the initial evaluation, 8 F_2 lines (4 chilling tolerant and 4 chilling sensitive) were selected and vegetatively propagated to generate 3 plants each. From each plant, a bundle of ~70 g of young or mature leaves from the same stems was harvested and stored in a marcro-perforated (8 holes of 5 mm diameter) polypropylene bag at 6°C for 5 days, then transferred to 17°C for 2 days and analyzed for browning, decay and leaf-abscission by visual ratings.

Preharvest visual ratings. CI was rated on a visual scale (0-4), modified from Ribeiro and Simon (2007). Individual seedlings received a rating of 0 for no necrotic lesions, 1 for 1-25% browning, 2 for 26-50% browning, 3 for 51-75% browning and 4 for >75% browning. Lower leaves were excluded due to pre-existing browning. Ratings were assigned immediately after refrigeration.

Postharvest image-based phenotyping. The CI of the stored basil leaves was evaluated by image processing to rapidly assess the entire F_2 population for necrosis. High quality images were acquired using a high resolution digital camera (Canon EOS 400D). The image of the leaf necrotic lesions was analyzed by the machine learning based digital image analysis platform, Leaf Necrosis Classifier (LNC; Obořil, 2017). The LNC detected the necrotic areas in the leaf surface by using a combination of multilayer perceptron (MLP) and self-organizing maps (SOM). The SOM was trained with the dataset on the basis of the leaf regions identified manually. The evaluation module detected the necrotic areas in the image on the basis of the trained SOM (Fig. 1). The results of the necrotic areas in the basil leaf were represented as percent (%) necrotic area of the whole leaf area. The mean of the leaves in a single bag was considered a single repeat. When a leaf was completely damaged it was considered to have 100% necrotic area.

Postharvest visual ratings. Browning or necrotic lesions, decay and leaf-abscission were evaluated to assess CI in the F_2 individuals selected for chilling tolerance and susceptibility following the methods of Aharoni et al. (2010). The degree of browning in the mature leaves and youngest leaves (less the 1 cm long), leaf abscission and decay were evaluated on a visual scale (1-5). Individuals received a rating of 1 if all leaves were symptom-free, 2 for 5% of the leaves showing damage, 3 for 20% of the leaves showing damage, 4 for most leaves showing damage and 5 for all leaves showing damage.

Statistics. Individual visual ratings were averaged for each line for each assay and standard error was determined. The GLIMMIX procedure of The SAS System (version 9.4) was used to fit a mixed effects model ANOVA to test for the main effect of variety and the variety by temperature interaction effect. The model included block as a random effect. The model was fit to individual observations on each plant subsample and the subsampling variation was partitioned out separately. One-way ANOVA was used to determine if locations in the refrigerator were significantly different in terms of temperature and humidity at each refrigerator setting. Statistical tests for the postharvest analyses were performed in JMP 15 (SAS corporation).

Figure 1. Postharvest image-based phenotyping. Example of evaluation of necrotic areas in four individual leaves as analyzed by Leaf Necrosis Classifier (Obořil, 2017). The original RGB images are presented at the top. The necrotic area analysis images are presented at the bottom.

RESULTS AND DISCUSSION

The preharvest chilling assay resulted in the two lines, CB15 and VDFSSP, exhibiting a differential response to chilling temperature. The leaves of VDFSSP consistently developed more necrotic lesions and received CI ratings of 3 or 4 after chilling compared to those of CB15, which had little to no damage and most often received ratings of 0 or 1 (Fig. 2). CB15 had significantly less CI than VDFSSP across all refrigerator settings of 0.56°C,

2°C and 3°C (Fig. 3). These refrigerator settings were lower than the experimental temperatures recorded by the thermometers (Tab. 1). The average experimental temperatures at settings 0.56°C, 2°C and 3°C were 2.7°C, 3.9 °C and 5.3 °C, respectively.

Different locations within the refrigerator were significantly different in terms of experimental temperatures (Fig. 4). The refrigerators maintained an interquartile range of ~1°C, however outliers usually occurred at higher temperatures, skewing the data toward a higher median. The differences highlight the importance of properly replicating and using several thermometers to record temperatures and potentially eliminate replicates in locations that fall outside of the intended temperature range.

The preharvest chilling assay method effectively distinguished between the phenotypic response of a cold tolerant and a cold sensitive line of basil to chilling temperatures (0-10°C). This method was selected as the chilling tolerant and chilling sensitive basil lines displayed the expected differential CI response. Variations of this method were trialed prior to this experiment and are not recommended as they reduced the expected differential response between lines. These variations included sowing seeds closer together, refrigerating the trays in the afternoon or evening and assaying at a younger or older leaf stage as they all reduced the difference between the cultivars (data not shown). These deviations from expected CI response are supported in the literature. Liu et al. (2018) reported that increasing plant density shelters plants from air flow, which would unevenly circulate cool air to plants in a refrigerator. Lange and Cameron (1994) recognized that basil harvested at 6 pm or 10 pm as compared to 2 am or 6 am increased the shelf life of the product by almost 100% when stored at 10°C, 15°C and 20°C. Cantwell and Reid (1993) found that young seedlings and multiply harvested field-grown basil were more susceptible to CI than basil harvested for the first time. However, differences in scaling or phenotyping may be considered (Aharoni et al., 2010; Dudai et al., 2017; Ribeiro and Simon, 2007).

The amount of hours or days that trays are refrigerated may need to be adjusted based on refrigeration unit. The NJAES greenhouse walk-in refrigerator (Mr. Winter, 1992, 593.7 ft^3) set to 3-5°C was used prior to the reach-in refrigerators (Avantco, 2020, 41.3 ft^3) used in this experiment. The walk-in refrigerators required 48-72 hours to induce a differential CI response, while the reach-in refrigerators required only 24 hours for the same population at the same temperatures (data not shown). The reach-in refrigerator is much smaller and has a different ventilation system, and thus likely keeps a consistently cooler temperature.

Trays were watered before the chilling assay to minimize drought stress and prevent wilting. Research has shown that wilting may still occur in some genotypes or with extended refrigeration due to physiological crosstalk between drought stress and chilling stress (Hussain et al., 2018). If so, an additional visual rating scale may be needed as wilting does not necessarily correlate with necrosis and structure may be restored when plants are rehydrated. For example, 0 would indicate no wilting, 1 would indicate wilting of the leaves and 2 would indicate wilting of the leaves and stem.

For the postharvest chilling assay, the rapid screen of the entire F_2 population by image-based estimation of necrotic area showed variation within the population (Fig. 5). The F_2 population displayed a continuous range of browning severity, supporting that response to cold is a quantitative trait. Twenty-six F_2 individulas were more sensitive to chilling than the Genovese basil parent, ‘Perrie’. On the other hand, only nine families were slighly more tolerant to chilling than the Thai basil parent, ‘Cardinal’. This suggests that chilling sensitivity may also be transgressive, possibly due to epistatic interactions. Thus, image-based phenotyping is a helpful tool when rapidly assessing for CI at the postharvest stage.

Four selected chilling tolerant and four selected chilling susceptible lines were further analyzed in a storage experiment of fresh-cut bundles (Fig. 6). The tolerant group showed significantly lower decay and leaf abscission damage compared to the sensitive group according to the Student’s *t*-test (Fig. 7A), yet browning damage did not differ between the two groups. Browning of the youngest leaves, which were the most sensitive to chilling damage, did not show a statistical difference between the chilling tolerant and chilling sensitive groups. Within the chilling sensitive group only, young leaves showed a higher degree of browning than mature leaves (Fig. 7B). This suggests that the fast Ziploc experiment is a helpful tool for decay and damage estimation that can rise from various parameters such as mechanical damage or infection due to *Botrytis cinerea* or

Erwinia carotovora (Aharoni et al., 2010). Yet, this method was not as effective as the image-based phenotyping at evaluating postharvest browning damage due to CI, because of these other factors that contribute to browning at the postharvest stage.

CONCLUSION

Chilling response is a complex, quantitative trait and therefore presents many challenges when breeding for cold tolerance in chilling sensitive plants, such as sweet basil. This paper demonstrates two methods to screen for cold tolerance in basil seedlings at the pre- and postharvest stages. A clear differential response was observed between a cold tolerant basil line and a cold sensitive basil line. Further studies are necessary to determine if cold tolerance of basil in the preharvest stage correlates to that in the postharvest stage.

A.

B.

Figure 2. Differential chilling response in cold tolerant line CB15 and cold sensitive line VDFSSP. A. All VDFSSP individuals (left) received a chilling injury (CI) rating of 4 on this tray while those of CB15 (right) received a CI rating between 0 and 2. The leaves of VDFSSP developed necrotic lesions or browning (left) while those of CB15 remained green and healthy (right) after the plants were refrigerated at <5°C for 24 hours. B. Close-up view of VDFSSP and CB15 in A.

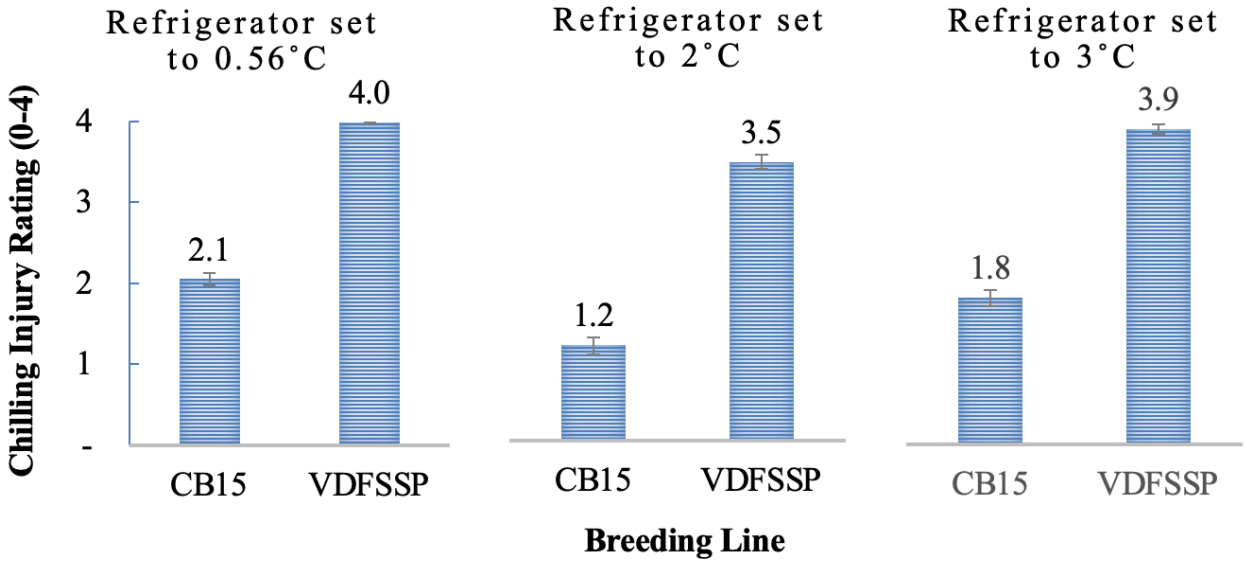

Figure 3. Visual chilling injury (CI) rating after refrigeration for 24h at three different refrigerator settings. CB15 had significantly less chilling injury across all refrigerator settings than VDFSSP with an average CI rating of 1.2-2.1 compared to 3.5-4.0 (ANOVA, *p*-value<0.0001). However, there was a significant interaction in that the difference between the two lines was greater at the 2°C refrigerator setting than the other settings (ANOVA, *p*-value=0.0054). Values are the mean of CI rating of each variety ± SE for refrigerator settings 0.56°C, 2°C and 3°C.

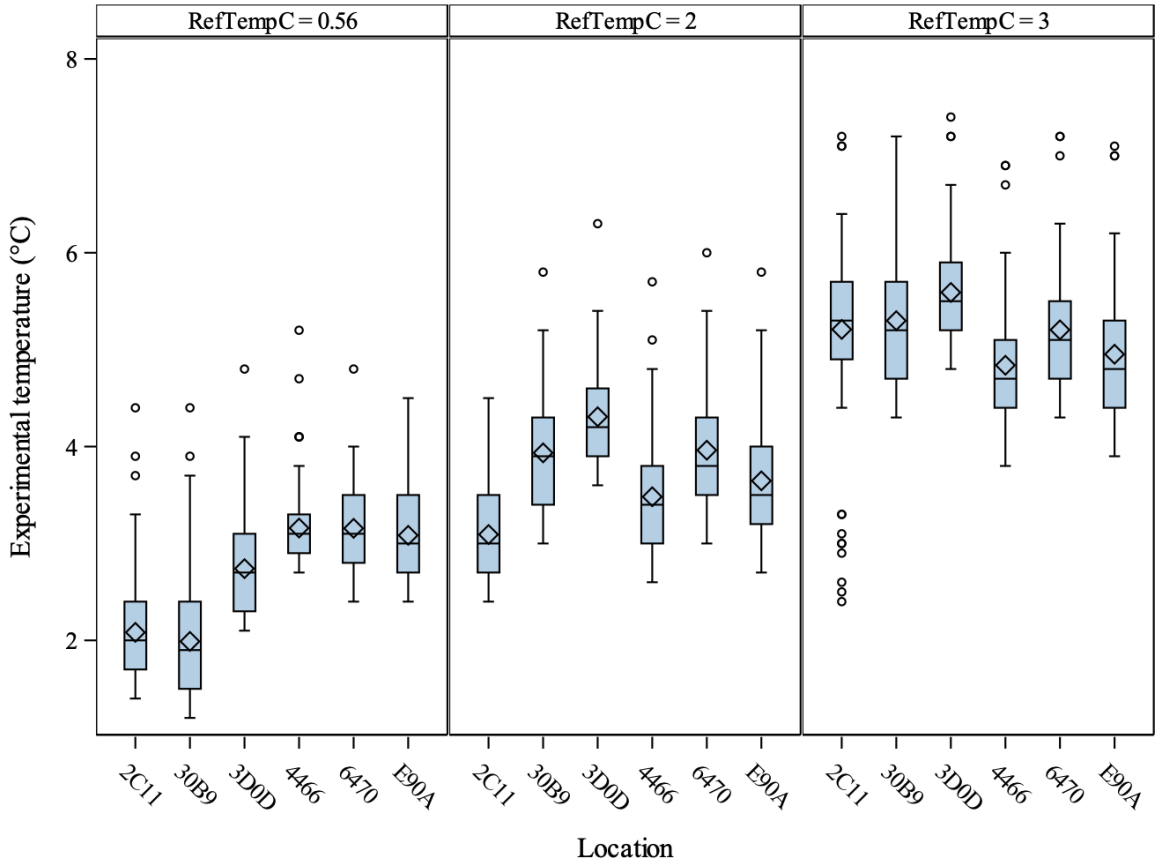

Figure 4. Experimental temperatures determined across different locations within reach-in refrigerator unit at three different refrigerator settings. Experimental temperatures were determined every 15 minutes by six thermometers (2C11, 30B9, 3D0D, 4466, 6470 and E90A) each placed in a different location in the refrigerator. Experimental temperatures were determined at three refrigerator settings (0.56°C, 2°C and 3°C), referred to as "RefTempC". Experimental temperatures occasionally formed outliers, skewing the data toward a higher median except in the case of thermometer 2C11 at 3°C, which measured 7 outlier anomalies. Temperatures were significantly different across location for each temperature (ANOVA, *p*-value<0.0001).

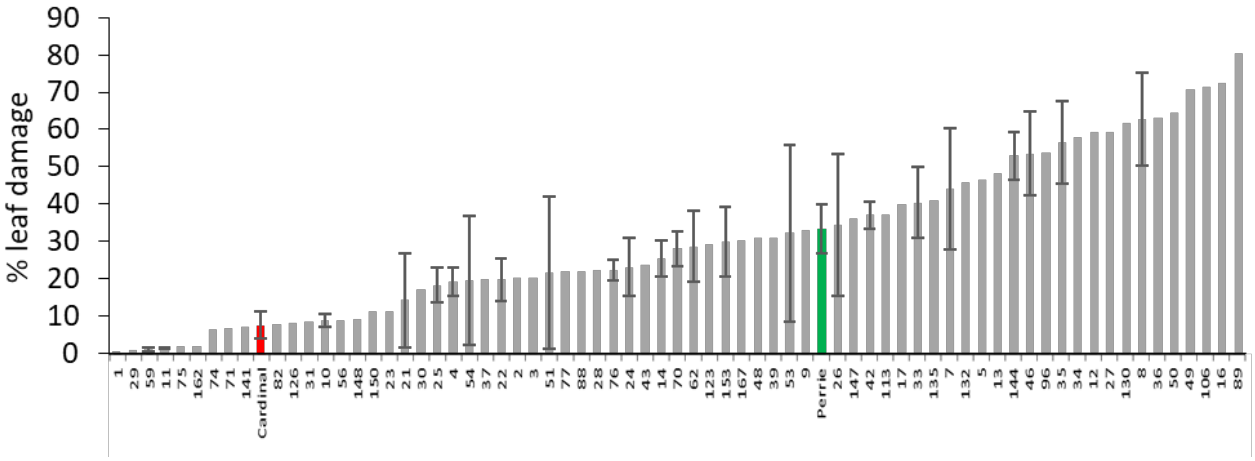

Figure 5. Chilling tolerance of postharvest fresh-cut basil leaves of F_2 population. Chilling damage of fresh-cut basil leaves stored at 4°C from F_2 plants (n=69) of a segregating population and from the population's parents. Leaf damage was determined by image analysis of necrotic areas of F_2 plants individuals (gray lines), 'Perrie' parent (green line) and 'Cardinal' parent (red line). Values are mean of 1-2 biological replicates ± SEM. Each replicate contained 4-5 leaves.

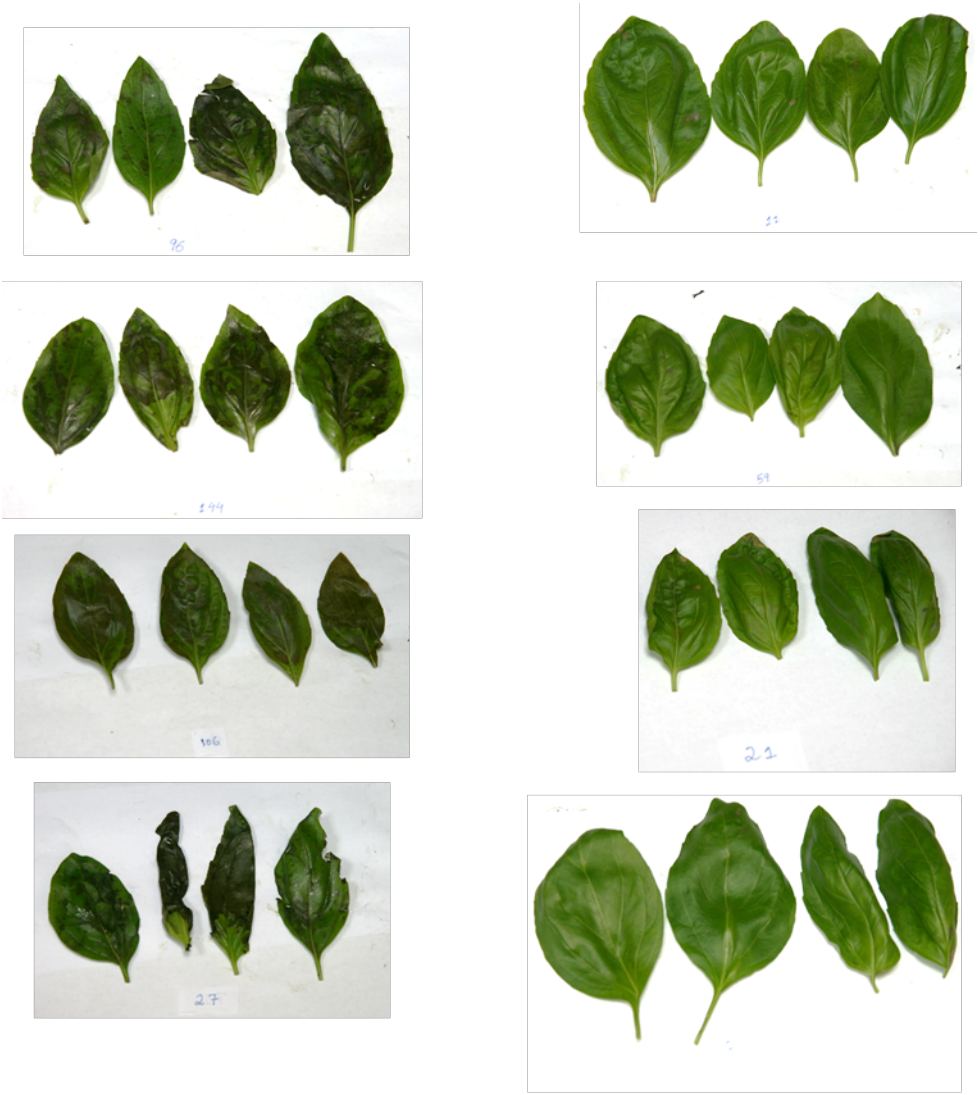

Figure 6. Chilling damage of individual leaves from selected F_2 chilling tolerant and susceptible individuals. Each image on the left represents an example of four leaves from a susceptible F_2 individual, while each image on the right represents an example of four leaves from a tolerant F_2 individual.

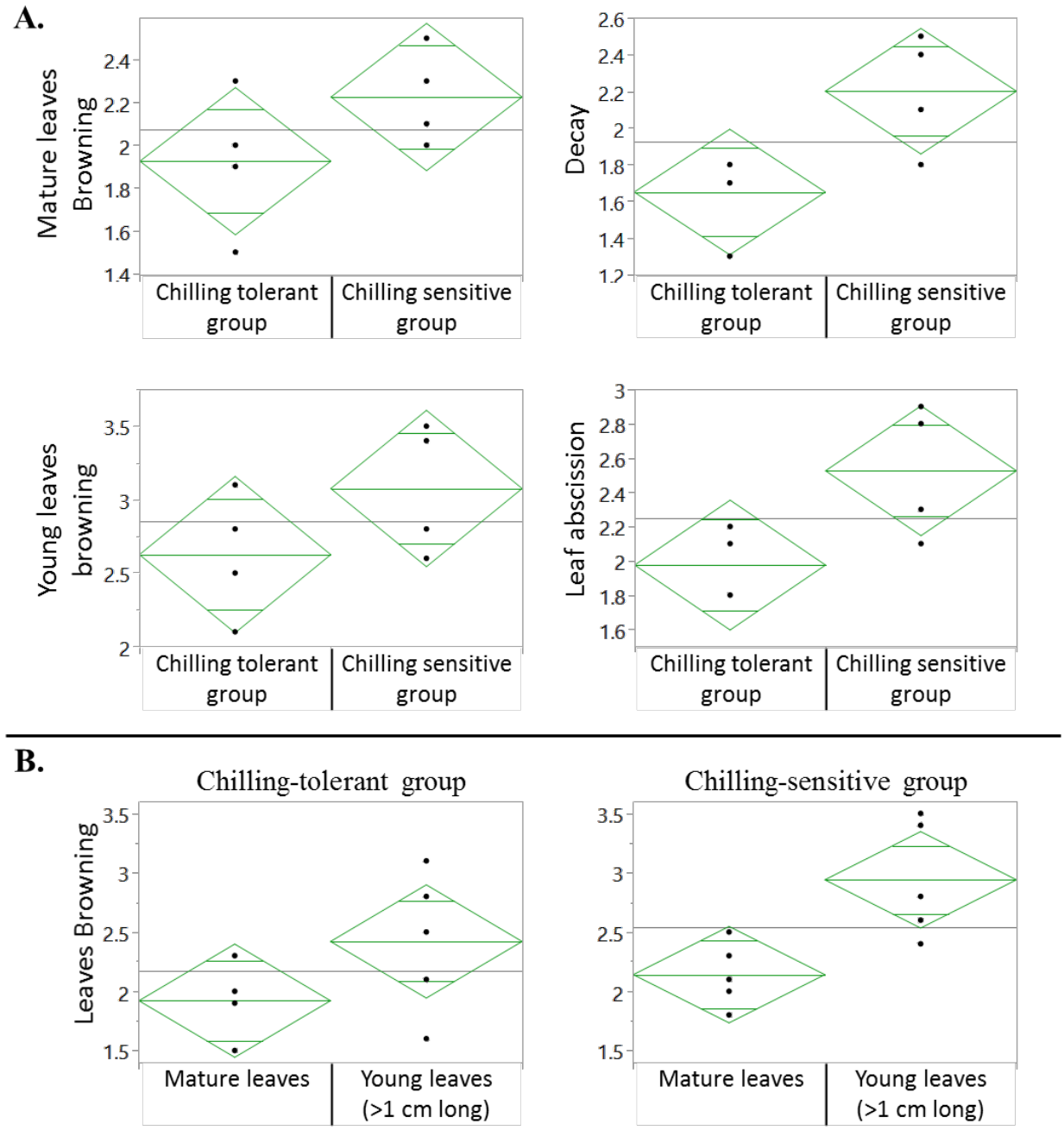

Figure 7. The effect of chilling stress on postharvest basil bundles from selected F_2 individuals. Fresh-cut bundles of leaves from mature basil plants that were vegetatively propagated from individual F_2 plants were stored at 6°C for 5 days, followed by 2 days at 17°C. The chilling tolerant group and chilling sensitive group each contained four lines. A. Comparison of browning between chilling tolerant and chilling sensitive groups. The difference between the tolerant and sensitive groups was significant only for decay and leaf abscission according to Student's *t*-test ($\alpha<0.05$). B. Comparison of browning between young and mature leaves within chilling tolerant and chilling sensitive groups. The difference between the browning of young and mature leaves was significant within the chilling sensitive group only (Student's *t*-test, $\alpha<0.05$).

Table 1. The average, minimum and maximum experimental temperature and humidity readings for three different refrigerator settings*.

Refrigerator setting (°C)	Average temperature (°C)	Minimum temperature (°C)	Maximum Temperature (°C)	Average Relative humidity (%)	Minimum Relative humidity (%)	Maximum Relative humidity (%)
0.56	2.7	1.2	5.2	77.1	63.4	97.4
2.00	3.9	2.6	6.3	78.8	64.5	94.8
3.00	5.3	3.8	8.8	76.5	64.3	95.4

*The thermometers measured minimum temperatures 0.6-0.8 °C, average temperatures 1.9-2.3°C and maximum temperatures 4.3-5.8°C higher than the refrigerator settings. The relative humidity was consistent across refrigerator settings.

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