Inheritance of heat tolerance in perennial ryegrass (*Lolium perenne*, Poaceae): evidence from progeny array analyses

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Background: Heat stress is considered one of the most important environmental factors influencing plant physiology, growth, development, and reproductive output. The occurrence and damage caused by heat stress will likely increase with global climate change. Thus, there an urgent need to better understand the genetic basis of heat tolerance, especially cool season plants.

Materials and methods: In this study, we assessed the inheritance of heat tolerance in perennial ryegrass (*Lolium perenne* L. subspecies *perenne)*, a cool season grass, through a comparison of two parental cultivars with their offspring. We crossed a heat tolerant cultivar (Kangaroo Valley) with a heat sensitive cultivar (Norlea), to generate 72 F1 progeny. Both parents and their progeny were then exposed to heat stress for 40 days, and their photosynthetic performance (Fv/Fm values) and leaf H_2O_2 concentrations were measured.

Results: As expected, Kangaroo Valley had significantly higher Fv/Fm values and significantly lower H_2O_2 concentrations than Norlea. For the F1 progeny, values of Fv/Fm decreased gradually with increasing exposure to heat stress, while the content of H_2O_2 increased. The progeny had a wide distribution of Fv/Fm and H_2O_2 values at 40 days of heat stress. Approximately 90% of the 72 progeny had Fv/Fm values that were intermediate to the values of the two parental cultivars and 67% of the progeny had H_2O_2 concentrations intermediate to their two parents.

Conclusion: Results of this study indicate considerable additive genetic variation for heat tolerance within the progeny array generated from this cross, and such diversity could be used to improve heat tolerance in perennial ryegrass cultivars. Our findings point to the benefits of combining physiological measurements within a genetic framework to assess the inheritance of heat tolerance, a complex phenotypic trait.

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Abstract

Heat stress is considered one of the most important environmental factors influencing plant physiology, growth, development, and reproductive output. The occurrence and damage caused by heat stress will likely increase with global climate change. Thus, there an urgent need to better understand the genetic basis of heat tolerance, especially cool season plants. In this study, we assessed the inheritance of heat tolerance in perennial ryegrass (*Lolium perenne* L. subspecies *perenne)*, a cool season grass, through a comparison of two parental cultivars with their offspring. We crossed a heat tolerant cultivar (Kangaroo Valley) with a heat sensitive cultivar (Norlea), to generate 72 F1 progeny. Both parents and their progeny were then exposed to heat

stress for 40 days, and their photosynthetic performance (Fv/Fm values) and leaf H_2O_2 concentrations were measured. As expected, Kangaroo Valley had significantly higher Fv/Fm values and significantly lower H_2O_2 concentrations than Norlea. For the F1 progeny, values of Fv/Fm decreased gradually with increasing exposure to heat stress, while the content of H_2O_2 increased. The progeny had a wide distribution of Fv/Fm and H_2O_2 values at 40 days of heat stress. Approximately 90% of the 72 progeny had Fv/Fm values that were intermediate to the values of the two parental cultivars and 67% of the progeny had H_2O_2 concentrations intermediate to their two parents. Results of this study indicate considerable additive genetic variation for heat tolerance within the progeny array generated from this cross, and such diversity could be used to improve heat tolerance in perennial ryegrass cultivars. Our findings point to the benefits of combining physiological measurements within a genetic framework to assess the inheritance of heat tolerance, a complex phenotypic trait.

Introduction

Based on the various habitats they occupy, plants require certain environmental conditions to maintain the abundance and persistence of their populations (Harper, 1977). During their lifetime, however, most plants experience abiotic stress when exposed to unfavorable chemical and physical environmental conditions such as heavy metals, high salinity, excessive solar radiation, freezing temperatures, severe drought, and extremely high temperatures (Nilsen and Orcutt, 1996). Of these stressors, drought and heat stress are among the two most important environmental factors influencing plant physiology, growth, development, and reproductive output (i.e., yield) (Jiang and Huang, 2001; Prasad et al., 2008, Jespersen et al., 2017).

55 According to Wahid et al. (2007), heat stress (or heat shock) in plants occurs when
56 temperatures rise above a threshold level for sufficient time to result in irreversible damage to
57 plant growth and development. Although heat stress usually occurs with an increase in
58 temperature of 10-15 °C above ambient; heat stress is also influenced by the intensity, duration,
59 and rate of increase in temperature (Wahid et al., 2012). Thus, heat stress in plants can occur on a
60 daily or seasonal basis and can vary from year-to-year. In addition, the occurrence and damage
61 caused by heat stress will likely increase with global climate change (Walter et al., 2013; Bita
62 and Gerats, 2013). Due to human activities, substantial increases in the concentration of
63 greenhouse gases are occurring, and global air temperatures are predicted to increase 1-4.5 °C
64 above the current level by 2100, depending on different carbon emission scenarios (Rogelj et al.,
65 2012, IPCC, 2019). Moreover, human-caused climate change is also associated with extreme
66 climate events such as precipitation extremes, flooding, frosts, drought, and excessive heat (Niu
67 et al., 2014; Stott, 2015). Thus, future climate change is expected to cause serious damage to the
68 growth and yield of native plants and crop plants, especially C_3 plants and crops (Lobell and
69 Field, 2007; Wahid et al., 2012).

70 Exposure of plants to excessively high temperatures can result in a range of complex
71 responses from molecular and cellular, to whole plant levels (Baniwal et al., 2004; Kotak et al.,
72 2007; Wahid et al., 2012; Prasad et al., 2008, Mittler et al., 2011; Soliman et al., 2011). Once leaf
73 temperatures rise above a threshold level (35 to 40 ºC for most plants), protein denaturation and
74 loss of cell membrane fluidity begins to take place and cell damage and programmed cell death
75 may occur (Huang and Xu, 2008; Horvath et al. 2012). Depending on the intensity and duration
76 of exposure to high temperatures, plant tissue type, and phenological stage, heat stress in plants
77 can induce the following responses: 1) loss of cell water content, 2) reduced photosynthetic

activity, 3) oxidative stress, 4) scorching of tissues and premature leaf senescence and abscission, 5) reduced growth rates through inhibition of shoot and root growth, 6) damage or alteration of floral (reproductive) tissues, and 7) reduced seed number and quality (see Figure 1 in Hasanuzzaman et al., 2013).

With heat stress, reductions in photosynthetic activity and efficiency may take place because high temperatures can lead to the dissociation or inhibition of oxygen evolving complexes (OEC) and reduce the activity of photosystem II (PSII) (Wahid et al., 2007). Photosynthetic performance during heat stress can be quantified by measuring chlorophyll fluorescence parameters (Baker and Oxborough 2004; Rosyara et al., 2010). One such parameter is Fv/Fm, which is calculated as the ratio between variable fluorescence (Fv = Fm - Fo) and maximum fluorescence (Fm). Exposure to high temperatures can also induce oxidative stress in plants by uncoupling enzymes and metabolic pathways which generates reactive oxygen species (ROS) that can damage multiple cellular organelles and physiological processes (Locato et al. 2008; Soliman et al., 2011; 2012; Hasanuzzaman et al., 2013). Reactive oxygen species (ROS) include singlet oxygen, superoxide radical, hydroxyl radical, and hydrogen peroxide (H_2O_2). Hydrogen peroxide is the most stable ROS and its adverse effects in plants include membrane lipid peroxidation, toxicity, and cell death. Interestingly, recent studies have highlighted the important role of H_2O_2 as a signaling molecule in plants, triggering tolerance responses to abiotic stresses (Suzuki et al., 2012; Baxter et al., 2014).

Plants are sessile organisms that cannot evade abiotic stressors. Thus, they have developed several mechanisms for mitigating and surviving heat stress (see Figure 4 in Hasanuzzaman et al., 2013). These mechanisms include short-term avoidance or acclimation mechanisms and long-term phenological and morphological adaptive traits such as early maturation, enhanced

root density and depth, changing leaf orientation and leaf rolling, transpirational cooling, and/or alteration of membrane lipid compositions (Wahid et al., 2007; 2012; Prasad et al., 2008; Hasanuzzaman et al., 2013; Jespersen et al., 2017). Additionally, plants have developed molecular, cellular, and physiological adaptations for tolerating heat stress (Wahid et al., 2012; Hasanuzzaman et al., 2013). These include signaling cascades and regulation of gene expression by transcription factors (Yang et al., 2014; Ohama et al., 2016; Jespersen et al., 2017), expression of heat shock proteins (HSPs) and molecular chaperones (Horvath et al., 2012; Davies et al., 2018), enzymatic and non-enzymatic antioxidant defense to prevent the harmful effects of ROS (Gulen and Eris, 2004), and the production of osmo-protectants or compatible solutes (Wahid et al., 2007; Hasanuzzaman et al., 2013). Clearly, heat tolerance in plants is controlled by a complex set of many genes and interacting mechanisms, and not just a single gene or mechanism (Erdayani et al., 2020).

Perennial ryegrass (*Lolium perenne* L. subspecies *perenne)* is a cool season (C_3), perennial grass that has a caespitose (bunch) growth form and can grow to a height of approximately 10-90 cm. Perennial ryegrass originated in the Middle East, and then dispersed across Europe and North Africa with the spread of agriculture (Balfourier et al. 2000). Perennial ryegrass has subsequently been introduced around the globe and is considered a weed, or an invasive species, in natural communities in many regions. It is also one of the most common pasture grasses in temperate climates regions where it is used as a forage grass for livestock and for hay production. In addition, it is widely used as a turf grass (Bolaric et al., 2005a; 2005b; Wang et al., 2009), and for restoration and conservation seedings. Perennial ryegrass is naturally a diploid species (2n = 2x = 14) (Bolaric et al., 2005b; Wang et al., 2009), but tetraploid (2n = 4x = 28) cultivars have also been developed (Nair, 2004). The grass has a two-locus self-

incompatibility system, which leads to an obligately outcrossing mating system (Cornish et al. 1979). This mating system ensures outbreeding among individuals and high amounts of genetic diversity within naturally occurring populations (Bolaric et al., 2005a; 2005b; Wang et al., 2009). Many cultivars of perennial ryegrass however are derived from a limited pool of foundational clones; and such cultivars typically exhibit a limited amount of genetic variation (Guthridge et al., 2001).

Because perennial ryegrass is a cool season grass of temperate regions, it is generally considered to be sensitive to heat stress (Li et al., 2020); although heat tolerant cultivars have been developed (Wilkins, 1991). In addition, because the grass is widely cultivated and has high economic value, experiments assessing heat stress in perennial ryegrass, especially comparisons of heat tolerant and heat sensitive cultivars, have been conducted (e.g., Wehner and Watschke, 1981; Jiang and Huang, 2001; Zhou and Abaraha, 2007; Wang et al., 2017; Sun et al., 2020; Li et al., 2020). For example, in a previous study we reported that a heat tolerant cultivar of perennial ryegrass (Yatugadake-24) exhibited significantly higher photosynthetic performance (i.e., they had higher Fv/Fm values) and lower leaf H_2O_2 content, compared to a heat sensitive cultivar (Norlea) (Soliman et al., 2011). In another study, Soliman et al. (2012) exposed 25 diploid and tetraploid cultivars of perennial ryegrass to prolonged heat stress and found that tetraploid cultivars had lower H_2O_2 content and experienced less oxidative stress than diploid cultivars. Taken together, these studies indicate considerable genetic variation in heat tolerance among perennial ryegrass cultivars and cytotypes. Yet, to the best of our knowledge, we are not aware of any assessment of the genetic basis of heat tolerance in perennial ryegrass.

In this study, we assessed the inheritance of heat tolerance in perennial ryegrass through a direct comparison of parental cultivars with their offspring, through progeny array analysis. This

147 was accomplished by crossing a heat tolerant cultivar of perennial ryegrass with a heat sensitive
148 cultivar, to generate F1 progeny. Both parents and their progeny were then exposed to long-term
149 heat stress, and their photosynthetic performance and leaf H_2O_2 concentrations were measured.
150 In addition, several leaf growth parameters were measured before the imposition of heat stress.
151 The specific goals of this research were to, 1) quantify the level of heat tolerance in the two
152 parental cultivars, 2) determine variation in heat tolerance among the F1 progeny, and 3)
153 compare the level of heat tolerance of the parents with their F1 progeny to assess the inheritance
154 of this complex and important phenotypic trait. Results of this study will improve our
155 understanding of the genetic basis of heat tolerance in perennial ryegrass, assist in estimating the
156 heritability of this trait, and aid in the identification and selection of plants with even higher
157 levels of heat tolerance for use in plant breeding programs.
158

159 # MATERIALS AN METHODS

160 ## Plant material

161 Two diploid perennial ryegrass cultivars were used as the parents in this study. Kangaroo Valley
162 (strain K7) is a heat tolerant cultivar developed in New South Wales, Australia, that is well-
163 suited to dry, hot regions (Wilkens, 1991; Blumenthal et al., 1996) and Norlea (strain N4) is a
164 heat sensitive cultivar developed in Canada (Soliman et al., 2011; 2012). Based on the breeding
165 programs that developed them, both strains of the two cultivars exhibit limited genetic diversity
166 (Blumenthal et al., 1996; Soliman et al., 2011). Flowers of each of these two cultivars were
167 crossed through hand-pollination, after they were emasculated. The Kangaroo Valley cultivar
168 was always used as the pollen donor, and the Norlea cultivar always served as the maternal

parent. These crosses were conducted at the Yamanashi Dairy Experimental Station, Yamanashi, Japan. Seventy-two full-sib, F1 progeny derived from this cross were used in this experiment.

Heat stress treatment

Our heat stress experiment was conducted using the procedures described by Soliman et al. (2012). The seeds/seedlings of the two perennial ryegrass strains (K7 and N4) used in the heat stress experiments were not the same individuals used to generate the progeny array; but because these two strains have limited genetic diversity, seeds of these two cultivars are genetically uniform. Seeds of the two parental cultivars and the progeny were germinated on wet filter paper in petri dishes. The grass does not require any other treatments to achieve high rates of germination. Seedlings were transplanted into pots (two seeds per pot), 7.5 cm in diameter and 8 cm deep, with a sandy loam potting soil containing 0.35 g of N, P2O5, and K2O for every kilogram of soil. The seedlings were grown in a controlled growth chamber with day/night temperatures of 23/16 °C, a 16h/8h day/night photoperiod (from 4:00-20:00 h), with photon flux density of 250 µmol m^{-2} s^{-1}, and a constant relative humidity of 70%. Forty days after transplanting, all the plants were exposed to 30 ºC for 3 days for acclimation to a higher temperature, after which the plants were exposed to heat treatments (36/30 °C, day/night temperatures) for 40 day. The heat stress experiment was set up in a randomized complete block design.

Leaf growth traits

Leaf growth traits were measured prior to the imposition of heat stress. For each seedling, the second fully matured leaf was selected for measurements. Specific leaf area (SLA) and its components were determined according to the method of Witkowski and Lamont (1991). Specific leaf area is calculated as the ratio of leaf area (LA) to leaf dry mass (LDM). Other leaf

measurements included leaf water content (LWC), leaf thickness (LT), and leaf density (LD). Before the start of the heat stress treatment, one mature leaf from each plant was harvested, its fresh weight was recorded, and it as immediately soaked in a 50 ml flask filled with water to perform measurements with leaves at full turgor. An image of each leaf was then digitally recorded using an optical scanner (D660U, Canon, Tokyo, Japan). The leaf area was calculated using Image J software (version 1.6, National Institutes of Health). The leaves were then oven dried at 80 °C for two days, and their dry weights were recorded. Leaf thickness was determined using microscopic observation of leaf transverse sections using MICROM (HM400R, Walldorf, Germany) as previously described (Soliman et al., 2012). Leaf density (mg/cm^{-3}), or dry matter concentration, was calculate by dividing leaf dry mass by leaf volume. Leaf volume was determined as the product of leaf area and mean leaf thickness.

Photosynthetic performance

Chlorophyll fluorescence (Fv/Fm) values were measured before the initiation of heat acclimation and at 10-day intervals thereafter. Individual seedlings were maintained in the dark for 20 min for dark adaptation and then the minimum (F_0) and maximal (Fm) levels of fluorescence were measured three times for each individual using a portable photosynthesis measuring system (LI-6400, Li-Cor, Lincoln, Nebraska, USA). Fv/Fm provides an estimate of the maximum quantum yield of PSII (Butler, 1978; Zhou et al., 2015); where heat tolerant plants typically exhibit higher Fv/Fm values (i.e., they have higher photosynthetic performance) than heat sensitive plants.

Oxidative stress

Heat sensitive plants experience greater oxidative stress than heat tolerant plants because plants that are heat sensitive (i.e., experiencing heat stress) produce higher concentrations of H_2O_2 than heat tolerant plants. Hydrogen peroxide (H_2O_2) concentration values were determined according

to the methods described by Soliman et al. (2011). There were two H_2O_2 measurement periods; before the imposition of heat stress and at 40 days of exposure to heat stress. Hydrogen peroxide (H_2O_2) content of leaves was measured using a modified version of the ferrous ammonium sulphate/xylenol orange (eFOX) method described by Cheeseman (2006) and Queval et al. (2008). Leaf extracts were obtained by grinding 50 mg of leaf tissue, first in liquid nitrogen and then in 500 µL of 0.1 M potassium phosphate buffer (pH 6.5) containing 5 mM NaN_3. Extracts were centrifuged at 10,000 rpm (8385 g) at 5ºC for 5 min. For every 200 µL of the extract, 5 mL of the solution containing 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 100 µM xylenol orange, 1% ethanol, and 25 mM H_2SO_4 were added. The assay consisted of measuring the difference in absorbance between 550 nm and 800 nm, after 15 min, with a spectrophotometer.

Statistical analyses

Analysis of variance (ANOVA) was used to test for significance differences between the two parental cultivars and among the progeny for leaf growth traits; and among the progeny for Fv/Fm values and H_2O_2 content, at different days of exposure to heat stress. Because the same plants were used to measure Fv/Fm values over time, and these data were not independent of each other, we used one-way repeated measures multivariate analysis of variance (MANOVA) to test whether the two cultivars were significantly different. We used a t-test to test for significant differences in the H_2O_2 content between two cultivars before and at 40 days of heat stress. A random-effects regression model was used to assess the relationship between Fv/Fm values and H_2O_2 content at 40 days after the imposition of heat stress, with Fv/Fm values and H_2O_2 content as random variables. All statistical analysis were carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

238

239 # Results

240 ## Prior to heat stress treatment

241 Before the imposition of heat stress, there were no statistically significant differences in
242 chlorophyll fluorescence (Fv/Fm) values and hydrogen peroxide (H_2O_2) concentration values for
243 the two parental cultivars (Figs. 1 and 2).

244 Prior to experiencing heat stress, the two parental cultivars did not exhibit significant
245 differences for three of five leaf growth traits (Fig. 3). The two exceptions to this patter were leaf
246 water content and leaf thickness. Conversely, significant variation was observed in all leaf traits
247 among the progeny (Table 1), and they exhibited a normal distribution for all five leaf growth
248 traits (Fig. 3). The majority of the 72 progeny had values of leaf growth traits beyond the values
249 of the two parental cultivars. However, for leaf water content, 65% of the progeny had values
250 that were intermediate to the values of the two parental cultivars (Fig. 3c). Seventy of 72 progeny
251 had larger leaf area values compared to the two parents (Figure 3a). Most of the progeny
252 generally had higher specific leaf area, thinner leaves, and higher leaf density compared to the
253 two parents (Figures 3b, 3d and 3e, respectively). There was no relationship (no significant
254 correlations) between leaf growth traits and Fv/Fm values and H_2O_2 concentrations at 40 days
255 after the imposition of heat stress (data not shown).

256 ## Response to heat stress: parental cultivars

257 The two parental cultivars did not exhibit statistically significant differences in Fv/Fm values for
258 the first three measurement periods (0 d) and after the imposition of heat stress (10 d and 20 d).
259 However, the two parents did show significant differences in their Fv/Fm values at 30 d and 40 d
260 of heat stress, with the Kangaroo Valley cultivar having higher values (i.e., higher photosynthetic

performance) (Figs. 1 and 4a). After 40 d of to heat stress, both parental cultivars had higher H_2O_2 values compared to before the imposition of stress. At 40 d of exposure to heat stress, Norlea, the heat-sensitive cultivar, had significantly higher H_2O_2 content than the Kangaroo Valley cultivar (Figs. 2 and 4b). This result indicates that the Norlea cultivar experienced more oxidative stress under these conditions.

Response to heat stress: progeny array

Fv/Fm values for the 72 progeny were significantly different for all time periods measured (0 d to 40 d) (Table 2). Fv/Fm values of the progeny decreased gradually with increased duration of heat stress (Table 2), and a broad distribution of Fv/Fm values was observed at 40 d (Fig. 4a). Approximately 90% of the progeny had Fv/Fm values that were intermediate to the values of the two parental cultivars.

The progeny had statistically significant variation in H_2O_2 values both without heat stress (at 0 d) and at 40 days after the imposition of heat stress (at 40 d). Additionally, the progeny undergoing heat stress experienced an increase in their H_2O_2 content (Table 2, Fig. 4b). At 40 days after the imposition of heat stress, approximately 67% of the progeny had H_2O_2 values that were equal to, or intermediate to, the two parental cultivars.

At 40 days of exposure to heat stress, the two cultivars and their progeny exhibited a significant inverse relationship ($r = -0.54$) between Fv/Fm values and H_2O_2 values (Fig. 5). Most of the data points for the progeny in Figure 5 cluster near the Kangaroo Valley cultivar, which served as the maternal parent in the cross that produced the progeny.

Discussion

Perennial ryegrass is one of the most common pasture and turf grasses in temperate climate regions around the globe (Bolaric et al., 2005a; 2005b; Wang et al., 2009). Because it is a cool-season grass, it is thought to be sensitive to heat stress (Li et al., 2020); however, plant breeders have also developed heat tolerant cultivars (Wilkins, 1991). In addition, because many strains of these cultivars are derived from a limited number of individuals, and possess limited genetic diversity (Guthridge et al., 2001), they can function similarly to inbred lines. These features of the two perennial ryegrass cultivars proved useful in designing our study. First, the Kangaroo Valley cultivar is heat tolerant and Norlea is heat sensitive; therefore, these two cultivars are genetically and phenotypically distinct. Second, different seeds of each of the two strains are genetically (and phenotypically) uniform, thus we could reliably use different seeds of each cultivar to generate the progeny and in the heat stress experiment.

The results of the current study are generally consistent with others that have assessed photosynthetic performance and oxidative stress with heat stress in heat tolerant and heat sensitive cultivars of perennial ryegrass (Wehner and Watschke, 1981; Jiang and Huang, 2001; Zhou and Abaraha, 2007; Soliman et al., 2011; 2012; Li et al., 2020). These results show that heat tolerant cultivars of perennial ryegrass had significantly higher photosynthetic performance (higher Fv/Fm values) and lower leaf H_2O_2 content, compared to heat sensitive cultivars. At 40 days of heat stress, approximately 90% of the progeny had Fv/Fm values that were intermediate to the values of the two parental cultivars and 67% of the progeny had H_2O_2 concentrations intermediate to their two parents. Conversely, other members of this progeny array have phenotypic trait values beyond their two parental cultivars.

The phenotypic trait distribution for the five leaf growth traits, Fv/Fm values, and H_2O_2 content for the 72 progeny is consistent with the distribution expected for traits that are

306 determined by multiple loci (i.e., they are quantitative genetic traits) (Falconer and Mackay,
307 1996). The distribution for these phenotypic traits indicates considerable additive genetic
308 variation within this progeny array, which resulted from crossing the Kangaroo Valley and
309 Norlea cultivars. This variation was generated by genetic recombination during gamete
310 formation by the parental plants. In addition, the clustering of many progeny data points near the
311 Kangaroo Valley cultivar, which served as the paternal parent in the cross, may signal the role of
312 dominance (the Kangaroo Valley cultivar possesses dominant alleles) or epistatic interactions in
313 the expression of photosynthetic performance and leaf H_2O_2 content in the progeny (Falconer
314 and Mackay, 1996). Determining the relative contributions of additive genetic variation, and
315 other genetic processes, in the expression of these two quantitative traits should be the focus of
316 future research.

317 The photochemical efficiency of photosystem II (PSII), measured by chlorophyll
318 fluorescence (Fv/Fm), is the most sensitive component associated with photosynthesis and it is
319 used commonly to evaluate heat tolerance in plants (Maxwell and Johnson, 2000). Under
320 elevated temperatures, ROS are produced through specific metabolic pathways such as
321 photosynthesis and photorespiration (Queval et al., 2008). The generation of ROS results from
322 disrupted balance between photochemical and biochemical reactions inhibiting the
323 photosynthesis process (Wahid et al., 2007). Plants however have developed several mechanisms
324 for tolerance to stress such as antioxidant enzymes and heat shock proteins.

325 The distribution of Fv/Fm and H_2O_2 values among the progeny suggests the genetic variation
326 for the genes responsible for heat tolerance. These genes control antioxidants activity and the
327 formation of heat shock proteins, which in turn inhibit the formation of ROS and maintain
328 membrane stability and thus increase photosynthetic efficiency, improve plant growth, and allow

329 plants to endure heat stress. These results suggest that the difference in heat tolerance shown by
330 the 72 progeny analyzed in this study is closely associated with the ability to suppress oxidative
331 stress. This is consistent with previous findings among cultivars of perennial ryegrass (Soliman
332 et al., 2011, 2012).

333 Leaf growth traits also play important roles in plant acclimation to environmental stress
334 (Terashima et al., 2018). We did not however detect a relationship between the leaf growth traits
335 we measure prior to the imposition of heat stress and Fv/Fm values and H_2O_2 content at 40 days
336 after the imposition of heat stress. Results of the current study differ from those of our previous
337 findings with other perennial ryegrass cultivars (Soliman et al., 2011), which showed significant
338 relationships between leaf traits, especially leaf thickness, and ROS generation and heat
339 tolerance. This discrepancy likely results from genetic difference of the parental cultivars used in
340 the previous study. Clearly, heat tolerance is a complex phenotypic trait governed by many
341 factors, not least of which is the genetic background of the plants (cultivars) being studied.
342

343 ## Conclusions

344 To the best of our knowledge, this study represents the first assessment of the genetic basis of
345 heat tolerance in perennial ryegrass. This study combined physiological measurements (Fv/Fm
346 and H_2O_2 content) within a genetic framework (i.e., parent-offspring comparison) to assess the
347 inheritance of heat tolerance in this grass. Based on the specific cross conducting in this study
348 (the Kangaroo Valley and Norlea cultivars), our results indicate considerable additive genetic
349 variation within this progeny array. This diversity could be used to improve heat tolerance in
350 cultivars of perennial ryegrass using conventional plant breeding, and could also facilitate
351 marker-assisted breeding and/or pave the way for characterizing the underlying genetic and

genomic factors which could be useful for developing plants with improved heat tolerance (Sreenivasulu et al., 2007; Barnabás et al. 2008; Tricker et al., 2018).

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Table 1(on next page)

Table 1. Analysis of Variance for chlorophyll fluorescence (Fv/Fm) and hydrogen peroxide content (H_2O_2, µmol $mgFW^{-1}$) among 72 genotypes at different durations of continuous exposure to heat stress.

1
2

Table 1. Analysis of Variance (ANOVA) for leaf growth traits for the two parental cultivars and the 72 F1 progeny, prior to the imposition of heat stress.

Leaf growth traits	Parental cultivars			72 F1 progeny	
	Norlea	Kangaroo	F value	Range	F value
Leaf area (cm^2)	1.72	1.81	0.07^{ns}	0.87 ~ 7.44	7.48^{***}
Specific leaf area ($mm^2\ mg^{-1}$)	26.96	24.43	1.01^{ns}	19.0 ~ 35.4	3.38^{***}
Leaf water content (%)	81.3	77.5	8.96^{*}	74.5 ~ 85.2	4.76^{***}
Leaf thickness (μm)	169	198	7.75^{*}	141 ~ 242	5.68^{***}
Leaf density ($mg\ cm^{-3}$)	221	211	0.27^{ns}	153 ~ 328	7.93^{***}

* and *** indicate the level of statistical significance at $P < 0.05$ and $P < 0.001$, respectively.
^{ns} indicates no statistical differences for the two parental cultivars for three leaf growth traits.

3

Table 2(on next page)

Table 2. Analysis of Variance for leaf traits between the two parents and among the 72 genotypes derived from them before exposure to heat stress.

1

Table 2. Analysis of Variance (ANOVA) for chlorophyll fluorescence (Fv/Fm) values and hydrogen peroxide (H_2O_2, μmol $mgFW^{-1}$) content among the 72 F1 progeny at different days of continuous exposure to heat stress.

Days of exposure	Range	F value
Chlorophyll fluorescence (Fv/Fm)		
0 day	0.762 ~ 0.807	2.71***
10 day	0.714 ~ 0.783	2.68***
20 day	0.603 ~ 0.778	3.01***
30 day	0.358 ~ 0.776	4.58***
40 day	0.483 ~ 0.767	25.43***
Hydrogen peroxide (H_2O_2)		
0 day	0.15 ~ 0.52	25.76***
40 day	0.32 ~ 1.74	27.95***

*** indicates the level of statistical significance at $P < 0.001$.

2

3

Figure 1

Temporal changes in chlorophyll florescence (Fv/Fm) values for the two parental cultivars, heat sensitive Norlea (N4) (●) and heat tolerant Kangaroo Valley (K7) (○), after imposition of heat stress. * represents the level of statistical significance at P

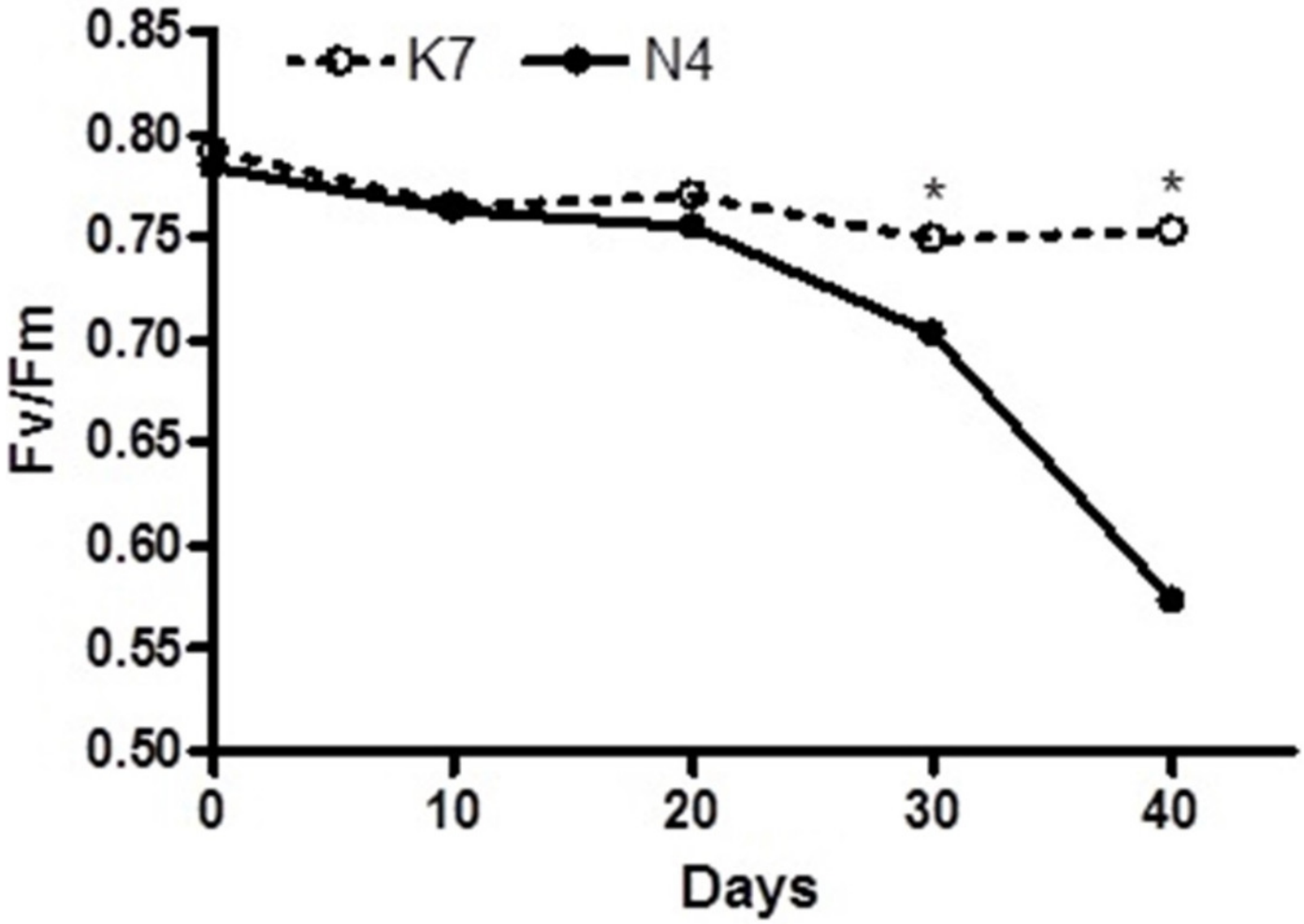


Figure 2

Hydrogen peroxide (H_2O_2) content before and after the imposition of heat stress in the two parental cultivars; heat sensitive Norlea (N4) and heat tolerant Kangaroo Valley (K7). * represents the level of statistical significance at P <

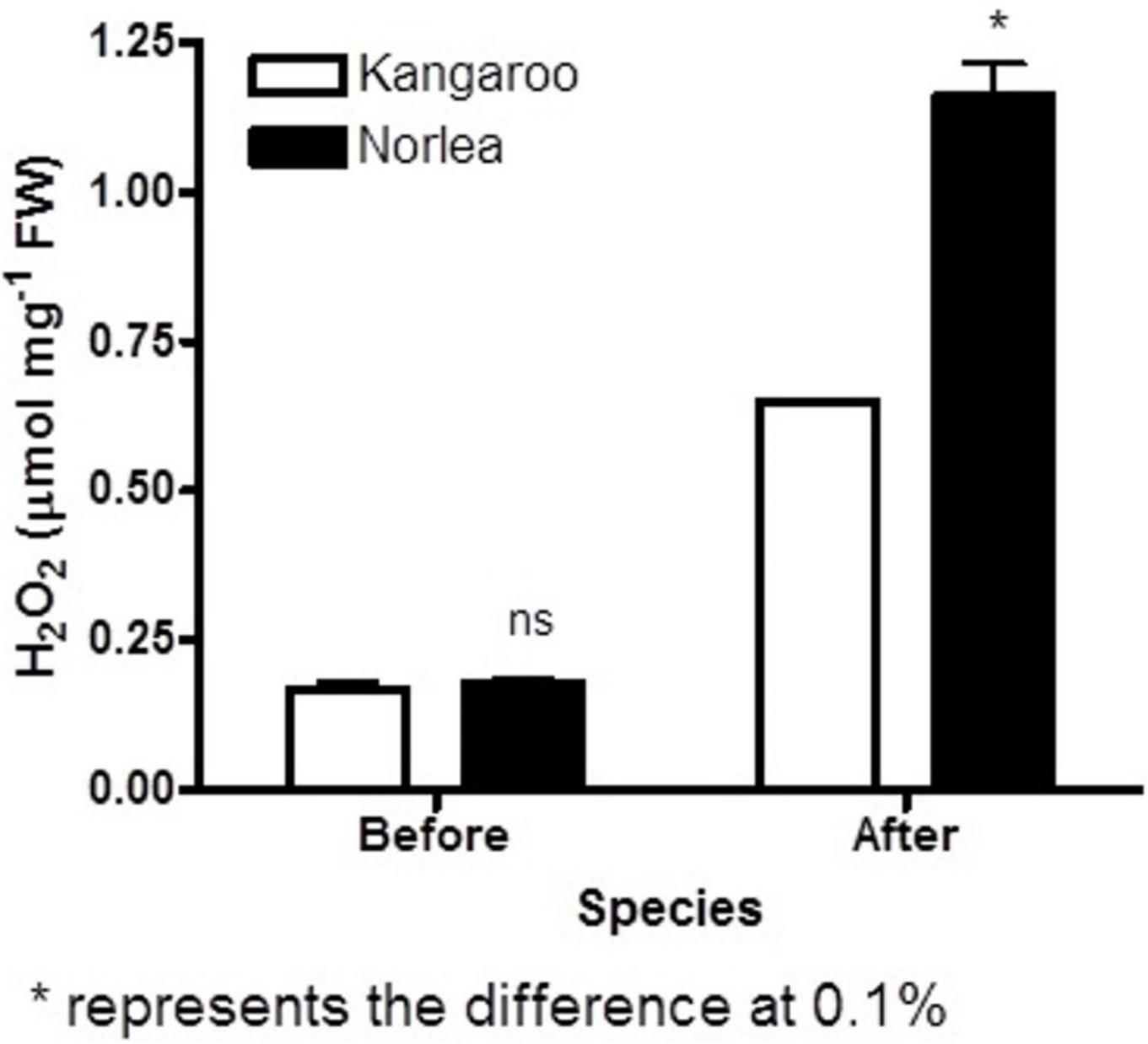


Figure 3

Frequency distributions of leaf area (a), specific leaf area (b), leaf water content (c), leaf thickness (d) and leaf density (e) for the 72 F1 progeny and the two parental cultivars, heat tolerant Kangaroo Valley (K7) and heat sensitive Norlea (N4), prio

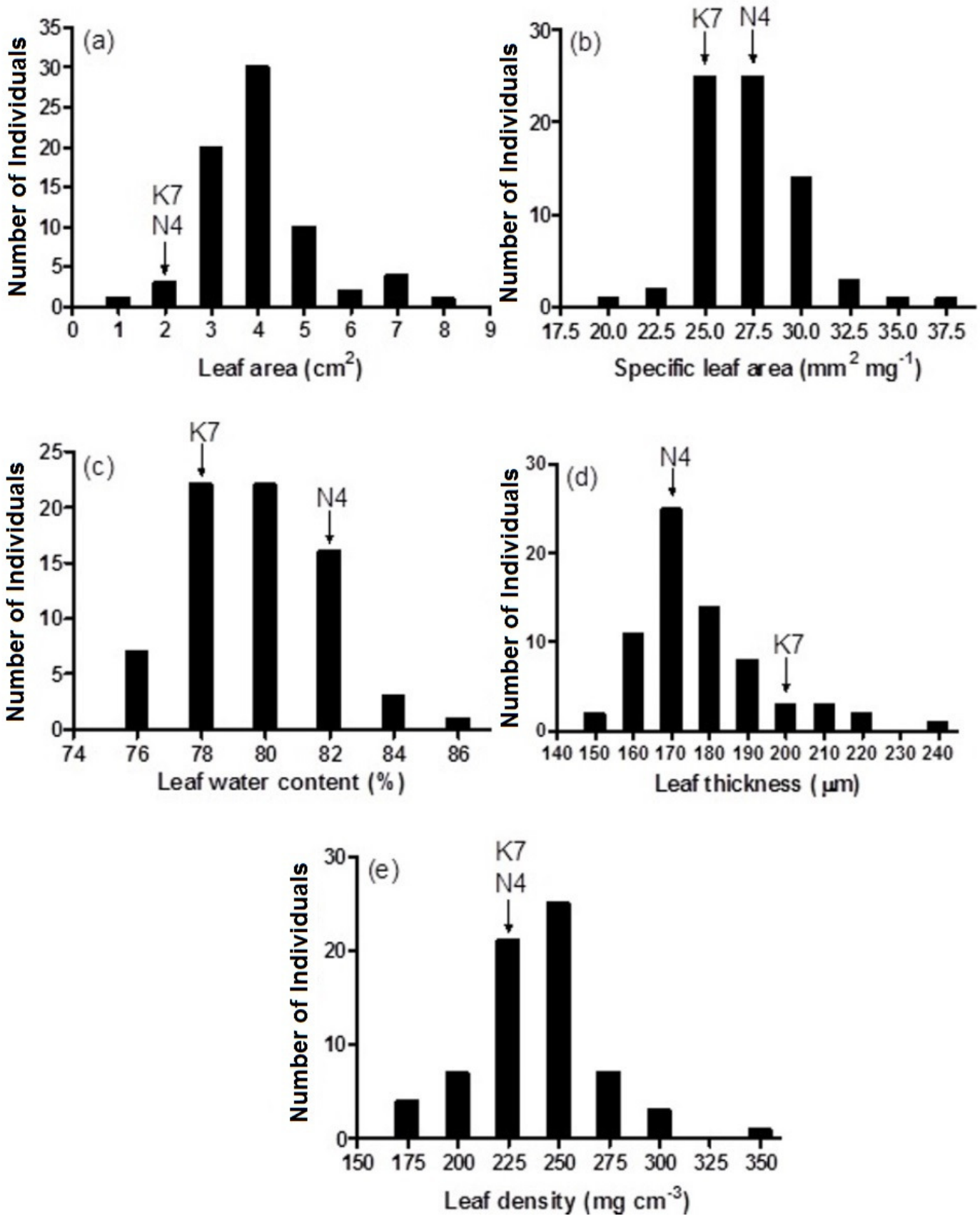
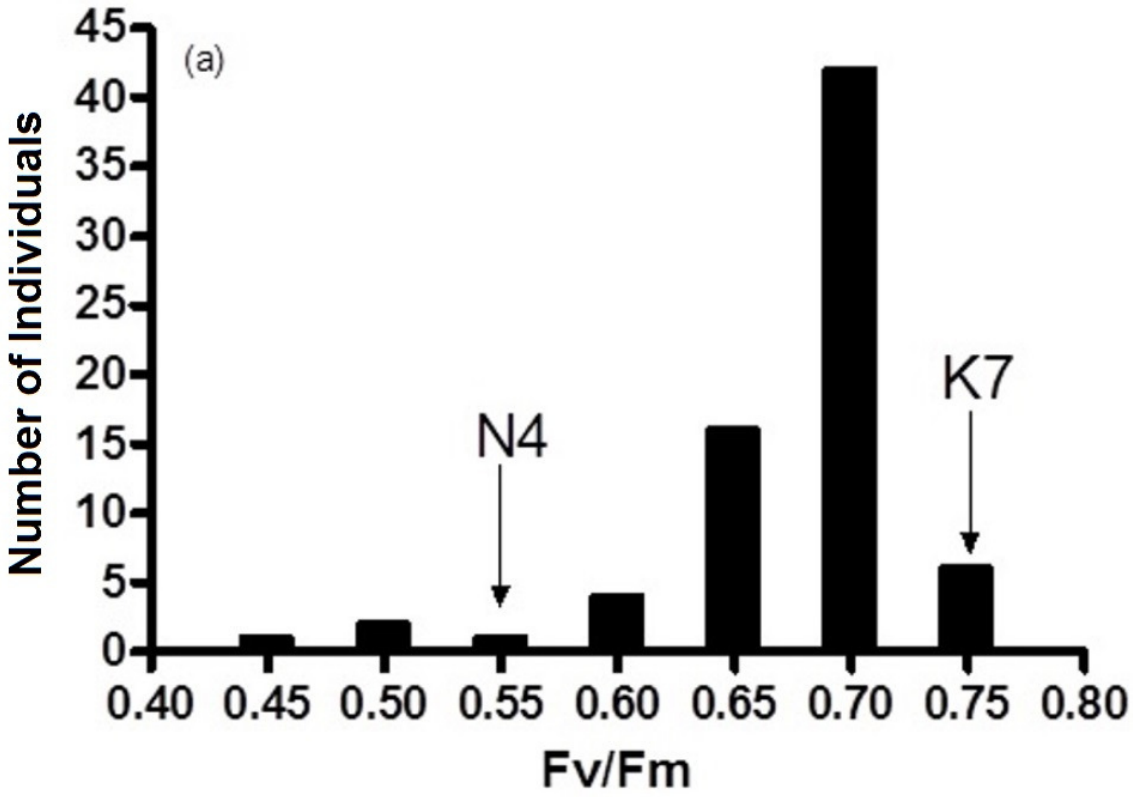

(a)
Number of Individuals
K7
N4
Leaf area (cm²)
(b)
K7
N4
Number of Individuals
Specific leaf area (mm² mg⁻¹)
(c)
K7
N4
Number of Individuals
Leaf water content (%)
(d)
N4
K7
Number of Individuals
Leaf thickness (μm)
(e)
K7
N4
Number of Individuals
Leaf density (mg cm⁻³)

Figure 4

Frequency distribution of chlorophyll fluorescence (Fv/Fm) values (a) and hydrogen peroxide (H_2O_2,) content (b) for the 72 F1 progeny and the two parental cultivars, heat tolerant Kangaroo Valley (K7) and heat sensitive Norlea (N4), at


(a)
Number of Individuals
45
40
35
30
25
20
15
10
5
0
N4
K7
0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80
Fv/Fm

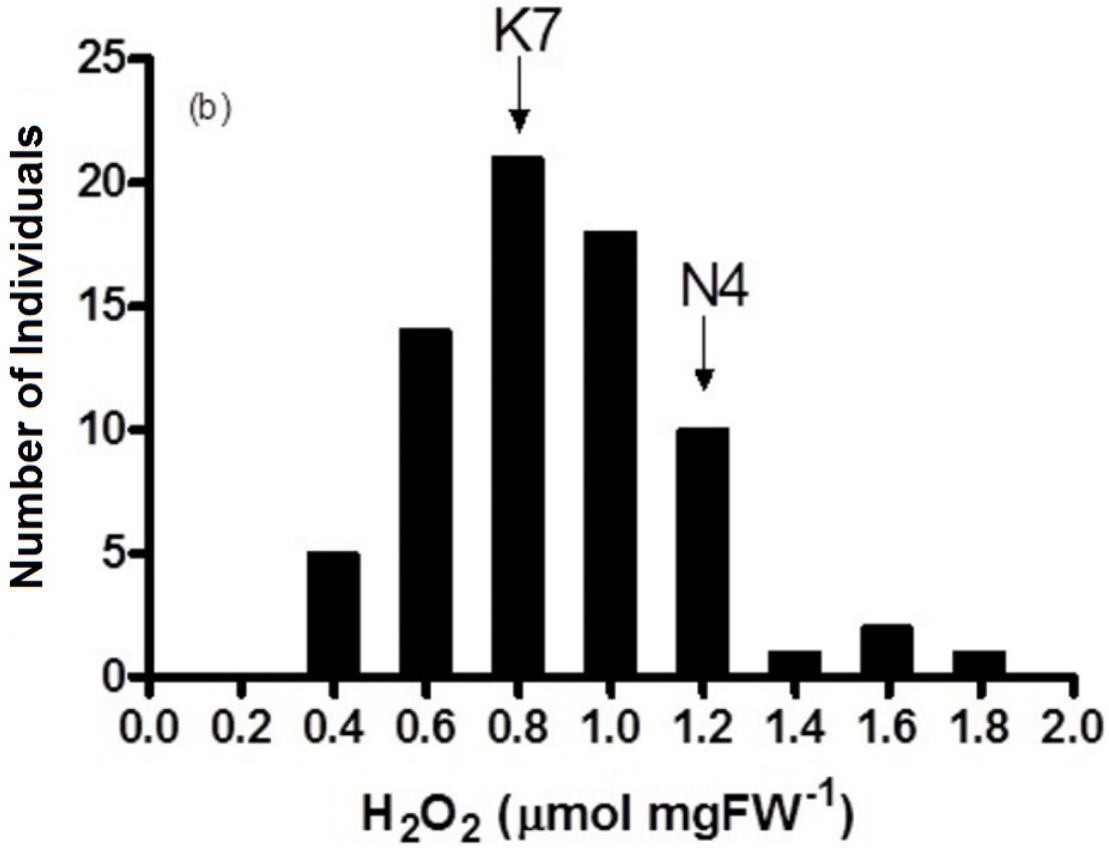

(b)
Number of Individuals
25
20
15
10
5
0
K7
N4
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
H2O2 (µmol mgFW-1)

Figure 5

Correlation between chlorophyll fluorescence (Fv/Fm) values and hydrogen peroxide (H_2O_2) content for the 72 F1 progeny and the two parental cultivars, heat tolerant Kangaroo Valley (K7) and heat sensitive Norlea (N4), at 40 days of exp