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Resilient Response to Combined Heat and Drought Stress Conditions of a Tomato Germplasm Collection, Including Natural and Ethyl Methanesulfonate-Induced Variants

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Abstract: Agricultural systems are currently facing significant issues, primarily due to population growth rates in the context of global climate change. Rising temperatures cause plant heat stress and impact crop yield, which in turn compromises global food production and safety. Climate change is also having a significant impact on water availability around the world, and droughts are becoming more frequent and severe in many regions. The combined effect of both heat and drought stresses increases plant damage, resulting in reduced plant development and productivity loss. Therefore, developing heat–drought-tolerant crop varieties is crucial for enhancing yield under these challenging conditions. Tomato (*Solanum lycopersicum* L.), a major vegetable crop highly appreciated for its nutritional qualities, is particularly sensitive to extreme temperatures, which have a significant negative impact on tomato fruit setting and cause male gametophyte abortion. In this work, a classical genetic approach was employed to identify tomato genotypes showing a resilient response to combined heat and drought stress conditions. A phenotype screening of a natural germplasm collection and an ethyl methanesulfonate (EMS) mutagenized population resulted in the identification of a significant number of tomato lines tolerant to combined heat and drought conditions, specifically 161 EMS lines and 24 natural accessions as tolerant. In addition, TILLING and Eco-TILLING analyses were used as proof-of-concept to isolate new genetic variants of genes previously reported as key regulators of abiotic stress responses in different species. The identification of these variants holds the potential to provide suitable plant material for breeding programs focused on enhancing tomato resilience to adverse climate conditions.

Keywords: EMS collection; heat and drought stress; natural accessions; *Solanum lycopersicum*; TILLING

1. Introduction

Global climate change is without a doubt the greatest challenge for humanity nowadays. This phenomenon causes global temperatures to gradually increase throughout the planet, most of the time accompanied by a reduction in precipitation, which diminishes

water availability and increases the occurrence of extreme weather events [1]. Given these challenging environmental conditions, agricultural systems face great difficulties in maintaining optimal crop production despite higher temperatures and water scarcity. Indeed, the combined stresses have been shown to adversely affect plant growth, as proved by the 21 and 40% yield reductions observed in wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) [2], as well as the 28% yield reduction accounted for in tomato [3]. Therefore, the development of new crop varieties better adapted to these extreme conditions has become crucial for enhancing crop productivity and ensuring access to food.

Tomato (*Solanum lycopersicum* L.) is a major crop with a global production of 186,107,972.48 million tons in 2022, according to FAO, and one of the most widely consumed as part of the Mediterranean diet [4], where its major production areas in Europe are located (https://www.fao.org/statistics/, accessed on 16 May 2024). It is also one of the most appreciated vegetables due to its nutritional qualities, like a higher content of antioxidants such as polyphenols, β-carotene, and lycopene, as well as essential minerals like manganese and zinc [5]. Tomatoes are grown in a variety of climate zones and are frequently subjected to extreme temperature stress, either in greenhouses or when grown outdoors [6]. Temperatures ranging from 25 to 30 °C during the day and 20 °C at night have been reported to be ideal for tomato growth [7]. Nevertheless, even a small increase in any of these values can have a severe impact, since fruit setting has been reported to be interrupted when day and night temperatures exceed 26 and 20 °C, respectively [8]. This sensitivity is reflected in alterations in male gametophyte development such as inadequate anther dehiscence and abnormal tapetum development in the early phases of pollen formation, ultimately causing male sterility, flower loss, and a significant decrease in yield [9]. Also, the impact of drought is quite significant in this crop since flower bud abscission and a reduction in photosynthetic parameters have been reported under reduced watering conditions [10].

Over the past few decades, huge efforts have been made to genetically improve this species, although breeding activities have been focused mainly on fruit quality traits [11]. Thus far, tomato heat tolerance breeding programs have been scarce and mostly carried out in the same production regions, thus not considering climate-adverse conditions [12]. Furthermore, these programs have outlined the limited genetic basis of cultivated tomatoes for heat tolerance, which has sparked interest in exploiting tomato wild relatives, frequently used as sources for abiotic and biotic stresses [13]. The challenges posed by climate change make it necessary to develop tomato varieties suitable for production under this agronomic scenario. One appropriate solution to improve tomato performance under heat-stress conditions and mitigate production losses is the exploration of new sources of tolerance. Natural germplasm is revealed to be a useful material for broadening the genetic basis of tomato abiotic stress tolerance by means of different breeding strategies. However, it may not be sufficient. The key lies in combining the exploration of natural variation with the implementation of large-scale mutagenesis programs, enabling the generation of new tolerance alleles and thereby increasing genetic diversity.

With the aim of identifying new sources of resilience to climate change impact, we evaluated germplasm collection under combined heat and drought stress conditions. This collection includes an ethyl methanesulfonate (EMS) mutagenized population [14] and natural germplasm accessions represented by wild and cultivated tomato species. The tolerant variants identified provide valuable insights into understanding the genetic basis of tomato thermotolerance and, simultaneously, serve as suitable materials for breeding programs aimed at increasing tomato resilience to adverse climate conditions.

2. Materials and Methods

2.1. Plant Material

EMS mutant population has been developed by a chemical mutagenesis program carried out in the cv. Moneymaker background of *S. lycopersicum* [14]. As a result, 7769 M2 families were phenotyped. Regarding the natural germplasm collection, we have screened a total number of 395 accessions, including wild accessions of *S. habrochaites*, *S. pimpinelli-*

folium, *S. galapagense*, *S. chilense*, *S. arcanum*, *S. chmielewskii*, *S. corneliomuelleri*, *S. neoricki*, *S. huaylense*, *S. peruvianum,* and *S. pennelli*, as well as *S. lycopersicum* var. *cerasiforme* accessions, landraces, modern cultivars, and advanced breeding lines. This plant material belongs to the germplasm collections of COMAV-Universitat Politècnica de València (UPV) and Consiglio per la Ricerca in Agricoltura e l'analisi dell'economia agraria (CREA) and is listed in Supplementary Table S1. Both collections were initially screened under natural field conditions during the summer seasons of 2018 and 2019.

2.2. Nursery Screening Trials

Nursery screenings were conducted under combined heat and drought stress conditions during the summer seasons of 2018 and 2019, named summer2018 and summer2019, respectively. Seeds were sown in polypropylene trays with 150 cells containing a substrate mixture of blond peat and coconut fiber in a 3:1 proportion. The germination rate was assessed two weeks after sowing. M2 families, each including 5–12 young plants, were then transplanted to individual pots to promote their vegetative growth. After a 14-day acclimation period, during which plants were allowed to root properly, stress treatment was implemented by ensuring minimum temperatures of 35 °C during the day and 25 °C at night. These temperatures exceed the optimal range of 25 °C to 30 °C during the day and 20 °C at night established for tomato cultivation [7]. In addition, combined drought stress was induced by reducing water supply to 50% for the first two weeks and then maintaining no irrigation conditions for an additional two weeks. M2 plants were grown in a commercial nursery greenhouse under natural photoperiod conditions. The effects of stress were assessed through visual observations of phenotypic traits related to plant development, such as plant growth reduction, decreased leaf development and expansion, leaf senescence and chlorosis, shoot apical meristem, and leaf necrosis. Temperature threshold values and relative humidity conditions were recorded throughout the entire trial using a Hobo MX2301 data logger.

2.3. Thermography Analysis

The tolerant phenotype of 17 EMS mutant lines, selected in the nursery trials, was further confirmed through thermography analysis under climate chamber conditions. After germination, seedlings were sown in plastic pots containing 180 g of substrate in an 8:3 (v/v) mixture of peat and perlite. Pots were covered with aluminum foil to prevent water losses through evaporation, enabling the determination of the amount of water transpired by each plant during the stress treatment, as assessed by weighting pots daily. Transpired water was calculated as W0-Wx, where Wx represents the pot weight determined each day of the stress treatment and W0 is the pot weight before the stress treatment was applied.

Plants were maintained under optimal conditions to allow proper growth, with a photoperiod cycle of 16 h of light and 8 h of dark, a temperature range of 23 °C to 25 °C, and a relative humidity of 50% to 60%. To implement stress conditions, photoperiod and relative humidity parameters were maintained, and the temperature was increased to 35 °C during the day and 25 °C during the dark period. Regarding plant irrigation, during the growing period, plants were irrigated every three days with 200 mL of half-strength Hoagland solution [15], previously calculated as saturation irrigation. For drought stress, irrigation was reduced to 1/3 of saturation irrigation (60 mL).

Phenotyping was extensively conducted to detect early stress symptoms such as necrosis, wilting, and loss of turgidity. Infrared thermography (IT) was used to measure leaf temperature. Thermal images were taken using an infrared camera, the FLIR T420BX (FLIR Systems, Wilsonville, OR, USA), equipped with a 10 mm lens, and processed with the FLIR ResearchIR software. Stress was maintained for an additional seven days before recovering the plants by watering them with saturation solutions (200 mL). Once the visual signs of stress were fully recovered, a second stress cycle was applied, consisting of withholding irrigation for three days to confirm the tolerant phenotype.

2.4. Combined Drought and Heat Stress Greenhouse Trials

Tomato variants selected as tolerant in the nursery screening trials were further characterized under greenhouse combined stress conditions in two summer trials conducted in 2020 and 2021, named the summer2020 and summer2021 trials, respectively. In this case, our purpose was to assess the effect of stress on reproductive traits, including flower and inflorescence morphology, viable pollen production, and fruit setting under commercial greenhouse conditions. Standard greenhouse fertilization was used until plants were grown and properly rooted in the substrate. Drought conditions were induced by gradually reducing irrigation over three weeks, from 50% to minimum values of 20%, thus reducing nutrient supply as well. Temperature thresholds and relative humidity values were continuously monitored using a Hobo MX2301 data logger. In the summer2020 trial, mean temperature values were 26.7 ± 6.6 °C, reaching maximum values of 45.3 °C, while in the summer2021 trial, they were 26.9 ± 6.6 °C, with maximum values of 44.3 °C. Regarding relative humidity, average values in the summer2020 trial were 62.9 ± 16.4%, whereas in the summer2021 trial, they were 62.6 ± 18.8%. Supplementary Figure S1 includes temperature and relative humidity records for both trials. To assess pollen viability, 2,3,5-triphenyltetrazolium chloride (TTC) staining was employed following the methodology described by Micol-Ponce et al. [16]. For statistical analysis, a completely randomized block design with two blocks of 8 plants each was used. Statistical analyses were performed using a one-way ANOVA and Tukey's HSD post hoc test for the detection of homogeneous groups, both included in the R package agricolae (version 1.3-7; https://cran.r-project.org/web/packages/agricolae/, accessed on 16 May 2024).

2.5. TILLING and EcoTILLING Analysis for Mutation Detection

Targeting-induced local lesions in genomes (TILLING) and ecotype TILLING (EcoTILLING) analyses were performed in the EMS mutant collection and in the natural germplasm collection, respectively. Genomic DNA was isolated from 3-week-old seedlings using the DNAzol® Reagent Kit following the manufacturer's instructions. DNA from individual M2 plants was grouped into 1440 pools, each comprising 32 M2 plants (8 plants × 4 M2 families). In the case of the core collection accessions, plant material was formed by 288 individual genotypes. Mutation detection was conducted using high-resolution melting (HRM) analysis using Idaho LightScanner genotyping equipment. Single nucleotide variants (SNVs) were detected based on the melting temperature difference in PCR fragments amplified from four amplicons designed in the coding sequence of the *SlCBL10* (*Solyc08g065330*) gene. Sequence information for the primer pairs used in HRM analysis is listed in Supplementary Table S2. After SNV detection by HRM, DNA from both individual M2 families and natural variants was analyzed by Sanger sequencing to identify the variant carrying SNV polymorphisms.

3. Results

3.1. Screening for Combined Heat and Drought Stress Tolerance in Nursery Conditions

With the aim of identifying new sources of tolerance to abiotic stress in tomatoes for their introduction into breeding programs, two trials were performed under combined heat and drought stress conditions in the summer cycles of 2018 and 2019. As part of these trials, we screened the tomato EMS mutant collection developed in the Moneymaker (MM) genetic background [14] and a natural germplasm collection. The cultivar MM was used as a control and grown alongside the other plants in each trial.

Trials were conducted under nursery conditions to detect phenotypic alterations caused by combined heat and drought stress in the early vegetative developmental stages. A total of 7769 M2 families and 395 natural accessions were sown for screening under these conditions. In the initial step, the germination rate was assessed, and only families and accessions with five or more plants (a germination rate higher than 40%) were evaluated. Thus, 72.6% and 91.7% of the total sown M2 families and natural accessions, respectively,

were assessed. Concretely, 3459 M2 families were screened in the summer2018 cycle and 2180 M2 families and 362 natural accessions in the summer2019 cycle.

At the initial stages of each trial, which took place in early summer, plants were cultivated until they developed a proper root system and 4–6 fully expanded leaves. At this point, stress was implemented by reducing water availability and increasing temperatures. Vegetative traits such as decreased growth rate, reduced leaf development and expansion, leaf senescence and chlorosis, leaf necrosis, and damage to the shoot apex were assessed. In addition, any other visual symptoms of hypersensitivity or tolerance responses were also noted. An overview of the combined heat and drought stress treatment effects, including phenotype features before and after stress condition implementation, can be observed in Figure 1.

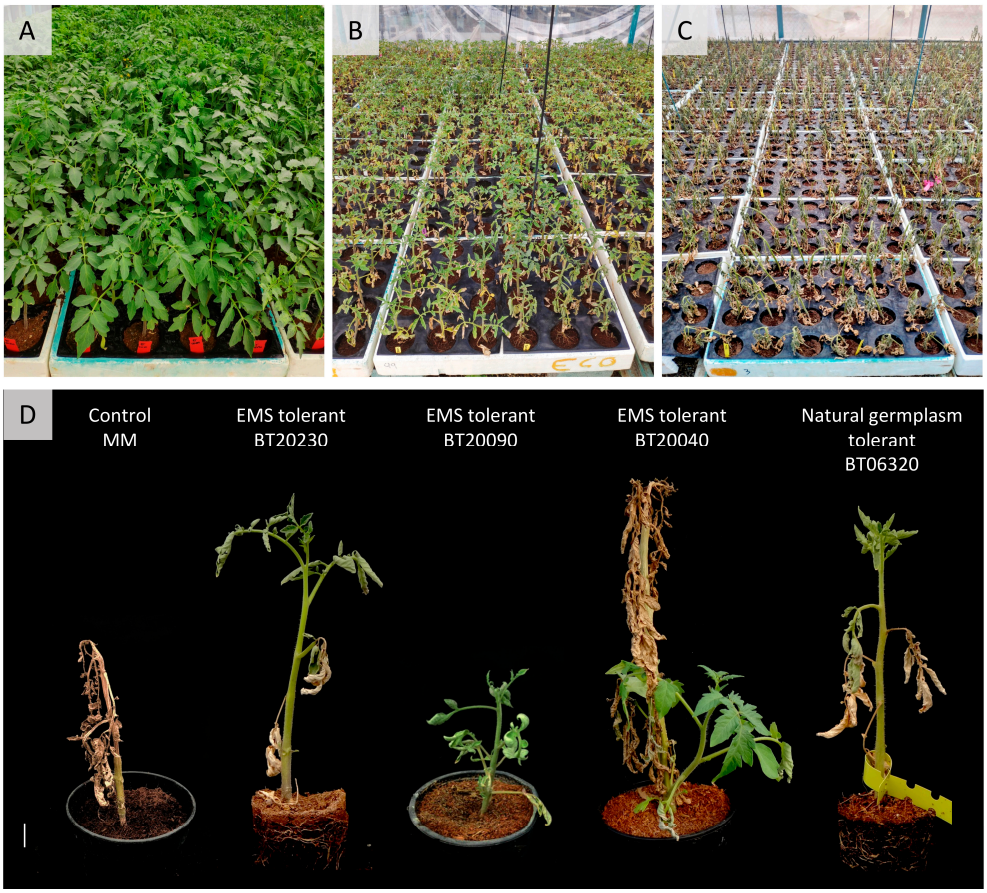

Figure 1. Phenotypic screening for heat and drought stress tolerance performed under nursery greenhouse conditions. (**A**) General aspect before stress implementation. (**B**) After one week of watering reduction to 50%, sensitive plants showed leaf chlorosis and necrosis in basal leaves. (**C**) Severe symptoms are evident after one week of water shortages of 50%, followed by another week of 25% watering. (**D**) Tolerant phenotypes identified during the nursery screenings. Tolerant phenotypes of the EMS BT20230, BT20090, and BT20040 lines, as well as the natural germplasm BT06320 line. BT20360 and BT06320 displayed green and turgid shoot apices, with reduced senescence and chlorosis symptoms. BT20090 showed compact vegetative growth, whereas BT20040 exhibited a regrowth phenotype characterized by the development of an axillary meristem. Scale bars apply to 1 cm.

After completing the stress period, which lasted 48 days in the summer2018 trial and 53 days in the summer2019 trial, most M2 families showed a hypersensitive response to the stress treatment since they showed premature death, extreme chlorosis, necrosis, and/or scarce leaf formation. However, 161 EMS M2 families (2.9% of the total assessed) were identified as including at least one tolerant plant among their members. This tolerance

response was primarily characterized by either the presence of green and turgid shoot apices, the absence of senescence, or reduced chlorosis symptoms in basal leaves. Some of the tolerant lines exhibited compact vegetative growth, while others were also able to develop new axillary shoots after the necrosis of the main shoot apex (Figure 1D). To obtain M3 progenies, tolerant plants were transplanted and grown to maturity under organic greenhouse conditions. Among these lines, 152 (94.4%) displayed a tolerant phenotype fitting a monogenic recessive inheritance pattern, while the remaining ones (5.6%) showed a phenotype segregation consistent with a single dominant mutation. Regarding the natural germplasm collection, we identified 24 tolerant accessions (6.1%) from the wild species *S. peruvianum*, *S. corneliomuelleri*, *S. pimpinellifolium*, and *S. huaylasense*, as well as from *S. lycorpesicum* var. *cerasiforme* and from the cultivated species *S. lycopersicum*. Among them, seven lines exhibited consistent and uniform tolerant behavior (29.2%). On the contrary, a heterogeneous stress response, from absolute susceptibility to great tolerance, was observed within the remaining 17 lines. The tolerant lines identified with both the EMS collection and the natural germplasm, along with the inheritance pattern and the number of detected tolerant plants, are displayed in Supplementary Tables S3 and S4.

3.2. Stress Tolerance of Selected Germplasm under Climatic Chamber Conditions

To further assess the stress tolerance phenotype of a set of 20 M2 families selected during the nursery trials, a new trial under controlled heat and drought conditions was set up. With this aim, 15 plants from each M2 family, along with control plants, were grown in a climatic chamber where growth parameters, i.e., photoperiod, temperature, water availability, and humidity, were precisely determined. When plants had developed four true leaves, a stress treatment consisting of a combination of high temperature and drought stresses was applied (for details, see the Material and Methods section). Daily phenotyping was carried out by assessing parameters such as leaf darkening, loss of turgidity, and symptoms of wilting and necrosis. Furthermore, leaf temperature, which is directly related to water loss by transpiration, was measured daily using an infrared thermography (IT) camera, a non-invasive method that allows the early identification of stress.

A representation of leaf temperature as recorded by the IT camera is shown in Figure 2A, which includes temperatures before the implementation of stress treatment (0 DAS) and those records corresponding to one to three days after stress treatment was applied (1–3 DAS). As a result of this chamber trial, a tolerant response was corroborated for the BT20250, BT20450, and BT20520 families. Whereas the leaf temperatures of sensitive plants continuously increased, the tolerant ones exhibited a thermographic profile characterized by lower temperature values (Figure 2B) and a reduced water evaporation rate (Supplementary Figure S2), even though stress symptoms were not yet evident in sensitive plants. After seven days of stress treatment, tolerant plants exhibited leaflets that remained bright green, showing no signs of wilting or other stress-related symptoms (Figure 2C). In contrast, control and sensitive plants displayed severe wilting and notable chlorophyll degradation, as evidenced by the dark brown color of the leaves and the entire plant (Figure 2C). Conclusive results were challenging to obtain for 11 M2 lines due to the unequal vegetative development exhibited by the plants at the time of assessment. Indeed, tolerance was only detected in the smaller plants, likely due to lower water evaporation. Finally, the stress-tolerant phenotype was uncertain for the remaining six M2 lines, as the plants with thermographic profiles showing lower temperature values ultimately exhibited sensitive symptoms at the end of the stress treatment.

3.3. Characterization of Stress Tolerance Response under Greenhouse Conditions

Given that reproductive traits such as flower number, pollen viability, and fruit setting have a direct impact on crop productivity, we conducted new trials under greenhouse conditions to assess the effects of combined heat and drought stresses on these reproductive traits. In addition, plant height and stem thickness were also assessed to determine the impact of stress on vegetative development. Field trials were carried out during the

summer2020 and summer2021 cycles using a random two-block design of eight M3 plants each. In both trials, M3 progenies, derived from 14 M2 plants identified as tolerant during the previous nursery screenings, were evaluated along with control plants. Temperature and humidity were constantly monitored to ensure that both day and night temperatures exceeded the established threshold values (25 °C during the day and 20 °C at night) [7].

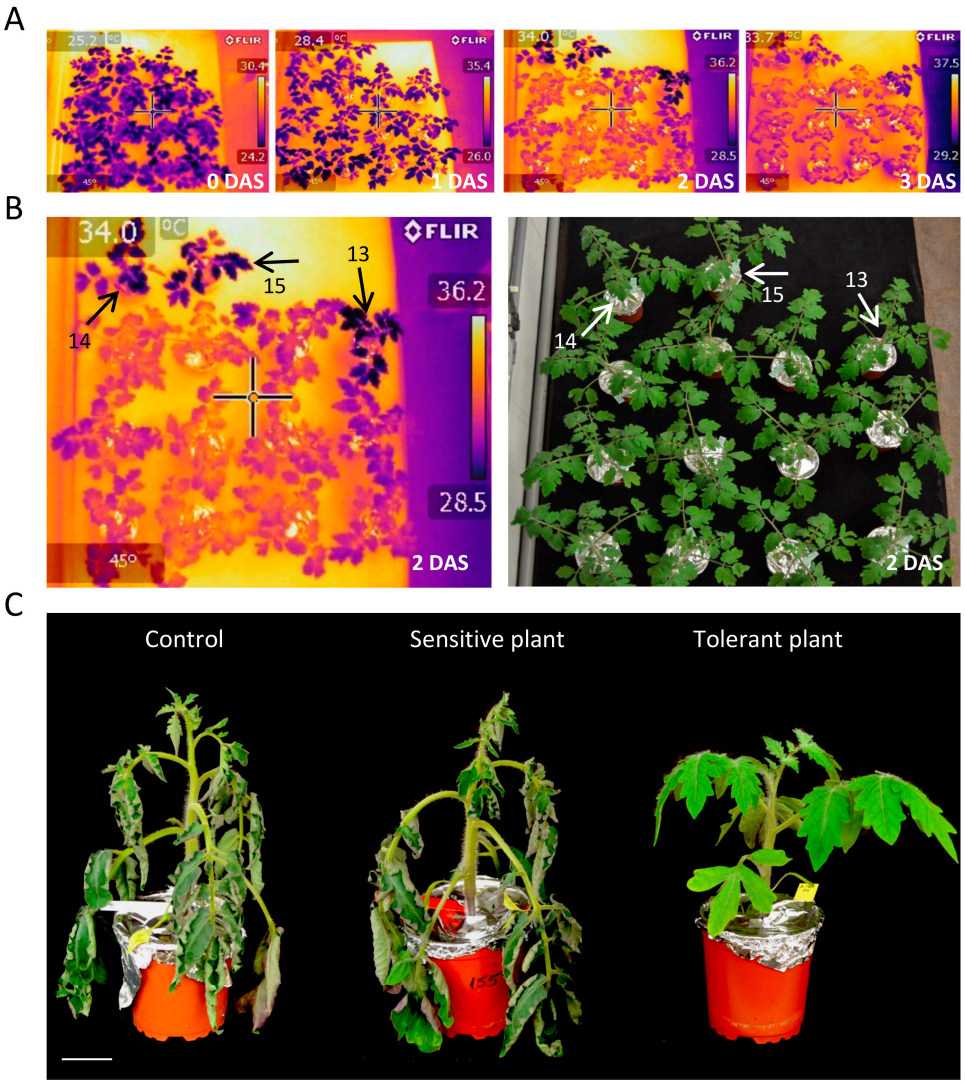

Figure 2. Thermography analysis and drought stress response phenotype of tomato plants grown under controlled chamber conditions. (**A**) Leaf temperature measured with an infrared thermography camera before the stress implementation and one to three days after stress (DAS). (**B**) A closer view of this parameter showed that tolerant plants, marked with arrows, exhibit a thermographic profile characterized by lower temperature values than sensitive plants. (**C**) Seven days after stress implementation, tolerant plants show no symptoms of stress, while control and sensitive plants exhibit evident signs of wilting and browning in their leaflets. Scale bars in (**C**) correspond to 1 cm.

After three weeks of growth under stress conditions, control plants began to display leaf chlorosis and reduced growth compared to tolerant lines (Figure 3A). Other stress-related features observed in control plants included senescence of the shoot apex, a burnt leaf appearance, and curly leaflets. In contrast, leaves of tolerant lines such as BT20330, BT20230, BT21400, BT20220, and BT21210 remained green, fully expanded, and lacked senescence symptoms (Figure 3B,C).

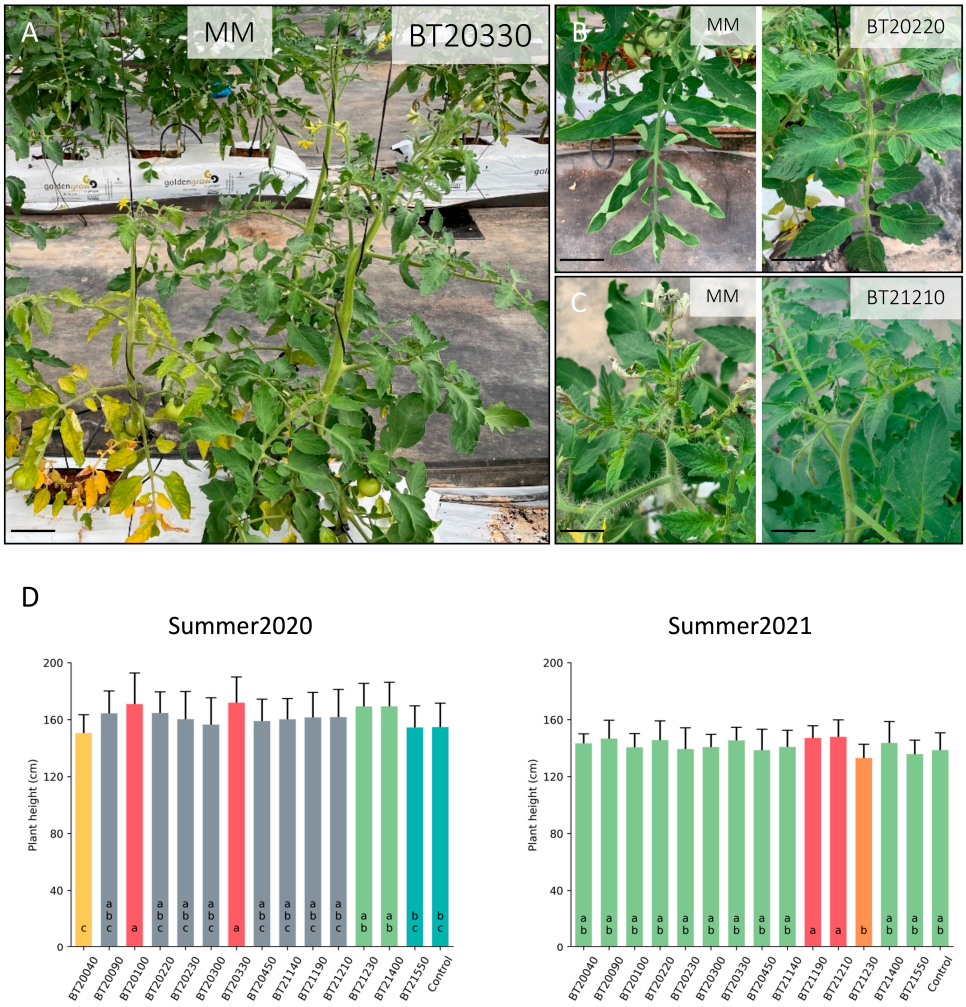

Figure 3. Vegetative traits screened during the summer2020 and summer2021 greenhouse trials. After three weeks of combined drought and heat stress conditions, Moneymaker control plants (MM) show reduced pant growth and evident symptoms of chlorosis when compared to tolerant accessions like BT20330 (**A**). Also, while leaflets of tolerant lines were fully open, in control plants they appeared raised upward on the adaxial side (**B**). Blazing and leaf damage were characteristic of the apical region of MM plants, but these symptoms were absent in tolerant lines (**C**). (**D**) Plant height was analyzed by one-way ANOVA with Tukey's HSD post hoc test for the detection of homogeneous groups (significant differences at $p < 0.05$). Different lowercase letters indicate significant differences among the genotypes. Scale bars correspond to 5 cm (**A**–**C**).

To ensure that observed phenotypic differences in plant height and stem thickness were solely attributed to the stress effect, these vegetative traits were measured before and after the implementation of stress conditions. No significant differences were found between the control and mutant genotypes before the stress conditions were implemented (Supplementary Figure S3). After the stress treatment, significant differences in plant height were observed in the BT20100 and BT20330 lines compared to control plants in the summer2020 trial, while no significant differences were observed for this trait during the summer2021 trial between EMS lines and control MM plants (Figure 3D; Supplementary Tables S5 and S6). It is worth mentioning that the latest trial was affected by an infestation of the *Tuta absoluta* pest, which could be responsible for some of the differences observed between the two trials. Additionally, significant differences in stem thickness were observed in the summer2020 trial for the lines BT20040, BT20090, BT20230, BT20330, and BT21140, which showed higher values than control plants under stress conditions. Interestingly,

similar differences were reproducible in the summer2021 trial for the BT20230, BT20330, and BT21140 lines. (Supplementary Figure S4; Supplementary Tables S5 and S6).

Regarding the assessment of reproductive traits, we focused on fruit setting, as well as the presence of flower and inflorescence damages such as burning and abortion. Three weeks after the onset of stress, unlike the observed response in tolerant lines like BT20230 (Figure 4B), some flowers of control plants began to show burning and inflorescence abortion (Figure 4A). Furthermore, to assess the impact of stress on pollen viability, a tetrazolium staining assay was performed on pollen grains harvested from the first inflorescence produced under these conditions (3rd truss). This analysis not only confirmed that heat and drought conditions have a severe effect on pollen viability (Figure 4C,D), but more importantly, it revealed that tolerant EMS lines identified in this study yielded an increased ratio of viable pollen compared to control plants (Figure 4E,F). As a consequence of the higher pollen viability, enhanced fruit setting was observed in tolerant plants such as BT20220, BT20300, BT20330, BT21210, BT21230, and BT21400 in the summer2020 trial. Despite the presence of the *T. absoluta* pest, significant differences in fruit setting were also noted for a few lines (BT20100, BT20300, and BT21400) during the summer2021 cycle (Figure 4G).

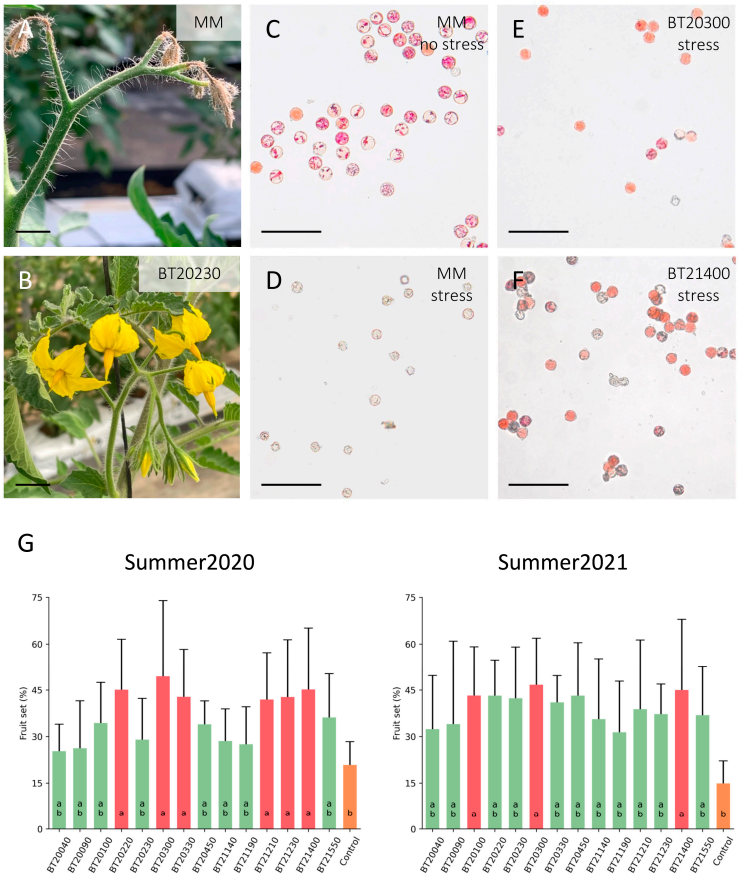

Figure 4. Reproductive traits screened during the summer2020 and summer2021 greenhouse trials. Three weeks after stress conditions were applied, the flowers of control Moneymaker (MM) plants exhibited inflorescence, with flowers showing signs of burning and abortion (**A**). In contrast, tolerant lines like BT20230 showed no damage to their flowers (**B**). Tetrazolium staining assays proved that pollen viability is severely affected in control plants subjected to stress conditions (**D**) compared to control conditions (**C**). Stress does not affect the pollen viability of tolerant accessions like BT20300 (**E**) and BT21400 (**F**). (**G**) Fruit setting percentage was analyzed by one-way ANOVA with Tukey's HSD post hoc test for the detection of homogeneous groups (significant differences at $p < 0.05$). Different lowercase letters indicate significant differences among the genotypes. Scale bars correspond to 1 cm in (**A**,**B**) and to 100 µm in (**C**–**F**).

3.4. TILLING and Eco-TILLING Analysis for Mutant Variant Detection

TILLING and Eco-TILLING analyses were applied as proof-of-concept to isolate new genetic variants that can be used for functional analysis of regulatory genes and as sources of tolerance to abiotic stress in tomato breeding programs. For this purpose, we screened both the EMS mutant and natural germplasm collections for polymorphisms in the CALCINEURIN B-LIKE PROTEIN 10 (SlCBL10) gene (Solyc08g065330). The loss-of-function mutant of this gene shows a hypersensitive phenotype when exposed to salt stress conditions, resulting in severe inhibition of vegetative development and collapse of the shoot apical meristem [17]. Furthermore, the pivotal role of SlCBL10 in ion homeostasis and its function as a positive regulator of the abiotic stress response in tomatoes have been demonstrated in recent years [18,19]. Therefore, the identification of new alleles of SlCBL10 through TILLING analysis could facilitate further research into its biological function and serve as genetic tools for the development of new sustainable tomato varieties.

Specific primers covering most of the coding regions of the *SlCBL10* (*Solyc08g065330*) gene were designed (Figure 5A; Supplementary Table S2). A workflow depicting the setup of the TILLING and EcoTILLING platforms, as well as the HRM analysis, is shown in Supplementary Figure S5. Our analysis revealed 11 putative mutations, with five identified in the natural germplasm accessions and six in the EMS collection. Subsequently, Sanger amplicon sequencing confirmed the presence of seven of these single-nucleotide mutations at the base-pair level in individual plants. Three of these mutations were located in the coding exons of the *SlCBL10* gene, resulting in one synonymous and two non-synonymous mutations (Figure 5B; Supplementary Table S7). Hence, our findings confirm that these reverse genetics strategies are an effective method for detecting mutations in tomato genes related to stress tolerance, and the populations used in this study have the potential to serve as a comprehensive platform for future reverse genetic studies in tomato.

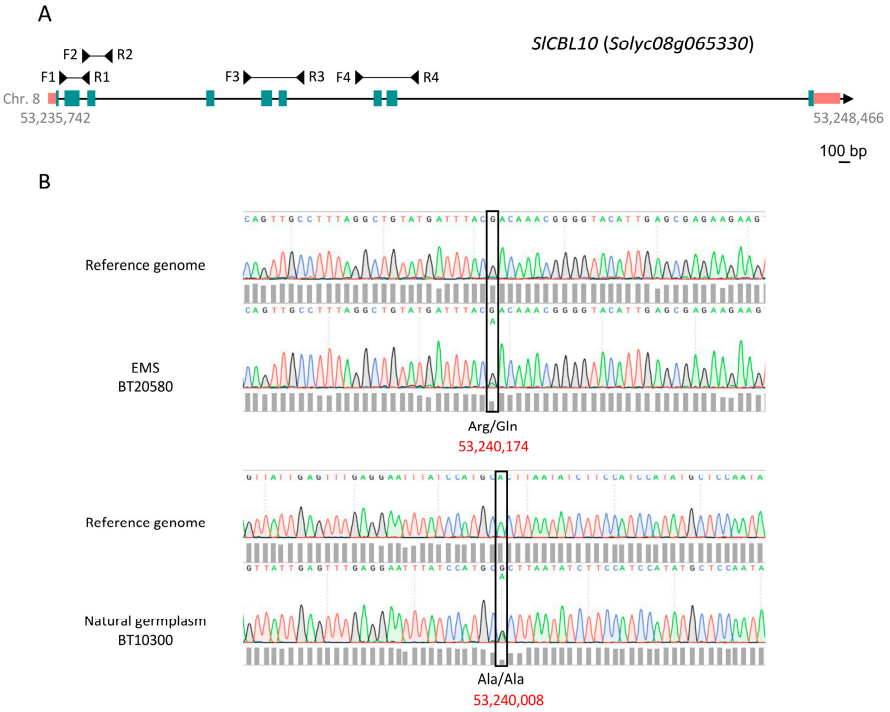

Figure 5. Single nucleotide variant detection by TILLING and EcoTILLING analysis of the *SlCBL10* gene. (**A**) Genomic organization of the *SlCBL10* (*Solyc08g065330*) gene. Coding exons are depicted as green boxes, introns as black lines, and 5′ and 3′ UTR regions as pink boxes. Chromosomic coordinates are also included, as is amplicon location. (**B**) Single nucleotide variants detected by means of High Resolution Melting (HRM) analysis were confirmed by Sanger sequencing in homozygosis in the EMS BT20580 line as well as in heterozygosis in the natural germplasm BT10300 accession.

4. Discussion

As assessed to date by several public and private institutions, global temperature data show a rising trend that makes heat stress a critical issue that must be addressed to ensure global crop yield and productivity [20]. This is particularly serious for tomatoes, a major vegetable crop whose proper growth is severely hampered by high temperatures [9]. Understanding the genetic and physiological responses to heat stress is crucial for improving their agronomic performance under the climate change scenario. With this aim, we characterized a natural germplasm collection, including a set of tomato wild relative accessions, and an EMS-mutagenized population, the latter developed from the commercial cv. Moneymaker, to identify new sources of genetic tolerance to high temperatures.

Some of the tolerant lines identified as part of our study displayed a compact phenotype. Previous works have reported this trait's occurrence, probably as a resource to reduce water evaporation to cope with abiotic stress conditions [21]. This is the case of the EMS heat-tolerant mutant line HT7, obtained from the ornamental Micro-Tom genetic background, which shows a compact phenotype as described by Pham et al. [22]. HT7 plants developed a narrow canopy and had less stomatal density than WT plants under stress conditions, which could contribute to a decreasing transpiration rate. Furthermore, HT7 plants produced a high number of viable pollen grains under long-term heat stress, resulting in high fruit-setting rates and increased fruit yield [22]. In our mutant collection, some tolerant lines, such as BT20090 (Figure 1D), exhibited compact vegetative growth when subjected to combined heat and drought stresses in nursery greenhouse conditions, which supports the idea that this strategy is effective for mitigating adverse climatic conditions.

The impact of heat stress on male gametophyte fertility has been broadly described to date [23–25]. In our greenhouse conditions, thermal stress induced several alterations in the reproductive traits of control and hypersensitive lines, including pollen number and viability. In contrast, tolerant lines showed higher pollen viability rates than control plants, where pollen grains were both fewer and non-viable. Previous studies have highlighted the particular sensitivity of flower buds to heat stress, especially during the 7 to 15 days before anthesis, a critical period that encompasses the meiosis phase [9,26]. Thus, when pollen mother cells are subjected to heat stress, the quality and quantity of pollen are significantly reduced [9]. In addition to reduced pollen viability, control and hypersensitive genotypes showed severe burning of entire inflorescences (Figure 4), which ultimately compromised their yield. Thus, the tolerant plants identified in our screenings demonstrated superior agronomic performance, particularly in terms of higher fruit setting when compared to control ones. Furthermore, under nursery conditions, we identified seven tolerant accessions from wild species such as *S. peruvianum*, *S. corneliomuelleri*, *S. pimpinellifolium,* and *S. huaylasense*. Despite their small fruit size and lack of commercial value, these accessions exhibited remarkable tolerance behavior and serve as valuable genetic resources for broadening the genetic base to improve abiotic stress tolerance in cultivated tomatoes through backcross breeding strategies.

A comprehensive study of the genetic basis of abiotic stress tolerance is essential for developing new resilient varieties. Given the polygenic nature of this trait, advanced QTL analyses have been conducted to dissect the genetic architecture of traits related to stress tolerance in tomatoes [27]. QTLs associated with several reproductive traits affected by temperature, such as flower number, fruit number per truss and fruit set percentage, stigma exertion, and pollen viability, have been identified [28]. Moreover, QTLs have been detected for traits that exhibit high plasticity under high temperatures, such as flowering time, fruit weight, and yield [29], underscoring the ability of tolerant accessions to display different phenotypic features depending on the environmental conditions. Notably, our greenhouse trials have revealed significant variability in the behavior of tolerant accessions from one season to another. This variability is likely attributed to the complexity of heat tolerance, a polygenic trait often associated with plasticity effects in response to changing environmental conditions. Therefore, gaining knowledge of the genomic regions and genes associated with the tolerant phenotype displayed by the accessions identified in our study, which

requires forward genetics analyses involving the development of mapping populations and whole-genome sequencing approaches, will contribute to a deeper understanding of heat and drought stress tolerance in tomatoes.

Furthermore, reverse genetics methodologies may be applied to isolate new drought and heat tolerance alleles. To achieve this goal, we combined extensive phenotyping with TILLING analysis to identify mutations in target genes previously involved in regulating the heat tolerance response in other plant model species. This is the case of the *SlCBL10* gene [17]. The TILLING strategy has proven useful in the past for identifying new mutant lines carrying SNVs in genes controlling tomato thermotolerance [30]. Through this approach, we identified seven new variants of the *SlCBL10* gene among both natural germplasm and EMS mutant collections. While this work focuses on this gene, our TILLING analysis has been extended to include other 14 genes, with the hope of identifying new tolerant variants of genes of interest. Therefore, our work proves that the combination of extensive phenotyping under controlled greenhouse conditions and the use of molecular biology techniques, such as TILLING, enables the identification of novel alleles associated with abiotic stress tolerance in tomatoes. These variants hold a great value for future breeding programs aimed at enhancing tomato resilience to adverse climate conditions.

5. Conclusions

To the best of our knowledge, no tomato accessions exhibiting tolerance to combined abiotic stress (heat and drought) have been reported to demonstrate their resilience during both early vegetative phases of development, carried out under nursery and controlled climatic chamber conditions, and reproductive development, assessed in greenhouse and natural conditions. The fact that these lines have successfully passed three stress screenings under different growing conditions underscores their value as a genetic resource in breeding programs aimed at developing tomato varieties with enhanced tolerance to combined heat and drought stress conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10060552/s1. Supplementary Figure S1: Temperature and relative humidity values recorded during the summer2020 and summer2021 greenhouse trials. Supplementary Figure S2: Water evaporation rate assessed in chamber-controlled conditions. Supplementary Figure S3: Vegetative traits assessed during the summer2020 greenhouse trial before stress treatment initiation. Supplementary Figure S4: Stem thickness values assessed during the summer2020 and summer2021 greenhouse trials. Supplementary Figure S5: General overview of TILLING and EcoTILLING analyses. Supplementary Table S1: Natural germplasm collection screened for heat and drought stress tolerance under nursery greenhouse conditions. Supplementary Table S2: Primers used for the HRM analysis of the *SlCBL10* gene. Supplementary Table S3: Tolerant M2 families identified during the screening of the EMS mutant collection under combined heat and drought stresses at nursery greenhouse conditions. Supplementary Table S4: Tolerant accessions identified during the screening of the natural germplasm collection under combined heat and drought stresses at nursery conditions. Supplementary Table S5: Phenotype traits evaluated during the summer2020 trial under combined heat and drought stresses. Mean ± standard deviations are included for plant height, stem thickness, and fruit setting. Supplementary Table S6: Phenotype traits evaluated during the summer2021 trial under combined heat and drought stresses. Mean ± standard deviations are included for plant height, stem thickness, and fruit setting. Supplementary Table S7. Single nucleotide variants of the *SlCBL10* gene were detected in the EMS and the natural germplasm collections.

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