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Arbuscular Mycorrhizal Inoculation Enhances Endurance to Severe Heat Stress in Three Horticultural Crops

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***Abstract*. Global climate change is increasing temperatures worldwide, which greatly affects all biological relationships. Plant and soil ecosystems are also suffering in this new scenario, especially in semi-arid areas where water resources are limited. Regarding agricultural crops, temperatures that increase dramatically negatively affect fruit production and quality, making it mandatory to find sustainable practices to cope with these new situations. Symbiotic microorganisms in general and arbuscular mycorrhizal fungi in particular have been revealed as promising methods of alleviating stress that are respectful of the environment and soil equilibrium. In this work, we demonstrate the suitability of an ultra-pure, in vitro-issued arbuscular mycorrhizal inoculant for alleviating severe heat stress when applied to three important agricultural crops (tomato: *Solanum lycopersicum* L.; pepper: *Capiscum annuum* L.; cucumber: *Cucumis sativus* L.) under agronomic conditions. Inoculated plants had greatly improved endurance under heat stress because of increased vigor, productivity, and fruit quality. Considering the actual scenario of global climate change, our results shed a light of hope and indicate more sustainable cultivation practices adapted to global change.**

Crop productivity is intimately linked to environmental conditions. For more than 11,000 years, humans have cultivated land to obtain food, and this cultivation is strongly dependent on rainfall, soil conditions, and temperature. New cultivation methods have improved throughout history, resulting in improved fruit production and quality and increased profitability. However, strong dependence on climatic conditions remains, and these conditions change with time, mostly due to the effects of human activity on Earth.

Global climate change is causing rapid variations in parameters such as soil and air temperature (Mellander et al., 2007). The rate of global warming is expected to continue increasing if no mitigation efforts are enabled (Teixeira et al., 2013). By 2080, it is expected that parameter values will reach record numbers in most cropping areas worldwide (Battisti and Naylor, 2009). Agricultural production and, consequently, food security, are strongly affected by temperature, which controls the rate of all metabolic processes leading to biomass and fruit and grain production (Hay and Walker, 1989). Moreover, high temperatures can increase the risk of drought, limit photosynthesis rates, and induce oxidative stress, and all of these negative effects are exacerbated in semi-arid and tropical areas. Of particular interest are the effects induced by short occurrences of extremely high temperatures, which are also known as heat stress (HS) events (Teixeira et al., 2013). Peaks of high temperatures, even when they occur for a few hours, may have dramatic effects on reduced crop production (Porter and Semenov, 2005). HS has both direct and indirect effects on plants, such as lower capacities for water acquisition, changes in Pi acquisition, metabolism, and homeostasis (Pacak et al., 2016), and severe damage to the plasma membrane of cells (Uemura et al., 2006; Zhu et al., 2017). Because HS events are likely to become more frequent with global warming (Tebaldi et al., 2006), it is important to find alternatives to protect crops under such situations.

Among agronomical practices introduced in semi-arid areas in the latest 30 years, the use of plastic greenhouses has become very popular and proved quite successful (Espí et al., 2006). Plastic increases production quality and quantity while reducing the consumption of valuable resources (such as water, pesticides, fertilizers, and energy). Plastics also retain CO_2, protect from wind, and warm the soil, thereby preserving humidity and reducing the leaching of pesticides and fertilizers. When whitened with lime, plastics reduce radiation, thereby lowering temperature up to 5 to 8 °C and protecting plants, roots, and soil structures. Some estimates indicate that without plastics, 60% of all fruit and vegetable production in these semi-arid and fragile areas would be endangered. A southeastern Spanish province, Almeria, is paradigmatic in the use of whitened plastic greenhouses. With a total of ≈32,000 ha covered (Tierra y Mar, 2019), this "plastic sea" that can be seen even from orbital satellites has changed the economy and agronomical practices of the zone, thereby becoming the most important production area for fruits, vegetables, and horts of Europe and one of the most important in the world. Moreover, because crop cultivation is performed during two cycles per year for most products, the real surface of production of this area per year is 43,086 ha. Of these, three of the top agricultural crops are tomato (*Solanum lycopersicum* L., 11,081 ha), pepper (*Capiscum annuum* L., 9325 ha), and cucumber (*Cucumis sativus* L., 4839 ha), rendering total production values of more than 1 million t, 650,000 t, and 450,000 t per year, respectively (data provided by Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía, Spain, 2013). The total income generated in this area by using under-plastic agronomic practices has reached €2333.2 million. It is easy to understand that all types of innovative techniques improving production,

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crop protection, and environmental management are of enormous interest at this zone and, by extension, in all similar agricultural areas.

Another agronomical practice introduced recently is the biological management of agriculture by using macro- and microorganisms (e.g., to induce pollenization, control pests, or reduce water and fertilizer use), which has become a real revolution in the latest years. Among the microorganisms used, arbuscular mycorrhizal fungi (AMF) have been revealed to be crucial in all sustainable practices (Duhamel and Vandenkoornhuyse, 2013; Pagano et al., 2016). This group of soil-borne microscopic fungi establishes an intimate symbiotic relationship with the immense majority of plant roots so that colonized plants no longer have a simple root; instead, they have a new and powerful supra-organ called arbuscular mycorrhiza (AM) for nutrient uptake (Azcón-Aguilar and Bago, 1994). AM are formed by most of the economically important crops, and their functioning consists of bidirectional nutrient transfer between the plant and fungal partner; while the plant provides the fungus with the necessary C resources to overcome its obligate biotrophic nature, the fungus provides the plant with water and mineral and organic nutrients (especially P) very efficiently from the soil via fungal hyphae (Smith and Smith, 2012). Furthermore, AM formation implicates a whole series of changes in plant capacities including, among others, enhanced resistance against pathogens (Pozo et al., 2013) and better adaptation to extreme environments (contaminated soils, drought, extreme temperatures) (Abdel-Latef et al., 2016; Lenoir et al., 2016). As a consequence of this, important amelioration of the plant nutritional status and physiological equilibrium, higher yield, and healthier and more sustainable crop production are obtained (Baum et al., 2015).

Different studies have revealed the important role that AM may have in temperature stress alleviation (Mathur et al., 2018; Zhu et al., 2017). To briefly summarize, and focusing on HS, AM formation increases plant biomass, thereby reducing leaf browning, and has better water use efficiency, water retention capacity, and relative water content. Increased antioxidative enzyme production (such as superoxide dismutase, catalase, and ascorbate peroxidase) and the generation of osmotic-active compounds (such as proline, trehalose, and glomalin) lead to better preservation of the plasma membrane of AM plant cells, thus protecting vegetal tissues against HS. AM plants subjected to HS also have greatly increased photosynthetic rates, stomatal conductivity, leaf transpiration, photosystem II efficiency, concentrations of chlorophyll (a and b) and carotenoids, and photochemical potential. Together with an increased rate in C-metabolism gene expression, soluble sugar content, better P uptake efficiency, assimilation, and use, and enhanced metabolism of N (nitrate, ammonium, and amino acids), AM plants are best-positioned to endure HS and overcome its negative effects. Moreover, mitigation of negative effects of combined drought and HS have been recently reported (Duc et al., 2018). However, to the best of our knowledge, no reports have been generated regarding the impact of AM symbiosis on fruit production and quality under severe HS situations, which are the most interesting issues for any producer.

This study aimed to fulfill that gap by studying, under agronomic plastic greenhouse conditions, the impact of AM inoculation on a severe (>45 °C) HS episode maintained for 11 d for three agricultural crops of key importance to horticulture: tomato, pepper, and cucumber. To achieve this, an ultra-pure, in vitro–issued, gel-based AM inoculant (MYCOGEL) that has largely demonstrated its efficacy in horticultural and extensive crop production in Almeria (Spain) was tested.

Materials and Methods

Experimental setup. The experiment was performed under agronomic conditions in a multi-tunnel plastic greenhouse (12–14 light hours/day; air humidity rates of 55% in the afternoon and 95% in the early morning; air T[a], see Fig. 1) at La Mojonera (Almeria, southeastern Spain). As indicated, this location is one of the highest-producing areas for horticultural crops in Spain, Europe, and the world.

Three different emblematic horticultural crops with high production at Almeria's plastic fields were selected: vine tomato (var. Guanche); orange pepper (var. Snacking); and cucumber (var. Mitre). Plants were acquired from Vicaplant Nursery (La Mojonera, Almeria, Spain) and transplanted individually when they were 54 (tomato), 62 (pepper), and 22 (cucumber) days old (following the common agronomic procedures at this zone) into 2-L pots. Growing substrate consisted of natural clay loam soil (44% sand, 28% loam, 28% clay) extracted from a soil quarry in the vicinity of the greenhouse. Soil analyses yielded the following results (in mg/L): NO_3^-, 14.0; SO_4^{2-}, 84.0; Cl^-, 25.0; Na^+, 12.0; K^+, 2.4; Ca^{2+}, 30.0; Mg^{2+}, 13.0; P (Olsen), 0.82 meq/L; and organic matter, 0.23% (pH 8.5). Pots were filled with the substrate; then, a thin layer of coconut fiber was placed on top of the substrate to avoid water loss. Nine replicates per crop and treatment were prepared. Pots were maintained on top of culture tables at the multi-tunnel greenhouse during the experiment. Therefore, the timing of the experiment started at transplanting, and results were noted to occur on days after transplanting (DAT).

Plants were subjected to two treatments: CONTROL treatment, which consisted of plants that received no AM inoculation (nonmycorrhizal plants); and TREATED treatment, which consisted of plants inoculated with 0.1 mL per plant of the AM commercial inoculant MYCOGEL (Agrocode Biosciences LTD, Almeria, Spain). MYCOGEL is an ultra-pure in vitro–produced concentrated gel containing more than 5×10^7 AMF propagules/L (spores, active extraradical hyphae, and aseptic root pieces colonized by AM intraradical mycelium) of the AMF *Rhizophagus irregularis* DAOM 240403. AM inoculation was performed during seedling transplanting by diluting the concentrated gel with tap water and then applying the resulting dilution (final propagule concentration per plant, 5×10^3 AMF propagules) with a pipette to the soil close to the vicinity of the roots, thus simulating drip irrigation. Special care was taken when facilitating fungal propagules so that they would be in close contact with roots, thus inducing quick colonization.

No P was amended to any of the treatments for 2 weeks after inoculation so that there would be no interference with AMF propagule development, growth, and installation within the root tissues. After this "protective" time, fertilization was applied to both treatments following the usual procedures and doses for these crops. Water management was also identical in both treatments and consisted of three applications per week

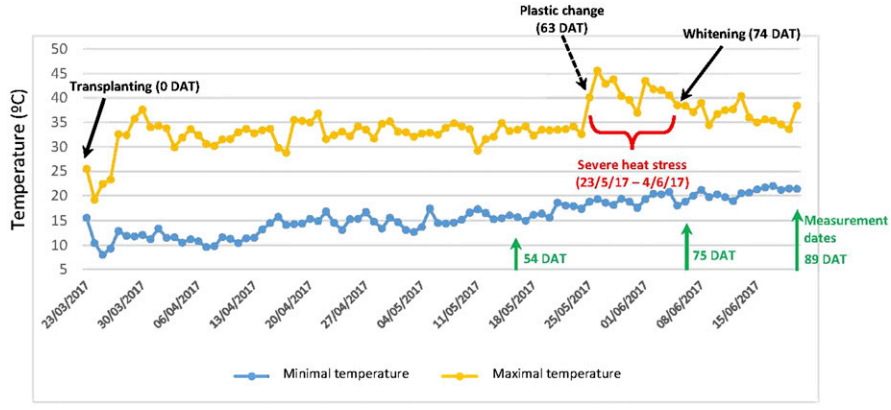

Fig. 1. Temperature records registered in the multi-tunnel greenhouse in which the experiment was performed. Relevant points of the experiment are indicated and described in black. Severe heat stress is indicated in red. Physiological measurement points over time are indicated in green.

of 200 mL of either tap water or 99 parts of tap water plus 1 part of a mixture of 4 fertilization solutions [0.5 mL of solution A containing 75 g/L $Ca(NO_3)_2$; 0.6 mL of solution B containing 50 g/L K_2SO_4; 0.3 mL of solution C containing 100 g/L KNO_3; 2.5 g/L microelements Fe (7%), Cu (0.4%), Mn (3.8%), Zn (0.6%), B (0.7%), and Mo (0.3%); 0.5 g/L Fe EDDHA (6%); and 0.6 mL of solution D containing 25 g/L KH_2PO_4 and 25 g/L complex fertilizer N13P40K13]. Pests control was performed following the usual plant producer (Vicaplant Nursery) practices. The experiment was initiated on 23 Mar. 2017, and it was ended on 20 June 2017 (i.e., 89 DAT).

Severe HS. On 25 May 2017 (63 DAT), the damaged plastic cover of the greenhouse was changed. No whitening of the new plastic section could be performed until 11 d after this change. This provoked a sharp and maintained temperature increase in the whole greenhouse during that period (25 May to 4 June), with temperatures increasing to a maximum of 45.6 °C (Fig. 1). This situation could be mitigated only by plastic whitening, which was performed on 5 June. From that point on, the temperature started to slowly decrease. It finally reached levels similar to those previous to the incident (Fig. 1).

Data recording and statistical analyses. At three time points during the experiment, physical, physiological, and production data were recorded: one previous to severe HS (54 DAT); one just after plastic whitening and, therefore, after severe HS (75 DAT); and one at 89 DAT, when temperature conditions were re-normalized. In vivo measurements performed during the experiment included plant height, leaf number and vigor, flower number, vigor and physiological stage, developing fruits, mature and fattening fruits, fruit ripening, and production (number of fruits, fruit weight). At the end of the experiment (89 DAT), comparative photographs of the general state of each crop subjected to the two treatments were obtained. Plants were then extracted from pots, shoots were severed, and the fresh weights of shoots and roots were measured. Plant material was then dried at 60 °C for 10 d with an Indelab drying stove (I.T.C., S.L., Lardero, Spain), and shoot and root dry weights were recorded. Plant shoot and root water contents were then calculated.

The percentage of AMF colonization of the roots was determined at three time points (28, 54, and 77 DAT) to assess mycorrhizal development. To achieve this, aliquots (≈0.5 g) of root material of both TREATED and CONTROL plants were carefully taken from soil and stained according to the Trypan blue staining technique (Phillips and Hayman, 1970). Stained samples were quantified for mycorrhizal frequency and intensity according to the method of Giovannetti and Mosse (1980). Photomicrographs of diagnostic structures of AMF growing within roots were obtained using an Eclipse CI-S microscope (Nikon Instruments Europe, B.V., Amsterdam, The Netherlands) and a digital camera Visicam 5.0 (VWR International Eurolab, S.L. Llinars del Vallès, Spain).

The experiment had a random block experimental design with nine replicates. Each experimental unit consisted of one plant in one pot. Statistical analysis was performed using an analysis of variance, to determine the mean, standard deviation, confidence intervals, and F and *P* values. Mean comparison by pairs was made using Fisher's least significant difference test at $\alpha = 0.05$. Graphics were created using Microsoft Word (version 1910 for Microsoft Office 365 Business; Microsoft, Redmond, WA).

Results

Results obtained during the experiment are shown in Figs. 2–6. Detailed data regarding crop production and plant water status are shown in Tables 1–3.

TREATED tomato plants became mycorrhizal before severe HS (Figs. 3A–C and 4A), reaching 2.1% mycorrhizal colonization at 28 DAT, which increased up to 12.8% at 54 DAT. After severe HS, the percentage of mycorrhizal colonization increased, reaching 16.6% at 77 DAT. Intraradical hyphae (Fig. 3A and B), numerous arbuscules (Fig. 3B and C) and vesicles/intraradical spores (Fig. 3A–C) were observed in these plants, indicating the establishment of functional symbiosis between partners. No mycorrhizal structures were detected in CONTROL tomato plants (not shown).

Severe HS induced dramatic changes in tomato CONTROL plants, whereas such changes were much less evident or even unnoticed in TREATED plants. Reductions in plant vigor and productivity were clearly visible in CONTROL plants (Fig. 2A–C), with an evident reduction in fruit production (Fig. 2A; Table 1) and increases in aborted flowers, fruits (Fig. 4F), and damaged and dead leaves (Figs. 2B and 4D). On the contrary, both the total number of leaves and number of green leaves were maintained or increased in TREATED plants after HS (Fig. 4B and C), with no apparent signs of being affected (Fig. 2C). The total numbers of flowers and fruits were also significantly increased upon mycorrhization (Fig. 4E). Both the number of fattening fruits and the number of ripe fruits were importantly increased by the mycorrhizal treatment (Figs. 4G and H).

Concerning fruit production, mycorrhizal plants dramatically increased the number of fattening fruits (Fig. 4G) and ripe fruits (Fig. 4H) after HS compared with CONTROL nonmycorrhizal plants. When considering the total weight of produced fruits of TREATED plants, this was 223% higher compared with that of CONTROL plants (Table 1). The mean weight per ripe fruit was also increased (+80%), although no statistical differences were obtained for this parameter.

Tomato plants showed important differences in total fresh and dry biomass and plant water status with different treatments after being subjected to severe HS. For TREATED plants, fresh biomass increased 29.28% (shoot) and 55.56% (root) compared with CONTROL plants; regarding dry biomass, these numbers were +18.30 and +48.61%, respectively. This translates to a significant increase in the water content in both shoot and root of mycorrhizal plants, which in environmental conditions such as Almeria's producing fields, where water is a precious treasure, is priceless.

Finally, the shoot-to-root ratio for dry biomass decreased to 24.49% in mycorrhizal plants compared with nonmycorrhizal ones; however, because of the larger range between data, these differences were not significant.

TREATED orange pepper plants also became mycorrhizal before severe HS (Figs. 3D–F and Fig. 5A), reaching 0.5% mycorrhizal colonization at 28 DAT (Fig. 5A), which increased up to 36.6% at 54 DAT. However, for this crop, after severe HS, the percentage of mycorrhizal colonization decreased, reaching 19% at 77 DAT. Diagnostic AM structures were clearly visible in trypan blue–stained roots, which showed intraradical hyphae (Fig. 2D), well-developed arbuscules (Fig. 2F), and vesicles/intraradical spores (Fig. 2E), suggesting functional symbiosis. No mycorrhizal structures were seen in CONTROL roots (not shown).

The effects of severe HS were also clearly visible in pepper CONTROL plants compared with TREATED plants: an obvious reduction in plant height and more effects on the fruit were observed (Figs. 2D, 5B, and 5H; Table 2). Fewer green leaves were evident on nonmycorrhizal plants (Fig. 5C) during the experiment. The number of active flowers (i.e., flowers that would develop into fruits) and the total number of flowers and fruits were significantly increased for TREATED plants when they had overcome HS (89 DAT) (Fig. 5D and E). Regarding fruit production, the number of collected fruits and the number of fruits at the fattening stage were increased for mycorrhizal plants (Fig. 5F and G); conversely, CONTROL plants had more physiopathic fruits and total aborted flowers and fruits (Fig. 5H).

The total production of fruits was statistically increased for TREATED orange pepper plants compared with CONTROL plants (Table 2), reaching a difference of +163% between treatments. The mean weight per commercial fruit was also higher for mycorrhizal orange pepper plants (+10.91%); however, in this case, differences were nonsignificant.

The water status of TREATED orange pepper plants was also preserved following severe HS as compared with CONTROL plants (Table 2). Significant differences in shoot fresh and dry weights indicated better management of water and biomass due to mycorrhizal colonization, which was confirmed by significant increases in both shoot and root water contents in mycorrhizal pepper. Although nonsignificant, an important decrease (–38.10%) in the shoot-to-root

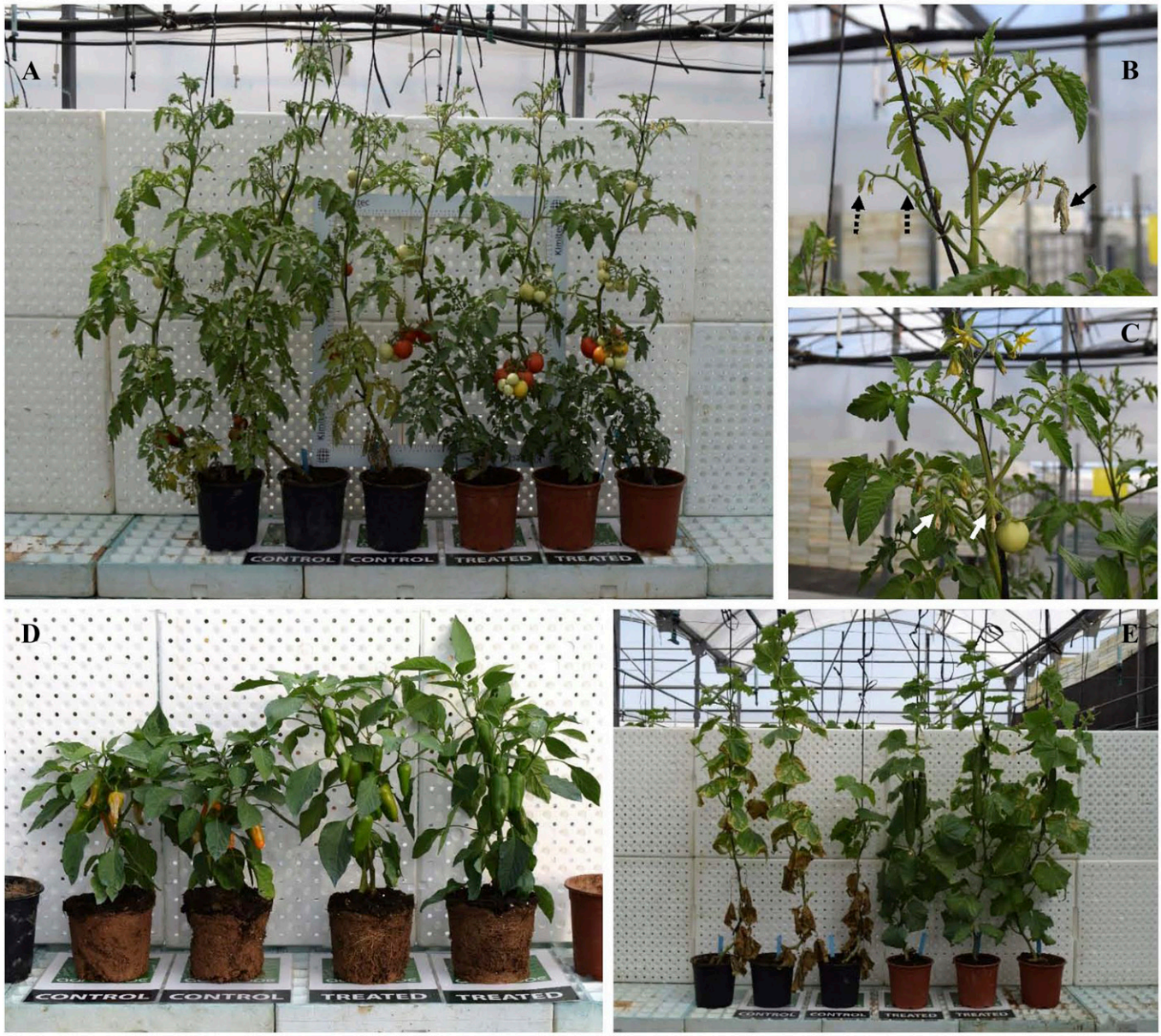

Fig. 2. Photographs of plants of the different crops tested at the end of the experiment at 89 d after transplanting (DAT). (**A**) Differences between treatments were clear in tomato plants, especially plant vigor, leaf status, and color and fruit production. (**B**) CONTROL tomato plants showed aborted flowers and fruits (dotted black arrows) as well as affected/dead leaves (solid black arrows) as a consequence of heat stress (HS). (**C**) TREATED tomato plants showed numerous fruits at a fattening stage that were still hanging on branches at the end of the experiment (solid white arrows), with leaves still vigorous and unaffected by stress. (**D**) Orange pepper CONTROL and TREATED plants showed important differences in vigor, leaf status, and fruit production at the end of the experiment (89 DAT). This was especially relevant for plant height, biomass, and fruit production. (**E**) Cucumber plants at 89 DAT exhibited strong differences between treatments: biomass, leaf status, and fruit production were either preserved or increased for TREATED plants after the severe HS episode, whereas CONTROL plants were unable to fully recover from such an event.

ratio of orange pepper mycorrhizal plants compared with nonmycorrhizal plants was also noted.

Figure 2E shows a comparison between CONTROL and TREATED cucumber plants at 89 DAT (25 d after being submitted to severe HS maintained for 11 d). Differences in plant vigor, biomass, and leaf status are evident, and such differences were confirmed by quantitative measurements (Fig. 6). Cucumber plants reached 5% root AM colonization at 28 DAT, increasing to a maximum of 25.9% at 54 DAT and decreasing to 18% at 77 DAT (Fig. 6A). As in the case of tomato and pepper plants, no mycorrhizal structures were seen in CONTROL roots. Mycorrhizal colonization was mainly arbuscular in cucumber TREATED roots (Fig. 3G–I), although some extra and intraradical hyphae and intraradical spores/vesicles were also visible (Fig. 3G).

Although no differences could be observed between treatments regarding the total number of leaves of cucumber plants during the experiments (Fig. 6B), differences were important if the physiological status of the leaves was considered; after severe HS, leaves of nonmycorrhizal cucumber were dramatically affected (Fig. 6C) and they did not recover during the remainder of the experiment, and damaged and dead leaves increased with time (Fig. 6D). However, mycorrhizal cucumber plants did recover after HS, maintaining their active green leaves over time (Fig. 6C). The number of active flowers was also preserved by AM colonization, in contrast to nonmycorrhizal plants, which experienced a sharp decrease after severe HS and did not recover (Fig. 6E).

Fruit production of cucumber mycorrhizal plants was unaffected after the severe HS episode (Fig. 6F); however, it was twice the amount produced by nonmycorrhizal plants at the end of the experiment. At that time

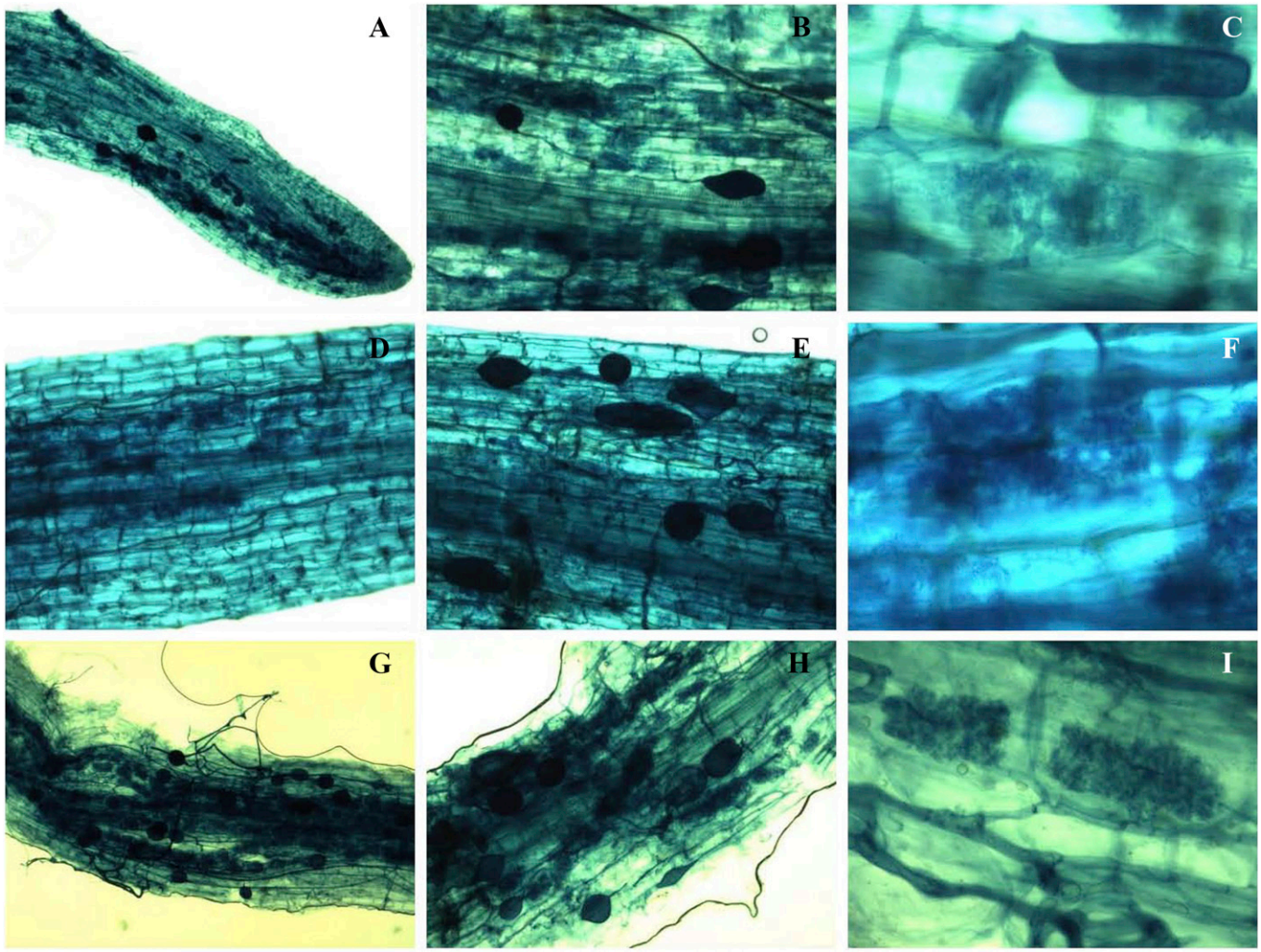

Fig. 3. Root photomicrographs of the different tested plants obtained at the end of the experiment at 89 d after transplanting (DAT) upon trypan blue staining. (**A–C**) tomato roots. Mycorrhizal colonization was mainly arbuscular (**B**, **C**), showing well-developed arbuscules within plant cortical cells (**C**). Some intraradical hyphae and vesicles/spores are also noted (**B**, **C**). (**D–F**) Pepper mycorrhizal roots. Colonization was also mainly arbuscular (**D**), with clear, highly branched arbuscules (**F**). Some intraradical vesicles/spores were also visible (**E**). (**G–I**) Cucumber mycorrhizal roots. (**G**) An entry point promotes a highly arbusculated colonization unit within the root. (**H**) Although mostly arbuscular, colonization features also include intraradical vesicles/spores. (**I**) Arbuscules are well-established within root cortical cells and appear highly functional.

(89 DAT), the number of fruits in the fattening stage was more than five-times higher for mycorrhizal plants (Fig. 6G), whereas the number of aborted fruits was clearly reduced by the mycorrhizal status (Fig. 6H).

The total production of cucumber fruits statistically increased for TREATED plants (+144.64%) compared with CONTROL plants (Table 3). At the end of the experiment, the total weight of fattening fruits remaining on plants was +456.80% higher for mycorrhizal plants, indicating that these differences in fruit production could have larger if the experiment had been prolonged. Regarding the water status of cucumber plants, both shoot and root fresh weights were statistically increased for TREATED plants, which provoked statistical differences in both shoot and root water contents between treatments. Finally, the shoot-to-root ratio experienced a sharp decrease (–226%) in mycorrhizal plants; however, because of plant variations, these data were not statistically significant.

Discussion

The AM has been considered one of the most promising solutions against the increasing use (and abuse) of chemical fertilizers, phytochemicals, and pesticides during plant production (Gianinazzi and Schuepp, 1994; Gianinazzi et al., 2010; Martín et al., 2018). The abilities of AMF to greatly enhance both nutrient uptake (especially phosphorous) and water uptake, transport and release to the host root via the extraradical hyphae (Smith and Smith, 2012), and the important role of AMF in alleviating both biotic and abiotic stresses in plants (Plouznikoff et al., 2016; Smith et al., 2010) have been widely reported, making this group of symbiotic fungi a target for research and technology. Nevertheless, after more than five decades, AM inoculation is not yet a worldwide technique used as a rule by all plant growers. The main reason for this has been the absence, for many years, of high-quality AMF inoculants that are easy to combine with general agricultural practices that render consistent positive results of crop production and quality (Martín et al., 2018). The fact that conventional AM inoculants were (and most of them still are) issued from open air pot cultures with no control of propagule quality and number and with almost assured contamination with undesired microorganisms has resulted in confusing, even contradictory, results, causing mistrust among potential users (Bago and Cano, 2006). Also, because different AMF isolates behave differently depending on environmental and soil conditions, as well as on the host plant and even the inoculum origin, all of this does not contribute to overcome such mistrust. Lack of knowledge of the basic AMF genetics and symbiotic functioning is probably at the base of these uncertainties.

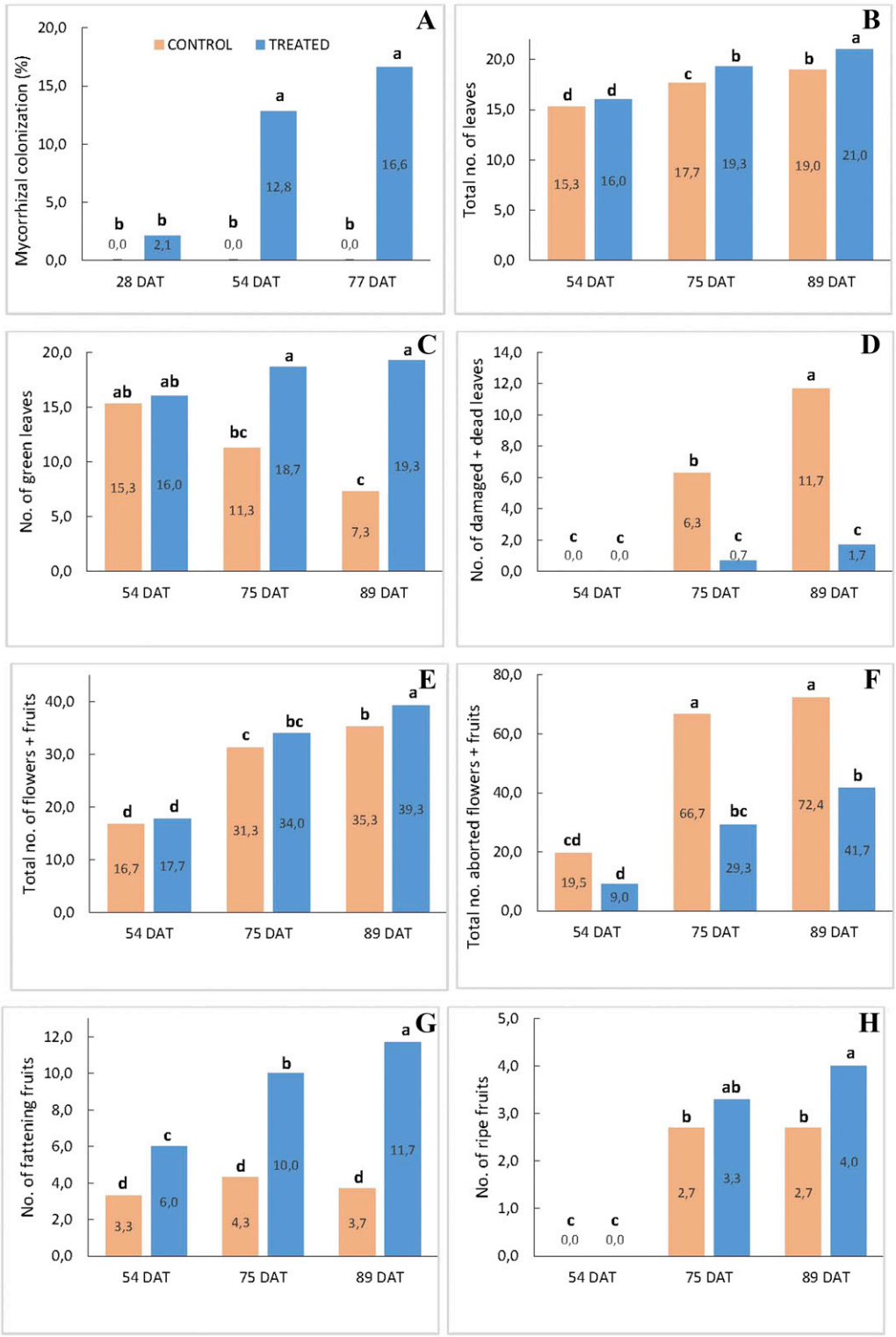

Fig. 4. Features of the tomato plants obtained during the experiment for both CONTROL (nonmycorrhizal, orange color) and TREATED (mycorrhizal, blue color) plants. (**A**) Arbuscular mycorrhizal root colonization intensity. (**B**) Total number of leaves. (**C**) Number of green leaves. (**D**) Sum of the number of damaged and dead leaves. (**E**) Total number of flowers and fruits. (**F**) Total numbers of aborted flowers and fruits. (**G**) Number of fattening fruits. (**H**) Number of ripe fruits. Different letters indicate statistically significant differences according to Fisher's least significant difference test, $\alpha = 0.05$.

Bago and Cano (2006) developed a protocol for the mass production of ultra-pure in vitro AMF propagules in a semi-solid gel-type substrate that resulted in a water-miscible, easy-to-use, reliable, ultra-pure AM inoculant. This inoculant can be formulated with clonal, well-characterized AMF ecotypes that were previously selected according to their abilities for (among others) crop production enhancement, drought resistance, heavy metal contamination endurance, pathogen resilience, and others while economizing precious water and nutrient resources. The use of such an inoculant (now commercialized under the name MYCOGEL) has changed the vision that many producers had of AM because it is now more prone to extensive use in all susceptible agricultural crops.

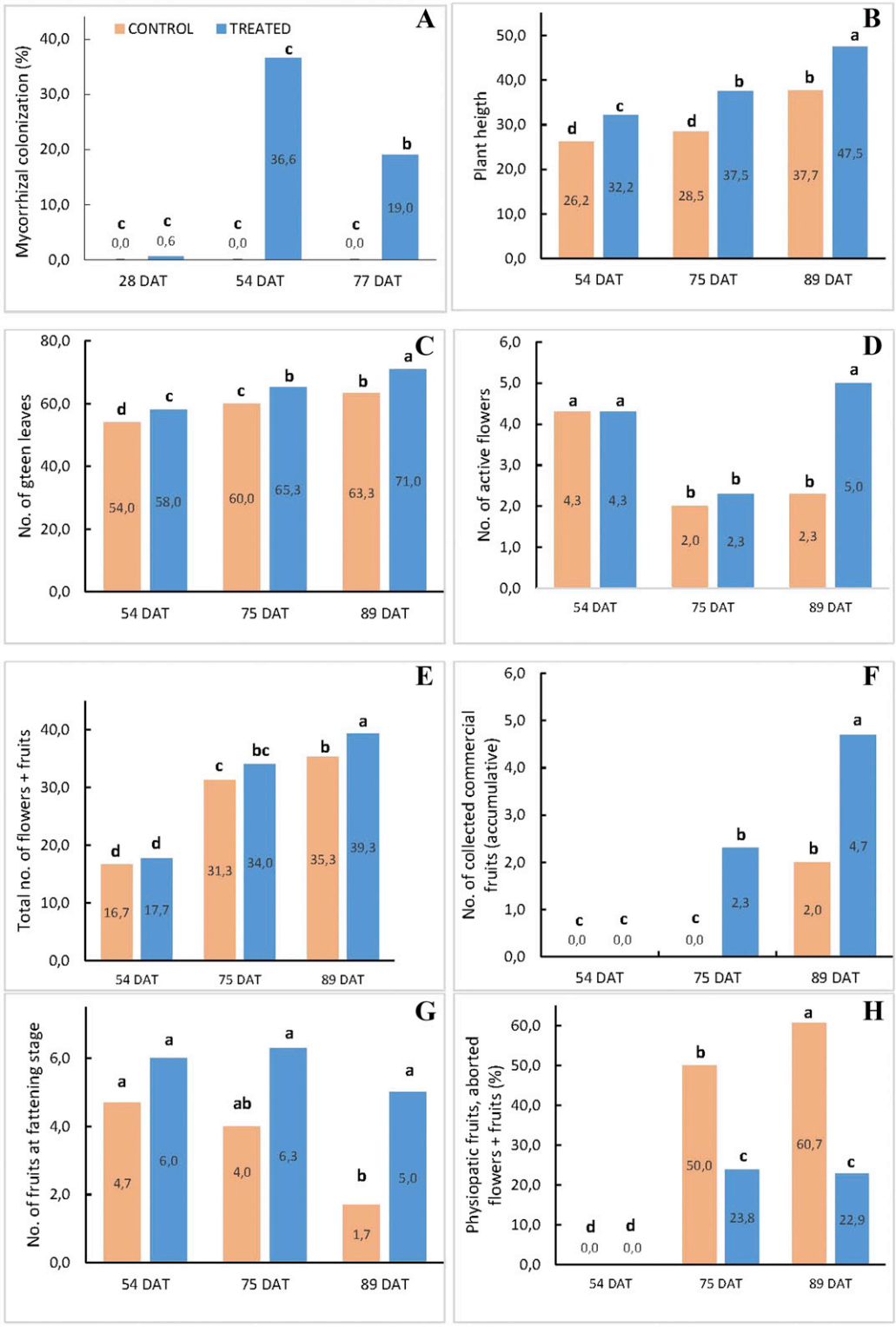

Fig. 5. Features of the orange pepper plants obtained during the experiment for both CONTROL (nonmycorrhizal, orange color) and TREATED (mycorrhizal, blue color) plants. (**A**) Arbuscular mycorrhizal root colonization intensity. (**B**) Plant height. (**C**) Number of green leaves. (**D**) Number of active flowers. (**E**) Total numbers of flowers and fruits. (**F**) Number of collected commercial fruits (accumulative). (**G**) Number of fruits at the fattening stage. (**H**) Numbers of physiopathic (discarded) fruits and aborted fruits and flowers. Different letters indicate statistically significant differences according to Fisher's least significant difference test, $\alpha = 0.05$.

Plants may greatly benefit from the protective effects that AM symbiosis confers against temperature stress (Zhu et al., 2017). Different studies have shown that physiological, biochemical, nutritional, and even reproductive conditions ameliorate in AM plants compared with nonmycorrhizal plants under low or high temperatures (Abdel-Latef and Chaoxing 2011; Atkin et al., 2009; Baon et al., 1994; Barrett et al., 2014; Bunn et al., 2009; Cabral et al., 2016; Chen et al., 2013, 2014; Duc et al., 2018; Gavito et al., 2005; Hawkes et al., 2008; Hu et al., 2015; Martin and Stutz 2004; Mathur et al., 2018; Matsubara et al., 2000, 2004; Maya and Matsubara 2013; Wu and Zou, 2010; Zhu et al., 2010a, 2010b, 2011). Most of these works also indicated that selection of the appropriated AMF isolate

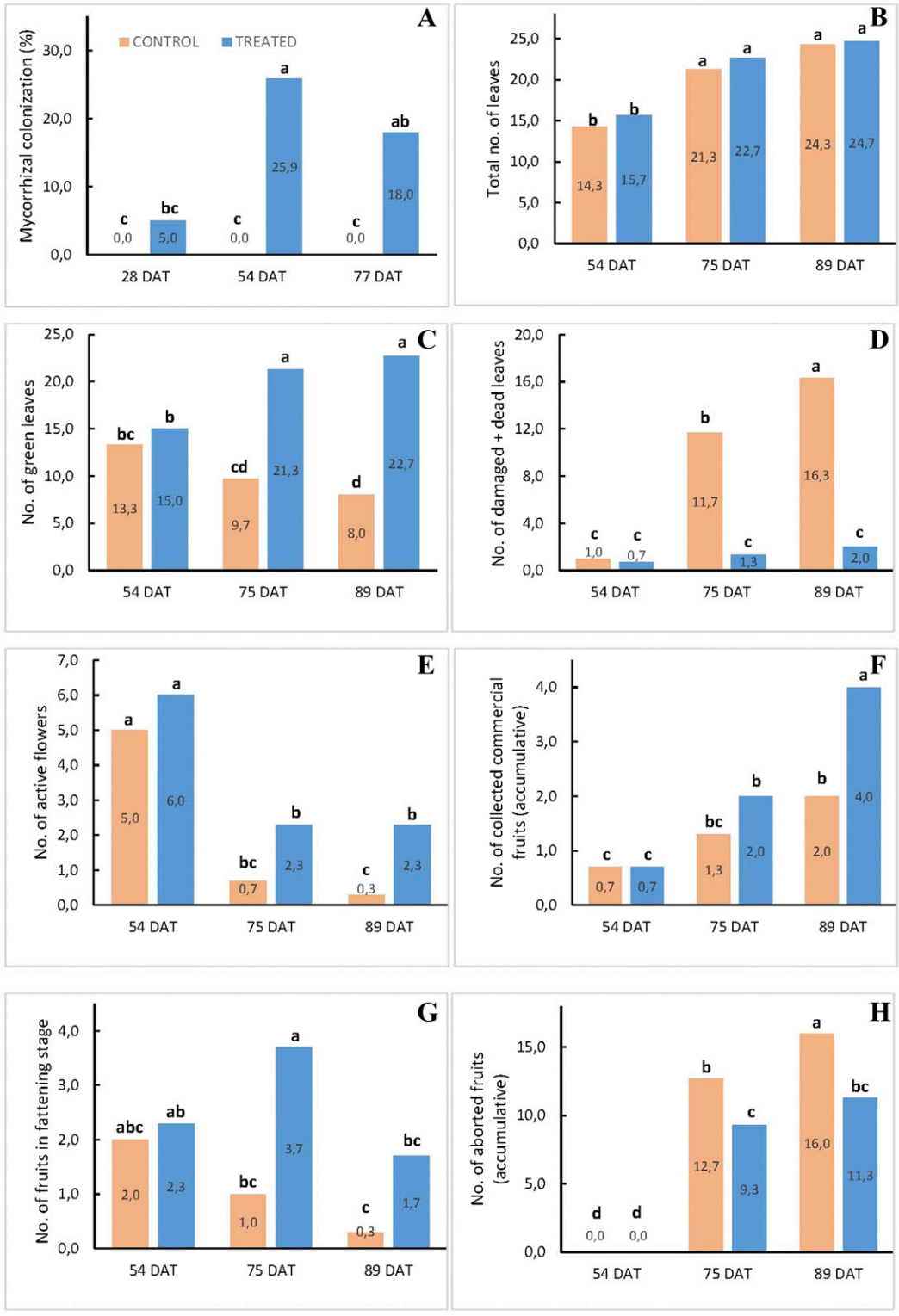

Fig. 6. Features of the cucumber plants obtained during the experiment for both CONTROL (nonmycorrhizal, orange color) and TREATED (mycorrhizal, blue color) plants. (**A**) Arbuscular mycorrhizal root colonization intensity. (**B**) Total number of leaves. (**C**) Number of green leaves. (**D**) Total number of damaged plus dead leaves. (**E**) Number of active flowers. (**F**) Number of collected commercial fruits (accumulative). (**G**) Number of fruits at the fattening stage. (**H**) Number of aborted fruits (accumulative). Different letters indicate statistically significant differences according to Fisher's least significant difference test, $\alpha = 0.05$.

is crucial to obtaining positive results. Therefore, in this study, we examined the suitability of applying the aforementioned mycorrhizal ultra-pure inoculum containing a selected AMF isolate to plants submitted to severe, prolonged HS under agronomic conditions. The fact that temperatures continue rising worldwide, thereby greatly affecting crop production, and the predictions indicating that they will continue increasing indicate that it is mandatory to develop new strategies or find new allies that could alleviate such situations.

Our results clearly indicate that the application of the ultra-pure AM inoculant MYCOGEL to three agriculturally important crops under agronomic conditions

Table 1. Fruit weight and shoot and root water contents of tomato plants at the end of the experiment at 89 d after transplanting.

	Total wt of fruits (g)	Mean wt per ripe fruit (g)	Root fresh wt (g)	Shoot fresh wt (g)	Root dry wt (g)	Shoot dry wt (g)	Shoot/root ratio (dry)	Root water content (%)	Shoot water content (%)
CONTROL	88.3	20.5	13.9	114.4	3.4	29.2	8.59	75.96	74.37
TREATED	285.4	36.8	21.7	147.9	5.0	34.5	6.90	76.98	76.66
Difference (%)	223.35	80.02	55.56	29.28	48.61	18.30	–24.49	1.34	3.08
CONTROL	b[z]	b	a	a	a	a	a	b	b
TREATED	a	a	a	a	a	a	a	a	a
P	0.0221	0.0189	0.0852	0.1983	0.1031	0.3365	0.184	0.0024	0.0331

[z]Fisher's least significant difference test, $\alpha = 0.05$.

Table 2. Fruit weight and shoot and root water contents of green pepper plants at the end of the experiment at 89 d after transplanting.

	Total wt of commercial fruits (g)	Mean wt per commercial fruit (g)	Root fresh wt (g)	Shoot fresh wt (g)	Root dry wt (g)	Shoot dry wt (g)	Shoot/root ratio (dry)	Root water content (%)	Shoot water content (%)
CONTROL	49.1	25.1	59.0	94.6	4.3	16.2	3.77	92.65	82.86
TREATED	129.4	27.9	106.1	117.3	7.0	19.1	2.73	93.36	83.65
Difference (%)	163.54	10.91	79.78	23.95	63.34	18.46	–38.10	0.77	0.95
CONTROL	b[z]	a	a	b	a	b	a	b	b
TREATED	a	a	a	a	a	a	a	a	a
P	0.0293	0.1209	0.0811	0.0206	0.0969	0.0405	0.1307	0.0486	0.0143

[z]Fisher's least significant difference test, $\alpha = 0.05$.

Table 3. Fruit weight and shoot and root water contents of cucumber plants at the end of the experiment at 89 d after transplanting.

	Total wt of collected fruits (g)	Total wt of fattening fruits left on plants (g)	Root fresh wt (g)	Shoot fresh wt (g)	Root dry wt (g)	Shoot dry wt (g)	Shoot/root ratio (dry)	Root water content (%)	Shoot water content (%)
CONTROL	413.7	8.1	4.9	75.7	1.6	28.5	17.81	66.9	62.1
TREATED	1012.0	44.9	15.7	122.7	4.6	36.2	7.87	70.4	70.5
Difference (%)	144.64	456.80	10.7	47.0	3.0	7.7	–55.74	3.6	8.4
CONTROL	b[z]	a	b	b	b	a	a	b	b
TREATED	a	a	a	a	a	a	a	a	a
P	0.0275	0.1195	0.0349	0.0237	0.0403	0.0873	0.0662	0.0024	0.0128

[z]Fisher's least significant difference test, $\alpha = 0.05$.

resulted in enhanced endurance of plants toward severe HS. Better shoot and root development and fitness of TREATED plants compared with CONTROL ones were obtained, yielding positive results for almost all of the parameters tested. These results are in agreement with those of most AM studies reported to date for different plants and soil and environmental conditions; these results included increased plant biomass and water content and fewer effects on AM plants with HS events (Zhu et al., 2017). However, our work also demonstrates that the use of the appropriate AM inoculant increases the efficacy of fruit production and quality, which are probably the two parameters most appreciated by both the plant (in reproductive terms) and producers (in terms of income). If we add to this the fact that AM promoted all these effects as a part of more respectful management of the soil, land, and environment, we can conclude that AM inoculation in agriculture, with the appropriate inoculum source, is one of the keystones to building our next defense wall against climate change.

In general, all three crops assayed greatly benefitted from AM inoculation after severe HS. This is especially evident in the reduced number of damaged/dead leaves of mycorrhizal plants, which is obviously a clue to maintaining their best photosynthetic and CO_2 fixation rates. The number of active flowers dramatically decreased for nonmycorrhizal plants following HS events, but it was maintained for TREATED plants, which is consistent with the increased fruit production, measured both as fattening fruits and ripe commercial fruits collected. Moreover, when considering the total weight of produced fruits for all three crops, and even the mean weight per ripe fruit in the case of tomato, very important differences are evident, which obviously translates to an important increase in benefits for the grower.

From economic and ecologic points of view, the shoot and root water contents should also be considered. Because water resources are important for agriculture and among the most affected by temperature increases in global climate change scenarios, the fact that AM inoculation statistically increased the water content in shoots from 1% (pepper) to more than 8% (cucumber) is quite relevant. It has been reported that temperature stress negatively affects water absorption in nonmycorrhizal plants (Zhu et al., 2011, 2017). However, AM plants overcome this situation, and this is associated with not only the increased ability of mycorrhizal roots to explore and explode soil water resources via the extraradical mycelium of the AM fungal partner (Barrett et al., 2014; Gavito et al., 2005) but also the ameliorated capacity of AM plants to manage water in an economic and more productive manner. Plant aquaporine gene expression and regulation, induced by AM establishment, are significantly related to this (Aroca et al., 2009; Luu and Maurel 2005).

In our study, AM symbiosis greatly reduced the shoot-to-root ratio compared with nonmycorrhizal plants. However, it has been reported that AM plants usually increase this parameter (Smith, 1980) because the fungus acts as ''root extensions'' (Bago et al., 1997) so that the plant should not invest carbohydrates and energy in root tissue for similar (or even greater) shoot development. These apparently contradictory results are easy to explain: the presence of functional AM symbiosis greatly ameliorated the stress situation of TREATED plants, allowing their roots to grow with almost no restrictions within its more HS-protected environment (the soil). The shoot, when directly confronted with the increased temperature, most probably changed its development route to invest the most in fruit production rather than in vegetative biomass. This is among the best proof of AM efficacy in the alleviation of HS.

It should be mentioned that inoculation with the commercial ultra-pure gel used resulted in extensive colonization of roots of all the three crops assayed. This is relevant because there are doubts regarding the colonization abilities of in vitro–issued AM propagules due to their ''artificial nature'', and these doubts, surprisingly, ranged from one extreme to another: either they were ''unable to colonize roots at all'' or they promoted an ''uncontrolled invasion'' and behaved as ''parasites'' completely invading and deforming roots, thus making them nonfunctional. Our results demonstrate that none of these situations is true, leading us to suspect that these doubts are ill-intentioned

commercial strategies. AM in vitro inoculants induce functional symbiosis at levels typically ranging from 5% to 40% root colonization (Cano and Bago, unpublished). These levels could be considered low when compared with those obtained for ex vitro/open-pot issued inoculants (usually ranging from 40% to 80%). However, the functionality, but not the amount of intraradical fungal tissue, is the clue to obtaining efficient AM symbiosis; it was pointed out that a single highly efficient "mycorrhizal unit" is enough to render benefits for the host plant (Smith and Read, 2010). The other doubts about the supposed "voracity" of the in vitro–produced AMF propagules have been clarified by our results; mainly arbuscular colonization, the paradigm of AM functionality, is the rule when using in vitro–produced AM inoculants with some vesicles/intraradical spores present in the root tissue as a part of normal fungal behavior and development.

It is also worth mentioning that in our experiment, the initial colonization of tomato plants increased from 2.1% (28 DAT) to 12.8% (54 DAT), reaching 16.8% at 77 DAT; in other words, no constraint to AM colonization was imposed by the severe HS episode that occurred from 63 to 74 DAT. However, we did observe such restrictions in pepper (36.6% at 54 DAT and 19% at 77 DAT) and cucumber (25% at 54 DAT and 18% at 77 DAT) plants. Although we should consider that these data might reflect dilution of AM colonization due to an increased rate of root growth not followed by the same extent of AM intraradical development, we should also consider that root colonization by AM is a balanced process in which plant and fungal necessities are working; perfect equilibrium is reached when the plant is colonized up to the correct point at which it can obtain the nutrients it needs without being overwhelmed, and when the fungus develops up to the correct point at which excessive, unnecessary biomass is not produced and it can obtain the carbohydrates it needs. It is evident that plant roots are prone to becoming AM-colonized under adverse conditions; however, they control fungal spreading more strictly if such a condition is maintained for long periods.

Finally, regarding the possibility of AM being affected by the soil nutrient content, it has been described that high nutrient (especially P) contents in the substrate impair AM colonization and functioning (Smith and Read, 2010). This is true during the initial steps of AM colonization, when the fungus has to develop from propagules and find a root to colonize (fungal asymbiotic growth); furthermore, the roots have to "choose" whether they will allow the fungus to enter and invade their tissues. In this situation, low P in the substrate is mandatory so that colonization is not affected. However, when colonization is fully established, the fungus is less affected by the P concentration in the substrate (Bago et al., unpublished). One of the most important recommendations when applying MYCOGEL to agricultural crops is that P fertilization should be avoided during the 2 weeks following inoculum application (see manufacturer's recommendations). Our results confirm the suitability of this practice, as shown both by the extensive symbiotic structure development (especially arbuscules) in TREATED plants and the benefits obtained in terms of fruit production.

In conclusion, this study demonstrates that inoculation with appropriate AM inoculants containing selected AMF isolates (such as MYCOGEL) greatly ameliorates plant endurance of HS by increasing its vigor, productivity, and fruit quality while creating more sustainable and durable agricultural practices. In actual global climate change scenarios, our results shed a light of hope and indicate how agriculture practice should proceed in the near future.

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