Journal of Horticultural Science & Biotechnology (2014) **89** (6) 679–685

Physiological and growth responses of young tomato seedlings to drip-irrigation containing two low doses of the arbuscular mycorrhizal fungus *Glomus iranicum* var. *tenuihypharum* sp. *nova*

By F. FERNÁNDEZ[1], J. VICENTE-SÁNCHEZ[1], J. F. MAESTRE-VALERO[2*], A. J. BERNABÉ[1], E. NICOLÁS[2], F. PEDRERO[2] and J. J. ALARCÓN[2]
[1]Symborg S.L., Campus de Espinardo, 7-Edificio CEEIM, 30100 Espinardo, Murcia, Spain
[2]Department of Irrigation, CEBAS-CSIC, Campus de Espinardo, 30100, Espinardo, Murcia, Spain
(e-mail: jmaestre@cebas.csic.es) (*Accepted 3 June 2014*)

SUMMARY
Two different doses of an arbuscular mycorrhizal fungus (AMF; *Glomus iranicum* var. *tenuihypharum* sp. *nova*) were applied through a drip irrigation system to evaluate their effects on the physiological, nutritional, and agronomic performance of tomato plants. Trials were conducted in south-eastern Spain under controlled greenhouse conditions from September – December 2012. The high rate of AMF colonisation at both doses applied improved plant growth performance, leading to significant increases in leaf macro- (N, P, K, Ca and Mg) and micro- (Fe, Cu, Zn, Mn and B) nutrient concentrations. The AMF present in colonised plants not only exerted some control over the rate of transpiration (stomatal control), but also maintained a higher rate of net photosynthesis and hence improved the intrinsic water use efficiency (computed from the ratio of the rate of net photosynthesis:stomatal conductance). In addition, a close relationship was found between the respiratory activity of the AMF and the rate of net photosynthesis, leaf N, P, Fe, and K concentrations, and fruit yield (expressed as the product of the average number of fruit × the average fruit weight in each treatment). In summary, the application of AMF was effective at improving the performance (i.e., the growth, nutrition, and yield) of tomato plants cultivated under an intensive fertigation regime.

Sustainable intensive agricultural production demands the development of new, inexpensive and environmentally-friendly strategies to improve competitiveness. Among such strategies, establishing mutually beneficial associations between plants and microorganisms in the rhizosphere has been shown to be an appropriate technique (Aroca *et al.*, 2013). Perhaps, the most widely studied plant–microorganism associations are those involving arbuscular mycorrhizal fungi (AMF; Smith and Read, 2008), since AMF are ubiquitous in terrestrial ecosystems and may establish symbiotic relationships with more than 85% of plant species of agricultural interest (Ruiz-Lozano and Azcón, 1996).

AMF play important roles in: (i) increasing plant root hydraulic conductivity (Aroca *et al.*, 2007); (ii) increasing plant growth and nutrient uptake (Hamel and Plenchette, 2007); and (iii) improving plant water relations (Sánchez-Blanco *et al.*, 2004), among other effects. Therefore, in order to promote sustainable agricultural systems, AMF should always be considered because of their ability to colonise root systems extensively and to participate actively in water and nutrient uptake (Sánchez-Blanco *et al.*, 2004; Rivera *et al.*, 2007). Moreover, there is evidence that AMF are significantly influenced by the physico-chemical properties of the soil (Gryndler *et al.*, 2009) as the soil not only provides AMF with mineral nutrients, but also constitutes the chemical and physical environment in which both the fungus and the plant co-exist. Research has shown that AMF work best within a specific range of soil conditions, therefore only AMF species that have adapted to the specific soil conditions that favour symbiosis with a given plant should be used.

AMF have been applied to different cultivation systems (Hamel and Plenchette, 2007) usually by placing an inoculum directly in the substrate, near the roots (Abdel-Latef, 2011; Aroca *et al.*, 2013) which is a laborious task under commercial-scale field conditions. For instance, Subramanian *et al.* (2006) concluded that the AMF, *Glomus intraradices* improved the nutritional and water status and growth of tomato plants and, as a result, fruit production and quality. In a study on the effect of *G. mosseae* on the agronomic and physiological performance of tomato plants, Abdel-Latef (2011) obtained similar results, and concluded that AMF inoculation significantly increased leaf P concentrations and fruit yields. However, to our knowledge, there have been few studies in which AMF inoculation was applied via a drip irrigation system (Vicente-Sánchez *et al.*, 2014). If successful, this would make the application of AMF much easier.

Previous studies agree that, despite the successful laboratory results obtained with certain strains of AMF, scale-up of their use for agriculture has been slow, probably due to the relative ineffectiveness of the inoculation process (Ryan and Graham, 2002), or other factors such as the technical difficulty involved in their application, their compatibility, field carrying capacity, abundance, and priority effects (i.e., the influence of

*Author for correspondence.

timing and competition on the establishment of alternative stable communities; Verbruggen *et al.*, 2013). It is clear that further research is required.

This study aimed to evaluate the effects of two levels of AMF inoculation, applied by means of a drip irrigation system, on the physiological and nutritional status of tomato plants cultivated in a mixture of organic matter and calcareous soil under Mediterranean conditions.

MATERIALS AND METHODS

Experimental design

This study was conducted at the experimental farm of the Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC) in Murcia, Spain (38º07' 18" N; 1º13' 15" W). The experiment lasted 90 d (September – December 2012) and was carried out under controlled conditions in a greenhouse equipped with a cooling system with a relative humidity of 55 – 65%, day/night temperatures of 25º/12ºC, and a 10 h photoperiod at a maximum photosynthetic photon flux density (PPFD) of 1,500 µmol m^{-2} s^{-1}, measured using an external light meter (LI-Quantum Q-40211; LI-COR Inc., Lincoln, NE, USA) during the gas exchange measurements.

A total of 120 pots, each containing 5 l of substrate composed of a 3:2:1 (v/v/v) mix of calcareous soil:sand:vermiculite were used in the experiment. The substrate had a pH of 7.1, with 1.7% (w/w) organic matter, 0.9% (w/w) total organic carbon, 0.1% (w/w) total nitrogen, and 196.6 mg kg^{-1} available phosphorus. The substrate was not sterilised, in order to simulate the real-life scenario of compatibility and competition between native and inoculated AMF. One seed of *Solanum lycopersicon*, 'Albatross' (Nuhnems-Bayer Crop Science, Valencia, Spain) was germinated per pot.

Hoagland and Arnon (1940) nutrient solution was applied twice a week throughout the experiment at 0.5 l per pot by drip irrigation with one pressure-compensated emitter per plant at a flow rate of 2 l h^{-1}.

Three different treatments were applied: Control (no AMF inoculation), D_1 (0.12 g of AMF inoculant $plant^{-1}$), and D_2 (0.20 g of AMF inoculant $plant^{-1}$). It was notable that, considering the characteristics of the AMF inoculant used in this experiment, the amount of AMF used was two orders of magnitude (i.e., 100 ×) lower than that reported previously for tomato plants by Subramanian *et al.* (2006) and Abdel-Latef (2011). For each treatment, the inoculant (*G. iranicum* var. *tenuihypharum* sp. *nova*; Fernández and Juárez, 2012) was delivered for 5 min through the drip irrigation system, using an injection pump set at 25 l h^{-1}, 5 d after sowing at the time of seed germination and appearance of the first roots.

Production of the arbuscular mycorrhizal inoculant

The AMF inoculant used in this study (*G. iranicum* var. *tenuihypharum* sp. *nova*) was obtained by isolating the fungus under extremely saline soil conditions (Solonetz Gley; a saline soil-type according to the FAO soil classification). Multiplication of the AMF strain was performed by cultivating propagules in a saline mineral substrate [clay soil with 66% (v/v) smectite clay] with a host plant (*Lolium perenne*; Fernández and Juárez, 2012). At the end of the host plant life-cycle, the substrate, containing colonised roots, mycelium, and spores, was ground to particles ≤ 50 µm, with an infection potential of 1.2×10^4 propagules 100 ml^{-1} of substrate.

The novelty of this finely-ground inoculant was that it could be applied either by spreading, or directly through the fertigation injection pumps. Substrate with a particle size (when dry) of < 100 µm did not block the pump filter, and all the AMF propagules (i.e., spores, mycelium, and colonised root fragments) remained in a dormant state until rehydrated. Thus, the propagules were activated as the inoculant was delivered through the irrigation system to the roots, where the propagules germinated and colonised the young rootlets.

Symbiotic development

Ten young tomato plants from each treatment were harvested 25 d and 50 d after germination (DAG) to measure the percentage of mycorrhizal colonisation (MC), the fungal intensity (FI) and the living respiratory activity (LRA) of the AMF. MC and FI values were obtained after clarifying and staining the roots with 0.05% (v/v) trypan blue in lactic acid (Phillips and Hayman, 1970), using the grid-line intersect method as described by Giovannetti and Mosse (1980) and modified by Trouvelot *et al.* (1986). LRA was determined using vital staining with a tetrazolium salt to measure succinate dehydrogenase activity (SDA) according to Smith and Gianinazzi-Pearson (1990).

Leaf nutrient concentrations

Twenty leaves from ten plants (two per plant) per treatment were collected at random 50 DAG. The leaves were washed with 0.1% (v/v) Alconox detergent (Proquilab S.A, Murcia, Spain), rinsed in tap water, cleared with 0.005% (v/v) HCl, rinsed in distilled water, left to drain on filter paper, and oven dried for at least 2 d at 65ºC. The dried leaves (0.2 g) were then ground and digested with a mix of 4 ml 69% (v/v) nitric acid and 1 ml 33% (v/v) hydrogen peroxide.

Macro- (N, P, K, Ca, and Mg) and micro- (Fe, Cu, Zn, Mn and B) nutrient concentrations were determined using an inductively-coupled plasma optical emission spectrometer (ICP-OES; IRIS Intrepid II XDL; Thermo-Fisher Scientific Inc., Loughborough, UK). Ion concentrations were compared with the ranges of macro- and micro-nutrients determined by the Diagnosis and Recommendation Integrated System (DRIS; Hartz *et al.*, 1998).

Leaf gas-exchange measurements

The instantaneous rate of net photosynthesis (A) and stomatal conductance (g_s) were measured at solar noon (12.00 h GMT) using an LI-6400 Portable Photosynthesis System (LI-COR Inc.) with an integrated leaf chamber fluorometer (LI-6400-40; LI-COR Inc.). All measurements were performed on ten tomato plants per treatment and on young, fully-expanded leaves from the middle part of each plant located on the side opposite the fruit, under a saturating PPFD (approx. 1,200 µmol m^{-2} s^{-1}) and almost constant ambient CO_2 concentration (400 µmol CO_2 mol^{-1}). Measurements were taken 25, 50, and 75 d after transplanting. The intrinsic water use efficiency (IWUE) was determined as the ratio of A:g_s (Alvarez *et al.*, 2012).

TABLE I

Mycorrhizal colonisation (MC), fungal intensity (FI), and vital staining for succinate dehydrogenase activity [living respiratory activity (LRA)] at two different doses of mycorrhizal inoculum 25 d and 50 d after germination of tomato seeds

Treatment[¶]	MC (%)[‡]		FI (%)		LRA (%)	
	25 d	50 d	25 d	50 d	25 d	50 d
Control	11.1 ± 2.2a[†]	20.7 ± 2.2a	0.1 ± 0.1a	1.5 ± 0.2a	8.6 ± 1.2a	14.7 ± 1.6a
D_1	63.3 ± 8.2b	83.1 ± 3.4b	3.3 ± 0.2b	17.5 ± 0.1c	39.1 ± 4.2b	62.1 ± 1.4b
D_2	49.2 ± 2.4c	85.2 ± 2.4b	4.6 ± 0.2c	13.7 ± 1.2b	35.0 ± 4.8b	58.2 ± 3.2b

[†]Each value is a mean (n = 10) ± SE. Mean values in each column followed by a different lower-case letter differ significantly ($P \leq 0.05$) as determined by Tukey's multiple range test.
[‡]MC, mycorrhizal colonisation; FI, fungal intensity; LRA, living respiratory activity.
[¶]D_1, inoculum dose of 0.12 g AMF plant^{-1}; and D_2, inoculum dose of 0.20 g AMF plant^{-1}.

Growth parameters and fruit yield

In order to determine the extent of tomato plant growth (i.e., root and shoot biomass accumulation), ten tomato plants per treatment were collected at 50 DAG and dried to constant weight for 48 h at 75ºC. Root and shoot dry weights (DWs) were measured, and root:shoot DW ratios were calculated.

In addition, at the end of the experiment (i.e., 90 DAG), tomato fruit from another set of ten plants per treatment were harvested and their individual fresh weights (FWs) were determined. The fruit yield for each treatment (in g FW plant^{-1}) was calculated as the product of the average number of fruit × the average FW of a fruit. Fruit with imperfections or symptoms of disease were discarded.

Statistical analysis.

In this experiment, 40 young tomato plants were attributed at random to each treatment (control, D_1 or D_2) and were used to analyse the effects of AMF inoculation via the drip fertigation system. The data were analysed by one-way analysis of variance (ANOVA) using SPSS Statistics software Version 21 for Windows (http://www.ibm.com/software/es/analytics/spss/) to detect significant differences between the parameters measured. When mean differences were significant, Tukey's multiple range test, at the 95 % confidence level, ($P \leq 0.05$) was applied. Percentage values for root colonisation were arc-sine [square root (X)] transformed before statistical analysis.

RESULTS AND DISCUSSION

Effect of AMF inoculant level on mycorrhizal colonisation

Table I shows the extent of mycorrhizal colonisation (MC), fungal intensity (FI), and living respiratory activity (LRA) determined by succinate dehydrogenase activity (SDA) in tomato seedling roots for the three treatments at 25 and 50 DAG.

As the substrate had not been sterilised, some proliferation of native AMF was detected in the non-inoculated control plants (11.1% and 20.7% of MC at 25 and 50 DAG, respectively). MC values increased gradually from the start of the experiment and, as expected, AMF-inoculated plants showed significantly higher MC values than non-inoculated plants. Twenty DAG, the highest MC level was observed in treatment D_1 (63.3%) although, at 50 DAG, no significant difference was seen between the two inoculation treatments (MC = approx. 85%; Table I). FI and LRA values were also notably higher in the AMF-inoculated treatments, in agreement with the MC values. Of particular interest was the observation that, at 50 DAG, D_2 plants did not show significantly higher MC, FI, or LRA values. This could indicate that there was an upper threshold of AMF-inoculum above which the extent of symbiotic development did not change.

Leaf nutrient concentrations

No significant differences in leaf nutrient concentrations were observed between the two AMF treatments, except for K and Ca, which showed significantly higher values in the D_2 treatment. Regardless of the dose applied, inoculation with AMF led to significant increases in almost all leaf macro- and micro-nutrient concentrations compared to the control treatment (Table II). These results agreed with previous research which showed that AMF associations usually enhanced the uptake of water and nutrients by roots (Ruiz-Lozano and Azcón, 1996). For instance, in the case of P, whose concentration was almost double that observed in the control treatment, AMF have been shown to improve the P-nutrition of host plants growing in soils with poorly-soluble forms of P (Shenoy and Kalagudi, 2005). In the case of N, its concentration was

TABLE II

Concentrations of macro- (N, P, K, Ca, and Mg) and micro- (Fe, Cu, Zn, Mn and B) nutrients in control or AMF-inoculated tomato leaves 50 d after germination

Treatment[¶]	Macronutrients					Micronutrients				
	N (mg 100 g^{-1} DW)	P (mg 100 g^{-1} DW)	K (mg 100 g^{-1} DW)	Ca (mg 100 g^{-1} DW)	Mg (mg 100 g^{-1} DW)	Fe (mg l^{-1})	Cu (mg l^{-1})	Zn (mg l^{-1})	Mn (mg l^{-1})	B (mg l^{-1})
Control	4.41 ± 0.24a[†]	0.36 ± 0.02a	2.04 ± 0.15a	3.52 ± 0.18a	0.80 ± 0.02a	336.7 ± 7.3a	16.7 ± 0.3a	23.6 ± 1.9a	86.9 ± 2.5a	26.1 ± 1.3a
D_1	5.63 ± 0.18b	0.54 ± 0.02b	3.15 ± 0.11b	5.03 ± 0.12b	0.99 ± 0.01b	661.7 ± 8.9c	23.4 ± 0.5b	31.8 ± 1.0b	100.5 ± 8.8b	36.9 ± 0.7b
D_2	5.40 ± 0.21b	0.52 ± 0.08b	3.97 ± 0.21c	5.96 ± 0.27c	0.98 ± 0.02b	621.8 ± 22.6b	23.9 ± 1.1b	32.9 ± 1.5b	98.9 ± 9.4b	37.6 ± 1.9b
Optimum ranges[‡]	3.50 – 4.50	0.25 – 0.41	1.60 – 3.10	1.80 – 3.60	0.40 – 0.60	–	–	–	–	–

[†]Each value is a mean (n = 10) ± SE. Mean values in each column followed by a different lower-case letter differ significantly ($P \leq 0.05$) as determined by Tukey's multiple range test.
[¶]D_1, inoculum dose of 0.12 g AMF plant^{-1}; D_2, inoculum dose of 0.20 g AMF plant^{-1}.
[‡]Optimum macronutrient ranges were calculated for tomato using the Diagnosis and Recommendation Integrated System (DRIS) proposed by Hartz *et al.* (1998).

27.7% higher in the D_1 and 22.4% higher in D_2 treatments than in the control treatment (Table II). This reflected the conclusions of previous research which highlighted that AMF can: (i) increase the use of different forms of N by plants (Hodge *et al.*, 2001); and (ii) take-up N directly and transfer it to roots of the host plant (Johansen *et al.*, 1996). All leaf macronutrient concentrations measured in AMF-inoculated plants were above the optimum ranges calculated by DRIS and proposed by Hartz *et al.* (1998; Table II).

As regards micronutrients, the greatest increases (compared to the control treatment) were observed for Fe (D_1 = 96.5% and D_2 = 84.7%), while the lowest increases were detected for Mn (D_1 = 15.5% and D_2 = 13.8%). These results agree with previous studies (Kothari *et al.*, 1990; Ortas and Akpinar, 2006) in which increases in the uptake of relatively immobile micronutrients such as Cu, Zn, Mn, and Fe were observed. However, the effect of AMF on the acquisition of these micronutrients by the host plant remains unclear, and some controversy exists. For instance, Kothari *et al.* (1991) demonstrated that Mn concentrations were lower in AMF-inoculated than in non-AMF plants; while Pacovsky and Fuller (1988) observed a decrease in Fe concentration in soybean plants inoculated with AMF. This variation in the acquisition of some mineral nutrients by AMF could be explained by the experimental conditions, the strain of fungus, and/or soil type, pH, nutrient supply, or soil temperature (Raju *et al.*, 1990).

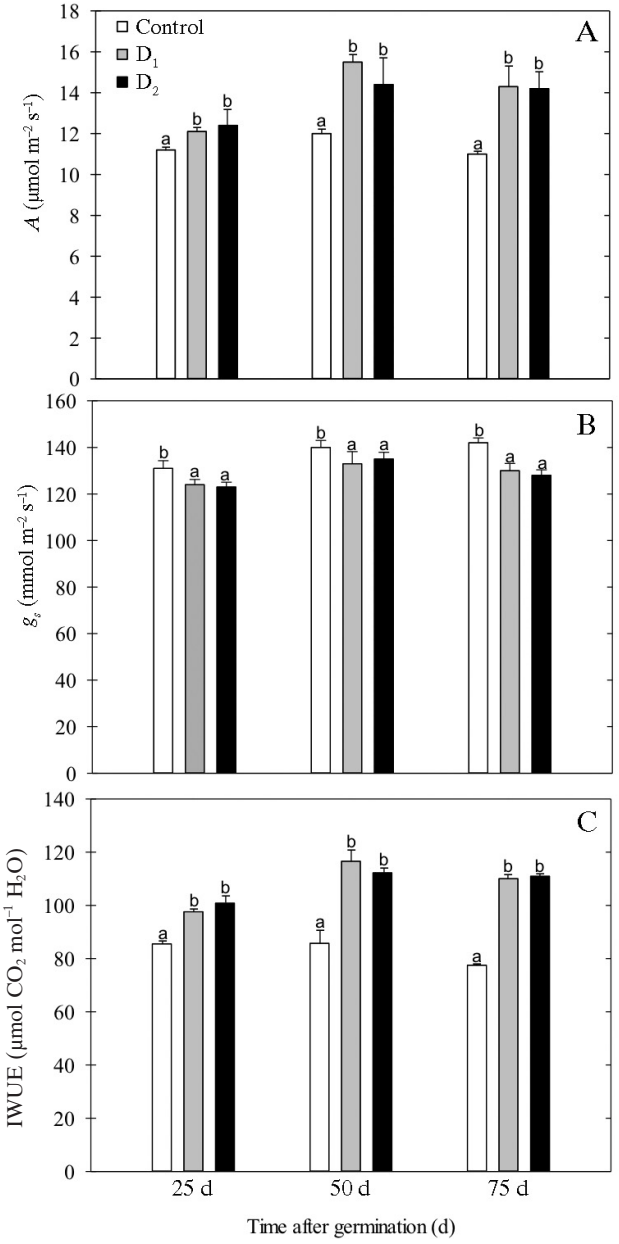

FIG. 1
Effect of two AMF inoculum doses (D_1 = 0.12 g plant^{-1} and D_2 = 0.20 g plant^{-1}) on selected physiological parameters in young tomato seedlings. Panel A, rate of net photosynthesis (A; µmol m^{-2} s^{-1}); Panel B, stomatal conductance (g_s; mmol m^{-2} s^{-1}); and Panel C, intrinsic water use efficiency (IWUE; µmol CO_2^{-1} mol H_2O) measured 25, 50, and 75 d after seed germination. Mean values (n = 10) ± SE in each Panel with a different lower-case letter differ significantly ($P \leq 0.05$) as determined by Tukey's multiple range test.

Leaf gas exchange

Our results confirmed that the rate of net photosynthesis (A) and stomatal conductance (g_s) were dependent on the level of AMF inoculation (Figure 1A, B). AMF-inoculated plants significantly reduced their g_s and increased their A and IWUE values in both the D_1 and D_2 treatments, but no significant differences were observed between the inoculation treatments on any measurement date (Figure 1). Seventy-five DAG, A values were 30.0% and 29.1% higher than in the control treatment in the D_1 and D_2 treatments, respectively (Figure 1A). It is likely that increases in Mn (a cofactor for enzyme pathways in photosynthesis) and, more especially, Fe (an important constituent of chlorophyll), increased the photosynthetic activity in AMF-inoculated plants (Table II), as reported elsewhere. For example, Ruiz Lozano (2003) observed that a mycorrhizal symbiosis with lettuce plants significantly improved plant growth, mineral uptake, the rate of CO_2 exchange, water use efficiency, transpiration, and stomatal conductance under drought conditions and when there was an adequate supply of water. Similarly, a mycorrhizal symbiosis with water-stressed *Rosmarinus officinalis* plants had a beneficial effect on plant water status and leaf gas exchange, improving root hydraulic conductivity and increasing photosynthetic activity (Sánchez-Blanco *et al.*, 2004).

In the case of g_s, the higher rate of net photosynthesis in plants inoculated with AMF (Figure 1A) cannot be attributed to increases in stomatal conductance. g_s values were always significantly lower in AMF-inoculated plants (e.g., 8.5% and 9.9% lower at 75 DAG in treatments D_1 and D_2, respectively, compared to the control treatment; Figure 1B). This is not surprising, as AMF may exert some control over transpiration, allowing AMF-inoculated plants, with no water restrictions, to maintain higher IWUE values (Figure 1C; Navarro-Ródenas *et al.*, 2013) and, consequently, a better plant water status (data not shown; Alvarez and Sánchez-Blanco, 2013).

Living respiratory activity of the AMF

Living respiratory activity (LRA) was determined by measuring the succinate dehydrogenase activity (SDA) of the AMF. The relationships between the SDA and A

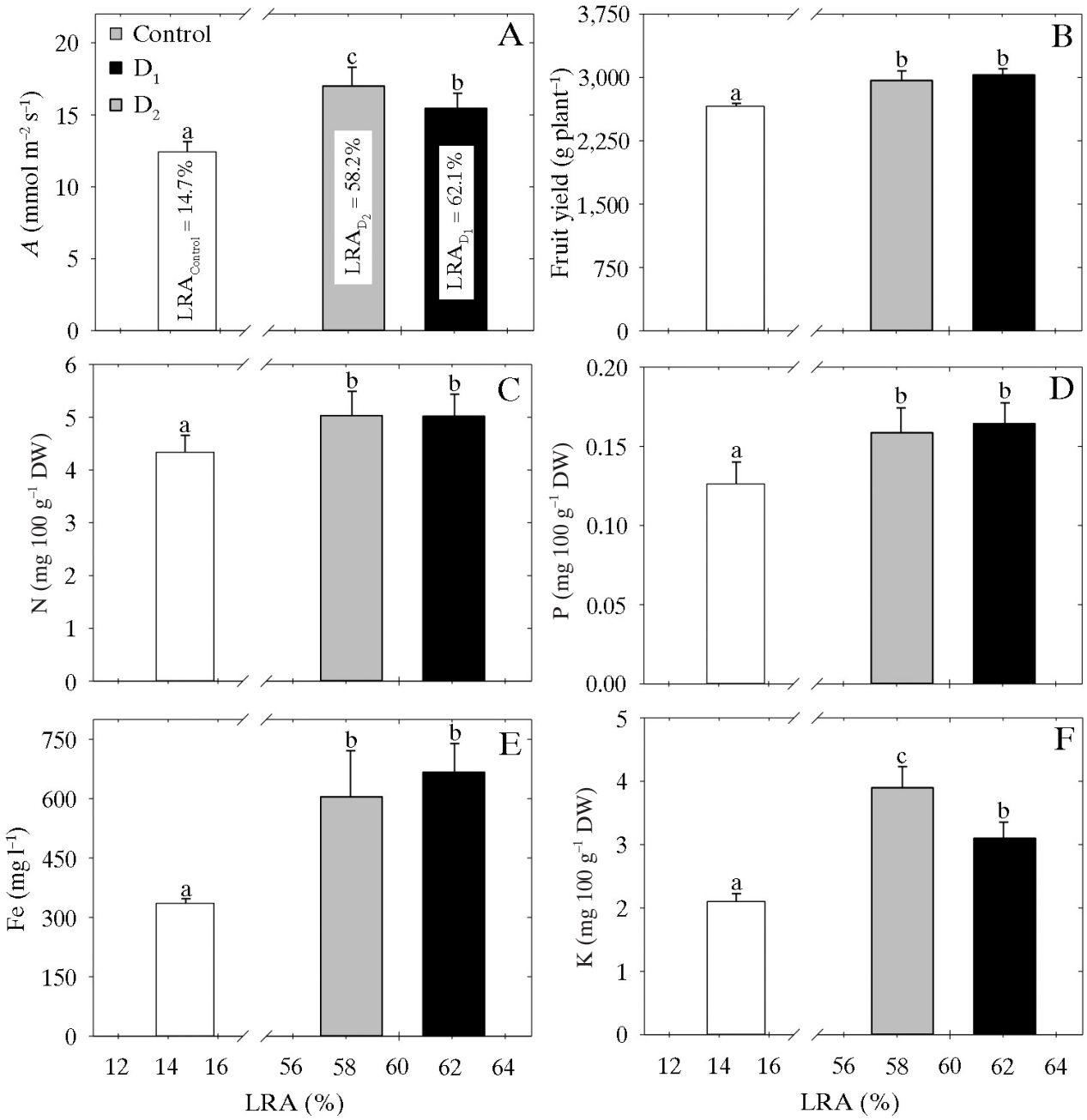

Fig. 2
Relationships between the living respiratory activity (LRA; %) of tomato seedling roots and selected physiological and agronomic parameters. Panel A, the rate of net photosynthesis (A; μmol m^{-2} s^{-1}); Panel B, fruit yield (g $plant^{-1}$); Panel C, N concentration; Panel D, P concentration; Panel E, Fe concentration (mg l^{-1}); and Panel F, K concentration. Values are means (n = 10) ± SE and mean values in each Panel with a different lower-case letter are significantly different at $P \leq 0.05$ by Tukey's multiple range test.

values, fruit yield, and leaf concentrations of N, P, Fe, and K are shown in Figure 2. The highest values of LRA were observed in the D_1 and D_2 treatments, and differed significantly compared to the LRA observed in the control treatment. Note that A values, fruit yield, and leaf concentrations of N, P, Fe, and K increased with LRA, emphasising the fact that AMF-inoculation improved the physiological and agronomic performance of tomato plants.

Plant growth and fruit yields

Inoculation of tomato plants with AMF improved their physiological and agronomic performance (Table III). Root dry biomass (DM) increased approx. three-fold and six-fold compared to the control treatment in the D_1 and D_2 treatments, respectively. The increase in shoot DM was lower than that detected for root DM, although shoot DM values were still significantly higher than those observed in the control treatment (increases of 48% in both the D_1 and D_2 treatments compared to the control treatment). Such growth-stimulating effects of AMF on shoot and root DMs have been reported previously (Terry *et al.*, 2001; Hamel and Plenchette, 2007) and have frequently been

TABLE III

Root and shoot dry biomass (DM) and root:shoot DM ratios 50 d after germination (DAG) and fruit yield (g plant^{-1}), and yield increase (%) compared to control tomato seedlings at harvest 90 DAG following two different doses of mycorrhizal inoculum

Treatment[¶]	Root dry biomass (g)	Shoot dry biomass (g)	Root:shoot DM ratio	Fruit yield (g plant^{-1})	Yield increase (%)
Control	0.4 ± 0.0a[†]	2.4 ± 0.2a	0.17 ± 0.01a	2,660.2 ± 85.3a	-
D_1	1.3 ± 0.1b	3.3 ± 0.1b	0.39 ± 0.02b	3,025.0 ± 73.6b	13.7
D_2	2.4 ± 0.1c	3.6 ± 0.2b	0.66 ± 0.02c	2,975.0 ± 96.2b	11.8

[†]Each value is a mean (n = 10) ± SE. Mean values in each column followed by a different lower-case letter differ significantly ($P \leq 0.05$) as determined by Tukey's multiple range test.
[¶]D_1, inoculum dose of 0.12 g AMF plant^{-1}; D_2, inoculum dose of 0.20 g AMF plant^{-1}.

attributed to an increase in the uptake of P and other elements (Herrera *et al.*, 2011).

The root:shoot DM ratio was also significantly higher in AMF-treated plants, which indicated that tomato plants inoculated with AMF accumulated more DM in their roots in order to maximise their capacity for nutrient and water uptake (Fernández *et al.*, 2011). In turn, this resulted in higher tomato fruit yields in AMF-treated plants than in untreated plants. This conclusion was confirmed by our results, in which a fruit yield increase of approx. 12% was seen in both the AMF treatments compared to the control treatment (Table III). This result also agreed with Hakan *et al.* (2011) for tomato plants pre-inoculated with AMF under saline conditions.

CONCLUSIONS

In this study, AMF-root colonisation, fungal intensity, and the living respiratory activity of the AMF improved the performance of tomato plants, including their growth, nutrition, and fruit yield (expressed as the average number of tomato fruit × the average fruit FW) in both treatments. There was also a strong correlation ($R^2 = 0.84$) between living respiratory activity and the rate of net photosynthesis. A higher dose of AMF inoculum did not necessarily result in increased symbiotic development, and there was an upper threshold above which plant performance was not improved.

These results confirmed the potential of applying AMF via a drip irrigation system and should encourage their use in sustainable agriculture in arid and semi-arid areas.

We thank CEBAS-CSIC and SYMBORG S.L. for the use of their facilities to conduct this research, and Christopher Heiss and David Walker for revision of this manuscript.

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