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Effects of nutrient solution strength, PGPB, and mycorrhizal inoculation on growth, yield, and quality of strawberry

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Abstract: Strawberry (*Fragaria* × *ananassa* Duch) cultivation is not without difficulty since its root environment is sensitive to many biotic and abiotic stresses. In addition, production is often phosphate limited, even at relatively high phosphorus levels, as the high fruit yields greatly exceed the capacity of the phosphorus supply by the root system. Arbuscular mycorrhizal fungi (AMF), plant growth promoting bacteria (PGPB), and combinations of both are known to mitigate stress and enhance nutrient uptake, particularly phosphorus. Therefore, we studied the effects of AMF (*Glomus mosseae*, and *Glomus intraradices*), PGPB (*Azospirillum lipoferum DSM1691*, and *Pseudomonas fluorescens DSM 50090*), and their combinations in a greenhouse under either optimal conditions, i.e. electrical conductivity (EC 1.3 dS.m^{-1}), or high nutrient solution strength, i.e. controlled stress (EC 1.9 dS.m^{-1}) conditions. Under optimal EC (1.3 dS.m^{-1}), AMF inoculation increased leaf phosphorus concentrations up to 25% and fruit yield up to 21%. Quality parameters like total phenolic, vitamin C, anthocyanin, fruit EC, and pH were also significantly increased. The high nutrient levels (EC 1.9 dS.m^{-1}) did increase leaf phosphorus but decreased leaf fresh weight (29%) and fruit yield (18%). Substrate inoculation with AMF partly mitigated the stress effect at 1.9 dS.m^{-1}; the plants appeared to be healthier, and the stress indicators – peroxidase activity (POD) and ion leakage – were reduced by AMF. Additionally, AMF inoculation at 1.9 dS.m^{-1} increased stomatal conductance, relative water content (RWC), chlorophyll index, fruit EC, leaf potassium, and leaf phosphate levels. Plants inoculated with PGPB did not show biologically relevant effects. In conclusion, AMF inoculation has a significantly beneficial effect on fruit yield and quality of substrate-cultured strawberries. These findings can assist growers in further boosting the trend of sustainable agriculture using microbial inoculants, even under optimal conditions of high-tech greenhouses and vertical farms.

Key words: Antioxidant activity, biofertilizer, electrical conductivity, salinity, soilless culture

1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch) is a tasty and nutritious fruit crop whose production is becoming more prominent. Global production over the past 25 years linearly increased by 0.21 million t per year, currently exceeding 9 million t per year (FAOSTAT, 2021). Strawberry cultivation is not without difficulty as its root environment is particularly sensitive to many biotic and abiotic stresses (Amil-Ruiz et al., 2011; Hautsalo et al., 2016; Keutgen and Pawelzik, 2008). For example, strawberry is very sensitive to osmotic stress caused by salinity (NaCl) (Kaya et al., 2002; Khayyat et al., 2007; Mozafari et al., 2019; Saied et al., 2005; Sarooshi and Cresswell, 1994). Yet, the use of greenhouse hydroponic systems can reduce stress and improve the productivity and quality of strawberries (Caruso et al., 2011; Hautsalo et al., 2016; Silber and Bar-Tal, 2008). This improvement is mainly accomplished by the use of clean growth substrates, proper irrigation regimes, and tailored nutrient solutions. Optimal nutrient solution formulations have been identified for many species (Steiner, 1961; Van Delden et al., 2020), including strawberries (Jun et al., 2013; Neocleous and Savvas, 2013; Sarooshi and Cresswell, 1994; Shirko et al., 2018). However, high-quality fertigation water is not always available and is forecasted to decline on a global scale (Hassani et al., 2020). Poor water quality, suboptimal watering frequency, or high fertilizer dosing, all lead to osmotic stress, i.e. high electrical conductivity (EC in dS.m^{-1}) in the rhizosphere, thereby compromising plant growth (Awang and Atherton, 1995; Kaya et al., 2002; Keutgen and Pawelzik, 2008; Khayyat et al., 2007; Mozafari et al., 2019; Saied et al., 2005; Sarooshi and Cresswell, 1994).

Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria (PGPB) are known to

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mitigate the effects of biotic (Li et al., 2006; Matsubara et al., 2004b; Norman et al., 2016) and abiotic stress (Fan et al., 2011; Matsubara et al., 2004a; Sinclair et al., 2013) in strawberry plants. These studies indicate that plants under stress conditions inoculated with either AMFs or PGPB have better root and shoot growth, nutrient uptake, hydration, chlorophyll content, and resistance to diseases. Increased nutrient uptake is partly due to better nutrient mobilization and retention but also selective uptake, such as against sodium and in favor of potassium (Wakeel et al., 2011). Both AMFs and PGPB can serve as biocontrol agents of phytopathogens by direct competition but also by the production of antibiotic metabolites (e.g., hydrogen cyanide (HCN), vitamin B groups, and amino acids), which reduce biotic stress and induce systemic resistance in plants (Nakkeeran et al., 2006; Rouphael et al., 2015; Tian et al., 2020). Moreover, AMFs and PGPB can induce the production of phytohormones (abscisic acid and ethylene) and antioxidative molecules (vitamin C, phenolic compounds) and enzymes (catalase and peroxidase), thereby reducing oxidative stress, supporting the plants defense systems, and potentially improving product quality (Gray and Smith, 2005; Nakkeeran et al., 2006).

Although a plethora of research on beneficial microorganisms has been performed, a huge amount of knowledge gaps need to be addressed to facilitate microorganisms commercialization in soilless agriculture (Azizoglu et al., 2021). Despite the frequently reported beneficial effects of AMF and PGPB on strawberry grown under stress conditions, some studies reported no effects (Calvo-Bado et al., 2006; Maboko et al., 2013; Martínez et al., 2015; Palencia et al., 2013) or even adverse effects under optimal conditions (Chávez, 1990; Sowik et al., 2016). This is because effects can be cultivar specific (Sowik et al., 2016; Vestberg, 1992a), substrate-specific (Vestberg et al., 2005; Vestberg, 1992b), and, in some cases, AMF and PGPB only work in a consortium (de Andrade et al., 2019; Trevizan Chiomento et al., 2019). In addition, the high nutrient levels are commonly used in commercial soilless culture, e.g., EC 1.3 $dS.m^{-1}$ (Caruso et al., 2011), cause inevitable stress for sensitive horticultural species such as strawberry. To date, only a few studies have been performed on using coinoculation of AMF and PGPB to mitigate yield reduction under these seemingly optimal horticultural conditions (Emmanuel and Babalola, 2020). Coinoculation might simply cumulate the benefits of AMF and PGPB but can also cause positive interactive effects, as PGPB has been shown to stimulate the beneficial role of AMF and vice versa (Selvakumar et al., 2016; Xie et al., 2018). Therefore, this study aims to clarify the effects of AMF (*Glomus mosseae*, and *Glomus intraradices*) and PGPB (*Azospirillum lipoferum DSM1691*, and *Pseudomonas fluorescens DSM 50090*), and their coinoculation on quality and productivity of greenhouse grown strawberry (*Fragaria x ananassa* Duch 'Selva') under controlled stress (EC = 1.9 $dS.m^{-1}$) and optimal (EC = 1.3 $dS.m^{-1}$) conditions.

2. Materials and methods

To attain our research aim we conducted a factorial study in a climate-controlled greenhouse from April to December. Each isolated PGPB strain (*Azospirillum lipoferum DSM1691*, or *Pseudomonas fluorescens DSM 50090*), and each isolated AMF strain (*Glomus mosseae*, or *Glomus intraradices*), and their combinations were used as inoculum for plants grown under either optimal (1.3 $dS.m^{-1}$) or moderate osmotic stress (1.9 $dS.m^{-1}$) (Table 1). Quality and productivity in response to these treatments were characterized by leaf and fruit fresh weight. To gain a better understanding of the underlying plant physiological response, we measured relative water content (RWC), membrane ion leakage, chlorophyll index (SPAD), stomatal conductance, and leaf nutrient status (N-P-K-Ca). To assess plant stress levels, catalase (CAT) and peroxidase (POD) were measured as indicators for enzymatic antioxidant capacity and leaf phenol and fruit vitamin C content as nonenzymatic antioxidant capacity; together with fruit pH, EC, and phenol content.

2.1. Growth conditions, plant material, and microorganism inoculation

This experiment was conducted in a climate-controlled greenhouse located in Mahabad, Iran; using a 10/14 h light/dark cycle with 65% ± 2% atmospheric humidity at 23/16 ± 2 °C light/dark temperature.

Strawberry (*Fragaria* × *ananassa* Duch 'Selva') propagules were transferred to autoclaved (120 °C at 1 bar for 1 h) pots (4 L, 1 plant per pot), filled with washed and autoclaved (120 °C at 1 bar for 1 h) cocofibre-perlite substrate (70: 30 V/V; Porous 66%). In more detail, autoclavable propylene nylon (25 cm × 50 cm dimension and 120-micron thickness) bags were used to autoclave the substrate media. Each bag was filled for 75% with the substrate (cocofibre-perlite) along with 300 mL of distilled water to ensure the vapor penetration within the media for 1 h (120 °C, 1 bar). After a cooling period, the substrate was inoculated with one of nine inoculum combinations (Table 1) established by using isolated AMF strains (either *Glomus mosseae*, or *Glomus intraradices*) or isolated PGPB strains (either *Azospirillum lipoferum DSM1691*, or *Pseudomonas fluorescens DSM 50090*). All strains were supplied by Water and Soil Institute, Karaj, Iran. Pot substrate inoculation with AMF was done by mixing 50 g inoculated sphagnum peat (1 g contains 1.6×10^4 spores) with the cocofibre-perlite substrate. PGPB inoculation was done by mixing 2 mL suspension (1 mL contains 9.8×10^7 colony forming units) with the cocofibre-perlite substrate

Table 1. Treatment overview with the combinations of applied electrical conductivity (EC), arbuscular mycorrhizal fungi (AMF), and plant growth promoting bacteria (PGPB) inoculums (n = 3 plants per treatment).

Treatment count	EC (dS. m^{-1})	Applied AMF species	Applied PGPB species
1	1.3	None	None
2	1.3	None	*A. lipoferum*
3	1.3	None	*P. fluorescens*
4	1.3	*G. mosseae*	None
5	1.3	*G. mosseae*	*A. lipoferum*
6	1.3	*G. mosseae*	*P. fluorescens*
7	1.3	*G. intraradices*	None
8	1.3	*G. intraradices*	*A. lipoferum*
9	1.3	*G. intraradices*	*P. fluorescens*
10	1.9	None	None
11	1.9	None	*A. lipoferum*
12	1.9	None	*P. fluorescens*
13	1.9	*G. mosseae*	None
14	1.9	*G. mosseae*	*A. lipoferum*
15	1.9	*G. mosseae*	*P. fluorescens*
16	1.9	*G. intraradices*	None
17	1.9	*G. intraradices*	*A. lipoferum*
18	1.9	*G. intraradices*	*P. fluorescens*

in the pot. Plants of all 9 treatments were grown at two EC levels (1.3 and 1.9 dS. m^{-1}) resulting in a total of 18 treatments that were replicated 3 times (n = 3 pots per treatment); one set of 18 treatments formed a statistical block (with n = 1), resulting in 3 blocks in total. To establish the two EC levels, plants were drip irrigated daily with a 300 mL nutrient solution of either 1.3 or 1.9 dS. m^{-1} (Table 2), and the nutrient solution was allowed to freely drain from the pots. To guarantee the set point EC within the substrate and prevent potential salt accumulation, an extra nutrient solution (>500 mL) was used weekly to wash the substrate until the set point EC was reached.

2.2. Plant physiological measurements

Leaf stomatal conductance (mM H_2O m^{-2} s^{-1}) (Leaf Porometer; SN: LP2402; Decagon, USA) and chlorophyll index (portable SPAD Opt science CCM-200: USA) were measured on two to three fully expanded mature leaves per plant. Measurements were conducted during fruiting waves, per block, and at fixed moments early in the morning.

Relative water content (RWC) was calculated based on Ritchie et al. (1990):

$$\mathrm{RWC} = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{SW} - \mathrm{DW}} \times 100$$

Where leaf fresh weight (FW) was represented by four leaf disks (1 cm diameter) punched and weighed directly after sampling; saturated weight (SW) was obtained by weighing these disks after a 4 h incubation period at 4 °C in a Petri dish filled with distilled water; before weighing, excessive water was removed by placing the disks shortly on filter paper; dry weight (DW) was obtained by drying the disks at 72 °C for 72 h.

Cell membrane stability was assessed by estimating ion leakage as described by Lutts et al. (1996). Briefly, 1 g of fresh leaf tissue was transferred into a falcon tube containing 20 mL of deionized water. After 24 h (25 °C), the ionic leakage of the samples (L_1) was measured by a conductivity meter (Aqualytic sensdirect, CD24). The samples were then autoclaved for 20 min at 120 °C and 1 bar. Samples were cooled down, the ionic leakage of the solution (L_2) was measured, and ion leakage (%) was calculated as

$$\mathrm{IL}(\%) = \frac{L_1}{L_2} \times 100$$

At the end of the experiment, plants were harvested per block, roots and leaves were separated. Fresh weight was directly measured, and leaves were counted; leaf area (LA) ($cm^2 \cdot plant^{-1}$) was measured using a Li-Cor-3100 (Li-Cor Biosciences, Lincoln, NE, USA). To obtain dry weights, aerial and underground biomass were dried separately at 70 °C until constant weight (approximately 3 days).

For analysis of mineral content, fully expanded mature leaves were sampled and washed using distilled water. Samples were oven dried (70 °C) for 48 h, ground, and divided into two subsamples. The first sample was used to determine nitrogen concentration using the Kjeldahl method (Jones, 1991). The second sample minerals were extracted using chloric acid to determine phosphate (P) and potassium (K) concentration using the Oliveira

Table 2. Macro and micro elements concentrations per electrical conductivity (EC) level of the used nutrient solutions (pH range 5.5–6.0).

EC*1(dS.m^{-1})	Macronutrients (mmol L^{-1})					Micronutrients (µmol L^{-1})					
	N*2	P	K	Ca	Mg	Fe	Cu	Mn	Zn	B	Mo
1.3	6.1	0.5	2.6	2	1.4	51.2	27.5	19.2	38.2	16.2	1.5
1.9	9.3	0.9	4.4	3	1.7	51.2	27.5	19.2	38.2	16.2	1.5

*1 Applied nutrient solution EC did not deviate more than ± 0.06 dS.m^{-1} from the set point.
*2 NH_4^+: NO_3^- ratio was 10:90%

method (Oliveira et al., 2010), and calcium (Ca) was determined using atomic absorption spectrophotometry (Perkin-Elmer Corporation 1964).

2.3. Plant chemical analysis

Phenolic content was measured as described by Velioglu et al. (1998). Briefly, 0.1 g of the fresh leaf tissue was ground with 5 mL of 80% methanol containing 1% choleric acid. The solution was mixed for 2 h at 51 rpm on a shaker and then centrifuged at 3000 rpm for 10 min, 100 µL of the obtained supernatant was mixed with 750 µL of folin reagent. After 5 min, 750 µL of 6% sodium carbonate was added to the mixture, and the absorption (UV/VIS Lambda25 Perkin Elmer) at 725 nm was measured after 90 min. The standard curve of gallic acid (Supplementary File Figure S1) was used to calculate the total phenolic content (mg gallic acid/g fresh weight).

Total protein was measured by grinding 1 g of fresh leaf tissue in 5 ml of 0.05 M Tris-HCL buffer, pH 7.5. The extraction was centrifuged for 25 min in 10,000 g at 4 °C (Hermle Z216 MK; Germany), and 0.1 mL of the supernatant and 5 mL biuret reagent were vortexed immediately. The biuret reagent was prepared beforehand by adding 0.1 g Coomassie brilliant blue G250 to 50 mL of 95% ethanol mixed with 100 mL of 85% phosphoric acid. This solution was filled to 1L using distilled water. The absorbance of the supernatant-biuret mixture was measured at 595 nm (Perkin Elmer UV/VIS Lambda 25; USA) on basis of Bradford (1976).

To measure the antioxidant enzyme activity, i.e. peroxidase (POD) (EC1.11.1.7) and catalase (CAT) (EC1.11.1.6) activity, enzymatic extractions were made from fully matured leaves that were uniform in colour and size. Leaves were directly stored after harvest in a –80 °C freezer. Frozen leaf tissue (1 g) was ground with 4 mL potassium-phosphate buffer (1% polyvinyl pyrrolidone and 0.1 mM EDTA, pH 7) in a chilled mortar; the obtained homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C (Hermle Z216 MK). This extract was used to measure the POD and CAT enzymatic activity.

POD activity was determined as described by Malik and Singh, (1980). Briefly, 3.5 mL of phosphate buffer (pH 6.5), 0.2 mL leaf extract, and 0.1 mL of freshly prepared o-dianisidine solution were pipetted in a dry cuvette. The mixture's temperature was raised to 28–30 °C then 0.2 mL of 0.2 M H_2O_2 was added. After mixing, the spectrophotometric (UV/VIS Lambda25 Perkin Elmer) absorbance at 430 nm was determined in 30 s intervals over 3 min. The POD activity was expressed as ΔOD430 min^{-1} mg^{-1} protein.

CAT activity was determined as described by Aebi (1984). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 7), 0.2 mL of 0.2 M H_2O_2, and 0.3 mL enzyme extract. CAT activity was measured as a decline in absorbance at 240 nm with an extinction coefficient (0.0436 mM^{-1} cm^{-1}). The CAT activity was expressed as ΔOD240 min^{-1}mg^{-1} protein.

2.4. Fruit yield and quality

Marketable fruit yield per individual plant was assessed by collecting, counting, and weighing ripe fruits throughout the whole experiment. Fruit quality was assessed by measuring fruit pH, EC, anthocyanin, and ascorbic acid (vitamin C) content.

The fruit pH and EC were measured in a filtered fruit extract using a pH meter (Mettler - S20 SevenEasy™ pH) and an EC meter (Sartorius-PT-20, Germany) (dS m^{-1}). The fruit extract was made by grinding fully grown, ripe, and uniform strawberries first and then suspending 10 g of fruit pulp in 100 mL distilled water.

The vitamin C content of the ripe fruits was measured using an HPLC (Unicam-cristal-200, UK) (Nisperos-Carriedo et al., 1992). Briefly, 1 g of fresh fruit tissue was ground and then centrifuged in a 40 mL buffer solution containing oxalic acid and sodium acetate; after separating the supernatant, 2% potassium phosphate was added. Samples were injected into HPLC that had an inner diameter and pillar length of 4.6 and 25 mm Supelcosil LC-18 respectively with a pillar washing solution of KH_2PO_4 at a speed of 0.5 mL per min and UV/Vis detector at 260 nm. The vitamin C content was determined based on output peak retention time, the area under the curve (AUC), and comparison with ascorbic acid standards/control samples.

Anthocyanin extraction of strawberry fruits was prepared based on the Kallithraka et al. (2005) method with some modifications. Briefly, 1 g of freeze-dried strawberry powder was added to 100 mL methanol containing 1% HCl and stirred for 48 h. This mixture was used for the HPLC

analysis and prepared three times per plant. Samples were diluted 1:3 with 0.1 M HCl and filtered through 0.45mm syringe filters before chromatography. Chromatography analyses (HP 1050 chromatography apparatus coupled to a diode array detector) were performed based on Arnous et al. (2002). Results were expressed as $mg \cdot kg^{-1}$ fresh fruit weight.

2.5. Statistical analysis

The experiment was setup as a randomized complete block design with 18 treatments and 3 blocks that each contained one replicate, resulting in a total of 3 replicates per treatment. All statistical analyses were done in R version 4.0.4. To test for the difference between means of the response variables, linear mixed effects regression models were used, i.e. the lmer function from the lme4 package version 1.1–26. Assessment for significant differences ($p > 0.05$) was done using Tukey adjusted least square means (emmeans version 1.5.5–1). PGPB was regarded as random variable and AMF treatments as the main effect ($n = 9$). This was reasonable because both the absolute PGPB effect and interactive effects between PGPB and AMF were minor (Supplementary File Figure S2) for interaction plots. Random variables at each harvest were tested for homogeneity of variance (Levene's test 3.0–10). The residuals of the lmer models were tested for normality (Shapiro–Wilkinson test, histogram and QQ-plots inspection) and homogeneity (Levene's test 3.0–10 and residual plots). The lmer models for stomatal conductance and vitamin C showed a minor deviation from normality; however, the residuals were homogenous, and we decided that it was safe to use the lmer models for these data. However, the data of leaf fresh weight, protein, and phenol content significantly violated the assumptions required for lmer models; therefore, a Kruskal–Wallis Rank Sum Test was used, followed by a Dunn test (FSA package version 0.8.32) to identify differences between groups. To get a better appreciation of the correlations between the response parameters we added Pearson correlation plots as supplementary material (Supplementary File Figure S3a,S3b,S3c).

3. Results

The nutrient solution concentration with a moderately elevated electrical conductivity (EC) (1.9 dS m^{-1}) reduced plant growth, i.e. leaf fresh (29%) and dry (18%) weight and fruit production (18%), compared to an optimal EC of 1.3 $dS \cdot m^{-1}$ (Figure 1). Many other morpho-physiological parameters were also significantly ($p < 0.001$) lower at elevated EC compared to optimal EC (Table 3, Figure 2): leaf relative water content (RWC) (40%), stomatal conductance (25%), chlorophyll index (24%), leaf protein level (31%), leaf phenolic compounds (10%), and catalase activity (CAT) (41%).

Plants at elevated EC were visibly stressed (chlorosis), as confirmed by increased peroxidase activity (POD) (61%), and ion leakage (30%). Elevating the EC did increase plant nitrogen (N) and potassium (K) levels but had no significant effect on leaf phosphate (P) and calcium (Ca) content (Table 4).

Not only fruit yield (Figure 1), but also fruit quality aspects such as fruit pH, anthocyanin, and vitamin C content were all significantly lower at elevated EC compared to optimal EC (Figure 2 and Table 3). Surprisingly, the nutrient solution EC did not significantly affect fruit EC (Table 3).

While the effect of plant growth-promoting bacteria (PGPB) (*Azospirillum lipoferum* and *Pseudomonas fluorescents*) were biologically insignificant (Supplementary File Figure S2), arbuscular mycorrhizal fungi (AMF) (*Glomus mosseae* and *Glomus intraradices*) did affect plant growth (Figure 1 and 2; Table 3 and 4). More specifically, inoculation with AMF at elevated EC resulted in significant beneficial effects on stomatal conductance, POD activity (Figure 1), RWC, ion leakage (Table 3), chlorophyll index, phosphate (P), and potassium (K) concentration (Table 4). Still, fruit yield, phenolic compounds, pH, and vitamin C content were not significantly affected by AMF at 1.9 dS m^{-1} (Figure 1 and 2). However, at optimal EC (1.3 dS m^{-1}) both AMF species were able to increase these parameters (Figure 1). *G. mosseae* did increase the fruit EC at both EC levels of the nutrient solution (Table 3). However, none of the measured fruit related parameters were affected by substrate inoculation with PGPB nor did PGPB have substantial interaction with AMF (Supplementary File Figure S2).

4. Discussion

Strawberry (*Fragaria* × *ananassa* Duch 'Selva') plants grown at a moderately elevated electrical conductivity (EC), 1.9 $dS \cdot m^{-1}$ instead of 1.3 $dS \cdot m^{-1}$, show a significant reduction in plant leaf fresh weight (29%) and fruit production (18%) (Figure 1). To some extend this finding is in line with earlier studies (Gallace et al., 2017; Jun et al., 2012, 2013) where the EC for optimal yield situates between 0.8 and 1.6 $dS \cdot m^{-1}$. However, the significant yield reduction (Figure 1) and physiological responses (Figure 2, Table 3 and 4) of cultivar 'Selva' with a moderate EC increase (0.6 $dS \cdot m^{-1}$) (Figure 1) show that 'Selva' is very sensitive to EC concentration when compared to other cultivars (Gallace et al., 2017; Jun et al., 2012, 2013). For example, the relative water content (RWC) of cultivar 'Selva' was reduced by 40% in response to a 0.6 $dS.m^{-1}$ EC increase (Table 3); whereas Karlidag et al. (2011) found that the RWC of cultivars 'Fern' and 'A6' was reduced by 11% and 13% in response to an increase of ~3.5 dS m^{-1} NaCl. The cause of salinity stress, i.e. NaCl or

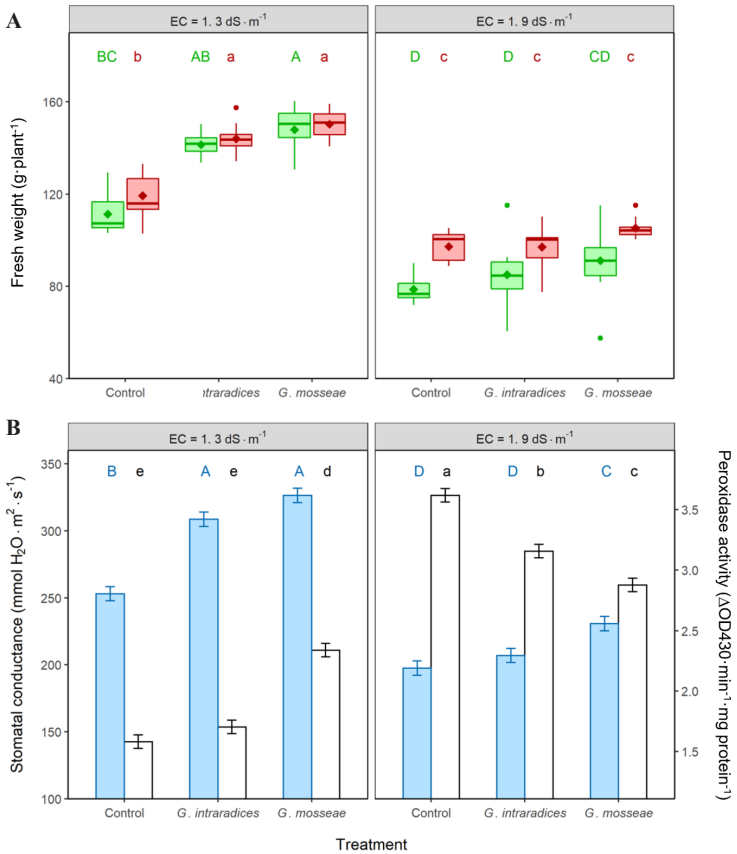

Figure 1. Leaf (light/green) and fruit (dark/red) fresh weight (A) and stomatal conductance (blue) and peroxidase activity [POD] (white) (B) for each AMF treatment. Boxplots (A) display data distribution (n = 9), from bottom to top: lower whisker as a minimum, bars as: the first quartile, median, and third quartile, and the upper whisker as maximum. The dots represent outliers, and diamonds are mean values. Bar size (B) corresponds with the least square (LS) means of AMF treatments modelled with PGPB as random variable, error bars represent the model standard error. Boxes or bars sharing the same letter are not significantly different $p > 0.05$. Tukey-adjusted comparisons were used for fruit fresh weight, stomatal conductance and POD; and a Kruskal–Wallis test was followed by the Dunn test for plant fresh weight.

macronutrient concentrations, might also play a role here. Under soilless conditions, macronutrients are typically the predominant cause of salinity, while most literature studies NaCl effects. Nevertheless, increasing the EC level does, in itself, limit water availability in the substrate (0.6 $dS.m^{-1}$ = −0.0216 MPa), which can directly affect root water uptake (Marschner, 2012). However, aside from reducing water availability, high nutrient concentrations are themselves also toxic (Marschner, 2012). This toxicity combined with lowered water availability can explain the observed chloroplast deterioration and the lower chlorophyll index. Furthermore, the stomata closure presumably results in a CO_2 deficiency in the leaf apoplast. Altogether these effects lower photosynthetic capacity (Figure 1, Table 3, and 4) and result in growth retardation (Razavi et al., 2008). Thus, our findings suggest a strong salinity effect of macronutrients on strawberry. However, the molecular reasons underlying strawberry's sensitivity were out of scope for this study and remain unclear.

The EC sensitivity was, to some extent, counteracted by substrate inoculation with arbuscular mycorrhizal fungi (AMF) (*Glomus mosseae* and *Glomus intraradices*). The AMF species *G.mosseae* was especially able to exhibit positive effects under elevated EC, as plants appeared to be healthier and stomatal conductance, RWC, chlorophyll index, fruit EC, leaf phosphate, and potassium concentration were all significantly elevated compared to the control. Additionally, the overall stress response was also reduced by AMF, based on the decrease in POD and ion leakage. Altogether, this indicates that AMF positively

Table 3. Effects of nutrient solution electrical conductivity (EC) and arbuscular mycorrhizal fungi (AMF) application on: catalase activity (CAT), cell ion leakage (CIL), relative water content (RWC), leaf dry weight (LDW), pH and EC of the fruits.

EC (dS m^{-1})	AMF treatment	CAT(μmol·min^{-1}·g^{-1})		CIL (%)		RWC (%)		LDW (g)		Fruit pH		Fruit EC(dS m^{-1})	
1.3	Control	2.8	b	43.6	c	61.1	b	31.5	b	3.53	bc	4.48	b
	G.intraradices	3.3	a	40.1	d	75.7	a	36.4	a	3.60	ab	4.48	b
	G.mosseae	3.5	a	38.3	d	79.0	a	38.2	a	3.69	a	4.63	a
1.9	Control	2.1	d	59.5	a	43.5	d	29.0	b	3.47	c	4.44	b
	G.intraradices	2.2	cd	56.3	a	46.8	cd	32.3	b	3.49	bc	4.42	b
	G.mosseae	2.4	c	50.0	b	50.7	c	36.4	a	3.58	abc	4.60	a
standard error		0.055		0.981		1.592		1.493		0.028		0.033	

The least square (LS) means (n = 9) of AMF treatments were modelled with PGPB as random variables. LS means sharing the same letter are not significantly different ($p > 0.05$, Tukey-adjusted comparisons).

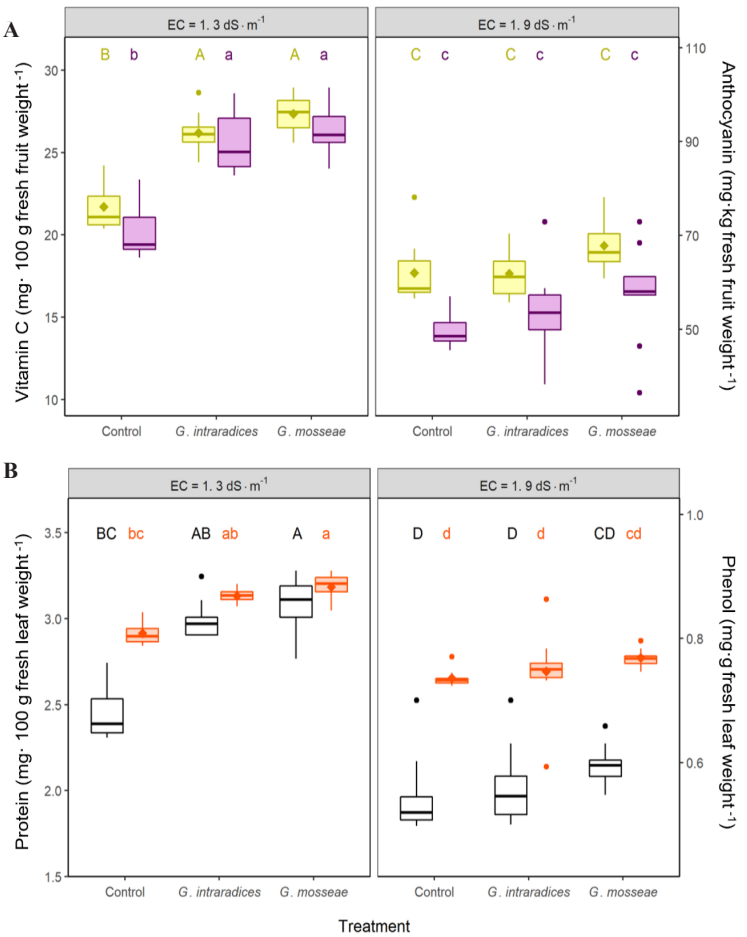

Figure 2. Fruit vitamin C (light/yellow) and anthocyanin (light/purple) content (A) and plant protein (white) and Phenol content (light/orange) (B) for each AMF treatment (regarding PGPB as random variable). Boxplots display data distribution (n = 9), with from bottom to top: lower whisker as a minimum, bars as: the first quartile, median, and third quartile, and the upper whisker as maximum. The dots represent outliers, and diamonds are mean values. Boxes sharing the same letter are not significantly different $p > 0.05$. Tukey-adjusted comparisons were used for vitamin C and Anthocyanin; and a Kruskal–Wallis test followed by the Dunn test for protein and phenol content.

Table 4. Effects of nutrient solution electrical conductivity (EC) and arbuscular mycorrhizal fungi (AMF) application on chlorophyll index (SPAD), and leave mineral element concentration: nitrogen (N), phosphate (P), potassium (K) and calcium (Ca) concentration.

EC (dS m^{-1})	AMF treatment	SPAD (%)		N (%)		P (%)		K (%)		Ca (%)	
1.3	Control	27	d	2.15	c	0.39	e	1.85	c	1.12	a
	G.intraradices	31	bc	2.35	bc	0.45	c	1.98	c	1.17	a
	G.mosseae	38	a	2.46	b	0.52	a	2.60	a	1.26	a
1.9	Control	22	e	2.70	a	0.43	d	2.14	b	1.12	a
	G.intraradices	29	c	2.92	a	0.48	b	2.25	b	1.17	a
	G.mosseae	32	b	2.86	a	0.53	a	2.70	a	1.22	a
standard error		0.979		0.054		0.008		0.065		0.075	

The least square (LS) means (n = 9) of AMF treatments were modelled with PGPB as a random variable. LS means sharing the same letter is not significantly different ($p > 0.05$, Tukey-adjusted comparisons).

affected plant nutrient and water status, cell membrane stability, and photosynthetic machinery. High EC lowered CAT activity (Table 3), which was also found for high salinity in rye (Feierabend and Dehne, 1996) and maize (Gondim et al., 2012), presumably due to the inability of catalase synthesis at high salt concentration. Hence, the increased CAT activity upon inoculation with AMF confirms that these fungi can alleviate EC stress to some extent.

Under optimal EC (1.3 dS m^{-1}), AMF, and especially *G. mosseae*, significantly increased leaf fresh weight (18%), fruit yield (20%), and fruit quality -- i.e. increased levels of vitamin C, anthocyanin, total phenolics, fruit EC, and pH (Figure 1 and 2, Table 3, and 4). Bona et al. (2015) found similar results at low nitrogen and phosphorus levels regarding increased fruit weight and vitamin C content. Cecatto et al., (2016) also found increased levels of anthocyanin and total phenolics upon AMF inoculation. Moreover, our study shows that inoculation with AMF increased both relative water content (RWC) (Table 3) and leaf nutrient levels, i.e. nitrogen, phosphate, and potassium (Table 4). These improvements might have contributed to the observed elevation of indicators for photosynthetic capacity, i.e. chlorophyll index (SPAD) (Table 4) and stomatal conductance (Figure 1). Most likely the combined effects of the increase in nutritional status, RWC, and indicators for photosynthetic capacity are the underlying cause for the increase in both plant and fruit biomass. These positive effects of AMF, resulting in higher photosynthetic capacity, have been reported for many species under suboptimal greenhouse conditions (Rouphael et al., 2015) and field conditions, e.g., for strawberry Borkowska (2002), but not under optimal conditions in the author's knowledge. Dunne and Fitter (1989) and Stewart et al. (2005) already predicted that even under optimal conditions strawberry might benefit from AMFs phosphorus delivering capacity. This is because the phosphorus demand caused by the high fruit production greatly exceeds the capacity of the root system, even at the relatively high phosphorus levels (Sharma and Adholeya, 2004) that are typically encountered in substrate culture. Our results confirm that AMF can increase leave phosphorus concentration, even under optimal conditions (Table 4), thereby alleviating this yield limiting factor. Interestingly, our data shows that under optimal conditions AMF concurrently causes a biomass increase and a mild stress response; as both enzymatic antioxidant capacity, i.e. catalase (CAT) (Table 3) and peroxidase (POD) (Figure 1) activity, and nonenzymatic antioxidant capacity, i.e. vitamin C, anthocyanin, and phenolic compounds (Figure 2) were significantly elevated. This is of interest for growers as higher levels of these antioxidants are generally associated with better fruit quality and nutritional value (Flores-Félix et al., 2015).

Yet, the findings of this study should be considered with some limitations. Firstly, the sample size of n = 3 is rather small. However, the PGPB treatments as a random variable result in a more robust sample size (n = 9). This is reasonable because both the absolute PGPB effect and interactive effects between PGPB and AMF were minor (supplementary file Figure S2). Secondly, no observations were made on the rooting system. In other words, the results reported here can only be related to substrate inoculation with AMF and PGPB, as no tests were done to identify whether the roots were hosting these micro-organisms.

Hence, the poor establishment of growth promoting bacteria in the substrate could be the reason behind the absence of biologically relevant effects of *Azospirillum lipoferum* DSM1691, and *Pseudomonas fluorescens* DSM 50090. We used cocofibre instead of peat as substrate, because peat is known to negatively affect strawberry's symbiosis with AMF (Vestberg et al., 2005; Vestberg, 1992b). However, the cocofibre might have hindered PGPB-root symbiosis. Karlidag et al. (2013) and Flores-Félix et al. (2015), who both used peat as substrate, did find significantly beneficial effects of PGPB, which is similar to many open-field soil based studies (Erturk

et al., 2012; Ipek et al., 2014; Kokalis-Burelle, 2003). Moreover, Pırlak and Köse, (2009)showed that, if PGPB root inoculation is ineffective, inoculating both roots and leaves may help to establish beneficial effects.

In essence, our study shows that AMF inoculation of substrate cultured strawberry in a greenhouse, increased fruit yield up to 21%, mitigated salinity stress, and significantly improved quality parameters like total phenolics, vitamin C, anthocyanin, fruit EC, and pH. Therefore, our findings are of direct interest to strawberry growers around the globe and challenge the current paradigm that AMF inoculation does not benefit strawberry yield under optimal conditions, e.g., in high tech greenhouses and vertical farms.

5. Conclusions

Inoculation with arbuscular mycorrhizal fungi (AMF) species *Glomus mosseae*, at optimal EC (1.3 $dS.m^{-1}$), significantly increases fruit yield (21%) and quality parameters like total phenolics, vitamin C, anthocyanin, fruit EC, and pH. Increasing nutrient solution strength from 1.3 to 1.9 $dS.m^{-1}$ decreases leaf fresh weight (29%) and fruit yield (18%) in strawberry (*Fragaria* × *ananassa* Duch 'Selva'). This stress effect at 1.9 $dS.m^{-1}$ can be partly mitigated by AMF; plants in inoculated pots appear to be healthier, and the stress indicators–peroxidase activity (POD) and ion leakage–are both reduced. Moreover, AMF inoculation also significantly elevates stomatal conductance, relative water content (RWC), chlorophyll index (SPAD), fruit EC, leaf phosphate, and leaf potassium levels. For pots inoculated with plant growth promoting bacteria (PGPB) (*Azospirillum lipoferum DSM1691*, or *Pseudomonas fluorescens DSM 50090*), no biologically relevant effects are observed.

Acknowledgments

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Appendices Files

S1. Standard absorbance curve gallic acid for total phenolic content

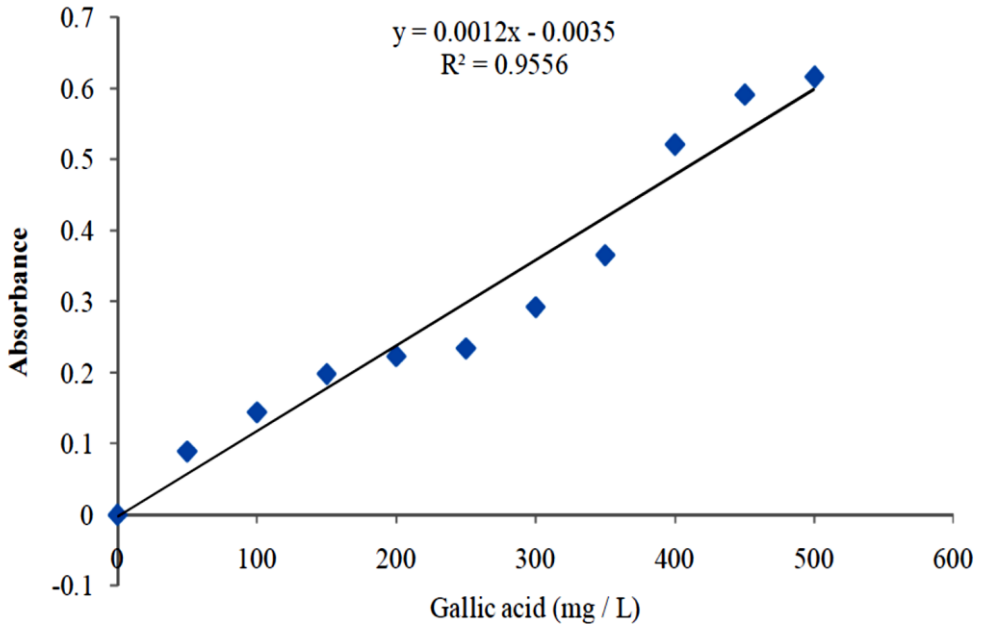
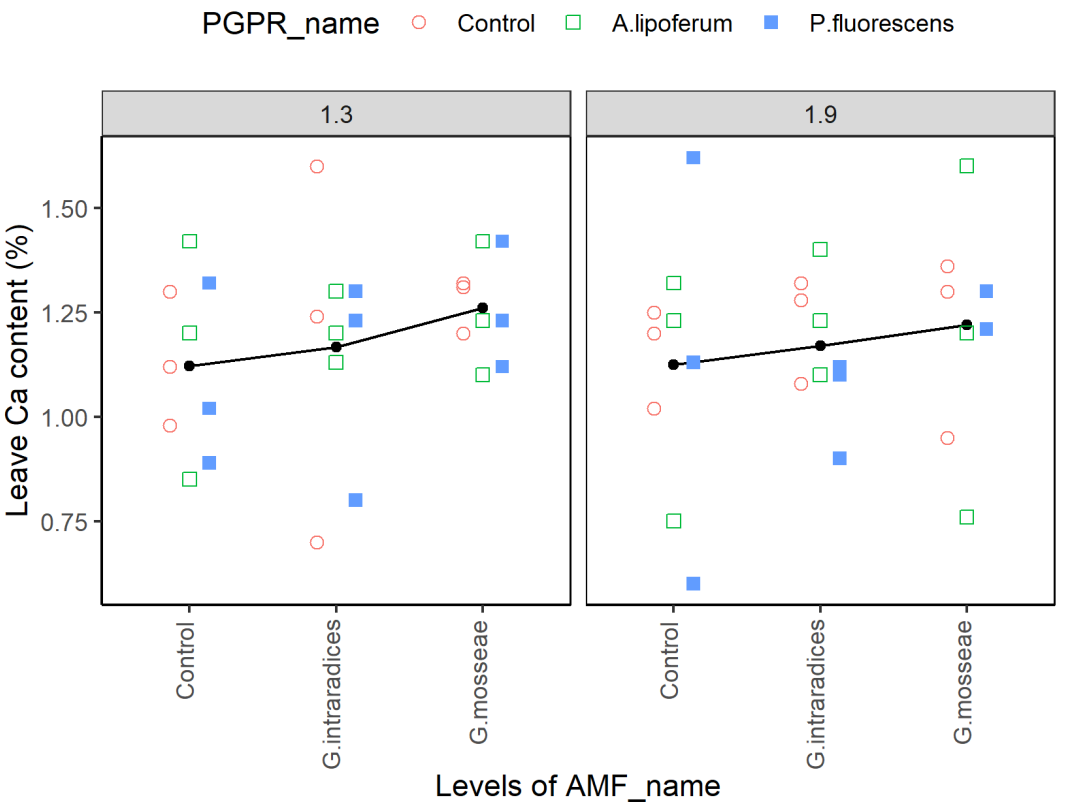
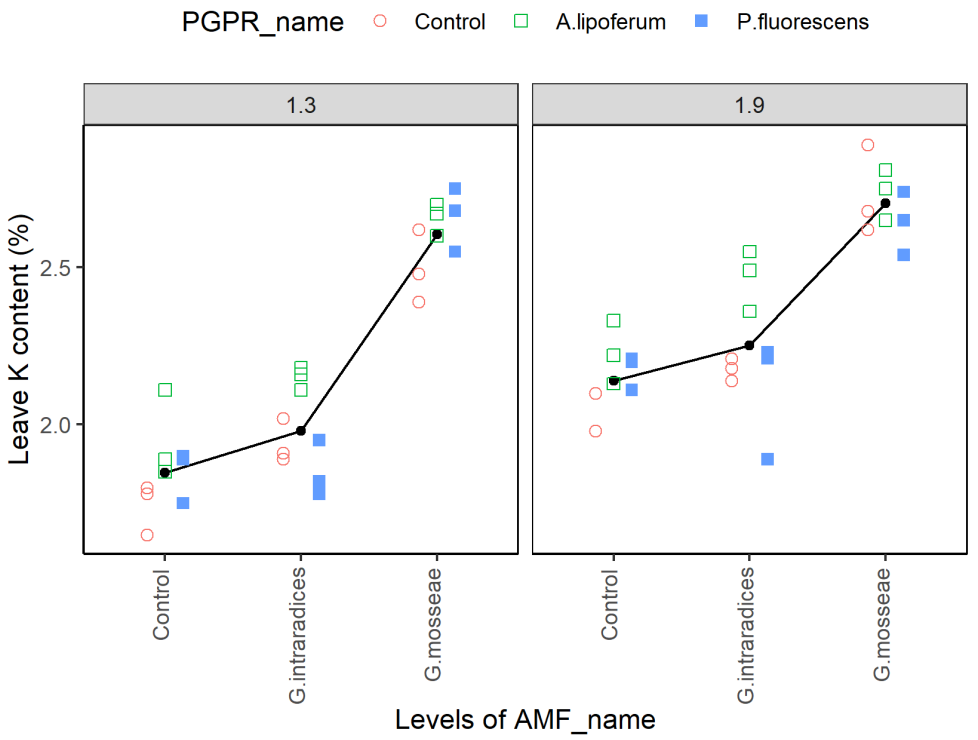
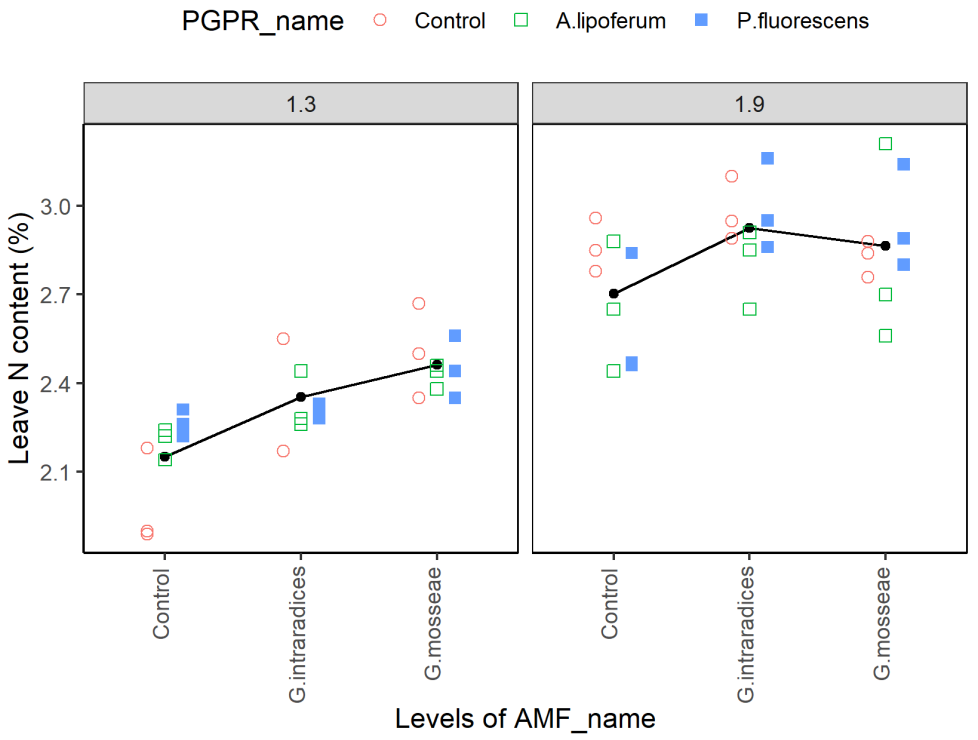


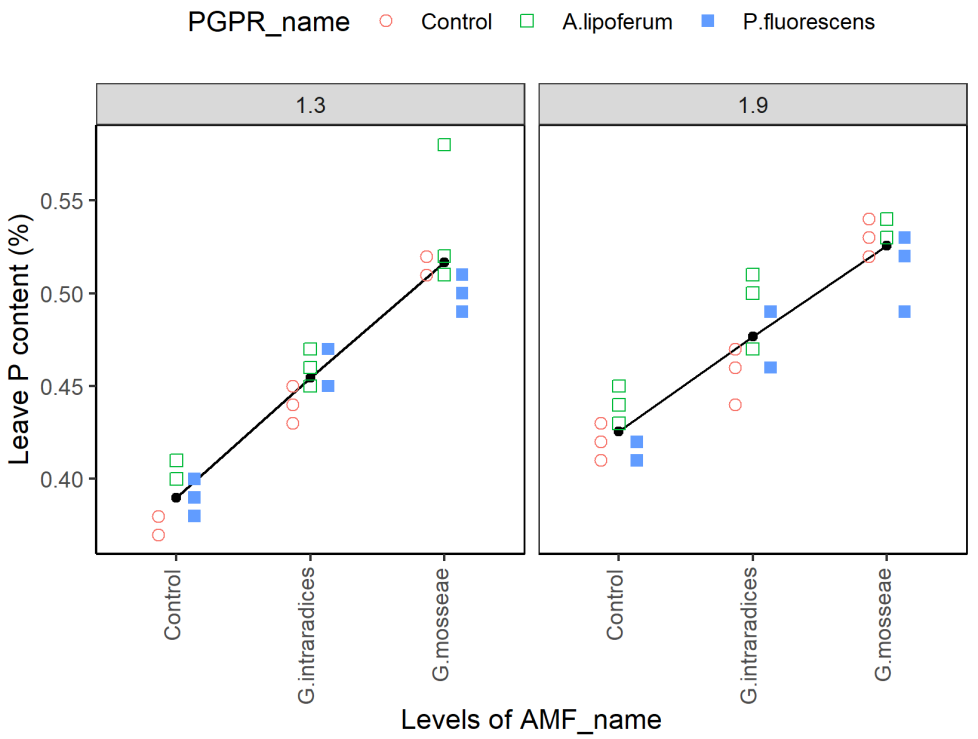
Figure S1. Standard absorbance curve of gallic acid to determine total phenolic content (mg Gallic acid/g fresh weight)

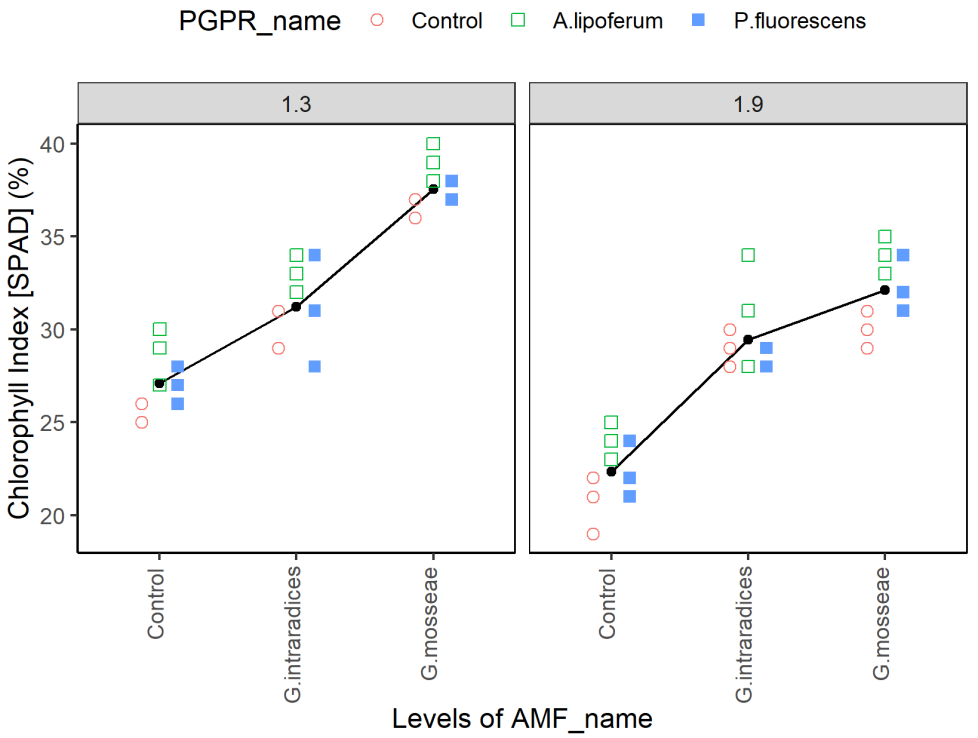
Effects plots of plant growth promoting bacteria (PGPR) (*Azospirillum lipoferum DSM1691*, and *Pseudomonas fluorescens DSM 50090*) under EC 1.3 and 1.9 dS m^{-1} in presence and absence of arbuscular mycorrhizal fungi (*Glomus mosseae*, and *Glomus intraradices*).

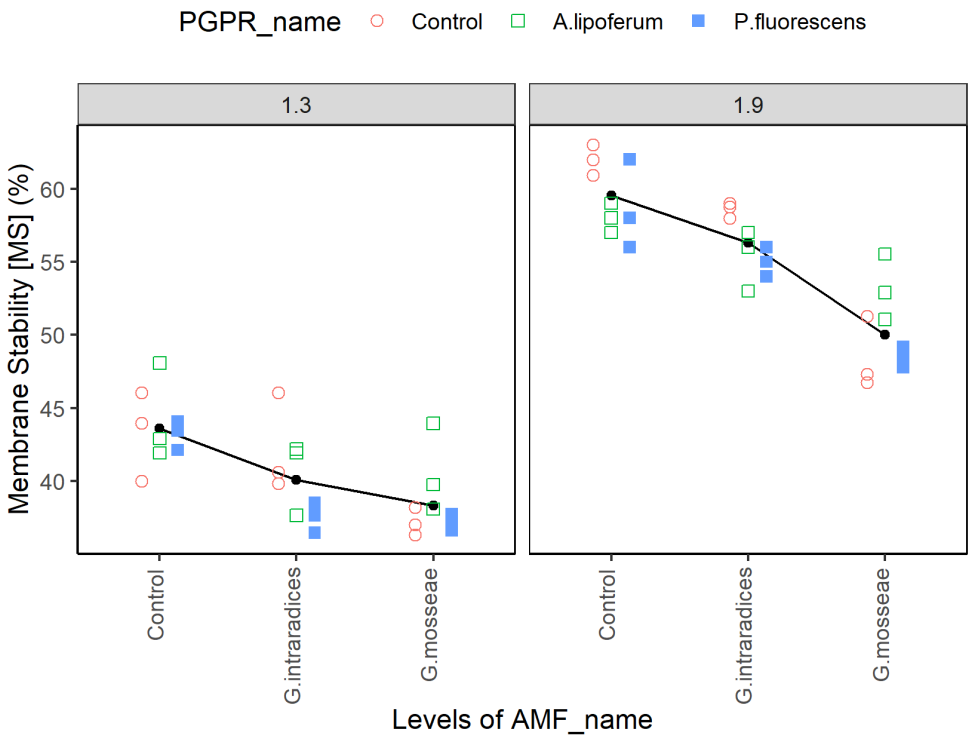


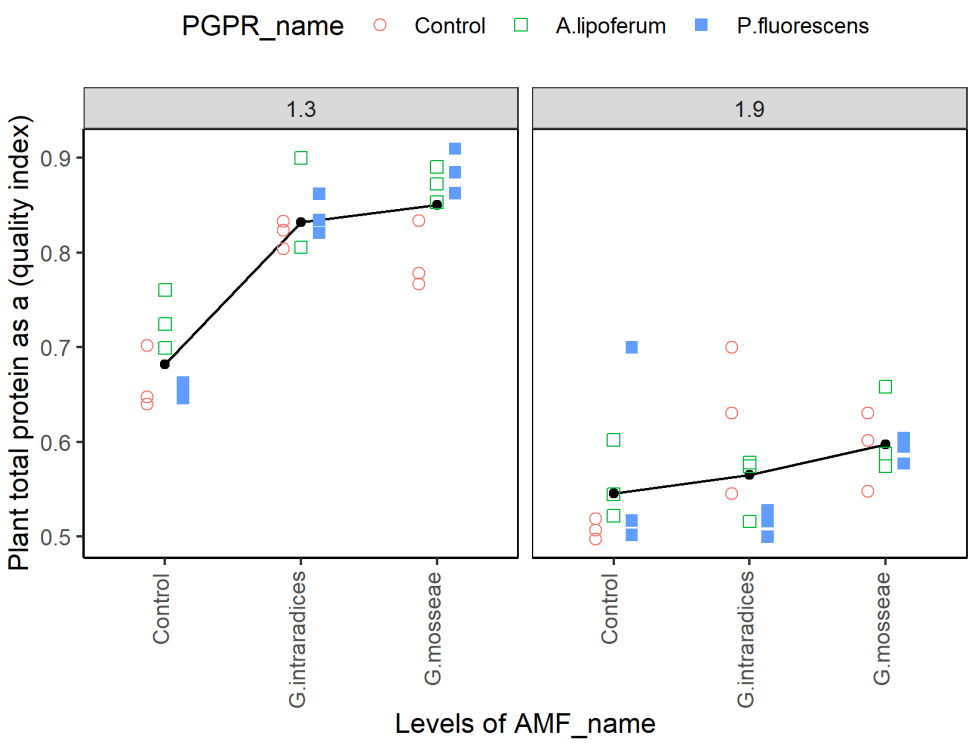

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Leave K content (%)
2.5
2.0
Control
G.intraradices
G.mosseae
Levels of AMF_name

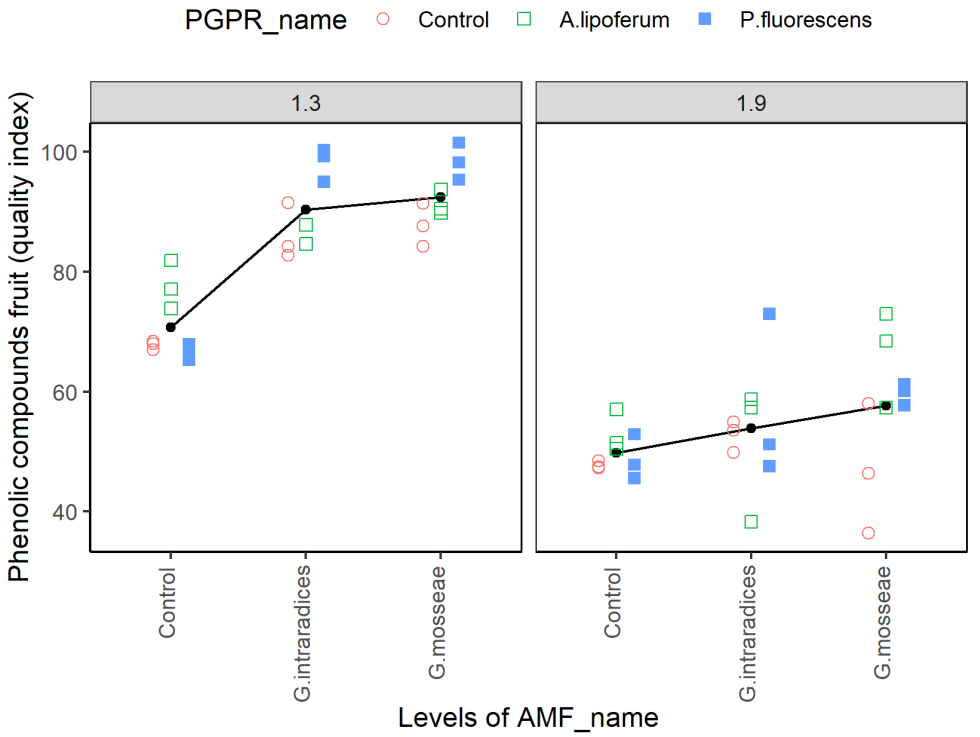

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Leave N content (%)
3.0
2.7
2.4
2.1
Control
G.intraradices
G.mosseae
Levels of AMF_name

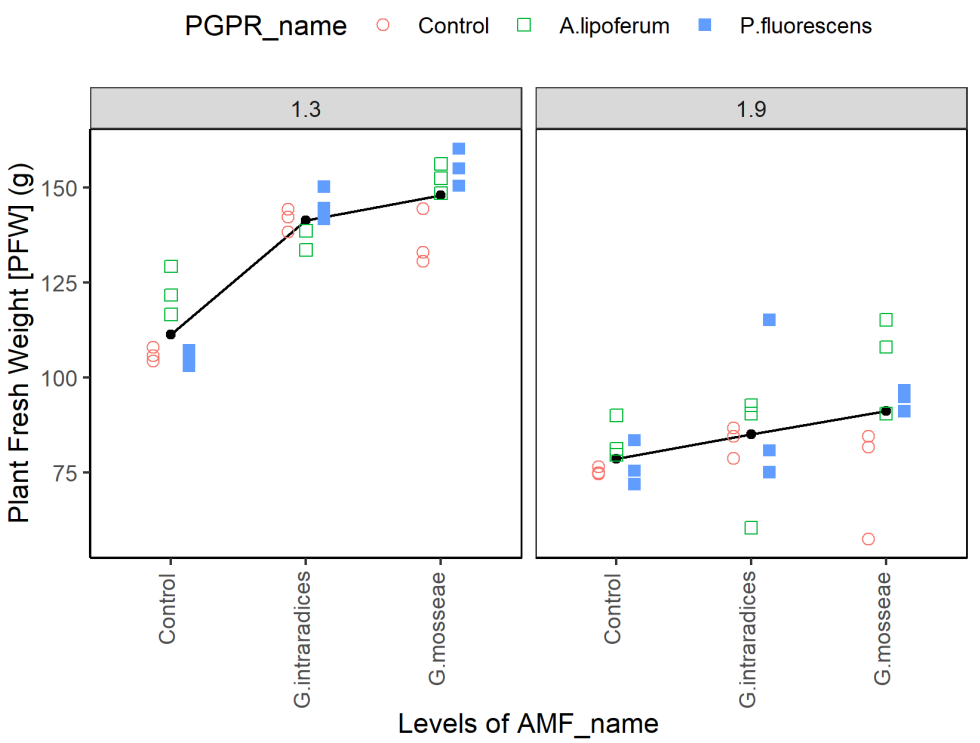

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Leave P content (%)
0.55
0.50
0.45
0.40
Control
G.intraradices
G.mosseae
Levels of AMF_name

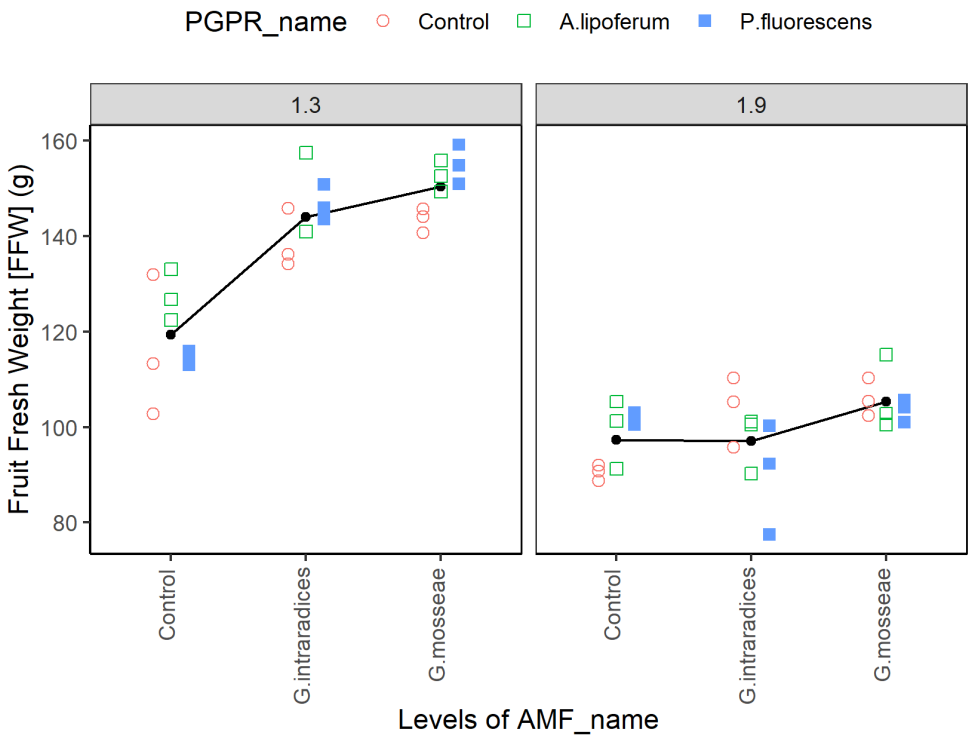

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Chlorophyll Index [SPAD] (%)
40
35
30
25
20
Control
G.intraradices
G.mosseae
Levels of AMF_name

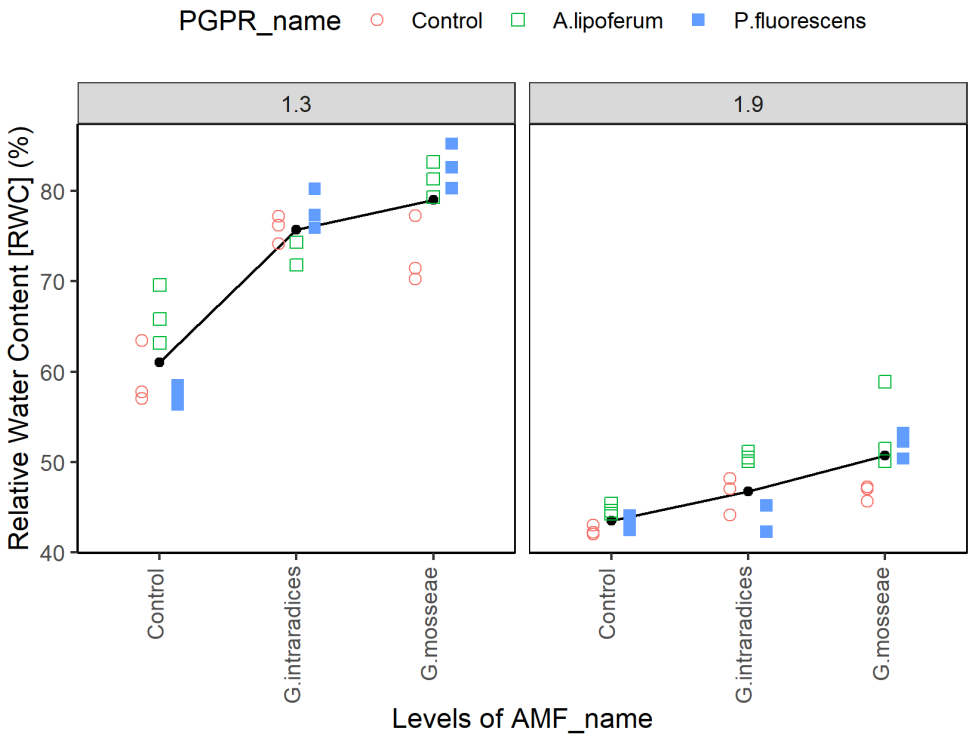

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Membrane Stability [MS] (%)
60
55
50
45
40
Control
G.intraradices
G.mosseae
Levels of AMF_name

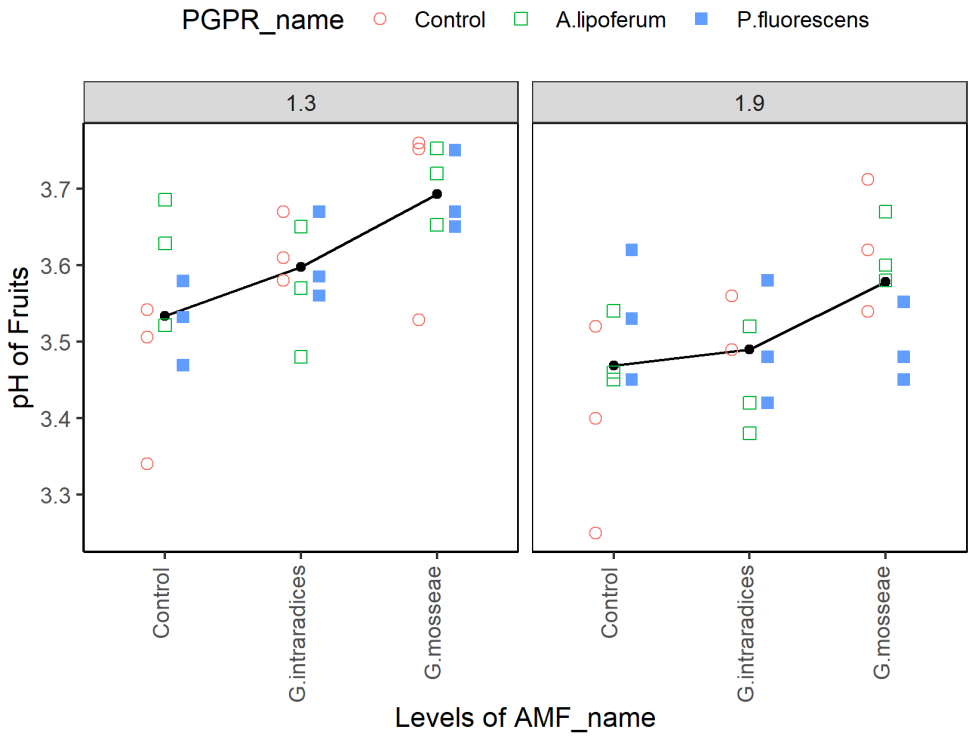

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Plant total protein as a (quality index)
0.9
0.8
0.7
0.6
0.5
Control
G.intraradices
G.mosseae
Levels of AMF_name

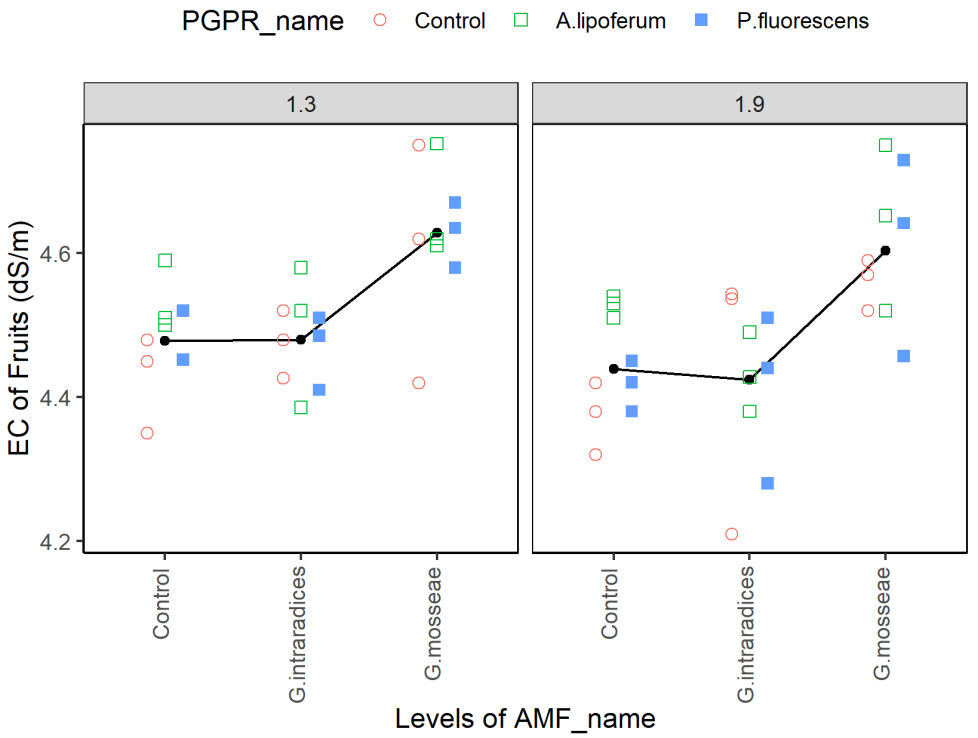

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Phenolic compounds fruit (quality index)
100
80
60
40
Control
G.intraradices
G.mosseae
Levels of AMF_name

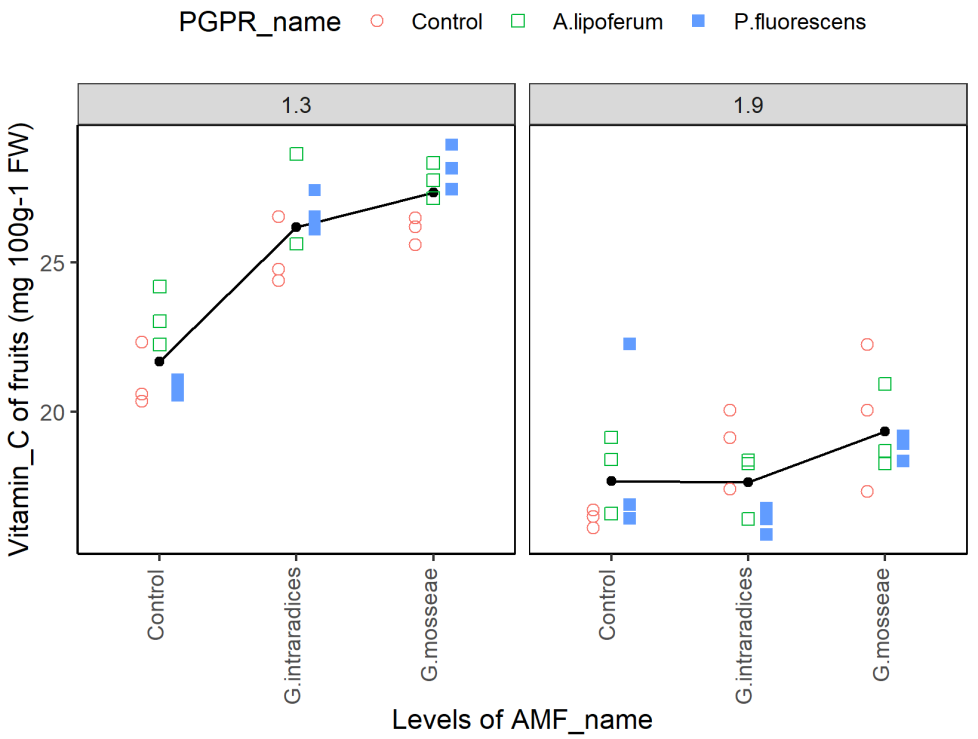

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Plant Fresh Weight [PFW] (g)
150
125
100
75
Control
G.intraradices
G.mosseae
Levels of AMF_name

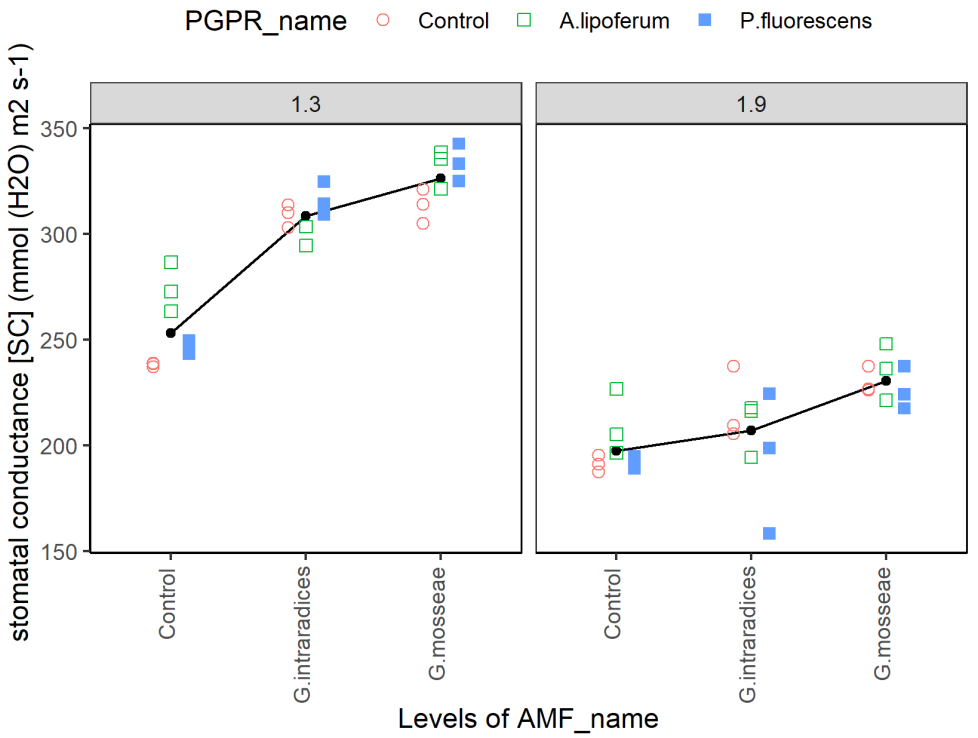

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Fruit Fresh Weight [FFW] (g)
160
140
120
100
80
Control
G.intraradices
G.mosseae
Levels of AMF_name

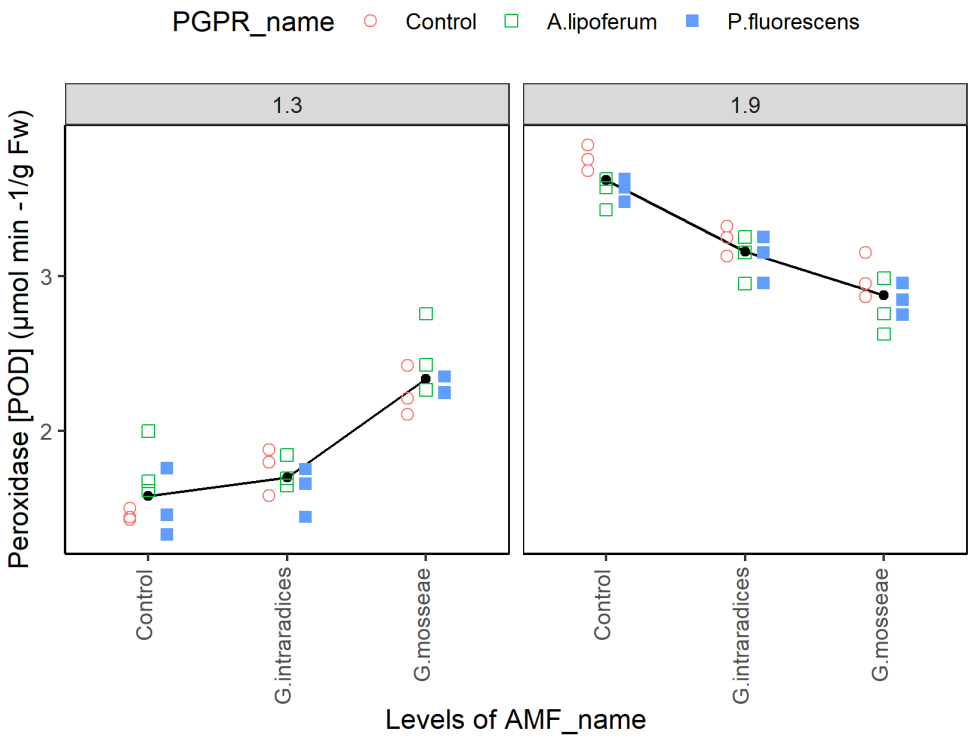

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Relative Water Content [RWC] (%)
80
70
60
50
40
Control
G.intraradices
G.mosseae
Levels of AMF_name

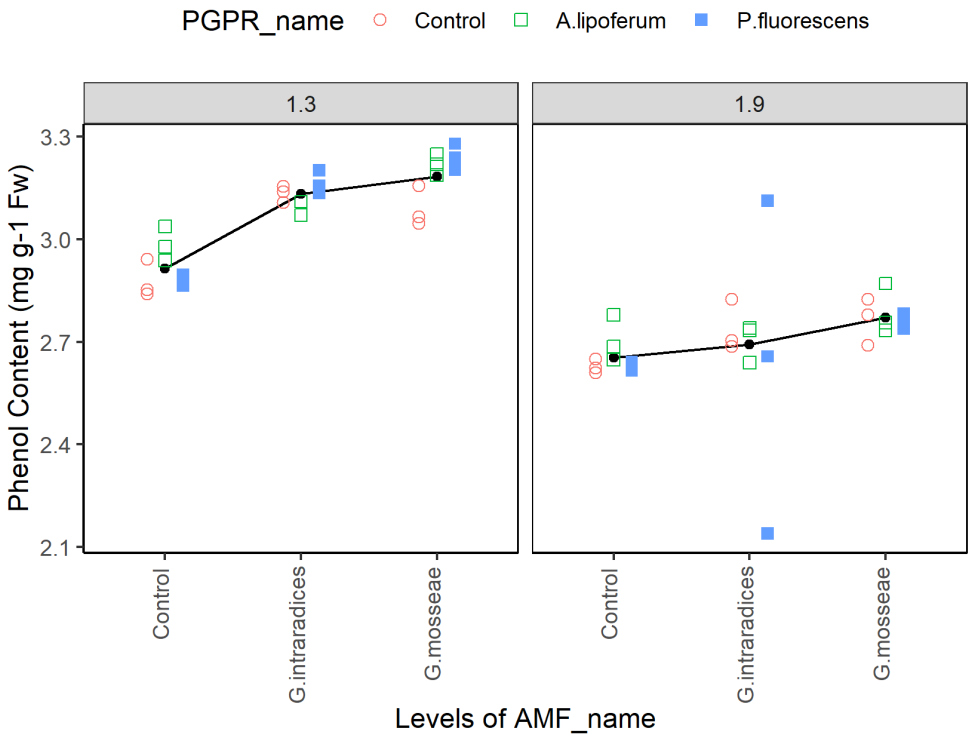

PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
pH of Fruits
3.7
3.6
3.5
3.4
3.3
Control
G.intraradices
G.mosseae
Levels of AMF_name


PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
EC of Fruits (dS/m)
4.6
4.4
4.2
Control
G.intraradices
G.mosseae
Levels of AMF_name


PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Vitamin_C of fruits (mg 100g-1 FW)
25
20
Control
G.intraradices
G.mosseae
Levels of AMF_name


PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
stomatal conductance [SC] (mmol (H2O) m2 s-1)
350
300
250
200
150
Control
G.intraradices
G.mosseae
Levels of AMF_name


PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Peroxidase [POD] (µmol min -1/g Fw)
3
2
Control
G.intraradices
G.mosseae
Levels of AMF_name


PGPR_name
Control
A.lipoferum
P.fluorescens
1.3
1.9
Phenol Content (mg g-1 Fw)
3.3
3.0
2.7
2.4
2.1
Control
G.intraradices
G.mosseae
Levels of AMF_name

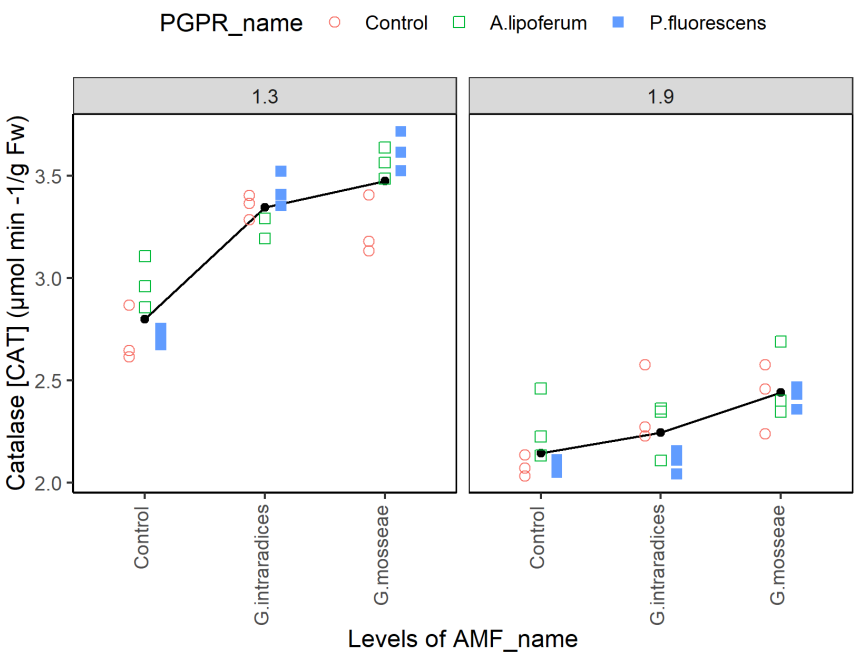


Figure S2. Effects plots of plant growth promoting bacteria (PGPR) (*Azospirillum lipoferum DSM1691*, and *Pseudomonas fluorescens DSM 50090*) under EC 1.3 and 1.9 dS m^{-1} in presence and absence of arbuscular mycorrhizal fungi (*Glomus mosseae*, and *Glomus intraradices*).

S3. Scatterplots and Pearson's correlations coefficients of the response variables

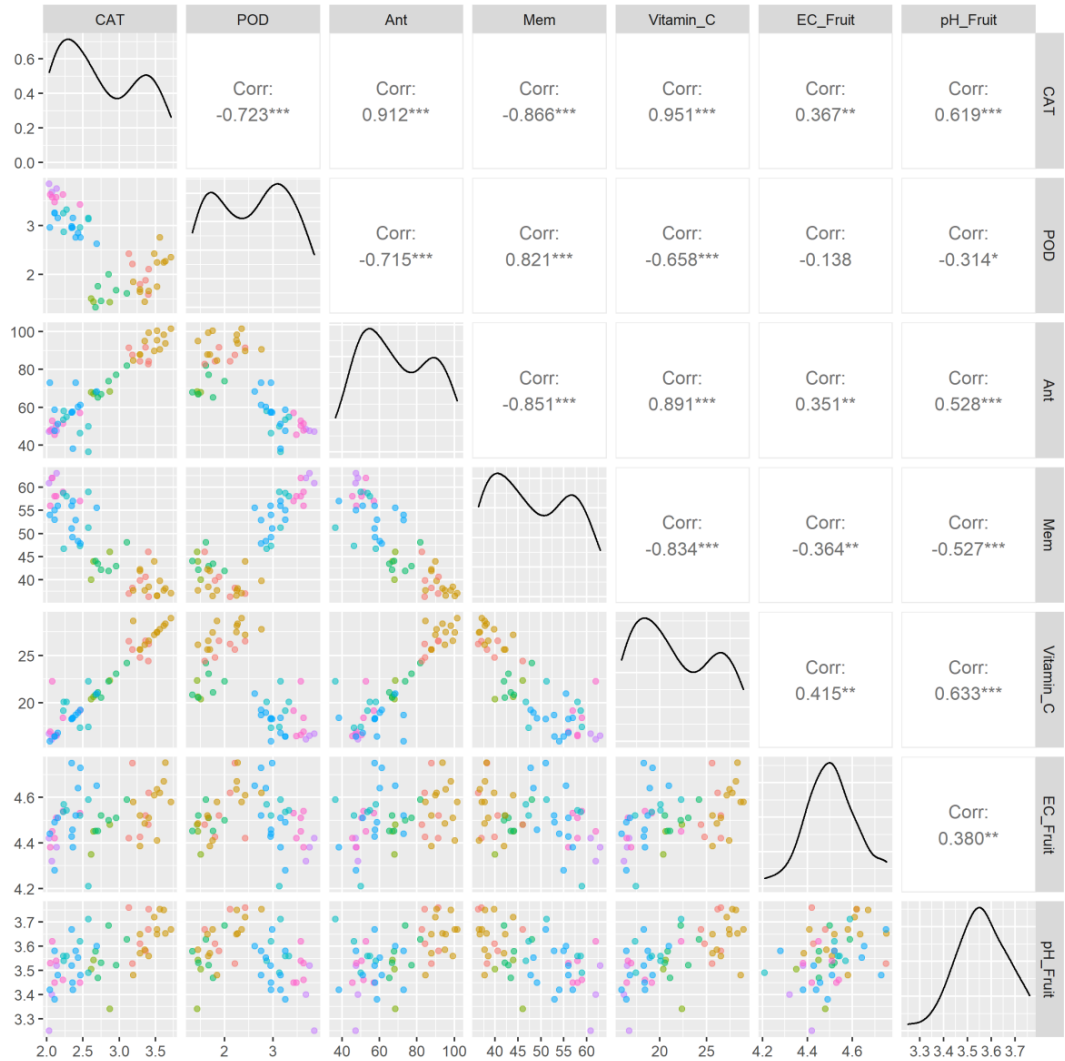


Figure S3a. Scatterplots and Pearson's correlations coefficients of the response variables: catalase (CAT), peroxidase activity (POD), anthocyanin concentration (Ant), membrane ion leakage (mem), Vitamin C content (Vitamin_C), Electrical Conductivity of Fruits (EC_Fruit), and the pH of the Fruits (pH_Fruit). Colors correspond to the different treatments, legend shown in Figure S2c.

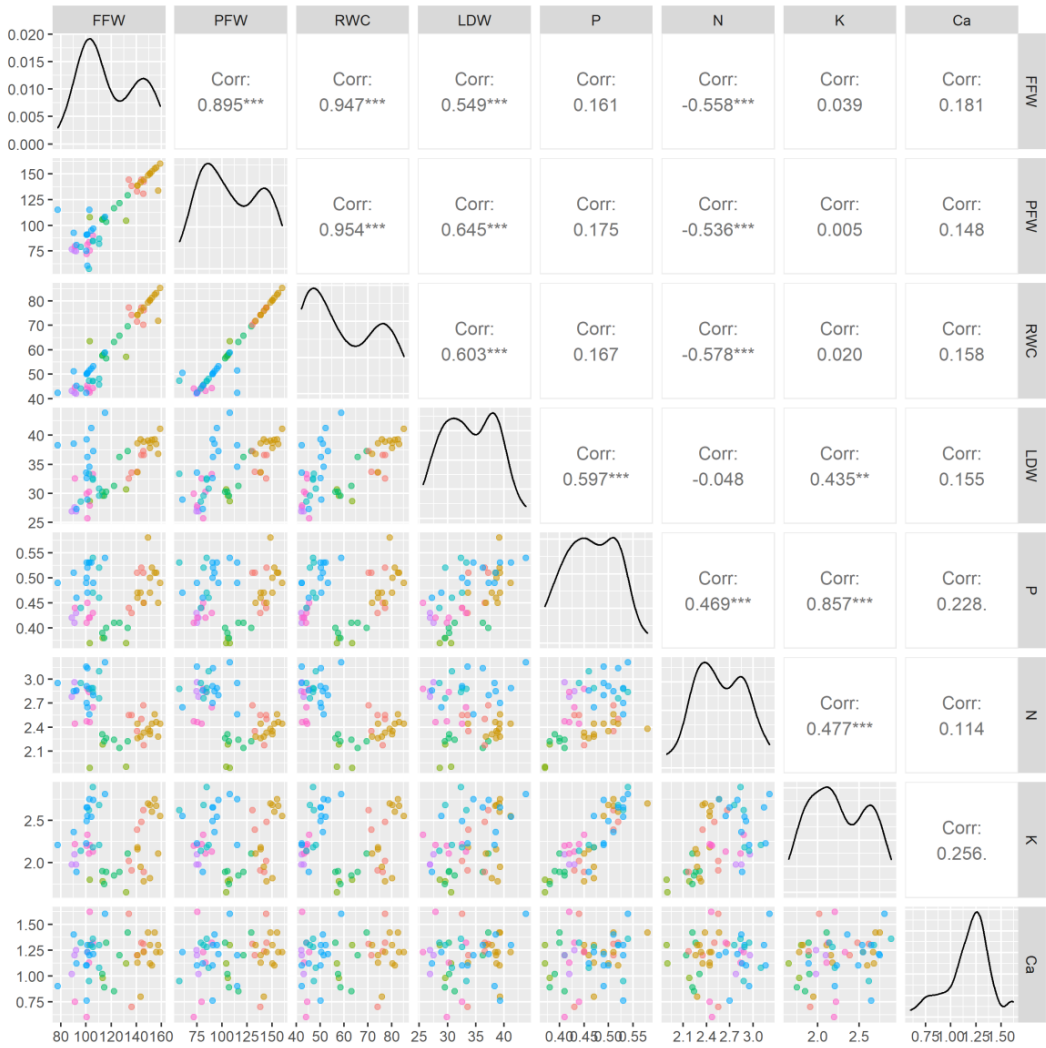


Figure S3b. Scatterplots and Pearson's correlations coefficients of the response variables: Fruit Fresh Weight (FFW), Leaf Fresh Weight (PFW), Relative Water Content (RWC), Leaf Dry Weight (LDW), and elemental leaf content of phosphorus (P), nitrogen (N), potassium (K), calcium (Ca). Colours correspond to the different treatments, legend shown in Figure S2c.

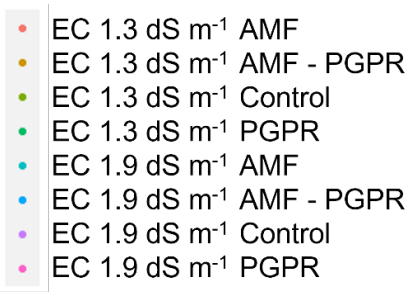


Figure S3c. Legend of colour codes used in Figure S2a and b for the different treatments.